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Mariana Sofia Silva Afonso

Role of glial cells in neuropathic pain: potential therapeutic advantages with use of opioids

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Nome: Mariana Sofia Silva Afonso

Endereço electrónico: m04013@med.up.pt

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Role of glial cells in neuropathic pain: Potential therapeutic advantages with use of opioids

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Glial cells have been the focus of scientific attention in the past 20 years. Their role as mere supportive elements of the central nervous system has definitely been abandoned, whereas their part as active mediators and modulators crucially involved in development and sustainment of neuropathic pain is currently well established. More recently, a link between glial activation and opioid actions is being progressively strengthened and there is growing awareness about glia's intervention on the installation of many adverse effects of opioids. There is hope that increasing knowledge of the terms of this interaction may allow development of new – or new uses for known – drugs, allowing enhancement of opioid analgesia and simultaneous inhibition of glial-mediated effects.

This review aims at condensing some of the most relevant data brought to light in these subjects, such as neuronal abnormal communication in abnormal pain states, microglial and astrocytic parts in intensification of painful stimuli transmission and, finaly, the relations between glia and opioid effects.

Key Words: Neuropathic pain, hyperalgesia, allodynia, astrocytes, microglia, glial activation, proinflammatory cytokines, opioids, tolerance, dependence, stereoselectivity.

Role of glial cells in neuropathic pain: Potential therapeutic advantages with use of opioids

Mariana Sofia Afonso[1], Fani Lourença Neto[2]

[1] Faculdade de Medicina, Universidade do Porto, Portugal

[2] Instituto de Histologia e Embriologia Abel Salazar, Faculdade de Medicina, Universidade do Porto, Portugal; IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal.

Corresponding author:

Fani Lourença Moreira Neto

Instituto de Histologia e Embriologia da Faculdade de Medicina da Universidade do Porto, Al. Prof. Hernâni Monteiro, 4200 – 319, Porto, Portugal;

Fax: +351 22 5513655; Telephone: +351 22 5513654

E-mail: fanineto@med.up.pt

Author's address:

Mariana Afonso

Faculdade de Medicina da Universidade do Porto

Al. Prof. Hernâni Monteiro, 4200 – 319, Porto, Portugal

Abstract

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1. Introduction

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Merskey, 2007). It not only involves the sensory perception, but also the cognitive analysis and emotional responses associated (Millan, 1999).

Pain processing is far from being simple. It is a redundant, multi-pathway and dynamic system, at which modifications to the signaling message could be made at any time or level (Millan, 1999; Milligan and Watkins, 2009). The traditional view is that pain results from the activation of specific primary afferent neurons whose cell body is located at dorsal root ganglions (DRGs). Detection of noxious stimulus is accomplished by the terminal ending of the nociceptor, a specialized region whose function is translating energy/chemical information into action potencials. Sensory messages are then conveyed to pain projection neurons located in the dorsal horn of the spinal cord. From the spinal cord multiple ascending pathways (usually fibers of the anterolateral system) relay information in the brainstem and thalamic nuclei, ultimately reaching cortical areas, where pain perception is integrated. In addition, the input that is transmitted to the cerebral cortex is modulated by several descending inhibitory and facilitatory pathways which terminate in the spinal cord, and also by spinal cord inhibitory interneurons (Millan, 1999; Milligan and Watkins, 2009). When the primary lesion or inflammation is prolonged these nociceptors undergo ongoing stimulation, which results in pathological pain through peripheral or central plasticity and sensitization. Typical manifestations of chronic pain include hyperalgesia, an increased response to a stimulus which is normally painful due to lowering of pain threshold, and allodynia, pain due to a stimulus which is normally innocuous

(Cavenagh et al., 2006). Therefore, pathological pain, which includes pain of neuropathic origin, has been classically viewed as being created and maintained solely by neurons (Watkins et al., 2001). As so, development of analgesics has focused on neuronal targets. However, particularly in the case of neuropathic pain, these proved to be only modestly, if at all, effective (Romero-Sandoval et al., 2008), leaving most patients with poorly controlled symptoms and a bad quality of life. Researchers begun then to wonder if there were aspects of pain processing they did not fully understand.

