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The role of neutrophils in neuro-immune modulation

Alexandre Kanashiro[a,b,*], **Carlos Hiroji Hiroki**[c], **Denise Morais da Fonseca**[d], **Alexander Birbrair**[e], **Raphael Gomes Ferreira**[f], **Gabriel Shimizu Bassi**[g], **Mirian D. Fonseca**[b], **Ricardo Kusuda**[b], **Guilherme Cesar Martelossi Cebinelli**[c], **Katiussia Pinho da Silva**[h], **Carlos Wagner Wanderley**[b], **Gustavo Batista Menezes**[i], **José Carlos Alves-Filho**[b], **André Gustavo Oliveira**[j], **Thiago M. Cunha**[b], **André Sampaio Pupo**[h], **Luis Ulloa**[g,**], **Fernando Queiroz Cunha**[b,**]

[a]Department of Neurosciences and Behavior, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil

[b]Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil

[c]Department of Immunology and Biochemistry, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil

[d]Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

[e]Department of Pathology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

[f]Araguaína Medical School, Federal University of Tocantins, Avenida Paraguai s/n, 77824-838, Araguaína, TO, Brazil

[g]Center for Perioperative Organ Protection, Department of Anesthesiology, Duke University, Durham, NC, 27710, USA

[h]Department of Pharmacology, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brazil

[i]Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

[j]Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

Neutrophils are peripheral immune cells that represent the first recruited innate immune defense against infections and tissue injury. However, these cells can also induce overzealous responses and cause tissue damage. Although the role of neutrophils activating the immune system is well established, only recently their critical implications in neuro-immune interactions are becoming more relevant. Here, we review several aspects of neutrophils in the bidirectional regulation

[*]Corresponding author at: Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes 3900, Vila Monte Alegre, CEP 14049-900, Ribeirão Preto, SP, Brazil. [**] Corresponding authors: alex_bioquimica@yahoo.com.br (A. Kanashiro), luis.Ulloa@Duke.edu (L. Ulloa), fdqcunha@fmrp.usp.br (F.Q. Cunha).

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between the nervous and immune systems. First, the role of neutrophils as a diffuse source of acetylcholine and catecholamines is controversial as well as the effects of these neurotransmitters in neutrophil's functions. Second, neutrophils contribute for the activation and sensitization of sensory neurons, and thereby, in events of nociception and pain. In addition, nociceptor activation promotes an axon reflex triggering a local release of neural mediators and provoking neutrophil activation. Third, the recruitment of neutrophils in inflammatory responses in the nervous system suggests these immune cells as innovative targets in the treatment of central infectious, neurological and neurodegenerative disorders. Multidisciplinary studies involving immunologists and neuroscientists are required to define the role of the neurons-neutrophils communication in the pathophysiology of infectious, inflammatory, and neurological disorders.

Keywords

Neutrophil; Neuroimmunomodulation; Nicotinic receptors; Adrenoceptors; Neuroinflammation; Neuroimmunology

1. Introduction

1.1. Neutrophils: an overview

Neutrophils are short-lived polymorphonuclear leukocytes that are continuously generated from myeloid precursors in the bone marrow. Neutrophils are activated by bacterial and tissue damaged products, such as cytokines, damage-associated molecular patterns (DAMPs), and growth factors. These factors increase the neutrophil lifespan and ensure their migration and infiltration into the inflammatory focus through a concentration gradient of chemotactic stimulus [1]. Neutrophils are a critical component of the innate immune system essential to fight microorganisms and clear cellular debris in both septic and aseptic processes. Neutrophils can kill pathogens through different mechanisms: phagocytosis, degranulation of proteinases, and the release of reactive oxygen/nitrogen species (ROS and RNS), and neutrophil extracellular traps (NETs). ROS are products of the "cellular respiratory burst", which is initiated by reducing oxygen to superoxide anions through the NADPH oxidase NOX2, an enzyme assembled in the phagosome membrane. From the formation of superoxide, hydrogen peroxide (H_2O_2) is produced and released into the phagosome space [213]. Neutrophils also release myeloperoxidase (MPO) into the phagosome by degranulated lysosomes. As a consequence, chloride ions are oxidized by H_2O_2 to generate hypochlorous acid (HOCl), a strong cell membrane oxidant [214]. The nitric oxide (NO) is produced by inducible NO synthase isoform (iNOS). iNOS produces high levels of NO in response to inflammatory mediators and/or to pathogen-associated molecular patterns (PAMPs). iNOS is regulated at transcriptional level and its activity is calcium-independent [2]. In addition to inflammation-induced iNOS expression, this enzyme has a constitutive expression in both murine and human resting neutrophils [3]. The iNOS-derived NO is a microbicidal and host cell-cytotoxic mediator by itself, but it can react with superoxide resulting in peroxynitrite, which is a stronger cytotoxic factor [4].

NETs were first described as a stick web of DNA conjugated with antimicrobial enzymes, such as elastase and MPO, that capture and kill bacteria in the extracellular milieu [5]. This

process is not specific for bacteria and many other pathogens including fungi, parasites, and viruses, can also activate neutrophils to produce NETs. Depending of the stimulus, NETosis can occur through different pathways. For example, incubation of neutrophils with phorbol-12-myristate-13-acetate (PMA) dissociates azyrophilic granules containing elastase and MPO via the oxidative burst. These enzymes are then translocated into the nucleus, where they activate the protein-arginine deiminase 4 (PAD4), which is responsible for the deamination of arginine into citrulline. This process results in chromatin decondensation, followed by cell membrane lyse, and NETs release. This pathway is known as a '*suicidal NETosis*', because it induces cell death [6]. By contrast, '*vital NETosis*' does not induce cell suicide. *Vital NETosis* occurs in response to bacteria and fungi, and results in the release of NETs via vesicles, allowing neutrophils to still perform phagocytosis and chemotaxis [7,8]. Although NETs release may help to control infection, it can also cause organ damage. In animal models of autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis, NETs are spontaneously induced causing tissue damage [9]. As described later in this review, NETs production has also implications in CNS disorders including multiple sclerosis (MS) [10,11], Alzheimer' disease [12] and stroke [13,14].

Neutrophils deficiency to kill microorganisms can cause immunosuppression and increases the risk of opportunistic infections. For example, individuals with chronic granulomatous disease, a hereditary condition impairing NADPH oxidase, are more susceptible to microbial infection and sepsis [15]. However, neutrophils' mediators are unspecific as they affect both microbial and host cells, leading to tissue and organ damage as found in auto-immune, infectious, and traumatic disorders [16]. Therefore, neutrophils are key players of the immune response being either a friend or foe for the host according to the inflammatory context.

