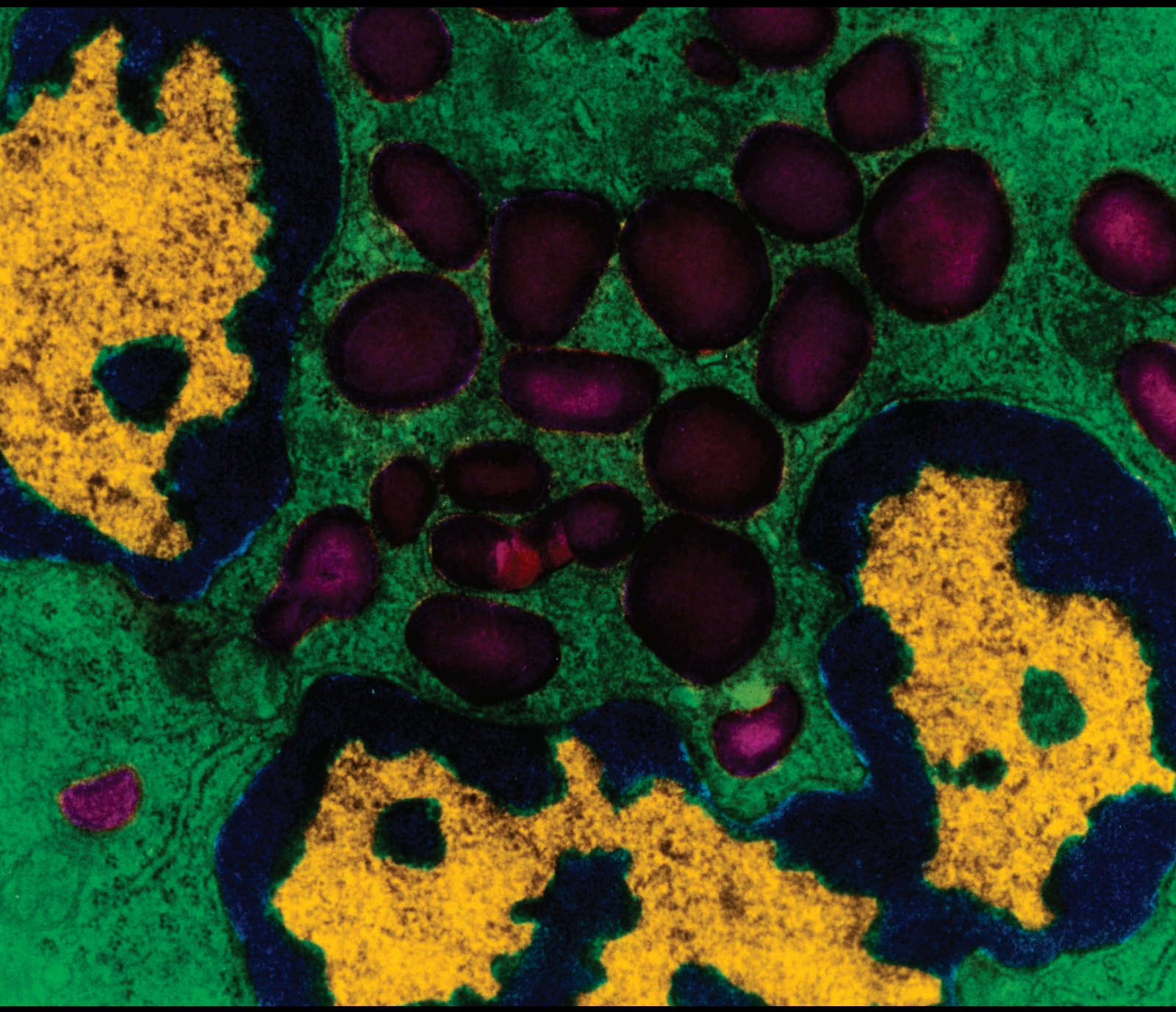
Mediators of Inflammation as Targets for Chronic Pain Treatment

Guest Editors: Metoda Lipnik-Stangelj, Mila Vlaskovska, Marshall Devor, Gila Moalem-Taylor, and Anna Maria Aloisi



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Editorial Board

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Editorial

Mediators of Inflammation as Targets for Chronic Pain Treatment

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Treatment of chronic pain remains an unresolved problem in human medicine which greatly impairs quality of life and prolongs treatment. Solving this problem is difficult due to a number of mechanisms and signalling pathways through which chronic pain is generated [1]. A common underlying mechanism of chronic pain is the presence of inflammation at the site of the damaged or affected tissue. The release of proinflammatory and immunoactive substances such as cytokines, neurotrophic factors, and chemokines initiates local actions and can result in a more generalized immune response that leads to the chronic pain condition. Clinical management of chronic pain, that is, neuropathic pain after nerve injury or cancer pain in tumour invasion represents therefore a real challenge due to our limited understanding of the cellular mechanisms that initiate and maintain chronic pain while many of them are closely overlapping with the processes of inflammation, immune response, endocrine and nerve system, and genetic factors as well [2, 3]. This special issue aims to bring a current knowledge of the role of mediators of inflammation in chronic pain, particularly molecular mechanisms, signalling molecules, and their role in the initiation and maintenance of chronic pain, as well as the diagnostic and therapeutic challenges on this field. The brief introductions of nine published papers are as follows.

The involvement of pro- and anti-inflammatory cytokines and angiogenic factors in the pain, associated with brain tumour progression, is shown in the paper entitled "*Cytokine patterns in brain tumour progression*." R. Albulescu et al. found significant changes in serum levels, with over threefold up-regulation of cytokines IL-6, IL-1β, TNF-α, and IL-10 and up to twofold up-regulation of cytokines IL-8, IL-2, and GM-CSF, and angiogenic factors VEGF and FGF-2. All these molecules are involved in tumour progression, and are also involved in a generation of pain, associated with disease. While pain is a frequent symptom caused by glioblastoma, the authors concluded that determination of selected cytokines could add to speed and accuracy, making thus possible earlier diagnostics and onset of therapy.

Activation of N-methyl-D-aspartate (NMDA) receptor leads to development of hyperalgesia. In the study called "*posttranslational nitration of tyrosine residues modulates glutamate transmission and contributes to N-methyl-D-aspartate-mediated thermal hyperalgesia*," C. Muscoli et al., showed that thermal hyperalgesia, induced by intrathecal administration of NMDA, is associated with spinal nitration of GluN1 and GluN2B receptor subunits, glutamine synthase, that normally convert glutamate into nontoxic glutamine, and glutamate transporter. Intrathecal injection of peroxynitrite decomposition catalyst FeTM-4-PyP5+ prevents nitration and inhibits NMDA-mediated thermal hyperalgesia. Their results support the hypothesis that nitration of key proteins involved in the regulation of glutamate transmission is a crucial pathway through which peroxynitrite mediates the development and maintenance of NMDA-mediated thermal hyperalgesia.

Inflammatory conditions, particularly in joint diseases, induce an increase in reactive oxygen substances which have a deleterious role in erosion, osteoarticular degeneration, and pain. L. Di Cesare Mannelli et al. in the psper "*Therapeutic effects of the superoxide dismutase mimetic compound $Mn^{II}Me_2DO2A$ on experimental articular pain in rats*" focused on superoxide dismutases which are decreased in pain conditions like joint inflammation, rheumatoid arthritis, and

osteoarthritis. They tested a superoxide dismutase mimetic compound 4,10-dimethyl-1,4,7,10 tetraazacyclododecane-1,7-diacetic acid Mn^{II} complex ($Mn^{II}Me_2DO2A$), which has potently relieved a pain in arthritis models, and they showed that the effect differed from a direct inhibition of cyclooxygenase enzymes. In chronic administration, it involved prevention of tissue degenerative alterations induced by the oxidative stress and reduction of a persistent inflammatory pain via a direct antioxidant mechanism, while in acute administration, it may decrease the nociceptive nervous fiber activation induced by the local production of reactive oxygen substances. Given these properties and the low toxicity of the molecule, $Mn^{II}Me_2DO2A$ represents a novel compound potentially suitable for the treatment of inflammatory and neuropathic pain.

In the paper "*Neurovascular unit in chronic pain*," B. M. Radu et al. focused on the role of blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) during the development of chronic pain. Reviewing several inflammatory- and nerve-injury-based pain models, they argue that the clarification of molecular BBB/BSCB permeabilization events is necessary for understanding chronic pain mechanisms. They proposed that the understanding of chronic pain mechanisms would benefit from the extension of research efforts to the neurovascular unit as a whole and reviewed the available evidence on the interaction between analgesic drugs and the neurovascular unit. Furthermore, they discussed chronic pain comorbidities, such as neuroinflammatory and neurodegenerative diseases, in a view of neurovascular unit changes, and innovative pharmacological solutions, targeting neurovascular unit components in chronic pain treatment.

Pain perception displays large interindividual variability in the population that affects selection of analgesics and their dosing. In the comprehensive paper "*Pharmacogenetics of chronic pain and its treatment*," S. Světlík et al. reviewed the most recognized pharmacogenetic areas and variables in the treatment of chronic pain. They focused on the impact of genetic variability of drug metabolizing enzymes, transporters, receptors, and pathways involved in chronic pain perception and on the efficacy and safety of analgesics and other drugs used for chronic pain treatment. Although several candidate genes have been identified in the literature, there is only limited clinical evidence substantiating for the penetration of the testing for these candidate biomarkers into the clinical practice. While the pain-perception regulation and modulation are still not fully understood, the authors have concluded that more complex knowledge of genetic and epigenetic background for analgesia will be needed prior to the clinical use of the candidate genetic biomarkers.

In autoimmune diseases of the nervous system, the neuropathic pain is frequently presented. In the review article "*Neuropathic pain in animal models of nervous system autoimmune diseases*," D. H. Tian et al. focused on neuropathic pain, associated with multiple sclerosis and Guillain-Barre syndrome, as well as with experimental autoimmune encephalomyelitis and experimental autoimmune neuritis, in animal models which enable investigations of behavioural changes, underlying mechanisms, and potential pharmacotherapeutic approaches for neuropathic pain, associated with these diseases. In this review, the symptoms, mechanisms, and clinical therapeutic options in these conditions are examined, and the value of experimental autoimmune encephalomyelitis and experimental autoimmune neuritis animal models for the study of neuropathic pain in multiple sclerosis and Guillain-Barre syndrome is highlighted.

In the paper "*Chronic pain treatment: the influence of tricyclic antidepressants on serotonin release and uptake in mast cells*," I. Ferjan M. Lipnik-Štangeli discussed the role of serotonin (5-HT), tricyclic antidepressants, and mast cells in the generation of chronic pain in the periphery and central nerve system. They showed that, besides inhibition of the pain stimuli in the central nerve system, 5-HT might be associated also by an increased pain transmission from the periphery, where mast cells play an important role. The authors demonstrated that tricyclic antidepressants are able to influence mast cell-derived 5-HT levels via at least three different mechanisms: secretion of 5-HT, uptake of exogenous 5-HT, and reuptake of secreted 5-HT. They concluded that analgesic effect of tricyclic antidepressants involved different mechanisms of action.

Current evidence indicates lines of the prominent role of gonadal hormones in affecting pain occurrence and intensity. In the review article "*Testosterone-induced effects on lipids and inflammation*," S. Vodo et al. described interesting aspects on the generation of chronic pain, influenced by androgen hormones, particularly testosterone, and lipids, whose altered metabolism is often accompanied by the release of interleukins and lipid-derived pro-inflammatory mediators, and based on interactions which are often not considered in chronic pain mechanisms. Also important is the ability of pain as well as pain therapies to affect gonadal hormone metabolism. The authors concluded that lower testosterone levels are associated with an increased metabolic risk, systemic inflammation, and chronic pain.

In the paper "*Inflammatory pain and corticosterone response in infant rats: effect of 5-HT1A agonist buspirone prior to gestational stress*," hypothalamo-pituitary-adrenal axis and serotonin system interactions in the chronic pain are discussed. I. P. Butkevich et al. presented the effect of buspirone on the dynamics of the inflammatory pain-like behaviour and stress response of corticosterone during the formalin test in the infant male rat offspring and evaluated the correlation between pain-like and hormonal parameters. They concluded that maternal buspirone, applicated before the stress during gestation, may enhance an adaptive mechanism of the inflammatory nociceptive system through activation of the hypothalamo-pituitary-adrenal axis peripheral link.

Acknowledgment

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Metoda Lipnik-Stangelj

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Research Article

Cytokine Patterns in Brain Tumour Progression

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Inflammation represents the immune system response to external or internal aggressors such as injury or infection in certain tissues. The body's response to cancer has many parallels with inflammation and repair; the inflammatory cells and cytokines present in tumours are more likely to contribute to tumour growth, progression, and immunosuppression, rather than in building an effective antitumour defence. Using new proteomic technology, we have investigated serum profile of pro- (IL-1β, IL-6, IL-8, IL-12, GM-CSF, and TNF-α) and anti-inflammatory cytokines (IL-4, IL-10), along with angiogenic factors (VEGF, bFGF) in order to assess tumoural aggressiveness. Our results indicate significant dysregulation in serum levels of cytokines and angiogenic factors, with over threefold upregulation of IL-6, IL-1β, TNF-α, and IL-10 and up to twofold upregulation of VEGF, FGF-2, IL-8, IL-2, and GM-CSF. These molecules are involved in tumour progression and aggressiveness, and are also involved in a generation of disease associated pain.

1. Introduction

Glioblastomas are the most aggressive type of intracranial tumours, highly resistant to combined treatment, in patients displaying a median survival time of 15 months [1]. The molecular mechanisms underlying these clinical features are the existence of specific genetic and molecular profiles of these tumour cells. Recent reports show genomic instability (especially in tumours from short-term survival patients), chromosomal alterations, somatic mutations, and polymorphisms [2]. Knowing this particular brain tumour cell, one can wonder if, besides the intrinsic cellular features, the inflammatory milieu triggered by the development of such a tumour cannot influence the particular clinical development in glioblastomas as well.

The relationship between inflammation and cancer has first been suggested in modern time, by Virchow in 1863, who found "lymphoreticular infiltrates" in neoplastic tissues, consequently suggesting that these reflect the origin of cancer of sites of chronic inflammation. Massive experimental proofs appeared in the recent years to support Virchow's concept [3].

In a synthetic formulation, inflammation is defined as "the seventh hallmark of cancer", by Colotta et al. [4]. The body's response to cancer has many analogies with inflammation and repair; the inflammatory cells and cytokines present in tumours are more likely to contribute to tumour growth, progression, and immunosuppression, rather than in building an effective antitumour defence. Cancer susceptibility and severity are often associated with functional polymorphisms in cytokine genes. As plastically described by Balkwill and Mantovani, if genetic damage is the "match that lights the fire" of cancer, some types of inflammation may provide the "fuel that feeds the flames" [3].

Tumour initiation and progression is a complex process involving genomic mutations, micro environmental

factors, and inflammatory mediators. Within the tumour environment inflammatory markers are responsible for cell proliferation, tumour invasion, marked angiogenesis, and suppression of certain immune functions [5].

Inflammation represents the immune system response to external or internal aggressors, such as injury or infection in certain tissues. Typical signs of inflammation include swelling, redness, pain, temperature rise, and subsequently loss of function. Numerous studies have shown that the majority of tumour tissues are associated with inflammatory signs. However, a clear connection between inflammation and cancer has yet to be demonstrated.

Glioblastoma represents the most common and lethal primary brain tumour. The prognosis is poor, especially for higher grade glioma—the most common primary neoplasm of the central nervous system, composing over 40% of all such tumours, with an incidence ranging from 8% to 27% [6].

A broad array of cytokines displays modified expression in cancers, including glioblastoma multiforme [7, 8].

The changes arise from the interaction of tumour cells and nontumour cells, like macrophages, lymphocytes, or stromal cells, and provide regulatory support for tumour growth, angiogenesis, invasion, and metastasis [8–10].

The vascular system of brain cancers inappropriately expresses membrane proteins, resulting in blood extravasation. The production of inflammatory mediators (such as cytokines and nitric oxide), and tumour hypoxia have been involved in these effects [11].

Pain belongs to the "classical" markers of inflammation, described over 2000 years ago by Aulus Celsus (*calor, rubor, tumour et dolor*). Various molecular actors of inflammation, including mediators of pain have been described in the recent years; a key role appears to be played by cytokines, which sometimes appear to conduct the orchestra of small molecule mediators, such as nitric oxide and prostaglandins. Many studies show that inflammation may be involved in different stages of tumour development. Several cancer risk factors like cigarette smoke, alcohol and growth factors can activate signaling pathways related with inflammation (such as NFκB and STAT3 signaling). Some chronic infections lead to inflammatory conditions and are associated with carcinogenesis (e.g., hepatitis B virus). Chemotherapeutic agents and gamma irradiation can also interfere in the regulation of expression of some genes implicated in inflammation, survival, proliferation, invasion, angiogenesis, and cancer metastasis [12]. Inflammation may be involved in carcinogenesis through mutations, genomic instability, and epigenetic modifications [13]. Also, inflammation can participate in premalignant cells proliferation, stimulate angiogenesis, and promote metastatic spread (Figure 1).

Among the cytokines, often found to be over expressed at tumour level, IL1-beta, TNF-alpha, IL-6, IL-10, IFN-gamma, CX3CL1, to name just a few, have been closely related to pain for a long time [14–16].

In the case of glioblastoma, headache is one of the most frequently claimed signs by the patients, but also, very often the diagnostic is set too late for a successful therapeutical approach.

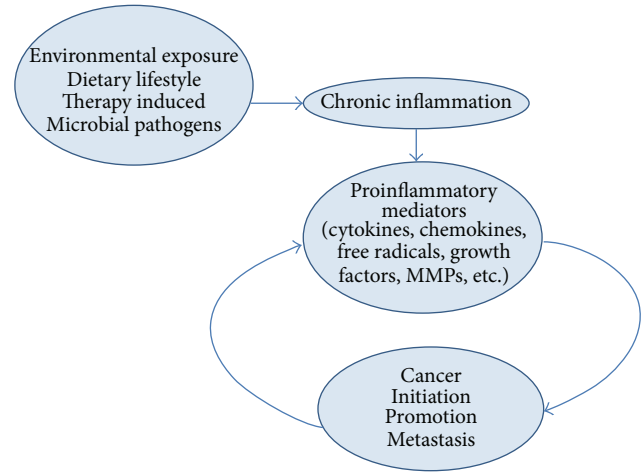

Figure 1: Implication of chronic inflammation in different stages of tumour development. Mediators of inflammation, triggered by different processes, may stimulate premalignant cell proliferation, angiogenesis, and metastasis. Reversely, tumour cells have the ability to stimulate other cells or to produce by themselves pro inflammatory and pro-angiogenic factors.

Acknowledging the worldwide research effort in the field of glioblastoma, we have embarked in the study of circulatory cytokines to pinpoint the serum inflammatory pattern that can characterize the glioblastoma patient's evolution. Therefore, our study investigated the serum levels of several pro- and anti-inflammatory cytokines and of angiogenic factors in brain tumour patients diagnosed in stages III and IV (glioblastoma), in order to establish their roles and behaviour in tumour progression.

2. Material and Method

2.1. Patients and Samples. Samples (serum) were collected from 55 patients with glioblastoma (28 men and 27 women; mean ages: 58 and 62 years, resp., range: 37–79 years) from Neurology and Neurovascular Diseases National Institute, Elias Hospital Neurosurgery Department and 20 controls (healthy individuals with no known history of inflammatory or neoplastic diseases, 12 men and 8 women; mean age: 57 years, range: 25–70 years). Written informed consent has been obtained upon sample prelevation according to Helsinki II Declaration and Ethics Committee of Victor Babes National Institute of Pathology that has approved the study. The collection of total peripheral blood from patients and controls has been achieved in vacutainers (Systems, Becton Dickinson) without anticoagulant. Serum was aliquoted and stored at −80°C until analysis.

2.2. xMAP Analysis and ELISA. The xMAP assay was performed according to the manufacturers' protocols, and the plates were analysed using Luminex 200 system. Cytokines levels and angiogenic factors were determined using the Human cytokine 12-plex Kit, with 12 analyte-specific bead

sets (simultaneous quantification)—pro-inflammatory IL-1β, IL-2, IL-6, IL-8, TNFα, GM-CSF, and INFγ, anti-inflammatory IL-4, IL-10, and IL-12, and angiogenic factors VEGF and FGF-2. Multiplex data acquisition and analysis were performed using STarStation 2.3. Triplicate samples were used for all specimens. Values for individual proteins measured by this multiplexed protein array technology have been shown to correlate with single ELISA measurements.

Immunoenzymatic ELISA analysis was performed with Quantikine (R&D Systems). Serum level of growth factors was determined according to the manufacturer's protocol. All samples were assayed in triplicate, and the mean values of cytokines were taken into account. Optical density was measured at 450 nm on an Anthos Zenith 3100 multimode micro plate reader. Minimum detectable concentrations were found to be less than 9.0 pg/mL for VEGF and less than 3.0 pg/mL for bFGF.

2.3. Statistical Analysis. Data were collected and expressed as the mean ± standard error of three independent repeats. Differences between groups were analysed by One Way Anova; P values less than 0.05 were considered statistically significant; Pearson correlation (r, p) was used to explore the association between cytokine expressions. Statistical analysis was performed using SPSS 19.0 software.

3. Results and Discussion

From multiplex assay (Luminex 200) a strong overexpression was detected for IL-6, IL-1β, TNF-α, and IL-10 (over 3-fold stimulation in glioblastoma patients). Significant up-regulation (up to 2-fold) was found for VEGF, FGF-2, IL-8, IL-2, and GM-CSF. Cytokines expression was significantly higher and strongly correlated with tumour grade, proliferation markers, and clinical aggressiveness in glioblastomas. Comparing the patient groups and control for growth factors, the obtained values by xMAP array were comparable to the outline obtained by the ELISA analysis.

Based on xMAP analysis, the changes in average serum levels (compared to the controls) are presented in Figure 2.

Several molecules display a modification in plasma levels of more than 2-fold, which is, as a general practice, a criterion of acceptance as potential marker. However, it also appears evident that, for several cytokines, the intervals of variations in patients were broad. Further details on expression are provided in Figures 3, 4, and 5, where the distributions can be better examined and also cover the behaviour of controls.

The enhanced expression of IL-1β appears to directly correlate with IL-6 and IL-8 levels and inversely correlate with IL-4. In brain, IL-1β regulates survival and invasiveness of glioblastoma cells, and anti-IL-1β antibodies inhibit both the growth and invasion of glioblastoma cells [17]. Wang et al. showed that in LN-229 glioma cell line, IL-1β and TGF-β can induce glioma stem cells phenotype and contribute to carcinogenesis [18]. Enhanced secretion of IL-1β, IL-6, and IL-8 by glioma cells was reported by Yeung et al. [19], and these cytokines are related with the expansion of GBM (glioblastoma multiforme). In other types of cancers (gastric and oesophageal), IL-1β was involved in carcinogenesis and proliferation and played a crucial role in the development of chemical carcinogen-induced tumours [20, 21].

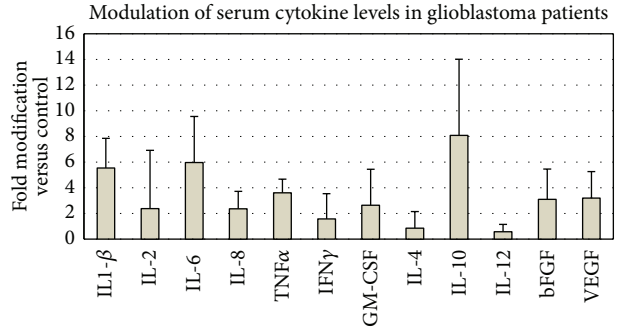

Figure 2: Modulation of serum cytokine levels in glioblastoma patients. The data represent group averages of fold modification versus controls + standard deviations. Statistical significance (one way ANOVA): pro-inflammatory cytokines, $P < 0.05$ for IL-1β, IL-6, and TNFα, GM-CSF; anti-inflammatory cytokines, $P < 0.05$ for IL-4, and IL-10; angiogenic factors, $P < 0.05$ for bFGF and VEGF. Expression levels of IL-2, IL-8, IFN-γ, and IL-12 were modified, but with low statistical significance.

IL-6 appeared overexpressed (average 4-fold) in glioblastoma patients. The determined serum levels are consistent with the ability of tumour cell to secrete pro-inflammatory cytokines, as well as with IL-6 role in stimulation of angiogenesis. According to our data, IL-6 expression correlates with IL-1β, IL-8, and IFN-γ. Ancrile et al. showed IL-6 is involved in carcinogenesis by angiogenesis and tumour growth and may be a potential anti-invasion target [22]. In U251, T98G and U87 MG glioblastoma cell lines, IL-6 promotes vascular endothelial cell migration and facilitates tumour angiogenesis and invasion [23, 24]. Amplification of the IL-6 gene in patients with glioblastoma multiforme is correlated with decreased survival [25].

Serum levels of TNFα appeared significantly enhanced ($P = 2.5E - 8$), suggesting a strong correlation with the disease; however, the correlation with other molecules is not so strong, suggesting its implication in distinct/complementary regulatory cascades. Hagemann et al. suggested that TNF is involved in tumour cell invasion through upregulation of migration-inhibitory factor (MIF) and through enhanced MMPs production in tumour cells via NF-$\kappa\beta$- and JNK-signalling [26]. In ovarian cancer, TNFα stimulated other cytokines (IL-6), angiogenic factors (VEGF), and chemokines (CCL2 and CXCL12) that promoted tumour growth and metastases [27]. Other studies have showed that TNF over-expression enhances migration and metastasis through induction of CXCR4, MCP-1, and IL-8 and matrix metalloproteinase [28, 29]. Recent studies on U373MG and C6 human glioma cell lines showed that TNF-α induces IL-6 synthesis through the JAK/STAT3 pathway and TNF inhibitors can reduce tumour cell invasion [30, 31].

IL-8 has been found to be up-regulated (fold stimulation 1.9) in patient sera, compared to controls. Many studies showed that IL8 is upregulated in gliomas and is involved in

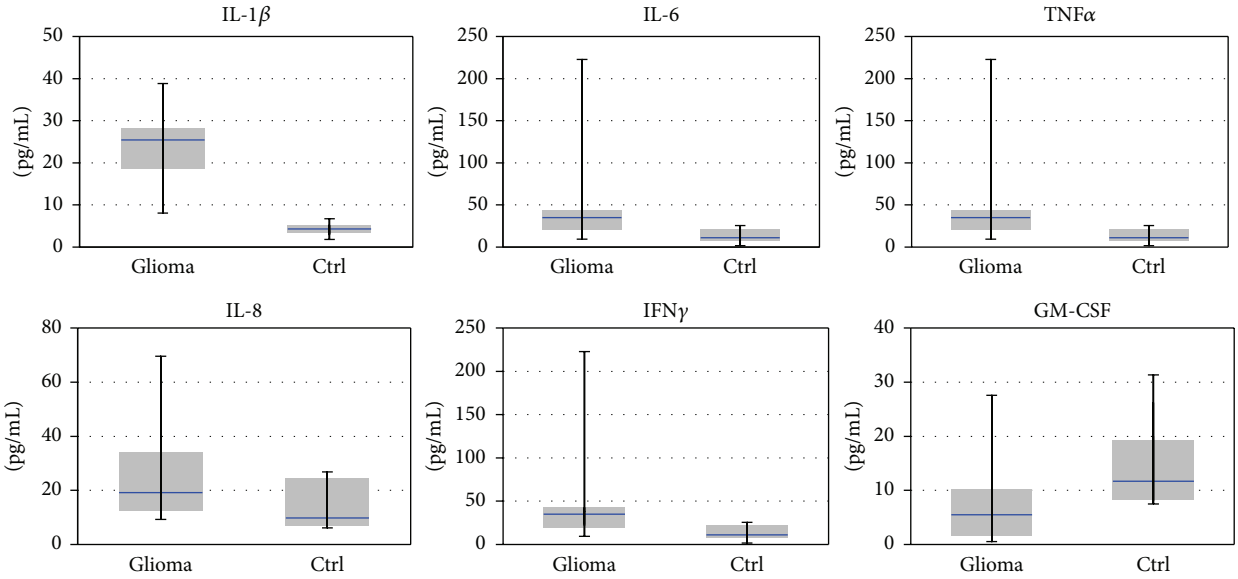

Figure 3: Serum levels of pro-inflammatory cytokines xMAP analysis. Statistical significance (one way Anova): $P < 0.05$ for over-expression of IL-1β, TNFα, IL-6, and GM-CSF in sera from glioblastoma patients compared to control.

the promotion of angiogenesis. In PTEN-deficient glioblastoma cells, repression of IL-8 can inhibit glioblastoma cell proliferation and invasiveness [32].

Studies on murine models showed that transplanted glioblastoma tumour cells, which express high levels of IL-2, IL-4, or GM-CSF show enhanced tumour survival. U87-MG glioblastoma cell line expressed high levels of GM-CSF, and GM-CSF over-expression is found exclusively in cultures derived from astrocytomas [33].

In glioblastoma patients, IL-2 overexpression averaged 2-fold, but the patient group has been distributed in one subgroup of patients (55%) who displayed strongly enhanced levels of expression (up to 5-fold) while 45% of the patients displayed a moderate enhancement of expression—on average 1.4-fold increase. The distribution could not be yet correlated with other clinical data. Statistical analysis (t-test) suggests that the two subgroups represent distinct subpopulation of glioblastoma patients, and so further investigations and integration of more data is required to consolidate and explain this segregation. IL-2 is reported in use for cancer treatment, as a stimulator of T-cell mediated anti-tumour activity.

Anti-inflammatory cytokines IL-4 and IL-12 appear at lower level in patient's sera at 57–80% compared to controls group (Figure 3). IL-4 is involved in inhibition of cell proliferation, regulation of adhesion molecules, and induction JAK/STAT signalling; IL-4 receptor is overexpressed in malignant glioma cell lines and tumour specimens from patients with glioblastoma, but his mechanism is still unclear [34]. Many studies on murine models showed that IL-12 is a powerful anticancer factor which can inhibit growth of implanted glioblastoma and the increase survival time [35].

IL-10 levels are significantly increased ($P < 0.001$). At a first glance, the strong increase in IL-10 looks paradoxical in the general balance of pro- and anti-inflammatory cytokines; however, the finding confirms previous reports of Kumar et al. [36], who reported significant increase of IL-10 serum levels in patients with anaplastic astrocytoma and glioblastoma. The increase in serum levels of IL-10 may also be correlated with glioma induced immunosuppression. The same study also detected significant decrease in serum levels of glioblastoma and anaplastic astrocytoma, suggesting a systemic impact of brain tumours on the immune system. RT-PCR and immunoassay studies on glioblastoma showed that IL-10 expression is significantly higher in stem-cell-derived tumour sphere cells than in primary cultured glioma cells from the same tumour [37]. IL-10 is significantly overexpressed in high grade tumours and can contribute to progression of astrocytomas [38].

Serum levels of angiogenic factors were considerably elevated in glioblastoma patients, as measured by xMAP analysis and confirmed by ELISA; both VEGF and bFGF were significantly overexpressed (bFGF, 3.05-fold modification, $P = 0.002$, VEGF, 3.2-fold modification, $P = 0.005$), see Figure 5. The expression and distribution profiles of angiogenic factors were similar in both detection methods, with an increase for bFGF of 2.99-fold in xMAP analysis and 3.22 in ELISA and for VEGF of 3.12 and 3.08, respectively.

In GBM, VEGF-VEGFR2 signalling is maintained by continuous secretion of VEGF ligand and promotes tumour growth, invasiveness and enhanced resistance to some treatments [39]. Anti-VEGF therapy and VEGFR inhibitors can delay progression of glioblastoma, but this mechanism is not well understood [40]. *In vitro* and *in vivo* studies showed that stem-cell-like glioma cells secrete elevated levels of VEGF induced by hypoxia, and anti-VEGF therapy cancel proangiogenic effects of glioma [41–43].

FGF-2 is involved in neoplastic transformation of glioma cells by activating Ras/Raf/ERK signalling and can stimulate angiogenesis in glioblastoma [44, 45].

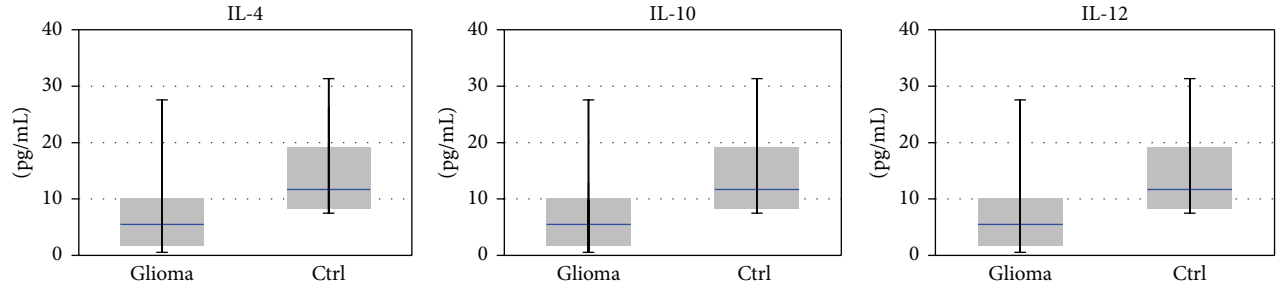

Figure 4: Expression levels of anti-inflammatory cytokines by xMAP analysis. Statistical significance (one way Anova): $P < 0.05$ for IL-4 and IL-10 modified levels in patients' sera versus controls.

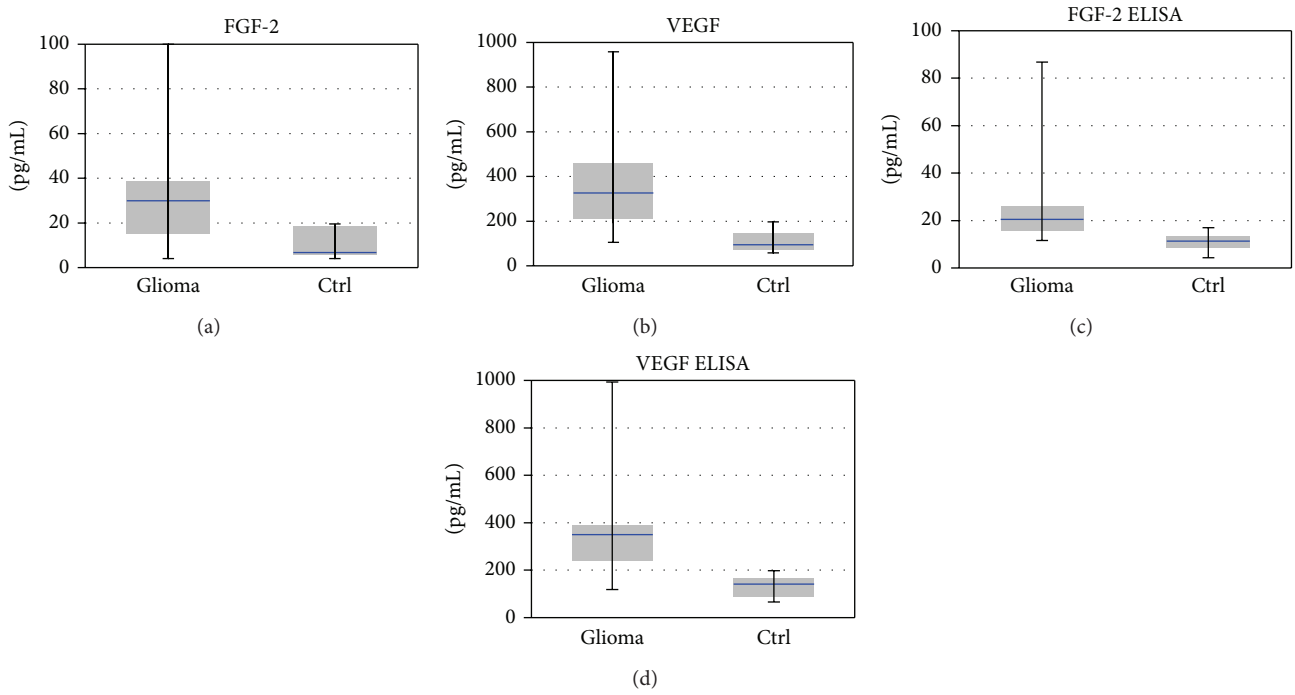

Figure 5: Expression levels of angiogenic factors, by xMAP analysis (a and b) and ELISA (c and d). Statistical significance (one way Anova): $P < 0.05$ for bFGF and VEGF over-expression in sera from glioblastoma patients versus control, by both methods.

Our study was primarily aimed on the estimation of serum level of several cytokines and angiogenic factors in glioblastoma patients, in order to assess their suitability as diagnostic, prognostic and monitoring biomarkers. Nevertheless, many components of the investigated panel are pleiotropic molecules, and, besides their primary role as regulators of cell behaviour, they also play major roles in inflammation and/or tumour related pain. The serum levels of IL-1β, TNFα, IFN-γ, and GM-CSF were significantly increased in glioblastoma patients. A previous study of Makimura et al. [46] investigated the plasma levels of 26 cytokines in cancer patients in correlation with responses to morphine treatment; they could not correlate the levels with pain levels, but were able to correlate some of the investigated molecules with responsiveness to morphine treatment. According to Kawasaki et al. [47] TNF and IL-1β cause an increase in the activity of AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid) or NMDA (N-methyl-D-aspartate) receptors, while IL-1β and IL-6 inhibit gamma-amino-butyric acid (GABA) and glycine-induced ion currents in Rexed lamina II nociceptors, demonstrating that these pro-inflammatory cytokines favour the increase in neuronal excitability. Several studies showed that pro-inflammatory and anti-inflammatory cytokines dysregulation is associated with carcinogenesis and tumour progression of solid cancers, like pancreatic and colorectal [48–50].

4. Conclusion

Our findings demonstrate that cytokines and angiogenic factors levels are closely linked to the brain tumour behaviour.

Out of all potential biomarkers for glioblastoma staging and prognostic, a panel of inflammatory cytokines and

angiogenic factors is more relevant than single molecules, as proven by our study. Moreover, further investigation could generate a multimolecular panel for better patient stratification and more adequate therapeutical approaches. The involvement of cytokines in inflammation and pain, as well as the relevance of pain in glioblastoma makes them reliable targets for investigation, with potential diagnostics and therapeutic applications.

xMAP technology might be a suitable tool for evaluation of tumoural development. The advantages of xMAP technology could be less invasive techniques, screening for molecular markers, and validation of putative therapeutic targets.

Further analysis on protein expression and signalling, protein interaction networks, associated with the implementation of a clinical panel for pain scoring, may lead to establish more clearly the connection between mediators of inflammation, signaling pathways and targets for tumoural progression, cancer therapy, and cancer pain therapy.

Pain is a frequent symptom accused by glioblastoma patients and one of the primary signs conducting to investigation and diagnostics, but, unfortunately, most often the diagnostics appear late or too late; the set of cytokines could add in speed and accuracy, making thus possible earlier diagnostics and onset of therapy.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Posttranslational Nitration of Tyrosine Residues Modulates Glutamate Transmission and Contributes to N-Methyl-D-aspartate-Mediated Thermal Hyperalgesia

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Activation of the N-methyl-D-aspartate receptor (NMDAR) is fundamental in the development of hyperalgesia. Overactivation of this receptor releases superoxide and nitric oxide that, in turn, forms peroxynitrite (PN). All of these events have been linked to neurotoxicity. The receptors and enzymes involved in the handling of glutamate pathway—specifically NMDARs, glutamate transporter, and glutamine synthase (GS)—have key tyrosine residues which are targets of the nitration process causing subsequent function modification. Our results demonstrate that the thermal hyperalgesia induced by intrathecal administration of NMDA is associated with spinal nitration of GluN1 and GluN2B receptor subunits, GS, that normally convert glutamate into nontoxic glutamine, and glutamate transporter GLT1. Intrathecal injection of PN decomposition catalyst FeTM-4-PyP^{5+} prevents nitration and overall inhibits NMDA-mediated thermal hyperalgesia. Our study supports the hypothesis that nitration of key proteins involved in the regulation of glutamate transmission is a crucial pathway used by PN to mediate the development and maintenance of NMDA-mediated thermal hyperalgesia. The broader implication of our findings reinforces the notion that free radicals may contribute to various forms of pain events and the importance of the development of new pharmacological tool that can modulate the glutamate transmission without blocking its actions directly.

1. Introduction

NMDARs in the spinal dorsal horns play a critical role in nociceptive transmission and modification [1, 2]. Glutamate-mediated activation of the NMDAR is fundamental in the development of hyperalgesic responses associated with pain of various etiologies [2–4]. Thus, the hyperalgesic responses detected in experimental models of acute inflammatory and neuropathic pain are blocked by intrathecal delivery of NMDAR antagonists [2, 3, 5–10].

We have reported that NMDAR activation releases superoxide (SO) which in turn is critical in mediating NMDA-mediated hyperalgesia [2, 11]. A key mechanism in maintaining and in sustaining high levels of SO at the sites of action is nitration of endogenous manganese superoxide dismutase (MnSOD), the enzyme that normally keeps SO under

tight control [12]. Nitration and subsequent deactivation of MnSOD are carried out by PN [13–16], a product from the reaction of SO with nitric oxide (NO) [17]. NMDAR activation favors the accumulation of PN by forming SO [2, 11, 18–20] and NO simultaneously [21–23]. Moreover, Muscoli and coworkers demonstrated that SO-mediated nitration and deactivation of spinal MnSOD are a novel pathway of NMDA-mediated spinal hyperalgesia and hence of central sensitization since it helps to maintain high levels of SO that in turn maintains the nociceptive signaling [2, 11]. The goals of this study were to elucidate how elevated levels of SO maintain nociceptive signaling in response to NMDA. To this end, we focused on the potential role of nitration of key proteins involved in glutamate transmission, namely, NMDAR, glutamate transporter, and glutamine synthase (GS). cDNA cloning has revealed that the NMDAR is formed by several NMDAR subunits. The coexpression of GluN1 with various GluN2 subunits is required for a fully functional ion channel receptor and the combined expression of GluN1 with different GluN2 subunits results in a channel with distinct pharmacological and physiological properties that define NMDAR heterogeneity [24, 25]. PN interacts with the NMDAR leading to nitration of the tyrosine residues present on the NMDAR subunits. This is an irreversible reaction that leads to a constant potentiation of the synaptic currents and calcium influx and ultimately excitotoxicity [26–28]. It has been demonstrated that nitration of tyrosine residues in proteins is sufficient to enhance the degradation of the modified proteins by the proteasome *in vivo* [29] and could be a critical event also for the turnover of the receptors. Intrathecal administration of NMDA releases glutamate in the synaptic cleft [30–32]. Thus, thermal hyperalgesia, in response to intrathecal injection of NMDA, results from a persistent state of NMDAR activation due to high levels of glutamate in the synaptic cleft [3]. Once released, glutamate is not metabolized by extracellular enzymes but is removed by cellular uptake via glutamate transporters. GLT1, a selective glial cells transporter, possesses an intracellular domain rich in amino-acid residues susceptible to oxidation such as cysteines and tyrosines [33, 34]. PN nitrates the glutamate transporter lowering its capacity to remove glutamate from the synaptic space and leading to neurotoxic concentration of this neurotransmitter [2, 35–37]. Once glutamate is taken up into glial cells, it is converted into nontoxic glutamine by the glia-specific enzyme GS [38, 39]. Excitotoxic stimulation occurring in brain tissues seems to inactivate GS leading to reduced ability of astroglial cells to regulate glutamate turnover via GS activity [40–42]. Inhibition of GS activity increases central sensitization associated with inflammatory hyperalgesia, neuropathic pain, and opioid tolerance [37, 43–45].