Since Garrison's breakthrough in identifying astrocytes's possible role in chronic pain (Garrison et al., 1991), glia is having an increasing attention from science, as it is now known to have a very important role in the regulation of synaptic strength and plasticity and the initiation of central sensitization (De Leo et al., 2006). Whatever the stimulus for glial activation – either neuropathy, damage or inflammation of peripheral tissues, immune activation in the spinal cord or morphine/other chronic drugs – it provokes release of substances that create pain enhancing changes, usually manifested as allodynia/hyperalgesia (Watkins et al., 2001). There are other non-neuronal cell types involved in pain modulation – endothelial cells, fibroblasts, mast cells, macrophages and oligodendroglia, to name some. However, their study has proven difficult, due to lack of upregulatable activation markers (Watkins et al., 2007a). As so, this review will consider only microglia and astrocytes and will focus primarily on the mechanisms involved in neuropathic pain that occur in the spinal cord, leaving aside peripheral and upper central nervous system events.

There is hope that with accurate knowledge about the several mechanisms in which glia influences pain, these can be targeted pharmacologically, allowing better pain management.

2. Neuropathic pain

Any type of chronic and/or recurrent painful sensation that lasts longer than 3 months and has unpredictable duration is considered to be pathological itself (Milligan and Watkins, 2009). Contrary to physiological (also termed acute) pain, which constitutes a warning against danger impelling the organism towards autoprotection and recovery, pathological pain does not have any apparent important biological role.

One type of pathological pain is that of neuropathic origin which is defined by IASP as pain initiated or caused by a primary lesion or dysfunction of the peripheral (PNS) or central nervous system (CNS) (Merskey, 2007). It can be caused by a large number of different lesions, such as infections, trauma, metabolic abnormalities, chemotherapy, surgery, radiation, neurotoxins, nerve compression or tumor infiltration, all resulting in a painful sensation that continues beyond the expected healing period for the injury that causes it (Cavenagh et al., 2006). It can prevail for years without its cause being definable or treated (Milligan and Watkins, 2009). Clinical pain syndromes occur in epidemic proportions worldwide (Watkins et al., 2007a) being estimated that about one sixth of the population worldwide is affected (Campbell and Meyer, 2006). These syndromes are likely to increase, following increasing prevalence of non-malignant and malignant diseases in current ageing populations, and also resulting of improved cancer treatments, augmenting the number of cancer survivors (Cavenagh et al., 2006).

Neuropathic pain is a relatively frequent occurrence in patients that suffer a partial denervation of a major peripheral territory, rather than a complete one (McMahon et al., 2005). It can present itself in various clinical manifestations, like allodynia, hyperalgesia or hyperpathia (explosive pain response when stimulus intensity exceeds sensory threshold),

to name a few (Cavenagh et al., 2006). The pain sensation may not limit itself to the enervation area of that specific nerve, but may also arise from tissues innervated by neighboring healthy nerves, a condition referred as extra-territorial pain, and also be perceived as originating in the undamaged contralateral part of the body – mirror pain (Watkins et al., 2001).

Amplification of pain may occur in multiple sites along the pain pathway. Considering the spinal cord, which is the focus of this review, amplification of pain takes place within the dorsal horn, a key site for the dynamic regulation of pain processing (Watkins et al., 2009).

3. Neuron

Neurons transform both functionally and anatomically in response to a peripheral nerve injury (Inoue and Tsuda, 2009). These transformations include differences in the ion channels, arising of new fibers or invasion of blood elements that alter the nerve's environment. The end result will be the amplification of pain messages. Several mechanisms are responsible for this amplification, some of which will now be described (Figure 1).