1.2. Neuro-immune interaction: neutrophils in a neuro-immune context

Emerging evidences show a complex and bidirectional communication between the nervous and the immune systems [17–21]. The nervous system encompasses both central (brain and spinal cord) and the peripheral (autonomic and enteric) systems. The autonomic nervous system controls organ functions through the balance between the *sympathetic* and *parasympathetic* systems. In the sympathetic network, preganglionic neurons originated along the thoracolumbar segments of the spinal cord synapse with ganglionic neurons in the pre- or paravertebral ganglia. These ganglionic neurons release norepinephrine on peripheral tissues and activate local adrenergic receptors. In the parasympathetic network, preganglionic neurons originated in the brainstem nuclei and along the sacral spinal cord synapse with ganglionic neurons located near the target organ. These ganglionic neurons release acetylcholine that subsequently activates local cholinergic receptors. The vagus nerve is the principal nerve of the parasympathetic system and plays a pivotal role connecting the brain with the most important organs including the heart, lungs, liver, and the adrenal glands. The adrenal medulla acts as a sympathetic ganglion releasing catecholamines directly into the bloodstream and inducing a systemic effect rather than modulating specific organs. Several studies demonstrated the regulation of the immune system by autonomic nervous networks. Most of these neuro-immune interactions has been described in monocytes/

macrophages and lymphocytes [22–24]. However, the role of neutrophils in the neuro-immune panorama in (patho)-physiological conditions is poorly understood.

Previous neuro-immune studies reported neutrophil recruitment as a response to pathological conditions, as determined by blood cytokine levels as inflammatory markers. We have used neutrophil recruitment as a biological signal of local/acute inflammation. We investigated neuromodulation of inflammation in experimental arthritis [25–28], using neutrophil migration as the main hallmark for local inflammation. Despite the key role of neutrophils in tissue damage, few studies investigated their role in the neural circuits, probably because of their short lifespan [29,30]. The half-life of neutrophils is approximately 10−19 h in mice and humans, and treatment with adrenergic or cholinergic drugs cannot be performed for long periods of time after their isolation from the blood. Moreover, mature neutrophils are found almost exclusively in the bloodstream and in inflamed tissue, but not in secondary lymphoid organs such as the lymph nodes or the spleen. The presence of mature neutrophils in the blood represents the first line of defense and, their quick migration into the injured site is essential to fight infections [31]. In contrast to neutrophils, direct interactions between the nervous and the immune systems are mediated through *neuro-immune synapses* between peripheral nerves and lymphocytes/macrophages. Lymphocytes are distributed in primary (thymus and bone marrow) and secondary (spleen and lymph nodes) lymphoid organs, which are innervated by post-ganglionic sympathetic nerves that interact with resident lymphocytes through synapsis-like structures [24,32,33]. In the thymus and spleen, these sympathetic innervations are responsible for the maturation of T and B lymphocytes [34,35], respectively. On the other hand, macrophages are present in many nonlymphoid organs, where they are regulated through direct sympathetic innervations as described in the liver, and intestine [22,36]. The barrier tissues are the major sites where immune cells traffic and reside; in particular, the intestinal mucosa alone harbors more lymphocytes than all the lymphoid organs combined. Therefore, the interference of such neural inputs in tissue-resident lymphoid populations cannot be excluded. Moreover, lymphoid structures rich in lymphocytes, such as thymus, are innervated by parasympathetic vagal fibers [37]. Moreover, considering the importance of chronic low-grade inflammation as a key factor in the development of cardiovascular diseases and metabolic syndrome [38–41], it is also essential to mention the implications of neuro-immune interface for many pathological states, such as obesity and insulin resistance, and their related diseases including hypertension, atherosclerosis, diabetes, and stroke [42–47].

From a clinical perspective, the study of neuro-immune interactions is allowing the design of new therapeutic strategies for infectious and inflammatory disorders. For example, electrical stimulation of the vagus nerve activates the splenic nerve to release norepinephrine, which in turn activates splenic lymphocytes to produce acetylcholine. Acetylcholine activates the alpha7 subunit of nicotinic acetylcholine receptors (α7nAChR) on macrophages and inhibits the production of inflammatory factors [17,24]. This neural circuits ("*inflammatory reflex*") inspired the design of bioelectronic devices for the treatment of autoimmune conditions such as rheumatoid arthritis and Crohn' disease [48–50]. Furthermore, the vagal signals to the spleen decrease the activation of circulating neutrophils by modulating the expression of CD11b [51]. These results evidence that vagal stimulation can be exploited to modulate neutrophil recruitment in infectious and inflammatory disorders.

In this review, the *first section* has focused on how neutrophils contribute to the neuronal regulation of the immune system in response to the catecholaminergic/ cholinergic neurotransmitters produced by specific neuronal networks. In return, neutrophils can also produce both neurotransmitters, to feedback the neuronal network, and cytokines to modulate the immune system. These atypical neural mechanisms are behind those classical anti- and pro-inflammatory mediators already described as chemokines and cytokines (Fig. 1A). The *second section* will discuss the bidirectional crosstalk between neutrophils and sensory neurons and their contribution to pain, and neurogenic inflammation. Pain, one of the cardinal points of inflammation, has relevant clinical importance that, together with fever, shows some neuroimmune peculiarities (Fig. 1B). Finally, the *third section* of this article discusses the role of neutrophils in neurologic and neurodegenerative disorders affecting the central nervous system (CNS) (Fig. 1C).

2. Neutrophils as an immunological and diffuse source of neurotransmitters

Recent studies show that immune cells are an important non-neuronal source of neurotransmitters that allow the bidirectional crosstalk between the nervous and the immune system. When activated, neutrophils produce acetylcholine and catecholamines that can feedback the original neuronal network and also to transfer the neuronal signal to other immune cells, including neutrophils themselves. In neurons, tyrosine hydroxylase (TH) initiates the synthesis of catecholamines converting the amino acid L-tyrosine to L-DOPA, the precursor for dopamine synthesis. Next, the vesicular monoamine transporter (VMAT) translocates this neurotransmitter into vesicles, where dopamine is hydroxylated in the β position by dopamine-β-hydroxylase to generate norepinephrine, which is converted into epinephrine by the phenylethanolamine N-methyltransferase. Likewise, neutrophils also have all the enzymatic machinery necessary for the synthesis, metabolism, storage, and uptake of catecholamines [52,53]. It has been detected and quantified the amounts of dopamine, norepinephrine, epinephrine, and their metabolites, such as DL-3,4-dihydroxyphenylglycol (DHPG; a norepinephrine metabolite) and metanephrine (MET; epinephrine metabolite), in human neutrophils isolated from peripheral blood by high performance liquid chromatography (HPLC) [53]. These levels are similar to those reported in rat neutrophils by using enzyme-linked immunosorbent assay (ELISA) [52]. Furthermore, rat neutrophils also produce mRNA for both TH and dopamine-β-hydroxylase [52]. In fact, the intracellular levels of dopamine, norepinephrine, and DHPG are reduced in neutrophils treated with α-metil-ρ-tyrosine, a classical inhibitor of TH [53]. Likewise, treatment of human neutrophils with reserpine, an VMAT inhibitor, reduces the intracellular concentrations of norepinephrine and dopamine [53]. Together, these results indicate that human, rat, and murine neutrophils produce catecholamines through a mechanism similar to that reported in neurons. After depolarization, catecholamines are released by the vesicles into the extracellular milieu to exert their effects until they are reuptake or processed into inactive metabolites. Incubation of human neutrophils with desipramine (inhibits monoamine reuptake) markedly decreases their intracellular levels of norepinephrine [53].