The glutamate pathway proteins have key tyrosine residues which can be nitrated by PN: the net result of the posttranslational modifications of proteins involved in the tight regulation of glutamate homeostasis such as NMDAR, GLT-1, and GS provide a unifying link in signaling events underlying the central sensitization. Central sensitization is one form of long-term plasticity in the central nervous system. Sustained activation of primary sensory fibers supplying dorsal horn can induce long-lasting increases in the discharge amplitude of primary afferent synapses [46]. Central sensitization is an excitatory state of spinal cord dorsal horn neurons that transmit nociception due to increased responsiveness to suprathreshold and/or a lowered threshold to nociceptive signals; this manifests behaviorally as hypersensitivity to noxious (hyperalgesia) and nonnoxious (allodynia) stimuli. This state is a result of physiologic, biochemical, and molecular changes within spinal and supraspinal nociceptive modulating centers in the CNS and is partly responsible for chronic pain pathology [47].

The results of our studies demonstrate that NMDA-induced PN production maintains central sensitization and hyperalgesia by modulating glutamate transmission through posttranslational nitration of the NMDAR subunits, GLT1, and GS.

2. Methods

2.1. Animals. Male Sprague-Dawley rats (225–250 g, Charles River) used for these studies were purchased with intrathecally implanted cannulas (32 gauge, polyurethane). For the intrathecal catheters, briefly, the animal's head was flexed forward in the stereotaxic apparatus, an incision was made in the skin at the back of the head and neck, and the cisternal membrane was exposed by sharp dissection. The membrane was gently punctured with the tip of a #15 scalpel blade, and the distal end of a 7.5 cm long PE-10 catheter was passed through the opening in the cisternal membrane, into the intrathecal space. The catheter was loosely sutured to subcutaneous tissue, leaving the proximal end external to the animal and accessible to the experimenter, and the skin was then approximated using 4–0 absorbable sutures (Ethicon). All animals were housed and cared for in accordance with the guidelines of the University of Magna Graecia, Catanzaro, Italy, as well as complied with the Italian regulations for the protection of animals used for experimental and other scientific purposes (D.M. 116192), and with European Economic Community regulations. The rats were maintained in a controlled environment (12 h light/dark cycle, room temperature, 50–60% relative humidity). All experiments took place during the light period between 7:00 am and 10:00 am in a quiet room.

2.2. Measurements of Thermal Hyperalgesia. Hyperalgesic responses to heat were determined as described by the Hargreaves method [48] and a cutoff latency of 20 sec was employed to prevent tissue damage in nonresponsive animals. Animals were allowed to acclimate for 30 minutes within a Plexiglas enclosure on a clear glass plate in a quiet testing room. A mobile unit consisting of a high intensity projector bulb was positioned to deliver a thermal stimulus directly to an individual hind paw from beneath the chamber. The withdrawal latency period of the right and left paw was determined to the nearest 0.1 sec with an electronic clock circuit and thermocouple. If the animal failed to respond within 20 sec, the test was terminated. Each point represents the change (sec) in withdrawal latency [(withdrawal latency

of right plus withdrawal latency of left paw)/2] at each time point. Results are expressed as paw withdrawal latency (sec). After thermal testing, all the animals were sacrificed and the lumbar spinal cord (block from L4 to L6) was removed, immediately frozen in liquid nitrogen, and was randomly distributed for further analysis.

2.3. NMDA-Induced Hyperalgesia. Six groups were used.

Group 1. FeTM-4-PyP^{5+} Vehicle + NMDA Vehicle: animals ($n = 8$) received an intrathecal injection (10 μL followed by a 10 μL flush) of saline followed by an intrathecal injection of 10 μL saline after 15 minutes which was followed by a 10 μL flush of saline.

Group 2. FeTM-4-PyP^{5+} + NMDA Vehicle (FeTM-4-PyP^{5+} was tested at the highest dose, 2 nmol): animals ($n = 8$) received an intrathecal injection of FeTM-4-PyP^{5+} (2 nmol, 10 μL followed by a 10 μL flush) followed by an intrathecal injection of 10 μL saline after 15 minutes which was followed by a 10 μL flush of saline.

Group 3. FeTM-4-PyP^{5+} Vehicle + NMDA: animals ($n = 8$) received an intrathecal injection (10 μL followed by a 10 μL flush) of saline followed by an intrathecal injection of NMDA (2 nmol in 10 μL, [49]) after 15 minutes which was followed by a 10 μL flush of saline.

Groups 4–6. FeTM-4-PyP^{5+} + NMDA (FeTM-4-PyP^{5+} was tested at 3 doses): animals ($n = 8$) received an intrathecal injection of 0.5, 1, and 2 nmol (10 μL followed by a 10 μL flush, $n = 8$ for each dose) of FeTM-4-PyP^{5+} followed by an intrathecal injection of NMDA (2 nmol in 10 μL) after 15 minutes which was followed by a 10 μL flush of saline.

The thermal stimulus was applied separately to the right and left hind paw and paw withdrawal latencies were assessed immediately before and subsequently at 10, 20, and 40 minutes after NMDA injection. Results are expressed as Paw withdrawal latency (sec); a decrease in paw withdrawal latency relative to baseline is indicative of hyperalgesia. Determination of antinociception was assessed between 7:00 am and 10:00 am (light period). In the behavioural study, one person prepared the drugs and the other, blind to the drugs and dosage, ran the behavioural observation. The blind observer was identical throughout the study.

2.4. Tissue Preparation for Cytosolic Extraction. For cytosolic extraction, tissues were homogenized with lysis buffer with a 1 : 3 w/v ratio. The lysis buffer (20 mM Tris-base, 150 mM NaCl, 10% glycerol, 0.1% Triton-X-100, 1% Chaps, 2 mM EGTA) contained 1% protease inhibitor cocktail (v/v). Solubilized extracts were sonicated (5 min) using a Sonicator (Fisher Scientific) and after 10 min of incubation in ice the lysates were centrifuged (12500 g, 30 min at 4°C). These supernatants were stored immediately at −80°C and were used to evaluate GS expression and activity. Protein concentration was determined using the Bicinchoninic Acid (BCA) protein assay (Pierce). All the experiments have been repeated at least twice for each different animal.

2.5. Synaptosome Preparation. P2 membranes were obtained as described before [50]. Briefly, the lumbar tract of the spinal cord was homogenized in an ice-cold buffer (0.32 M sucrose, 100 μM sodium orthovanadate, 0.02 M glycerophosphate, and 1% protease inhibitor cocktail, Sigma) in a glass homogenizer. The homogenates were centrifuged at 800 g for 10 min at 4°C. The resulting pellets were rehomogenized and centrifuged as before. The supernatants were combined and centrifuged at 12500 g at 4°C for 30 min to obtain the P2 pellet. This pellet was resuspended in homogenization buffer and protein concentrations were determined using BCA protein assay (Pierce). Samples were stored at −80°C and were used to determine NMDAR subunits and GLT1 expression following western blotting protocol as described below. All the experiments have been repeated at least twice for each different animal.

2.6. Immunoprecipitation and Western Blot Analyses. Cytosolic fractions and P2 membranes obtained as previously described were used for immunoprecipitation and Western blot analyses. For immunoprecipitation 300 μg of the solubilized proteins were incubated with 10 μg of agarose-conjugated anti-nitrotyrosine antibody (Upstate Biotechnology) overnight at 4°C. Agarose beads were collected by centrifugation (1 min at 12000 ×g at 4°C) and washed in PBS (pH 7.4) three times. The mixture of the beads-antibody and binding proteins were resuspended in 50 μL of sample buffer [2x, 0.5 M Tris·HCl, (pH 6.8) 2.5% glycerol/0.5% SDS/200 mM 2-mercaptoethanol/0.001% bromophenol blue] and heated at 95°C (5 min). To determine whether GS, GLT-1, and NMDAR subunits were nitrated, western blot of immunoprecipitated protein complex and total lysates were made using antibodies specific to these proteins. In brief, the samples were loaded in 10% SDS-PAGE minigels for GS detection and in 7.5% SDS-PAGE minigels for NMDAR and GLT1 detection (Bio-Rad).

After separating by SDS/PAGE, proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad). Ponceau red (Sigma) staining was used to ensure successful protein transfer. Membranes were blocked (1 hr, room temperature) with 1% Bovine Serum Albumin (BSA)/0.1% Thimerosal in 50 mM Tris·HCl, (pH 7.4)/150 mM NaCl/0.01% Tween 20 (TBS/T). Membranes were incubated with mouse monoclonal anti-GS (O/N, 4°C, 1 : 1000 dilution; Transduction Laboratories), mouse monoclonal GluN1 anti-body and rabbit polyclonal GluN2B (O/N, 4°C, 1 : 1000 dilution; Upstate Biotechnology), and rabbit polyclonal GLT1 (O/N, 4°C, 1 : 1000 dilution; US Biological). After washing with TBS/T, the membranes were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1 : 15000 dilution or 1 : 10000 resp.; Amersham) and the specific complex was detected by an enhanced chemiluminescence detection system (ECL, Amersham). Quantitation of nitration levels was then performed by

densitometry using ImageQuant 5.2 software by Molecular Dynamics (Molecular Dynamics). Equal protein loading was determined using β-actin expression as housekeeping gene. SDS/PAGE was performed using 40 μg of solubilized protein and subsequent transfer to nitrocellulose membrane (Bio-Rad). Membranes were blocked (1 h, room temperature) with blocking solution and then incubated with mouse monoclonal anti-β actin (2 h, room temperature, 1 : 5000 dilution; Sigma). After washing with TBS/T, the membranes were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1 : 15000 dilution; Amersham) and the specific complex was detected by an enhanced chemiluminescence detection system. No difference for β-actin was detected among the lanes. All the densitometry units have been normalized against actin for each lane and are expressed as the ratio of nitrated to unnitrated proteins.

2.7. Glutamine Synthase Activity. GS activity was determined using a Glutamine/Glutamate Determination Kit (Sigma) following the manufacturer's protocol. In brief, samples (25 μL) in a final volume of 200 μL were incubated with Acetate Buffer and Glutaminase for 1 hour at 37°C followed by incubation with Tris-EDTA-hydrazine buffer, NAD solution, ADP solution, and Glutamic Dehydrogenase for 40 minutes at room temperature. To evaluate the conversion of NAD^+ to NADH an absorbance of 340 nm was imposed. All the experiments have been repeated at least twice for each different animal.

2.8. Statistical Analysis. Results are given as mean ± SEM. Statistical analysis was performed using ANOVA followed by Student-Newman-Keuls. $P < 0.05$ was considered statistically significant.

3. Results

3.1. FeTM-4-PyP^{5+} Inhibits NMDA Mediated Thermal Hyperalgesia. Intrathecal injection of NMDA in rats (2 nmol; [49]) produces a time-dependent development of thermal hyperalgesia (Figure 1). Pretreatment of rats with the PN decomposition catalyst FeTM-4-PyP^{5+} (0.5–2 nmol, given intrathecally 15 minutes before NMDA) reduced the NMDA-evoked thermal hyperalgesia in a dose-dependent fashion (Figure 1). These results confirm our previous observations [11] and emphasize the fact that free radicals are important mediators of hyperalgesia induced by glutamate receptor activation.

3.2. Intrathecal NMDA Induces Spinal GluN1 and GluN2B Tyrosine Nitration. Nitration of the tyrosine residues on the GluN1 (Table 1) and GluN2B (Table 1) subunits of the NMDAR occurred following thermal hyperalgesia that was induced by intrathecal injection of NMDA (2 nmol) as assessed by immunoprecipitation and western blot analysis (Figures 2 and 3). This effect was significantly reduced by pretreatment of the rats with FeTM-4-PyP^{5+} (2 nmol, given intrathecally 15 min before NMDA) (Table 1).

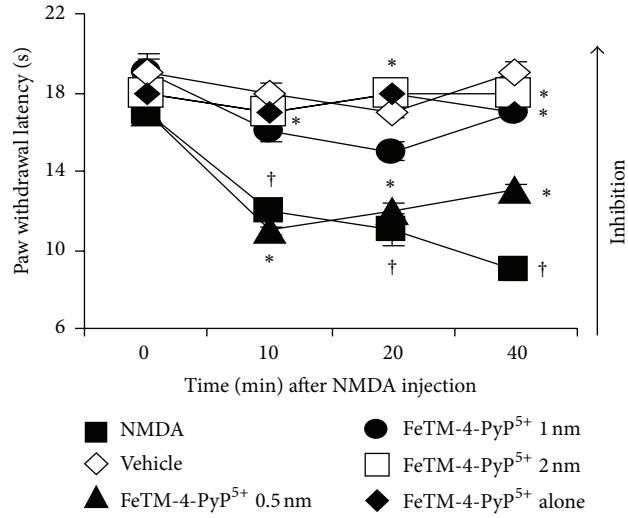

Figure 1: Intrathecal injection of NMDA (2 nmol, ■) causes thermal hyperalgesia when compared to vehicle ◊, and this response is blocked by FeTM-4-PyP^{5+} in a dose-dependent manner (0.5 nmol (▲), 1 nmol (•), and 2 nmol (□), given intrathecally 15 min before NMDA). Intrathecal injection of FeTM-4-PyP^{5+} alone (⧫, 2 nmol) did not exert any effect. Results are expressed as mean ± SEM for 8 rats; $^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

3.3. Superoxide-Mediated Nitration of Glutamate Transporter GLT-1 Is Reversed by FeTM-4-PyP^{5+}. Intrathecal NMDA injection (2 nmol) leads to nitration of the glutamate transporter GLT1 observed by immunoprecipitation assay in the lumbar tract of the spinal cord (Table 1, Figure 4). Pretreatment of the rats with FeTM-4-PyP^{5+} (given intrathecally 15 min before NMDA) prevents GLT1 nitration (Table 1, Figure 4) together with the thermal hyperalgesia (Figure 1).

3.4. Intrathecal NMDA Induces Nitration of Glutamine Synthase in Lumbar Tract of the Spinal Cord. In addition to NMDAR subunits and GLT1, the intrathecal NMDA injection (2 nmol) also induces nitration of the tyrosine residues of GS. This enzyme is found almost exclusively in astrocytes and normally converts the synaptically released glutamate into nontoxic glutamine. Tyrosine-nitrated proteins were immunoprecipitated and analyzed by western blot for the presence of nitrated GS. NMDA (2 nmol, given intrathecally) induces nitration of spinal GS (Table 1, Figure 5), and its inactivation was shown by a significant reduction of glutamine formation (Figure 6). FeTM-4-PyP^{5+} (2 nmol, given intrathecally 15 min before NMDA) blocked PN-mediated nitration (Table 1) and restored its enzymatic activity (Figure 6).

These data suggest that PN formation induced upon NMDAR activation leads to posttranslational modification of important proteins involved in the glutamate turnover contributing to the nociceptive pathway.

Table 1: Densitometry data expressed in %.

	Treatment	Nitrated protein	Total lysate	β-actin
GluN1	Naive	17.00 ± 3.58	31.56 ± 7.8	32.26 ± 7.3
	NMDA (2 nmol, i.t.)	60.00 ± 2.94†	35.44 ± 5.5	33.53 ± 7.5
	NMDA + FeTMPyP (2 nmol, i.t.)	23.48 ± 1.43*	33.00 ± 3.5	34.21 ± 6.2
GluN2B	Naive	14.67 ± 1.96	34.00 ± 3.5	32.26 ± 7.3
	NMDA (2 nmol, i.t.)	65.58 ± 3.35†	33.73 ± 3.7	33.53 ± 7.5
	NMDA + FeTMPyP (2 nmol, i.t.)	19.75 ± 2.28*	32.27 ± 2.5	34.21 ± 6.2
GLT-1	Naive	14.78 ± 2.34	34.22 ± 3.5	33.98 ± 2.3
	NMDA (2 nmol, i.t.)	70.31 ± 2.35†	34.36 ± 4.5	33.10 ± 5.8
	NMDA + FeTMPyP (2 nmol, i.t.)	14.91 ± 2.18*	31.42 ± 4.8	32.92 ± 6.8
GS	Naive	5.14 ± 1.12	33.10 ± 4.2	32.78 ± 4.4
	NMDA (2 nmol, i.t.)	84.46 ± 2.19†	33.40 ± 3.1	32.67 ± 6.7
	NMDA + FeTMPyP (2 nmol, i.t.)	10.40 ± 0.1*	33.50 ± 2.8	34.55 ± 4.3

$^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

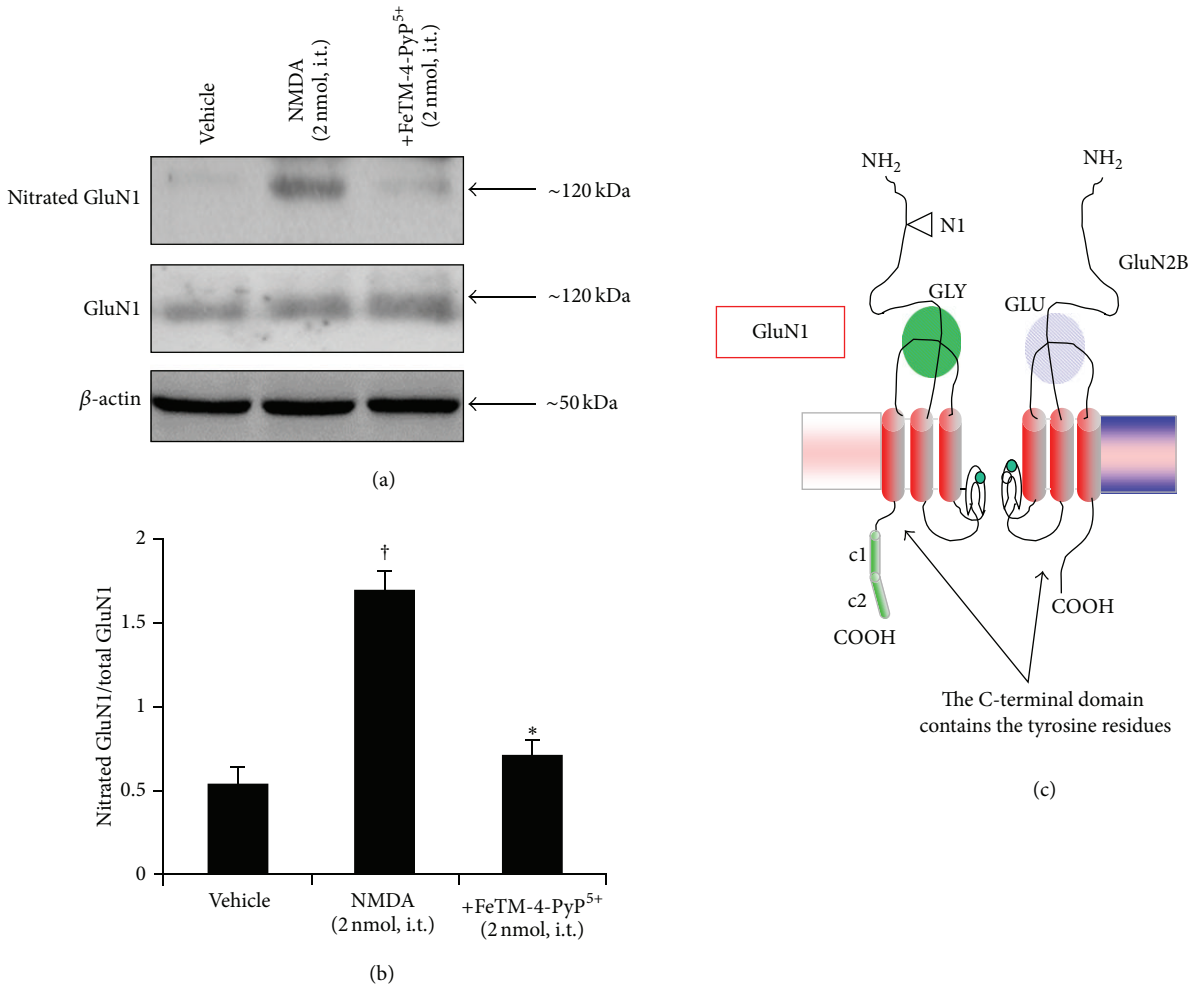

Figure 2: Inhibition of NMDA-induced hyperalgesia by FeTM-4-PyP^{5+} is associated with the inhibition of spinal protein nitration ((a)–(c)). As shown by immunoprecipitation, at the time of maximal NMDA mediated hyperalgesia (40 min), nitration of GluN1 was observed at the level of the spinal cord ((a), (b)). FeTM-4-PyP^{5+} (2 nmol given 15 min before NMDA) attenuates spinal GluN1 nitration ((a), (b)). Immunoprecipitation data shown in (a) are representative of at least 6 gels from 3 different animals performed on different days. Bar graph in (b) represents quantification by densitometric analysis. No difference for GluN1 or β-actin expression was detected among the lanes in these conditions. $^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

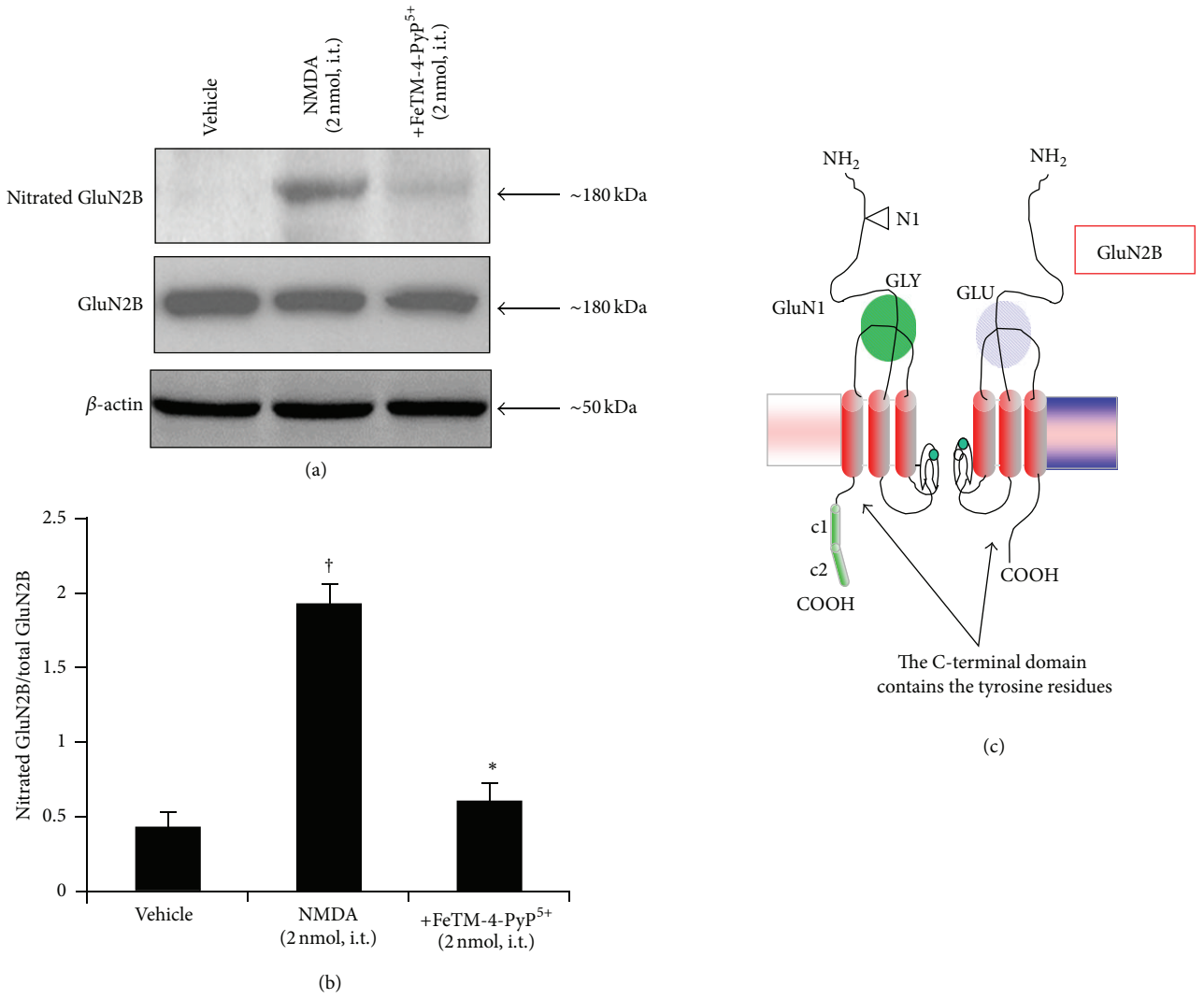

FIGURE 3: Inhibition of NMDA-induced hyperalgesia by FeTM-4-PyP^{5+} is associated with inhibition of spinal protein nitration ((a)–(c)). As shown by immunoprecipitation, the time at which the NMDA mediated hyperalgesia was at its peak (40 minutes), nitration of GluN2B was observed at the level of the spinal cord ((a), (b)). FeTM-4-PyP^{5+} (2 nmol given 15 min before NMDA) attenuates spinal GluN2B nitration ((a), (b)). Immunoprecipitation data shown in (a) are representative of at least 6 gels from 3 different animals performed on different days. Bar graph in (b) represents quantification by densitometric analysis. No difference for GluN2B or β-actin expression was detected among the lanes in these conditions. $^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

4. Discussion

The dorsal horn of the spinal cord is the site where the modulation of incoming pain information takes place through the release of glutamate by the C-fiber nociceptors. Here we have shown that once released, glutamate exerts its action on NMDAR increasing the production of reactive oxygen species such as SO, NO, and in turn PN which leads to nitration of tyrosine residues of key elements in the glutamate transmission. After intrathecal NMDA administration, nitration of NMDAR subunits, glutamate transporter GLT1, and GS synthase was observed in the spinal cord and these events were associated with enhanced hyperalgesic response to heat.

The spinal cord neurons express three subtypes of glutamate receptors: the NMDA and the kainate (KA)/the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), which are both ligand-gated ion channels and the metabotropic receptors (mGluRs) [51]. On the other hand, the NMDAR activation is highly regulated and requires several conditions to occur. Channel opening needs the presence of depolarization, induced by the early activation of AMPA receptors by primary afferent fibres, in order to remove the magnesium physiological blockage that plugs the channel in a voltage-dependent manner [52] and the simultaneous activation by two agonists (glutamate and glycine) [51, 53]. The enhanced sensitivity of the postsynaptic cells evoked by glutamate, an event known as central sensitization, occurs by either the removal of the magnesium block in the NMDAR ion channel or via a posttranslational changes mediated by the phosphorylation of the receptor

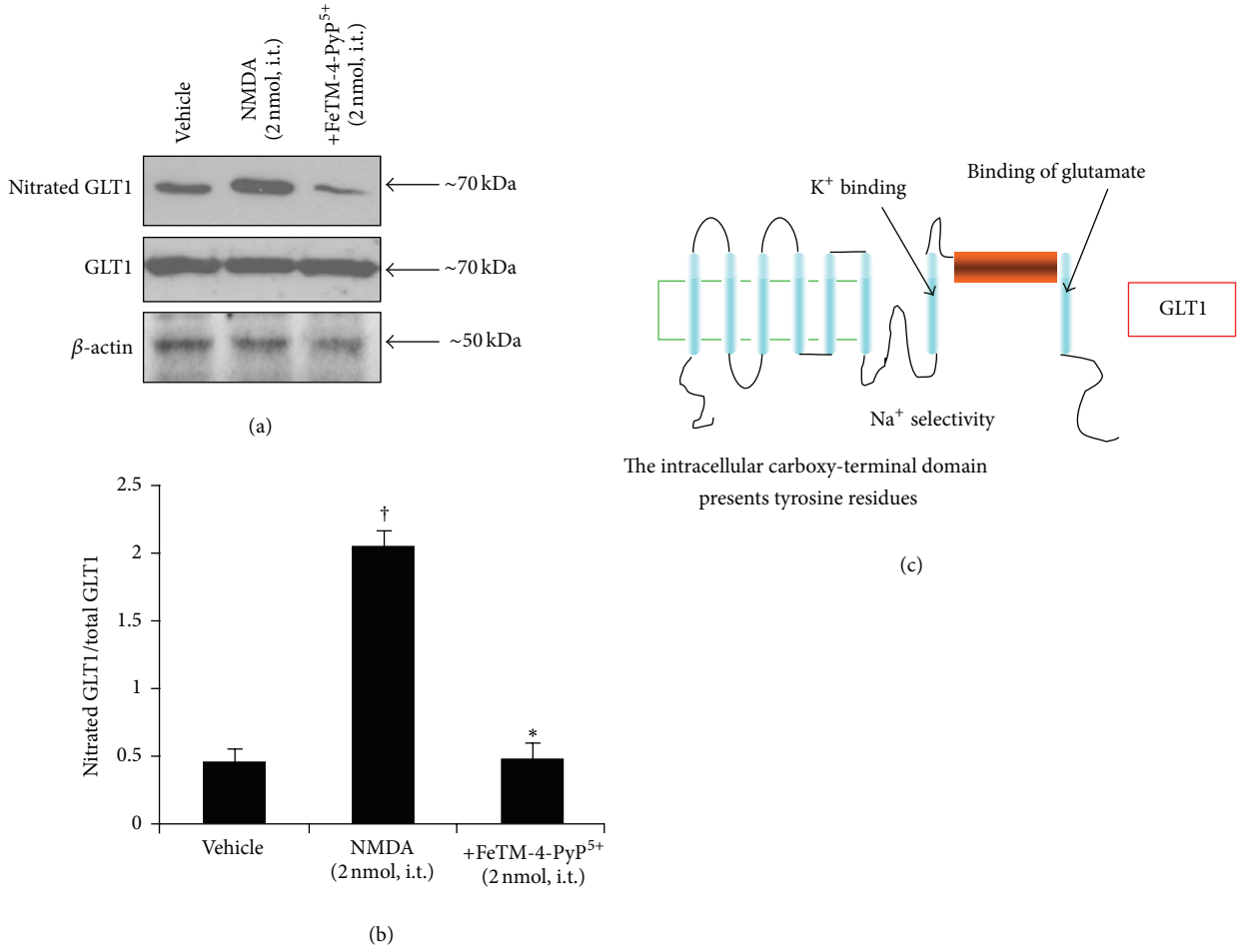

Figure 4: NMDA (2 nmol) induced thermal hyperalgesia was also associated with nitration of the glutamate transporter GLT1 ((a)–(c)). At the time at which hyperalgesia was at its peak (40 minutes), immunoprecipitation analysis revealed that FeTM-4-PyP^{5+} (2 nmol given 15 min before NMDA) reduced the NMDA mediated nitration of GLT1 at the level of the spinal cord ((a), (b)). Immunoprecipitation data are representative of at least 6 gels from 3 different animals performed on different days. Bar graph in (b) represents quantification by densitometric analysis. No difference for GLT-1 or β-actin expression was detected among the lanes in these conditions. $^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

and this appears largely to be the mechanism involved in the maintenance of central sensitization [54]. The balance between phosphorylation/dephosphorylation on the tyrosine residues of the NMDAR subunits is known to regulate the activity of the receptor [55]. Receptor phosphorylation potentiates synaptic currents, calcium influx, and AMPA-receptor mediated responses known to be dependent on NMDAR activation [51]. Phosphorylation of the NMDAR on its tyrosine residues occurs via activation of Src kinase family, which is highly activated by PN [56]. Tyrosine nitration may keep the protein from performing the task of the phosphorylated form or it may mimic the structural changes imposed by phosphorylation and therefore imitate the consequences of phosphorylation with the difference that the nitration of the tyrosine residues is an irreversible process and can alter the normal protein's function by enhancing or inhibiting their activity [57]. NMDAR presents potential site for nitration and it has been previously demonstrated that the nitration of the NMDAR subunits observed *in vitro* and *in vivo* in a model of hypoxia leads to an increased glutamate binding to the NMDAR and consequently an increase in calcium influx and synaptic current [43, 58]. We have previously shown that during central sensitization there is an increased production of SO and in turn PN that leads to nitration and deactivation of MnSOD [2, 11, 59]. Deactivation of the endogenous scavenger of SO leads to an increased production of free radicals that, at least in part, contributes to the maintenance of the hyperalgesic state.

Glutamate metabolism takes place only within the glial cells where the presence of specific transporters permits glutamate removal from the synaptic cleft. It is known that the uptake of glutamate by glutamate transporter system is impaired by PN [35, 37]. Most likely, loss in glutamate transporter activity is due to a posttranslational modification since it is neither associated with a decrease of mRNA nor to genomic mutations [60, 61]. PN lowers the capacity of the glutamate transporters to remove glutamate from the synaptic space leading to neurotoxic concentration of

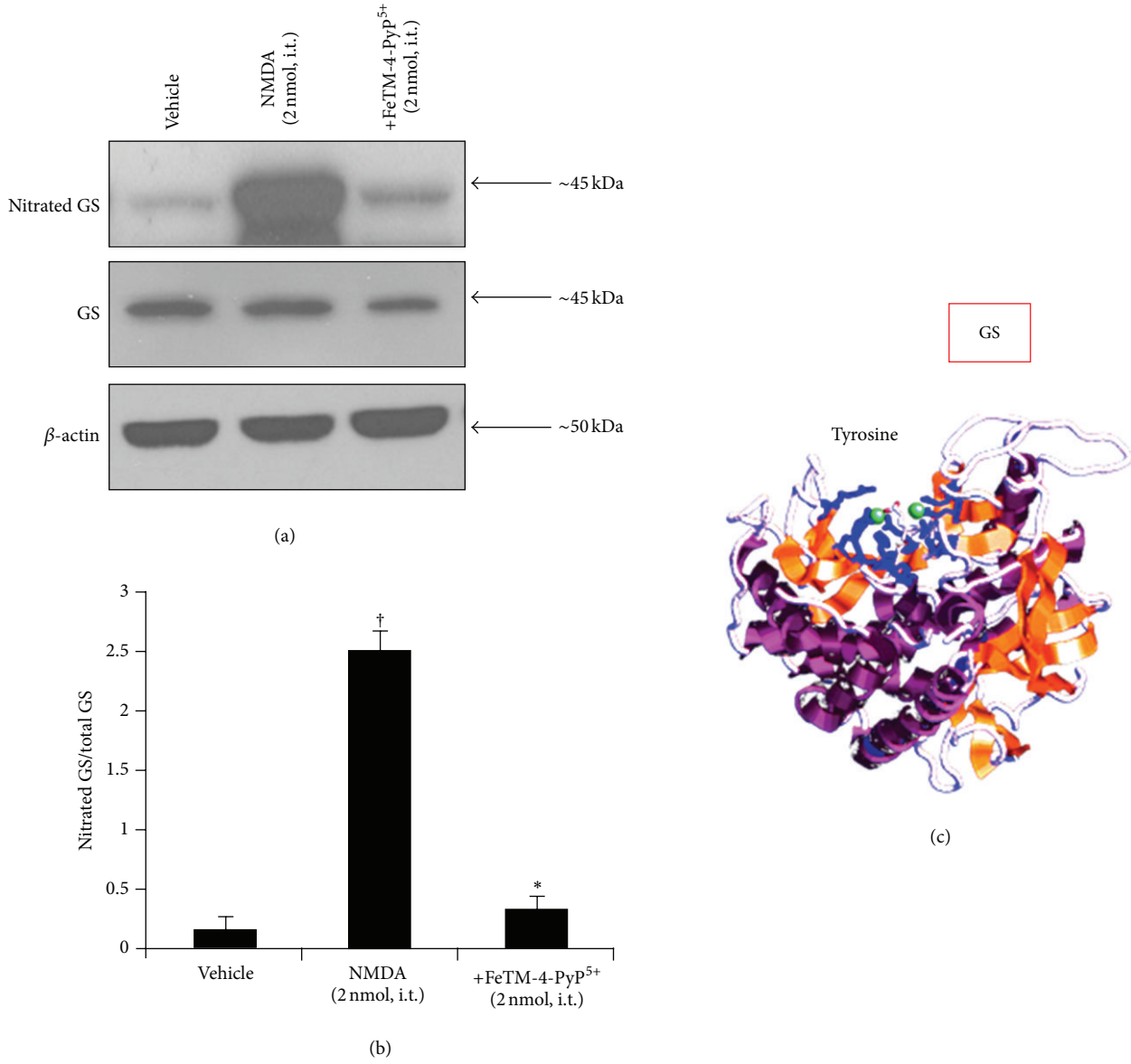

FIGURE 5: Nitration of glutamine synthase occurs following NMDA (2 nmol) induced thermal hyperalgesia ((a)–(c)). The time at which the NMDA mediated hyperalgesia was at its peak (40 minutes), immunoprecipitation analysis revealed that FeTM-4-PyP^{5+} (2 nmol given 15 min before NMDA) reduced the NMDA mediated nitration of GS at the level of the spinal cord ((a)–(b)). Immunoprecipitation data are representative of at least 6 gels from 3 different animals performed on different days. Bar graph in (b) represents quantification by densitometric analysis. No difference for GS or β-actin expression was detected among the lanes in these conditions. $^{\dagger}P < 0.001$ compared to vehicle and $^{*}P < 0.001$ compared to NMDA alone.

this neurotransmitter [35]. Dithiothreitol (DTT), a specific disulphide reducing agent and Mn(III) TBAP, a nonselective antioxidant, restored the transporter activity [62, 63]. Tyrosine nitration is a posttranslational modification that enhances susceptibility to degradation by the proteasome [29]. During NMDA-mediated hyperalgesia, we found that the transporter GLT-1 undergoes SO/PN attack that finally led to nitration of this protein.

Within the glial cells, glutamate catabolism occurs mainly via glutamine formation by GS which is the only enzyme in the CNS that is able to deactivate this excitatory amino acid. GS is inactivated by free radicals attack [64] leading to accumulation of synaptic glutamate and therefore prolonged NMDAR stimulation. Glutamate neurotransmission mediated via NMDAR plays a critical role in the development of central sensitization. Spinal release of glutamate and subsequent NMDAR activation favors PN accumulation by forming $O_2^{\bullet-}$ and NO simultaneously. Moreover, formation of NO, $O_2^{\bullet-}$, and PN in spinal cord contributes to the development of hyperalgesia that results from intrathecal delivery of NMDA [2]. GS activity is regulated by adenylation on the tyrosine residue in each of the 12 identical subunits of the enzyme

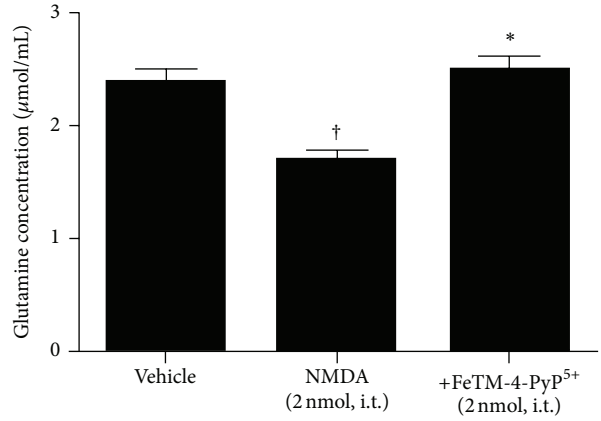

Figure 6: Inhibition of NMDA-induced hyperalgesia by FeTM-4-PyP^{5+} (2 nmol, given 15 min before NMDA) modulates GS activity. The amount of glutamine is highly decreased in animals treated with NMDA while it is restored by FeTM-4-PyP^{5+} treatment. Results are expressed as mean ± SEM for 3 rats; $^{\dagger}P < 0.001$ compared to vehicle; $^{*}P < 0.001$ compared to NMDA alone.

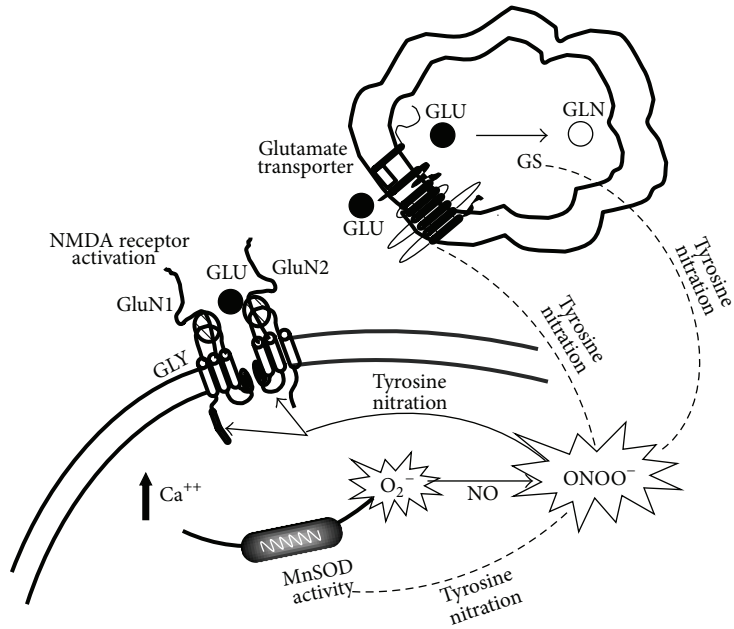

Figure 7: Nitration of tyrosine residues modulates glutamate transmission in the spinal cord. NMDAR activation increases intracellular calcium influx and leads to the production of peroxynitrite which in turn contributes to the hyperalgesic state by nitrating and subsequently activating NMDAR subunits while inhibiting GLT-1 and GS. Removal of PN by antioxidant abolished NMDA-mediated hyperalgesia by preventing tyrosine residues nitration of the glutamate pathway.

[65]. Nitration of the tyrosine residues leads to complete loss of the catalytic activity of the adenylylated enzyme *in vitro* [65, 66] and loss of GS activity was observed during ischemia/reperfusion injury in a gerbil model [67]. During enhanced pain, neuroplastic changes occur in the spinal and supraspinal nociceptive modulating centers and may result in a hypersensitive state termed as central sensitization, which is thought to contribute to chronic pain states [47].

We have previously documented the role of PN in the nociceptive cascade [2, 11, 37, 44, 59, 68]. Here we have demonstrated that PN maintains central sensitization and hyperalgesia by modulating glutamate transmission through posttranslational nitration of the NMDAR subunits, GLT1, and GS. These events are fundamental for the regulation of glutamate turnover and consequently for the modulation of the spinal neurons responsiveness to the inputs that regulate the central sensitization as depicted in Figure 7.

The broader implication of our findings is that PN may contribute to various forms of centrally induced hyperalgesia that are driven by NMDAR activation. This data together with our findings on the identification of free radicals scavengers as novel nonnarcotic agents [11, 37, 59, 69] strongly supports the notion that SO/PN is a viable therapeutic target for the development of nonnarcotic analgesics in pain of various

etiologies. In fact, we observed that spinal administration of NMDA leads to GLT1, GS nitration and imbalance in glutamine production that is associated with development of thermal hyperalgesia.

Conflict of Interests

The authors hereby declare no conflict of interests.

Acknowledgments

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Research Article

Therapeutic Effects of the Superoxide Dismutase Mimetic Compound $Mn^{II}Me_2DO2A$ on Experimental Articular Pain in Rats

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Superoxide anion ($O_2^{\bullet-}$) is overproduced in joint inflammation, rheumatoid arthritis, and osteoarthritis. Increased $O_2^{\bullet-}$ production leads to tissue damage, articular degeneration, and pain. In these conditions, the physiological defense against $O_2^{\bullet-}$, superoxide dismutases (SOD) are decreased. The Mn^{II} complex MnL4 is a potent SOD mimetic, and in this study it was tested in inflammatory and osteoarticular rat pain models. *In vivo* protocols were approved by the animal Ethical Committee of the University of Florence. Pain was measured by paw pressure and hind limb weight bearing alterations tests. MnL4 ($15\,mg\,kg^{-1}$) acutely administered, significantly reduced pain induced by carrageenan, complete Freund's adjuvant (CFA), and sodium monoiodoacetate (MIA). In CFA and MIA protocols, it ameliorated the alteration of postural equilibrium. When administered by osmotic pump in the MIA osteoarthritis, MnL4 reduced pain, articular derangement, plasma TNF alpha levels, and protein carbonylation. The scaffold ring was ineffective. MnL4 (10^{-7} M) prevented the lipid peroxidation of isolated human chondrocytes when $O_2^{\bullet-}$ was produced by RAW 264.7. MnL4 behaves as a potent pain reliever in acute inflammatory and chronic articular pain, being its efficacy related to antioxidant property. Therefore MnL4 appears as a novel protective compound potentially suitable for the treatment of joint diseases.