One of such mechanisms involves the neuron's sodium channels (Figure 1A). There are two types of sodium channels – one involved in normal physiological action potentials of all nerves, and other more specific to nociceptive neurons, related to the processing of neuropathic pain states. After nervous injury, both types are upregulated, leading to generalized hyper-excitability (Woolf and Mannion, 1999). This hyper-excitability is characterized by an increased response to stimuli, increase in the receptive fields, reduction

in the threshold of activation and increased spontaneous activity (Baranauskas and Nistri, 1998). Also leading to enhanced excitability following persistent painful stimuli is the sustained depolarization of spinal cord neurons (Figure 1B). The increased and continuous release of glutamate and substance P (SP) that occurs in the primary afferent pre-synaptic terminal leads to a sustained depolarization due to increased intracellular Ca^{2+} and Na^{+} concentrations. As a consequence, there is unplugging of N-methyl-D-aspartate (NMDA) receptor channels, usually blocked by an Mg^{2+} ion. NMDA receptors activation by glutamate causes high Ca^{2+} influx leading to increased intracellular Ca^{2+} concentration and therefore enhanced enzyme activity. This triggers, among other events, increased expression of prostaglandins and nitric oxide. Besides increasing excitability of spinal cord neurons, these mediators cause an exaggerated release of neurotransmitters from sensory neuron pre-synaptic terminals to the spinal cord, contributing to the pain message amplification (Milligan and Watkins, 2009). Increased phosphorylation of certain receptors and ionic channels also occurs. This process is usually known as central sensitization, and from the moment it sets in pain stops acting as a defensive, protective mechanism it is supposed to be, and becomes pathological (Milligan and Watkins, 2009).

Injury may also result in sprouting of sympathetic axons into the DRGs. This, combined with the expression of α-adrenegic receptors at the injured neurons, may be a potential mechanism for sympathetically maintained pain. Also, lamina II interneurons, which play an inhibitory influence on the propagation of the algic stimulus, die some time after peripheral nerve injury, allowing more traffic to get through (Figure 1B). Aβ fibres, that normally relay non-painful stimuli to lamina IV, can start to sprout into lamina II, leading the brain to interpret it as pain. These fibers also shift their usual neurotransmitters release, complementing dying Aδ and C fibers production of SP and calcitonin-gene related

peptide (CGRP), and thus contributing to increased central excitability (Woolf and Mannion, 1999).

Production of growth factor from the enervation territory is also crucial to the development of neuropathic pain. Disconnection of damaged sensory axons from peripheral targets interrupts the retrograde trophic support these neurons normally receive from the periphery (Figure 1A). Absence of nerve growth factor (NGF), neurotrophin-3 (NT3) and/or glial cell line-derived neurotrophic factor (GDNF) being relayed to the cell bodies of neurons causes a disruption in the normal expression of genes, namely of neuromodulators and receptors, resulting in abnormal sensory nerve function (McMahon et al., 2005). Some studies proved that exogenous supply of such factors diminishes or reverses these changes, like treatment with NT3 (Munson et al., 1997; Ohara et al., 1995) or with GDNF (Bennett et al., 1998; Boucher et al., 2000). After peripheral nerve injury, both Schwann cells distal to the injury site and satellite glial cells in DRG produce some quantity of these trophic factors, although they are not enough to compensate, either because of insufficient quantity or unavailability to neurons. Still, these are probably responsible for the sympathetic fibers sprouting into the DRGs, contributing to neuropathic pain (McMahon et al., 2005). Brain-derived neurotrophic factor (BDNF) is synthesized in sensory neurons themselves, and their activity is presumed to be dependent on accumulation and subsequent release upon activity from primary afferent terminals in the dorsal horn (McMahon et al., 2005). Experiments in which sequestering of centrally released BDNF result in attenuation of neuropathic pain manifestations suggest that this member of the neurotrophin family is likely to be involved in the maintenance of neuropathic pain (Theodosiou et al., 1999; Yajima et al., 2002).

Usually, not all neurons belonging to the injured nerve are affected. These spared neurons will receive an increased quantity of neurotrophic factors, not only because the

expression of target-derived factors does not seem to depend on innervation density, but also because the process of degeneration also implicates expression of the same factors either by Schwann cells or fibroblasts and macrophages invading the dying nerve, subsequent to the disruption of the blood-nerve barrier (Figure 1B). NGF induces potent thermal and mechanical hyperalgesia, dependent of activation of trkA receptors in the sensory neurons and on mast cells, which proliferate and desgranulate, releasing inflammatory mediators such as interleukin 1 beta (IL-1β), serotonin (5-HT) and tumor necrosis factor alfa (TNFα). It also stimulates production of BDNF in C-fibers, which also contributes to hyperalgesia (McMahon et al., 2005), as proved by increased mechanical and thermal hyperalgesia following intrathecal administration of BDNF (Zhou et al., 2000).

