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2022/2023

David Ferreira Ramos

The role of microglia in chronic neuropathic pain pathways associated with spinal cord injury - A systematic review.

Março, 2023

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The role of microglia in chronic neuropathic pain pathways associated with spinal cord injury - A systematic review.

Mestrado Integrado em Medicina

Área: Medicina Básica

Tipologia: Dissertação

Trabalho efetuado sob a Orientação de:

Doutora Célia da Conceição Duarte Cruz

E sob a Coorientação de:
Doutor João Carlos Bettencourt de Medeiros Relvas

Trabalho organizado de acordo com as normas da revista:
Reviews in the Neurosciences

Março, 2023

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UC Dissertação/Projeto (6º Ano) - DECLARAÇÃO DE INTEGRIDADE

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Medicina Básica

TÍTULO DISSERTAÇÃO/~~MONOGRAFIA~~ (riscar o que não interessa)

The role of microglia in chronic pain pathways associated with spinal cord injury – A systematic review.

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Faculdade de Medicina da Universidade do Porto, 24/03/2023

Assinatura conforme cartão de identificação: David Ferreira Ramos

Agradecimentos

À professora Célia, pela dedicação que forneceu, pelo conhecimento que me ensinou, pela orientação que me deu e pela energia que me passou. Que aceitou fazer parte do final deste meu percurso académico e me ajudou a contactar e preparar com alguma da prática e muitos dos valores profissionais, de melhoria e de superação que me aguardam na próxima fase.

A todos os meus amigos. Aos que tive a indescritível felicidade de ter e manter desde a adolescência. Aos que conheci no início deste percurso e com quem estive lado a lado nos muitos desafios que atravessámos. Aos que se mantiveram presentes até hoje e aos que conheci ao longo deste percurso, nas mais simples, caricatas ou inesperadas situações, que me acompanharam e ajudaram por rotas de constante mudança, crescimento e expansão de horizontes.

A quem tenho próximo, com plena confiança e suporte. Quem me ajudou a mudar e crescer, a ultrapassar obstáculos e partilhar os melhores momentos. A quem posso recorrer para o que houver.

À minha família que fez possível eu seguir este caminho e que me ajudou a ter o que precisei para prosseguir e crescer.

A todos os professores de toda a vida que não só me ensinaram, mas que me apoiaram e ajudaram. Aos que tiveram carinho perante as dificuldades; aos que foram duros perante as necessidades. Aos que se preocuparam, aos que tanto se esforçaram, aos que me prepararam.

Aos presentes ao longo da minha aprendizagem de medicina. Aos que se disponibilizaram e sacrificaram um pouco de si para me orientar e oferecer oportunidades. Aos que garantem o funcionamento dos estabelecimentos de saúde. Aos professores, médicos, enfermeiros, auxiliares e secretários que direta ou indiretamente cuidaram dos espaços e criaram as condições para a minha formação. Aos pacientes que despenderam do seu tempo para a minha formação, aos tive o prazer de conhecer, aos que me inspiraram, àqueles com quem partilhei sorrisos e àqueles com quem evoquei esperança.

“A verdadeira grandiosidade está naquilo que fazemos com as cartas que recebemos” [Victor Sullivan]

(Former title)

The role of microglia in chronic neuropathic pain pathways associated with spinal cord injury - A systematic review.

(Title after peer review)

Involvement of microglia in chronic neuropathic pain associated with spinal cord injury - A systematic review.

By

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Running title: Microglia in chronic pain after spinal cord injury

Key Words: microglia, spinal cord injury, pain, chronic pain, neuropathic pain

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Abstract

In recent decades microglia have been found to have a central role in the development of chronic neuropathic pain after injury to the peripheral nervous system. It is widely accepted that peripheral nerve injury triggers microglial activation in the spinal cord, which contributes to heightened pain sensation and eventually chronic pain states. The contribution of microglia to chronic pain arising after injury to the central nervous system, such as spinal cord injury (SCI), has been less studied, but there is evidence supporting microglial contribution to central neuropathic pain. In this systematic review, we focused on post-SCI microglial activation and how it is linked to emergence and maintenance of chronic neuropathic pain arising after SCI. We found that the number of studies using animal SCI models addressing microglial activity is still small, compared with the ones using peripheral nerve injury models. We have collected 20 studies for full inclusion in this review. Many mechanisms and cellular interactions are yet to be fully understood, although several studies report an increase of density and activity of microglia in the spinal cord, both in the vicinity of the injury and in the spared spinal tissue, as well as in the brain. Changes in microglial activity come with several molecular changes, including expression of receptors and activation of signalling pathways. As with peripheral neuropathic pain, microglia seem to be important players and might become a therapeutic target in the future.

Keywords: microglia, spinal cord injury, pain, chronic pain, neuropathic pain

INTRODUCTION

It is estimated that approximately 27 million people live worldwide with life-long consequences of spinal cord injury (SCI) (James et al. 2019) and that every year between 250 000 and 500 000 people become spinal cord injured (World Health Organization. and International Spinal Cord Society. 2013). SCI causes range from motor vehicle accidents and community violence to recreational activities and workplace-related injuries. While traffic accidents remain the most frequent cause of SCI in young adults, for the elderly population, above 65 years old, SCI mostly results from falls (Sekhon and Fehlings 2001). Typically, young adults (30-40 years old) comprise the majority of SCI patients and, of these, approximately 80% are male. The ratio of men to women is typically 3 to 4:1 and males are consistently at greater risk of morbidity and mortality from SCI across all age groups (Kraus et al. 1975, Griffin et al. 1985, Stover and Fine 1987).

SCI causes major disturbances in sensory, motor and autonomic function, strongly impacting the physical, psychological and social well-being of patients and caregivers (Braaf et al. 2017). In general, SCI healing after a traumatic spinal injury involves complex tissue remodelling (Ramer et al. 2005, Cregg et al. 2014, Alizadeh et al. 2019). Most spinal neurons located below the lesion, particularly those at the lumbosacral segments, lose contact with supraspinal projections. Some of these descending tracts provide inhibitory input, critical for endogenous pain control (Tavares et al. 2021). Moreover, sensory afferents, which extend long processes into the spinal grey matter, also experience morphological and neurochemical modifications. These neuroplastic events result in the establishment of new circuits, mostly independent of supraspinal control.

Activation of these circuits has been linked to adverse events, most importantly development of chronic pain.

After traumatic SCI, pain is reported at all stages of disease progression. Acute pain is present during early stages of disease progression, receding with tissue scarring, whereas chronic pain emerges due to maladaptive neuroplasticity. Chronic pain has life-long consequences, strongly impairing patients' quality of life and often exceeding the impact of other functional disabilities. More than 50% of SCI patients report chronic neuropathic pain within a year of SCI (Dijkers et al. 2009, Finnerup 2013). Neuropathic pain is highly prevalent and reflects SCI-induced damage in the somatosensory system. It is typically of central origin, felt diffusely below the level of injury and appearing when chronicity has set (Siddall et al. 2003, Finnerup 2013). Gabapentin, opioids and pregabalin remain gold standard for SCI-associated pain treatment, but are often ineffective and do not prevent pain worsening (Widerström-Noga 2017).

Current therapies to control pain are designed to target neurons. Yet, the role of glial cells, particularly microglia, in regulating synaptic communication is increasingly well established but its manipulation remains largely unexplored in the context of SCI-induced pain. The involvement of microglial cells in peripheral neuropathic pain is now well acknowledged (Donnelly et al. 2020), either by direct modulation of synaptic communication by acting on neuronal terminals or by activating astrocytes, which release interleukins that further potentiate neurotransmission by nociceptive neurotransmission (Matejuk and Ransohoff 2020). Microglia activation can also be enhanced by astrocytes, maintaining the activity of this quad-partite synapse (Schafer et al. 2013) and perpetuating pain (Donnelly et al. 2020). While not fully investigated in the context of chronic post-SCI central neuropathic pain, there is evidence

demonstrating that microglial mechanisms may be critical. In this context, this systematic review aims to collect information from studies documenting the involvement of microglial cells in the emergence and maintenance of central neuropathic pain associated with SCI.

METHODS

This systematic review was elaborated following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0 were considered as an additional checklist for a more thorough and quality-improved analysis of the included articles, considering they referred to animal models of disease. The ARRIVE guidelines were originally developed in 2010 to improve the reporting of animal research (Kilkenny et al. 2010), and updated in 2020 under the affix 2.0. The ARRIVE guidelines consist of a checklist of information to include in publications describing *in vivo* experiments to enable others to scrutinize data adequately, evaluate its methodological rigor, and reproduce the methods and results (Percie du Sert et al. 2020). The risk of Bias was assessed using SYRCLE'S Risk of Bias tool (Figure 2). This tool is adapted to experimental animal studies from Cochrane Risk of Bias Tool. The SYRCLE'S Risk of Bias tool encompasses multiple questions and topics that aim to evaluate and report selection bias, performance bias, detection bias and attrition bias (Hooijmans et al. 2014). The Cochrane Risk of Bias (Rob) checklist was also consulted (Higgins et al. 2011).