Neutrophils can also produce acetylcholine. Peripheral human granulocytes are a non-neural source to produce and storage acetylcholine, but they do not synthetize significant amounts of acetylcholine as compared with lymphocytes [54]. Norepinephrine can stimulate splenic modulatory T lymphocytes expressing choline acetyltransferase to produce acetylcholine, which inhibits the production of inflammatory cytokines from splenic resident macrophages and prevent systemic inflammation in experimental sepsis [24]. Future studies are needed to determine the condition by which neutrophil-derived acetylcholine can induce (*i*) pro-inflammatory effects, as described for catecholamines [52], or (*ii*) an anti-inflammatory mechanism, as described for T lymphocytes in the spleen [24].

2.1. Catecholamines and their effects in neutrophils

Catecholamines have a critical role mediating the crosstalk between the nervous and the immune systems. Epinephrine and norepinephrine bind to either α (α_{1A}, α_{1B}, α_{1D}, and α_{2A}, α_{2B}, α_{2C}) or β (β_1, β_2, and β_3) adrenoceptors (ARs), two families of adrenoceptors with distinct structural and pharmacological properties [55]. ARs are coupled to G proteins as their principal second messengers. α_1ARs activate $G_{q/11}$, a subfamily of heterotrimeric G proteins that activates the phospholipase C (PLC)-calcium-diacyl glycerol (DAG)- protein kinase C (PKC) pathway in vascular (α_{1A}, α_{1B}, α_{1D}ARs) [56] and non-vascular systems (α_{1A}ARs). α_2ARs activates G_i proteins to decrease the outflow of catecholamines (α_{2A} and α_{2C}ARs) and modulate cognitive and behavioral disorders (α_{2A}, α_{2B} and α_{2C}ARs). βARs are coupled to G_s proteins, which activate the adenylyl cyclase and increase the intracellular cAMP levels. Thus, βARs can activate protein kinase A (PKA) to increase cardiac contractility (mainly β_1ARs) and relax bronchial smooth muscles (mainly β_2ARs) [57]. Due to the vast array of biological functions regulated by the adrenoceptors, it is easy to understand the importance of these receptors as potential therapeutic targets in multiple pathologies.

Recent studies showed that neutrophils express both αARs and βARs including α_{1A}, α_{2C}, α_{1D}, β_1, β_2, and β_3ARs but not α_{2B}ARs mRNA [52,58–63]. The expression of αARs in neutrophils has been confirmed in multiple studies related to diverse physiological conditions [52,61]. For instance, α_1 and α_2ARs exert opposite effects in neutrophils as they increase and decrease CD11b expression, respectively [62]. On the other hand, catecholamines can be produced by neutrophils increasing the extension of tissue damage through a autocrine mechanism via α_2ARs activation [52].

βARs are predominantly anti-inflammatory. *in vitro* studies showed that βARs modulate neutrophil oxidative burst, chemotaxis, NET formation, and the expression of adhesion molecules, leukotriene B_4 (LTB_4), and chemokines/cytokines [62–65]. The intracellular βAR signaling, especially those associated with cAMP and PKA activation, modulate most to the neutrophil functions [66,67]. For example, isoproterenol, a β-adrenergic agonist, inhibits neutrophil oxidative burst induced by N-Formyl-methionyl-leucyl-phenylalanine (fMLP), a chemotactic peptide, or calcium ionophores by increasing intracellular calcium and cAMP levels [65]. However, a recent study also showed that βARs inhibit superoxide production in human neutrophils via a cAMP-independent mechanism [68]. Finally,

adrenergic agents selectively inhibit the oxidative burst of human neutrophils without affecting elastase release [64].

Treatment of neutrophils with adrenergic agonists can attenuate cellular responses by inducing the desensitization and internalization of the adrenoceptors [69]. From a clinical perspective, neutrophils from patients treated with adrenergic agonists or presenting elevated levels of endogenous catecholamines are less capable to migrate or generate microbicidal agents. Furthermore, incubation of neutrophils with norepinephrine reduces cell chemotaxis by impairing the cytoskeleton remodeling [70]. Neutrophil phagocytosis and chemotaxis is also impaired in animals that underwent experimental stroke [70], a wellknown condition that induces a sustained sympathetic activation [71]. Thus, the use of β-blockers could prevent the immunosuppression and subsequent infection that usually follows stroke conditions [47], as isoproterenol reduced the expression of βARs in neutrophils [72].

Several sympathetic dysfunctions are mediated by end-target βARs and therefore neutrophils could be a useful model to study autonomic alterations. For example, reduction in βARs expression and receptor responsiveness in peripheral neutrophils have been reported in neonates and elderly, respectively. These results indicate that adrenergic signaling can change during development, depending on the physiological homeostasis or sympathetic dysfunctions [73,74]. Other studies also reported that β_2ARs are reduced in neutrophils from diabetic children and hypertensive subjects, but increased in patients with post-traumatic stress disorder [60,75,76]. The potential role of β_2ARs in other conditions such as psoriasis and atopic dermatitis appears to be controversial. Although there are some indications suggesting an irregularity in the function or expression of neutrophil β_2ARs in psoriasis [77], posterior studies reported no change in the density and affinity of these receptor in neutrophils isolated of patients with psoriasis [78] or atopic dermatitis [59]. Moreover, neutrophil count in peripheral blood is also modulated by circadian oscillations by down-regulating CXCL-12 in the bone marrow, which can allow neutrophil release. This down-regulation is mediated by sunlight stimulation of β_3ARs [79].

Another catecholamine that showed a great anti-inflammatory potential is dopamine [80,81]. There are two families of dopaminergic receptors: D1-type (including D_1 and D_5 subtypes) and D2-type (D_2, D_3, and D_4 subtypes) [82]. D_3 and D_5 receptors are commonly expressed in human neutrophils, whereas D_2 and D_4 expression showed significant variability [83]. Dopaminergic receptors seem to be functional and interfere in neutrophil activity (e.g. phagocytosis) as observed in sympathectomized mice [84]. The uptake, synthesis, storage, and release of dopamine in neutrophils support the hypothesis of dopaminergic regulation of human neutrophil functions [53]. Dopaminergic agonists decreases nitric oxide production [85], and dopamine decreases ROS production in stimulated neutrophils [86–88], but only high concentrations of dopamine impairs neutrophil phagocytosis of bacterial pathogens [88].