1. Introduction

Reactive oxygen species (ROS) are by-products of cellular metabolism and can behave as second messengers in physiological conditions. However, in degenerative and inflammatory diseases, ROS production is dramatically increased and can induce cell, tissue, and organ toxicity [1]. In particular, ROS overproduction is a typical hallmark of rheumatoid arthritis [2, 3] and osteoarthritis [4, 5]. In addition, ROS are involved in pain sensation [6–9].

Superoxide anion ($O_2^{\bullet-}$) is one of the most harmful oxidant species identified in the above pathological conditions [2]. The superoxide dismutase enzymes (SOD) can reduce $O_2^{\bullet-}$ toxicity. Three SOD families have been characterized: the cytosolic Cu/Zn-SOD1, the matrix mitochondrial Mn-SOD2, and the extracellular EC-SOD3.

The importance of ROS in joint degeneration is indicated by the finding that EC-SOD3-deficient mice show increased severity of collagen-induced arthritis [10]. Moreover, in inflammatory conditions, $O_2^{\bullet-}$ reacts with nitric oxide to form peroxynitrite which can decrease SOD functionality [9]. In addition, Mn-SOD2 is downregulated in osteoarticular cartilage [11, 12]. EC-SOD3 is also decreased in the cartilage of osteoarthritic patients and in a mouse model of osteoarthritis

[13]. Of note, decreased SOD2 and SOD3 expression precedes the appearance of histological lesions in osteoarticular cartilage [14].

All these data emphasize the concept that, during inflammation and degenerative arthritis, the physiological defences against $O_2^{\bullet-}$ are reduced, suggesting that compounds able to decompose $O_2^{\bullet-}$ may be pharmacological aids for the treatment of articular pain.

We have described the $O_2^{\bullet-}$ scavenging activity of some polyamine-polycarboxylate-Mn^{II} complexes [15]. Among tested compounds, the 4,10-dimethyl-1,4,7,10 tetraazacyclododecane-1,7-diacetic acid Mn^{II} complex ($Mn^{II}Me_2DO2A$, herein indicated as MnL4, Supplementary Material, See Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/905360) is the most potent agent of the series.

In a cellular environment, MnL4 (1 μM–10 nM) dose-dependently reduces $O_2^{\bullet-}$ generated enzymatically (xanthine/xanthine oxidase) or by formyl-methionyl-leucyl-phenylalanine- (fMLP-) activated macrophages. MnL4 (100 nM) can cross cell membranes and significantly reduces oxidative injury in cells exposed to $O_2^{\bullet-}$. Systemically administered to mice (5–15 mg kg^{-1} body weight), MnL4 reduces the acute pain induced by acetic acid (writhing test). Since this anti-inflammatory effect has been observed with both intraperitoneal and oral administration, MnL4 demonstrates a favourable pharmacokinetic profile [15]. Moreover, MnL4 markedly reduces lung inflammation, oxidative, injury, and breathing dysfunction induced by exposure to the airborne allergen in sensitized guinea pigs [16].

Compared with other SOD mimetics, MnL4 would have the advantage of being a smaller, more lipophilic molecule, capable of readily entering cells and decomposing $O_2^{\bullet-}$ at cytoplasmic sites of generation [17]. The present study was therefore designed to test the action of MnL4 in rodent models currently used to reproduce acute inflammation, rheumatoid arthritis, or osteoarthritis. According to the high potency of this SOD mimetic compound, we used the low dosage 15 mg kg^{-1}.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used for the experiments. Four animals per cage were housed at 23 ± 1°C under a 12 h light/dark cycle; they were fed with standard laboratory diet and tap water *ad libitum* and used at least one week after their arrival. The experimental protocol complied with the European Community guidelines for animal care (DL 116/92, the European Communities Council Directive of 24 November 1986: 86/609/EEC) and was approved by the animal subject reviews' board of the University of Florence. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; University of Florence assurance number: A5278-01). Animals were anesthetized with 2% isoflurane before the surgical procedures and sacrifice, which was performed by cervical dislocation. All efforts were made to minimize suffering and reduce the number of animals used. Rats were randomly assigned to each experimental group and individually habituated to handling before testing.

2.2. Drug Administration. MnL4, H_2L4, diclofenac, ibuprofen, and gabapentin were dissolved in sterile saline solution. In a first experimental set, the abovementioned compounds were administered acutely by intraperitoneal (i.p.) injections at the indicated doses. MnL4 and H_2L4 dosages were chosen on the base of previous experiments [15]; typical doses were chosen for diclofenac, ibuprofen, and gabapentin [18–20].

In a second experimental set, MnL4 was given by continuous subcutaneous (s.c.) delivery using an osmotic minipump (Alzet 2002, Palo Alto, CA, USA) implanted on the back and filled to deliver a daily dose of 15 mg kg^{-1} for 14 days.

The organic ligand 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetic acid (H_2L4) was synthesized as previously reported. This compound was isolated as trihydrochloride salt ($H_2L4{\cdot}3HCl$ [15]). Its Mn^{II} complex, MnL4, was obtained by reaction of $MnSO_4$ with $H_2L4{\cdot}3HCl$ (1 : 1 molar ratio) in aqueous solution at neutral pH under nitrogen atmosphere; the MnL4 complex was then isolated as a white solid by precipitation with an ethanol/diethyl ether 2 : 1 mixture, according to a previously described procedure [15]. The complex was further purified by recrystallization with water/ethanol. The purity of both compounds was ascertained by elemental analysis. Diclofenac, ibuprofen, gabapentin, and fMLP were purchased from Sigma-Aldrich, Milan, Italy.

2.3. Carrageenan-Induced Acute Inflammatory Pain. 100 μL of carrageenan solution (Sigma-Aldrich; 1% in saline) was injected intraplantarly into the left hindpaw. Three hours after carrageen injection, MnL4, H_2L4 (15 mg kg^{-1}), diclofenac, ibuprofen (15 and 100 mg kg^{-1}), or saline was i.p. administered and their antihyperalgesic effect was measured along the following 45 minutes (at time 15, 30, and 45 minutes) by the paw pressure test. Control rats received 100 μL of saline solution intraplantarly and saline i.p.

2.4. Freund's Adjuvant-Induced Inflammatory Arthritis. Articular damage was induced by injection of complete Freund's adjuvant (CFA, Sigma-Aldrich) into the tibiotarsal joint [21]. Briefly, the rats were slightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol, and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. A volume of 50 μL of CFA was then injected (day 0). The paw pressure and the incapacitance tests (see below) were performed 7 days after CFA administration. MnL4 (5 and 15 mg kg^{-1}), H_2Me_2DO2A (15 mg kg^{-1}), ibuprofen (15 and 100 mg kg^{-1}), diclofenac (15 and 100 mg kg^{-1}), or saline was i.p.

administered. Control rats received 50 μL of saline solution (day 0) in the tibiotarsal joint and saline i.p. at day 7.

2.5. Monoiodoacetate-Induced Osteoarthritis. Unilateral osteoarthritis was also induced by injection of monoiodoacetate (MIA, Sigma-Aldrich) into the tibiotarsal joint. On day 0, rats were slightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol, and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. 2 mg MIA in 25 μL saline was delivered into the left articular cavity. The paw pressure and the incapacitance tests (see below) were performed at day 14. MnL4 (15 mg kg^{-1}), H_2Me_2DO2A (15 mg kg^{-1}), gabapentin (70 mg kg^{-1}), or saline was i.p. administered. Control rats received 25 μL of saline solution (day 0) in the tibiotarsal joint and saline i.p. at day 14. To evaluate its preventive effect, MnL4 was administered by continuous s.c. infusion, from day 0 to day 14, using the Alzet 2002 osmotic minipump (15 mg kg^{-1} day).

2.6. Paw Pressure Test. The pain threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy) as described [22]. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe. Pressure was increased until a vocalization or a withdrawal reflex occurred. The withdrawal threshold was expressed in grams, the test was repeated twice, and the mean was considered as the value for each paw. Before starting experimental protocols, pain threshold was evaluated and rats scoring below 50 g or over 80 g were discarded. These limits assured a more precise determination of mechanical withdrawal threshold in experiments aimed to determine the effect of treatments. Mechanical pressure application was stopped at 150 g independently of rat reflex. Blind experiments were performed. In the saline + saline, carrageenan + saline, CFA + saline, and MIA + saline treated groups, recorded pressure values did not vary when repetitively measured during the experimental session.

2.7. Incapacitance Test. Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury [23]. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value considered for each animal was the mean of 5 consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating a postural equilibrium, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. Data are expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one. Blind experiments were performed.

2.8. Histopathological and Biochemical Evaluations. Tissues of rats used to study the preventive effect of MnL4 (minipump infused) and their controls were analyzed as follows. (a) Legs were cut under the knee, flayed, and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 48 h. Samples were then rinsed in PBS and placed in decalcifying solution (4N formic acid in distilled water), which was changed every 7 days until bone demineralization was complete (42 days). Samples were dehydrated in graded ethanol, embedded in paraffin wax, cut into 6 μm thick sections, and stained with hematoxylin and eosin. Histological sections taken in the midst of the tibiotarsal joint were viewed and photographed under a light microscope equipped with a digital camera. (b) After sacrifice, blood was collected in heparin-treated tubes and plasma fraction was isolated by centrifugation. Plasmatic TNF-α levels were evaluated by ELISA method (eBioscience, San Diego, CA, USA), using a specific antirat polyclonal antibody. In order to obtain detectable levels of TNF plasma samples were lyophilized and reconstituted in 1/5 of the initial volume. Range sensitivity was 11.2–2.500 pg mL^{-1}. (c) Total plasma proteins was quantified by bicinchoninic acid (BCA; Sigma-Aldrich) assay. Then, 20 μg of each sample was denatured by 6% SDS and derivatized by 15 min incubation with 2–4 dinitrophenyl hydrazine (DNPH; Sigma-Aldrich) at room temperature in order to evaluate carbonylated protein evaluation. Samples were separated on a 10% sodium dodecyl sulphate- (SDS-) polyacrylamide gel by electrophoresis and blotted onto nitrocellulose membranes (BioRad, Milan, Italy). Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and then incubated overnight with anti-DNPH primary antibodies (Sigma-Aldrich; 1 : 5000 in PBST added with 5% non-fat dry milk). After washing with PBST, the membranes were incubated for 1 h in PBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1 : 5000; Cell Signalling, USA) and thoroughly washed [24]. The chemiluminescent substrate ECL (Pierce, USA) was used to visualize the peroxidase-coated bands. Densitometric analysis was performed using the free-share Scion Image 4.03 image analysis software (Scion Corp., Frederick, MD, USA). Ponceau-stained membranes were used as loading control [25].

2.9. Patient's Characteristics and Isolation of Human Chondrocytes. Human chondrocytes used for the experiments were isolated from 3 patients requiring arthroplasty for degenerative disorders of the knee. Slices of articular cartilage were obtained from a peripheral zone of the affected joint, outside regions with macroscopic degeneration but close to the calcified cartilage layer, after administration of an informed consent approved by the Local Ethical Committee.

Human chondrocytes (HCs) were isolated and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco, Invitrogen, Italy), 2 mM L-glutamine, 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin in 5% CO_2 atmosphere at 37°C as described [26].

2.10. Human Chondrocyte Lipid Peroxidation Induced by Stimulated RAW264.7. For the experiments, HCs at the 3rd culture passage were used in 5 separate experiments. They were grown to 90% confluence on 6-well cell culture plates (Corning, Italy) and starved for 18 h in serum-free medium. The mouse leukemic monocyte macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (Rockville, MD, USA). RAW 264.7 were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU mL^{-1} penicillin, and 100 $\mu g\, mL^{-1}$ streptomycin in 5% CO_2 atmosphere at 37°C. 72 h before experiments, cells were detached, plated on the upper layer of polycarbonate transwell dishes with a pore diameter of 3 μm (Corning, Italy), and starved in serum-free medium for the last 18 h. The transwells were then placed into HCs-containing wells, and cells were coincubated together in DMEM without phenol red for 30 min in the absence or presence of 10^{-7} M MnL4. Then, RAW 264.7 were activated with 10^{-7} M fMLP (dissolved in DMEM), while in control samples the same volume of DMEM was added. According to previous data [15], fMLP-activated RAW 264.7 produced a significant and reproducible amount of $O_2^{\bullet -}$. The basal value of HCs lipid peroxidation was obtained in cells not cocultured with RAW 264.7. After 4 h, the reaction was stopped on ice; the upper layer with RAW 264.7 was removed. HCs were scraped in 1 mL of cold PBS, and the cell suspension was used to measure the thiobarbituric acid reactive substances (TBARS), assumed as a marker of cell oxidative injury. Briefly, the suspensions were mixed with 4 mL thiobarbituric acid (36 mM in acetic acid/sodium acetate, adjusted to pH 4 with NaOH) and boiled for 1 h. After cooling on ice, the mixture was centrifuged at 5000 ×g for 10 min and the absorbance of the supernatant was spectrophotometrically evaluated at the 532 nm wavelength against a standard curve of 1,1,3,3-tetramethoxypropane. Protein concentration in the samples was determined using the Coomassie protein assay (Pierce, Rockford, IL, USA). TBARS values were expressed as μmol mg^{-1} of proteins. All reagents used were of the highest purity grade.

2.11. Statistical Analysis. All experiments were evaluated blind. Results were expressed as the means ± s.e.m. Statistical analysis of differences among the experimental groups was performed using one-way ANOVA followed by Student-Newman-Keuls *post hoc* test. A P value ≤ 0.05 was considered significant.

3. Results

3.1. Effects of MnL4 on Carrageenan-Induced Acute Inflammatory Pain. Three hours after the administration of carrageenan, all inflammatory signs were observed (paw swelling hyperaemia and hyperalgesia). The paw pressure test was used to measure pain. In ipsilateral paw (carrageenan + saline), the mechanical withdrawal threshold was significantly decreased as compared to the controlateral paw and control animals (saline + saline, Figure 1) and remained to the same value for at least 1 h. MnL4 (15 mg kg^{-1}) significantly

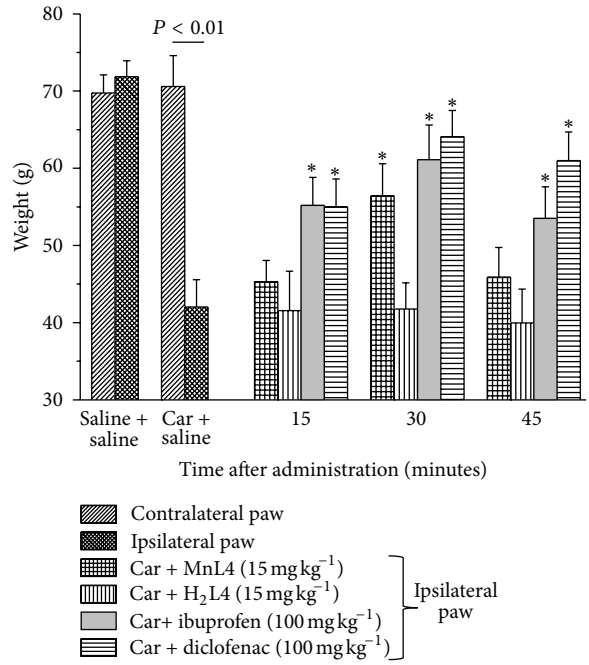


Figure 1: Effect of MnL4, H_2L4, ibuprofen, and diclofenac on carrageenan-induced acute inflammatory pain. Carrageenan (Car, 1%, 100 μL) was injected in the left posterior sole 3 hours before pain evaluation by the paw pressure test. Molecules or saline was administered i.p. at time 0 and measures were performed at time 15, 30, and 45 min. Values are the mean ± s.e.m. of 6 animals. $^*P < 0.05$ versus the ipsilateral paw of carrageenan + saline group.

increased mechanical withdrawal threshold in the ipsilateral (carrageenan + MnL4) paw 30 min after its i.p. administration (Figure 1), but not modify the contralateral one (data not shown). Neither H_2L4 (Figure 1), nor ibuprofen or diclofenac (not shown) at the same dosage of MnL4 were active, whereas ibuprofen and diclofenac at 100 mg kg^{-1} i.p. were effective (Figure 1).

3.2. Effects of MnL4 on CFA-Induced Inflammatory Arthritis. With the aim of testing the pharmacological activity of MnL4 in articular inflammatory damage resembling human rheumatoid arthritis [27], the SOD mimetic compound was evaluated in the CFA-model. The pain threshold was measured 7 days after intra-articular CFA injection by paw pressure and incapacitance tests. The mechanical withdrawal threshold in ipsilateral- (CFA + saline) treated paw was significantly reduced as compared to the controlateral paw and control animals (saline + saline). MnL4 (15 mg kg^{-1}), 15 minutes after i.p. administration, increased the withdrawal threshold and was still effective after 45 minutes while at the dose of 5 mg kg^{-1} was effective 30 min after administration (Figure 2). Ibuprofen and diclofenac at 100 mg kg^{-1} i.p. were also active (Figure 2). H_2L4 (Figure 2), ibuprofen, or diclofenac (not shown) was ineffective at 15 mg kg^{-1}. Moreover, MnL4 significantly reduced hind paw unbalance in a time-dependent manner, being particularly effective 30 min after i.p. injection (Table 1).

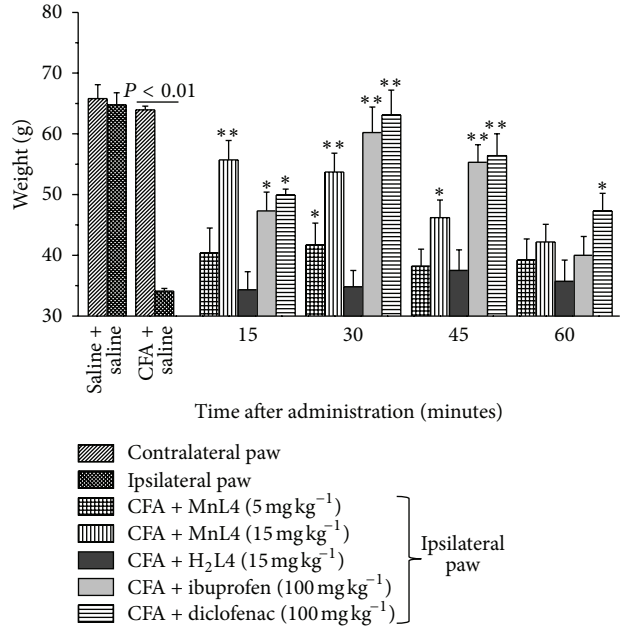


Figure 2: Effect of MnL4, H_2L4, ibuprofen, and diclofenac on complete Freund's adjuvant-induced inflammatory arthritis. Complete Freund's adjuvant (CFA, 50 μL) was injected in the left posterior tibiotarsal articulation 7 days before the pain evaluation by the paw pressure test. Molecules or saline was administered i.p. at the indicated doses at time 0 and measures were performed at time 15, 30, 45, and 60 min. Values are the mean ± s.e.m. of 6 animals. $^*P < 0.05$ and $^{**}P < 0.01$ versus the ipsilateral paw of CFA + saline group.

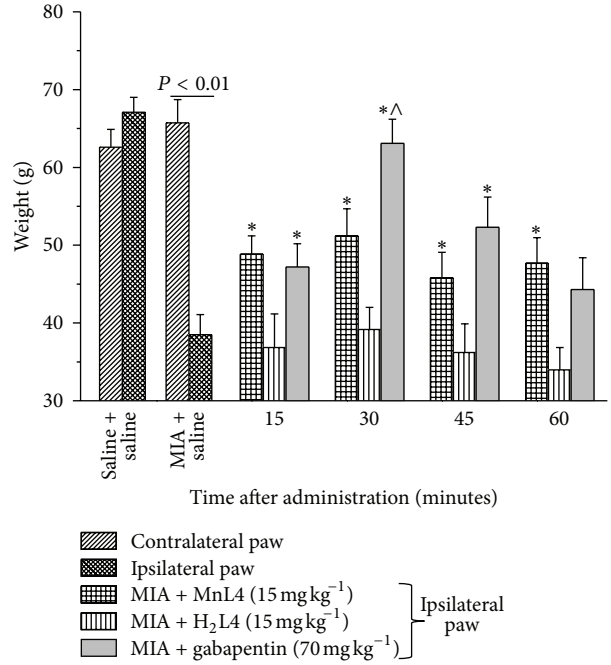


Figure 3: Effect of MnL4, H_2L4, and gabapentin (acutely administered) on monoiodoacetate- (MIA-) induced unilateral osteoarthritis. MIA (2 mg/25 μL) was injected in the left posterior tibiotarsal articulation 14 days before pain evaluation by the paw pressure test. Molecules or saline was administered i.p. at time 0 and measures were performed at time 15, 30, 45, and 60 min. Values are the mean ± s.e.m. of 6 animals. $^*P < 0.05$ versus the ipsilateral paw of MIA + saline group, $^{\wedge}P < 0.05$ versus MIA + MnL4 at the same time.

3.3. Effect of MnL4 on MIA-Induced Osteoarthritis. The effectiveness of MnL4 was evaluated in the rat unilateral osteoarthritis induced by MIA according to two different protocols: acute i.p. administration (15 mg kg^{-1}, 15–60 minutes before the test) or continuous subcutaneous infusion by osmotic minipumps (15 mg kg^{-1} day^{-1} for 14 days). Fourteen days after MIA, the weight tolerated on the ipsilateral paw (MIA + saline) was significantly reduced as compared to the contralateral paw and control animals (saline + saline, Figure 3). MnL4 (15 mg kg^{-1}), 15 minutes after i.p. administration, increased the withdrawal threshold and was still effective after 60 minutes. At the same dosage, H_2L4 was ineffective (Figure 3). Gabapentin (70 mg kg^{-1}) showed a higher effectiveness than MnL4 30 min after administration, but was similarly active at the other times (Figure 3).

Moreover, MnL4 significantly reduced hind limb weight bearing alterations, being particularly effective 30 min after i.p. injection (Table 1).

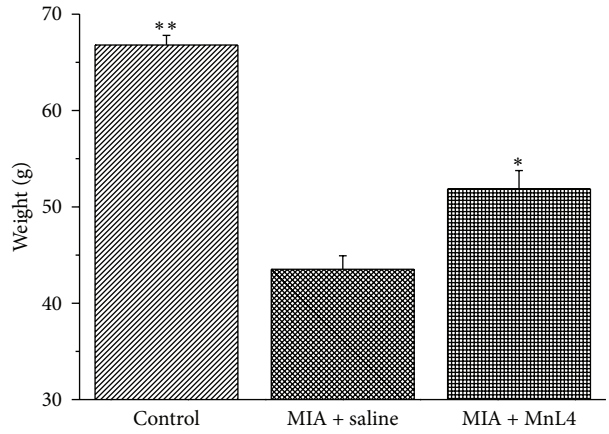
MnL4 (15 mg kg^{-1} day^{-1}) was also effective when continuously administered by s.c. route for 14 days (Figure 4(a)). This functional effect was accompanied by a substantial improvement of joint histopathology. Figure 4(b) shows representative pictures of hematoxylin-eosin-stained longitudinal sections of tibiotarsal joints in the different experimental conditions: 14 days after injection, MIA caused intra-articular fibrin accumulation and extensive degeneration of the articular cartilage, that is, overall thinning, ulceration, and scarring. These changes resulted in a marked reduction of the intra-articular space compared to the normal joint (contralateral, control). Continuous s.c. administration of MnL4 prevented the appearance of these cartilage abnormalities and improved the intra-articular space. This beneficial effect of long-term MnL4 treatment was confirmed by the dosage of TNF-α plasma levels. As reported in Table 2, TNF-α was significantly increased in MIA + saline-treated rats at day 14 compared to naïve animals; MnL4 completely prevented MIA-induced TNF-α elevation. Moreover, in MIA + saline-treated rats, systemic oxidative damage was also present, as evaluated by the carbonylation of plasma proteins. In fact, on the 14th day, plasma-carbonylated proteins increased up to twice the basal level of naive animals (5.9 ± 0.29; densitometric arbitrary units). MnL4 (15 mg kg^{-1} day^{-1}) significantly reduced this oxidation parameter (Figure 5).

3.4. Effect of MnL4 on Lipid Peroxidation in Human-Cultured Chondrocytes. In order to study the effect of MnL4 on an ROS attack in HCs, we performed experiments in a coculture system of HCs and mouse leukaemic monocyte macrophage cells (RAW 264.7). This experimental set allowed us to study the effect of RAW 264.7-produced $O_2^{\bullet -}$ on HCs lipid peroxidation. In basal conditions (without coculture with RAW

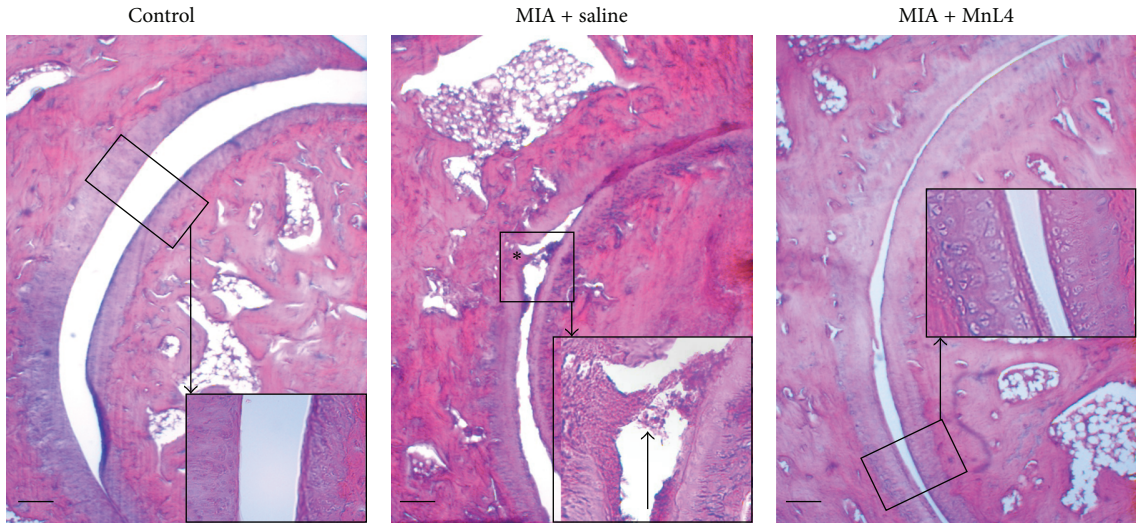
Table 1: Effect of MnL4 on hind limb weight bearing alterations induced by CFA or MIA.

Treatment	Δ Weight (g) (contralateral minus ipsilateral paw)				
	0 min	15 min	30 min	45 min	60 min
Control (saline + saline)	5.2 ± 2.3	3.1 ± 1.9	3.8 ± 2.5	4.2 ± 3.0	6.3 ± 3.1
CFA + 15 mg kg^{-1} MnL4	58.8 ± 1.6$^{\wedge\wedge}$	37.9 ± 2.1*	28.7 ± 1.9**	34.8 ± 2.0*	40.6 ± 2.3*
Control (saline + saline)	3.2 ± 1.2	−2.3 ± 2.1	1.5 ± 3.1	−3.0 ± 2.8	3.8 ± 2.5
MIA + 15 mg kg^{-1} MnL4	61.3 ± 2.3$^{\wedge\wedge}$	30.2 ± 3.1*	20.1 ± 3.6**	40.3 ± 1.1*	55.3 ± 2.9

Hind limb weight bearing alterations were evaluated in rats by incapacitance test. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. CFA was injected 7 days before the test, MnL4 was acutely i.p. administered at time 0 min; MIA was injected 14 days before the test, MnL4 was acutely i.p. administered at time 0 min.
$^{\wedge\wedge}P < 0.01$ in respect to control (saline + saline) group; $^{**}P < 0.01$ and $^{*}P < 0.05$ with respect to the 0 min value of the same treatment.



(a)



(b)

Figure 4: Effect of MnL4 (continuously infused for 15 days) on monoiodoacetate- (MIA-) induced unilateral osteoarthritis. MIA (2 mg/25 μL) was injected in the left posterior tibiotarsal articulation 14 days before the pain evaluation by the paw pressure test. At day 0, a minipump containing MnL4 solution (15 mg kg^{-1} day) was implanted on the back of MnL4-treated rats. Values are the mean ± s.e.m. of 5 animals. Panel (a): pain behavior; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus MIA + saline. Panel (b): effect of MnL4 (continuously infused for 15 days) on tibiotarsal articulation histopathology on MIA-induced osteoarthritis. Hematoxilin and eosin staining of longitudinal section of tibiotarsal joint. Pictures are representative of histological preparations from 5 animals per group. Bars = 100 μm.

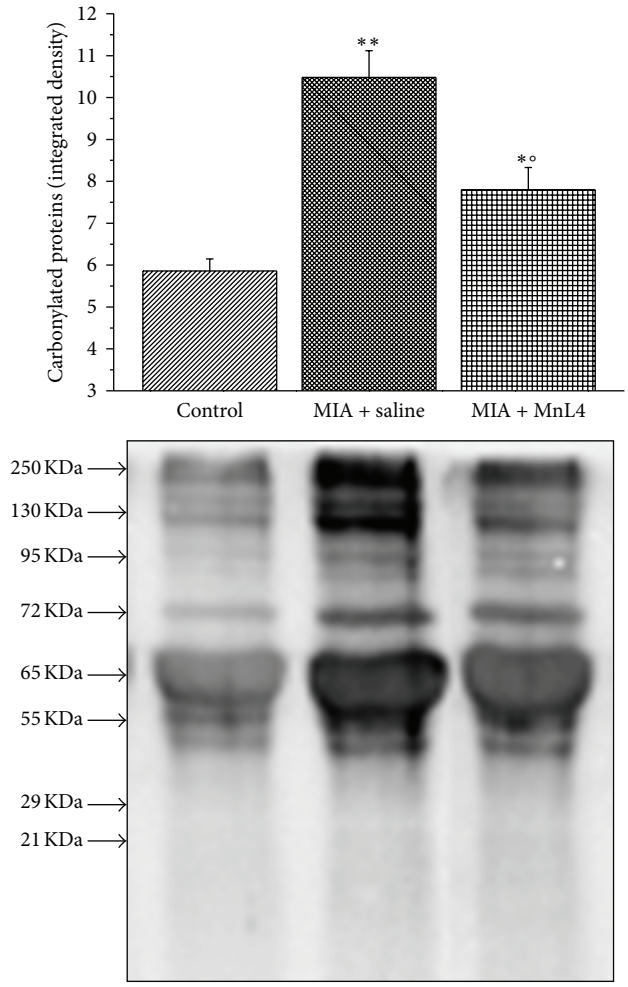


Figure 5: Effect of MnL4 (15 mg kg^{-1} continuously infused for 15 days) on plasma oxidation levels 14 days after MIA injection (2 mg/25 μL in the left posterior tibiotarsal articulation on day 0). Immunoblot analysis was performed after a reaction with dinitrophenylhydrazine. Densitometric analysis and representative Western blot are shown. Ponceau-stained membranes were used as loading control. Each value represents the mean of 4 biological samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control rats; $^{\circ}P < 0.05$ versus MIA + saline treatment.

Table 2: TNFα plasma levels in control, MIA- and MnL4 + MIA-treated rats.

	Control (saline + saline)	Saline + MIA	MnL4 + MIA
pg/mL	5.37 ± 1.77	16.86 ± 2.02*	4.75 ± 1.69

Monoiodoacetate (MIA, 2 mg/25 μL) or saline was injected in the left posterior tibiotarsal articulation 14 days before the test. At day 0, a minipump containing MnL4 solution (15 mg kg^{-1} day) was implanted on the back of MnL4 + MIA-treated rats. TNFα levels were measured in plasma samples by ELISA.

$^{*}P < 0.01$ versus saline + saline and the MnL4 + MIA groups.

264.7), membrane lipid peroxidation of HCs (expressed as TBARS) was 1.24±0.14 μmol mg^{-1} of proteins. This value was not significantly modified when HCs were incubated with unstimulated RAW 264.7 (1.65 ± 0.18 μmol mg^{-1} of proteins, control) but was markedly and significantly increased up to 3.0 ± 0.54 μmol mg^{-1} of proteins when RAW 264.7 were stimulated with 10^{-7} M fMLP (Figure 6). When cells were preincubated with 10^{-7} M MnL4, lipid peroxidation was totally prevented.

4. Discussion

Inflammatory conditions (and in particular, joint diseases) induce an increase in ROS which have a deleterious role in erosion, osteoarticular degeneration, and pain. Conversely, ROS increase inflammatory mediators [28]. ROS are also implicated in persistent pain behavior as already demonstrated by several authors [25, 29, 30]. Therefore, molecules able to reduce $O_2^{\bullet -}$ can be used to reduce pain and inflammation.

Following this line of reasoning, extractive or recombinant SOD seems to be the most valid choice for such a targeted therapeutic approach [31]. However, its clinical use is hampered by multiple factors, including instability, limited cellular accessibility, immunogenicity, short half-life, and high production costs [32, 33]. Because of these limitations,

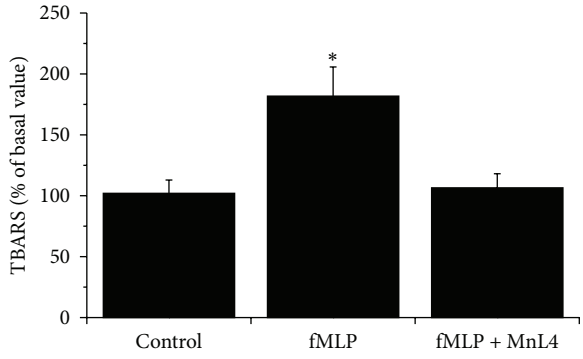


FIGURE 6: Effect of 10^{-7} M MnL4 on the lipid peroxidation (TBARS) induced by superoxide anion in human chondrocytes. Human chondrocytes were coincubated with RAW 264.7 with or without 10^{-7} M MnL4. RAW 264.7 were activated by 10^{-7} M fMLP for 4 h to produce superoxide anions. Values are expressed as percentage of control values (human chondrocytes coincubated with unstimulated RAW 264.7). TBARS in control value was $1.65 \pm 0.18\ \mu$ moles mg^{-1} of total proteins. Each value represents the mean of 5 experiments, performed using chondrocytes isolated from 3 patients. $^{*}P < 0.05$ versus all other conditions.

SOD mimetic compounds have been proposed as appropriate strategies in many degenerative pathological conditions [32], and pharmacological research has highlighted low molecular weight compounds, such as the antioxidant Tempol [34] and the Mn^{II} chelates with organic scaffolds [17, 35], capable of catalyzing $O_2^{\bullet -}$ decomposition like authentic SOD. Tempol and Mn^{II} complexes with pentaazamacrocycles, salen-, and porphyrin-based scaffolds have been reported to reduce inflammation and pain in different animal models of articular diseases [9, 34, 36, 37]. The SOD mimetic compound MnL4 has already been characterized as a membrane-permeable, highly effective scavenger compound [15], possessing anti-inflammatory properties in a model of allergic asthma [16]. Therefore, we studied it using a panel of *in vivo* rat models of articular pain induced by acute and chronic inflammation.

Intra-articular injection of MIA provides a rodent model of monolateral osteoarthritis with features resembling those seen clinically. These include synovial thickening, loss of cartilage, formation of osteophytes, and eventual fibrillation of cartilage [18, 38, 39]. Morphological alterations are associated with a persistent inflammatory pain which, starting from the 14th day after MIA injection, possesses a neuropathic component [20]. Nonsteroidal anti-inflammatory drugs such as diclofenac can reduce MIA-dependent pain during the first inflammatory phase, but they are ineffective in the degenerative neuropathic phase [40], while gabapentin, an antiepileptic molecule widely used to treat neuropathic pain in adult patients [41], is effective [20].

In the MIA model, acutely administered MnL4 ($15\ mg\ kg^{-1}$) causes a prolonged (60 min) reduction of pain sensitivity during the phase when the neuropathic component prevails over the inflammatory one and its efficacy is quite similar to that of gabapentin administered at typical dosage [20]. Its parent compound, H_2L4, which lacks ROS-scavenging effects, is totally ineffective.

Although we injected MIA in tibiotarsal articulation, several characteristics of our model resemble those observed after MIA knee injection. Indeed, after 14 days from MIA injection, the neuropathic component of pain predominates as demonstrated by the high effectiveness of gabapentin. Moreover, the histological analysis confirms a degeneration pattern of the tibiotarsal joint similar to that described for knee [23, 42]. The performed model permits us to directly compare MnL4 as pain reliever in MIA and CFA.

In the same osteoarthritis model, MnL4, continuously infused by an osmotic pump (chronic administration), increases the pain threshold and ameliorates tibiotarsal joint histopathological parameters. Moreover, in blood samples obtained at the same stage of joint degenerative changes, the SOD mimetic compound prevents the significant, TNF-α increase induced by MIA and reduces protein carbonylation. The proinflammatory cytokine, TNF-α, is a critical mediator in osteoarthritis and rheumatic disease. Its serum level is linearly related to disease activity clinical score in patients with rheumatoid arthritis, and it has been proposed as clinical marker of this pathology [43]. TNF-α upregulation is a consequence of NFκB nuclear translocation which can be due to the ROS-activated intracellular signaling cascade [44]. Carbonylation of proteins is an irreversible oxidative damage. Carbonyl groups are introduced into protein side chains by a site-specific mechanism often leading to a loss of protein function. It is considered a widespread indicator of severe oxidative damage and disease-derived protein dysfunction [45]; its increase has been described in the plasma of human subject affected by systemic rheumatic diseases [46].

CFA-induced inflammatory arthritis in rats presents similar features to rheumatoid arthritis [27]. CFA-induced inflammatory arthritis starts between the 3rd and 7th days after inoculation; at this time, the pain threshold is significantly decreased [47], while sensory neuron firing is increased [48] leading to changes in gene expression and sensitization of the nervous system. These functional alterations contribute to the pain associated with joint injuries [49]. Seven days after CFA injection, $15\ mg\ kg^{-1}$ MnL4, acutely administered before the behavioral tests, increases the pain threshold for at least 45 min and prevents the hind limb weight bearing alterations whereas the scaffold congener of MnL4 is totally ineffective.

Since acute inflammation occurs at the initial stage of articular diseases, we tested MnL4 in carrageenan-induced paw acute edema. In this condition, 30 min after administration, MnL4 enhances the pain threshold, decreasing mechanical hypersensitivity by about 50%. However, the effectiveness of MnL4 in this model is short lasting. At the same dosage ($15\ mg\ kg^{-1}$), the well-known anti-inflammatory NSAIDs ibuprofen and diclofenac are ineffective, being their anti-inflammatory activity observed at higher dosages ($100\ mg\ kg^{-1}$) currently used in animal tests [18, 19].

Many of the effects of MnL4 are in agreement with the antioxidant property of the compound [15, 16]: accumulating evidence indicates that the production of ROS is increased in the nociceptive system during persistent inflammatory and neuropathic pain [50]. Since ROS have also been implicated

in chondrocyte degeneration and death [4], we tested MnL4 activity against the oxidative stress induced by $O_2^{\bullet-}$ in isolated human chondrocytes. According to previous data of our laboratory on reproducibility and effectiveness of RAW 264-7 in producing $O_2^{\bullet-}$ after fMLP stimulation [15], we coincubated human chondrocytes with RAW 264-7. This experimental condition simulates an ROS attack on chondrocytes by infiltrating inflammatory cells. MnL4 at 10^{-7} M can totally prevent lipid peroxidation, suggesting an important contribution to joint protection.

In conclusion, MnL4 behaves as a potent pain reliever compound both in arthritis models and, to a lesser extent, in acute inflammation. This effect is not related to a direct inhibition of cyclooxygenase enzymes as already described [15] but, conceivably, related to the SOD mimetic property of the molecule as also demonstrated on HCs. The mechanism by which MnL4 acts after chronic and acute administration may be somewhat different. Namely, chronically administered MnL4 may prevent tissue degenerative alterations induced by the oxidative stress and reduce a persistent inflammatory pain via a direct antioxidant mechanism; while in acute administration, it may decrease the nociceptive nervous fiber activation induced by the local production of ROS [28, 50]. Given these properties and the low toxicity of the molecule, MnL4 is a novel compound potentially suited for the treatment of inflammatory and neuropathic pain.

Disclosure

The authors declare that they have no financial competing interests. Dr. Daniele Bani is recipient of a Grant from General Project for studies on biomedical devices, not related with the current investigation. Financial support for the research was obtained from the Italian Ministry of Scientific Research, University of Florence, and F.I.R.M.O. Fondazione Raffaella Becagli (to Maria Luisa Brandi). Barbara Valtancoli gratefully acknowledges the financial contribution from Ente Cassa di Risparmio di Firenze.

Authors' Contribution

Lorenzo Di Cesare Mannelli carried out the *in vivo* experiments. Daniele Bani and Miriam Cantore performed tissue histology. Andrea Bencini and Barbara Valtancoli prepared and purified HL4 and MnL4. Miriam Cantore and Anna Maria Carossino performed *in vitro* experiments on cells. Maria Luisa Brandi and Carla Ghelardini participated in the design of the study and helped to draft the paper. Paola Failli conceived of the study, planned its design, and drafted the paper. All authors read and approved the final paper.

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Review Article

Neurovascular Unit in Chronic Pain

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Chronic pain is a debilitating condition with major socioeconomic impact, whose neurobiological basis is still not clear. An involvement of the neurovascular unit (NVU) has been recently proposed. In particular, the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB), two NVU key players, may be affected during the development of chronic pain; in particular, transient permeabilization of the barrier is suggested by several inflammatory- and nerve-injury-based pain models, and we argue that the clarification of molecular BBB/BSCB permeabilization events will shed new light in understanding chronic pain mechanisms. Possible biases in experiments supporting this theory and its translational potentials are discussed. Moving beyond an exclusive focus on the role of the endothelium, we propose that our understanding of the mechanisms subserving chronic pain will benefit from the extension of research efforts to the NVU as a whole. In this view, the available evidence on the interaction between analgesic drugs and the NVU is here reviewed. Chronic pain comorbidities, such as neuroinflammatory and neurodegenerative diseases, are also discussed in view of NVU changes, together with innovative pharmacological solutions targeting NVU components in chronic pain treatment.

1. Introduction

According to the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [1].

Chronic pain onset can be sudden or slow and progressive, varies in intensity from mild to severe, and its end cannot be predicted. The diagnosis of chronic pain requires that the condition lasts longer than 3–6 months. Chronic pain can be a debilitating condition with potentially devastating impact on the quality of life [2]. It occurs in a wide variety of conditions, including peripheral neuropathy, stump pain, phantom pain, complex regional pain syndrome, central pain, polymyalgia rheumatica, fibromyalgia, pain of psychological origin, and epilepsy. The recently revised taxonomy includes several new conditions, such as chronic paroxysmal hemicrania: remitting form, hemicrania continua, postlumbar puncture headache, and so forth [1].

According to a report released in June 2011 by the Institute of Medicine of the National Academies, chronic pain affects about 100 million American adults—more than the total affected by heart disease, cancer, and diabetes combined [3]. The 2010 Patient Protection and Affordable Care Act required the Department of Health and Human Services of the United States of America to consider pain as a public health problem.

A 2006 study in 15 European countries and Israel indicates that chronic pain of moderate to severe intensity occurs in 19% of adult Europeans, seriously affecting the quality of their social and working lives [4]. A more recent evaluation

of chronic pain in the European Union reports an even higher impact on the general adult population, with an average prevalence of 27%, similar to the that of other common chronic conditions [5].

Understanding the biological, cognitive, and psychological underpinnings of chronic pain represents a major research challenge. From a neurobiological standpoint, the cellular and molecular communication between the central nervous system (CNS) parenchyma and the circulating mediators of the immune and inflammatory response is at the core of such challenge. Indeed, an increasingly compelling body of evidence highlights a major role for the role of nonneuronal cells and diffusible mediators in the functional state of the brain, including neuronal excitability. The concept is captured in the term "neurovascular unit" (NVU), an ensemble of cellular and noncellular players (neurons, endothelial cells, glial cells, pericytes, the extracellular matrix, immune cells, inflammatory mediators) which form an integrated functional unit [6, 7].