Nerve trauma initiates the release of TNFα from mast cells, macrophages and Schwann cells, which can sensitize nearby neurons. They also produce interleukin 1 (IL-1) and interleukin 6 (IL-6). Inhibiting these cytokines (TNFα and IL-1) action with neutralizing antibodies results in reduced behavioral signs of neuropathic pain (Myers et al., 1996); also, knockout IL-6 mice do not exhibit neuropathic pain after injury (Murphy et al., 1999; Ramer et al., 1998). Thus there is increasing evidence that cytokines and chemokines have a crucial role in the establishment/maintenance of neuropathic pain following nerve injury/degeneration (for review see(McMahon et al., 2005)).

4. Glial cells

Over 70% of the cell population of the spinal cord and brain are glial cells, classified into astrocytes, oligodendrocytes and microglia. Once thought of as mere housekeepers of neurons, and each with their distinct functions (microglia as host defense,

astrocytes with synapse function control and oligodendrocytes for myelination) (De Leo et al., 2006), they are now recognized as key neuromodulatory, neurotrophic and neuroimmune elements in the central nervous system (Romero-Sandoval et al., 2008), in constant dynamic communication with neurons (Watkins et al., 2001). Even their functions appear to be greater than they looked in the first place – for example astrocytes, usually classified as non-immune cells, can become immunocompetent, releasing immune mediators in pathological states (De Leo et al., 2006).

Both microglia and astrocytes have innate immunity functions, thanks to possession of pattern-recognition receptors that enable them to recognize pathogens by identifying specific molecular patterns of the pathogens proteins and toxic cell debris. Activation of such receptors, like toll-like receptors (TLRs), evokes an immune response whose end actions are release of proinflammatory substances, creating an excitatory positive-feedback loop and thus controlling the developing infection. Phagocytosis and tissue debris clearance are also part of their functions (Miller et al., 2009). This glial response to a peripheral immune challenge also elicits typical sickness responses, like fever, increased sleepiness, decreases in food and water intake, generalized suppression of behavior and enhanced pain response (Watkins et al., 2007a).

Glia is implicated in the modulation of pain, as proved by their activation after experiments that are known to enhance pain, like spinal cord trauma (Popovich et al., 1997), spinal root constriction (Hashizume et al., 2000) or peripheral nerve trauma (Sweitzer et al., 1999). Classical activation markers for glia include glial-fibrillary acidic protein (GFAP) for astrocytes and complement receptor type 3 (CR3) for microglia, which have increased expression in activated states (Watkins et al., 2001). Activation of glia *in vivo* follows upon contact with pathogens, as previously stated, as well as in response to

communication by both primary afferent terminals (upon release of SP, CGRP, ATP and excitatory aminoacids) and pain transmission neurons (which release fractalkine, nitric oxide, prostaglandins) (Watkins et al., 2001). In the activated state, glia produces several neuroactive substances, like reactive oxygen species, nitric oxide, arachidonic acid, leukotrienes, prostaglandins, excitatory amino acids (such as glutamate, aspartate, cysteine and quinolinic acid), nerve growth factors and enkephalinases (Watkins et al., 2001). This glial-neuron communication is facilitated by the anatomical position of glia, which encapsulates synapses, thus being easily able to regulate these messengers (Watkins et al., 2001). The group of substances released by activated glia also includes proinflammatory cytokines, like TNFα, IL-β and IL-6, which act in an autocrine/paracrine fashion (many times activating TLRs (Milligan and Watkins, 2009)), capable of using their own perserverative release (Watkins et al., 2001). Proving their modulation role, it is known that drugs that inhibit proinflammatory products block and/or reverse such pain states (Watkins et al., 2009), as observed by prevention (Tan et al., 2009) or reversal (Marchand et al., 2009) of pain facilitation by minocycline (an antibiotic that precludes microglia activation) in models of spinal cord injury.