2.2. Cholinergic and nicotinic receptors in neutrophils

Acetylcholine (ACh) is a neurotransmitter found in central and peripheral synapses that binds to muscarinic (mAChRs) and nicotinic (nAChRs) cholinergic receptors [89]. nAChRs are cationic channels composed of five homologous subunits, which are encoded by a large

multi-gene family [18]. Although nicotine, a component of tobacco and the canonical nAChR agonist, displays potent anti-inflammatory effects when signaling through α7nAChRs in macrophages [90]. These receptors are considered a central component of the "inflammatory reflex" [90]. α7nAChRs are found in neurons and non-neuronal cells (e.g. microglia, astroglia, oligodendrocytes, endothelial and leukocytes) [89] and appear to be the main functional cholinergic receptors modulating the immune system. The expression of nAChRs in neutrophil' surface has been studied by high affinity binding assays [91,92]. In humans, it has already been described that neutrophils isolated from blood also express α7nAChRs and α3β4 nAChRs [91]. More recently, the expression of mRNA for nAChR subunits, mostly α2–9 and β2–4, were detected in stimulated murine neutrophils [92]. The expression of these nAChRs strongly suggests the regulation of neutrophils by nicotinic agonists. Treatment of neutrophils with nicotine diminishes the expression of integrin adhesion molecules (CD11b and L-selectin) and endothelial intercellular adhesion molecule 1 (ICAM-1) [93], and inhibits their microbicidal properties, such as phagocytosis and chemotaxis, without affecting superoxide production [94]. Treatment with nicotine enhances elastase degranulation and generation of eicosanoids, such as prostaglandin E_2 and LTB_4, in these cells [94], but other studies showed opposite effects [95–97]. For example, treatment with nicotine interferes with the ability of neutrophils to kill periodontal pathogens, without affecting bacteria uptake [95]. Nicotine can also halt the degranulation and the production ROS, including superoxide, in stimulated neutrophils [95–97]. By contrast, cholinergic antagonists decreases neutrophil phagocytic migration activity without affecting cell oxidative burst [54] and, although nicotine did not affect the oxidative burst, it reacts with neutrophil-derived HOCl to generate nicotinechloramine, a membrane pore-forming compound [98]. Noteworthy, all these studies were performed under viable nicotine concentrations that failed to induce cellular cytotoxicity in neutrophils [94,95,97]. The different results in these studies can be due to the incubation times and concentration of nicotine, source of neutrophil, medium composition, and specificity and particularities of the assays utilized. In summary, despite the controversy, most studies show that nicotine substantially impairs neutrophil functions that may contribute to the higher susceptibility to infections reported in tobacco smokers.

Recent studies showed that the cholinergic regulation of neutrophils is not exclusively mediated by extracellular receptors of the cell membrane. For example, nicotine still modulates neutrophil activity even in the presence of first generation hydrophilic muscarinic or nicotinic antagonists [96]. In fact, acetylcholine and acetylcholine-like structures have lipophilic characteristics, allowing their translocation into the cytoplasm where α7nAchRs are also found (e.g. mitochondria) [99–101]. Thus, extracellular ligands can also target intracellular nicotinic receptors exerting particular biological properties in neutrophils, where each specific subtype of nicotinic receptor could exert distinct and opposite functions.

The immune properties of nicotine on neutrophils have clinical implication for infectious disorders. For example, nicotine increases survival in endotoxemia and experimental sepsis by inhibiting the production and release of systemic inflammatory mediators, such as tumor necrosis factor (TNF) and high mobility group box protein 1 (HMGB-1) [102]. Other studies showed that nicotine also inhibits neutrophil infiltration into vital organs, reducing organ damage and failure [103,104]. On the other hand, multiple studies showed that decreasing

neutrophil recruitment to the infected area impairs bacterial clearance in experimental peritonitis induced by *E. coli* inoculation [105] and pneumonia [106]. In fact, α7nAchR-deficient mice show accentuated neutrophil migration toward the infectious focus and improved bacterial clearance [107]. These contradictory effects appear to depend on exposure range to α7nAchR agonists at different time points after the infection. Activation of nicotinic receptors in early phases of the infection prevent neutrophil recruitment into the infectious site allowing the spread of microorganisms; whereas nicotinic activation at later time points, can inhibit massive neutrophil recruitment into vital organs preserving tissue integrity [20]. Other studies reported that the disparity between the pro- and anti-inflammatory effects of nicotine is due to the differences in the period of exposition. The pre-treatment (before the arthritis induction; mimicking long-term exposition) exacerbated arthritis severity, whereas the post-treatment (therapeutic use) improved the inflammatory and clinical signs in arthritic rats [108]. Future studies will be needed to determine whether how the dosage and method of administration (bolus injection *vs.* continuous infusion) affect these results [109].

The chronic exposure to nicotine, as observed in smokers, exacerbates neutrophil activity as observed in patients with rheumatoid arthritis, and periodontal and lung diseases. Chronic stimulation of nAChRs in neutrophil increases IL-8 production by peroxynitrite generation, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and inhibitor of kB (IκB) degradation [110]. Indeed, smokers have higher plasma levels of IL-8 associated to blood neutropenia. Nicotine expands the neutrophil lifespan by inhibiting the diphospho-inositol pentakisphosphate (InsP7) and Akt pathway [111,112], contributing to cell accumulation and tissue damage in emphysema and bronchitis [113]. Chronic activation of α7nAchRs also increases NETs release via Akt and PAD4 activation [114]. Treatment with nicotine worsens the clinical score in murine collagen-induced arthritis, by increasing plasma levels of MPO-DNA complex, a known marker for NETs [109]. The authors also reported higher plasmatic levels of MPO-DNA complex in smoker subjects, and that nicotine acts as a potent inducer of NETosis via α7AChR activation. These results are consistent with the epidemiological studies showing that cigarette smoking predispose to rheumatoid arthritis development [114].

3. Bidirectional regulation of sensory neuron-neutrophil functions

The tissue injury caused by physical trauma or infection generates a local synthesis of mediators and DAMPs by neuronal and non-neuronal cells. In an initial stage, the generation of arachidonic acid metabolites enhances the production of prostanoids, a subclass of eicosanoids (e.g., prostaglandin E_2), which increase vascular tissue permeability and activate afferent neurons (Fig. 1A–C). These events are associated with the appearance of critical cardinal signs of inflammation, including redness, heat, swelling, and pain. Physiologically, pain protects the inflamed tissue by sending a warning (nociceptive) signal to the brain [115,116]. The activation and/or sensitization of the peripheral endings of nsensory neurons (mostly nociceptors) by inflammatory mediators alerts the organism about an infection or a tissue injury, shunning it from further damage for proper healing [117]. Thereafter, leukocytes are attracted to the site of the injury (*i*) to fight against infection, to repair tissue damage, and (*ii*) to mediate a complex neuro-immune interaction with the sensory neurons

[118]. From a clinical perspective, dysregulation in the resolution of acute inflammation may result in persistent and exacerbated inflammatory disorders, as observed in rheumatoid arthritis and neuropathic pain [119,120].