In the context of the NVU, an obviously crucial role is played by the blood-brain barrier (BBB) and of the blood-spinal cord barrier (BSCB), both in general and with respect to the pathophysiology of chronic pain.

The purpose of this review is to explore the role played in the establishment and maintenance of chronic pain by the NVU, emphasizing (but not limited to) BBB and/or BSCB permeabilization phenomena. Chronic pain has a significant prevalence in neurodegenerative and neuroinflammatory pathologies, and BBB/BSCB permeabilization is discussed in this extended context. Finally, novel strategies targeting the NVU are considered for chronic pain relief.

2. BBB and BSCB in the Neurovascular Unit

The importance of a full understanding of BBB/BSCB function is emphasized by its well-known role in regulating paracellular and transcellular drug transport, thus preventing or allowing CNS-acting drugs for chronic pain relief to reach their intended target [8]. In addition, there is a possibility that BBB/BSCB permeability may be altered in association with the development of chronic pain [9–12].

2.1. Anatomical Structure of Blood-Brain Barrier and Blood-Spinal Cord Barrier. The BBB is the regulating interface between circulating blood and brain parenchyma. Endothelial cells of brain capillaries, unlike those of the peripheral circulation, are characterized by the absence of cell membrane fenestrations, the presence of tight junctions, having a high number of cytosolic mitochondria, and minimal pinocytotic activity [7]. As an exception, the so-called circumventricular organs (CVOs) do possess fenestrated vasculature. In particular, secretory CVOs (median eminence and neurohypophysis) present a higher vascular permeability for low-molecular-mass tracers compared to sensory CVOs (organum vasculosum of lamina terminalis, subfornical organ, and area postrema) [13].

The surface area of the BBB, depending on the anatomical region, is between 150 and 200 cm^2/g of tissue, resulting in a total area for blood-brain exchange between 12 and 18 m^2 for the average human adult [14].

A functional equivalent of the BBB is the blood-spinal cord barrier (BSCB), constituted by nonfenestrated endothelial cells, basement membrane, pericytes, and astrocytic feet processes [15]. Several aspects distinguish BSCB from BBB, such as the glycogen deposits in the superficial vessels of the spinal cord [16], increased permeability to tracers and cytokines [17–19], decreased expression of tight-junction proteins and adherens junction proteins [20]. Such differences should be taken into account when these barriers are targeted for chronic pain treatment.

2.2. Mechanisms of Transport through Blood-Brain Barrier. The BBB has low passive permeability to many essential water soluble nutrients and metabolites required by the nervous tissue. However, in healthy conditions, the BBB shows temporary increases in permeability, allowing access to nutrients and oxygen. Since no brain cell is farther than about 15 μm from a capillary [21], drugs and other solutes can rapidly reach all neurons and glial cell bodies, once the BBB has been crossed. Exchange of small organic compounds between blood and brain is regulated by plasma membrane transporters working either in the blood-to-brain direction, the brain-to-blood direction, or both. The directionality of transport is set by the subcellular location of the transport system (blood-facing or brain-facing membrane of the endothelial cells) and by the transport mechanism [8]. Several transport pathways have been identified in the BBB, such as (i) passive diffusion into brain of lipid soluble molecules (e.g., oxygen and carbon dioxide); (ii) ATP-binding cassette transporters (ABC-transporter, see below) efflux (P-glycoprotein (P-gp), multidrug resistance protein (MRP) 1–6, and breast cancer resistance protein transporters (BCRP)); (iii) solute carriers—SLC (transporters of glucose, amino acids, nucleosides, monocarboxylic acids, thyroid hormone, organic anions, organic cations, amine, and choline); (iv) transcytosis of macromolecules by receptor-mediated or adsorptive-mediated mechanism (transport of transferrins, lipoproteins, glycosylated proteins, IgG, insulin, leptin, tumor necrosis factor-alpha (TNF-α), EGF, LIF, cationised albumin, cell penetrating peptides); and (v) mononuclear leukocyte migration [22, 23]. In this review, particular attention will be devoted to ABC transporters with regard to chronic pain and BBB, as the majority of the analgesics are substrates for these transporters, especially for P-gp transporter [24–28].

The endothelial cells of capillary vessels play a major role in BBB physiology. The flattened cells present a luminal and an abluminal surface, separated by a 300–500 nm thick cytoplasm in human brain microvessels [29]. The tight junctions (TJs) connecting adjacent cells represent the most significant BBB structure and serve a dual purpose. On one hand, by sealing the intercellular space, they control the paracellular transport pathway ("gate function"). On the other hand, they effectively subdivide the membrane into two distinct functional domains ("fence function") [30]. The endothelial cell polarization arises in particular from the differential expression of specific transporter proteins on

either surface. The TJ-associated membrane proteins comprise occludin, tricellulin (also called marvelD2), cingulin, claudins (CL-1, CL-3, CL-5), junction-associated molecules of the immunoglobulin superfamily (JAMs), zona occludens proteins (ZO-1, ZO-2, ZO-3), 7H6, and AF-6 [7, 31, 32]. Signaling pathways involved in TJs regulation include G-proteins, serine-, threonine- and tyrosine-kinases, extra- and intracellular calcium levels, cAMP levels, proteases, and cytokines, and all these pathways share the modulation of cytoskeletal elements and the connection of TJs' transmembrane molecules to the cytoskeleton [31].

In pathological states, such as neurodegenerative diseases (including stroke, multiple sclerosis, rheumatoid arthritis, and AIDS dementia) or neuroinflammation, BBB has an uncontrolled and prolonged increase in permeability that results in vasogenic edema and leakage of neurotoxic plasma constituents [33].

2.3. ABC Transporters in BBB and BSCB. ABC transporters represent the largest family of transmembrane proteins. Upon binding ATP, these proteins translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids and sterols, and drugs [34]. Tight junctions and ABC transporters expressed in the brain and spinal cord endothelial capillaries represent the major "guardians" of the transport through BBB/BSCB endothelium. ABC transporters, such as P-gp, MRP 1–6, and BCRP, are expressed in the barriers endothelium both in humans and rodents [27].

It is presumed that only efflux transporters located on the luminal (apical) side of the endothelium can restrict drug uptake into the brain [25]. However, transport balance (influx/efflux) is dramatically affected by pathological stressors, such as *status epilepticus* and neurodegenerative diseases [47–49], and has been suggested to be also partly modified in inflammatory pain [26, 36, 37]. P-gp and BCRP expression in the BBB is regulated during early inflammatory stages by TNF-α and IL-1β [50].

Table 1 summarizes some of the links established so far between ABC transporters in BBB/BSCB and chronic pain.

In conclusion, ABC transporters appear to play an important role in inflammatory pain and in analgesia (opioids or nonsteroidal anti-inflammatory drugs). Knocking out genes encoding ABC transporters has consequences in inflammatory pain or analgesic profile. For example, knocking out the gene encoding for MRP4 increases inflammatory pain threshold [45] and knocking out the gene encoding for MRP3 alters morphine pharmacokinetics [44]. Therefore, these transporters, in particular P-gp, represent key molecules that might contribute to BBB/BSCB permeabilization induced by inflammation-like stimuli in various pain syndromes [26, 36, 37].

3. Cross Talk between NVU Partners in Chronic Pain

In vitro and *in vivo* animal studies have confirmed NVU cellular crosstalk in inflammation-induced hyperalgesia or nerve injury models and results can be extrapolated to chronic pain.

3.1. Glia-Neuron Interactions. Glia are significantly activated in response to trauma, ischemia, and invading pathogens by means of cytokine release (IL-1β, TNF-α) and may contribute to the maintenance of chronic pain [51, 52]. In addition to proinflammatory cytokine release at the peripheral site of injury, release also takes place in the CNS (spinal cord, brainstem, and forebrain) [53–55]. Released cytokines together with activated glia have been proved to influence and modulate neurons in the trigeminal nucleus region in a trigeminal model of inflammatory hyperalgesia [56]. On the other hand, different signaling pathways mediate IL-1β actions in hippocampal neurons compared to astrocytes [57].

Glia activation within the CNS has been suggested to maintain the pain sensation, even after the original injury or inflammation has healed, and convert it into chronic pain by altering neuronal excitability [58]. In a peripheral nerve injury pain model, the inhibition of microglia after four weeks from nerve injury normalized the pain threshold, while removing the inhibitor immediately restored pain-related phenomena [52].

3.2. Microglia-Astrocytes Interactions. Both *in vitro* and *in vivo* data provide clues on how the crosstalk between microglia and astrocytes may play a role in chronic pain maintenance [59–63]. The activation of microglia has been shown to cause astrocytic activation, with a delay of about 4 days [54, 64]. Preventing microglial activation (and subsequent astrocyte activation) inhibits hyperalgesia or allodynia [59, 61]. Once the astrocytes are activated, inhibiting microglia has no effect on pain [59, 60]. On the other hand, brain astrocytes can be activated in response to peripheral nerve injury without prior microglia differentiation [65]. A dialogue between microglia expressing IL-18 and astrocytes expressing its receptor (IL-18R) was suggested to be important in tolerance to morphine analgesia, by means of a P2X7R/IL-18/D-serine/N-methyl-D-aspartate receptor (NMDAR)/PKCγ-mediated signaling pathway [62], but also for tactile allodynia after nerve injury [66].

Increased monocyte chemotactic protein 3 (MCP-3, known as CCL7) expression associated with IL-6-dependent epigenetic modification at the MCP-3 promoter after nerve injury, mostly in spinal astrocytes, may serve to facilitate astrocyte-microglia interaction in the spinal cord and could play a critical role in the neuropathic pain-like state [63].

Some studies suggest the importance of the triad neuron-astrocyte-microglia in physiological and pathological inflammatory states [67].

3.3. Astrocyte-Endothelial Cell Interactions. Astroglial-endothelial signalling is altered under pathological conditions, such as infection, inflammation, stroke, or trauma, leading to BBB opening [6]. The coupling between the abluminal capillary cell membrane and the surrounding glial end-foot processes is reduced in pathological conditions [68, 69]. Stimulation of astrocytes, in coculture with brain endothelial cells,

Table 1: ABC transporters presence in BBB and BSCB and their potential role in pain.

ABC transporter (gene)	BBB	BSCB	Localization in brain capillary endothelium	Direction of efflux/influx	Implications in pain/analgesics or anti-inflammatory drugs versus ABC transporters
P-gp (ABCB1)	Yes [24]	Yes [35]	Luminal [25]	Blood [25]	There is an increased P-gp expression and dynamic redistribution between membrane domains of P-glycoprotein and caveolin-1 in peripheral inflammatory pain [26, 36, 37]. P-gp is involved in pain control with opioid analgesics [38]. Diclofenac is not transported by P-gp [39].
MRP1 (ABCC1)	Yes [24, 25, 40]	Yes [40]	Luminal [25] Abluminal [24]	Blood [25] Brain [24]	The nonsteroidal anti-inflammatory drug indomethacin, an efficient analgesic in some forms of trigeminal autonomic cephalalgias (e.g., paroxysmal hemicrania) [41], was proved to inhibit MRP1 function and expression in cancer cell lines [42]. Most probably indomethachin inhibits MRP1 in BBB. Diclofenac, rofecoxib, and celecoxib are poor inhibitors of MRP1 in HEK293 cells [43].
MRP2 (ABCC2)	Yes [24, 27]	Yes [35]	Luminal [25]	Blood [25]	Diclofenac is not transported by MRP2 [39].
MRP3 (ABCC3)	Yes [24]	?	Abluminal [24]	Brain [24]	Mice lacking MRP3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception [44].
MRP4 (ABCC4)	Yes [24, 27]	?	Luminal [25] Abluminal [24, 25]	Blood [25] Brain [25]	Mice lacking MRP4 show increases in inflammatory pain threshold compared to wild-type mice [45]. MRP4 acts as a prostaglandin efflux transporter and is inhibited by nonsteroidal anti-inflammatory drugs (e.g., indomethacin, indoprofen, ketoprofen, and flurbiprofen) [43]. Diclofenac, rofecoxib, and celecoxib are poor inhibitors of MRP4 [43].
MRP5 (ABCC5)	Yes [24, 27]	?	Luminal [25] Abluminal [24]	Blood [25] Brain [24]	?
MRP6 (ABCC6)	Yes [24, 27]	Possible [46]	Abluminal [24]	Brain [24]	?
BCRP (ABCG2)	Yes [24]	Yes [35]	Luminal [25]	Blood [25]	Diclofenac, an analgesic mainly used against cancer-associated chronic pain, is efficiently transported by murine BCRP1 and moderately by human BCRP [39].

with 5-hydroxytryptamine (5-HT) generated a pronounced increase in intracellular Ca^{2+} release in the presence of inflammatory or pain-mediating activators, such as substance P, calcitonin gene-related peptide (CGRP), lipopolysaccharide (LPS), or leptin [70]. Mu-opioid agonists inhibit the enhanced intracellular Ca^{2+} responses in inflammatory-activated astrocytes cocultured with brain endothelial cells [70]. Overexpression of endothelin-1 in astrocytes, but not in endothelial cells, ameliorates inflammatory pain response after formalin injection [71]. The role played in chronic pain development by *in vivo* endothelial-astrocyte interaction at the barrier has not been investigated yet.

3.4. Pericyte-Endothelial Cell Interactions. *In vivo* studies in wild-type mice have shown that pericytes are more numerous in the brain than in the spinal cord [72]. Whereas brain regions such as the neocortex, hippocampus, and caudate nucleus show almost uniform presence of pericytes, the spinal cord shows significantly nonuniform distributions along the rostrocaudal extent, with the thoracic region being richer in pericytes, but with no more than 70% of brain levels. This reduced number of pericytes in the spinal cord correlates with (i) a higher BSCB permeability, as probed by fluorescent dextran and (ii) a diminished expression of tight junction proteins ZO-1, occluding, and claudin-5. Compared to wild-type mice, in $Pdgfr\beta^{F7/F7}$ pericyte-deficient mice, pericytes are reduced more in spinal cord capillaries, leading to BSCB disruption to serum proteins. ZO-1 and occludin are also reduced, and the accumulation in motor neurons of cytotoxic thrombin and fibrin leads to motor neuron loss [72]. In another pericyte-deficient model, the $Pdgfb^{ret/ret}$ mouse, an increase in BBB permeability to water and to a range of low- as well as high-molecular-mass tracers has been shown [73]. Pericytes express MRP1, MRP4, and MRP5 transporters, which might imply a role played by these cells in regulating xenobiotic transport through the BBB [74].

Abnormal interactions between pericytes and endothelial cells have been implicated in a number of human pathological conditions, including tumor angiogenesis, diabetic microangiopathy, ectopic tissue calcification, and stroke and dementia syndrome CADASIL [75]. In pathological conditions implying BBB damage, such as stroke, hypoxia, and traumatic brain

injury, the pericytes migrate away from brain microvessels wall and it seems to have an important role in neurovascular unit repair [76–78].

Morphine potentiates endothelial-pericyte interaction via platelet-derived growth factor-BB (PDGF-BB)/PDGF receptor-β (PDGFR-β) signaling and promotes tumor angiogenesis, pericyte recruitment, and coverage of tumor vessels [79]. The role of pericyte-endothelial cell interaction in chronic pain development and the role of pericytes during BBB disruption are still open topics.

4. BBB and BSCB in Chronic Pain: "To Be or Not to Be" Permeabilized/Disrupted

4.1. Acute/Chronic Pain Induces Changes at the BBB and BSCB Level. Does chronic pain cause BBB/BSCB permeabilization or disruption? The variety and complexity of the clinical conditions that involve chronic pain make a simple answer impossible, and studies on animal models of acute/chronic pain provide controversial responses to this hypothesis.

In the literature, the terms BBB "opening," "leakage," and "breakdown" are often used interchangeably, but more caution should be paid, when choosing between them [80]. A distinction should be made between BBB "permeabilization" and BBB "disruption" in experimental animal models. For the purpose of this review, the term BBB permeabilization refers to leukocytic recruitment associated with increased endothelial permeability, with no tight junction opening or altered efflux transport. As BBB "opening" or "permeabilization" is a physiological phenomenon, it should be reserved to transient processes [80]. On the other hand, we consider the BBB "disrupted" if Evans blue (EB) or albumins are extravasated into brain or spinal cord parenchyma. BBB "disruption" or "breakdown" represents a long-term opening associated with often-irreversible phenomena [80–82].

4.1.1. BSCB Permeabilization in Neuropathic Pain and Disruption in Chronic Pain Animal Models. Peripheral nerve injury produced by either sciatic nerve constriction or selective transection (peroneal and tibial nerve branches, but not sural branch) causes a transient increase in BSCB permeability in the lumbar and thoracic spinal cord, peaking about 24–48 h. after injury and returning to normal levels after 7 days, as assessed by EB dye or horseradish peroxidase accumulation in the parenchyma [10]. BSCB permeability was also increased 24 hours after electrical stimulation of the sciatic nerve at intensity sufficient to activate C-fibers, but not A-fibers, or after capsaicin application on the sciatic nerve [10].

Partial sciatic nerve ligation in rats, a model of neuropathic pain, triggers an increase of BSCB permeability in the lumbar, but not in the thoracic, spinal cord to tracers of different size (e.g., EB, sodium fluorescein), which was prominent between day 3 and day 7, stayed significant for at least 4 weeks after injury, and returned to normal levels after 2 months [11]. Contrasting results on BSCB permeability in extralumbar spinal cord regions (e.g., thoracic) [10, 11] could likely be attributed to EB protocol differences.

Plasma proteins (IgG and fibronectin) immunopositive deposits in the ipsilateral side of the spinal parenchyma and downregulation of tight junction proteins (ZO-1, occludin-1, and caveolin-1) in isolated microvessels of the spinal cord were found 3 days after injury [11]. BSCB permeabilization occurs independently of the activation of resident microglial cells, EB extravasation being present while a microglial inhibitor minocycline is infused intrathecally from day 0 to day 7 [11]. Additionally, it was shown that the administration in rats of high doses of IL-1β (intravenous) impairs BSCB disruption, while TGF-β1 and IL-10 (intrathecal) shut down the openings in BSCB [11].

In a recent study of perispinal inflammation induced by applying the toll-like receptor (TLR)-2 agonist zymosan to the dorsal dural surface of the L1/L2 spinal cord, the lack of BSCB permeabilization was inferred from the lack of serum proteins in the spinal parenchyma 24 h after surgery [12]. No immunohistological evidence of T-cell or Mac-1-positive macrophages crossing into the parenchyma was found, but ATF-3 (a transcription factor that is also a sensitive indicator of neuronal injury) expression was observed in the dorsal horn of the same spinal cord segments after 1 day [12]. Thus, inflammatory signals are indeed transduced across the BSCB at the site of the inflammatory stimulus, within a 400–500 μm radius. Astrocyte activation and gliosis are significantly increased in the superficial dorsal horn 1–7 days after surgery, with a transient recovery after 14 days, while resident microglia cells show a steady increase in staining density within the superficial dorsal horn beginning 1 day after surgery [12].

Neuropathic pain induced by L4 spinal nerve lesions in animal models is accompanied by astrocyte activation and albumin leakage, revealing BSCB disruption more prominent in the gray matter of the lesioned side compared to the contralateral in both dorsal and ventral horns [83]. Inflammatory events and changes in astrocyte and microglia reactivity at the spinal level in response to injury or disease are important processes that can initiate pain hypersensitivity [84, 85]. Studies conducted in a T-cell-deficient Rag1-null adult mouse have shown that T-cell infiltration and activation in the dorsal horn of the spinal cord following peripheral nerve injury contribute to the evolution of neuropathic pain-like hypersensitivity [86]. Most likely, the T-cell infiltration into the spinal cord is higher than normal in the nerve-injured animals, a fact that may be correlated with an increase in BSCB permeability.

BSCB permeabilization is a delayed event with respect to the initial injury and has a transient character. Studies addressing the role of the endothelium in BSCB disruption have been carried out, but the inclusion of the NVU as a whole is needed [87]. While the activation of glia may be important for the development of chronic pain, it is still unclear if the activation is required for BSCB disruption or if the two phenomena are independent. Peripheral inflammation or nerve injury in animal models induces astrocytes and microglia activation in the spinal cord [52, 88–91], but in these studies evidence regarding BSCB permeabilization is not available. In this view, new approaches connecting glia activation to BSCB opening would be very useful.

4.1.2. BBB Permeabilization in Animal Models of Inflammatory Pain. Inflammation induced by an intraplantar injection of λ-carrageenan into the rat hindpaw causes increased brain uptake (*in situ* brain perfusion) of [^{14}C]sucrose at 1, 3, 6 and 48 h after injection [92]. In the same study, Western blot analysis on isolated cerebral microvessels indicated a transitory increase in ZO-1 expression (increase after 1–6 h, returned to control after 12 h.) and a reduction in occludin expression (after 1, 3, 6, 12, and 48 h) [92]. These expression patterns indicate increased BBB permeability and suggest a link with the development of inflammatory pain. In another study devoted to inflammatory pain, [^{14}C]sucrose *in situ* brain uptake, [^{3}H] *in situ* cerebral flow, and Western blot analysis (occludin, ZO-1, CL-1, and actin expression) were performed 1 h after formalin injection, 3 h after λ-carrageenan injection and 3 days after complete Freund's adjuvant (CFA) injection, and BBB permeabilization was observed [93].

In a rat model of inflammatory pain (injection of CFA into the plantar hindpaw), significant edema formation and hyperalgesia were observed 72 h. after treatment, together with significant increases in brain sucrose uptake. Expression of the transmembrane TJ proteins occludin, claudin-3 and -5, and junction adhesion molecule-1 (JAM-1) significantly changed 24–72 h after CFA injection, as proved by Western blotting [9] and confocal microscopy [94].

The induction of peripheral inflammatory pain through the injection of λ-carrageenan was associated with increased BBB permeability in a study that showed, by means of SDS-PAGE/Western blot analysis, a significant change in the relative amounts of oligomeric, dimeric, and monomeric occludin isoforms in BBB endothelial cells, presumably promoted by the disruption of disulfide-bonded occludin oligomeric assemblies [95].

Expression of organic anion-transporting polypeptide 1a4 (Oatp1a4) is upregulated after 3 h exposure to λ-carrageenan; the upregulation is prevented by diclofenac, suggesting the implication of acute/chronic inflammatory pain [36]. This modulation of BBB permeability in inflammatory pain appears to be controlled by the TGF-beta/activin receptor-like kinase-5 (ALK5) signaling pathway [96]. λ-carrageenan-induced peripheral inflammatory pain generates increased [^{14}C]sucrose and [^{3}H]codeine *in situ* brain uptake, and rats pretreated (10 min before λ-carrageenan injection) with tempol, a pharmacological ROS scavenger, have an attenuated radiotracers uptake [97]. In the same study, other indirect pieces of evidence for BBB modulation have been presented consisting in increase of the nitrosylated proteins in isolated brain vessels extract.

In a λ-carrageenan inflammatory pain model, unidirectional permeability coefficients for several selected brain regions (hypothalamus, cerebellum, midbrain, cerebrum, hippocampus, brainstem, and thalamus) were calculated. Three hours after λ-carrageenan injection, the BBB resulted in an increased permeability in cerebrum and brainstem; diclofenac administration reversed this effect [98]. Western blot analysis of occludin expression in the same brain regions, however, did not reveal any significant changes [98]. In conclusion, correlating occludin expression changes with BBB "permeabilization" is problematic on the basis of the available data.

Administration of EB, which readily binds to serum albumins, is "classically" employed to assess BBB integrity, since in normal conditions the dye should not be found in the brain parenchyma [10]. However, in order to be revealed by EB, BBB disruption must be of a substantial degree (e.g., ischemic stroke [99]), while inflammatory pain per se does not constitute sufficient stimulus [100]. Inflammatory pain is more likely related to BBB permeabilization, as suggested by [^{14}C]sucrose transport through the BBB using *in situ* brain perfusion [9].

Despite the valuable information contained in the above described studies, there are several experimental pitfalls to be considered. First, only indirect pieces of evidences are available in support of the idea of BBB permeabilization in inflammatory or chronic inflammatory pain. It is difficult to assess BBB permeabilization based on changes in TJ protein expression in an homogenate of isolated brain capillaries or to expand results from *in situ* brain perfusion with radioactive tracers to the BBB permeabilization. Another problem is that relatively short experimental durations (such as 24–72 h) are considered equivalent to a "chronic" pain state [9], while similar experiments on BSCB permeabilization were carried out over a significantly longer time scale (1 week–2 months) [10–12]. More consistent studies, based on *in vivo* brain uptake of Evans blue or [^{14}C]sucrose should be done in order to prove BBB permeability changes. Alternative *in vivo* methods, such as intravital microscopy [101] or nuclear imaging of radioisotope-labeled leukocytes [72], are still unexplored in the field of chronic pain. A regional brain mapping of BBB permeabilization from the initial acute pain induction to the late chronic pain phase would be of significant use. In any case, clinical translation of the results obtained with experimental inflammatory pain models is still far from accomplished.

Possible changes in BBB and/or BSCB permeability as a result of acute and chronic pain are shown in Figure 1.

4.2. Chronic Pain Treatments and NVU. Two major classes of analgesic drugs are currently in use for chronic pain treatment: opioids and nonsteroid anti-inflammatory drugs (NSAIDs). NSAIDs are used to treat chronic mild to moderate pain, while opioids are powerful analgesic agents used to treat moderate to severe chronic pain [102].

On the other hand, beside a wide range of adverse effects, long-term clinical administration of opioids (e.g., morphine) in chronic pain therapy is prevented by tolerance and dependence [102]. A classical dogma holds that agonist-induced μ-opioid receptor internalization contributes directly to functional receptor desensitization and opioid tolerance [103]. By contrast, other studies suggest that opioid receptor internalization can reduce opioid tolerance *in vivo* (reviewed by [103]). Beside neurons, other NVU players (e.g., glial cells, pericytes) have been considered to contribute to opioid tolerance development [62, 79, 104]. Endothelial cell lining represents the first "defence" to be crossed by opioids before

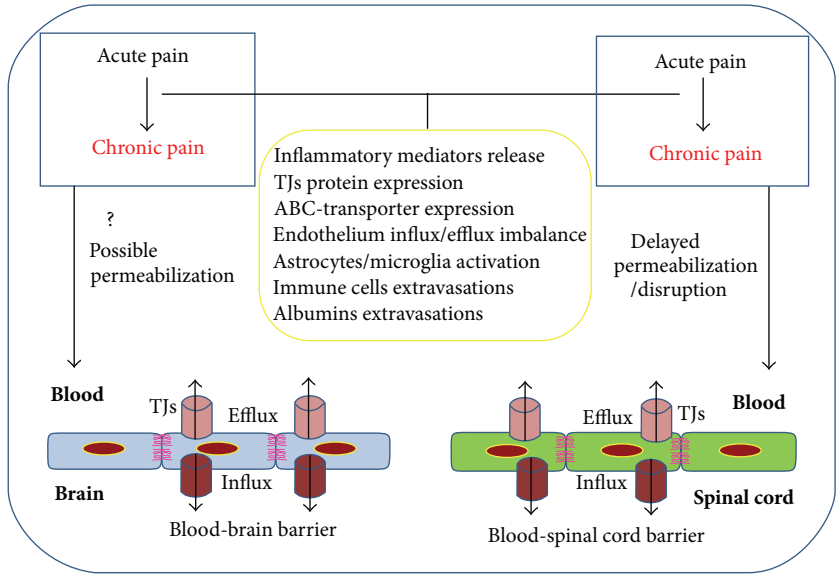

FIGURE 1: Acute pain occurs in a first step as a result of peripheral injury and/or inflammation. Chronic pain appears as a delayed event associated with permeabilization or brain/spinal cord capillary endothelium disruption. Different processes, such as inflammatory mediator release, changes in TJs protein and ABC transporters expression, activation of microglia and/or astrocytes, immune cells and albumin extravasation, may occur independently or in an "orchestrated" manner, and might contribute to the process of BBB/BSCB permeabilization or disruption.

interacting with CNS cells and therefore efflux alterations at this level are crucial in opioid tolerance.

In the λ-carrageenan rat model, acute inflammatory pain generates an increased functional expression and trafficking to membrane domains of endothelial efflux transporters (e.g., P-gp) in the BBB microvasculature [26, 37]. On the other hand, the same rats treated with morphine show reduced brain uptake of the drug due to increased P-gp activity [26]. Coadministration of cyclosporine A (P-gp inhibitor) with morphine in rats increased morphine transport through the BBB in a dose-dependent manner [26]. In the clinical practice, reducing tolerance to morphine by co-administration with cyclosporine A is unfeasible due to severe side effects (nephro- and neurotoxicity) [105, 106].

Chronic morphine treatment induced an increase in the expression of interleukin (IL)-18 by microglia, IL-18 receptor (IL-18R) by astrocytes, and protein kinase Cγ (PKCγ) by neurons in the spinal dorsal horn. The results were interpreted by the authors as signs of a complex glia-neuron dialogue in the process of developing tolerance to morphine [62]. Morphine also potentiates endothelial-pericyte interaction via PDGF-BB/PDGFR-β signaling [79]. Morphine upregulates sphingolipid ceramide (in spinal astrocytes and microglia, but not in neurons) and spinal sphingosine-1-phosphate [104]. In turn, sphingosine-1-phosphate modulates spinal glial function, increasing the production of glial-related proinflammatory cytokines, in particular TNF-α, IL-1β, and IL6 [104].

Another major line of chronic pain treatment is represented by nonopioid analgesics such as NSAIDs. These drugs have several side effects, the most important being the risk of serious upper gastrointestinal complications, including bleeding, ulcers, and perforation [102]. NSAIDs act on the descending pain control system, which includes the periaqueductal gray matter and rostral ventromedial region of the medulla, which are also targets for endogenous opioids. Therefore, repeated administration of NSAIDs (e.g., metamizol, lysine-acetylsalicylate, analgine, ketorolac, and xefocam) to rats induces tolerance to themselves and cross-tolerance to opioids [107, 108].

Studies suggest that NSAIDs interact in several different ways with the brain endothelium, either by reducing edema and BBB/BSCB permeabilization [109, 110] or by inhibiting endothelial ABC transporters (e.g., MRP1, MRP4) [42, 43]. Diclofenac attenuates edema and hyperalgesia induced by λ-carrageenan in the cerebral and brainstem regions [98]. Indomethacin, an inhibitor of cyclooxygenase (COX)-1 and COX-2, reduces BBB damage induced by intracerebral injection of TNF-α [109]. Pretreatment with p-chlorophenylalanine, indomethacin, ibuprofen, and nimodipine of rats with spinal cord injury, reduced edema formation, BSCB permeabilization, and blood flow [110]. Indomethacin was shown to be an inhibitor of MRP1 function [42] and indomethacin, indoprofen, ketoprofen, and flurbiprofen inhibit MRP4 [43]. Diclofenac is transported by BCRP, but not by P-gp [39].

5. Comorbidities of Chronic Pain with Neuroinflammatory and Neurodegenerative Diseases: Role of the Neurovascular Unit

Chronic pain has an extensive palette of comorbidities, but only neuroinflammatory and neurodegenerative diseases with known alterations of the NVU are here discussed (Figure 2). High prevalence of chronic pain can be

observed in all these CNS pathologies. BBB/BSCB alterations in epilepsy, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis will be briefly described, with regard to chronic pain syndromes. Understanding the exact role played by each pathology in permeabilizing/disrupting brain and spinal cord capillaries' endothelium is a crucial step in finding better therapeutic solutions.

5.1. Epilepsy and Chronic Pain. Epilepsy is a set of chronic neurological disorders characterized by abnormal, excessive, or hypersynchronous neuronal activity in the brain. The Epilepsy Comorbidities and Health (EPIC) Survey recently performed in the United States indicated that epilepsy is comorbid with several pain disorders, such as migraine, chronic pain, fibromyalgia, and neuropathic pain [111]. Additionally, the EPIC study indicated that chronic pain is prevalent in 25.4% of epileptic versus 17.7% of nonepileptic survey responders [111]. Chronic pain and fibromyalgia may be related to physical inactivity, which is more prevalent among adults with a history of epilepsy than among those without epilepsy [112].

The recent IASP taxonomy includes epilepsy in the list of generalized syndromes of chronic pain and includes chronic paroxysmal hemicrania—remitting form and hemicrania continua in the list of the chronic pain conditions [1]. Epilepsy and ictal epileptic headache share several pathophysiological mechanisms, such as (i) EEG abnormalities—lateralized or generalized, ipsilateral or contralateral, with focal theta activity or generalized spike-waves, and brief or longer-lasting episodes and (ii) headache and EEG anomalies resolve within minutes of i.v. antiepileptic medication administration [113]. The overlap between migraine and epilepsy may be partial or complete, not necessarily synchronous (preictal, ictal, or postictal), and in some cases the headache may represent the only ictal phenomenon [113]. In pediatrics studies, 3.1% of the patients suffered from idiopathic headache and idiopathic or cryptogenic epilepsy or unprovoked seizures [114]. The same study showed a strong association between migraine and epilepsy: in migraineurs the risk of epilepsy was 3.2 times higher when compared to tension-type headache, and children with epilepsy had a 4.5-fold increased risk of developing migraine than tension-type headache [114]. Postictal headache occurred in 41% of temporal lobe epilepsy patients, 40% of frontal lobe epilepsy patients, and 59% of occipital lobe epilepsy patients [115].

Several mechanisms have been proposed to explain comorbidity of epilepsy and chronic pain (such as that characterizing migraine), such as (i) the essential role of glutamate as a mediator of the hyperexcitability in both focal seizures and migraine, considering that seizure generation and spread are mediated by synaptically released glutamate acting on AMPA receptors, while triggering of cortical spreading depression depends on NMDA receptors and spread does not require synaptic transmission; (ii) mutations in genes for the membrane ion transport proteins CACNA1A (P/Q-type voltage-gated calcium channel), ATP1A2 (Na+-K+ ATPase), and SCN1A (voltage-gated sodium channel) [116].

Another important mechanism implied in chronic pain comorbidity with epilepsy is NVU activation. In this respect, brain endothelium seems to play an important role.

BBB disruption induces epileptiform activity [117–122]. We have previously shown that BBB leakage is induced by acute seizure activity but prevented by blockade of leukocyte-vascular adhesion, either with blocking antibodies or by genetically interfering with P-selectin glycoprotein ligand-1 (PSGL-1) function in mice [123]. Endothelial proinflammatory chemokines induce complex signal transduction pathways leading to integrin activation and controlling leukocyte recruitment, and therefore play a critical role in epileptogenesis [124, 125].

ABC transporters in the BBB are also affected in epilepsy. Shortly after *status epilepticus*, MRP1, MRP2, and BCRP are upregulated in astrocytes within several limbic structures, including hippocampus [47]. In chronic epileptic rats, these proteins are overexpressed in the parahippocampal cortex, specifically in blood vessels and astrocytes surrounding these vessels [47].

Whether transient BBB opening occurs during migraine attacks is controversial. Some magnetic resonance imaging studies have reported negative results [126, 127] while others have found indications of BBB leakage [128]. In migraine, indirect evidence for BBB permeabilization is provided by increased circulating levels of matrix metalloproteinases (MMPs) 2 [129] and 9 [130] that have been attributed to MMPs release from the extracellular matrix of the neurovascular unit.

5.2. Alzheimer's Disease and Chronic Pain. Alzheimer's disease (AD) is the most common cause of dementia. It is a neurodegenerative disorder characterized by synaptic and neuronal loss, by the accumulation in the extracellular matrix of beta-amyloid deposits, and by the presence of abnormal aggregates of microtubule-associated proteins, the so-called neurofibrillary tangles, in neuronal cell bodies.

Prevalence of pain in AD was estimated at 57% of all patients [131], although such assessment is complicated by two factors. First, pain processing may be altered in dementias [132, 133] including AD [134]. Second, the primary method for pain assessment is patient reporting [135], but pain affects cognitive function [136, 137] and cognitive function in turn affects pain [133], which makes pain assessment in AD very difficult.

Astrocytes tend to localize around fibrillar amyloid plaques, suggesting that Aβ deposition is a potent trigger of astroglial activation in the AD brain [138]. Additionally, an increase in the number of IL-1 immunoreactive microglia associated with AD plaques has been shown [139]. A variety of biomarkers for microglial activation in AD have been proposed, such as chitotriosidase, CCL18 (pulmonary activation-regulated chemokine; PARC), YKL-40, CCL2 (monocyte chemoattractant protein 1; MCP-1), CD14, and neopterin [140].

Immunohistochemistry on postmortem human brains affected by AD or vascular dementia indicated an increased expression of CL-2, Cl-5, and CL-11 in neurons and of CL-2

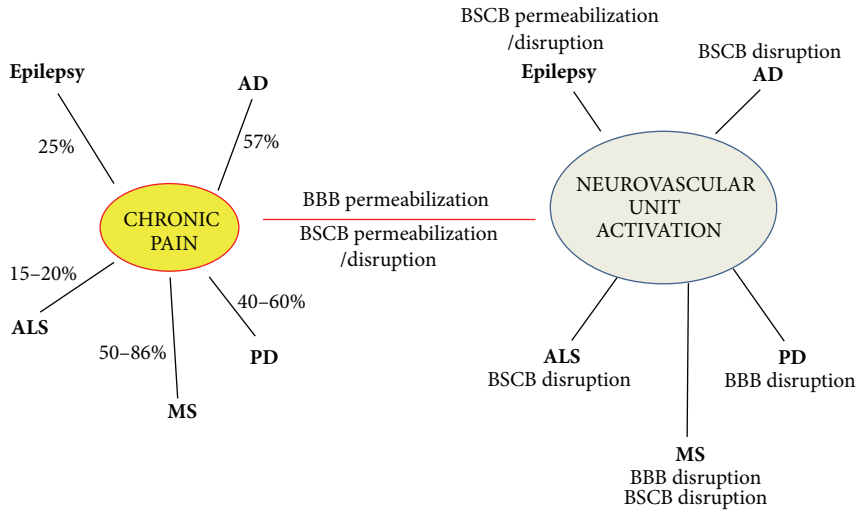

Figure 2: Similarities between chronic pain and NVU activation. Studies from the literature indicate that different NVU components are activated in a given pathology (e.g., epilepsy, Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, chronic pain) with a special focus on BBB/BSCB permeability alterations. Neuroinflammatory/neurodegenerative diseases are associated with chronic pain (see indicated percentages), but further studies are necessary to establish whether NVU activation may represent a "missing link" in the association. While the intrinsic mechanisms relating NVU activation, chronic pain, and neuroinflammatory/neurodegenerative disorders remain unclear, BBB/BSCB permeabilization appears to play a role.

and CL-11 in astrocytes and oligodendrocytes [141]. There is a strong relationship between neurodegeneration, cognitive decline, and BBB disruption in AD [142]. It was suggested that during neurodegeneration the receptor for advanced glycation end products (RAGE), which mediates transfer of amyloid-β to the brain through the endothelial cells, can be upregulated [143]. In AD transgenic mice, BBB alteration was proven to precede accumulation of senile plaques [144].

Neuroinflammation represents a crucial part in the pathogenesis of AD and other neurodegenerative diseases [145]. Inflammatory mediators, such as IL-1β, IL-6, TNF-α, IL-8, transforming growth factor-β (TGF-β), and macrophage inflammatory protein-1α (MIP-1α), are upregulated in AD [146].

5.3. Parkinson's Disease and Chronic Pain. Parkinson's disease (PD) is a degenerative disorder of the CNS, mainly characterized by loss of dopamine-generating cells in the substantia nigra. Prevalence of pain (musculoskeletal pain, neuritic or radicular pain, dystonia-associated pain, primary or central pain, and akathitic discomfort) in PD is estimated around 40–60% [147, 148]. See [149] for a comprehensive review of pain in PD.

Impairment of BBB function has been implicated in the pathogenesis of PD. Accumulation of verapamil (normally extruded from the brain by P-gp) in the brain of PD patients proves a dysfunction of BBB [150]. Injection of dopamine neurotoxin 6-hydroxydopamine (6OHDA, which produces Parkinson's-like dopaminergic neuron lesions) into the striatum of rats induced FITC-labeled albumin leakage in areas of the brain that are not protected by the BBB (e.g., the hypothalamus around the third ventricle and area postrema along the floor of the fourth ventricle) but no leakage in BBB-protected areas (e.g., ipsilateral parietal cortex or hippocampus, or into contralateral structures) [151]. The presence of neuroinflammatory markers, such as activated microglia or astrocytes, is also an important feature of PD [152]. Microglia activation and upregulation of inflammatory mediators can be induced by α-synuclein and contributes to PD pathogenesis [153]. On the other hand, astrocyte activation in PD is still under debate [152, 154–158]. In a recent study performed on aged c-rel$^{-/-}$ mice developing PD-like degeneration of substantia nigra pars compacta, we observed a marked microglia activation in the substantia nigra pars compacta and striatum, but no GFAP-positive astrocyte activation [159].

5.4. Multiple Sclerosis and Chronic Pain. Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS, which leads to demyelination, neurodegeneration, perivascular edema, and inflammatory infiltrates [160]. Prevalence of pain in MS is estimated around 50–86% [161, 162]. A recent classification based on pathophysiological mechanisms and response to treatment identified nine types of MS-related pain: trigeminal neuralgia and Lhermitte's phenomenon (paroxysmal neuropathic pain due to ectopic impulse generation along primary afferents), ongoing extremity pain (deafferentation pain secondary to lesion in the spinothalamocortical pathways), painful tonic spasms and spasticity pain (mixed pains secondary to lesions in the central motor pathways but mediated by muscle nociceptors), pain associated with optic neuritis (nerve trunk pain originating from nervi nervorum), musculoskeletal pains (nociceptive pain arising from postural abnormalities secondary to motor disorders), migraine (nociceptive pain favored by predisposing factors or

secondary to midbrain lesions), and treatment-induced pains [163].

BBB disruption is an early event in the progression of MS, as proved by magnetic resonance imaging studies [164, 165]. Diapedesis of monocytes and subsequent trafficking of monocyte-derived macrophages into the brain are key mediators of demyelination and axonal damage in MS. Endothelin 1 (ET-1), its type B receptor (ET(B)) and endothelin-converting enzyme-1 (ECE-1) are mediators for monocyte diapedesis through the human BBB and play a key role in demyelination and axonal damage in MS [166]. In experimental models of MS, such as experimental autoimmune encephalomyelitis (EAE), BBB disruption is induced by T-cells in conjunction with antigen-presenting dendritic cells [167, 168], and monocytes [169]. MS lesions are often found in proximity to blood vessels [170], associated with loss of occludin and ZO-1 in the microvasculature [171–173]. Leukocyte extravasation through BBB is mediated by cytokines: TNF-α, IL-1B, and interferon-γ [174]. Infiltration of inflammatory cells are localized perivascularly, but can also be located in the CNS parenchyma. In acute inflammatory lesions, $CD4^+$ and $CD8^+$ T cells and B cells infiltrate the lesion site. Lesions at later MS stages show an abundance of macrophages with internalized myelin degradation products and reactive proliferating astrocytes [175]. Sodium channels contribute to activation of microglia and macrophages in EAE [176].

MS is also characterized by significant changes in the composition and dynamics of the BSCB [177]. CD3-positive T-cells accumulate within the dorsal horn in mice with EAE, early in the disease course when cold and tactile allodynia are observed [178]. BSCB disruption is greatest at disease onset, followed by inflammation and demyelination, indicating that increased BSCB permeability precedes the destructive inflammatory process [177]. A recent study showed that autoreactive T cells access CNS via the fifth lumbar spinal cord in EAE mouse model [179].

In an EAE model, a recent study suggested a signalling role for Wnt (a family of secreted signaling proteins) in MS-associated chronic pain pathogenesis, although only neurons and glial cells were examined [180]. On the other hand, Wnt signaling contributes to brain angiogenesis, BBB formation, influences vascular sprouting, remodelling, and arteriovenous specification by modulating the Notch pathway [181]. Therefore, further studies on Wnt signalling in brain microvasculature could bring new insights in to MS-related pain syndromes.