The query "Spinal Cord Injury" AND "Microglia" AND "Chronic Pain" was searched in three databases: PubMed Central (via PubMed); and Medline and Embase (via Scopus). No MeSH terms were used as Scopus does not support them and as their use in PubMed

reduced the number of articles presented substantially. The Boolean operator “AND” were chosen as such to include exclusively the indispensable keywords for this review. These terms were searched within the keywords, title or abstract of the studies, as it was intended that these would be one or the main focus of the study. The search was conducted on the 11th of October 2022, limited to articles published between 2012 and 2022. The year 2012 was chosen as specific automated devices for spinal injury became available in that year (Cheriyan et al. 2014). No further restrictions or filters were used. The inclusion criteria were (1) the inclusion of an experimental group of SCI animals; (2) description of density, morphology and metabolic characteristics of microglial cells; and (3) studies published in English. The exclusion criteria were: (1) non-original studies (reviews, conference abstracts, and editorials); (2) studies conducted in human population, such as case reports and clinical trials; (3) presence of peripheral nerve injury (Spared Nerve Injury (SNI) or peripheral nerve CCI (Chronic Compression Injury) and not SCI; (4) impossibility of obtaining the full-text article (even after contacting the authors). All articles were submitted to full-text screening if all inclusion criteria were present and if no exclusion criterion was met.

For data extraction the following outcomes were sought: (1) Study characteristics (author and year of publication); (2) Type of SCI; (3) Experimental and control groups; (4) Effects of microglia modulation; (5) Changes in microglia’s activity and (6) Other results and conclusions. These outcomes are indicated in Table 1. Both researchers evaluated all articles and worked independently without using automatic tools. Both researchers performed data analysis and extraction independently. When conflicts were found in this process, both performed a second analysis together and discussed the conflicting points.

RESULTS

A total of 854 articles were found, 341 via PubMed and 513 via Scopus. The number of duplicate papers was 288. These were excluded, generating a list of 566 publications. Articles were then independently screened by two investigators using the title and abstract. After initial screening, 4 articles in the PubMed group and 20 articles in the Scopus group met all the requirements for posterior analysis and possible inclusion in this review. Further screening of the publications included reading of the full text and sought to identify information, which was not clear in the abstract, namely if the methods included an SCI procedure and if microglia's analysis was present.

Further in-depth analysis showed that 2 studies did not use any SCI model (Batti et al. 2016, Turcato et al. 2019) and, for that reason, were excluded. Furthermore, 2 other articles were also excluded for not demonstrating effects of SCI on microglial cells (Yu et al. 2014, Sabirzhanov et al. 2019). As such, 20 articles were deemed to full inclusion and analysis (Figure 1). A moderate risk of bias was found (Figure 2).

The experimental unit of all articles was a single mouse or rat. A single study used both rat and mice models (CD1 mice and Wistar rats) (Martini et al. 2016). Furthermore, mice were the most used experimental animal, having been used in 10 other studies (50%), with C57BL/6 being the preferred strain. Other mice strains used included the BALB/c strain (Tenorio et al. 2013); spinal hyperostotic tiptoe walking (*ttw/ttw*) and ICR mice (Takeura et al. 2019) and *plt* (paucity of lymph node T cell) mice (Honjoh et al. 2019). On the other hand, rats were used in 9 other studies (45%). Males were used in the majority of studies, as 14 publications (70%) reported their exclusive use. On another hand, 4 studies (20%) exclusively used females and 2 publications reported the use of female and male mice (10%).

The methods used to induce SCI were varied. Spinal cord contusion was used in 13 (65%) studies and spinal cord compression was used in 4 studies (20%). Three articles (15%) used less common approaches and included perispinal zymogen-induced inflammation (Tenorio et al. 2013), unilateral electrolytic lesion focused on the spinothalamic tract (Naseri et al. 2013) and spinal cord hemisection (Martini et al. 2016). Regarding the level of spinal lesion, SCI were induced at low thoracic levels (T7-T12) in 15 (75%) articles, whilst in 3 (15%) SCI was induced at cervical level. Injection of zymosan was conducted at L1-L2 spinal level. One additional study (Li et al. 2019) did not specify the location of SCI and referred to a paper's described methods which we could not obtain (Sekiguchi et al. 2011).

SCI-related damage and microglia reactions were comparable across all types of SCI used in the included papers. Chronic compression injury caused histopathological and pathophysiological changes significantly similar to those found in traumatic SCI, such as degeneration and demyelination of neurons (Takeura et al. 2019). There was also chronic brain neurodegeneration in the cortex, thalamus, and hippocampus at week 10, which correlated with microglial activation (Wu et al. 2014).

The use of the ARRIVE Guidelines was mentioned by 2 studies (10%). Blinding of the investigators for results analysis was only clearly mentioned in 13 articles (65%), only being referred for the analysis of motor activity of the animals and no other measured parameters, such as immunofluorescence or histological analysis. Randomization procedures of mice and rats based in random number generation for the attribution to groups was mentioned in 5 articles (25%). As reported, age matching was conducted in 12 articles (60%).

Exclusion of experimental animals from analysis was also performed. Exclusion parameters reported included surgical complications, poor tissue dissection and tissue damage (Li et al. 2021); mice that did not develop pain post-SCI (Crown et al. 2012); the extension of the lesion beyond the intended area or lack of the intended lesion (Naseri et al. 2013); animals that presented severe motor impairment post-SCI (Naseri et al. 2013); animals presenting signs of infection (Li et al. 2020) and animals that died during surgery (Yao et al. 2022).

All included articles reported analysis and interpretations of changes in numbers, density or activation of microglia and, frequently, other phagocytic immune cells. Expression of inflammatory and anti-inflammatory mediators were also analysed, aiming to establish a correlation among changes in cutaneous sensitivity and motor activity. Studies demonstrate an upregulation of microglial markers after SCI in the superficial dorsal horn (sDH), both close to the lesion and caudal to it, and in the brain (cortex, hippocampus and thalamus). While only two articles used male and female animals, it seems there are no gender-related differences in microglial changes post-SCI (Li et al. 2021).

Regarding the time frames of the microglia reactions post-SCI, these were reported with varied evidence. After perispinal inflammation, there was a steady increase in microglia numbers and activity from day 1 post-SCI (Tenorio et al. 2013), whilst after contusion SCI Iba1-positive cells started to increase from day 10 post-SCI (Brown et al. 2021). In the model of unilateral electrolytic lesion, there were two isolated significant increases at 3 and 7 days post-SCI (Naseri et al. 2013). Microglia and infiltrated macrophages on-site were reported to peak at 14 days post-SCI (Honjoh et al. 2019, Nakajima et al. 2020), correlating with the peak of pain and hypersensitivity symptoms

(Nakajima et al. 2020). Long-term post-SCI increases in spinal microglia were observed at 28 days (Honjoh et al. 2019), 35 days (Brown et al. 2021) and 6 weeks after SCI (Li et al. 2020). In the studied brain regions (cerebral cortex, hippocampus and thalamus), no significant changes were reported at 7 days post-SCI (Wu et al. 2014, Li et al. 2020), only being observed at 8 to 10 weeks post-SCI, with very significant increases in microglial activation and density in all areas except in the centrolateral thalamic nucleus (Wu et al. 2013, Wu et al. 2014). It should be noted that 7 days post-SCI was a key time point in this model, as the microglial phenotype changed from neuroprotective to neurotoxic (Li et al. 2020).

Changes in density and location of microglial cells after SCI were also analysed. All studies included in the present review reported a significant increase post-SCI in microglia whether on-site or in areas further away from injury. The density of microglial cells was upregulated after SCI, irrespective of the type of injury. In many cases, such as spinal cord compression, there was a constant increase in microglia at the lesion site up to the 14th day post-SCI, correlating with the severity of degree of compression (Tenorio et al. 2013, Takeura et al. 2019). The density of microglial cells was further exacerbated by noxious stimulation (Garraway et al. 2014).

Several approaches to reduce microglial numbers were tested and presented positive results, including acupuncture (whereas simulated acupuncture did not produce any significant effects) (Choi et al. 2012); treatment with L1 agonists, such as Duloxetine and L1Fc (Kataria et al. 2016), being L1 a recognition molecule, expressed in neurons and glial cells, that plays a crucial role in neuronal cell migration and survival, neurite outgrowth, axon guidance and fasciculation, myelination, synaptic plasticity, and regeneration after injury (Barbin et al. 2004); inhibition of the HV1 receptor, which

participates in NOX2-dependent extrusion of protons to alleviate intracellular acidosis during phagocytic processes (Li et al. 2021); and inhibition of the chemokine CCL21, which induces upregulation of the P2X4 receptor in microglia and macrophages, linked to the regulation of release of pro-nociceptive factors such as brain-derived neurotrophic factors (BDNF) and inflammatory cytokines (TNF-α and IFN-ɣ) (Honjoh et al. 2019); long-term inhibition of the colony-stimulating factor 1 receptor (CSF1R) with PLX5622 (as CSF1R signalling is essential for microglia proliferation) (Li et al. 2020); treatment with selective cyclin dependent kinase (CDK) inhibitors (Wu et al. 2013, Wu et al. 2014); treatment with LXA4 (Martini et al. 2016) (an endogenous lipid mediator with an anti-inflammatory properties that affects cell cycle progression via CyclinD1/P16INK4A, ERK1/2, and NF-κB signal transduction pathways (Hu et al. 2015, Hu et al. 2015)); and minocycline administration (Aceves et al. 2019) (a tetracycline antibiotic with anti-inflammatory, immunomodulatory and neuroprotective properties via inhibition of iNOS, MMPs, PLA2, caspase-1 and caspase 3 pathways (Garrido-Mesa et al. 2013)).