Besides pathogens and messages from abnormally functioning neurons, glia also responds to generalized cell stress/damage, independently of the production of classic neurotransmitters or neuromodulators. These signals, called "endogenous danger signals" or "alarmins" include degradation products of the extracellular matrix, components of circulating blood (that usually do not reach the extracellular space), and substances released by injured and dying cells – nuclear protein High Mobility Box Group 1 (HMBG1), heat shock proteins (HSPs), DNA and related "self" substances. These are recognized by a type of immune pattern recognition receptors, the TLRs (Watkins et al., 2009).

Glial products can have multiple functions: enhance neurons' excitability, increase sensory afferents' release of pain-associated neurotransmitters, upregulate calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors, as well as enhance their conductance, potentiate inward currents in tetrodoxin-resistant sodium channels, downregulate γ-aminobutyric acid (GABA) receptors, decrease outward potassium currents, and downregulate glial glutamate transporters (Watkins et al., 2009). Particularly in enhanced neuronal excitability, there is increased neuronal firing or decreased thresholds to firing, thus producing heightened responses to noxious and non-noxious stimuli characteristic of neuropathic pain (De Leo et al., 2006). Explaining in a simplified manner, injured/overstimulated neurons release ATP, glutamate, SP, and other mediators above mentioned, stimulating glia to become activated. In turn, astrocytes and microglia release substances that stimulate neurons and nearby glia to be further activated (Milligan and Watkins, 2009).

The release of glial substances can explain phenomenon like extra-territorial pain and mirror pain. Autocrine/paracrine self-stimulation accounts not only for a diffusion to surrounding glial cells, making them active, even when being remote from the lesion site, but also for the communication between different groups of glia, via electrical gap junctions or propagating calcium waves. When these distant glial populations are activated and have healthy neuronal terminals nearby, pain sensation will be transmitted, and the brain will interpret as if these areas are also damaged (Watkins et al., 2001). Proving all this, extraterritorial pain and mirror pain are disrupted by intrathecal administration of glia or IL-1 inhibitors (Watkins et al., 2003).

4.1 Microglia

Microglial cells, accounting for 5-10% of the total glial population, derive from mesodermal precursor cells of hematopoietic lineage. They are considered the resident macrophages of the CNS (Inoue and Tsuda, 2009), given their monocytic origin and being recognized by the same antibodies as macrophages (ED1, ED2, major histocompatibility complex - MHC, type 3 complement receptor - CR3/CD11b) and also the surveillance role they play in the CNS, forming a regularly spaced network of resident cells (De Leo et al., 2006). When in basal conditions, microglia does not produce neuroexcitatory substances (Watkins et al., 2009). Morphologically, they are identified as cells with small somata bearing thin and branched processes (Inoue and Tsuda, 2009) and may be easily visualized with immunohistochemistry techniques by using OX-42, an antibody that recognizes CR3 in mononuclear phagocytes and is commonly used as a specific marker of microglia (Ling et al., 1990).

Microglial cells are highly dynamic in the brain, and rapidly move toward the site of injury (Inoue and Tsuda, 2009), making them the first cell types to respond to several forms of injury (Romero-Sandoval et al., 2008). They perform an important function as antigen recognizers and presenters (Romero-Sandoval et al., 2008). In face of threatens to physiological homeostasis, they become activated and show long-term responses, such as changes in morphology, number, function and gene expression (Inoue and Tsuda, 2009). Their processes become thickened and retracted, and their number increases by two to four folds, as shown by their positivity to proliferation markers such as 5-bromo-2-deoxyuridine (BrdU) 2-3 days after nerve injury. BrdU is a thymidine analog, incorporated into DNA during cell replication, in the S phase, as investigated and used by Eidinoff or Poot and

colleagues (Eidinoff et al., 1959; Inoue and Tsuda, 2009; Poot et al., 2001). Microglia express many of the same neurotransmitter receptors as astrocytes and neurons, and once activated they produce several substances – the same released under immunogenic conditions - that influence surrounding astrocytes, neurons and other microglial cells (Milligan and Watkins, 2009).

In experimental models of neuropathic pain, dorsal horn areas that show activated microglia coincide with areas that receive projections from injured nociceptors, showing that the signals sent by the injured neuron are crucial for their activation (Inoue and Tsuda, 2009). These signals can be neuronal depolarization combined with extracellular ion changes, for example, or substances produced by the neuron (De Leo et al., 2006; Romero-Sandoval et al., 2008), or even byproducts of their destruction, such as aggregated or misfolded proteins or nuclear factors (Milligan and Watkins, 2009).