Neutrophil migration is an early event in the cellular phase of inflammation and it is responsible for the elimination of infectious agents and cellular debris. Within a few hours after the tissue damage, neutrophils are the most predominant leukocytes that migrate to the injured site, fighting the infection, and orchestrating the wound healing. Furthermore, they are also an important source of chemical mediators that affect the sensitivity of primary afferents neurons, such as cytokines and chemokines [121,122] (Fig. 1D–E). Cytokine-stimulated neutrophils induce, in turn, the release of additional mediators and trigger a complement alternative pathway amplifying nociception [123,124]. The pronociceptive action of neutrophils was first reported by our group showing that neutrophil recruitment toward the joints of dogs enhanced articular nociception promoted by the administration of lipopolysaccharides. Subsequent studies showed that intra-plantar administration of LTB_4 and the complement component 5a (C5a) in rat paw elicited mechanical hypernociception by a mechanism dependent on neutrophil migration [125,126]. Furthermore, the administration of an allosteric C5aR antagonist inhibited C5a-induced neutrophil migration reducing mechanical hyperalgesia in experimental models of inflammatory and neuropathic pain [127]. Our studies were confirmed by other investigators showing that neutrophils participate in the genesis of different pathological types of pain [128–132]. Regarding inflammatory pain models, treatment with fucoidin reduces neutrophil recruitment and mechanical allodynia during carrageenan-induced inflammation [133], and the depletion of neutrophils with vinblastine sulfate or anti-neutrophil antibody decreases mechanical hyperalgesia induced by paw incision in mice [134]. In humans, there is a strong relationship between the hyperalgesic effects of LTB_4 and the kinetic of neutrophil migration [135,136]. Other studies have observed that a neutrophil infiltration into the joint of patients suffering from arthritis precedes the clinical signs of inflammation and, therefore, this cellular event could be considered a predictive signal of pain development [137,138]. Antibody-induced neutropenia inhibits edema formation, but not the mechanical and thermal thresholds on complete Freund's adjuvant (CFA)- and zymosan-induced pain [139,140]. Although these studies used different methods to induce neutropenia, these data suggest that neutrophils may not be the only leukocytes modulating nociception in these inflammatory conditions, although future studies would be required to confirm this hypothesis.

Other studies suggest that neutrophils can also contribute to neuropathic pain. Neutrophils are almost absent in an intact nerve, but a significant infiltration of neutrophils and macrophages have been observed at the site of the nerve lesion in experimental models of neuropathy [141]. A substantial endoneurial neutrophil invasion was reported at the site of a partial transection of the sciatic nerve, and the depletion of circulating neutrophils reduced the development of thermal hyperalgesia [131]. Further studies indicated that genetic ablation of mediators or receptors mediating neutrophils adhesion and migration improves mechanical hyperalgesia in experimental neuropathic pain [142,143]. Another interesting study showed that chronic constriction injury of peripheral nerve induces neutrophil infiltration into the dorsal root ganglia (DRG), ipsilateral to the nerve lesion, and it correlates with an increase in MCP-1 expression [144]. Moreover, we also reported that

fucoidin or depletion of neutrophils with antibody anti-Ly6G inhibited the expression of TNF in DRGs and reduced the mechanical threshold after infection with Herpes Simplex Virus Type-1 in a murine model of acute herpetic neuralgia [145]. Finally, although the functional implications of neutrophils on neuronal axon and soma remain unknown, some studies suggest that neutrophil-derived elastase present in DRG and nerves is an important mediator for an induction of pain hypersensitivity in experimental models of neuropathy [146,147]. Taken together, these data support a direct role of neutrophils and its mediators in etiology and maintenance of neuropathic pain.

At the lesion site, neutrophils release inflammatory mediators, such as cytokines, that activate other cells, inducing edema, hyperalgesia, and migration of other leukocytes. However, the hypernociceptive potential of neutrophils is not directly associated with the release of nociceptive cytokines [148,149]. On the contrary, this effect depends on the potential of neutrophils to release direct-acting mediators through a coordinated cascade of factors. In fact, an inflammatory stimulus in the rodent paw induces an initial formation of bradykinin that triggers resident cells and neutrophils to release inflammatory factors, PGE_2, and sympathetic amines that will have a direct action on neurons and nociception [130,150–153]. We reported that *in vitro* neutrophils stimulated with IL-1β produced PGE_2 through a mechanism that is inhibited by fucoidin [133]. These results evidence that infiltrated neutrophils contribute to mechanical hypernociception, at least by releasing direct-acting mediators, such as PGE_2 and sympathetic amines. Neutrophils also modulate other peripheral mechanisms of inflammatory pain including the production of ROS, metalloproteases, and hydrogen protons [154–157]. Furthermore, neutrophils may generate endothelins, that synergize with other hyperalgesic mediators and increases the nociceptors excitability and, consequently, pain sensitivity [158]..

Finally, nociceptors activation promotes an axon reflex and generates action potentials that propagate antidromically, triggering a rapid and local release of neural mediators, such as substance P (SP), neuropeptides calcitonin gene related peptide (CGRP), vasoactive intestinal peptide (VIP), and gastrin releasing peptide (GRP). These neuronal mediators produce an independent inflammatory response similar to the innate immune system. This process is known as "*neurogenic inflammation*" [159–162] (Fig. 2C). Neuropeptides released by sensory neurons, such as GRP and VIP, induce neutrophil chemotaxis and can also act as anti-microbicidal components [163–165]. On the other hand, recent studies showed that CGRP released from sensory neurons during host-pathogen interactions reduces neutrophil recruitment as well as their microbicidal activities and thereby promoting immunosuppression [166,167]. Although most of the studies suggest an indirect contribution of neutrophils to hyperalgesia, the development and use of drugs to mudulate neutrophil migration to the focus of lesions should be considered as a potential strategy against inflammatory persistent pain.

4. Neutrophil in the central nervous system

Early studies suggested that the CNS is an "immune privileged site" due to the impenetrable blood-brain barrier (BBB), and the lack of antigen-presenting cells and lymphatic vasculature [168]. Nonetheless, this concept has been changed over the past few years [169],

and recent studies show the presence of lymphatic vessels in the brain, and alterations of the BBB during CNS disorders allows a bidirectional communication between peripheral leukocytes and CNS [170,171]. Neutrophils are immune cells with a short lifespan that can contribute to the brain damage during the acute stage of cerebral injury. Under physiological conditions, neutrophils are hardly found in brain parenchyma due the BBB [172]. However, a small number of neutrophils are present in the meninges, pia membrane, and the cerebrospinal fluid. During CNS disorders, including during central infections, immune and non-immune resident cells of the cerebral tissue generate chemoattractant mediators that induce neutrophil infiltration into the cerebral tissue [168] (Fig. 3A–C). In fact, avoiding neutrophil infiltration into the CNS after brain injuries diminishes neuronal damage [173]. On the other hand, inhibition of neutrophils in patients may increase the risk of opportunistic infections [174]. Therefore, future studies will require determining the role of neutrophils in specific neurological disorders. This could lead to the design of specific therapies against the detrimental effects of the neutrophils, without affecting their beneficial protective roles. In this review, we discussed the participation of CNS-infiltrating neutrophils addressing the main aspects of inflammatory mediators and cellular types involved as well as the damaged structures in autoimmune (e.g. multiple sclerosis), neurodegenerative (e.g. *Alzheimer' disease*) and infectious (e.g. sepsis, fungal and parasite infections) diseases and ischemic stroke.