Microglial phenotypes were also analysed in SCI tissue. At the lesion site, microglia were predominantly M1 type (Honjoh et al. 2019, Nakajima et al. 2020, Yao et al. 2022). M1 microglia cells were upregulated by NOX2, a NADPH oxidase critical to induce ROS in microglia/macrophages immune response, and contributed to inflammatory-mediated neurological dysfunction (Sabirzhanov et al. 2019). In contrast, microglial cells present in spared spinal tissue caudal to lesion belonged to the M2 type (Nakajima et al. 2020).

Analysis of microglia activation phenotypes was also conducted. Reports documented the presence of activated microglial cells, characterized by large cell bodies

and retracted processes. These analyses were performed exclusively in select brain regions and not in the spinal cord. The regions analysed were: the posterior thalamic nucleus, the ventral posterolateral nucleus of the thalamus (Wu et al. 2013), the cerebral cortex, the hippocampus and the cerebellum (Wu et al. 2014). These activation phenotypes remained up to 8 and 10 weeks post-SCI (Wu et al. 2013, Wu et al. 2014). This activation and the corresponding mechanical allodynia were significantly reduced by CR8 treatment (Wu et al. 2013).

After SCI, several receptors and signalling pathways were upregulated in microglial cells. The list is long and includes TNF-α (Wu et al. 2014, Li et al. 2019, Yao et al. 2022), ROS (Yao et al. 2022), IL-1α , Igf1, the microglial receptors Csf1r, P2ry12, Trem2 and Itgam (Li et al. 2020) and TREM1 (Li et al. 2019). Importantly, TNF-α was decreased in *plt* mice, which do not express the chemokine CCL21 (Honjoh et al. 2019); after TREM1 inhibition (Li et al. 2019) and after ALX/FPR2 receptor activation (Martini et al. 2016). Furthermore, ROS were decreased following acupuncture (Choi et al. 2012); by Hv1 inhibition (Li et al. 2021) and by microglia depletion via PLX5622 treatment (Li et al. 2020).

Studies included in this review were able to positively correlate the presence of mechanical allodynia and thermal hypersensitivity, that are main features in neuropathic pain, with increased density and activation of microglial cells. In most studies mechanical hypersensitivity was tested using von Frey filaments, while thermal hyperalgesia was evaluated with the Hargreaves method (Hargreaves et al. 1988). Alleviation of mechanical allodynia and thermal hyperalgesia were reported after acupuncture (Choi et al. 2012); deletion of CCL21 (alteration present in *plt* mice) (Honjoh et al. 2019); CR8 treatment (Wu et al. 2013); inhibition of Calcium/calmodulin-

dependent protein kinase II (CaMKII) (Crown et al. 2012); administration of huperzine-A (HUP-A) (a potent reversible inhibitor of acetylcholinesterase and NMDA receptors, which contributes to inhibition of microglial-driven inflammation) (Yu et al. 2013) and administration of LXA4 (which didn't have any effect in mice receiving receptor ALX/FPR2 inhibitory treatment) (Martini et al. 2016). In all cases, the studies report a downregulation of microglial activity.

Motor function was also evaluated in some studies. Mice in the SCI groups showed poor motor functions, but showed improvement across time with the repetition of motor tests (Yao et al. 2022). Motor function was also improved after treatment with L1 agonists, namely Duloxetine and L1Fc (Kataria et al. 2016); and in Hv1-knockout mice (Li et al. 2021). Interestingly, mice with perispinal zymosan-induced spinal inflammation did not perform worse at the rotarod test than control animals (Tenorio et al. 2013), suggesting physical trauma is the main cause of loss of motor function post-SCI.

Finally, it is important to refer to tools used to validate results and for bias analysis. Overall, the application of the SYRCLE'S Risk of Bias (RoB) tool showed that a moderate risk of bias may be present in these studies (Figure 2), but much of the information required to fully use both the RoB tool and the ARRIVE Guidelines was missing or not clear. The results were not quantified, as the RoB tool defines that it is not recommended to calculate a summary score for each individual study when using this tool, as a summary score inevitably involves assigning "weights" to specific domains in the tool, and it is difficult to justify the weights assigned (Hooijmans et al. 2014). The relevant lacking information that increases the risk of bias is mostly related to a set of reasons, including lack of information regarding randomization; the fact that blinding

was only reported in 65% of articles; and reasons relating to husbandry and testing conditions.

Finally, in many studies, reference to the use of the ARRIVE Guidelines was left incomplete. In most studies the time frames and order of each of the procedures were not explicit, as well as it was not clear the total number of animals used and if there had been exclusion of animals from experimental groups.

DISCUSSION

This paper aimed to collect and organize relevant and available information about the involvement of microglia in post-SCI neuropathic pain. Studies reviewed demonstrate that multiple microglia-related mechanisms and are critical, but many remain to be fully investigated, as a multitude of interactions between different microglia pathways and with other CNS cells might be at play. We found that many modulators show promise to be explored as potential treatments for chronic pain, such as minocycline which is already included in clinical trials. The set of studies analysed point to microglia being a potential therapeutic target, with further studies being warranted for a full clinical translation and a significant improvement of the quality of life of many SCI patients that live with chronic neuropathic pain.

Traumatic SCI injury induces an irreversible damage of descending and ascending tracts in the neuroaxis, triggering a wide spectrum of sensorimotor dysfunctions as spasticity and chronic neuropathic pain (Crone et al. 2008, David and Steward 2010, Masri and Keller 2012), with highly deleterious consequences on quality of life of SCI patients. Most available treatments for chronic neuropathic pain are medications aiming to block or reduce excitatory neurotransmission, such as botulinum toxin A (Finnerup et

al. 2015), which exerts its effects by entering the pre-synaptic neuronal terminal and promoting long-term cleaving of synaptic proteins necessary for docking and fusion of neurotransmitter vesicles (Rossetto et al. 2021). Gabapentin, also widely used in treatment of chronic neuropathic pain (Finnerup et al. 2015), is a ligand of the α2δ calcium channel subunit that indirectly blocks channels in neurons present in the central nervous system (CNS), impairing nociceptive neurotransmission (Chincholkar 2018, Manville and Abbott 2018, Taylor and Harris 2020). In most cases, prolonged treatments with these medications bring about undesirable side-effects and/or decrease in efficacy. There is a need to identify new therapeutic targets to improve treatment.

In recent years, glial cells have been receiving increased attention in fields as varied as pain, neurodegenerative and mood disorders. Astrocytes and microglia are now recognized as potent regulators of synapses, either potentiating or reducing neurotransmission (Matejuk and Ransohoff 2020). Microglial cells are the archetypical resident macrophages of the CNS and display various phenotypes according to their surrounding environment. The involvement of glial cells, particularly microglia, has been demonstrated in cases of chronic peripheral neuropathic pain (Morgado et al. 2011) and their involvement in SCI-induced neuropathic pain is now indicated by several studies. Here, we performed a systematic review providing evidence of the importance of microglial cells in this context and analysed the effects of manipulation of these glial cells. The studies analysed here used spinal contusions and spinal transections, which are commonly used to produce spinal injury (Cheriyan et al. 2014).

Analysis of the animal models used

In our search, we found that the most used animals are rats and mice. These animals are widely used in fundamental and translational research as they are easily

manipulated, there is a wealth of knowledge of their biology and use as animal models of disease and, particularly mice, offer the opportunity of genetic manipulation (Bradley et al. 1992). Most studies used male animals, which might facilitate clinical translation, as the majority (78%) of new SCI cases are males (Lo et al. 2021). The maximal duration of the experimental period was 10 weeks, which could be a concern, as chronic pain is a long-term condition of patients post-SCI. It would be relevant to extend the periods of animal observation to potentiate the identification of long-term changes and effects of microglial manipulation.