5.5. Amyotrophic Lateral Sclerosis and Chronic Pain. Amyotrophic lateral sclerosis (ALS) is a chronic, progressive, and ultimately fatal neurodegenerative disease of motor neurons in the brain and spinal cord [182]. Prevalence of chronic pain (especially located at the arms level) in ALS is estimated around 15–20% [183, 184].

Increased permeability of the BSCB has been implicated in the pathogenesis of ALS [185]. Studies conducted in the ALS mouse model SOD1-G93A have shown BBB and BSCB disruption [186, 187], in areas of motor neuron degeneration (early and late ALS stages) [186] and capillary rupture in brainstem (early symptomatic ALS stage) [186]. Some studies indicate reduction in tight junction proteins (ZO-1, occluding, and claudin-5) before motor neuron loss, in presymptomatic ALS stages [188], while other data point out the reduction in tight junctions proteins (ZO-1 and occludin) and basement membrane protein agrin in symptomatic ALS stages [187]. Therefore, it is still controversial if the BBB/BSCB disruption is the cause or the consequence of ALS development. In SOD1-G93A mice, an increase was detected in mRNA and protein levels for P-gp and BCRP at the level of capillary endothelium in several regions, such as whole spinal cord, cerebral cortex, and cerebellum [189]. Additionally, the transport activity of P-gp and BCRP increased with ALS progression in spinal cord and cerebral cortex capillaries [189].

T lymphocytes are able to cross into the brain and spinal cord parenchyma, where they interact with resident microglia, inducing them to adopt either an M1 (cytotoxic) or M2 (protective) phenotype, depending on ALS stage [190]. Clinical studies evidenced perivascular and intraparenchymal $CD4^+$ T-lymphocytes in the proximity of degenerating corticospinal tracts and ventral horns in two-thirds of ALS patients [191]. $CD4^+$ T-lymphocytes slow disease progression, modify the microglial phenotypes, and extend survival [192, 193]. A potential mechanism behind the longer life expectancy may be mediated by the augmented secretion of IL-4 from mutant Cu^{2+}/Zn^{2+} superoxide dismutase regulatory T lymphocytes that directly suppressed the toxic properties of microglia [193]. It was suggested that $CD4^+CD25^{High}FoxP3^+$ regulatory T lymphocytes (Tregs) are neuroprotective and slow ALS progression [194].

6. Potential Strategies Targeting BBB or BSCB for Chronic Pain Relief

The molecular mechanisms of BBB/BSCB permeabilization due to chronic pain have yet to be clarified. Nevertheless, the barriers represent promising targets in designing new therapeutic strategies for chronic pain. Several approaches tested in preclinical and clinical studies, such as the use of Rho-kinase inhibitors, antiepileptic compounds, and statins, might turn out to be viable solutions in the future.

6.1. Rho-Kinase Inhibitor. Rho kinase (ROCK) is in- volved in various physiological functions, including cell motility, vasoconstriction, and neurite extension. ROCK inhibition reduces tissue-type plasminogen activator (t-PA)/plasminogen-mediated increase in permeability of *in vitro* models of the BBB [195]. Fasudil, a specific ROCK inhibitor, partly alleviates EAE-dependent damage by decreasing BBB and BSCB permeability [196]. In preclinical models of pain, fasudil (30 mg/kg) significantly attenuated mechanical allodynia in spinal-nerve ligation, chronic constriction injury, capsaicin-induced secondary mechanical hypersensitivity, sodium iodoacetate-induced pain, and capsaicin-induced acute flinching behaviors, but failed to attenuate or had only modest effects on inflammatory

thermal hyperalgesia following carrageenan injection and mechanical allodynia following complete Freund's Adjuvant injection [197]. Fasudil also proved to be efficient in adjuvant-induced arthritis model (inflammatory arthritis model) and a monoiodoacetate-induced arthritis model (noninflammatory arthritis model) [198].

6.2. Antiepileptic Drugs. It is difficult to consider currently market-available antiepileptic drugs (AEDs) as an alternative for classical analgesics because of their side effects, potential drug interactions, and unsatisfactory efficacy (epilepsy resistance). Between 1990 and 2012, 16 new AEDs were approved, most of them developed using mechanism-unbiased anticonvulsant animal models [199]. In order to be attractive for the pharmaceutical industry, the future design of new AEDs must also include a potential in nonepileptic CNS disorders, such as bipolar disorder and neuropathic pain [199]. Resistance to AEDs is encountered in more than 40% of epileptic patients [25], probably due to upregulation of the efflux transporters in brain capillary endothelium [200].

Only three AEDs are currently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of neuropathic pain: carbamazepine (CBZ), gabapentin (GBP), and pregabalin (PGB), all of them considered first-line treatment options for several neuropathic pain conditions (reviewed by [199]). Randomized clinical trials in spinal cord injury-related pain indicate gabapentin and pregabalin as powerful analgesics [201]. Cochrane Library reports based on extended clinical trials indicate GBP, PGB, and lacosamide, but not valproic acid, to be efficient against neuropathic pain or fibromyalgia [202–205].

Levetiracetam (LEV) may constitute a novel approach for BBB protection [206]. Clinical studies have evidenced the effects of levetiracetam (LEV) in various pain conditions, such as postmastectomy pain syndrome, trigeminal neuralgia, chronic general or central pain in MS, lumbar radiculopathy, chronic daily headache, polyneuropathy, and central poststroke pain [207–212]. In a rat model of hypothermia-induced cortical dysplasia, LEV and topiramate were found to protect the BBB [212]. However, a recent clinical trial failed to reveal significant effects of LEV against spinal cord injury-related pain [213]. Despite LEV's protective properties on the BBB, clinical efficacy against chronic pain is still controversial.

6.3. Statins. Beside the well-known efficacy of statins (inhibitors of HMGCoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase) in lowering plasma cholesterol levels, these compounds show a large palette of pleiotropic effects. Statins can improve endothelial function (thereby regulating the BBB permeability), decrease the oxidative stress and inflammation, and generally have a beneficial effect on the immune system, central nervous system, and bone [214]. Some of these effects point out statins as good candidates for chronic pain treatment. *In vivo* preclinical tests showed that Atorvastatin (a lipophilic statin) restored the BBB permeability in mice fed with saturated fatty acids (which compromised BBB integrity) [215]. In primary human skeletal muscle myoblast cells, atorvastatin and rosuvastatin proved to be substrates for MRP1, MRP4, and MRP5 transporters [216].

An analgesic effect was revealed by hot-plate test for some statins [217]. Preclinical tests have been performed to evaluate statin efficacy in neuropathic pain. Daily administration of statin for two weeks completely prevented the development of mechanical allodynia and thermal hyperalgesia in a nerve injury model [218]. Such approaches provide promising results for considering statins as a possible future generation of drugs against chronic pain, especially for patients with dislipidemy.

7. Future Perspectives

General mechanisms of chronic pain onset, development, and maintenance still await clarification, and the particular relationship between chronic pain and NVU function is an especially complex issue. Whether permeabilization/disruption of the endothelial barrier in brain or spinal cord could be a cause and/or a consequence of chronic pain is an open topic. Clearly, a better knowledge of the neurovascular unit contribution to chronic pain physiopathology would be highly beneficial in the clinical practice, especially in view of pharmacological targeting of the NVU.

The use of currently available analgesics (opioids and NSAIDs, in particular) in chronic pain is limited by their side effects and by the induction of tolerance and/or dependence. In this review, we have described some aspects of the neurobiological mechanisms of chronic pain, with particular emphasis on NVU players' interactions, also in view of present and future treatments. Future strategies against chronic pain should take into account the essential role played by the neurovascular unit in the efficacy of analgesics in an effort to overcome the already-known problems.

As many neurodegenerative/neuroinflammatory pathologies are comorbid with chronic pain in a significant number of patients, the identification of dual-target therapeutic strategies should be considered a priority.

With the NVU as an increasingly relevant target for the treatment of chronic pain, development of immunologically based strategies for preventing BBB and/or BSCB permeabilization or disruption would also represent an opportunity.

Authors' Contribution

Giuseppe Bertini and Paolo Francesco Fabene share the senior authorship position.

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Review Article

Pharmacogenetics of Chronic Pain and Its Treatment

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This paper reviews the impact of genetic variability of drug metabolizing enzymes, transporters, receptors, and pathways involved in chronic pain perception on the efficacy and safety of analgesics and other drugs used for chronic pain treatment. Several candidate genes have been identified in the literature, while there is usually only limited clinical evidence substantiating for the penetration of the testing for these candidate biomarkers into the clinical practice. Further, the pain-perception regulation and modulation are still not fully understood, and thus more complex knowledge of genetic and epigenetic background for analgesia will be needed prior to the clinical use of the candidate genetic biomarkers.

1. Introduction

It is well recognized that pain perception as well as pain relief after analgesic treatment display, large interindividual variability in the population that affects selection of analgesics and their dosing in the population. Age, gender, ethnicity, and actual level of stress, mood, or diseases may modify individual pain perception. This alters also the response to drug treatment, which represents a complex interaction between analgesic medication and organism. Several mechanisms may be involved in the pain relief either as drug targets or as drug metabolizing enzymes/transporters, and the genetic variability in these processes influence the analgesic efficacy in individual patients. This review is focused on highlighting the genetic variability reported to affect chronic pain treatment efficacy. This paper does not provide exhaustive list of polymorphisms reported but focuses on the current status of the most recognized pharmacogenetic areas and variables in the treatment of chronic pain.

2. Neurotransmitters

At least 100 substances can act as neurotransmitters, some of them being released after stimulation of sensory receptors, for example, catecholamines, GABA, and serotonin. Genes associated with synthesis, release, or target proteins for these pain neurotransmitters all represent candidate genes for chronic pain treatment variability. Variation in these pain-associated genes may result not only in variable pain perception but also in variable drug efficacy. Chronic pain and its association with gene polymorphism involved in neurotransmission have been widely studied in animal models. So far, 371 candidate genes have been identified in mice (http://www.jbldesign.com/jmogil/enter.html, accessed in December 2012), and some of them have been also shown to be of clinical relevance for man. Overview of major recently studied pain-associated genes in humans is presented in Table 1. However, the clinical data suggesting possible routine use of all these genetic biomarkers is unconvincing. Studies describing an association of various neurotransmission-related gene polymorphisms with variability of drug response in the treatment of chronic pain are listed in Table 2.

Recently, Klepstad et al. analyzed 112 SNPs in 25 candidate genes involved in opioid neurotransmission (OPRM1, OPRD1, OPRK1, ARRB2, GNAZ, HINT1, Stat6, ABCB1, COMT, HRH1, ADRA2A, MC1R, TACR1, GCH1, DRD2, DRD3, HTR3A, HTR3B, HTR2A, HTR3C, HTR3D, HTR3E, HTR1, and CNR1) in a large cohort of oncologic patients [22]. No association of these SNPs with opioid dosing (oxycodone, morphine, and fentanyl) was observed. However, haplotypes

Table 1: Overview of recently released (2010–2012) studies assessing the influence of various gene polymorphisms on pain perception in humans.

Gene	Refrence	Polymorphisms	Populations	Results
COMT	Hocking et al., 2010 [1]	Totally 11 SNPs	n = 8572, 1958 British birth cohort (83% Caucasians)	No associations of either chronic widespread pain or pain status with COMT genotypes or haplotypes
	Finan et al., 2010 [2]	rs4680	n = 46 female fibromyalgia patients (93.0% Caucasians)	Individuals with met/met genotype experienced a greater decline in positive effect on days when pain was elevated more than did either val/met or val/val individuals, *COMT* genotype contributing 1% of variance over and above the effect of pain on PA
	Fijal et al., 2010 [3]	rs6269, rs4633, rs4818, rs4680, and haplotypes	n = 159/93 female/male Caucasians with major depressive disorder	Associations between a haplotype created using rs6269, rs4633, rs4818, and rs4680, and the proportion of female patients with "Pain While Awake" and "Overall Pain" at baseline. No association was found in males
	Fernandez-de-las-Penas et al., 2011 [4]	rs4680	n = 70 children with chronic tension type headache, n = 70 healthy children	Children with chronic tension type headache (CTTH) met/met genotype-longer headache history compared with met/val (P = 0.001) or val/val (P = 0.002), children with CTTH, met/met genotype showed lower pressure pain test score over upper trapezius and temporalis muscles than children with CTTH with met/val or val/val genotype.
	Barbosa et al., 2012 [5]	rs4680 and rs4818	n = 112 fibromyalgia patients n = 110 healthy individuals	SNP rs4818, the frequency of variant genotype CC was 73.21 and 39.09% for patients with FS and controls, respectively, Fibromyalgia Impact Questionnaire score was higher in patients with the homozygous variant genotype for SNPs rs4680 (87.92 points) and rs4818 (86.14 points)
	Loggia et al., 2011 [6]	rs4680	n = 54 healthy subjects	met/met subjects exhibited stronger pain-related fMRI signals than val/val in several brain structures, including the periaqueductal gray matter, lingual gyrus, cerebellum, hippocampal formation, and precuneus
	Dai et al., 2010 [7]	rs6269, rs4633, rs4818, rs4680, and haplotypes	n = 69 patients with low back pain who underwent an intervention	rs4633 T allele—greater improvement in ODI (Oswestry disability index) score 1 year after surgery ATCA haplotype-APS-average pain sensitivity (9.3% in the study population)—greater improvement in ODI. The greatest mean improvement in ODI-ATCA-homozygotes
	Omair et al., 2012 [8]	rs4633, rs4680, rs4818, rs6269, rs2097603, and haplotypes	N = 93 patients with low back pain	Association of rs4633 and rs4680 with posttreatment improvement in VAS, for better improvement among heterozygous patients compared to the homozygous ones, no association was observed for the analysis of the common haplotypes
	Martinez-Jauand et al., 2013 [9]	rs6269, rs4633, rs4818 rs4680, and haplotypes	N = 113 fibromyalgia patients n = 65 healthy controls	Fibromyalgia individuals with the met/met genotype (Val158Met SNP) or the high- and average-pain sensitivity-associated haplotypes showed higher sensitivity to thermal and pressure pain stimuli than patients carrying the LPS haplotype or val alleles (Val158Met SNP)
OPRM1	Klepstad et al., 2004 [10]	A118G (rs1799971)	N = 99 Caucasians	Brief pain inventory average pain scores higher in AG heterozygotes
	Olsen et al., 2012 [11]	A118G	N = 258 patients with lumbar disc herniation and sciatic pain, Caucasians	∗/G women had 2.3 times as much pain as the ∗/G men 12 months after the disc herniation, while A/A women and A/A men had almost exactly the same recovery rate

Table 1: Continued.

Gene	Refrence	Polymorphisms	Populations	Results
OPRM1	Menon et al., 2012 [12]	A118G	$n = 153$ chronic migraine females, Caucasians	G118 allele carriers were more likely to be high pain sufferers compared to homozygous carriers of the A118 allele (OR = 3.125, 95% CI = 1.41, 6.93, $P = 0.0037$)
	Finan et al., 2010 [2]	A118G	$n = 46$ female patients with fibromyalgia 93.0% Caucasians	Patients with an 118G allele reported higher positive affect score across diary days than those homozygous for 118A
	Janicki et al., 2006 [13]	A118G	$n = 121$ chronic, non-cancer pain patients, $n = 101$ opioid-naive subjects with acute postoperative pain, Caucasians	The frequency of 118G is significantly lower in the subjects with chronic pain than in the group with acute postoperative pain—0.079 versus 0.158; $P = 0.009$
GCH1	Heddini et al., 2012 [14]	rs8007267 rs3783641 rs10483639	$n = 98$ women with provoked vestibulodynia, healthy controls $n = 102$	Significant interaction effect of GCH1 gene polymorphism and hormonal contraceptive therapy on coital pain among patients with current treatment ($n = 36$)
TRPV1	Carreno et al., 2012 [15]	rs222741	$n = 1040$ cases—Caucasians, 1037 controls	Association of rs222741 with the overall migraine group
SCN9A	Reeder et al., 2013 [16]	rs6746030	$n = 53$ biopsy specimens, $n = 26$ control specimens	AA or AG genotypes were present in 39.6% patients with cystitis/bladder pain syndrome—statistically significant difference compared with the controls: 11.5%
KCNS1	Costigan et al., 2010 [17]	rs734784	$n = 1359$ six independent cohorts	rs734784 significantly associated with higher pain scores in five of six independent patient cohorts, lumbar back pain with disc herniation—association with greater pain outcome in homozygote patients. The combined P value for pain association in all six cohorts
CACNG2	Nissenbaum et al., 2010 [18]	Totally 12 SNPs	$n = 549$ breast cancer patients: $n = 215$ control group $n = 334$	rs4820242, rs2284015, rs2284017, rs2284018, and rs1883988 showed significant association with chronic pain
ADRβ2	Diatchenko et al., 2006 [19]	Totally 8 SNPs and their haplotypes H1, H2, and H3	$n = 181$ cohort of females (Caucasians)	H1/H2 and/or H1/H3—lowest temporomandibular disorder incidence—1.3%, H1/H1 elevated risk of developing temporomandibular disorder (RR = 8.0, 95% CI = 1.2–52.2, 99% CI = 0.815–79.7), H3/H3, H2/H3, and H2/H3 H1 elevated risk of developing temporomandibular disorder (RR = 11.3, 95% CI = 1.95–67.9, and 99% CI = 1.38–102
	Hocking et al., 2010 [1]	rs12654778 and rs1042713	$n = 8572$, 1958 British birth cohort (82.6% Caucasians)	ADRB2 SNPs rs12654778 and rs1042713 were associated either with chronic widespread pain alone or with pain status
HTR2A	Nicholl et al., 2011 [20]	Totally 47 SNPs	$n = 164$, control group $n = 172$	rs12584920T (T/$*$, T/T) increased likelihood of having chronic widespread pain (OR) = 1.64, 95% confidence interval (95% CI) = 1.01–2.60 ($P = 0.03$) in the discovery cohort, and OR = 1.46, 95% CI = 1.07–2.00 ($P = 0.018$) in the validation cohort, similar association between rs17289394 and the maximum number of pain sites reported in both cohorts

VAS: visual analogue scale, OR: odds ratio, RR: relative risk, CI: confidence interval, SNP: single-nucleotide polymorphism, fMRI: functional magnetic resonance. GCH1: GTP cyclohydrolase 1, the rate limiting enzyme in the biosynthesis of tetrahydrobiopterin is an essential cofactor in the synthesis of serotonin, nitric oxide, and catecholamines. These neurotransmitters are known to modulate pain perception. TRPV1: transient receptor potential cation channel, subfamily V, member 1, acts as an integrator of multiple painful stimuli in chronic pain conditions. SCN9A: sodium channel, voltage-gated, type IX, alpha subunit encodes the voltage-gated sodium channel. Homozygotes with 2 loss-of-function alleles are congenitally indifferent to pain without other neurological deficit. KCNS1: voltage-gated potassium channel 1. CACNG2: calcium channel, voltage-dependent, gamma-subunit 2, encodes the gamma-2 transmembrane AMPA receptor protein (TARP) stargazin. This protein is known to be involved in the modulation of the ion channel function of glutamatergic AMPA receptors. ADRB2-beta2-adrenergic receptor is a target for epinephrine. HTR2A: 5-hydroxytryptamine (serotonin) receptor 2A. P2X7: cAMP responsive element binding protein 1.

Table 2: Trials assessing the influence of gene polymorphisms associated with neurotransmission on drug response in humans.

Gene	Refernces	Drugs	Polymorphisms	Populations	Results
COMT	Laugsand et al., 2011 [21]	Opioids (morphine, oxycodone, fentanyl, others)	rs4680, rs4633	n = 1579 cancer patients (European Caucasians) from the cohort of [22]	C allele of rs165722, the T allele of rs4633 and the G allele of rs4680 had less nausea/vomiting
	Reyes-Gibby et al., 2007 [23]	Morphine	rs4680	n = 207 cancer	Carriers of val/val and val/met genotype required 63% and 23%, respectively, higher morphine dose compared to carriers of met/met genotype
	Lötsch et al., 2009 [24]	Various opioids		n = 352 patients with chronic pain of various origin	No association
	Klepstad et al., 2011 [22]	Morphine (n = 830), oxycodone (n = 446), fentanyl (n = 699), or other opioids (n = 234)	112 SNPs in the 25 candidate genes including OPRM1 A118G	n = 2294 cancer patients, European Caucasians	None of SNPs in the candidate genes *OPRM1, OPRD1, OPRK1, ARRB2, GNAZ, HINT1, Stat6, ABCB1, COMT, HRH1, ADRA2A, MC1R, TACR1, GCH1, DRD2, DRD3, HTR3A, HTR3B, HTR2A, HTR3C, HTR3D, HTR3E, HTR1,* or *CNR1* showed significant associations with opioid dose
	Rakvåg et al., 2008 [25]	Morphine	11 SNP and haplotypes, including rs4680, rs4633 not included	n = 197 Caucasian cancer patient cohort receiving oral morphine treatment for cancer pain	The most frequent haplotype (34.5% rs2075507, rs737866, rs7287550R, rs5746849, rs740603, rs6269, rs2239393, rs4818, rs4680 (Val158Met) rs174699, rs165728 GACAAAACATT) associated with lower morphine doses, with a reduction factor of 0.71
	Ross et al., 2008 [26]	Morphine	13 SNPs, rs4818 not included	n = 228 cancer patients on morphine	Haplotype in intron 1 (AATTGAAATAATT) and 4873G genotype (10% is strongly associated with somnolence), hallucinations and confusion after treatment with morphine (protective effect). ABCB1 genotypes and haplotypes investigated in the study as well allele 21/2677G and 12/1236C associated with somnolence, hallucinations, and confusion after treatment with morphine (protective effect)
OPRM1	Reyes-Gibby et al., 2007 [23]	Morphine	A118G	n = 207 cancer patients, Caucasians	GG genotype required 93% higher morphine dose compared to carriers of AA genotypes (P = 0.012)
	Klepstad et al., 2004 [10]	Morphine	A118G	n = 99 cancer patients, Caucasians	No association with the intensities of symptoms such as fatigue, nausea and vomiting, dyspnea, sleep disturbance, loss of appetite, and constipation were similar between the three cohorts, The serum concentrations of morphine, M6G, and M3G were higher in patients homozygous for the 118G allele
	Campa et al., 2008 [27]	Morphine	A118G	n = 145 Italian Caucasians	Significant association of pain relief after treatment with morphine with the allele. The association improved with the combination of the allele and polymorphism in ABCB1 detection of three groups: strong responders, responders, and nonresponders, sensitivity $\rightarrow \rightarrow$ 100%, specificity > 70%
	Lötsch et al., 2009 [24]	Various opioids	A118G	n = 352 patients with chronic pain of various origin	Tendency towards increased pain in dose-dependent manner with the μ-opioid receptor variant 118G. Daily opioid doses significantly decreased in a gene dose-dependent manner with the P-glycoprotein variant ABCB1 3435C>T

Table 2: Continued.

Gene	Refernces	Drugs	Polymorphisms	Populations	Results
	Liu and Wang 2012 [28]	Acetaminophen/ tramadol	A118G	$n = 96$ patients with adenocarcinoma of the colon or rectum ($n = 84$), or stomach ($n = 12$) who developed oxaliplatin-induced painful neuropathy	The requirement for rescue analgesia higher for patients with G allele, AA genotype-better analgesic effect than G allele variants (AG or GG genotypes). Pretreatment and posttreatment VAS scores for patients with G allele variants were 3.1 and 2.6, respectively; for patients with AA genotype, pretreatment and posttreatment VAS scores were 3.0 and 0.9
OPRM1	Janicki et al., 2006 [13]	Morphine	A118G	$n = 121$ chronic, noncancer pain patients, Caucasians	The mean opioid dose is significantly larger in the homozygous carriers of the wild-type 118A allele when compared with the carriers of the variant allele
	Klepstad et al., 2011 [22]	Morphine ($n = 830$), oxycodone ($n = 446$), fentanyl ($n = 699$), or other opioids ($n = 234$)	112 SNPs in the 25 candidate genes including OPRM1 A118G	$n = 2294$ cancer patients, European Caucasians	None of SNPs in the candidate genes *OPRM1, OPRD1, OPRK1, ARRB2, GNAZ, HINT1, Stat6, ABCB1, COMT, HRH1, ADRA2A, MC1R, TACR1, GCH1, DRD2, DRD3, HTR3A, HTR3B, HTR2A, HTR3C, HTR3D, HTR3E, HTR1,* or *CNR1* showed significant associations with opioid dose
	Droney et al., 2013 [29]	Morphine	A118G	$n = 264$ cancer patients taking oral morphine	Genetic factors only accounted for 12% of variability in residual pain on morphine and 3% of variability in central side effects
CREB1	Nishizawa et al., 2012 [30]	Opioids			rs2952768 was associated with more analgesic requirements, and consistent results were obtained in patients who underwent abdominal surgery
HTR3B	Laugsand et al., 2011 [21]	Opioids (morphine, oxycodone, fentanyl, and others)	rs1176744, rs3782025 rs1672717	$n = 1579$ cancer patients (European Caucasians)	G allele of rs1176744, the T allele of rs3782025, and the T allele of rs1672717 were associated with less nausea/vomiting
CHRM3	Laugsand et al., 2011 [21]	Opioids (morphine, oxycodone, fentanyl, and others)	rs10802789 rs685550	$n = 1579$ cancer patients (European Caucasians)	T allele of rs10802789 associated with more nausea/vomiting
KCNJ6	Lötsch et al., 2010 [31]	Methadone	rs2070995	$n = 352$ opioid-treated chronic pain patients	The daily methadone substitution doses during the first therapy year were larger in the rs2070995 AA genotype ($n = 4$, 119.7 ± 49.6 mg/day) than in other rs2070995 genotypes (77.5 ± 26.2 mg/day, $P = 0.003$)
DRD4	Ho et al., 2008 [32]	Heroin	−521C/T	$n = 43$ current heroin uses, 66 controls	TT control subjects had lower pain threshold versus CC/CT controls and versus TT addicts
HTR2C	Brash-Andersen et al., 2011 [33]	Escitalopram	rs6318	$n = 34$ patients with peripheral neuropathic pain	rs6318 (Cys23Ser) in the HTR2C gene showed significant association with treatment response in men, with 75% carrying the C allele being responders. The same tendency was seen in women

VAS: visual analogue scale. CREB1: cAMP responsive element binding protein 1 encodes a transcription factor, a member of the leucine zipper family of DNA binding proteins. HTR3B: 5-hydroxytryptamine (serotonin) receptor 3B encodes subunit B of the type 3 receptor for serotonin (neurotransmitter, hormone, and mitogen). Activation of the receptor leads to fast depolarizing responses in neurons. Pentaheteromeric complex with subunit A (HTR3A) displays the full functional features of this receptor. HTR2C encodes the 2C subtype of serotonin receptor. CHRM3: the muscarinic cholinergic receptor 3, G-protein-coupled receptor controls smooth muscle contraction, and its stimulation increases secretion of glandular tissue. KCNJ6: gene for potassium inwardly rectifying channels, subfamily J, member 6 (Kir3.2, GIRK2). This G channel is important for opioid receptor transmission and is involved in opioid effects on postsynaptic inhibition [34]. DRD4: dopamine receptor D4 belongs to the dopamine receptor D2-like family, which mediates reward and reinforcement effects (e.g., of heroin) [35].

were not analyzed in this study. Laugsand et al. analyzed 96 single-nucleotide polymorphisms (SNPs) in 16 candidate genes related to opioid or nausea/vomiting signaling pathways (ABCB1, OPRM1, OPRK1, ARRB2, STAT6, COMT, CHRM3, CHRM5, HRH1, DRD2, DRD3, TACR1, HTR3A, HTR3B, HTR3C, and CNR1) for the association with nausea and vomiting in the same cohort of cancer patients. Totally 8 SNPs in 3 genes, COMT, HTR3B, CHRM3 (rs1176744, rs3782025, rs1672717, rs165722, rs4680, rs4633, rs10802789, rs685550), were significantly associated with the interindividual differences in nausea and vomiting among cancer patients treated with opioids [21].

Two candidate genes have been clinically studied most widely so far (OPRM1 and COMT).

2.1. OPRM1. The μ-opioid receptor gene, OPRM1, is the most widely studied gene in association with different aspects of chronic pain. Probable effect of its polymorphism A118G (rs1799971, Asn-40 $\rightarrow$ Asp) is recognized. In 1998, Bond et al. demonstrated that Asp substitution on the extracellular N-terminal of the receptor determines the same binding affinity for endo- and exogenous opioids (morphine, fentanyl, methadone, naloxone, and met- and leu-enkephalins) with one exception; β-endorphin showed higher affinity to the receptor in the 118G variant carriers [68]. This finding allowed the authors to propose a hypothesis that there is a possible connection between the allele and addiction. Somewhat later, Zhang et al. found a 2-fold higher expression of μ-opioid receptor in brains of 118G heterozygotes [69]. In the study by Oertel et al., a significant reduction in effectivity of subsequent signaling pathways after the binding of a specific agonist DAMGO was observed. Rate of G-protein coupling in carriers of the G allele reached only 57% in comparison with AA homozygotes [70]. Recently, 4-fold increase in inhibition of Ca channels in the carriers of G-allele was also demonstrated [71]. Contrary to the preclinical data, the results of conducted clinical trials provide unconvincing evidence only. Recent meta-analysis and in particular large-scale cohort study found no evidence for an effect of this polymorphism on opioid dose (oxycodone, morphine, and fentanyl) in oncologic patients [22], although less frequent nausea and vomiting were associated with the polymorphism in the meta-analysis [72].

2.2. COMT. *Catechol O-methyl transferase* plays a central role in extracellular inactivation of catecholamine neurotransmitters, including dopamine and norepinephrine, in the central nervous system. Val158Met variant (rs4680, G1947A) showed higher enzymatic activity compared to wild type in postmortem human brains [113]. It is associated with a three-to-four-fold variation in COMT enzyme activity and also with individual variation in COMT thermal instability. Lower dopamine levels in carriers of this polymorphism were associated with lower levels of enkephalins in animal models, which in turn lead to downregulation of μ-opioid receptor [114, 115]. However, the clinical relevance of these findings is still questionable. Polymorphism rs4680 did not result in variable opioid dosing in the treatment of pain in oncologic patients [22]. Meta-analysis studying rs4680 in different types of chronic pain demonstrated that fibromyalgia or chronic widespread pain could be influenced by the presence of the variant allele. No association was observed with migraineous headache or chronic musculoskeletal pain conditions. According to systematic review of the literature, low COMT activity enhances opioid analgesia and adverse effects in some cancer pains via increasing the absolute amount of opioid receptors [116]. Reyes-Gibby et al. observed significantly lower doses of morphine in cancer patients, carriers of OPRM1 118AA and COMT rs4680 met/met ($P < 0.012$) [23].

Three haplotypes (containing alleles rs6269, rs4633, rs4818, and rs4680) which accounted for 96% of all haplotypes observed in the Caucasian population have been identified [79, 117]. Some haplotypes were associated with different phenotypes: low-pain sensitivity, average-pain sensitivity, and high-pain sensitivity, while the risk of developing temporomandibular disorder could be predicted by a single haplotype in this study [79]. Surprisingly, both the low- and high-pain sensitivity-associated haplotypes possessed the val variant of rs4680. According to Nackley et al., interaction of rs4680 with other SNPs (silent mutations: rs6269, rs4633, and rs4818) determines the changes in the secondary structure of the messenger RNA, and these may modify the protein translation and the real *in vivo* activity of the enzyme [118]. The average-pain sensitivity-associated haplotype was modestly associated with greater improvement on a long-term VAS 1 year after lumbar surgery in patients with disc herniation [8]. In another recent study, high-pain sensitivity associated haplotype was associated with moderate or severe headache and moderate or severe dizziness in patients after motor vehicle collision [119]. However, Nicholl et al. found no correlation between COMT "pain sensitivity" haplotypes (rs6269, rs4633, rs4818 and rs4680 alleles) and chronic wide spread pain in two case-control studies (cases $n_1 = 164$, $n_2 = 172$; controls $n_1 = 204$, $n_2 = 935$) [120].

2.3. Candidate Genes in New Drug Development. With regard to the preclinical studies, TPRV1 gene product (transient receptor potential cation channel, subfamily V, member 1) appears to be the most promising as a potential target for therapeutic intervention. It is a polymodal nociceptor, the expression of which is upregulated in several painful disorders. Analysis of its function (including knockout mice) revealed that it plays a crucial role in integrating multiple painful stimuli in chronic pain conditions [121]. *TRPV1* gene polymorphism might be an underlying cause of the inter-subject variability in pain sensation and response to TRPV1 antagonists [15]. TRPV1 antagonists are undergoing clinical trials in patients with chronic pain at present (reviewed in [122]).

3. Proinflammatory Cytokines

Peripheral nociceptors are sensitized by proinflammatory cytokines that are produced by inflammatory cells (CD4+ and CD8+ T cells) in response to disease as cancer or

Table 3: Impact of genetic variability in genes for proinflammatory cytokines.

Gene product	Genetic variability	Effect	Reference
TNFR2	TNFR2(−/−) mice	Attenuated hyperalgesia	[36]
TNF α	A allele in −308G/A (rs1800629)	Higher pain severity	[37–39]
	G allele in −308G/A (rs1800629)	Anti-inflammatory treatment success with phytotheraphy	[40]
LTA	Variant allele in rs5275	Lower pain scores	[41]
COX 2	CC in rs5275	Lower risk of severe pain	[39]
	G allele in rs5277	Higher pain scores	[41]
IL-1α	C889-T (rs1800587)	Pain intensity	[42]
IL-1β	C3954-T (rs1143634)	Occurrence of low back pain, the number of days with pain, and the number of days with limitations in daily activities due to pain	
IL-1RN	G1812-A		
IL1-receptor 1	Variant allele in rs2110726	Less frequent breast pain	[43]
IL-4	Varant allele in rs2243248	More frequent pain	[44]
IL-6	−174G/G (rs1800795)	Pain	[45]
	−174C/C (rs1800795)	Higher opioid dosage	[37, 38]
IL-8	T allele in 251T/A (rs4073)	More frequent severe pain	[46]
	251T/T (rs4073)	Least frequent severe pain	[37]

its treatment, that is, cytostatics. This is one of the direct mechanisms leading to hyperalgesia in chronic diseases [123]. Therefore, the research attributed to the polymorphisms in genes coding for these cytokines and their relationship to various diseases including pain and its treatment arises [124–129].

Summary of known implications of genetic variability in genes for proinflammatory cytokines is given in Table 3.

3.1. TNF-α. TNF-α is known to contribute to hyperalgesia associated with chronic illness. After administration of bacterial endotoxin, hyperalgesia can be blocked by functional antagonists of TNF-α, for example, TNF-α binding protein [130]. Deletion of the tumor necrosis factor receptor type 2 (TNFR2) gene attenuated heat hyperalgesia in tumor-bearing mice, whereas TNFR1 gene deletion played only a minor role [36]. There are few clinical trials suggesting that TNF α-308 G/A (rs1800629) is associated with chronic pain perception and treatment success. Variant alleles in TNF α-308 G/A were significantly associated with higher pain severity in a study with 140 Caucasians newly diagnosed with nonsmall cell lung cancer [37, 38], and this has been confirmed in another study in newly diagnosed non-Hispanic Caucasian lung cancer patients ($n = 667$) [39]. Higher chance for success of anti-inflammatory phytotherapy treatment in TNF α-308 wild-type allele carriers with chronic pelvic pain syndrome has been also proposed in a small study [40].

3.2. LTA. Lymphotoxin-alpha (LTA), also known as TNF-β, as a member of TNF family is also an important inflammatory marker. In a recent study with lung cancer patients, variant allele in rs5275 was associated with lower pain scores in patients surviving for more than 5 years [41].

3.3. COX-2. Cyclooxygenase 2, a product of prostaglandin-endoperoxide synthase 2 (PTGS2), is an enzyme responsible for the production of prostaglandins and represents the target for NSAIDs. As such, it plays a significant role in inflammation and chronic, particularly cancer, pain [131, 132].

Two recent studies reported an association between PTGS2 polymorphisms and pain. In study [39], CC genotypes for rs5275 were at lower risk for severe pain. Close SNP rs5277 was found to predict pain intensity in 1149 Caucasian lung cancer patients in the Mayo Clinic Lung Cancer Epidemiology Project. People carrying one or two minor (G) alleles reported higher pain scores [41].

3.4. IL-1. IL-1 is a family of 11 members produced during neuropathic pain and inflammation [133, 134]. Its involvement with pain mediation is undisputable as its, IL-1β to be precise, intrathecal injection produces hyperalgesia [135, 136].

Polymorphisms in their genes, as well as in genes of their receptor,have been reported to affect nociceptive response. It was shown that the IL-1α (C889–T) (rs1800587) and IL-1β (C3954–T) (rs1143634) [137, 138] polymorphisms, and an 86-base pair repeat (VNTR polymorphism) in the IL-1 receptor antagonist (IL-1Ra) [139] genes are associated with the regulation of the IL-1 and IL-1Ra production. Association between IL-1α (C889–T) polymorphism with pain intensity was revealed in study in Finnish men ($n = 1832$) with low back pain. Moreover, it was implicated that IL-1β (C3954–T) and the IL-1RN (G1812–A) polymorphisms, and their composite genotype, are related to the 12-month occurrence of low back pain, the number of days with pain, and the number of days with limitations in daily activities due to pain [42]. Carriers of variant allele in IL1-receptor 1 (rs2110726) were less likely to report breast pain prior to surgery in a study with 398 women [43].

Table 4: The principal polymorphic DMEs involved in the metabolism of drugs used for chronic pain.

Enzymes	Important gene variants	Influenced drug group	Proven effect on PK or efficacy/safety in clinical trials	References
CYP2D6	CYP2D6*1-wt CYP2D6*3 2549A>del CYP2D6*4 1846G>A CYP2D6*5 CYP2D6*6 1707T>del MxN CYP2D6*10	TCA Opioids SSRI	Opioids (codeine, tramadol, hydrocodone, and oxycodone), TCA (amitriptyline, nortriptyline, imipramine, and desipramine), and SSRI (fluoxetine, paroxetine, and citalopram)	[47, 48]
CYP2C9	CYP2C9*1-wt CYP2C9*2 (Cys144Arg) CYP2C9*3 (Leu359Iso) CYP2C9*5	NSAIDs SSRIs SNRIs	Coxibs (celecoxib)	[48–50]
CYP2C19	CYP2C19*1-wt CYP2C19*2 CYP2C19*3 CYP2C19*17-Ums	NSAID Antidepressants	SSRI (citalopram)	[48, 51, 52]
CYP2C8	CYP2C8*1-wt CYP2C8*2 (Ile269Phe) CYP2C8*3 (Arg139Lys, Lys399Arg)	NSAID	NSAID (ibuprofen and diclofenac)	[48, 53–55]
CYP3A4	CYP3A4*1 (2023G>A) CYP3A4*2 CYP3A4*10 CYP3A4*17	Opioids	Opioids (methadone and fentanyl)	[48, 56–58]
UGT1A6	UGT1A6*1 UGT1A6*2	NSAIDs, anticonvulsants	NSAID (acetylsalicylic acid)	[59, 60]
UGT2B7	UGT2B7*2 (802C>T, H268Y), 161C>T	NSAID Opioids Anticonvulsants	Opioids (morphine) and anticonvulsants (lamotrigine and valproic acid)	[61–65]
UGT1A1	UGT1A1*28	Paracetamol Opioids	Paracetamol	[62, 66]
SULT1A1	SULT1A1*2 (G638A; Arg213His) SULT1A1*3 (A667; Met223Val)	Paracetamol	Paracetamol	[67]

3.5. IL-4. IL-4 is produced by T cells, mast cells, eosinophilis, and basophiles [140]. It coregulates the inflammatory response by suppressing TNF-α and IL-1 expressions [141] and by modulating B cells to undergo Ig isotype switching to IgE [142]. Homozygotes for variant allele (rs2243248) in the gene coding for this anti-inflammatory cytokine were found to be more likely in patients with high degree of depression and pain in a recent study with oncology patients (n = 168) and their family caregivers (n = 85) [44].

3.6. IL-6. IL-6 has a role in the regulation of inflammatory response. IL-6 knockout mice have had significantly higher levels of other cytokines in response to endotoxin [143]. In patients with juvenile rheumatoid arthritis, IL-6 genotype −174G/G (rs1800795) was positively correlated with pain [45]. Homozygous carriers of the IL-6 −174C allele required 4.7 times higher dose of opioids for pain relief relative as compared with GG and GC newly diagnosed patients with nonsmall cell lung cancer [37, 38].

3.7. IL-8. IL-8 attracts the neutrophiles to the site of infection or injury [144]. Its elevated concentrations are found in various diseases, particularly associated with inflammation, such as rheumatoid arthritis [145]. There are however very few discrepant data on SNP IL8-251T/A (rs4073), where wild-type allele was a predictor for severe pain in 168 Caucasian patients—TT or AT subjects had more than a threefold risk (OR = 3.23, 95% CI = 1.4, 4.7) for severe pain compared to the AA patients [46]; while in another study, TT homozygotes had the least frequency of severe pain [37].

4. Drug Metabolism

The pharmacokinetics of drugs is subject to a large interindividual variability, which is important cause for adverse drug reactions and lack of drug response. In therapy of pain, numerous genetic polymorphisms affecting pharmacokinetics of drugs have been shown to contribute in part to interindividual variability in drug efficacy

Table 5: Drugs used in pain treatment and its major DMEs emphazing FDA recommendations for genetic testing [73–75].

Drug class	Drug	Major enzymes			FDA drug labels including pharmacogenetics information
		CYPs	UGBTs	SULTs	
Analgesic/ antipyretics	Paracetamol	3A4, 2E1, 2A6, 1A2, 2D6, 2C19, 2C9, 2E1, 2A6	1A6, 1A9	1A1	In combination with tramadol CYP2D6
NSAIDs	Ibuprofen	2C9, 2C8, 3A4, 2C19, 2C9, 2C8, 3A4, 2C19	2B7, 1A9, 1A3, 2B4		
	Diclofenac	2C9, 2C8, 3A4, 2C19	2B7, 2B4, 1A3, 1A9		
	Naproxen	2C9, 1A2			
	Ketoprofen		2B7		
	Meloxicam	2C9, 3A4			
Coxibs	Celecoxib	2C9, 3A4			CYP2C9
	Etoricoxib	3A4, 2C9, 2D6, 1A2, 2C19			
TCAs	Amitriptyline	2C19, 2C8, 2C9, 1A2, 2D6, 3B6, 3A4	1A3, 1A4		CYP2D6
	Nortiyptyline	2D6, 3A4			CYP2D6
	Imipramine	2D6, 2C19, 1A2			CYP2D6
	Desipramine	2D6			CYP2D6
SNRIs	Duloxetine	2D6, 1A2			
	Venlafaxine	2D6, 2C19, 2C9			CYP2D6
SSRIs	Fluoxetine	2C9, 3A4, 2D6, 2C19, 1A2			In combination with olanzapine, and CYP2D6
	Paroxetine	2D6			CYP2D6
	Citalopram	3A4, 2C19, 2D6			CYPs 2C19, and 2D6
Antiepileptics	Carbamazepine	3A4, 2C8	2B7		
	Valproate		2B7, 1A6, 1A9		
Opioids	Buprenorphine	3A4, 2C8	1A1, 2B7, 1A3		
	Codeine	2D6, 3A4	2B7, 2B4		CYP2D6
	Dihydrocodeine	2D6, 3A4	2B7		
	Morphine	3A4, 2C8	2B7, 1A8, 1A1, 1A3, 1A10, 1A6, 1A1		
	Oxycodone	2D6, 3A4	2B7		
	Pethidine	3A4, 2B6, 2C19			
	Tilidine	3A			
	Tramadol	3A4, 2B6			In combination with paracetamol CYP2D6

and safety [146]. The most important drug metabolizing enzymes for pain treatment are cytochromes P450 (P450), UDP-glucuronyltransferases (UGTs), and sulfotransferases (SULTs) [59, 147]. Table 4 summarizes the principal polymorphic DMEs with its most important genetic variants involved in the metabolism of drugs used for chronic pain. Table 5 shows the drugs used in pain treatment and its major DMEs emphasizing FDA recommendations. An example in which pharmacogenetic testing of DMEs could be clinically relevant is P450 and UDP-glucuronyltransferase [59].