Currently, little is known about the involvement of neutrophils in the pathogenesis of multiple sclerosis (MS). Neutrophils are recruited to the CNS through the release of chemokines, such as CXCL1 and CXCL5, which are produced different cells, including the astrocytes - via Th17-derived IL-17 - and mast cells from the meninges [175,176]. IL-17$^{-/-}$ mice show reduced neutrophil infiltration into the brain, but not in the spinal cord. In this case, astrocytes express CXCL2 and CXCL5 mediated by IFN-γ. Curiously, IFN-γ did not induce chemokine production by astrocytes *in vitro*, suggesting that it might act indirectly. Clinical studies reported that MS patients have increased levels of neutrophils and systemic NETs [10,11], and the plasma levels of CXCL1, CXCL5, and elastase correlated with clinical disability [177]. Interestingly, neutrophil recruitment into the CNS differ between IL-17 (to the brain) or interferon (IFN)-γ (to the spinal cord) production in experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis model [178]. When in the CNS, neutrophils increase the permeability of the BBB and disrupt myelination [179]. Thus, neutrophil infiltration into the CNS leads to demyelination and axonal loss (Fig. 3D).

Neutrophil infiltration into the CNS also appears to contribute to the development of neurodegenerative disorders, such as Alzheimer' *disease*. Neutrophil infiltration occurs in the regions with amyloid-β deposits by a LFA-1 integrin mechanism, and further brain injury appears following NETs and IL-17 release [12]. Different studies demonstrated that Alzheimer's patients present high number of neutrophils in the peripheral blood [180]. TNF levels modulation using isoindolin-1,3 dithione increased the presence of "healthy" neutrophils in the CNS, and improved the clinical score of 3xTgAD mice [181]. Of note, an increasing number of investigators propose that targeting α7AChR in neutrophils may modulate neuro-inflammatory and cognitive dysfunctions in Alzheimer's disease [182]. Multiple studies showed that activation of α7- and α9nAChR reduces the levels of CXCL12

and CCL2 in the CNS, and improves the clinical outcomes by preventing neutrophil infiltration [182,183] (Fig. 3E).

During infections by microorganism that invade the CNS, neutrophils can perform protective or deleterious roles depending on the nature of the pathogen and host defense. Infection susceptibility is increased in immunosuppressed individuals, as usually observed in neutropenia or infected HIV patients. For example, fungal CNS infection by *Candida albicans* promotes a protective recruitment of CXCR2-expressing neutrophils through microglia-induced IL-1β and CXCL1 [184,185]. *Toxoplasma gondii* can lead to cerebral toxoplasmosis, and the infiltration of neutrophil promotes host defense contributing to IFN-γ production and parasite control during the early stages of infection [186]. On the other hand, during sepsis, a systemic infectious condition, multiple vital organs are damaged by neutrophil infiltration, including the brain. Sepsis can also induce cognitive dysfunction and neurological disorders that appears to be associated with neutrophil filtration. Neutrophil adheres to the cerebrovascular endothelium via β2-integrins [187]. Recent studies showed that natural killer (NK) cells have an essential role in neutrophil recruitment into the brain during sepsis via chemokine production and microglial interaction [188]. Recent studies found neutrophils in the CNS, even fourteen days after the septic challenge, that are associated wih high levels of TNF and CXCL1 along with behavioral alterations [189].

In animal models of ischemic stroke, injury induces damaged cells to release DAMPs, which activate resident cells to produce chemokines, such as CXCL2 and CXCL8, resulting in neutrophil recruitment into the CNS. Neutrophils activation starts in the peripheral blood by systemic HMGB1, and then they infiltrate into the CNS due to an increased expression of very late antigen-4 (VLA-4), allowing their migration and adhesion to the brain blood vessels [173,190]. In the brain, neutrophils are activated releasing NETs, and also interacting with microglia, which results in tissue damage and the disruption of the BBB [13,14] (Fig. 3D). Of note, patients who suffered hemorrhagic stroke showed low levels of oxidative respiratory in the isolated neutrophils, which inversely correlated with the plasma levels of norepinephrine [191]. This suppression could explain the higher susceptibility to infections in post-stroke patients. The susceptibility to nosocomial infections due to reduced neutrophil infiltration after stroke was improved by α7nAChR pharmacologic blockage or genetic deletion [192]. Hypoperfusion enhances the interaction of neutrophils with the vasculature and promotes their adhesion by inducing the expression of selectins in the surface of the endothelial cells [193]. Neutrophils first accumulated within hours in the leptomeninges and perivascular spaces before they infiltrate into the parenchyma [194]. Neutropenic animals display reduced blood flow in the injured hemisphere after traumatic brain injury [195]. Hypoperfusion and neutrophils adherence promote ischemia and even early coagulopathy [196]. Likewise, neutrophils also contribute to vascular dysfunction during and after the injury in a hypoxia-ischemia model [197]. The interaction between activated neutrophils and endothelium is normally associated with secondary injury after traumatic brain injury [171].

Other neurological disorders are also associated by neutrophil infiltration into the CNS, including viral infection [198], febrile seizure [199], and Entero-Behçet's disease [200]. However, the molecular mechanisms mediating the role of neutrophils in the pathogenesis of

CNS disorders are not well-known, and future research is needed to determine the specific contribution of neutrophils to distinct neurological disorders.

5. Conclusion and perspectives

Although neutrophils are classical innate immune cells, they are a significant non-neural source of neurotransmitters (e.g. catecholamines and acetylcholine) exerting both paracrine and autocrine, self-regulatory modulation in inflammatory conditions. From a clinical perspective, neutrophils can interact with the central and peripheral nervous systems being responsible for the genesis of central inflammatory/neurodegenerative conditions and pain, respectively. Thus, inhibition of neutrophils could be a promising strategy against inflammatory and neurogenic pain or brain damage. The main findings in the current literature that demonstrate the role of neutrophils in a neuro-immune context are shown in the Table 1.

Neutrophil represents a heterogeneous population of cells with different characteristics. They can be subdivided into at least two distinct subpopulations [201,202] named N1 and N2 neutrophils and represent their immune-stimulating or suppressive potential, respectively. These two subpopulations of neutrophils have significant clinical implications in multiple infectious and inflammatory disorders, including autoimmunity and cancer. Unfortunately, there are no specific molecular markers yet to purify and study these subpopulations. Also, the specific brain micro-environmental cues that regulate their activities remain to be elucidated. Further understanding of these cues will allow the use of neutrophils as specific CNS targets to control inflammation in neurological and neurodegenerative disorders.