Increases in microglia density positively correlate with tissue damage

It was documented that after SCI there is an increase in density of microglial cells and/or proteins specific to their structure and metabolism, such as Iba-1. These increases were established in many locations, such as the injury site (Naseri et al. 2013, Garraway et al. 2014, Aceves et al. 2019, Takeura et al. 2019, Nakajima et al. 2020, Brown et al. 2021), distant sites of the spinal cord (Wu et al. 2013, Nakajima et al. 2020) and brain (Wu et al. 2013, Wu et al. 2014). Experiments with PLX5622 suggest that microglia found at early stages of disease progression have a neuroprotective role, while when present at later stages they favour neurodegeneration (Li et al. 2020). Density of microglia and their activity correlated positively with the amount of neural tissue damage and with poor prognostic in the recovery of symptoms post-SCI, which suggests that microglial cells are involved in maladaptive neuroplasticity.

Changes in microglia-related signalling

Increased numbers of microglial cells coursed with increased activation of these cells, as shown by upregulation of several intracellular signalling proteins such as Iba-1 (Wu et al. 2013, Wu et al. 2014, Kataria et al. 2016, Martini et al. 2016, Li et al. 2019,

Sabirzhanov et al. 2019), MAPK, ERK, pCaMKII and CCL21 (Choi et al. 2012, Crown et al. 2012, Wu et al. 2014, Honjoh et al. 2019). Another indication of increased microglial activation is the high level of mediators present in the spinal tissue, including pro- and anti-inflammatory interleukins (TNF-α, caspase proteins, BAX, NOX proteins, IL-1β, IL-6, IL-18, IL-10, CD86, CD68, SOCS and iNOS (Yu et al. 2013, Garraway et al. 2014, Wu et al. 2014, Martini et al. 2016, Honjoh et al. 2019, Li et al. 2019, Sabirzhanov et al. 2019, Li et al. 2020, Li et al. 2021, Yao et al. 2022)) and proteins involved in neuroplasticity associated with neuropathic pain (Pezet and McMahon 2006, Thakkar and Acevedo 2023), such as BDNF (Garraway et al. 2014).

Increased microglia activity post-SCI correlated with increased mechanical allodynia and thermal hyperalgesia

All studies showing mechanical allodynia and/or thermal hyperalgesia post-SCI demonstrated positive correlation with increased density and activity of microglial cells (Choi et al. 2012, Tenorio et al. 2013, Wu et al. 2013, Yu et al. 2013, Garraway et al. 2014, Martini et al. 2016, Honjoh et al. 2019, Li et al. 2019). Mechanical allodynia was assessed by the paw withdrawal threshold (PWT) in response to probing with a series of calibrated von Frey filaments, whilst heat sensitivity was assessed according to the Hargreaves method (Hargreaves et al. 1988) to determine paw withdrawal latency (PWL) in response to a radiant heat (Choi et al. 2012, Honjoh et al. 2019). Taken together, these observations indicate that microglial cells are involved in chronic neuropathic pain arising after SCI.

Procedures that reduced microglia activity and/or pain

Many studies investigated the effects of microglia modulation on pain levels. Overall, microglia inhibition in SCI animals reduced cutaneous hypersensitivity. The procedures

presented by studies that achieved a reduction both in microglia activity and in pain sensitivity were: acupuncture (Choi et al. 2012) and TREM1 inhibition (Li et al. 2019). Other studies did not test the pain perceived by the animals, but equally documented a reduction in microglia and inflammation-derived tissue damage. These were: the administration of minocycline to reduce microglia activation (Aceves et al. 2019); the use of PLX5622, a specific microglial inhibitor (Li et al. 2020); administration of LXA4 (Martini et al. 2016) and HUP-A administration (Yu et al. 2013). In many studies, the activation of signalling pathways (see above) activated in and by microglial cells in SCI animals was downregulation after intervention, reducing pain levels.

Assessment of bias and heterogeneity of results

In this review, bias was assessed using the SYRCLE'S Risk of Bias (RoB) tool and evaluating if studies followed the ARRIVE guidelines. The SYRCLE'S Risk of Bias (RoB) tool showed a moderate risk of bias. Overall, there was a significant amount of information lacking in the methods section of several studies. Performance bias was likely present significantly as there was a high variety of SCI procedures whose results might not be entirely comparable and the methods by which the microglia were identified and analysed varied significantly. Furthermore, the variations in signalling molecules and inflammation mediators could not be considered directly or exclusively conveyed by the microglia, as tissue samples analysed certainly contained other cell types beyond microglial cells.

Despite a moderate risk of bias, studies were consistent as variations density and activity of the microglia were similarly reported, as well as for signalling molecules and inflammation mediators assessed in multiple studies such as TNF-α, caspase and NOX proteins, IL-6, IL-10, CD86, CD68, SOCS and iNOS. Some variation was found relating to

the time point of disease progression when SCI animals were studied. The type of SCI, strain and/or sex and different husbandry conditions might also have contributed to some variability between studies. Many of these factors were frequently not reported, which may constitute and an important source of bias.

In what refers to the ARRIVE guidelines, they were proposed in 2010 (Kilkenny et al. 2010) and updated in 2020 (Percie du Sert et al. 2020, Percie du Sert et al. 2020). These guidelines describe a checklist of information that should be included in publications using animal research to ensure transparency and reproducibility. While animal models of disease are critical to expand basic and clinical knowledge, most studies analysed here do not present evidence of quality of reporting and adherence to the ARRIVE guidelines, which reflected on the moderate bias assessed by the SYRCLE'S Risk of Bias (RoB).

CONCLUSION

In recent decades, our understanding about the role and the importance of microglial cells in the spinal cord has greatly increased. Several studies demonstrate their involvement in peripheral neuropathic pain, a condition in which they participate by directly regulating neurotransmission and local interaction with other glial cells. In this systematic review, we found studies demonstrating that microglial cells are also relevant in the pathophysiology of post-SCI neuropathic pain. There is evidence showing these cells might be potential targets for pharmacological treatment of chronic central neuropathic pain. This treatment would be based on glial inhibitors, which can block membrane receptors and/or intracellular signalling pathways. Of all compounds used, minocycline is the only one which is currently being evaluated in clinical trials, in this

case, for its use in lower back pain (Loggia et al. 2017). If this trial is successful, this could mean that minocycline could eventually be used in clinical context to treat pain.

CONFLICT OF INTERESTS

The authors declare they have no conflicts of interests.