4.1.1 Neuron to microglia communication (Figure 2)

4.1.1.1 *Neuronal produced substances*

MCP-1

Monocyte chemoattractant protein-1 (MCP-1/CCL2), a chemokine, is not usually expressed in neurons (Milligan and Watkins, 2009). Only in unhealthy conditions it is upregulated in DRG neurons, in a similar time-course to that of microglial activation, making it a probable signaling molecule between injured neurons and microglia (Inoue and Tsuda, 2009). Additionally, it has been observed that mice not possessing the correspondent receptor (chemotactic cytokine receptor 2 – CCR2) have reduced microglial activation after nerve injury (Zhang et al., 2007), the same happening when a MCP-1-

neutralizing antibody was used intrathecally (Thacker et al., 2009). The importance of spinal MCP-1 is also shown by intrathecal injection of MCP-1 into normal rats, causing microglial proliferation and behavioral allodynia (Thacker et al., 2009), events prevented by a selective antagonist for CCR2 (Dansereau et al., 2008). CCR2 protein is expressed in microglia, but also in neurons, in the rat's paw skin, in the peripheral nerve, at the site of injury and in DRG. Since knockout mice for CCR2 show reduced allodynia, it is suggested that this receptor is crucial for the development of neuropathic pain (Inoue and Tsuda, 2009).

Fractalkine (CX3CL1)

This chemokine, a protein located on the outer surface of neurons (Watkins et al., 2001), is expressed in naïve DRG and dorsal horn neurons, as well as in dorsal horn astrocytes following nerve injury. When its receptor, CX3CR1, only expressed in microglia, is inhibited by an intrathecally delivered neutralizing antibody, development and maintenance of allodynia are disrupted (Milligan et al., 2004; Zhuang et al., 2007). Additionally, CX3CR1 expression is enhanced after nerve injury (Lindia et al., 2005; Zhuang et al., 2007). Also, intrathecal fractalkine activates p38 and nuclear factor-κB (NFκB) - a transcription factor controlling the expression of genes involved in immune responses, apoptosis, and cell cycle - and this activation following nerve injury is suppressed by a CX3CR1 neutralizing antibody (Zhuang et al., 2007). Fractalkine is cleaved by cathepsin S, a cysteine protease expressed in spinal microglia (Inoue and Tsuda, 2009), although it can also be cleaved by metalloproteinase-9 (MMP-9) (Milligan and Watkins, 2009). Cathepsin S intrathecal injection activated p38 and induced allodynia, and both were prevented by a fractalkine neutralizing antibody (Clark et al., 2007). When a

cathepsin inhibitor was used, the established allodynia was reversed, but its development was not affected, suggesting a role for cathepsin only in the maintenance of neuropathic pain, but not in its establishment (Inoue and Tsuda, 2009). It is known that fractalkine provokes pain enhancement through release of proinflammatory cytokines and nitric oxide. It increases the numbers of neurons exhibiting prolonged after-discharges which is indicative of central sensitization and spontaneous pain (Watkins et al., 2007a).

Secondary lymphoid tissue chemokines (CCL21) and Interpheron-inducible protein of 10kDa (CXCL10)

These chemokines are rapidly induced in damaged neurons and released inside vesicles, being then transported to distant pre-synaptic terminals. This allows distant activation of glia, since both astrocytes and microglia express the common receptor CXCR3. This stimulation also induces calcium and chloride transients and chemotaxis, indicating activation (Watkins et al., 2007a). In addition it has been observed that mice with no CXCR3 do not display secondary activation of microglia (Biber et al., 2002; Rappert et al., 2002), suggesting that activation of this receptor by these chemokines might be an important neuron-glia signaling pathway leading to microglia activation.

MMP-9

Expression of metalloproteinase-9 is enhanced in damaged DRG neurons 6-24h after injury, as well as in Schwann cells. Knockout mice for MMP-9 show reduced expression of injury-induced microglial activation markers (p38 phosphorylation, Iba1 immunofluorescence and IL-1β expression), while intrathecal administration of MMP-9 into normal rats induced the activation markers (Suter et al., 2007). Substrates for this activation are probably fractalkine, IL-1β and TNFα (Inoue and Tsuda, 2009).