Recently, comprehensive and in-depth monography concerning drug metabolism (including impact of genetic polymorphisms) was published [73].

4.1. P450. Cytochrome P450 (P450) consists of heme-containing monooxygenase enzymes located on the smooth endoplasmic reticulum membranes of liver hepatocytes and along the mucosal surface of the intestinal tract and several other tissues including kidney, heart, and brain. Research on human P450 polymorphisms began in the 1970s and continues till now [73]. Actual information concerning P450 polymorphisms is compiled on the website http://www.cypalleles.ki.se/.

4.2. CYP2D6. CYP2D6 accounts for 2–5% of the total hepatic P450 enzymes; however, it is involved in the metabolism of 25% of all drugs administered in clinical practice [47].

It is also important for many drugs used in pain and palliative medicine as it is responsible for metabolizing certain analgesics as opioids (codeine, tramadol, hydrocodone, oxycodone), neuroleptics, and antidepressants; see Table 5. CYP2D6 plays an important role not only in the metabolism of exogenous opioids but also in the endogenous morphine synthesis pathway. CYP2D6-metabolized drugs exhibit nonlinear saturable kinetics owing to the low capacity of CYP2D6. The existence of almost 80 CYP2D6 allelic variants is known to lead to phenotype diversity within populations [148]. Approximately 7–10% of people may be classified as poor metabolizers (PM) and 3% as ultrarapid metabolisers (UM) in the Caucasian populations [149, 150]. An example in which pharmacogenetic testing of CYP2D6 is clinically relevant is codeine. Codeine as a prodrug requires *O*-demethylation catalyzed by CYP2D6 to be converted into morphine and become analgesic. This metabolite pathway accounts for 10% of codeine clearance in EMs but is much more pronounced in UMs and far less pronounced in PMs; so PMs suffer from a lack of analgesia, while UMs have been shown to be more likely to experience side effects and have 50% higher plasma concentration of morphine compared to EMs [151]. Codeine as a weak opioid was believed to be a relatively safe analgesic. However, after the death of breastfed neonate through morphine overdose by his mother taking codeine, the safety profile of codeine was reevaluated and FDA published a warning on codeine use in nursing mothers [152]. It is suggested that codeine should be avoided in breastfeeding mothers, who are extensive metabolizers (EMs) or UMs of CYP2D6 [153, 154]. The European Medicines Agency started a review of codeine-containing medicines on October 3, 2012, as well [155]. Young and obese children with history of sleep apnea are also at higher risk of developing severe opioid-related respiratory depression. The adverse outcomes of codeine treatment could be avoided and the safety of pain management could be improved by CYP2D6 genetic testing before prescribing the drug (tramadol, hydrocodone, or oxycodone) or by using alternative analgesics [156]. Another analgesic agent which is metabolized by CYP2D6 and where genetic examination is proposed is tramadol. The main metabolite is *O*-desmethyltramadol; (+)-*O*-desmethyltramadol has 300–400 times greater affinity for μ-opioid receptors than tramadol, whereas (−)-*O*-desmethyltramadol mainly inhibits noradrenalin reuptake [157]. Production of *O*-desmethyltramadol through mono-*O*-demethylation is mediated by polymorphic CYP2D6. As consequence, PMs need approximately 30% higher tramadol doses compared to EMs, and UMs are at greater risk to develop adverse effects of tramadol [47, 158]. Genetic testing of variants CYP2D6 is commercially available [159].

4.3. CYP2C. The group of CYP2C subfamily consists of four members: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. These enzymes metabolize approximately 20% of clinically available drugs. Their genes are tandemly located at 10q24 and there is a linkage between them. Genetic variants in CYP2C8, CYP2C9, and CYP2C19 have been shown to have clinical consequences. Among pain treatment, NSAIDs represent typical substrates for CYP2C enzymes; however, the relative role of CYP2C enzymes in primary metabolism differs among different NSAIDs.

Common CYP2C8 and CYP2C9 polymorphisms were studied by Blanco et al. in a cross-sectional study, involving 134 NSAID-related bleeding patients and 177 patients receiving NSAID with no adverse effects [160]. Among patients with bleeding after NSAID (CYP2C8/9 substrates), the frequencies of variant alleles carriers versus control patients were 0.50 versus 0.23 (odds ratio (OR); 95% confidence interval (CI) = 3.4; 1.5–7.5; $P = 0.002$), 0.48 versus 0.26 (OR; 95% CI = 2.7; 1.2–5.8; $P = 0.013$), and 0.24 versus 0.20 (OR; 95% CI = 1.3; 0.5–3.1; $P = 0.578$) for CYP2C8*3, CYP2C9*2, and CYP2C9*3, respectively. These findings were not influenced by gender, age, smoking, or drinking habits. Among bleeding patients receiving NSAID that are not extensively metabolized by CYP2C8/9, no differences in genotypes or allele frequencies were observed as compared to control patients. Similar findings have been shown by other authors; individuals carrying the gene variants CYP2C8*3 (rs11572080; rs10509681), CYP2C9*2 (rs1799853), or CYP2C9*3 (rs1057910) show increased risk of developing acute gastrointestinal bleeding during the use of NSAID that are CYP2C8 or CYP2C9 substrates [161, 162].

4.4. CYP2C19. Totally 36 alleles of gene CYP2C19 have been identified and described so far [48]. CYP2C19 is responsible for the metabolism of several clinically important drugs as citalopram, barbiturates, diazepam, and other drugs [52]. The roles of the cytochrome P450 2C19 enzyme and cytochrome P450 2D6 enzyme in citalopram metabolism were studied [163]. The inactive CYP2C19*2 (rs4244285) allele was associated with lower odd ratios for tolerance. The estimated dose adjustments for CYP2C19 poor metabolizers suggest using approximately 60% of the standard dose of citalopram [164]. Also the allelic variant CYP2C19*3 (rs4986893 or rs57081121) influences the total concentration of the active compounds venlafaxine and its active metabolite *O*-desmethylvenlafaxine. Thus, CYP2C19 genotypes (together with CYP2D6 genotypes) should be considered for dose alterations of venlafaxine [165].

4.5. CYP2C9. The pharmacokinetics of ibuprofen is strongly related to CYP2C8 and CYP2C9 genotypes. The effect of CYP2C8*3 (rs10509681 or rs11572080) on ibuprofen clearance is prominent; heterozygous and homozygous carriers of this variant allele display clearance reduced to approximately 62% and 10% as compared to individuals lacking any variants within CYP2C8 and CYP2C9 genes [166]. Although initial findings indicated association of CYP2C9*2 (rs1799853) genotypes with ibuprofen clearance, it has been shown that CYP2C9*2 alone, when it is not linked to CYP2C8*3, does not translate into a major impairment of ibuprofen clearance. Clearance values in subjects heterozygous and homozygous for CYP2C9*2 not carrying any other mutations are 96 and 84%, respectively, as compared to individuals lacking any mutations in CYP2C8 and CYP2C9 genes. Individuals

carrying CYP2C9*3 (rs1057910) variant alleles display a mean reduction of clearance of ~65% and 17% for heterozygous and homozygous individuals, respectively [167].

Studies with tenoxicam have indicated that oral clearance among carriers of CYP2C9*2 and CYP2C9*3 decreases to ~70 and 55% [168]; however, efficacy or safety data are not available yet.

4.6. CYP2C8. CYP2C8 comprises 7% of the total hepatic CYP content and plays an important role in the metabolism of a diverse number of exogenous (e.g., NSAIDs, carbamazepine, diltiazem, methadone, morphine, and zopiclone) and endogenous compounds (e.g., arachidonic acid) [55]. A number of common SNPs have been identified including CYP2C8*2 (Ile269Phe and rs11572103), CYP2C8*3 (linked polymorphism Arg139Lys and Lys399Arg, rs10509681, or rs11572080), and CYP2C8*4 (Ile264Met and rs1058930). One of the drugs implicated as CYP2C8 substrate is methadone. *In vitro*, CYP2C8 was shown to metabolize both the R- and S-enantiomers of methadone, with a greater selectivity for R-enantiomer [169]. Considering that the R-enantiomer is the more pharmacologically active form *in vivo*, the potential influence of CYP2C8 polymorphism on the metabolism of the R-enantiomer may be clinically significant and warrants further studies.

Allelic variants of CYP2C8, UGT2B7, and ABCC2, which may predispose for the formation and accumulation of reactive diclofenac metabolites, are associated with diclofenac hepatotoxicity [55]. Daly et al. showed that UGT2B7*2 allele (rs7439366) was more common in patients with diclofenac-induced hepatotoxicity when compared with hospital controls (OR, 8.5, $P = 0.03$) or healthy controls (OR, 7.7, $P = 0.03$). Further, the ABCC2 C-24T (rs717620) variant was more common in patients with hepatotoxicity compared with hospital (OR 5.0, $P = 0.005$) and healthy controls (OR 6.3, $P = 0.0002$). Haplotype distributions for CYP2C8 were different between patients and hospital controls ($P = 0.04$).

4.7. CYP3A4. CYP3A4, coded by the gene located on chromosome 7q21.1, is involved in the oxidation of the largest range of substrates of all the CYPs. CYP3A4 plays a role in the metabolism of some opioids as fentanyl, oxycodone, and methadone along with the other CYPs [148]. There is little conclusive information about the importance of genetic variation in the CYP3A pathway, but some studies of postmortem forensic toxicology propose pharmacogenomics of CYP3A4 as a kind of molecular autopsy in the analysis of pain-medications-related deaths. In studies of fentanyl-, oxycodone- or methadone-related deaths, the PM status was a clear risk factor [170–172].

4.8. UDP-Glucuronyltransferase. Glucuronidation is an important pathway of human metabolism that leads to the formation of water soluble glucuronides. The substrates for glucuronidation include both endogenous substances, such as bilirubin, steroid hormones, and bile acids, and exogenous substances such as morphine, antidepressants, or nonsteroidal anti-inflammatory drugs. The human genome codes for at least 19 different UDP-glucuronosyltransferases (UGTs) classified within three subfamilies, UGT1A, 2A, and 2B [173]. Genetic polymorphisms have been reported in virtually every UGT family member and many of them have potential clinical consequences. For example, morphine undergoes extensive metabolism by glucuronidation to form morphine-3-glucuronide and morphine-6-glucuronide which possess significant analgesic activity. The ability to glucuronidate morphine varies substantially between individuals. The major enzyme responsible for glucuronidation of morphine is UGT2B7. The variant homozygotes for T/T802 (Y/Y268) displayed the strongest catalyzing abilities toward morphine, and this genotype has been considered as a one of the possible causes of interindividual variability in therapeutic response to morphine [174].

A small cross-sectional study observed faster *in vivo* conjugation of salicylic acid in patients genotyped UGT1A6*2/*2 (rs2070959) than in the wild-type carriers. The faster conjugation may subsequently influence the therapeutic response to aspirin [175].

5. Drug Transport

There are many families of transporters and some of them are known to be interacting with pathways of pain-transporting analgesics, prostaglandins [22]. So far the most known and best studied transporter is P-glycoprotein (Pgp), the first-studied member of ATP-binding cassette (ABC) superfamily. In humans, Pgp consists of two isoforms with 78% amino acid homology. Overexpression of isoform I (ABCB1) in cancer cells was linked with resistance to multiple drugs, hence the name for this transporter multidrug resistance protein 1 (MDR1). Isoform II (MDR2/ABCB4) transports phosphatidylcholine into the bile and is not involved in drug transport [176, 177].

Summary of known implications of genetic variability in genes for drug and neurotransmitter transporters is given in Tables 6 and 7, respectively.

5.1. MDR1/ABCB1. Pgp is known to show extremely broad substrate specificity, including peptides, steroids, therapeutic drug from very large and complex ones as paclitaxel [178] to relatively simple as phenytoin, opioids, and other analgesics [179] or even ions [180–186]. Substrates are often, but not always (e.g., colchicine), amphipathic and relatively hydrophobic. Planar aromatic ring and tertiary amino group were also proposed as required structure elements, but many peptides do not have them, and yet they are substrates of Pgp [187].

In addition to this broad substrate specificity, Pgp is also very abundant in the body as it can be found in most tissues [188], although in significantly larger amount on the apical surface of the endothelial cells lining the small intestine, colon, kidney, adrenal gland, bile ductules, thus in the tissues with excretory (or absorptive) function in general. In addition, also in cells with "barrier" function, that is, cells in blood brain [189], blood testis [190], blood

Table 6: Impact of genetic variability in genes for drug transporters.

Transporter	Drug	Genetic variability	Effect	Reference
MDR1/ABCB1	Methadone	61 (rs9282564), 1199 (rs2229109), 1236 (rs1128503), 2677 (rs2032582), and 3435 (rs1045642)	Lower dosage	[76]
		C1236 (rs1128503)	Higher dosage	[77]
		61A (rs9282564) : 1199G (rs2229109) : 1236C (rs1128503) : 2677T (rs2032582) : 3435T (rs1045642)	Higher dosage	[78]
		61A (rs9282564) : 1199G (rs2229109) : 1236C (rs1128503) : 2677T (rs2032582) : 3435T (rs1045642)	Lower through concentrations	[78]
		61G (rs9282564) and 3435T (rs1045642)	Lower through concentrations	[79]
		3435T (rs1045642)	Higher dosage	[80]
		C3435T (rs1045642)	Pain relief	[27]
		3435TT (rs1045642)	Higher CSF concentrations	[81]
	Morphine	CC3435 (rs1045642)	Higher CSF concentrations of morphine glucuronides	[81]
		GG2677 (rs2032582) and CC3435 (rs1045642)	Fewer side effects	[82]
		G2677 (rs2032582) and C3435 (rs1045642)	Vomiting	[61]
		3435T (rs1045642)	Less frequent pain scores >6	[83]
	Fentanyl	3435T (rs1045642)	Suppression of respiratory rate	[84]
	Tramadol	3435TT (rs1045642)	Higher C_{max}	[85]
	Oxycodone	3435T, 2677A (rs2032582)	Fewer side effects	[86]
		2677A (rs2032582)	Better analgesic activity	[86]
	Lamotrigine	C1236 (rs1128503)	Higher dose corrected concentrations	[87]
	Gabapentin	2677T/A (rs2032582)	Trend towards higher AUC(0–1.5 h)	[88]
		CC1236 (rs1128503)	Significantly lower clearance	[89]
	Carbamazepine	CC3435 (rs1045642)	Lowest plasma levels	[90]
		3435TT (rs1045642)	Decreased plasma levels	[91]
	Venlafaxine	MDR1/ABCB1(−/−) mice	Higher plasma levels	[92–94]
	Venlafaxine's metabolites	MDR1/ABCB1(−/−) mice	Higher plasma levels	
	Venlafaxine	TT in rs2232583	Higher plasma levels	[95]
	Trimipramine	MDR1/ABCB1(−/−) mice	Higher plasma levels	[96]
	Amitriptyline	MDR1/ABCB1(−/−) mice	Higher plasma levels	[97, 98]
MRP2/ABCC2	Diclofenac	MRP2/ABCC2(−/−) mice	Impaired clearance	[99]
	Diclofenac	24T (rs717620)	Hepatotoxicity	[55]
	Carbamazepine	AA + AG in rs2273697	Higher clearance	
		AA + AG in rs4148386	Higher clearance	[89]
	—	rs2756109	Pain	[100]
MRP3/ABCC3	Morphine	MRP3/ABCC3(−/−) mice	Increase in plasma levels of its glucuronides	[101]
MRP4/ABCC4	—	MRP4/ABCC4(−/−) mice	Decreased pain responsiveness	[102]
	—	rs9524885	Pain	[100]

Table 7: Impact of genetic variability in genes for transporters of neurotransmitters.

Transporter	Drug	Genetic variability	Effect	Reference
5-HTT	Remifentanil	Triallelic 5-HTTLPR	Better analgesic effect	[103]
	—	Low 5-HTT-expressing	Higher pain thresholds	[104, 105]
		Tandem-repeat polymorphism 2.10	Lesser temporomandibular joint pain and dysfunction	[106, 107]
		10/12 and 10/10 STin2.12 alleles	Protective effect against migraine	[108]
		14/14 sequence repeats	Higher frequency of abdominal pain	[109]
			More frequent in fibromyalgia patients	[110]
DAT	—	DAT*10	More frequent in migraine-without-aura-group	[111]
EAAT2	Analgesics	A allele in −181A/C	Higher usage	[112]

mammary tissue [191], blood inner ear barrier [192], and in placenta [193], protecting respective tissues (or fetus [194]) from toxins in the blood. Recent studies show that this may not be the only one physiological function of Pgp or even the crucial one. It seems that Pgp is involved in the inhibition of apoptosis induced by a number of factors as tumor necrosis factor and ultraviolet and gamma radiations [195]. Further, it was shown that blocking Pgp by antibodies induced apoptosis of activated lymphocytes in peripheral blood, and MDR1/ABCB1 seems to regulate even stem cells [196]. Secretion of various cytokines (interleukin 2 and 4, interferon γ) is mediated by Pgp [197].

Pgp is a product of the ATP-binding cassette, subfamily B (MDR/TAP), member 1 gene (*ABCB1*), gene of 209617 bp with 29 exons of total length 4872 bp located on chromosome 7q21.12. There are 1425 known SNPs to date with average. distance of 161 bp; 46 of them are nonsynonymous: two of them in introns and 46 in coding sequence of exons [198]. The synonymous SNPs and SNPs in promoter regions could influence the expression level of MDR1/ABCB1, for protein activity, that is, its substrate binding, ATP hydrolysis and folding, are most important probably the nonsynonymous SNPs [195]. Because of the important role of Pgp in drug disposition, it seems as a fair presumption that such SNPs could have clinical importance in drug pharmacokinetics and, by extension, in pharmacodynamics (and even direct impact on PD in tumor cells with overexpression of MDR1/ABCB1). Campa et al. found that variability of pain relief in 145 patients on morphine treatment was significantly associated with SNP C3435T (rs1045642). The association was stronger; when C3435T was combined with A80G in OPRM1 SNPs were taken into account [27]. There were significant C3435T-dependent differences in morphine concentrations in cerebrospinal fluid (CSF) with the highest levels in CSF in TT carriers of SNP C3435T and the highest morphine-6- and 3-glucuronide concentrations in CSF in wild-type homozygotes [81]. Response to morphine was dependent on SNP C3435T in children in a recent study using Faces Pain Scale (FPS). Scores >6 were more frequent in 11 checks during 24 hours after orthopedic or abdominal surgery in carriers for the wild-type alleles (adjusted risk ratio = 4.5; 95% confidence interval (CI), 1.5–13.4; corrected CI for multiple comparisons, 0.98–20.55) [83].

For fentanyl, variability in suppression of respiratory rate (significant only for C3435T and diplotype) and need for oxygen (increased in carriers of 1236T (rs1128503) and 3435T alleles, $P = 0.0847$) were observed, and significant differences in the level of respiratory suppression were found in patients with linked 3435T and 2677T (rs2032582) alleles [84].

The atypical opioid tramadol was proposed to be a subject of P-glycoprotein-dependent transport, as there were significant differences in its C_{max} and borderline significant differences in AUC_{0-24} amongst different genotypes for MDR1/ABCB1 in CYP2D6 poor metabolizers [85]. Conversely, no significant differences among MDR1/ABCB1 subgroups with regards of pain difference, drug consumption, reporting of adverse reactions, need for rescue analgesic medication, or verbal description of pain were observed [158]. For oxycodone, strong associations between variant alleles 3435T and 2677A and less adverse drug reactions and better analgesic effect and variant 2677A were found in study with 33 healthy volunteers and experimental pain [86].

Apart from opioids, Pgp is believed to transport several antiepileptic drugs (AED), for example, lamotrigine, gabapentin, topiramate, valproic acid, and carbamazepine and its ketoanalog oxcarbazepine, although there is no consensus about this and studies with positive [199–201] or negative results [202–204] may be found. The efflux may play facilitatory role in refractory epilepsy [205–207], although contradictory results are also available [208]. These agents are widely used in the treatment of neuropathic pain. There are only pharmacogenetic studies in epilepsy in association with MDR1/ABCB1 polymorphisms, but their results could give some guidance about the impact of SNPs in MDR1/ABCB1 in the treatment of neuropathic pain.

In the case of lamotrigine, homozygotes for the C allele in C1236T have had significantly higher lamotrigine dose corrected concentrations ($0.068\ \mu mol \cdot l^{-1} \cdot mg^{-1}$) than subjects with CT or TT ($0.053\ \mu mol \cdot l^{-1} \cdot mg^{-1}$). Furthermore, 1236C-2677G-3435C carriers have had higher lamotrigine concentrations than 1236T-2677G-3435T carriers ($P < 0.001$), followed by 1236T-2677T-3435C carriers ($P < 0.001$) [87].

Change in gabapentin's disposition due to 2677T/A MDR1/ABCB1 alleles was less pronounced resulting only in trend toward higher values of the absorptive phase characterized by the AUC (0-1 h) and AUC (0–1.5 h) [88].

SNP C3435T did not influence disposition of valproic acid as its serum concentrations of the patients with CT, TT and CC genotypes were 72.92 ± 20.55, 80.47 ± 14.01, and 68.29±12.17 μg/mL, respectively, and there was no significant difference [209].

Carbamazepine appears to be subject of Pgp transport, its clearance was associated with rs1128503, being significantly lower in subjects with alleles CC versus CT + TT [89]. The median total carbamazepine plasma levels were the lowest in CC (20 μmol/L) homozygotes followed by CT (23 μmol/L) and TT (29 μmol/L) carriers of SNP 3435 [90]. However, Meng et al. suggested that ABCB1 3435TT is associated with decreased plasma carbamazepine levels in Chinese patients with epilepsy [91]. On the other hand, Hung et al. did not find any difference in carbamazepine levels among genotype groups for SNPs C1236T, G2677T/A, and C3435T in MDR1/ABCB1 [210]. Moreover, some studies did not found any association between C3435T and epilepsy treatment response [211] or with dosage [212].

Speaking of neuropathic pain, even tricyclic antidepressant drugs, such as amitriptyline, nortriptyline, desipramine, and SNRI (venlafaxine and duloxetine), are being used for treating this condition, all of which are subject to the Pgp mediated efflux [213–219], which was implicated to be associated with refractory depression [220].

In knockout mice, venlafaxine and its three demethylated metabolites reached significantly higher concentrations than in wild-type mice [92–94]. Similar results were obtained with trimipramine [96] and amitriptyline [97, 98].

Case study was reported in which SNP rs2232583 in MDR1/ABCB1 apparently resulted in excessive plasma levels of venlafaxine and its metabolite desmethylvenlafaxine in the patient [95].

5.2. MRP. Multidrug resistance-associated proteins (MRP) also belong to the ABC transporters family, subfamily ABCC, which consists of 12 members, nine of them are MRPs. Similar to MDR1/ABCB1, they utilize ATP but share only 24% of amino acid sequence homology [221]. Thus, there are distinct substrate specificity, inhibitors, and tissue distribution as compared to P-glycoprotein. Mainly, three of MRPs are known to interact with the pathways of pain: MRP2/ABCC2, which transfers diclofenac's metabolites [55, 99], MRP3/ABCC3, which was shown to transfer morphine [101], and diclofenac's glucuronides [99], and MRP4/ABCC4, which transports most prostaglandins [222] (even proposed as prostanoid export pump [223, 224]) and acetylsalicylic acid [225, 226].

As for MRP2/ABCC2, genetic variability could lead to impaired clearance of diclofenac and its glucuronides and hence to hepatotoxicity, as it was shown with knockout mouse [99]. One SNP (C24T, rs717620) in 5′-untranslated region was associated with decreased mRNA expression [227, 228], although many other SNPs have been found [229, 230]. Allele 24T was found to be more prevalent in patients with hepatotoxicity as compared with patients taking diclofenac for 0.3–20 years ($n = 48$) without hepatotoxicity (OR 5.0, $P = 0.005$) and healthy controls (OR 6.3, $P = 0.0002$) [55].

Carbamazepine appears to be subject of ABCC2 transport, as its clearance was significantly higher in subjects with alleles AA + AG versus GG in rs2273697 and rs4148386 [89]. Ufer et al. strengthen this assumption since in their study carriers of the ABCC2 1249G>A (rs934847) variant were more frequently classified as responders to treatment and this impact was even more pronounced among 64 patients receiving carbamazepine or oxcarbazepine [231].

Involvement of MRP4/ABCC4 in export of prostanoids could have significant clinical implication for nociception and analgesia, as was shown in MRP4/ABCC4 knockout mice in study by Lin et al., where disruption of MRP4/ABCC4 resulted in decreased pain responsiveness [102]. Further, recent study associated SNP rs9524885 in MRP4/ABCC4 (as well as rs2756109 in MRP2/ABCC2) with pain in nonsmall-cell lung cancer patients [100].

5.3. SLC22A6. Another drug transporter known to transport analgesics, particularly NSAIDs [232], is SLC22A6, human organic anion transporter 1. However, the clinical relevance for chronic pain treatment of its variation has not been clarified yet.

5.4. SLCO1B1. SLCO1B1, also known as OATP2, was shown to transport opioid peptides across blood brain barrier [233] therefore, it is possible that genetic variability may have influence on the pain perception. This has not been clinically assessed yet, but immunofluorescence microscopy and uptake measurements were used to study localization and transport properties. The polymorphisms SLC21A6*1b and SLC21A6*4 have been associated with altered transport of cholyltaurine and 17 beta-glucuronosyl estradiol [234].

5.5. 5-HTT. Genetic polymorphism of serotonin transporter (5-HTT) has also been associated with alteration of pain pathways. For example, study with 43 healthy volunteers found that subjects with the triallelic 5-HTTLPR genotype coding for low 5-HTT expression gained better analgesic effect of remifentanil compared to those homozygous for the 5-HTTLPR LA allele, although the baseline sensitivity to heat pain was not affected by the triallelic 5-HTTLPR polymorphism [103]. Low 5-HTT-expressing group compared to the high 5-HTT-expressing group exhibited significantly increased pressure pain and heat-pain thresholds [104], while contradicting results have been described by Aoki et al. [235]. Association between inferred low 5-HTT expression and elevated thresholds to thermal pain was found in 44 healthy nondepressed individuals [105]. Similarly, homozygotes for variable-number tandem-repeat polymorphism 2.10 suffered less from temporomandibular joint pain and dysfunction [106, 107]. Two recent meta-analyses found that non-STin2.12 alleles possess protective effect compared to STin2.12 alleles, respectively, 10/12 and 10/10 genotypes compared to the 12/12 genotype against migraine among populations of European descent [108],

while no overall association between the SLC6A4 5-HTTLPR polymorphism and migraine among Europeans and Asians was found, though gender and migraine aura status may have modifying roles among Europeans [236].

Repeat variation polymorphism in 5-HTT gene consists of a short (s) variation of 14 repeats of a sequence and a long (l) variation of 16 repeats. Subjects with irritable-bowel syndrome with s/s genotype for 5-HTT have suffered more often from abdominal pain than l/s and l/l [109]. A significantly higher frequency of the s/s genotype of the serotonin transporter promoter region was found in fibromyalgia patients (31%) compared with healthy controls (16%) in study with 62 patients and 110 healthy controls [110], but this was later contradicted in different study with 53 mentally healthy subset of fibromyalgia patients and 60 healthy controls [237].

5.6. Others. Dopamine transporter (DAT, SLC6A3) and glutamate transporter protein excitatory amino acid transporter 2 (SLC1A2, EAAT2) polymorphisms have also been reported to affect pain perception. Allele DAT*10 was significantly underrepresented in patients with chronic daily headache associated with drug abuse when compared with the migraine-without-aura group [111] and A allele carriers of −181 A/C in EAAT2 polymorphism used significantly more analgesics than non-A carriers in migraine patients with chronic daily headache [112].

6. Conclusion

There is number of candidate genes whose genetic variability may translate in either individual variation of chronic pain perception or treatment response. The clinical data from pharmacogenetic studies is still very limited and heterogenous as a result of various methodologies used in different studies, generally small sample sizes and heterogenous patient populations. Therefore, there is still a need for further clarifications of the clinical importance for all these findings, but the recent research in the field that encompasses larger studies and larger-scale genome perspectives may bring more promising findings in the future.

Abbreviations

CYP: Cytochromes P450
DME: Drug-metabolizing enzyme
EM: Extensive metabolizer
FDA: Food and drug administration
IM: Intermediate metabolizer
NSAID: Nonsteroidal anti-inflammatory drug
PK: Pharmacokinetics
PM: Poor metabolizer
SNP: Single-nucleotide polymorphism
SNRI: Serotonin-norepinephrine reuptake inhibitor
SSRI: Selective serotonin reuptake inhibitor
SULT: Sulfotransferase
TCA: Tricyclic antidepressant
UGT: UDP-glucuronyltransferase
UM: Ultrarapid metabolizer.

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Review Article

Neuropathic Pain in Animal Models of Nervous System Autoimmune Diseases

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Neuropathic pain is a frequent chronic presentation in autoimmune diseases of the nervous system, such as multiple sclerosis (MS) and Guillain-Barre syndrome (GBS), causing significant individual disablement and suffering. Animal models of experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN) mimic many aspects of MS and GBS, respectively, and are well suited to study the pathophysiology of these autoimmune diseases. However, while much attention has been devoted to curative options, research into neuropathic pain mechanisms and relief has been somewhat lacking. Recent studies have demonstrated a variety of sensory abnormalities in different EAE and EAN models, which enable investigations of behavioural changes, underlying mechanisms, and potential pharmacotherapies for neuropathic pain associated with these diseases. This review examines the symptoms, mechanisms, and clinical therapeutic options in these conditions and highlights the value of EAE and EAN animal models for the study of neuropathic pain in MS and GBS.

1. Introduction

Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system either at the peripheral or central level and is a frequent presentation in a myriad of medical conditions [1]. It is characterised by abnormal sensations or hypersensitivity in the affected area, which is often combined with, or is adjacent to, areas of sensory deficit [2]. Symptoms include tactile or thermal hypoaesthesia (reduced sensation to nonpainful stimuli), hypoalgesia (reduced sensation to painful stimuli), loss of sensation, paraesthesia (abnormal sensations such as skin crawling or tingling), paroxysmal pain (e.g., shooting, electric shock-like sensations), spontaneous ongoing pain (not induced by stimulus e.g., burning sensation), and evoked pain (i.e., stimulus-induced pain), the last of which includes hyperalgesia (increased sensitivity to painful stimuli) and allodynia (perception of innocuous/non-painful stimuli as painful) [2]. In particular, neuropathic pain is common in autoimmune demyelinating diseases of the nervous system, such as multiple sclerosis (MS) and Guillain-Barre syndrome (GBS), and adversely affects millions of sufferers worldwide [3, 4].

Thus far, several animal models have been established to mimic features of MS and GBS, so as to better enable researchers to understand the underlying pathophysiology and immune mechanisms and to investigate better therapeutic options. For example, experimental autoimmune encephalomyelitis (EAE) serves as the classic animal model of multiple sclerosis, whereas experimental autoimmune neuritis (EAN) mimics acute inflammatory demyelinating polyneuropathy, the most common subtype of GBS [5–7]. These two models are the most widely used and accepted analogues of MS and GBS and provide many immunological parallels. In this review, we discuss the symptoms, mechanisms, and potential therapeutic strategies in neuropathic pain associated with EAE and EAN.

2. Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Multiple sclerosis is a chronic, T-cell mediated autoimmune inflammatory disease of the central nervous system (CNS) that predominantly affects the myelin sheath. It is the most common cause of acquired disability in young adults in the western world [8–10]. Among the many sensory disturbances present in MS, pain—nociceptive, neuropathic, or mixed—is a highly prevalent symptom, reported by 25 to 90% of patients

[3, 11–17]. It negatively impacts on general health, energy and vitality, mental health, and social functioning [17, 18], as well as impinges on daily life [12, 14, 19]. Despite its prevalence, the specific underlying mechanisms of MS pain are still not well understood [20], although elucidation has been sought through recent studies in animal models [21–23].

Experimental autoimmune encephalomyelitis has frequently served as an animal model of MS. EAE is commonly induced in genetically susceptible animal strains by immunisation with a self-antigenic epitope of myelin, which causes characteristic breakdown of the blood-brain barrier and multifocal infiltration of activated immune cells that attack the myelin sheath [8]. The ensuing immunologic response leads to chronic neuroinflammation, demyelination, and neuronal damage in the CNS. The species-specific disease course exhibits close clinical and histopathological similarities to various forms of MS [24–26], thereby presenting EAE as a suitable model to study multiple sclerosis [27, 28].

3. Symptoms of Neuropathic Pain in MS and EAE

MS patients often experience a wide range of neuropathic pain symptoms. This includes ongoing extremity pain (characterised by constant pain in the legs and feet), trigeminal neuralgia (characterised by paroxysmal attacks of electric-shock-like sensations in specific facial or intraoral areas), Lhermitte's phenomenon (characterised by a transient electrical sensation that runs down the back and is related to neck movement), and thermal and mechanical sensory abnormalities [18, 29]. As behavioural models of ongoing extremity pain and paroxysmal pain in animals are currently unavailable, most animal studies have focused on thermal and mechanical abnormalities (Table 1). EAE has thus far served as the basis for preclinical research into the mechanisms of these abnormalities. Genetic, clinical, and histopathological heterogeneities of EAE models produce different sensory and pathological changes, allowing for robust representation of the various forms of pain in MS [30].

3.1. Heat Disturbances. In multiple sclerosis, neuroinflammatory lesions in the CNS produce significant somatosensory deficits, particularly for temperature discrimination, such as paradoxical heat sensations and altered heat/cold thresholds [38–40]. Up to 58% of MS patients have reported suffering from heat sensitivities, which is a significant cause of fatigue, concentration problems, and pain [41]. Such abnormalities have been paralleled in several EAE studies.

In an early study, Duckers and associates noted 23%–58% prolongation of reaction time to noxious heat at 10 and 18 weeks following EAE induction in Lewis rats, suggestive of chronic hypoalgesia [31]. More recently, Aicher and colleagues observed a dynamic thermal response in a chronic relapsing-remitting form of EAE induced by a myelin proteolipid protein (PLP). In both male and female SJL mice, initial thermal hypoalgesia occurred concurrent with onset of clinical symptoms, later manifesting as chronic thermal hyperalgesia of the tail. Hyperalgesia was more sustained in female mice [21], reflecting a sex-linked disease profile [42]. While the magnitude and duration of tail hyperalgesia were seen to be related to the severity of motor symptoms, thus potentially cofounding the results, it was noted that the onset of hypoalgesic nociceptive responses preceded motor dysfunctions by several days [21].

Similar studies in chronic EAE induced in C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) also reported heat hypoalgesia in the hindpaws, developing subsequent to symptomatic onset of disease, although this was also theorised by the investigators to be affected by concurrent gross locomotor disabilities [22]. In contrast, thermal hyperalgesia in the hindpaws developed during the chronic disease phase in SJL and C57BL/6 mice immunised with PLP or MOG, respectively [30]. In a study of both acute and chronic EAE induced by myelin basic protein (MBP) in rats, comparable tail heat allodynia was reported, with the onset of thermal abnormalities appearing prior to the development of clinical signs [23].

These findings show differential thermal responses and concur with case reports of heat hypoalgesia [43], as well as thermal hyperalgesia [18, 44, 45] in MS patients.

3.2. Cold Disturbances. Cold allodynia, a reported sensory disturbance in MS patients [18], has been observed in several EAE models. In particular, cold allodynia in response to application of acetone to the hindpaws has been demonstrated in mice with a MOG-induced chronic-relapsing EAE prior to and during onset of motor disturbances [22]. Similarly, cold sensitivity at the level of the hindpaws was noted in EAE rats that were tested on a cold plate, starting before and lasting during and after clinical signs [23]. In the latter investigation, cold hyperalgesia at the level of the tail was also observed, although this was only present prior to clinical onset of EAE. Thibault and colleagues also detected no significant differences in cold allodynia and hyperalgesia between both acute and chronic EAE models, suggesting that abnormalities to cold sensitivities are independent of EAE phenotype [23]. The early onset of cold allodynia parallels the observation that neuropathic pain in MS patients often precedes or is present at clinical onset [11].

3.3. Mechanical Disturbances. In addition to thermal abnormalities, MS patients often experience tactile allodynia [11, 18, 46]. For example, both tactile hypoesthesia (reduced sensation to touch) and allodynia have been reported in relapsing remitting forms of MS [45, 47, 48]. A recent study reported high prevalence of hypoesthesia and hyperesthesia (61% and 34%) in patients with MS and central neuropathic pain, although this was similar to a control group of MS patients with painless sensory symptoms [40].

In chronic relapsing EAE models (using MOG_{35-55} in mice), robust mechanical allodynia became apparent prior to clinical signs [22], although the response times to tactile stimuli increased during disease peak (hypoalgesia), and reduced following partial amelioration of motor dysfunction. Again, this suggests confounding influence of mechanical paralysis. Similar studies using the same encephalitogenic

Table 1: Summary of pain symptoms observed in EAE models and potential therapeutic options for treating MS pain.

EAE	Antigen	Animal model	Test site	Test	Symptoms during disease	Drug	Effects of drug	References
Acute	500 μg MBP	♀ 5 wk Lewis rats	Tail	Water bath immersion	Heat allodynia (before clinical signs and after resolution)[1]			[23]
			Hindpaw	Cold plate analgesia meter	Cold hyperalgesia[2] (before clinical signs)			
					Cold allodynia[3]			
					Cold hyperalgesia[3] (during clinical signs)			
				Pinch test	Mechanical hyperalgesia (after emergence of clinical signs)			
				Electronic von Frey	No significant difference			
CREAE	500 μg MBP+ Cyclosporine A	5 wk ♀ Lewis rats	Tail	Water bath immersion	Heat allodynia (before clinical signs and after resolution)[1]	Gabapentin	Reduce mechanical hyperalgesia	[23]
			Hindpaw	Cold plate analgesia meter	Cold hyperalgesia (before clinical signs)[2]			
					Cold allodynia[3]	Tramadol	Reduce thermal and mechanical hyperalgesia; reduce cold allodynia; known analgesics	
					Cold hyperalgesia[3] (during recovery)			
				Pinch test	Mechanical hyperalgesia (at disease peak and recovery)			
				Electronic von Frey	Mechanical allodynia (after clinical signs)	Duloxetine	Reduce thermal hyperalgesia; reduce cold allodynia	
CEAE	100 μg GPSCH	♀ Lewis rats	Paws	Hot plate	23% prolonged reaction to noxious heat (hypoalgesia @ wk 10)[4]	ACTH4-9[5]	Reduce neurological signs and facilitate recovery of sensory function	[31]
					58% prolonged reaction to noxious heat (hypoalgesia @ wk 23)[4]			
CREAE	50 μg MOG_{35-55}	♀ Lewis rats	Hindpaw	Hargreaves test	Heat hypoalgesia at disease peak and recovery			[22]
				Acetone application	Cold allodynia before clinical signs and at disease onset			
				Manual von Frey	Tactile allodynia at disease onset			
CREAE	50 μg GPMBP	Lewis rats	Tail	Vocalisation to noxious mechanical stimuli	Ascending pattern of absent vocalisation			[32]
CREAE	150 μg $PLP_{139-151}$	M/F SJL mice	Tail	Hargreaves test	Heat hypoalgesia at disease peak followed by heat hyperalgesia at disease recovery			[21]

Table 1: Continued.

EAE	Antigen	Animal model	Test site	Test	Symptoms during disease	Drug	Effects of drug	References
CREAE	40×10^6 T cell blasts[6]	♀ SJL mice	Tail	Hargreaves test	Heat hypoalgesia at disease peak followed by heat hyperalgesia at disease recovery			[21]
CREAE	100 μg MOG_{35-55}	♀ C56BL/6J mice	Hindpaw	Electronic von Frey	Mechanical allodynia before clinical signs			[33]
CREAE	50 μg MOG_{35-55}	♀ C56BL/6J mice	Hindpaw	Formalin injection	Hyponociception before clinical signs			[34]
RREAE	35 μg MOG_{1-125}	♂ Dark Agouti rats	Hindpaw	Manual von Frey	Mechanical allodynia (before clinical signs and during recovery)	IL-10 gene therapy[7] (plasmid DNA)	Simulates anti-inflammatory response Significant decrease of disease severity Prevention of mechanical allodynia	[35]
CEAE	300 μg MOG_{35-55}	♀ C56BL/6J mice	Paw	Manual von Frey	Mechanical allodynia (during disease course)	Rapamycin[8]	Blocks cytokine-driven T-cell proliferation Reduced EAE development Inhibition of mechanical allodynia after development of clinical signs	[36]
RREAE	8.75 μg MOG_{35-55}	♂ Dark Agouti rats	Paw	Manual von Frey	Mechanical allodynia (at disease onset)	Ceftriaxone[9]	Upregulates glutamate transporter to reduce excess intracellular glutamate and excitotoxicity Significant decrease of disease severity Reverse tactile allodynia	[37]

CEAE: chronic experimental autoimmune encephalomyelitis; CREAE: chronic relapsing experimental autoimmune encephalomyelitis; RREAE: relapsing remitting experimental autoimmune encephalomyelitis; MBP: myelin basic peptide; MOG: myelin oligodendrocyte glycoprotein; PLP: proteolipid peptide; GPSCH: guinea pig spinal cell homogenate.
[1]42°C; [2]2°C; [3]cooled from 22°C to 0°C, demonstrating hyperalgesic or allodynic nociceptive behaviour; [4]54°C; [5]repeated s.c. injections of ACTH4-9 peptide; [6]passive immunisation with transfer of $PLP_{139-151}$ specific splenocytes from EAE-induced animals; [7]intrathecal plasmid DNA IL-10F129S, 100 μL at disease onset and 25 μL 3 days later; [8]1 mg/kg i.p. Rapamycin on D11, and every 2 days thereafter; [9]daily intrathecal 150 μg ceftriaxone injection at symptom onset.

antigen (MOG_{35-55}) elicited comparable results of hypernociception [33, 36], although without hypoalgesia at disease peak. A recent study has demonstrated that the development of mechanical sensitivity is dependent upon the EAE model used; whereas SJL mice immunised with MOG developed marked mechanical allodynia during the chronic phase of the disease, C57BL/6 mice immunised with PLP developed only minor mechanical allodynia during disease onset and peak phases [30].

The robust nociceptive changes were similarly observed in a study using a rat model of MOG-induced EAE, showing periods of both decreased sensitivity to touch prior to the onset of hindlimb paralysis and increased sensitivity to touch (mechanical allodynia) during symptomatic remission [35]. Interestingly, a study using 2 doses of MOG in rats established that a 12.5% reduction in the dosage of the encephalitogenic peptide was sufficient to significantly ameliorate motor deficit profiles but did not significantly alter the robust pain states, thereby highlighting the partial independence of evoked pain presentations to motor dysfunctions [37]. This is a concept previously established through a novel study, whereby the investigator observed the absence of vocalised pain response despite noxious mechanical stimulation of the paralysed tail [32]. As vocalisation reflex can occur unhindered by tail paralysis, it can be surmised that motor paralysis (in this case, of the tail) is not the sole cause of diminished pain behaviours.

4. Potential Mechanisms of Neuropathic Pain in MS and EAE

As the importance of pain as a functional disability of multiple sclerosis has only recently been recognised, a clear understanding of its pathogenesis is still absent. Several theories exist to explain its mechanism, including lesions of CNS areas that process pain information, generation of enhanced response to painful stimuli due to loss of descending inhibitory nociceptive pathways, damage to somatosensory nerves, and inflammation of the spinal cord [10, 21, 29, 49].