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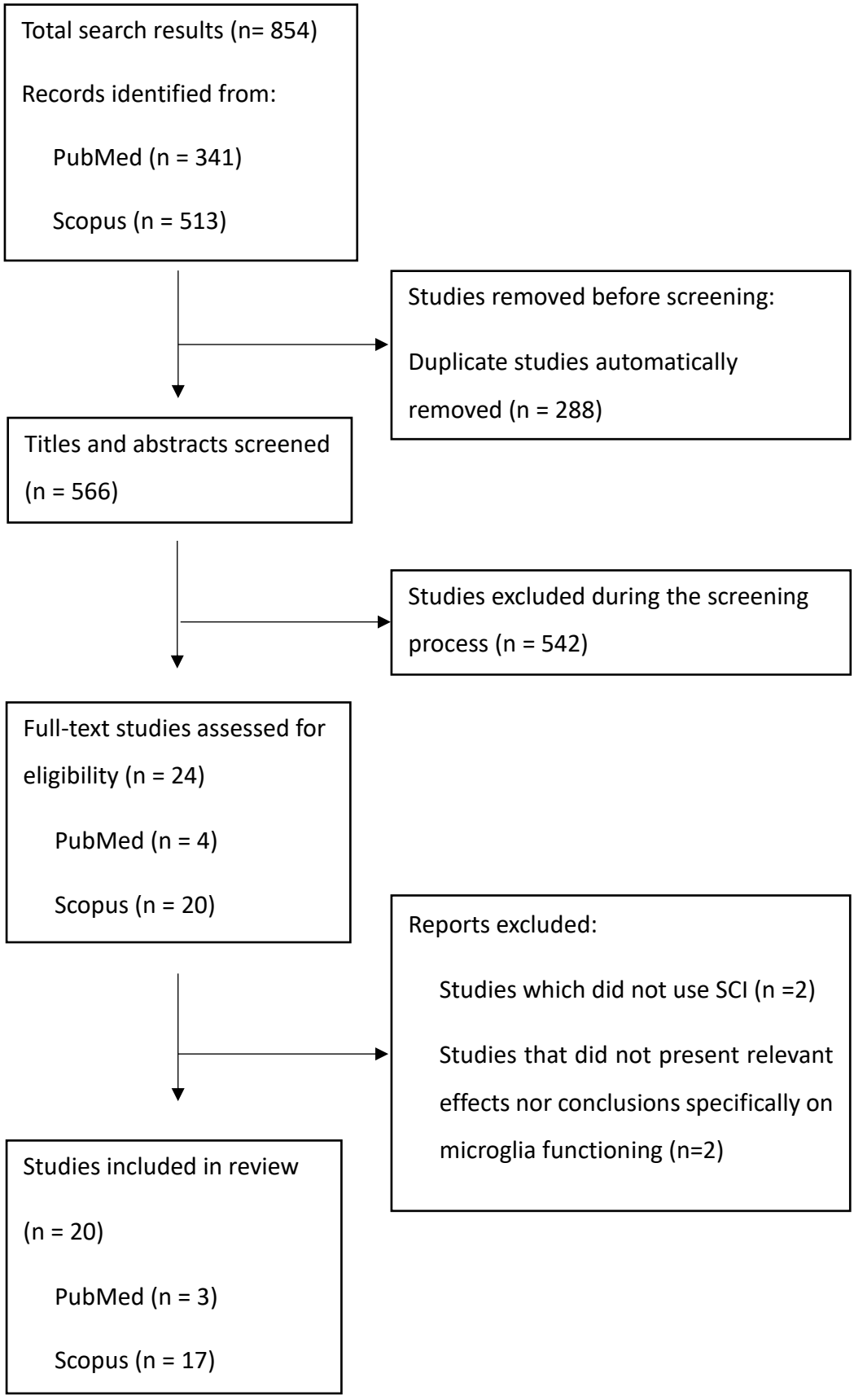
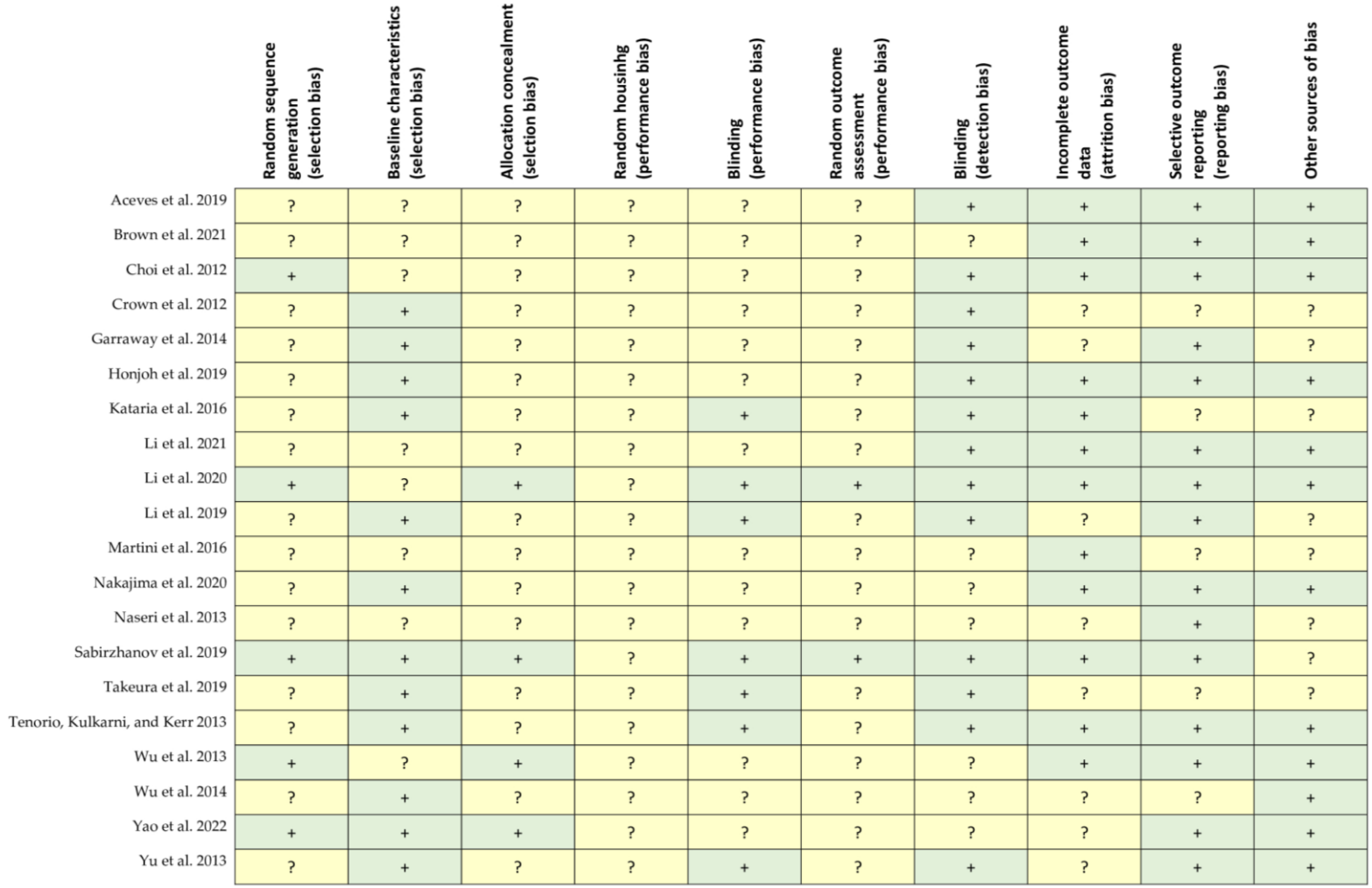


Figure 1: PRISMA flow diagram with the screening process for this systematic review.



	Random sequence generation (selection bias)	Baseline characteristics (selection bias)	Allocation concealment (selction bias)	Random housinhg (performance bias)	Blinding (performance bias)	Random outcome assessment (performance bias)	Blinding (detection bias)	Incomplete outcome data (attrition bias)	Selective outcome reporting (reporting bias)	Other sources of bias
Aceves et al. 2019	?	?	?	?	?	?	+	+	+	+
Brown et al. 2021	?	?	?	?	?	?	?	+	+	+
Choi et al. 2012	+	?	?	?	?	?	+	+	+	+
Crown et al. 2012	?	+	?	?	?	?	+	?	?	?
Garraway et al. 2014	?	+	?	?	?	?	+	?	+	?
Honjoh et al. 2019	?	+	?	?	?	?	+	+	+	+
Kataria et al. 2016	?	+	?	?	+	?	+	+	?	?
Li et al. 2021	?	?	?	?	?	?	+	+	+	+
Li et al. 2020	+	?	+	?	+	+	+	+	+	+
Li et al. 2019	?	+	?	?	+	?	+	?	+	?
Martini et al. 2016	?	?	?	?	?	?	?	+	?	?
Nakajima et al. 2020	?	+	?	?	?	?	?	+	+	+
Naseri et al. 2013	?	?	?	?	?	?	?	?	+	?
Sabirzhanov et al. 2019	+	+	+	?	+	+	+	+	+	?
Takeura et al. 2019	?	+	?	?	+	?	+	?	?	?
Tenorio, Kulkarni, and Kerr 2013	?	+	?	?	+	?	+	+	+	+
Wu et al. 2013	+	?	+	?	?	?	?	+	+	+
Wu et al. 2014	?	+	?	?	?	?	?	?	?	+
Yao et al. 2022	+	+	+	?	?	?	?	?	+	+
Yu et al. 2013	?	+	?	?	+	?	+	?	+	+

Figure 2: SYRCLE's risk of bias tool. Every article included in this systematic review was subjected to risk of bias assessment tool developed by Hooijmans et al. + (green background): low risk of bias; ? (yellow background): unclear risk of bias; - (red background): high risk of bias.

	Aim of the study	Type and location of SCI	Experimental and control groups	Effects of microglia modulation	Further changes associated with microglia activity
Aceves et al. 2019	Assessment of immune cells increase upon morphine administration and the effect of prior minocycline treatment.	Contusion (T12)	[1]: Sham; Morphine; Minocycline post-SCI. [2]: Minocycline pre-SCI (0, 50 and 100μg); Morphine (0 and 90μg).	Morphine significantly increased the number of microglia present in the spinal cord regardless of injury. Pre-treatment with minocycline reduced the morphine-induced increase in microglia present at the site of injury without compromising the immune response necessary for recovery of function.	
Brown et al. 2021	Characterization of the superficial dorsal horn response of microglia and macrophages within intact cervical spinal cord segments post-SCI caudal to the lesion.	Contusion (Right side of C5-C6)	SCI; Sham.	Cervical contusion SCI induces a heterogeneous inflammatory activity of phagocytic cells in the sDH 1 day post-SCI. Iba1-positive cells increase from day 10 post-SCI and persist for at least 35 days. There were no significant cellular changes in the lumbar sDH post-SCI at any time point.	Contusion SCI caused a significant increase of IL-1β and CD86, but not of TNFα, iNOS, IL-10. This increase happened caudal to the lesion within intact tissue of the posterior spinal cord. An increase in CD68+ cells in the sDH was verified as soon as 1 day post-SCI.