Importantly, it is not clear for how long the neutrophils survive in the brain. Recent studies indicate that their lifespan may significantly vary in distinct disease conditions [203,204]. Neutrophil survival time in the brain can depend on specific factors, such as adenosine, ATP, glutamate, and hypoxia [205–207]. Neutrophils can also modulate other CNS cells such as astrocytes and induce a complex cellular process modulating neuro-inflammation in neurological and neurodegenerative disorders [208]. Future studies are also needed to determine whether neutrophil recruitment into the brain is similar to that in peripheral tissues. Recent studies suggest that the rolling and migration of neutrophils in the brain venue are coordinated by different mechanisms. However, the specific molecules involved in the adhesion and transmigration of neutrophils in these vessels are still not known and require future explorations [1].

It is also unknown whether neutrophils that enter the brain could return to the bloodstream, and consequently to peripheral tissues. Interestingly, brain cells and neurotransmitters can affect neutrophil migration and prolong their survival time [209,210]. Peripheral neutrophils can be reprogrammed into a phenotype with specific enhanced function [201]. Recent studies have described a phenomenon called ‘reverse migration’ (when neutrophils migrate from the organ that they infiltrated back to the blood vessels) [211]. It is unknown whether this phenomenon also happens in the CNS. Interestingly, lung tumor induces osteoblastic cells in bone marrow to release primed neutrophils, which promote tumor development

[212]. Future studies will explore whether pathological brain tissue may prime neutrophils and change their function in peripheral organs.

Finally, experimental and clinical studies will be needed to determine the clinical implications of neutrophils in chronic neurological and neurodegenerative disorders. Further studies are needed to describe whether neutrophil infiltration is a process involving specific regions or a global event in the brain as observed in EAE model [178]. These studies could enable the development of new therapies to target the recruitment of neutrophil in neurodegenerative or infectious diseases. This approach could avoid the systemic immunosuppression induced by a general depletion of neutrophils. Moreover, the modulatory role of neurotransmitters on neutrophils is still largely controversial and may be explored. Therefore, multidisciplinary studies involving immunologists and neuroscientists will be required to define the role of the neurons/neutrophils communication in the pathophysiology of infectious, inflammatory, and neurological disorders.

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Abbreviations

ACh	acetylcholine
ARs	adrenoceptors
βARs	beta-adrenoceptors
BBB	blood-brain barrier
C5a	complement component 5a
CFA	complete Freund’s adjuvant
CGD	chronic granulomatous disease
CGRP	calcitonin gene-related peptide
CNS	central nervous system
DAMPs	damage-associated molecular patterns
DHPG	DL-3,4-dihydroxyphenylglycol
DRG	dorsal root ganglia
ELISA	enzyme-linked immunosorbent assay
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GRP	gastrin releasing peptide

H^+	hydrogen protons
H_2O_2	hydrogen peroxide
HMGB-1	high mobility group box protein 1
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
iNOS	inducible NO synthase
InsP7	diphospho-inositol pentakisphosphate
IκB	inhibitor of kB
LTB_4	leukotriene B_4
mAChRs	muscarinic receptors
MET	methanephrine hydrochloride
MPO	myeloperoxidase
MS	multiple sclerosis
nAChRs	nicotinic receptors
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NO	nitric oxide
PAMPs	pathogen-associated molecular patterns
PGE_2	prostaglandin E_2
PMA	phorbol-12-myristate-13-acetate
RNS	reactive nitrogen species
ROS	reactive oxygen species
SP	substance P
TH	tyrosine hydroxylase
TNF-α	tumor necrosis factor α

VIP	vasoactive intestinal peptide
VLA-4	very late antigen-4
VMAT	vesicular monoamine transporter
α7nAChR	alpha7 subunit of nicotinic acetylcholine receptors

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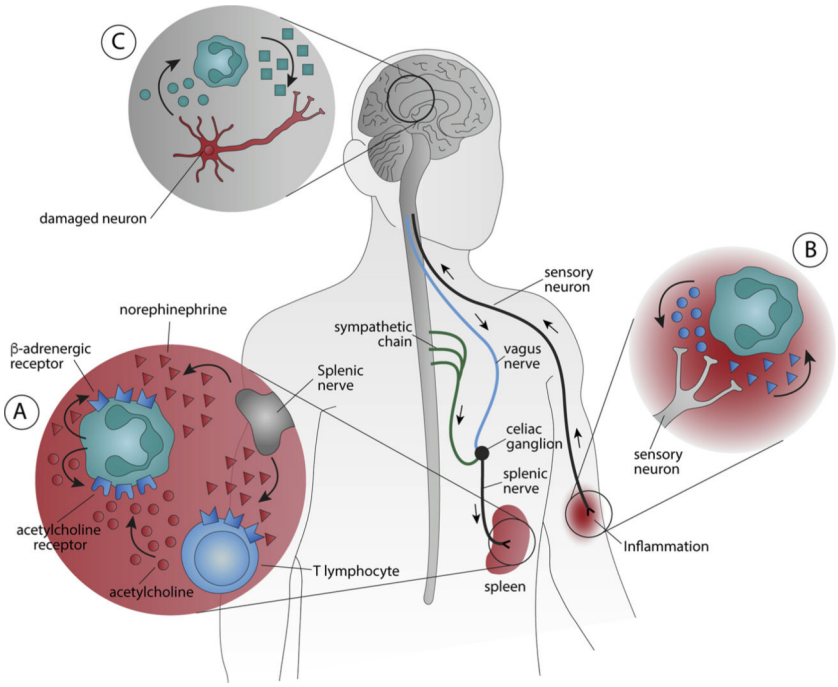

Fig. 1. The neuro-immune interactions of neutrophils.

(A) Neutrophils contribute to the neuronal regulation of the immune system. Neurotransmitters produced by either neuronal pathways (such as norepinephrine produced by the splenic nerve) or immune cells (acetylcholine produced by T lymphocytes) modulate neutrophil activity and the production of secondary messengers that, in turn, regulate both neurons and immune cells (including neutrophils by autocrine mechanism). (B) Neutrophils also modulate neuronal sensory signals by producing neurotransmitters to regulate the activation, sensitization, and maintenance of sensory neurons as observed in pain. Sensory neurons can also modulate neutrophils inhibiting their microbicidal functions by releasing neuropeptides. (C) Neutrophils can also cross the blood-brain-barrier (BBB) and release cytotoxic and inflammatory factors that induce neuronal damage in the central nervous system and contribute to neurological and neurodegenerative disorders.