Some of the proposed mechanisms of neuropathic pain in MS patients include thalamic or cortical deafferentation due to multiple lesions along the spinothalamocortical pathways generating ongoing extremity pain, high-frequency ectopic discharges due to demyelination of the trigeminal afferents producing symptoms of trigeminal neuralgia, and high-frequency ectopic discharges due to demyelination of the dorsal column primary afferents causing Lhermitte's phenomenon [10]. While these mechanisms have not yet been validated through animal studies, preclinical studies suggest that inflammation and gliosis are key mediators in changes in sensory functions (such as cold and tactile allodynia) seen in EAE.

It is well accepted that inflammatory cells and immune-like glial cells and their mediators facilitate central sensitisation and contribute to neuropathic pain symptoms [50]. Indeed, a recent study has shown that animals with EAE did not have altered expression of sensory neuropeptides but had a significant influx of CD3+ T cells and increased astrocyte and microglia/macrophage reactivity in the superficial dorsal horn of the spinal cord, an area associated with pain processing [22]. Furthermore, a significant increase in the level of tumour necrosis factor α (TNF) expression in the dorsal root ganglia (DRG) of EAE animals was found at disease peak [51]. A later study confirmed a correlation between the increase in TNF gene and protein expression in the DRG and spinal cord with the onset of neuropathic pain in rats with EAE [52]. Similar increases in the gene expression of cytokines interleukin (IL)-1β and IL-6 in the spinal cords of EAE mice coincided with increased nociceptive sensitivity and deficits in object recognition [53]. Gene therapy with anti-inflammatory IL-10 in animals with EAE improved motor and sensory function, prevented allodynia, and reduced glial activation in the lumbar spinal cord [35].

Further mechanisms have implicated the accumulation of infiltrating macrophages expressing purinergic $P2X_4$ receptors ($P2X_4R$) in CNS lesions of EAE animals [54]. As activation of these receptors by adenosine triphosphate is implicated in the microglial response to peripheral nerve injury and neuropathic pain symptoms, an association between $P2X_4R$ and neuropathic pain in EAE is suggested [55]. Additionally, increased phosphorylation of transcription factor cyclic AMP response element-binding protein (CREB) has also been observed at disease peak in EAE lesions, particularly in the dorsal horn sensory neurons [56], which are associated with the generation and maintenance of neuropathic pain. Similar involvement of chemokines in leukocyte recruitment, immune regulation, and T-cell polarisation is believed to significantly impact on pain regulation. For example, CCL2, a chemokine with elevated levels in MS patients, amplifies inflammatory responses in EAE [57, 58], while intrathecal administration of CCL2 chemokine is sufficient to induce mechanical allodynia in naïve animals but not in CCL2-receptor knockout mice [59–61].

Dysregulation of the glutamatergic system, caused by reduced glutamate transporter expression in spinal cords, has been implicated in abnormal pain sensitivity in mice with MOG-induced EAE. For example, EAE mice showed a lack of behavioural response to formalin stimulation, a behavioural model of injury-induced central sensitization. This hyporesponsiveness was attributed to a decreased expression of the glutamate transporters EAAT-1 and EAAT-2 in the spinal cord [34]. Furthermore, pharmacological treatment to upregulate the levels of EAAT-2 in mice with EAE resulted in prevention of tactile hypersensitivity and normalisation of performance in cognitive assays [53].

5. Clinical Applications of EAE Neuropathic Pain Models

EAE has proven to be a successful therapeutic preclinical model for MS. Indeed, a number of approved drugs and current phase II and III trials for MS were first examined in EAE models [62].

Several pharmacotherapies used to treat pain in multiple sclerosis have shown similar efficacies in EAE. For example, Gabapentin, a γ-aminobutyric acid (GABA) analogue used

by up to 19% of MS sufferers [63, 64], is highly effective in ameliorating pain symptoms in MS, such as trigeminal neuralgia and tonic spasms [65–68]. Moreover, using Gabapentin, Thibault and colleagues demonstrated a significant reduction of mechanical hyperalgesia in EAE murine models, highlighting the effectiveness of GABA analogues and their therapeutic potentials on neuropathic pain in EAE models [23].

There also exist several promising avenues of pharmaceutical research. Lisi and associates have established that prophylactic Rapamycin administration, a macrocyclic antibiotic with immunosuppressive activity, is able to reduce disease severity and ameliorate pain behaviour in EAE animals [36], confirming similar rodent studies [69–71]. It is theorised that by regulating effector T cell and regulatory T-cell function [72], Rapamycin is able to modulate cytokine release, particularly interferon (IFN)-γ [73], a potent cytokine implicated in neuropathic pain [50].

Another promising candidate for MS pain amelioration targets glutamate transporters. MS patients are known to have an elevated concentration and/or altered transport of glutamate in the CNS [74–78], partly due to glutamate released by invading T cells and macrophages [79, 80]. This increases extracellular accumulation of glutamate through the downregulation of glutamate transporters and impairment of glial glutamate uptake [81]. The excess glutamate concentrations allow for prolongation of calcium-permeable ionotropic glutamate receptor activation on neural and glial cells, leading to excitotoxic CNS tissue damage [82, 83]. Studies in EAE rodent models have demonstrated 50% reduction in glial glutamate transporter (GLT-1) spinal expression compared to normal animals [37, 84]. In chronic EAE models, administration of ceftriaxone, a third-generation cephalosporin antibiotic which upregulates CNS glutamate transporters, has not only shown to limit and attenuate clinical symptoms [37, 85] but also shown to significantly reverse tactile allodynia [37] and normalise facets of cognitive functioning [53]. Normalisation of pain behaviour has been confirmed using other compounds known to promote glutamate transporter activity in EAE models, such as MS-153 [34].

As MS is a predominantly proinflammatory disease, anti-inflammatory agents predictably demonstrate significant therapeutic potential. Currently, several drugs exist that effectively target the inflammatory process in MS patients [86–89]. In EAE, lumbar intrathecal injections of a plasmid DNA with mutated IL-10 gene, designed to stimulate an anti-inflammatory response, reduced disease course and prevented mechanical allodynia [35]. Furthermore, FTY720, a sphingosine 1-phosphate receptor modulator, has been shown to suppress EAE development in several rodent models [90–93] by reducing the infiltration of CD4+ T cells, macrophages, and proinflammatory cytokines [93–96], as well as by modulating signalling pathways on glial cells [97, 98]. In addition to confining lymphocytes to lymphoid tissue [94] and preventing and reversing pathological disturbances to pre- and postsynaptic glutamate transmission [99], FTY720 is thought to induce endogenous repair mechanisms in the CNS, as it preferentially localises to myelin sheath [100]. Clinically, FTY720 has reduced MS relapse rates and lesion frequency [101–103]. While these studies focus on disease amelioration, Balatoni and associates have demonstrated in a chronic EAE model that prophylactic application of FTY720 prevented evoked potential disturbances of the somatosensory system [104], raising the possibility of using FTY720 to modulate neuropathic pain. In support of this, a recent study has shown that administration of FTY720 reduces mechanical and thermal allodynia in animals with neuropathic pain caused by peripheral nerve injury [105].

6. Guillain-Barre Syndrome and Experimental Autoimmune Neuritis

Guillain-Barre syndrome is the most common acute inflammatory demyelinating neuropathy in the peripheral nervous system (PNS), and as such can almost be considered a counterpart to multiple sclerosis. It affects 1-2 individuals per 100,000, with a greater disposition towards men [106]. GBS is a common cause of neuromuscular paralysis, characterised by areflexia or acute hyperreflexia, and can be effectively treated with immunotherapies such as intravenous immunoglobulin. However, despite immunotherapy, GBS has a 5% mortality rate, with up to 20% of patients remaining severely disabled [107]. Other symptoms of GBS include sensory impairments, such as moderate to severe nociceptive and neuropathic pain [4, 108, 109]. In fact, pain is a highly prevalent symptom, with 55–85% of sufferers complaining of paraesthesia/dysaesthesia, backache and sciatica, neck pain, muscle pain, joint pain, and visceral pain [4, 109].

Experimental autoimmune neuritis is a T-cell-mediated acute demyelinating inflammatory disease of the PNS widely used as an animal model of the acute inflammatory demyelinating polyneuropathy, the most common form of GBS [110]. First successfully induced in rabbits by Waksman and Adams in 1955, EAN is characterised by degeneration of myelin sheaths, proliferation of histiocytes, breakdown of blood-nerve barrier, and localised PNS inflammation with infiltration of lymphocytic and mononuclear cells [6].

EAN can be induced by immunisation with neuritogenic peripheral nerve myelin components, purified myelin proteins (such as P0, P2, or PMP-22), or synthetic peptides of myelin proteins [111, 112], or by passive transfer of T cells sensitised to these proteins. Susceptible animals (such as rats, mice, rabbits, and guinea pigs) induced with EAN develop monophasic disease characterised by weight loss, ascending progressive paralysis, and spontaneous recovery.

7. Symptoms of Neuropathic Pain in GBS and EAN

Neuropathic pain, primarily affecting the distal extremities, represents a common and severe symptom in patients with GBS and is more common and persistent than nonneuropathic pain [113]. Dysaesthetic extremity pain, described as burning, tingling, or shock-like sensations, has been reported in up to 49% of GBS patients [109]. GBS patients also experience altered thermal sensations, with significantly higher

warm threshold temperatures and lower cold threshold temperatures as compared to age- and gender-matched controls [114]. In support of this, a recent study has shown that GBS patients have a significantly more severe impairment of cold detection thresholds, heat pain thresholds, and responses to suprathreshold heat stimuli in the foot, as compared to patients with nonneuropathic pain or without pain [113]. In addition, GBS patients suffer from brush-induced allodynia [113].

The thermal and tactile sensory abnormalities evident in GBS are reflected in EAN models (Table 2). Behavioural tests of pain hypersensitivity in EAN, including thermal hyperalgesia and mechanical allodynia, have frequently served as tools to study GBS sensory dysfunctions. For example, Moalem-Taylor and colleagues were able to observe significant mechanical allodynia and thermal hyperalgesia in both hindpaws and forepaws of rats with EAN [115]. A subsequent study confirmed the development of neuropathic pain in EAN animals and further demonstrated that mechanical allodynia preceded the onset of neurological signs and persisted after cessation of locomotor deficit [116].

8. Potential Mechanisms of Neuropathic Pain in GBS and EAN

Despite its prevalence, the mechanisms of neuropathic pain in GBS patients remain unknown. It has been suggested that in the acute phase of GBS, neuropathic pain results from nerve inflammation, whereas in the chronic phase of the disease, neuropathic pain results from degeneration of sensory nerve fibres [121]. Recently, it has been shown that a considerable reduction in intraepidermal nerve fibre density at the distal leg is evident early in the disease and correlates with pain intensity in the acute phase of GBS [122]. Furthermore, impairment of small myelinated and unmyelinated nociceptive fibres is significantly greater in GBS patients with neuropathic pain than in those without neuropathic pain. The severity of such impairment during the acute phase of GBS is predictive of chronic neuropathic pain [113].

To date, very few research laboratories have studied the mechanisms underlying neuropathic pain in EAN animals. However, existing studies have implicated several inflammatory mediators and cells in the initiation and maintenance of neuropathic pain in EAN through secretion of inflammatory mediators that sensitise nociceptors to amplify pain hypersensitivity. For example, greater numbers of T cells, antigen-presenting cells, and macrophages were observed in peripheral nerves of EAN animals [115]. These infiltrating leukocytes in the PNS may play a role in EAN-induced pain by releasing proinflammatory cytokines such as IL-18 (an IFN-γ inducing factor, produced by macrophages) with significantly greater IL-18 expression observed in nerve roots of EAN rats and significantly higher serum levels of IL-18 detected in GBS patients as compared to control subjects [123]. Cells immunoreactive for inducible nitric oxide synthase and TNF have been also observed in the DRGs of animals with EAN [124].

Additionally, there exists accumulating evidence that microglia become activated following PNS damage and contribute to sensitisation of central nociceptors through the production of proinflammatory cytokines, chemokines, and extracellular proteases [50]. Indeed, an increase in the number of microglial cells has been demonstrated in rats with EAN [116, 120]. In particular, the association of the time course of mechanical allodynia and spinal upregulation of $P2X_4R$ on spinal microglia in lumbar dorsal horns in EAN rats has been successfully observed by Zhang and colleagues [116]. This suggests that activation of $P2X_4R$ drives the release of brain-derived neurotrophic factor from spinal microglia, a cellular substrate that causes disinhibition of pain-transmitting spinal lamina I neurons and mediates aberrant nociceptive processing in the spinal cord [125]. The involvement of transmembrane chemokines such as CX3CL1 (fractalkine) has also been implicated, as it plays a key role in mediating neuron-microglia interactions in nociceptive transmission. Elevated levels of CX3CL1 have been recorded in GBS patients [126], while in EAN rats, extensive upregulation of immunoreactivity for CX3CL1 and its receptor CX3CR1 in the dorsal horn has been shown to correlate with the establishment of mechanical allodynia [120].

Taken together, development and maintenance of neuropathic pain in EAN models may result from (a) demyelination and degeneration of sensory nerve fibres, (b) autoimmune inflammation in the PNS, and (c) spinal glial activation in the CNS, therefore providing a useful model for finding novel therapeutic approaches for GBS-related pain.

9. Clinical Applications of EAN Neuropathic Pain Models

Although the studies on pain in EAN are inadequate to date, there are a few significant approaches that may be therapeutic in relieving pain in GBS patients.

Firstly, immunotherapeutic approaches that enhance the numbers of immunosuppressive $FoxP3^+$ regulatory T (Treg) cells and decrease neuroinflammation have demonstrated potential. Recently, it has been shown that treatment with CD28 superagonist, a Treg cell expander, resulted in a significant amelioration of EAN severity and mechanical allodynia, with associated reduction of neuroinflammatory responses [119]. Treatment with Compound A, a plant-derived ligand of glucocorticoid receptors that enhances Treg cells in blood of EAN animals, is also able to attenuate mechanical allodynia [118]. The same study further observed that Compound A reduced microglial activation and IL-1β and TNF upregulation in the spinal cord, increased the numbers of anti-inflammatory M2 macrophages in sciatic nerves, and modulated lymphoid cytokines to an anti-inflammatory profile [118].

Furthermore, statins, which are used to treat hypercholesterolaemia in humans, are reported to potentiate anti-inflammatory and immunomodulatory effects, including deviation of helper T cell Type 1 (Th1) mediated proinflammatory response to Th2 mediated anti-inflammatory response,

Table 2: Summary of pain symptoms observed in EAN models and potential therapeutic options for treating GBS pain.

EAN antigen	Animal model	Test site	Test	Symptoms during disease	Drug	Mode of action and effects of drug	References
200 μg $P2_{(57–81)}$	♂ Lewis rats	Forepaws & hindpaws	Hargreaves test	Thermal hyperalgesia (during disease course)			[115]
			Electronic von Frey	Mechanical allodynia (during disease course)			
80 μg $P2_{(57–81)}$	♂ Lewis rats	Hindpaw	Electronic von Frey	Mechanical allodynia (before clinical signs, during disease, and after recovery)			[116]
80 μg $P2_{(53–78)}$	♂ Lewis rats	Hindpaw	Electronic von Frey	Mechanical allodynia (during disease)	Minocycline (an antibiotic of tetracycline family)	Inhibition of expression of MMPs and infiltration of immune cells to PNS Significant decrease of disease severity Attenuate mechanical allodynia in EAN rats	[117]
100 μg $P2_{(57–81)}$	♂ Lewis rats	Hindpaw	Electronic von Frey	Mechanical allodynia (before clinical signs and during disease)	Compound A (a plant-derived glucocorticoid receptor ligand)	A selective glucocorticoid receptor ligand with anti-inflammatory action Attenuate neurological signs Reduce mechanical allodynia in rats	[118]
200 μg $P2_{(57–81)}$	♂ Lewis rats	Hindpaw	Dynamic plantar aesthesiometer	Mechanical allodynia (during disease and recovery)	CD28 superagonist (CD28SupA)	Expands regulatory T-cell population Ameliorate disease severity Reduction of mechanical pain hypersensitivity in rats	[119]
25 μg bovine peripheral myelin	♀ Lewis rats	Hindpaw	Dynamic plantar aesthesiometer	Mechanical allodynia (before clinical signs)			[120]

inhibition of Th1 and Th17 mediated autoimmune response, inhibition of maturation and activation of antigen presenting cells, and increasing the numbers of $CD4^+CD25^+FoxP3^+$ Treg cells [127]. Recent studies have found that administration of atorvastatin reduces EAN severity through a similar mechanism [127], while treatment with rosuvastatin and simvastatin prevents the development of thermal hyperalgesia and mechanical allodynia and significantly reduces spinal glial activation following peripheral nerve injury [128]. This highlights the potential for statins to manage neuropathic pain in GBS.

In addition, the existing evidence for the role of microglia in neuropathic pain suggests that controlling spinal glial activation may result in pain amelioration in GBS. In particular, inhibition of microglial activation and alleviation of mechanical allodynia has been successfully observed by peritoneal administration of minocycline, which attenuates TNF and decreases proinflammatory cytokine response [117, 129]. Elevated levels of CX3CL1 in GBS suggest that inhibiting CX3CL1/CX3CR1 interactions will negatively affect microglial activation [120] and might prevent the development of neuropathic pain.

Other possible therapeutic approaches include inhibiting matrix metalloproteinases (MMPs). MMPs comprise a large family of proteases that have been implicated in the generation of neuroinflammation and the development of neuropathic pain through the cleavage of extracellular matrix proteins, cytokines, and chemokines [130]. MMP-9 and MMP-7 were found to be selectively upregulated during EAN and expressed in nerves of GBS patients [131]. BB-1101, a broad spectrum MMP inhibitor, has already demonstrated potential in preventing the development of EAN [130], and reduced expression of MMP-9 by treatment with minocycline was associated with improved EAN outcome and reduced mechanical allodynia [117].

10. Summary

Although much has been uncovered in the past few decades about the nervous system autoimmune disorders of MS and GBS, the clear pathogenesis of these diseases has not been fully elucidated. However, utilisation of animal models, in particular EAE and EAN, has significantly advanced our understanding and provided a platform for development and investigation of new therapies. Recently it has become clear that neuropathic pain is a common debilitating symptom in MS and GBS and that some of the changes in pain sensitivity observed in these patients can be mimicked in EAE and EAN animals. Tables 1 and 2 summarise neuropathic pain symptoms observed in EAE and EAN, respectively, and the therapeutic agents tested in these animal models. Many complex mechanisms are involved in mediating the various sensory changes, and we are only now beginning to understand the mechanisms underlying neuropathic pain in MS and GBS. A recent study in humans has demonstrated an autoimmune basis for some types of chronic idiopathic pain highlighting the role of autoimmune antibodies and cells in pain mediation [132]. A concerted effort is required to elicit more information regarding the mechanisms underlying neuropathic pain in MS and GBS to better enable the development of more effective treatments.

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Research Article

Chronic Pain Treatment: The Influence of Tricyclic Antidepressants on Serotonin Release and Uptake in Mast Cells

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The involvement of serotonin (5-HT) in chronic pain mechanisms is established. 5-HT inhibits central painful stimuli, but recent data suggests that 5-HT could also enhance pain stimulus from the periphery, where mast cells play an important role. We aimed in our study to clarify the influence of selected tricyclic antidepressants (TCAs) on mast cell function: secretion, uptake, and reuptake of 5-HT, that could interfere with 5-HT levels and in this way contribute to the generation of pain. As an experimental model, we used isolated rat peritoneal mast cells and incubated them with selected TCAs (clomipramine, amitriptyline, doxepin, and imipramine) under different experimental conditions. 5-HT release, uptake, and reuptake were determined spectrofluorometrically. We showed that TCAs were able to inhibit 5-HT secretion from mast cells, as well as uptake of exogenous 5-HT and reuptake of secreted 5-HT back into mast cells. The effects of TCAs were concentration dependent; higher concentrations of TCAs inhibited the secretion of 5-HT induced by compound 48/80, whereas lower concentrations of TCAs inhibited 5-HT uptake. The most effective TCA was halogenated clomipramine. As TCAs are well introduced in chronic pain treatment, the insight into mechanisms of action is important for an understanding of their effect in various pain conditions.

1. Introduction

Chronic pain is a complex neurobiological phenomenon with a variety of factors contributing to peripheral and central pain-signaling mechanisms. A common underlying mechanism of chronic pain is the presence of inflammation at the site of the damaged or affected tissue which causes release of several inflammatory mediators such a prostaglandins, bradykinin, and histamine. These agents increase the sensitivity of primary sensory neurons to painful stimuli [1]. Strong activation by proinflammatory mediators also drives the opening of voltage-gated sodium channels (VGSCs) that are crucial for central and peripheral sensitization and the excitability of neurons in the central and peripheral nervous systems [2–4]. The release of proinflammatory and immunoactive substances initiates therefore local actions and can result in a more generalized response that leads to a chronic pain condition.

Besides peripheral sensory pathways, there are central inhibitory or facilitatory pathways where various neurotransmitters and signaling molecules can contribute to the generation and/or maintenance of central as well as peripheral painful stimuli [1]. Among them, serotonin (5-HT) plays a complex role. In the central nervous system, monoaminergic (noradrenaline and 5-HT) and opioidergic neurons from descending pathways are inhibitory for pain transmission; in neuropathic pain, persistent pain is thought to be principally due to activation of descending pain facilitatory pathways and deactivation of descending pain inhibitory pathways [5–9]. In the spinal cord, convergence of peripheral inputs and descending pathways occurs. Here, the inhibitory molecules such as gamma-aminobutyric acid (GABA), endogenous opioids, and monoamines control the transmission of noxious stimuli [10, 11].

On the contrary of the inhibitory effect of 5-HT on central painful stimuli, recent findings suggest that 5-HT

might enhance a pain stimulus from the periphery. It has been found that the association between increased 5-HT levels and increased number of mast cells in patients with chronic abdominal pain [12–14]. A possible relationship between the number of mucosal mast cells and rectal sensitivity has also been demonstrated in humans [14]. There is also evidence of a significant increase in mast cell numbers in patients with intestinal bowel syndrome. Along with increased mast cell counts, there is support that mast cell numbers directly correlate with abdominal pain in those patients [15]. On the other hand, we have only limited data about the role of mast cells in the central nervous system in the occurrence of chronic pain. The precise role of the mast cell-derived 5-HT in the chronic pain mechanisms is therefore still unknown.

To date, selected antidepressants are considered as an essential component of the therapeutic strategy for treatment of different types of persistent pain like neuropathic pain, painful polyneuropathy [16, 17], postherpetic neuralgia [18, 19] as well as rheumatoid arthritis, ankylosing spondylitis [20], and fibromyalgia [21], although the exact mechanisms involved in these processes are not fully known (for review see [1]). The main mechanism of action of antidepressants involves reinforcement of the descending inhibitory pathways by increasing the amount of noradrenaline and 5-HT in the synaptic cleft at both supraspinal and spinal levels. Further studies have demonstrated a critical role of VGSCs in different types of chronic pain syndromes; in this sense, antidepressants with property of blocking sodium channel have been shown to be effective in suppression of persistent pain signal [1]. We found in our previous studies that some antidepressants are able to influence 5-HT secretion from the mast cells [22, 23]. Since the impact of the mast-cells derived 5-HT in the persistent pain might be important, we were interested in present work to clarify the influence of selected antidepressants on different processes, controlled by mast cells, like secretion, uptake, and reuptake that could interfere with 5-HT levels and therefore with the generation and/or maintenance of pain.

2. Materials and Methods

2.1. Materials. Serotonin, amitriptyline, doxepin, imipramine, and clomipramine were obtained from Sigma, Steinheim, Germany. Compound 48/80, concanavalin A, bovine serum albumin, glucose, Tris-HCl, and phthaldialdehyde (OPT) were also obtained from Sigma Chemicals, Steinheim, Germany. HEPES was purchased from Merck, Darmstadt, Germany, and Percoll was obtained from Amersham Biosciences, Uppsala, Sweden. All other chemicals were of analytical grade. Spectrofluorometry was carried out on the spectrofluorometer Shimadzu RF-1501.

2.2. Animals. Wistar rats (200–350 g) were obtained from our own breeding colony. They were maintained under constant environmental conditions, with an ambient temperature of 22 ± 1°C, a relative humidity of 55 ± 10%, and a natural regimen of light-dark cycle. The animals were kept in cages Ehret type 4 (Germany); bedding material was Lignocel 3/4. They received standard rodent diet Altormin (Germany) and have free access to food and water. We used two animals for each experiment. All animal procedures have been approved by the National Animal Ethical Committee of the Republic of Slovenia and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123).

2.3. Isolation of Mast Cells. Rat peritoneal mast cells were isolated from peritoneal cavity as follows: 10 mL of buffered salt solution was injected into the peritoneal cavity, and then the abdomen was gently massaged for 1.5 min. Mixed rat peritoneal cells were suspended in buffered salt solution with the following composition (mmol/L): NaCl 134.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.0, Tris-HCl 12.5, bovine albumin 1 mg/mL, and pH 7.4. The cell suspension was then centrifuged at 220 g for 10 min, and supernatants discarded. The collected cells were resuspended in buffered salt solution and centrifuged at 220 g for 10 min. For the preparation of purified mast cells (>98%), the cells were transferred to a HEPES-buffered (32 mmol/L) Percoll solution. A gradient of Percoll was created by centrifugation at 21000 g for 30 min at 4°C. After the centrifugation, Percoll was removed by washing the mast cell fraction in buffered salt solution, and additional centrifugation of the fraction, containing mast cells.

2.4. Treatment of Mast Cells with TCAs. Mast cells were resuspended in buffered salt solution (pH = 7.2) having the following composition (mmol/L): Na_2HPO_4 6.7, KH_2PO_4 6.7, NaCl 137, KCl 2.7, $CaCl_2$ 1.0, bovine albumin 0.5 mg/mL, and glucose 1 g/L. Each sample contains between 5.10^5 and 2.10^6 mast cells.

(1) In the secretion experiments, mast cells were preincubated with different concentrations (10^{-8}–10^{-4} mol/L) of selected TCAs (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min and then incubated in the presence of compound 48/80 (0.1 μg/mL) for additional 10 min.

(2) In the uptake experiments, mast cells were incubated with 5-HT (250 ng/sample) for 10, 30, or 60 min. The experiments were performed at 37°C or at 0°C in the presence of extracellular Ca^{2+} ions (10^{-3} mol/L) or in Ca^{2+}-free medium. In the next group of experiments, mast cells were preincubated with different concentrations (10^{-8}–10^{-4} mol/L) of selected TCAs (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min and then incubated with 5-HT (250 ng/sample) for additional 30 min.

(3) In the reuptake experiments, mast cells were incubated with compound 48/80 (0.2 μg/mL) for 10, 30, or 60 min. In the next set of experiments, mast cells were preincubated with different concentrations (10^{-8}–10^{-4} mol/L) of selected TCAs (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min

and then incubated in the presence of compound 48/80 (0.2 μg/mL) or concanavalin A (100.0 μg/mL) for additional 60 min.

After the incubation, the secretion, uptake, or reuptake of 5-HT was stopped by cooling the tubes in an ice-cold bath.

2.5. Determination of 5-HT Secretion, Uptake, and Reuptake. 5-HT was determined in the supernatants and in the cell fraction, using a spectrofluorometric method and omitting the extraction procedure (for details see [24]). Samples (1 mL) were warmed in the presence of 0.05 mL cysteine (3%), 1.1 mL HCl (37%), and 0.07 mL OPT (0.2%) at 75°C for 15 min. After that they were cooled in an ice-cold bath, and 5-HT was measured spectrofluorometrically at excitation wavelength 360 nm and emission wavelength 478 nm. 5-HT was determined in the supernatants and in the cell fraction. 5-HT release was expressed as a percentage of the total 5-HT in the sample. All values were corrected for spontaneous 5-HT release, which was always <7.0%.

2.6. Statistical Analyses. Determinations of 5-HT content are shown as means ± standard error of the mean (SEM) of five independent assays. For each treatment and controls, four samples were analyzed. Student's t-test was used for statistical analysis. For all tests, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Inhibitory Effect of Antidepressants on 5-HT Release. The secretagogue, compound 48/80, releases 5-HT from mast cells. After 10 min of incubation of mast cells with compound 48/80 (0.1 μg/mL) 5-HT release is approximately 42%. The results show that TCAs are able to inhibit 5-HT secretion, induced by compound 48/80 from mast cells. The effect is dose dependent and occurs at higher concentrations of TCAs only. The inhibitory effect of TCAs depends on the polarity of the drug; the halogenated derivative clomipramine is significantly more potent than other used antidepressants (Figure 1).

3.2. The Effect of Antidepressants on 5-HT Uptake and Reuptake into Mast Cells. The results show that mast cells are capable to remove exogenous 5-HT from incubation medium. The uptake involves an active process which depends on temperature and time of incubation of mast cells with exogenous 5-HT. At 37°C it increases with time of incubation of mast cells with exogenous 5-HT, whereas at 0°C it is inhibited (Figure 2(a)). The uptake requires the presence of extracellular Ca^{2+} ions. In the medium, containing extracellular Ca^{2+} ions (10^{-3} mol/L), the uptake increases with time of incubation. In contrast, the uptake is significantly inhibited in Ca^{2+}-free medium (Figure 2(b)).

In the presence of extracellular Ca^{2+} ions (10^{-3} mol/L), TCAs inhibit 5-HT uptake into mast cells in a dose-dependent manner. The most potent compound is halogenated antidepressant clomipramine, where inhibition of exogenous 5-HT uptake is observed at concentration 10^{-8} mol/L (Figure 3).

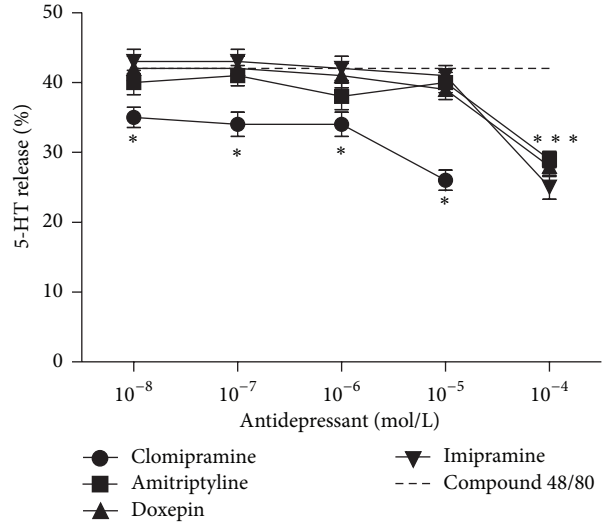

Figure 1: The influence of selected TCAs on 5-HT release from mast cells after stimulation of the cells with compound 48/80 (0.1 μg/mL). Mast cells were preincubated with different concentrations (10^{-8}–10^{-4} mol/L) of antidepressants (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min and then incubated with compound 48/80 for further 10 min. Results are expressed as a percentage of the total 5-HT in the sample. Each point represents mean ± SEM of 5 experiments. $^*P < 0.05$ versus compound 48/80.

In the next group of experiments, we demonstrated that mast cells are able to reuptake released 5-HT after stimulation of mast cells with compound 48/80. The reuptake is time dependent; after 10 min of incubation of mast cells with compound 48/80 (0.2 μg/mL), it releases an average 60% of the total 5-HT. After 60 min of incubation, the amount of 5-HT was significantly reduced in comparison to 10 min incubation, which indicates that mast cells are capable to reuptake released 5-HT from the medium (Figure 4).

In further experiments, we examined the influence of selected TCAs on reuptake of 5-HT into mast cells after long-term (60 min) incubation of mast cells with different secretagogues, compound 48/80, and concanavalin A. Our results show that preincubation of mast cells with selected TCAs leads to inhibition of 5-HT reuptake into mast cells. The inhibition is dose dependent and differs between used TCAs; the most potent is halogenated antidepressant clomipramine. In Figure 5, we show that 60 min after the stimulation of mast cells by secretagogues (compound 48/80 and concanavalin A), the released 5-HT in the medium represents 36% and 49%, respectively, in comparison to the total 5-HT of the sample. The preincubation of mast cells with selected TCAs in concentration range from 10^{-8} to 10^{-5} mol/L leads to inhibition of 5-HT reuptake into mast cells, in a dose-dependent manner. Therefore, after 60 min preincubation of mast cells with increasing concentrations of TCA, we observed higher concentrations of released 5-HT in the medium in comparison to the mast cell which have not been preincubated with TCA (Figure 5).

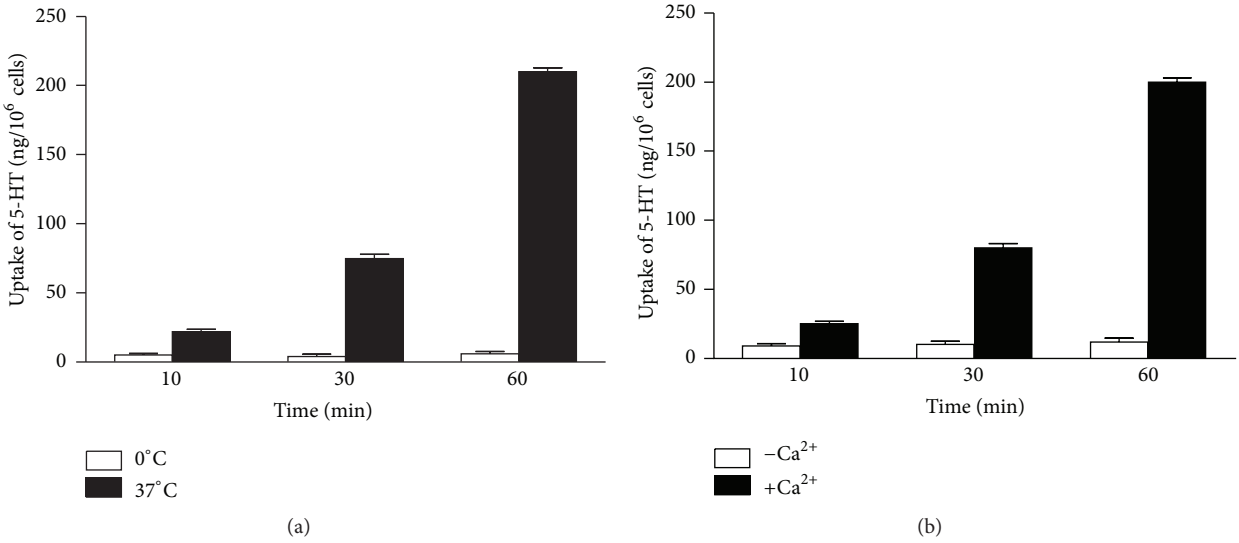

Figure 2: The effect of time of incubation on 5-HT uptake into mast cells. The mast cells were incubated with exogenous 5-HT (250 ng/sample) for 10, 30, or 60 min. (a) The effect of temperature of the medium on 5-HT uptake: mast cells were incubated with 5-HT at 37°C or at 0°C. (b) The effect of extracellular Ca^{2+} ions on 5-HT uptake: mast cells were incubated with 5-HT in the presence of extracellular Ca^{2+} ions (10^{-3} mol/L) or in Ca^{2+}-free medium. Each bar represents mean ± SEM of 5 experiments.

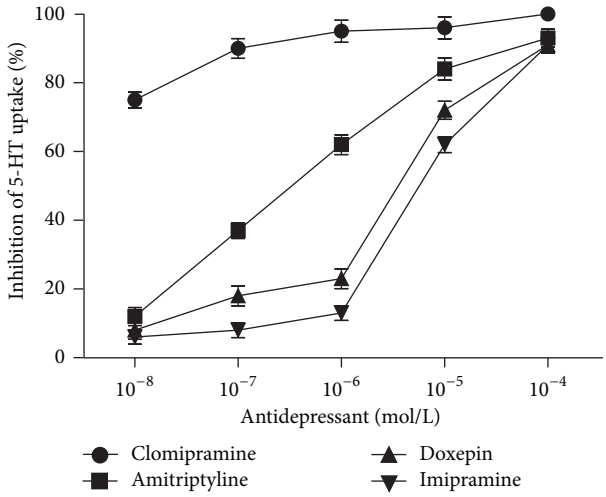

Figure 3: The influence of selected TCAs on the uptake of 5-HT into mast cells. Mast cells were preincubated with different concentrations (10^{-8}–10^{-4} mol/L) of antidepressants (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min. After that, mast cells were incubated with exogenous 5-HT (250 ng/sample) for the next 30 min. Each point represents mean ± SEM of 5 experiments.

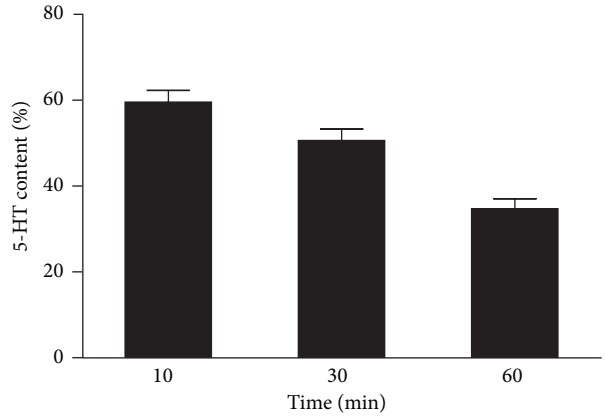

Figure 4: The effect of time of incubation on 5-HT content in the medium after stimulation of mast cells with compound 48/80. Mast cells were incubated with compound 48/80 (0.2 μg/mL) for 10, 30, or 60 min. Each point represents mean ± SEM of 5 experiments.

4. Discussion

Recent studies have indicated a strong communication between immune, endocrine, and nervous systems in the maintenance of chronic pain, where 5-HT plays significant role [25]. So far, we believed that 5-HT inhibited the generation of painful stimuli on the central nervous system level, but recent evidence indicates that 5-HT might be associated also by an increase pain transmission from the periphery, where mast cells play an important role [26, 27].

Using rat mast cells from peritoneal cavity, we show that TCAs influence mast cell-derived 5-HT levels via at least three different mechanisms: secretion of 5-HT, uptake of exogenous 5-HT, and reuptake of secreted 5-HT. At first, selected TCAs are able to inhibit the secretion of 5-HT from mast cells. The inhibition is dose dependent, and halogenated clomipramine has been found to be the most potent in comparison to imipramine, doxepin, and amitriptyline. The inhibition of 5-HT secretion from mast cells contributes to lower concentration of 5-HT at periphery and therefore could diminish sensitization of sensory nerve endings by 5-HT, which is important for the generation of peripheral painful

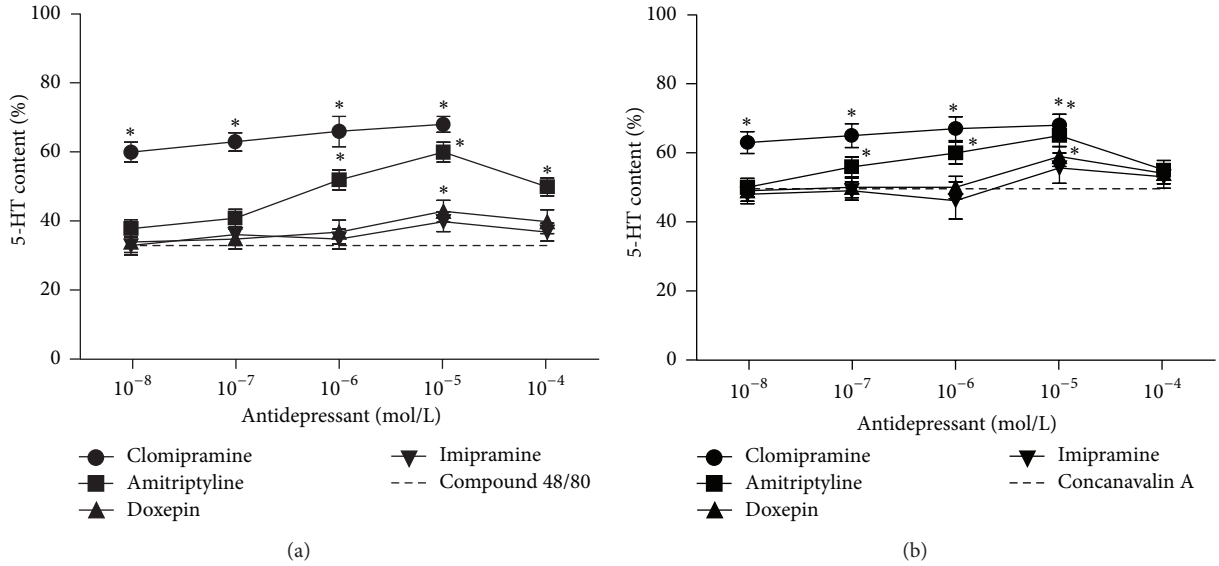

Figure 5: The influence of selected TCAs on 5-HT content after stimulation of mast cells with different secretagogues. (a) Mast cells were preincubated with increasing concentrations (10^{-8}–10^{-4} mol/L) of antidepressants (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min and then incubated with compound 48/80 (0.2 μg/mL) for the next 60 min. (b) Mast cells were preincubated with increasing concentrations (10^{-8}–10^{-4} mol/L) of antidepressants (amitriptyline, doxepin, imipramine, and clomipramine) for 10 min and then incubated with concanavalin A (100 μg/mL) for the next 60 min. Each point represents mean ± SEM of 5 experiments. $^{*}P < 0.05$ versus compound 48/80 (a) or concanavalin (b).

stimuli [28, 29]. It is already known that approximately 95% of 5-HT in the body is produced in the peritoneal cavity, and inhibition of 5-HT secretion from mast cells might be beneficial in the treatment of chronic abdominal pain [12]. Our results support recent findings, where the association between enhanced mast cells number and 5-HT levels has been suggested in patients with chronic abdominal pain [14, 15]. With this regard, 5-HT has been proposed as an important mast cell mediator which could interact with peripheral nerves leading to increased sensitivity in the gut and chronic abdominal pain [30–33].

However, the precise role of mast cells in these cases has not been clarified yet, and several issues remain to be addressed. Beside 5-HT, mast cells release several mediators like histamine, tryptase, proteoglycans, leukotriene C4, platelet activating factor, and prostaglandin D2. All of them can activate sensory nerves, leading to visceral hyperalgesia/allodynia [29]. On the other hand, mast cells not only degranulate and release proinflammatory substances but also may be in closer proximity to the cholinergic nerves thereby altering GI motility and hypersensitivity (i.e., increased abdominal pain). The detection of abnormalities of 5-HT metabolism in the peritoneal cavity has therefore generated a particular interest [34–36].

In the central nervous system, 5-HT contributes to the inhibition of the pain signal transmission. In this process, serotonergic neurons from descending inhibitory pathways, and not mast cells, are crucial to derive 5-HT for synaptic transmission. It is already known that TCAs inhibit 5-HT uptake into serotonergic neurons and on this way enhance the concentration of 5-HT in synaptic cleft and inhibition of central painful stimuli. Moreover, the antidepressants with a property of blocking sodium channel (i.e., VGSCs) have been shown to be effective in suppression of persistent pain signal because these channels play a fundamental role in the excitability of neurons in the central and peripheral nervous system, as well [25]. In addition, we show in our study that TCAs are able to inhibit uptake of 5-HT into mast cells that could also contribute to higher concentrations of 5-HT in the central nervous system.

At the periphery, TCAs effects seem much more complex. They inhibit secretion of 5-HT from mast cells, which leads to diminished concentrations of 5-HT. In addition, they are also able to inhibit an uptake of exogenous 5-HT, as well as reuptake of secreted 5-HT from mast cells back into mast cells, which causes higher levels of 5-HT in the environment. In the peritoneal cavity, mast cells represent an important source of 5-HT, and when the secretion of 5-HT from mast cells is inhibited, the 5-HT-mediated sensitization of sensory might be inhibited as well.