Choi et al. 2012	Assessment of analgesic effect of acupuncture on SCI-induced neuropathic pain.	Contusion (T9-T10)	SCI; Sham; SCI + Vehicle; SCI + Minocycline SCI + Acupuncture; SCI + PD98059; SCI + PD98059 + Acupuncture; SCI + SB203580; SCI + Acupuncture + SB203580; Simulated Acupuncture.	Acupuncture decreased activated microglia numbers, ERK, PGE2, p38MAPK and production of ROS post-SCI on-site. Specific ERK inhibition reduced levels of PGE2.	Mice post-SCI developed mechanical allodynia and thermal hyperalgesia. Acupuncture alleviated mechanical allodynia and thermal hyperalgesia. Higher presence of activated microglia numbers, ERK, PGE2, p38MAPK and production of ROS post-SCI on-site correlated with stronger symptoms of mechanical allodynia and thermal hyperalgesia. Simulated acupuncture was reported to no show any of the effects present in all acupuncture groups. Cessation of acupuncture or minocycline treatment resulted in loss of their antinociceptive effect.
Crown et al. 2012	Identify the presence, changes	Contusion (T10)	Naïve; Sham (Kn-93 (100µM); Kn-92	The microglia of the sDH did not show any changes in levels	SCI caused at-level mechanical allodynia

	and effects of chronically activated pCaMKII post-SCI by comparison with a control model with CaMKII inhibitor Kn-93 and its enantiomer Kn-92.		(100μM)); SCI (KN-93 (1, 10 and 100μM); KN-92 (100μM)).	of pCaMKII post-SCI similarly to astrogliocytes, but contrary to the dorsal column oligodendrocytes (which had pCaMKII upregulated).	accompanied by CaMKII activation of spinal grey matter neurons and oligodendrocytes. At-level mechanical allodynia was decreased by inhibition of CaMKII for as long as 35 days post-SCI.
Garraway et al. 2014	To study the effect of noxious input post-SCI on the development and maintenance of chronic neuropathic pain and effects on the temporal and spatial expression of TNFα, TNF receptors and respective downstream targets.	Contusion (T12)	Sham; SCI; SCI + noxious tail stimulation.	Noxious stimulation post-SCI augments, further than SCI by itself, the microglia morphological signs of apoptosis.	Noxious stimulation post-SCI increases, more than SCI by itself, the expression of TNFα and caspase-3 at the superficial dorsal and ventral lesioned spinal cord. Peripheral noxious input post-SCI caused an increase in mechanical allodynia, TNFα and caspase-8 signalling. Significant mechanical allodynia developed from 24h onward post-SCI.

Honjoh et al. 2019	Evaluation of post-SCI neuropathic pain, expression of microglia and macrophages and inflammatory cytokines at the injured site and lumbar enlargement in deficient CCL21 mice.	Contusion (T9-T10)	Sham; wild-type; *plt*-mice.	Microglia and macrophages peaked at 14 days post-SCI on-site. However, these were significantly suppressed at days 4 and 14 in *plt* mice. In the lumbar enlargement microglia and macrophages were diminished in all 4, 14 and 28 days post-SCI in *plt* mice. Phenotype of microglia and macrophages was M1 type-dominant in both types of mice at the lesion site and lumbar enlargement. The M1 type microglia numbers decreased only in *plt* mice.	Mechanical and thermal hypersensitivities had more significant improvements in *plt* mice 2 weeks post-SCI compared to wild-type. M1-induced cytokines (TNF-a and IFN-g) were decreased in *plt* mice, while M2-induced cytokines (IL-4 and IL-10) did not differ. Locomotor recovery post-SCI did not differ between *wild-type* and *plt* mice.
Kataria et al. 2016	To analyse the effect of agonists of the cell adhesion molecule L1 on the inflammation, motor recovery and allodynia development post-compression SCI in mice.	Compression (T7-T9)	Mock (phosphate-buffered saline solution control); duloxetine; phenelzine sulphate; tacrine hydrochloride; etinylestradiol; L1Fc; crotamiton; honokiol;	Treatment of primary neurons with all L1 agonists post-SCI decreased astrocytes and microglia, while Duloxetine and L1Fc were the ones with the most significant effect.	Treatment of primary neurons with all L1 agonists post-SCI, namely Duloxetine and L1Fc, improved locomotor recovery, enhanced survival, outgrowth and remyelination of motoneurons and increased number of perisomatic synaptic terminals.

			trimebutine maleate; piceid.		
Li et al. 2021	To understand whether the phagocyte-specific proton channel Hv1 mediates hydrogen proton extrusion post-SCI and the assessment of its contribution to extracellular acidosis and poor prognostic.	Contusion (T10)	Sham/WT; SCI/WT; Sham/Hv1-KO; SCI/Hv1-KO.	Hv1 deficiency, at 3 days post-SCI and compared to WT, reduced microglia proliferation and leukocyte infiltration. Hv1 was exclusively expressed in microglia within the CNS. NOX2 expression post-SCI was increased in microglia, but comparably reduced in SCI/Hv1 KO mice.	SCI significantly increased Hv1 mRNA expression in the CD11b+ cells. Depletion of Hv1 attenuated tissue acidosis, NOX2 expression, CD68 levels, phagocytic oxidative burst, and ROS production at 3 days post-SCI. Hv1 KO mice exhibited improved locomotor function, reduced cellular inflammation and ROS production. Hv1 KO mice showed a recovery in fine motor control with significant improvement at 21 days, which remained during the studied 42 days post-SCI. SCI/Hv1 KO group revealed less myelin damage and higher volume of spared white matter.

Li et al. 2020	To determine the effects of microglia depletion on CNS-infiltrating cells post-SCI. To test whether PLX5622-mediated microglial depletion after injury affects the acute inflammatory response. To investigate whether such microglial depletion post-SCI affects neurological function. To determine whether the effect of PLX5622 on locomotor function was injury severity dependent.	Contusion (T10)	Sham/Vehicle; SCI/Vehicle; Sham/PLX; SCI/PLX.	SCI did not change the number of CD11b+CD45int microglia at 7 days post-SCI in the cerebral cortex. SCI changes the microglia's phenotype from a neurorestorative and neuroprotective to a neurotoxic one. PLX5622 pre-treatment reduced number of microglia by >94% in the brain and 70% in the spinal cord in 7 days and >90% overall in 6 weeks of treatment. Mice depleted of microglia post-SCI reduced markedly the SCI-induced expression of reduced gene expression of Casp1, Casp4, Casp6, Casp7, Casp8, Bax, IL-1α, Inpp5d, Csf3r, Vav1, Il1a and Lrrc25.	Mice depleted of microglia before SCI exhibited more severe locomotor deficits 1 day post-SCI. PLX5622 reduced infiltrating monocytes and neutrophils and production of both ROS and TNFα in these cells on-site at 2-days post-SCI. 6 weeks of PLX5622 treatment were associated with improved neuronal survival in the brain and neurological recovery. Treatment caused Bcl2 and Igf1 reduction in the cortex post-SCI which correlated with reduced neuronal survival in the thalamus and hippocampus, as well as reduced fine motor dysfunction, cognitive dysfunction and depressive like behaviour. Mice depleted of microglia post-SCI had markedly reduced the microglial

					receptors Csf1r, P2ry12, Trem2 and Itgam and the genes Il1a, Lrrc25 and IL-1α and Lrrc25. Genes upregulated post-SCI were Atm, Atr, Prkdc, Bcl2, Il1a, Trp53bp2, Lrrc25 and IL-1α Chn2, Chst8, Epsti1 and Kcnk13. PLX5622 treatment also significantly reduced the genes Chn2, Chst8, Epsti1, Kcnk13.

Li et al. 2019	To understand the role of TREM1 post-SCI.	Contusion SCI + in vitro TREM/NC with siRNA transfection (control)	Sham/WT; Sham/TREM1KO; SCI/WT; SCI/TREM1KO.	Post-SCI, TREM1 was over-expressed in the injured spinal cord tissues, and LPS-stimulated astrocytes and microglia.	TREM1 inhibition down-regulated the pro-inflammatory mediators TNF-α, IL-1β, IL-6, IL-18, CD86, iNOS, CD68, CCL3, IRF5, IRF7, MCP-1 and MCP-3 with associated decreased inflammatory damage. TREM1KO mice exhibited improved locomotor function, mechanical and thermal hypersensitivity in the hindpaws.
Martini et al. 2016	To study the effects of lipoxin A4 (LXA4) on spinal neuroinflammation and chronic pain post-SCI.	Spinal Cord Hemisection (Left side T10)	Sham; SCI + vehicle; SCI + LXA4.	LXA4 directly interacts with microglia through ALX/FPR2 receptors and reduces both its activation and TNF-α release.	Mechanical hypersensitivity post-unilateral hemisection SCI developed in both ipsilateral and contralateral hindpaws. LXA4, administered both pre-SCI and exclusively 35 days post-SCI, reduced the intensity of mechanical allodynia. In mice, SCI upregulates mRNA levels of ALX/ FPR2 receptor, GFAP, IBA-1, P2Y12, TNF-α, IL-6, iNOS, and TGF-β 7 days post-SCI.