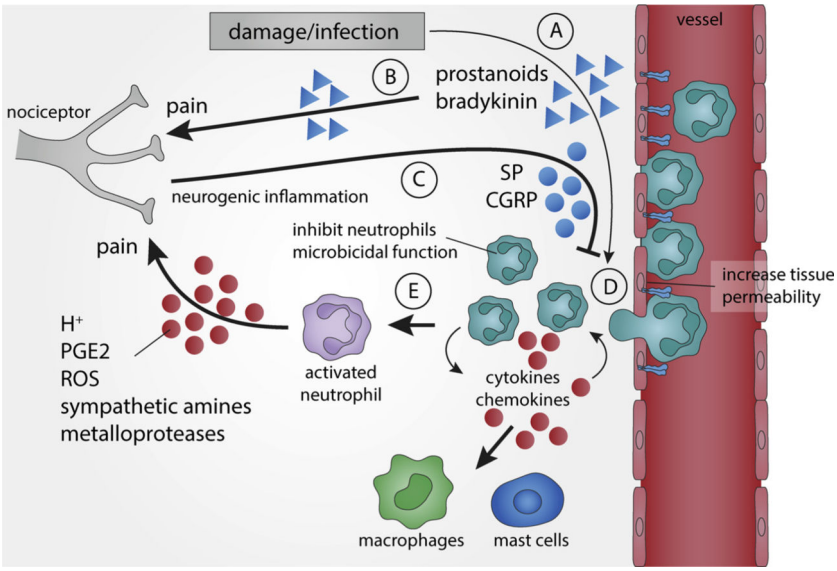

Fig. 2. Bidirectional regulation of sensory neuron-neutrophil functions.

Infection or tissue damage induces the production of inflammatory factors such as arachidonic acid metabolites and prostanoids that (A) increase tissue permeability to circulating neutrophils, and (B) can induce nociception and pain by activating sensory neuronal pathways. (C) The activation of nociceptive terminals triggers an axonal reflex that generates neurogenic substance P (SP) and calcitonin gene-related peptide (CGRP) increasing tissue permeability and inhibiting neutrophil microbicidal functions (neurogenic inflammation). (D) Neutrophils migrate into tissue to produce cytokines and chemokines that attract other leukocytes such as mast cells and circulating neutrophils. (E) Infiltrated neutrophils are activated and can produce multiple factors such as prostaglandin E_2 (PGE_2), sympathetic amines, reactive oxygen species (ROS), hydrogen protons (H^+), and metalloproteases that modulate nociceptors and inflammatory pain.

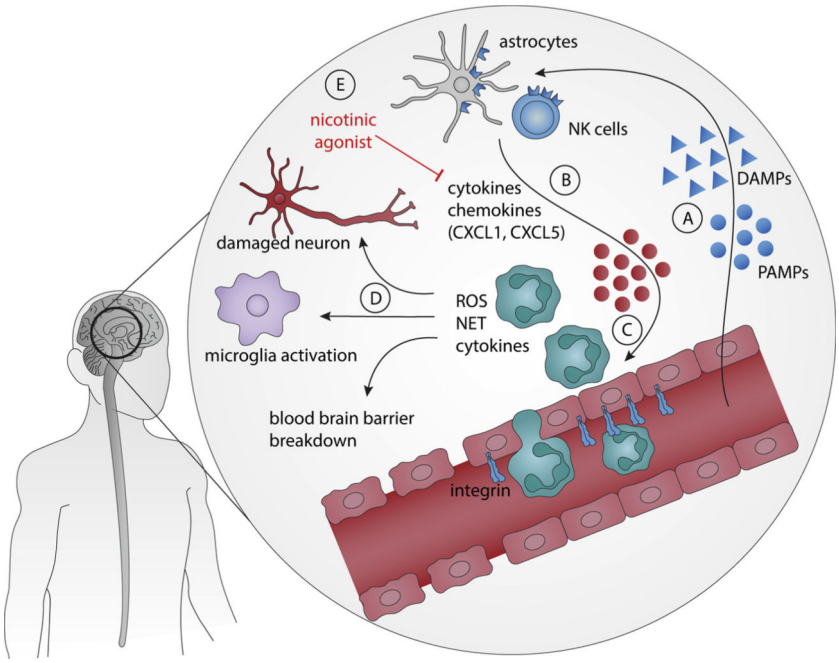

Fig. 3. Role of neutrophils in the central nervous system.

A) During the presence of neurodegenerative (e.g. Alzheimer), inflammatory (e.g. stroke) or infectious (e.g. sepsis, viral infection) conditions in the central nervous system (CNS). (B) Resident cells, such as astrocytes and NK cells, release different chemotactic substances, mainly chemokines. (C) These chemical mediators allow neutrophils to cross the blood-brain barrier (BBB) and produce high quantities of harmful mediators to neurons, such as reactive oxygen and nitrogen species (ROS and RNS), neutrophil extracellular traps (NETs) and cytokines. (D) In addition, these mediators induce microglial activation, ruptures on BBB, and demyelination and axonal loss. (E) The pharmacological activation of nicotinic receptors by acetylcholine or nicotinic agonists decreases neutrophil infiltration to the CNS by reducing the levels of chemokines.

Table 1

The main findings showing the role of neutrophils in a neuro-immune context.

Experimental Condition	Stimulus	Neutrophil Target	Neutrophil Release Factors	Effect	Ref.
Arthritis	Vagus Nerve Stimulation or β-adrenoceptor Agonists	β-Adrenoceptors	–	Vagal stimulation reduced neutrophil migration and arthritic joint inflammation by activating specific sympatho-excitatory brain nuclei	[26,27,28]
Acute lung injury	LPS	TLR4	Norepinephrine and Epinephrine	Neutrophil-derived catecholamine mediate lung injury	[52]
Stroke	Norephnephrine	Adrenoceptors	–	Activation of sympathetic nervous system weakens neutrophil migration, activation, and phagocytosis, inhibiting their critical host defense functions	[70]
Sepsis	Nicotine	α7-Nicotinic Receptors		Nicotine reduces neutrophil recruitment to the infected area during sepsis development	[106]
Pain	IL-1β	–	PGE_2, ROS, Elastase	Neutrophils participate in the cascade of events leading to inflammatory hypernociception	[133,144,146]
Multiple Esclerosis	IL-17	–	–	Neutrophils are required for tissue damage in the brain and development of experimental encephalomyelitis	[175,176]
Alzheimer's disease	–	–	IL-17, NETs	Neutrophils contribute to Alzheimer's disease pathogenesis and cognitive impairment	[12]
Experimental models of inflammation (air-pouch and peritonitis)	α2-Adrenoceptor Agonist	α2-Adrenoceptors	–	α2-Adrenoceptor agonists prevent the accumulation of neutrophils in inflammatory focus *in vivo*	[61]
In vitro assay	Ephnephrine, Epinephrine and Isoprenaline (β-Agonist)	Adrenoceptors	–	Adrenergic agents reduced neutrophil migration mainly through β-adrenoceptor activation and inhibit neutrophil extracellular traps	[62,63]