5. Conclusions

In summary, we have found that TCAs are able to inhibit 5-HT secretion from mast cells, as well as uptake of exogenous 5-HT and reuptake of secreted 5-HT back into mast cells. All of these events influence 5-HT levels and as a consequence could contribute to a generation and maintenance of painful stimuli in the body. As TCAs are well established in the chronic pain treatment, the insight into their mechanisms of action is crucial for an understanding of their effects

in various pain conditions. In this respect, our study provides a simple *in vitro* approach for the mechanistic studies of compounds, aimed for the modulation of 5-HT levels by mast cells.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Review Article

Testosterone-Induced Effects on Lipids and Inflammation

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Chronic pain has to be considered in all respects a debilitating disease and 10–20% of the world's adult population is affected by this disease. In the most general terms, pain is symptomatic of some form of dysfunction and (often) the resulting inflammatory processes in the body. In the study of pain, great attention has been paid to the possible involvement of gonadal hormones, especially in recent years. In particular, testosterone, the main androgen, is thought to play a beneficial, protective role in the body. Other important elements to be related to pain, inflammation, and hormones are lipids, heterogenic molecules whose altered metabolism is often accompanied by the release of interleukins, and lipid-derived proinflammatory mediators. Here we report data on interactions often not considered in chronic pain mechanisms.

1. Introduction

Chronic pain and inflammation involve multiple pathophysiological systems described or only suggested to be involved in their modulation, from genetic to environmental/cultural influences. Among all these actors, gonadal hormones have repeatedly been suggested to play a prominent role. Indeed, a number of studies have shown the ability of gonadal hormones to affect pain intensity and occurrence, for example [1]. Also important is the ability of pain (and pain therapies) to affect gonadal hormone metabolism, as recently reported by our group [2–4]. Patients often suffer complex side effects (fatigue, depression, osteoporosis, etc.) attributed to the original disease and not to the drug-induced endocrinopathies, and thus not adequately treated.

Gonadal hormones, androgen, and estrogen in particular are steroids present in both male and female subjects at different concentrations (Table 1), which depend mainly on age but are also highly sensitive to many internal and external factors. In both sexes, androgens are primarily synthesized in the gonads but also by the reticular portion of the adrenal gland as dehydroepiandrosterone (DHEA). The amount of testosterone (T) synthesized is regulated by the hypothalamic-pituitary-gonadal axis [5]. In males, T is reduced to 5α-dihydrotestosterone (DHT) by 5α-reductase (about 7%), an enzyme highly expressed in the urogenital tract, hair follicles, skin, liver, and brain [6]. In addition, 0.3% of T is converted to estradiol (E2) [7] by the enzyme aromatase, a member of the cytochrome P450 superfamily expressed in brain, liver, and adipose tissue. Testosterone and DHT bind to androgen receptors (AR) mostly located in the brain, skin, muscle, kidney, liver, and bone [8]. E2 is the most potent estrogen and targets a variety of tissues in the reproductive tracts, mammary gland and skeletal and cardiovascular systems. E2 acts by binding to its specific receptors (ER α and β).

In the "classic" pathway of action, steroid hormones bind to their specific ligands and interact through the DNA binding domain with specific DNA sequences, activating or repressing transcription of target genes [9]. In addition to these well-known genomic effects of gonadal hormones, rapid effects appearing between seconds to a few minutes from stimulation have been described in different cell models [10].

Among the many effects of androgens and estrogens on body functions, we have concentrated on that between T and

Table 1: Hormone levels commonly recorded in adult men and women. In females, the high variability of estradiol concentration is due to the menstrual cycle variations. Note that testosterone is expressed in ng/mL and estradiol in pg/mL (1 ng = 1000 pg).

Hormones	Adult men	Adult women
Testosterone (ng/mL)	3–8	0.5–1
Estradiol (pg/mL)	<50	20–400
Estriol (mg/dL)	<2	<2
Estrone (pg/mL)	15–65	Pre-menopausal: 15–200 Post-menopausal: 15–55
Androstenedione ng/dL	50–220	30–285
SHBG nmol/L	14–71	20–155
DHEA ng/dL	180–1250	130–980
DHEA-Sulfate μg/dL	10–619	Pre-menopausal: 12–535 Post-menopausal: 30–260

lipids, particularly in view of their involvement in inflammation and pain. Firstly, T is described as being involved in lipid modulation of inflammatory processes. Secondly, since obesity and other pathological or physiological conditions like aging can be accompanied by a hypogonadic state, we report data on the possible role played by this condition in the development of inflammation and pain.

2. Lipids and Testosterone

The first step to be considered is the possible interactions between T and the other steroids, starting with cholesterol, its precursor. Cholesterol is the major constituent of cell membranes and serves as a precursor of important hormones and other substances. Cholesterol is insoluble in blood and is transported in the circulatory system bound to different lipoproteins. Low-density lipoproteins (LDL-C) carry cholesterol from the liver to cells of the body, particularly to organs that require it in large amounts (such as endocrine glands synthesizing steroids). The denser but smaller high-density lipoproteins (HDL-C), mainly consisting of lipoproteins and only a small cholesterol fraction, collect cholesterol from peripheral tissue and take it to the liver where it is metabolized [11]. It has been suggested that HDL-C and their protein and lipid constituents participate in body functions related to oxidation, inflammation, coagulation, and platelet aggregation [12].

The different concentrations of gonadal hormones in men and women are thought to be important factors contributing to the sex difference in lipoprotein profiles [13]. Epidemiological data suggest that T levels are negatively associated with total cholesterol, LDL-C, and triglyceride (TG) [14], while in men T levels appear to have a complicated and controversial relationship with HDL-C levels and cardiovascular risk. In fact, androgen levels within the normal adult male range were found to have a suppressive effect on HDL-C [15]. On the other hand, several studies on patients with coronary artery disease have shown that higher T levels are associated with higher HDL-C concentrations [16]. In particular, it was found that two genes involved in the catabolism of HDL-C are upregulated by T, namely, hepatic lipase (HL) and scavenger receptor B1 (SR-B1). SR-B1 mediates the selective uptake of HDL-C lipids into hepatocytes and steroidogenic cells, including Sertoli and Leydig cells of the testes, as well as cholesterol efflux from peripheral cells [5]. T upregulates SR-B1 in the human hepatocyte and in macrophages and thereby stimulates selective cholesterol uptake and cholesterol efflux, respectively. HL hydrolyzes phospholipids on the surface of HDL-C, facilitating the selective uptake of HDL-C lipids by SR-B1. The activity of HL is increased after administration of exogenous T [17]. The increases in both SR-B1 and HL activities are consistent with the total cholesterol lowering effect of T [5].

Obesity, and particularly visceral fat excess, is associated with insulin resistance, hyperglycemia, atherogenic dyslipidemia, and hypertension, as well as prothrombotic and proinflammatory states. Adiposity, with its associated hyperinsulinism, suppresses sex hormone-binding globulin (SHBG) synthesis and therewith the levels of circulating total T [18]. It may also decrease the strength of luteinizing hormone (LH) signaling to the testis [19]. In addition, insulin and leptin have a suppressive effect on testicular steroidogenesis [20, 21]. Visceral fat cells secrete a large number of cytokines which impair testicular steroidogenesis [22]. Hence there are reasons to believe that adiposity is a significant factor in lowering circulating levels of T. Furthermore, white adipose tissue, found in high levels in obese men, exhibits elevated aromatase activity and secretes adipose-derived hormones as well as adipokines. High levels of estrogens in obese males result from the increased conversion of androgens to estrogens, owing to the high bioavailability of these aromatase enzymes [23]. Hammoud et al. [24] recently discovered that an aromatase polymorphism modulates the relationship between weight and E2 levels in obese men. Abdominal or visceral fat is more likely to lead to changes in hormone levels and to cause inflammation than fat stored in other parts of the body [25]. An increase in aromatase activity also causes an alteration in the estrogen/T ratio, which may contribute to decreased androgen production.

Aromatase inhibitors were found to be an effective treatment in restoring normal hormone levels: this led to normalization of the patient's T, LH and FSH hormone levels, as well as suppression of the serum E2 levels [26].

3. Inflammation and Testosterone

Inflammation is the body's response to cellular injury. The inflammation process involves several reciprocally modulating actors, from chemical factors derived from plasma proteins to cells that mediate vascular and cellular inflammatory reactions. To appreciate the inflammatory process, it is important to understand the role of chemical mediators such as eicosanoids, kinins, complement proteins, histamine, monokines, and cytokines, a group of soluble polypeptides. Even excess body fat can produce inflammation [27]. These inflammatory mediators act synergistically in the development of pain and hyperalgesia [28–30]. Cytokines are polypeptides produced by cells of both the innate and specific compartments of the immune system. There are various types of cytokines with widespread actions in the body. Many

of these cytokines are produced by leukocytes, on which they also exert their key actions; it is common to call them interleukins (IL followed by a number). Although each one has a specific function, it is possible to identify common basic features: short period and self-limiting secretion, molecular weight between 10 and 50 kD, pleiotropic and redundant actions, influence on other cytokines (synthesis; action), systemic and local action, binding to membrane cell receptors [31]. These substances are known to be involved in changes to vascular permeability, the oxidative burst, and chemotaxis of leukocytes.

In some cases, especially in the elderly, the body loses its ability to stop the cytokine secretion [32]; indeed, aging is accompanied by a pro-inflammatory state expressed by the increasing levels of several cytokines, including interleukin-6 (IL-6). The need to focus attention on aging derives from the evidence that in men over 45–50 years there is a progressive, slow, but continuous decrease of serum T levels, and androgens have been shown to inhibit the expression and release of cytokines and chemokines [33, 34]. This relationship is supported by the finding that androgen deprivation therapy is associated with increased levels of pro-inflammatory factors and decreased levels of anti-inflammatory cytokines [35, 36], while observational and interventional studies indicate that T supplementation reduces inflammatory markers in both young and old hypogonadal men [35].

Moreover, several lines of evidence support a close association between T levels, the evolution of diabetes secondary to hyperglycemia and hyperlipidemia and oxidative stress [37]. This association is most likely the result of elevated metabolic rates required to maintain normal biological processes and an increased level of stress in the local testicular environment, both of which naturally produce reactive oxygen species (ROS).

As ROS are generated mainly as by-products of mitochondrial respiration, mitochondria are thought to be the primary target of oxidative damage and play an important role in aging. Emerging evidence has linked mitochondrial dysfunction to a variety of age-related diseases, including neurodegenerative diseases, cancer, and chronic inflammation [38].

Oxidative stress is the result of an imbalance between the production of ROS and antioxidant defenses [39, 40]. In particular, ROS and reactive nitrogen species (RNS) are unstable and very reactive by-products of normal metabolism, leading to lipid peroxidation, nucleic acid oxidation (including DNA modification and DNA strand breaks), protein oxidation, and enzyme inactivation [39, 41–43].

Lipid peroxidation refers to the addition of oxygen to unsaturated fatty acids to form organic hydroperoxides (ROOH). Organic peroxyl ($ROO^{\bullet}$) radicals arise during the radical-initiated and O_2-dependent peroxidation of lipids, which can also produce alkoxyl radicals ($RO^{\bullet}$) in metal-catalyzed reactions [44]. The oxidation of membrane phospholipids in the plasma membrane, as well as within internal organelle membranes such as the mitochondria, leads to biophysical changes that disrupt membrane and organelle function. While these processes may stimulate cellular signaling pathways, they are generally associated with the promotion of cell death. Breakdown of lipid peroxidation yields additional reactive species (e.g., 4-hydroxynonenal, 4-HNE and malonyldialdehyde), which may contribute to toxicity and/or cellular signaling [45]. In addition, an increase in lipid peroxidation may be one of the factors responsible for the disruption of the normal feedback mechanism in the hypothalamus-pituitary-gonadal (HPG) axis [46].

Since T usually enhances the metabolic rate [47, 48], it could be expected that high T levels might alter the balance between ROS production and antioxidant defenses, resulting in an enhanced risk of oxidative stress [49, 50]. Yet, closer scrutiny of the available data reveals a more complex pattern, and different studies indicate that the relationship between T and oxidative stress can be more complex than previously thought, as it is tissue- and gender-dependent [51, 52].

4. Testosterone, Aging, and Inflammation

Aging is associated with a decrease in circulating T levels. This characteristic hormonal change of male aging is of interest because lower T concentrations are commonly associated with a number of clinical conditions of particular importance such as metabolic syndrome, type 2 diabetes, carotid intima-media thickness, and aortic and lower limb arterial disease [53–55]. The effects related to the cardiovascular system are particularly important because of the high personal and economic costs. Putative mechanisms by which lower T levels could contribute to an increased burden of cardiovascular disease range from the loss of beneficial effects of T on endothelial function and vasodilation to epidemiological correlations between T and more favorable lipid profiles [56, 57]. Indeed, lower T is associated with higher body mass index and fat mass, which are recognized cardiovascular risk factors. A study by Nettleship et al. [58] provided evidence that low serum T is linked to increased fatty streak formation. Moreover, as already reported, many of these conditions present in the elderly are accompanied by a pro-inflammatory state expressed by the increasing levels of inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha), and interleukin-1 beta (IL-1beta).

These inflammatory cytokines are known to modulate lipid homeostasis, vascular endothelial function, plaque, and atherosclerosis. During inflammation, peroxynitrite, a potent pro-inflammatory nitro-oxidative species with an established role in inflammation [59], induces endothelial cell damage and increased microvascular permeability [60] and activates redox-sensitive transcription factors, including NF-κB and AP-1, which in turn regulate genes encoding the pro-inflammatory and pronociceptive cytokines such as IL-1β, TNF-α, and IL-6 [61, 62]. Peroxynitrite also upregulates adhesion molecules such as ICAM-1 and P-selectin to recruit neutrophils at sites of inflammation [63] and autocatalyzes the destruction of neurotransmitters and hormones such as norepinephrine and epinephrine [64]. Age-associated induction of NF-κB activation is especially interesting since it seems to contribute significantly to endothelial activation in aged vessels, a critical initial step in the development of atherogenesis [65]. A significant clinical example of the possible interaction between these factors is peripheral artery

disease (PAD), consisting of partial or complete obstruction of the arteries in the lower limbs; it is one of the most common manifestations of atherosclerosis and is more frequent in aging men. Patients often describe claudication pain as episodic, which may be accompanied by physical findings of foot blanching and disappearance of pedal pulses. This was attributed primarily to a flow-limiting stenosis or occlusion of a conduit artery that limits oxygen delivery during exercise. A large body of evidence indicates that, with exercise, limb ischemia evokes an acute systemic response characterized by increased oxidative stress, local and systemic inflammation and endothelial dysfunction [66, 67]. In patients with claudication, these inflammatory responses to exercise may have adverse interactions with both the microcirculation and skeletal muscle metabolism, which could further compromise exercise performance and increase pain.

5. Vitamin D, Testosterone, and Inflammation

Vitamin D, in particular its metabolite 25-hydroxyvitamin D (25[OH]D), is widely recognized for its involvement in calcium homeostasis and immunomodulatory effects. Its hormonal action decreases the risk of many chronic illnesses, including osteoporosis, osteoarthritis, metabolic syndrome, fibromyalgia, and chronic fatigue syndrome [68–70]. Vitamin D can be synthesized in the skin from sun exposure and is found in salmon, mushrooms, eggs, and dairy products.Biological actions of vitamin D are mediated through the vitamin D receptor (VDR). The VDR is almost ubiquitously expressed in human cells, which underlines the clinical significance of the vitamin D endocrine system [68]. Altered vitamin D homeostasis is associated with increased risk of developing obesity [71, 72], hypertension [73], glucose intolerance, and metabolic syndrome [74]. Indeed, plasma vitamin D levels were associated inversely with body mass index (BMI) and fat levels and positively with HDL cholesterol [75]. Furthermore, visceral adipose tissue was higher in vitamin D deficient subjects. Sequestration of vitamin D in body fat stores and its consequent reduced bioavailability offer a plausible explanation for this association [76, 77]. Recent research revealed that calcitriol also exhibits multiple anti-inflammatory effects. First, calcitriol inhibits the synthesis and biological actions of pro-inflammatory prostaglandins (PGs) by three mechanisms: suppression of the expression of cyclooxygenase-2, the enzyme that synthesizes PGs; upregulation of the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme that inactivates PGs; and downregulation of the expression of PG receptors that are essential for PG signaling [78]. Moreover, vitamin D is able to suppress the release of TNF-α and to enhance synthesis of the anti-inflammatory cytokine IL-10 [79, 80]. Finally, vitamin D enhances the effect of anti-estrogen-like substances. In addition to these general/indirect effects, it has been shown that vitamin D increases T levels. This is primarily due to vitamin D being able to decrease the enzyme aromatase, which converts T into E2.

In fact, vitamin D reduces the production of E2 itself and blocks the production of the alpha-E2 receptor [81]. Thus, vitamin D increases T levels, as further confirmed by a study in which men with sufficient 25(OH)D levels had significantly higher levels of T and significantly lower levels of SHBG than 25(OH)D-insufficient men [82]. Moreover, Pilz and colleagues reported that vitamin D supplementation increases T levels [83]. Symptoms of T deficiency, which may be indirectly contributed to by a lack of vitamin D, include fatigue, depression, and muscle wasting. This reduced muscle mass could promote pain in muscles, causing older men to attribute muscle aches and pains to the aging process.

6. Clinical Aspects

As we have shown, there are various problems related to androgen dysfunction and inflammation such as fatigue, obesity, glycemic imbalance and altered immunity. These may represent the precursors of more severe conditions leading to disease in many individuals [84–86].

The neurodegenerative disorder X-linked-adrenoleukodystrophy (X-ALD) is an example of interesting links between T, lipid metabolism and inflammation. In X-ALD, a certain percentage of patients present hypogonadism. Moreover, due to the mutation of a peroxisomal transport protein, the metabolic pathways of specific long chain fatty acids (FA, very long chain fatty acids) are impaired [87, 88]. These FA accumulate abnormally in plasma and in all tissues, although the most affected ones are the nervous system, the adrenal and the testis, all characterized by elevated steroidogenesis. FA can be esterified in different forms, an important component being FA esterified with cholesterol. They are vehicled by lipoproteins. The adrenal cortex and testis of affected patients contain intracytoplasmic lamellar inclusions consisting of FA-cholesteryl esters [89]. Cholesterol, as mentioned above, can be metabolized into androgens. In steroidogenic tissues, free cholesterol can be obtained in three ways: after cholesteryl ester hydrolysis, de novo synthesis from acetate, or mainly imported from lipoproteins by specific receptor-mediated pathways. In the adrenals, this mechanism is mediated by adrenocorticotropic hormone (ACTH).

In X-ALD, since cholesterol is entrapped as esters in the lamellar inclusions, it cannot be normally metabolized into T. Moreover, the functionality of the T-converting enzyme 5α-reductase is altered in X-ALD [90, 91], indicating an alteration of the homeostasis of androgens. In X-ALD and in other chronic disorders, alterations of lipid metabolism, such as FA peroxisomal catabolism and esterification processes, and the presence of secondary inflammation, augmented by the release of interleukins and lipid-derived pro-inflammatory mediators, can contribute to a T deficit or generally to an alteration of T homeostasis and to the consequent clinical symptoms of the patients.

7. Conclusion (See Figure 1)

Androgens are large functional molecules able to greatly affect body functions. In this paper, we have considered the relationships between the main androgen hormone, T, and some aspects of inflammatory processes in order to highlight possible mechanisms able to affect pain chronicization. Indeed, it is becoming increasingly clear that inflammation,

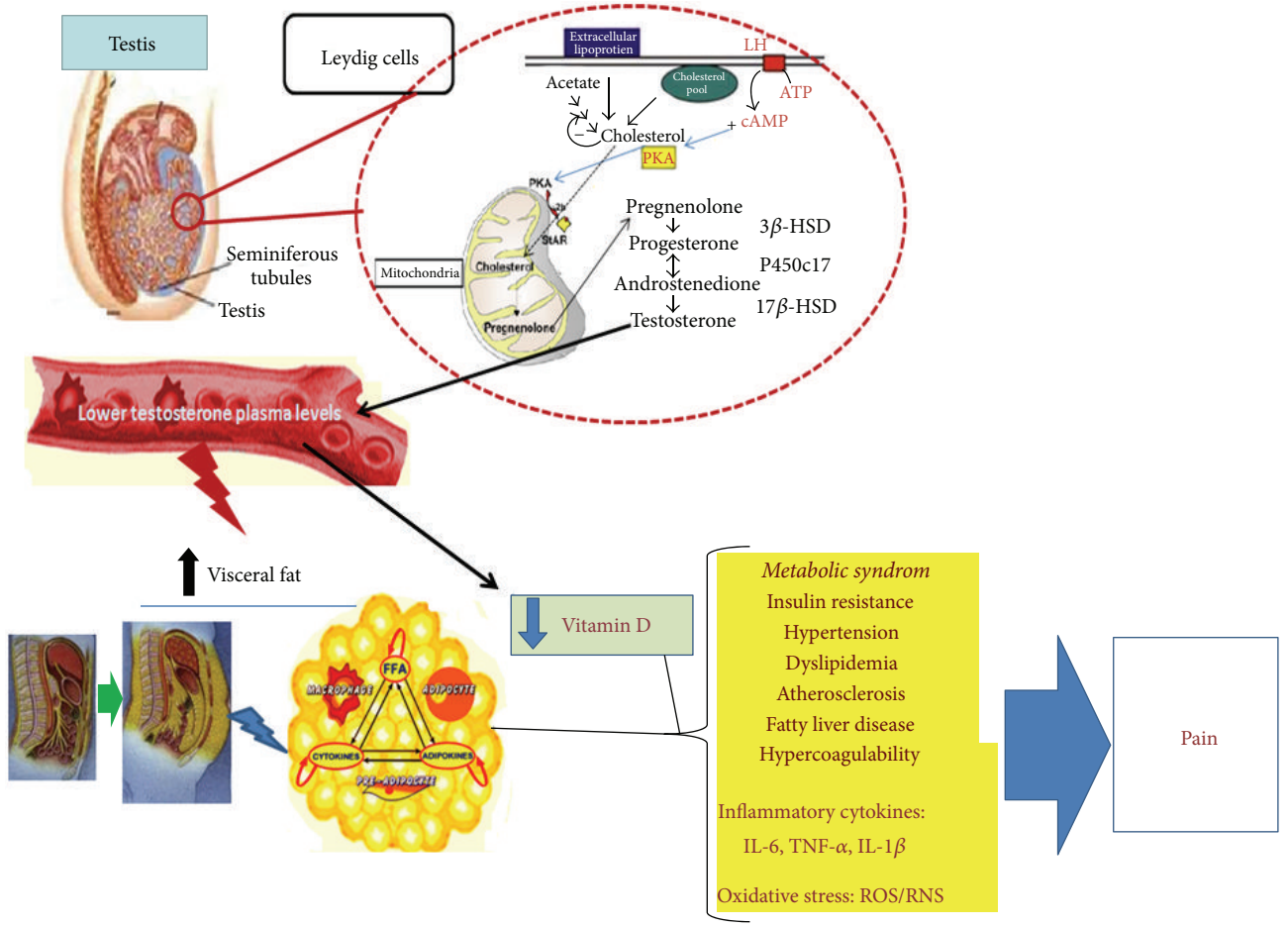

Figure 1: Representative schema of the clinical consequences suggested to be related to androgen deficiency. Lower testosterone levels are associated with an increased metabolic risk, systemic inflammation, and chronic pain.

often not clearly acknowledged, is involved in many chronic painful syndromes still far from being explained by the "usual" pain system alterations.

Database

The methodology utilized here follows a narrative review process. Some aspects of the systematic review process were derived from observational studies along with previous systematic reviews. The search involved multiple sources including PubMed. The search terminology included testosterone, lipids, and inflammation.

Conflict of Interests

All authors declare no conflict of interests.

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Research Article

Inflammatory Pain and Corticosterone Response in Infant Rats: Effect of 5-HT1A Agonist Buspirone Prior to Gestational Stress

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Our researches have shown that gestational stress causes exacerbation of inflammatory pain in the offspring; the maternal 5-HT1A agonist buspirone before the stress prevents the adverse effect. The serotonergic system and hypothalamo-pituitary-adrenal (HPA) axis are closely interrelated. However, interrelations between inflammatory pain and the HPA axis during the hyporeactive period of the latter have not been studied. The present research demonstrates that formalin-induced pain causes a gradual and prolonged increase in plasma corticosterone level in 7-day-old male rats; twenty-four hours after injection of formalin, the basal corticosterone level still exceeds the initial basal corticosterone value. Chronic treatments of rat dams with buspirone before restraint stress during gestation normalize in the offspring pain-like behavior and induce during the acute phase in the formalin test the stronger corticosterone increase as compared to the stress hormonal elevation in animals with other prenatal treatments. Negative correlation between plasma corticosterone level and the number of flexes+shakes is revealed in buspirone+stress rats. The new data enhance the idea about relativity of the HPA axis hyporeactive period and suggest that maternal buspirone prior to stress during gestation may enhance an adaptive mechanism of the inflammatory nociceptive system in the infant male offspring through activation of the HPA axis peripheral link.

1. Introduction

Interrelations between the serotonergic system and hypothalamo-pituitary-adrenal (HPA) axis determine the formation of mechanisms of stress adaptation [1–6]. Pain is a stress and therefore can activate the HPA axis [7–15]. In this case, inflammatory pain is still not clearly understood, and the data available are inconsistent [9, 14–17]. In a widely used model of inflammatory pain, the formalin test, activation of the HPA axis with the nociceptive stimulus formalin has been shown on adult awake rodents and differences in the dynamics of release of ACTH and corticosterone in response to pain impact found [8, 9, 17–19]. The HPA axis during the postnatal development goes through the period of hyporesponsiveness, which extends from the second to fourteenth postnatal days and is characterized by a low level of the response of adrenals to many stress stimuli [20]. Investigations of formalin-induced pain effects on the HPA axis in infant rats could elucidate unexplored previously interrelations between the tonic nociceptive and stress systems during the period of hyporesponsiveness of the latter. We revealed for the first time that prenatal stress induces strengthening inflammatory pain-related response in the formalin test and decrease of adaptive capacities in

infant rats; chronic injections of an agonist of serotonin (5-HT) receptors 1A (5-HT1A) buspirone to dams prior to stress during gestation cancel the adverse consequences of the stress in the offspring [21]. In prenatally stressed individuals, abnormalities in the HPA axis function [22] and neurotransmitter systems including the serotonergic one [23] were shown.

Serotonin acts as a growth factor in early cell division, migration, and differentiation in the brain specifically in development of the serotonergic system [24–27]. Many of regulator influences on developing neurons 5-HT mediates through presynaptic 5-HT1A autoreceptors in the raphe nuclei [28, 29]. Later 5-HT and 5-HT1A receptors take part in many kinds of behavior. The serotonergic system plays an important role in pain transmission, its processing and regulation [30–33]. Buspirone, serotoninergic anxiolytic and antidepressant, mediates its effect through the serotonergic system and the HPA axis. There are synergistic interrelations between these systems impaired in prenatally stressed individuals [23, 34–36]. A peculiar mechanism of buspirone action has not been completely understood; it is also true for its analgesic effect. Studies of effects of buspirone, an agonist of presynaptic and a partial agonist of 5-HT1A receptors, on the nociceptive system are limited, and the results obtained do not coincide [37, 38]. Prenatal effect of buspirone on the nociceptive system has not been studied until our researches. Activation of the antinociceptive descending serotonergic system and the decrease in hyperactivity of the HPA axis are considered as potential mechanisms of analgesic action of antidepressants. Activating effects of buspirone on the HPA system are found in adult persons [39]. It may be suggested that the period of hyporesponsiveness of the HPA, which is characterized by a low level of reaction of adrenals in response to many stress stimuli [20], will allow to prevent activating influences of buspirone on the HPA axis.

The aim of our work was to study effects of maternal buspirone prior to stress during gestation on the dynamics of the inflammatory pain-like behavior and stress response of corticosterone during the formalin test in the infant male rat offspring and also to evaluate correlation between pain-like and hormonal parameters.

2. Materials and Methods

2.1. Animals. All experimental procedures were approved by the Local Ethics Committee for Animal Experiments of the I. P. Pavlov Institute of Physiology and followed the guidelines published by the Committee for Research and Ethical Issues of the IASP on ethical standards for investigations of experimental pain in animals.

Adult female rats and male rats (Wistar) at the age of 90 days were obtained from the vivarium of the I. P. Pavlov Institute of Physiology RAN, St. Petersburg, Russia. Two days after adaptation, the rats were mated. The days of insemination and delivery were considered as gestational day (GD) 0 and postnatal day (PD) 0, respectively. All animals were maintained at constant temperature (20–22°C) under the standard light-dark cycle (8.00 AM–8.00 PM) with unrestricted access to food and water. Seventeen rat dams (controls) were not exposed to any impacts during gestation. The equal number of remaining dams ($n = 68$) was randomly treated with the 5-HT1A agonist buspirone (buspirone hydrochloride, Sigma, 3 mg/kg, 1 mL, i.p. at 9 AM) or with injection of saline (control animals from the same litters, in the same conditions of injections) from GD9 to GD21. A half of the treated rats from each group were randomly exposed to restraint stress for 60 min (in 5 min after buspirone injection) from GD15 to GD21. All influences on gestational females were identical to those used in our previous study [21]. The dose of buspirone was sufficient for inducing an anxiolytic effect in adult rats [40] and did not exceed the dose used for pregnant rat dams to protect the fetal serotonergic system against damaging effects of in utero ethanol exposure [41]. It should be noted that such dose of buspirone was not able to implicate dopamine and norepinephrine in the mediation of buspirone effects [42, 43]. Litters were called to 8 pups (4 females and 4 males, as far as possible) in 48 hours after birth. In the study, 7-day-old males born to the dams with the above-mentioned treatments during gestation and to control dams were used; females and remaining males were used in other researches. There were 245 males offspring of control, saline, saline + stress, buspirone, and buspirone + stress dams in the study with formalin injection (about 6–8 males per a group, no more than 3 animals from one dam); in addition, 80 male rats from the same litters were used as control for the formalin, with saline injection into the hind limb.

2.2. Experimental Formalin-Induced Inflammatory Pain in Infant Male Rats. Formalin test is widely used for evaluation of tonic inflammatory pain and analgesic effects of various pharmacological drugs [9, 44–47]. Flexing and shaking behaviors are the specific expression of inflammatory pain-related behavior in the formalin test in both infant and adult rats [44, 45, 48, 49]; we used the formalin test as previously described [21]. The formalin test allows evaluating acute nociception (the first phase, 5–10 min after formalin injection), tonic persistent nociception (the second phase about 30–40 min), and functional activity of the descending serotonergic inhibitory system (the interphase about 3–10 min). The second phase appears during postnatal development when the descending serotonergic inhibitory system matures [45, 46]. Characteristics of the phases depend on many factors including age and sex [50].

Each male rat was taken from the nest, injected intraplantarly to the left hindpaw with formalin solution (2.5%, 10 μL), and placed singly in a warm (25°C) chamber ($25 \times 20 \times 10$ cm) with transparent glass walls encircled by mirrors to improve the observation of the animal's behavior [21, 51]. The number of flexes + shakes was recorded using a computer program that allows recording, quantifying, and analyzing the pain-related behavior. In each group of the males, the number of flexes + shakes was averaged for 3, 9, 21, 30, and 60 min after formalin injection. Each animal was used only once.

2.3. Corticosterone Determination in Infant Male Rats. Blood samples were collected by decapitation in the rats with different prenatal treatments and in controls at 09:00-10:00 before

and 24 hours after the formalin test for determination of basal plasma corticosterone levels. During the formalin-induced pain, blood sampling by decapitation occurred at 3, 9, 21, 30, and 60 min after formalin injection. The blood samples were centrifuged and the plasma was kept at −20°C no more than a week. The plasma corticosterone (SIGMA-ALDRICH, USA) levels (μg/dL) were measured by microfluorometry [52].

2.4. Statistical Analysis. Data are presented as mean ± S.D. Formalin-evoked flexing + shaking, and corticosterone responses were analyzed by two-way ANOVA, with treatment (control, saline, saline + stress, buspirone, and buspirone + stress) and time as factors. Behavioral and corticosterone responses during 3, 9, 21, 30, and 60 minutes were separately evaluated. Comparisons between the basal levels and the data over time as well as comparisons between groups with different types of treatment were conducted using tests of simple effects. Besides pairwise comparisons, *t*-test and Mann-Whitney test were performed. Pearson and Spearman Correlations were calculated to estimate relationships between behavioral and hormonal variables. For all tests, $P < 0.05$ was considered to be statistically significant.

3. Results

Two-way ANOVA applied to pain-like responses (Figure 1) resulted in a significant effect of the factor prenatal treatments ($F(4,114) = 5.094$, $P = 0.001$); ($F(3,114) = 56.545$, $P < 0.001$). Tests of simple effects showed a significant increase in the number of flexes + shakes at 9, 30, and 60 min after formalin injection in saline + stress as compared to saline ($P < 0.05$, $P < 0.05$, and $P = 0.014$, resp.) and in saline + stress as compared to the control ($P = 0.002$, $P = 0.03$, resp.) (Figure 1). Tests of simple effects showed a decrease in the number of flexes + shakes at 9, 21, 30, and 60 min after formalin injection in buspirone + stress as compared to saline + stress ($P = 0.002$, $P < 0.05$, $P < 0.05$, and $P = 0.002$, resp.) (Figure 1).

Two-way ANOVA applied to the level of plasma corticosterone (Figure 2) resulted in a significant effect of the factor prenatal treatments ($F(4,170) = 2.706$, $P = 0.002$) and time ($F(5,170) = 22.574$, $P < 0.001$). The significant effects of factor prenatal treatment on dependent variable corticosterone were revealed at 3 and 9 min ($F(4,170) = 2.322$, $P < 0.05$; $F(4,170) = 3.634$, $P < 0.007$, resp.). Tests of simple effects showed the corticosterone level at 3 min after formalin injection was higher than basal level ($P = 0.037$) in buspirone + stress males; during the following time periods (9, 21, 30, and 60 min), corticosterone was higher than basal level in animals with all prenatal treatments ($P < 0.05$) (Figure 2). Tests of simple effects (pairwise comparisons) or/and Mann-Whitney test showed that only in buspirone + stress males at 3 and 9 min after formalin injection, the corticosterone level was higher than similar hormonal level in animals with all different prenatal treatments ($P < 0.05$). During the following time course of formalin-induced pain, there were no differences in the stress level of hormone between animals with different prenatal treatments. Tests of simple effects (pairwise comparisons) or/and Mann-Whitney test showed that in the course of inflammatory pain, the level of plasma corticosterone gradually increased ($F5,170) = 22.574$, $P < 0.001$) and to the end of the formalin test (at 60 min) was significantly higher than basal level ($P = 0.001$). Pairwise comparisons showed that basal level 24 h after the formalin test was greater than that prior to the formalin test ($P = 0.001$) in animals with all prenatal treatments ($P < 0.05$) (Figure 2).

There were no significant differences in indices under study in buspirone, saline, and control animals.

Correlation between plasma corticosterone level and the number of flexes + shakes was revealed in buspirone + stress male rats at 3 ($-r = 0.925$, $P = 0.008$), 9 min ($-r = 0.937$, $P = 0.002$), and 60 min ($-r = 0.690$, $P = 0.05$) and in control rats at 3 min ($-r = 0.90$, $P = 0.037$) after injection of formalin.

Injection of saline to the left hindpaw (controls for formalin injection) resulted in a few weak flexes + shakes during some first minutes after injection only in prenatally stressed males. Corticosterone response to pain was a specific reaction; in control animals, an increase in the plasma corticosterone in response to the procedure of saline injection into the paw was less prolonged (no more than 30 min) and did not exceed the value of corticosterone response to formalin-induced pain (the data are not shown in the table).

4. Discussion

The dynamics of corticosterone stress response to inflammatory pain and participation of 5-HT1A receptors in it were investigated in the present study in 7-day-old male rats with various prenatal treatments. Evidence of increased formalin-induced pain in prenatally stressed animals is in agreement with the data that we obtained earlier [21]. Chronic treatments of rat dams with the 5-HT1A agonist buspirone prior to stress during gestational period increased resistance of the tonic nociceptive system normalizing behavior in the inflammatory pain model and changed the time course of stress corticosterone response to formalin-induced pain in the offspring.

Before our studies, in a widely accepted model of inflammatory pain, the formalin test, it was demonstrated in adult awake rats that the nociceptive stimulus formalin induced activation of the HPA axis and increased concentration of ACTH and corticosterone [9, 13, 19]. Interestingly, the authors that found the peak of the corticosterone release at 30 min and its restored level at 80 min after formalin injection concluded that the resulting release of corticosterone is not antinociceptive as neither adrenalectomy nor high-dose dexamethasone changed behavioral nociceptive responses [9]. It is worthy to note that peaked time in release of corticosterone in response to the formalin test as well as the time of the hormonal restoration level after formalin injection vary according to the authors from the 15–60 min to 60–120 min, respectively [9, 13, 19]. These differences may be attributed to peculiarities of the formalin test. The behavioral response in the formalin test, represented by acute and tonic phases of different chemical nature, depends on concentrations and volumes of formalin solution, a place

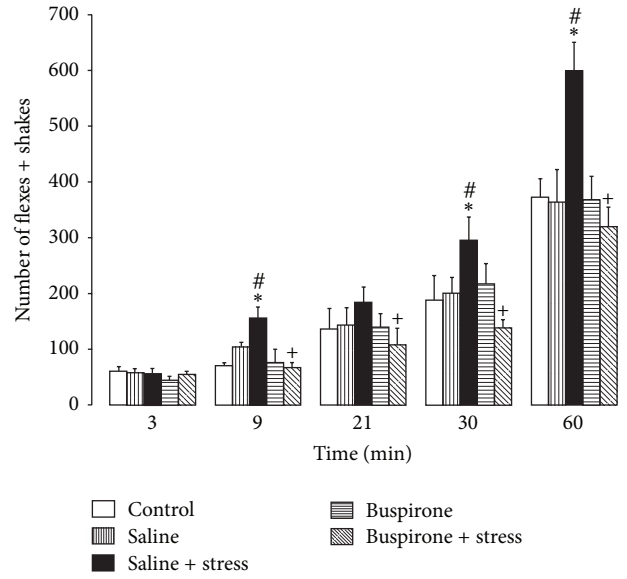

Figure 1: Pain-like responses recorded over different periods of time (3, 9, 21, 30, and 60 min) after injection of formalin (mean ± SEM) in 7-day-old male rats with different prenatal treatments. $^{*}P < 0.05$ different from saline; $^{+}P < 0.05$ different from saline + stress; $^{\#}P < 0.05$ different from controls.

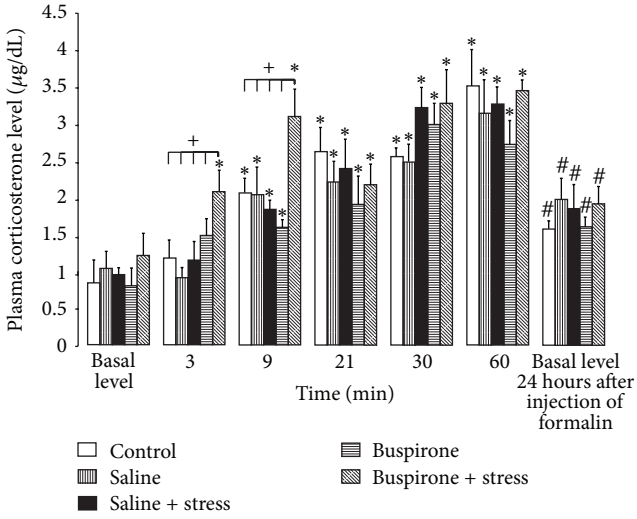

Figure 2: Basal levels of corticosterone and plasma corticosterone levels (μg/dl) determined 3, 9, 21, 30, and 60 min after injection of formalin (mean ± SEM) in 7-day-old male rats with different prenatal treatments. $^{*}P < 0.05$ different from the basal level; $^{+}P < 0.05$ different from the control, saline, saline + stress, and buspirone; $^{\#}P < 0.05$ different from the basal level before injection of formalin.

of its injection, temperature in the room, a strain of rats, and conditions of experimental performances [9, 50, 53, 54]. These factors determine involvement in the response of various mediators influencing the intensity and dynamics of release of corticosterone.

Our study is the first to evaluate the dynamics of corticosterone release in conditions of inflammatory pain in infant rats during the hyporesponsive period of the HPA axis [20]. The new data obtained testify that inflammatory formalin-induced pain evokes the stress response of corticosterone in male rats during the hyporesponsive period of the HPA axis. This reaction is a specific reaction to pain; in control animals, an increase in the plasma corticosterone in response to the procedure of saline injection into the paw was less prolonged and weaker than the hormonal response to formalin-induced pain. We have revealed that the characteristic feature of the dynamics of corticosterone response to inflammatory pain in infant rats is a gradual increase of hormonal release in the formalin test, so to the end of the response the level of corticosterone considerably exceeded its initial level. Most importantly, the results indicate that 24 hours after the formalin test, the corticosterone level still exceeds the basal corticosterone value before the formalin test in males of all the groups under study. This fact cannot be associated with an increase in corticosterone basal level in intact 8-day-old male rats as compared to that in 7-day-olds, as available data and our own results indicate equal value in the basal level of plasma corticosterone in 5–8-day-old male rats that were not exposed to any prenatal impacts [55]. Based on these results, we conclude that the peripheral link of the HPA axis responds to inflammatory pain in the formalin test in infant rats with a prolonged reaction. Experimental data reported here enhance the idea of relativity of hyporesponsive period of the HPA axis [20]. Up to now, there has not been any detailed work done to find a clear explanation for this period in the development of the HPA axis.

The results obtained provide new important information that maternal 5-HT1A agonist buspirone prior to stress during gestation induces in the offspring during the acute phase in the formalin test the stronger corticosterone increase as compared to the stress hormonal elevation in animals with other prenatal treatments. In the following time periods of formalin-induced pain, the animals with different prenatal treatments do not show significant differences in stress corticosterone level. Negative correlation revealed between the corticosterone concentration and the number of flexes + shakes during the first nine minutes after injection of formalin in buspirone + stress male rats is noteworthy. These results suggest that activation of the corticosterone release via 5-HT1A receptors may facilitate some adaptive mechanisms associated with a decrease of inflammatory pain in buspirone + stress rats.

There are multiple pathways through which 5-HT and its agonists may stimulate the HPA axis [56]. It is shown that formalin activates ascending ways to the HPA [57]. The chemical stimulus formalin induces appearance of "inflammatory soup" from various chemical substances including 5-HT released from platelets and also activation of neutrophils and leucocytes that produce proinflammatory cytokines IL-6 [17]. Cytokines contribute to the increase in ACTH and corticosterone [17] and to the exacerbation of nociceptive processing [58]. Interaction between the HPA axis and 5-HT system would be dependent on concentration of 5-HT released from platelets during inflammation which reaches the central nervous sites, but this question is poorly known. Both systems are highly plastic during maturation [6], and

prenatal stress impairs interaction between the HPA axis and 5-HT system and alters their functional activity in adults [2, 35]. The expression of 5-HT1A receptors is found during the initial stages of prenatal development of the hippocampus [59] and prenatal stress impairs their development [60]. There is evidence that buspirone penetrates through the placental and blood brain barriers [61] and is able to exert the protective effects presumably through its ability to overcome the deficit of fetal serotonin and to stimulate fetal 5-HT1A receptors [62]. Further studies are needed to evaluate influences of maternal buspirone prior to stress during gestation to the HPA axis response during the inflammatory pain immediately after finishing the period of responsiveness in the HPA axis development. Thus, new data indicate an important role of 5-HT1A receptors in the development of close relationships between the HPA axis and tonic nociceptive system that mediate adaptation of organism to extreme conditions.

5. Summary

The formalin-induced pain causes a gradual and prolonged increase in plasma corticosterone level during the persistent pain-like behavior in 7-day-old male rats. Chronic treatments of rat dams with buspirone before restraint stress during gestation normalize in the offspring inflammatory pain behavior and induce during the acute phase in the formalin test the stronger corticosterone increase as compared to the stress hormonal elevation in animals with other prenatal treatments. Buspirone + stress rats display the negative correlation between plasma corticosterone level and the number of flexes + shakes. Thus, the new data enhance the idea about relativity of the HPA axis hyporeactive period and suggest that maternal buspirone prior to stress during gestation may enhance an adaptive mechanism of the inflammatory nociceptive system in the infant male offspring through activation of the HPA axis peripheral link.

Conflict of Interest

The authors declare that they have no conflict of interest.

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