					In rats, SCI elevates IL-6, IL1β and TNF-α while IL-10 doesn't change.
Nakajima et al. 2020	To investigate, after lower thoracic SCI, the roles and differences between infiltrated macrophages and activated microglia on-site and at the lumbar enlargement at various times.	Contusion (T9-T10)	SCI (Exclusively Experimental).	Prevalence of microglia and infiltrating macrophages peaked at day 14 post-SCI, which coincided with the time of the most severe pain hypersensitivity. This rise was mainly composed of macrophages at the lesion location and microglia at the lumbar enlargement. The dominant microglia phenotypes were M1 at the injured site and M2 at the lumbar enlargement.	Expression of TNF-α peaked at 4 days on-site and at 14 days at the lumbar enlargement. TNF-α fluctuations and peaks correlated with the severity of neuropathic pain (NeP).
Naseri et al. 2013	To investigate the role of glial cells in the process of central pain syndrome induced by unilateral electrolytic lesion of spinothalamic tract (STT).	Unilateral electrolytic lesion of the spinothalamic tract (T8-T9)	Spinothalamic tract (STT) injured; Sham.	There was an increase of microglia at days 3 and 7 post-SCI on-site.	Unilateral SCI causes ipsilateral and contralateral glial proliferation and extension of pain to the contralateral side.

Sabirzhanov et al. 2019	To understand the mechanisms and effects of NOX2 on the symptoms following SCI contusion.	Contusion (T10)	Sham/WT; Sham NOX2-/-; SCI/WT; SCI/NOX2-/-.	SCI elevates NOX2 expression in microglia and macrophages up to 8 weeks post-SCI.	Deletion of NOX2 reduces ROS production at 8 weeks post-SCI and macrophage infiltration 1 day post-SCI. miR-155 expression is increased by SCI and lowered, exclusively in microglia and macrophages, by NOX2 depletion. Despite increases of Arg1 and Ym1 mRNA, their protein levels by western blot were not detectable until 3 days post-SCI. Deletion of NOX2 increases Arginase-1, YM1 and IL-10. The IL-10 increase is greater in NOX2 -/- mice. Increased NOX2 activity post-SCI correlates with M1-like cells activation and upregulation and neurological dysfunction. SCI increases mRNA levels of Ym1, NOX4, Arg1, SOCS3, IL-10 and IL-4Rα but decreases SOCS1.

					NOX2 depletion increases mRNA of Ym1, IL-10, Arg1, SOCS3 and IL-4Rα, but decreases that of SOCS1 and NOX4.
Takeura et al. 2019	To determine the roles of microglia and infiltrating macrophages in NeP in chronic compression SCI in mice.	Chronic compression (C2-C3)	*ttw/tww* mice; ICR mice (control).	Compression SCI increases the number of macrophages and activated microglia. Level of compression correlates with higher activated microglia numbers on-site.	Compression SCI caused overexpression of p-38 MAPK and p-ERK1/2 in these cells in the dorsal horn on-site. Compression SCI causes chronic neuropathic pain. The histopathological and pathophysiological changes found in these mice are similar to those found in traumatic SCI, such as degeneration and demyelination of neurons.

Tenorio, Kulkarni, and Kerr 2013	To create a model of perispinal inflammation which could be used equally to other SCI models for investigation purposes.	Zymosan-induced perispinal inflammation (L1-L2)	Perispinal inflammation-induced; phosphate buffered saline (control).	Perispinal inflammation increases microglia in a slow constant rate and macrophage density, all of which remain at levels higher than control at all times within the sDH from 1 day post-SCI onward.	Both pP38 MAPK and cFOS expression increased at 1 day post-SCI and normalized by day 14. Perispinal zymosan-induced inflammation does not disrupt the integrity of the BSCB, nor cause T-Cells or Mac-1-positive macrophages to migrate to the spinal parenchyma. Mice treated with zymosan did not perform worse at the rotarod test and had reduced withdrawal thresholds to both tactile and heat stimuli.
Wu et al. 2013	To understand whether cell cycle activation post-contusion SCI on-site contributes to neuronal hyperexcitability of the posterior thalamic nucleus, microglial and astroglia activation	Contusion (T10)	Sham-vehicle; Sham-CR8; SCI-vehicle; SCI-CR8.	In vitro, pre-treatment with CR8 in cultured microglia significantly attenuated LPS-induced microglial proliferation and NO release in a dose-dependent manner. Post-SCI, microglia exhibited activation phenotypes such as larger cell bodies with shorter and thicker projections and retraction of processes at 7	SCI causes up-regulation of Cyclin D1 and CCL21 expression in the posterior thalamic nucleus. Cyclin D1+ cells were co-labelled with CCL21. Treatment with CR8 reduced cyclin D1+ cells in the posterior thalamic and ventral

	and hyperpathia. Assess the influence of CDK inhibition on microglial activation of the thalamus.			days post-SCI both in the posterior thalamic nucleus and in the ventral posterolateral nucleus of the thalamus and remained as such for as long as 8 weeks post-SCI. No difference was observed in microglia in the adjacent centrolateral nucleus post-SCI. In the sDH of spared tissue in CR8-treated animals, activated microglia phenotypes and microglia numbers were reduced and correlated with below-level mechanical allodynia.	posterolateral nucleus of the thalamus. Testing to mechanical stimuli started at 3 weeks post-SCI, at which point enhanced pain sensitivity was reported and was correlated with increased posterior thalamic nucleus neuronal activity. CR8 treated mice had reduced hyperesthesia and a reduction of CCL21 signal at 7 days post-SCI.
Wu et al. 2014	To study the effects of SCI in rats on cognition, brain inflammation, and neurodegeneration and the role of CCA in the observed changes.	Contusion (T8)	Sham-vehicle; Sham-CR8; SCI-vehicle; SCI-CR8.	SCI causes microglial activation in the brain, namely it's cortex, hippocampus and cerebellum. There is an increase in microglia activation at 7 days post-SCI, but it is higher and associated with increased numbers of microglia at week 10 post-SCI. CR8 treatment decreased activated microglia numbers at	Post-SCI there was an increase of cell cycle-related genes and proteins in the hippocampus and cortex. SCI increases local expression of TNFα, iNOS, IL6, Arg-1 and YM1. Arg-1 increases gradually up to the week 9. YM1 increases at day 1 followed by a decline.

				day 7 and microglia with active phenotypes at week 10 post-SCI. Activated microglia show increased CCL21 expression.	
Yao et al. 2022	The establishment of an animal model of double-level cervical cord compression by using a new type of hydrophilic expanding polymer to better mimic clinical compression.	Compression (C3-C4 and C5-C6)	Sham; Screw compression; Hydrogel compression.	Post-SCI on-site microglia were bigger and rounder and had higher expression of iNOS, IL-1β, IL-6 and TNF-α related to M1 phenotype, as well as Arg-1, CD206, and TGF-β related to M2 phenotype. Microglia and macrophages with M1 phenotype marker were far more frequent than M2 phenotype post-SCI.	Post-SCI on-site there was an increase of ROS. Mice in the SCI groups showed poorer motor functions than those of sham.

Yu et al. 2013	To assess whether HUP-A could mitigate pain signalling, microglial inflammation and NMDA-mediated central hypersensitization without invoking drug tolerance or dependence.	Compression (T10)	HUP-A-treated; Saline-treated.	HUP-A reduced demyelization resulting from neuroinflammation that is mediated largely by locally activated microglia, astroglia and by macrophage invasion.	HUP-A reduced CD68+ M1 macrophages, in the lumbar spinal cord. Continuous intrathecal administration of HUP-A induces sustainable suppression of post-SCI hypersensitivity. HUP-A acts in a cholinergic dependent way, as atropine abolishes its effect. HUP-A administration does not cause tolerance. HUP-A decreased iNOS and CD68 levels in the cervical and lumbar spinal cord.

Table 1 – Summary of study characteristics (aim of the study; type and location of SCI and experimental and control groups) and of main conclusions achieved (effects of microglia modulation, changes in microglia activity and other results and conclusions) distributed by each study included in the review.

Apêndice 1 – Reporting Guidelines - PRISMA 2020 for Abstracts Checklist

PRISMA 2020 for Abstracts Checklist

Section and Topic	Item#	Checklist item	Reported (Yes/No)
TITLE			
Title	1	Identify the report as a systematic review.	Yes
BACKGROUND			
Objectives	2	Provide an explicit statement of the main objective(s) or question(s) the review addresses.	Yes
METHODS			
Eligibility criteria	3	Specify the inclusion and exclusion criteria for the review.	No
Information sources	4	Specify the information sources (e.g. databases, registers) used to identify studies and the date when each was last searched.	No
Risk of bias	5	Specify the methods used to assess risk of bias in the included studies.	No
Synthesis of results	6	Specify the methods used to present and synthesise results.	No
RESULTS			
Included studies	7	Give the total number of included studies and participants and summarise relevant characteristics of studies.	Yes
Synthesis of results	8	Present results for main outcomes, preferably indicating the number of included studies and participants for each. If meta-analysis was done, report the summary estimate and confidence/credible interval. If comparing groups, indicate the direction of the effect (i.e. which group is favoured).	Yes
DISCUSSION			
Limitations of evidence	9	Provide a brief summary of the limitations of the evidence included in the review (e.g. study risk of bias, inconsistency and imprecision).	No
Interpretation	10	Provide a general interpretation of the results and important implications.	Yes
OTHER			
Funding	11	Specify the primary source of funding for the review.	Not applicable
Registration	12	Provide the register name and registration number.	Not applicable

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

Apêndice 2 – Reporting Guidelines - PRISMA 2020 Checklist

PRISMA 2020 Checklist

Section and Topic	Item#	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Pages 3-5
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 5
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 6
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Pages 5-6
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 5-6
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 6 – last paragraph
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 6 – last paragraph
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 6 – last paragraph
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Page 6 – last paragraph
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 5
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Not applicable – No Meta-analysis was performed

Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Page 6 – last paragraph
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Page 6 – last paragraph
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 6 – last paragraph
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Not applicable
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Not applicable – No Meta-analysis was performed
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not applicable – No Meta-analysis was performed
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 5
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not applicable – No Meta-analysis was performed
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 7 and 31
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Pages 7 (2nd paragraph) and 31
Study	17	Cite each included study and present its characteristics.	Pages 33-47
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Pages 32
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	(a) Pages 33-47; (b) Not applicable – No Meta-analysis was performed
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Pages 13-14

	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Not applicable – No Meta-analysis was performed
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Not applicable – No Meta-analysis was performed
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not applicable – No Meta-analysis was performed
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Pages 13-14
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not applicable – No Meta-analysis was performed
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 14 - 15
	23b	Discuss any limitations of the evidence included in the review.	Page 18
	23c	Discuss any limitations of the review processes used.	Not applicable
	23d	Discuss implications of the results for practice, policy, and future research.	Page 19
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not applicable
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Not applicable
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not applicable
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Not applicable
Competing interests	26	Declare any competing interests of review authors.	Page 20
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Not applicable

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi:10.1136/bmj.n71

Apêndice 3 – Regras de formatação da revista *Reviews in the Neurosciences (REVNEURO)*

Reviews in the Neurosciences

Information for Authors

Aims and Scope

Reviews in the Neurosciences (*Rev. Neurosci*.) provides a forum for reviews, critical evaluations and theoretical treatment of selective topics in the neurosciences. The journal is meant to provide an authoritative reference work for those interested in the structure and functions of the nervous system at all levels of analysis, including the genetic, molecular, cellular, behavioral, cognitive and clinical neurosciences. Contributions should contain a critical appraisal of specific areas and not simply a compilation of published articles.

Readership includes: neurologists, psychologists, psychiatrists, neuropharmacologists, neurochemists, neurophysiologists, neuroanatomists, neuroendocrinologists, neurogeneticists and behavioral neuroscientists.

Submission of a manuscript to Reviews in the Neurosciences implies that it has not been published before and is not under consideration for publication elsewhere. It is the corresponding author's responsibility to ensure that all authors approve of the manuscript's submission for publication. Once the manuscript is accepted, it may not be published elsewhere without the consent of the copyright holders.

Submission of manuscripts

Manuscripts may be submitted online at the following URL:

https://mc.manuscriptcentral.com/revneuro.

Refereeing of manuscripts

Submitted manuscripts will undergo a peer review process. During online submission, please indicate the names and e-mail addresses of at least three potential referees who are not members of the Editorial Board of this journal. Revised manuscripts must be submitted within eight weeks of the authors' notification of conditional acceptance.

Preparation of manuscripts

Manuscripts must be written in clear and concise English and should be regarded as final texts as no changes are possible at the proof stage other than correction of printer's errors.

General format

Manuscripts (including table legends, figure legends and references) should be typed double-spaced with font size of 12 pt letters. Pages should be numbered (with the title page as 1) and have margins of 2.5 cm (1 inch) on all sides. Footnotes in the text should be avoided in favor of parentheses.

Sections

Manuscripts should be organized into: Title page, Abstract, Key words, Body with subsections (Body may be preceded by an outline of the structure of the review), Acknowledgments, References, Tables and Figure legends.

Title page

The Title page should include (a) an informative title; (b) names of all authors (with one first name in full for each author), followed by their affiliations (department, institution, city with postal code, country); (c) the mailing address, fax, phone number and e-mail address of the corresponding author; (d) a running title of up to 50 characters. If more than one institution is involved in the work, the authors' names should be linked by superscript consecutive numbers to the appropriate institutions. If required, small superscript letters should be used to indicate present addresses.

Abstract and Keywords

The second page of the manuscript should contain the Abstract and the Key words. The Abstract should be a single paragraph of no more than 250 words. Abbreviations and reference citations should be avoided. Below the Abstract provide up to six Key Words, which are not part of the title, listed in alphabetical order and separated by semicolons.

Acknowledgments

Acknowledgments should be placed at the end of the text. Names of funding organizations should be written in their entirety.

References

For citations authors are encouraged to rely as far as possible upon articles published in primary research journals. Unpublished results and personal communications should be cited as such within the text; Meeting abstracts may not be cited. Within the text references should be cited by author and date; et al. should be used if there are more than two authors, e.g. (Gerfen and Bolam 2010; Hogan et al. 1986). At the end of the text the citation list should be in alphabetical order. Journal names should be given by employing commonly used abbreviations. Citations should be in accordance with the following examples:

Journal article: Nikolaus, S., Mamlins, E., Hautzel, H. and Müller, H.-W. (2019). Acute anxiety disorder, major depressive disorder, bipolar disorder and schizophrenia are related to different patterns of nigrostriatal and mesolimbic dopamine dysfunction. Rev. Neurosci. 30: 381–426.

Book chapter: Gerfen, C.R. and Bolam, J.P. (2010). The Neuroanatomical Organization of the Basal Ganglia. In: Steiner, H. and Tseng, K.Y. (Eds.). *Handbook of Basal Ganglia Structure and Function*. Elsevier/Academic Press, pp. 3–28.

Book/monograph: Hogan, B., Costantini, F., and Lacy, E. (1986). *Manipulating the Mouse Embryo*: *A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA).

Tables

Tables should be typed on separate pages and be numbered consecutively using Arabic numerals. A short descriptive title, column headings, and (if necessary) footnotes should make each table self-explanatory. Please indicate in the manuscript the approximate position of each table.

Illustrations

Electronic files containing illustrations should be provided in a generic graphics format (tif or jpg preferred; use PowerPoint or eps files only if no other format is available). For reproduction, high-resolution (approx. 400 dpi and better) images are required. All figures will be reduced in size to fit, wherever possible, the width of a single column, i.e. 80 mm, or a double column, i.e. 168 mm, of text. Ideally, single column figures should be submitted with a width of 100 mm, double column figures with a width of 210 mm. Lettering of all figures within the article should be uniform in style (preferably a sans serif typeface) and of sufficient size (so that the final height will be approximately 2 mm). Upper-case letters A, B, C etc. should be used to identify individual parts of multipart figures. All figures must be cited in the text in numerical order. Reference to figures is to be made as Figure 1 etc. in the text and captions.

Color figures: Authors are encouraged to submit illustrations in color if necessary for conveying their scientific content. Publication of color figures is provided free of charge both in online and print editions.

Line drawings: These should also be provided as high-resolution files. No additional artwork, redrawing or typesetting will be done by the publisher. Note the faint shading or stippling may be lost upon reproduction, heavy staining or stippling may appear black.

Figure legends: These should be provided on separate, numbered manuscript pages. All symbols and abbreviations used in the figures must be explained, except for standard abbreviations or others defined in the preceding text.

Abbreviations

No separate list of abbreviations is accepted. Abbreviations and acronyms should be defined parenthetically within the text upon first appearance.

Author photo and short CV

Authors may submit a portrait photograph and a short scientific CV (4-6 sentences, written in 3rd person singular) for articles with 4 authors or less. Please use the appropriate “file designation” terms for the CV text file(s) and author photograph(s) during online submission. This information will be published in the article after the References section.

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Please contact the journal staff if you have any further questions (e-mail: RNS@uni-duesseldorf.de or rev.neurosci.editorial@ degruyter.com). We will do our best to assist you.

Apêndice 4 – Aceitação de publicação pela revista *Reviews in the Neurosciences (REVNEURO)*

RE: RNS.2023.0031.R2 - Decision Accepted

Para:David Ramos <david.f.ramos@hotmail.com>
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-----Original Message-----
From: Reviews in the Neurosciences <onbehalfof@manuscriptcentral.com> Sent: 10 de junho de 2023 12:53
To: Célia Cruz <ccruz@med.up.pt>
Subject: RNS.2023.0031.R2 - Decision Accepted

10-Jun-2023

Dear Prof. Cruz:

I would like to thank you for submitting your manuscript to Reviews in the Neurosciences (REVNEURO). It is a pleasure to accept your manuscript no. RNS.2023.0031.R2 entitled "Involvement of microglia in chronic neuropathic pain associated with spinal cord injury - A systematic review." in its current form for publication in REVNEURO.

The REVNEURO production office will contact you for proofreading in the near future. Your article will be published ahead of print as soon as possible, and in the printed edition at a later time.

Thank you for your fine contribution. On behalf of the Editors of Reviews in the Neurosciences we look forward to your continued contributions to the Journal.

With kind regards
Joseph P. Huston
Editor in Chief, Reviews in the Neurosciences