ATP

ATP may act through activation of purinergic receptors, which are classified in two distinct groups: the ionotropic or ligand-gated cation channel receptors, $P2X_{1-7}R$, and the metabotropic or G-protein coupled receptors, $P2Y_{1, 2, 4, 6, 11-14}R$ (Inoue and Tsuda, 2009).

In microglia, expression of some subtypes of purinergic receptors has been found, as is the case of $P2X_4R$ .Tonic activation of $P2X_4R$ is necessary for allodynia maintenance, as proved by the reversal of established allodynia after $P2X_4R$ acute pharmacological blockade. Moreover, mice lacking expression of $P2X_4R$ do not show allodynia after nerve injury, whereas spinal administration of $P2X_4R$-stimulated microglia caused normal rats to develop allodynia (Inoue and Tsuda, 2009), suggesting that activation of these receptors in microglia is crucial for allodynia development. It has been found that $P2X_4R$ is upregulated following nerve injury, and although the mechanisms by which this occurs are not completely established at the moment, it is speculated that fibronectin protein might be involved. In fact, the evidences pointing to it are numerous. Accordingly, the levels of this extracellular matrix protein are elevated 3 to 7 days after injury (Nasu-Tada et al., 2006), corresponding to the time when $P2X_4R$ protein levels start to increase (Tsuda et al., 2003). In addition, blockade of the fibronectin receptor diminished $P2X_4R$ upregulation and allodynia (Tsuda et al., 2008), and fibronectin delivered intrathecally resulted in increased $P2X_4R$ expression and allodynia, a behavior that is not verified in $P2X_4R$ deficient mice. Moreover, when cultured microglia was stimulated by fibronectin it showed enhanced levels of $P2X_4R$ protein and of ATP-induced Ca^{2+} influx (Nasu-Tada et al., 2006). Therefore, all these studies allow concluding that the expression of functional $P2X_4R$ in microglia is upregulated by fibronectin stimulation (Inoue and Tsuda, 2009). These effects are thought to be mediated by TLR4, which is activated by fibronectin, causing $P2X_4R$

upregulation (Milligan and Watkins, 2009). The calcium influx that occurs will probably lead to intracellular signaling cascades mediated by p38 mitogen activated protein kinases (MAPK) (Milligan and Watkins, 2009).

$P2X_7R$ may also be involved in neuropathic pain, since knockout mice for these receptors show reduced thermal and mechanical hyperalgesia after nerve injury (Chessell et al., 2005), and systemic administration of antagonists to these receptors (A-740003 and A-438079) reduces tactile allodynia in different neuropathic pain models (Honore et al., 2006; Inoue and Tsuda, 2009).

Experiments similar to those performed for $P2X_4R$ demonstrated that $P2Y_{12}R$ is another purinergic receptor subtype that is important in neuropathic pain processing. In fact it has been observed enhanced expression of $P2Y_{12}R$ after nerve injury, reduced hyperalgesia upon their inhibition (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008), failure to develop allodynia in $P2Y_{12}R$ knockout mice, and alleviation of established allodynia upon blockade by $P2Y_{12}R$ antagonists (Tozaki-Saitoh et al., 2008). However, since no differences were detected in the morphological and numerical alterations of microglia between normal and knockout mice, it is suggested $P2Y_{12}R$ may not be necessary for the microglial activation, but is probably implicated in the motility of microglial cell bodies and processes, as other lines of evidence point to (Inoue and Tsuda, 2009).

Activation of microglia by ATP leads to release of ATP and BDNF by microglia, causing a depolarization shift that inverts the polarity of currents activated by the inhibitory neurotransmitter GABA in spinal lamina neurons (Milligan and Watkins, 2009).

4.1.1.2 *Microglia receptors*

In addition to the numerous receptors existent in microglia already stated, it is important to mention toll-like receptors (TLRs). TLR 2-4 (expressed in microglia as well as astrocytes in neuroinflammatory conditions) (Watkins et al., 2009) are shown to be upregulated in the spinal cord following nerve injury (Inoue and Tsuda, 2009), and are key receptors in initiating allodynia and hyperalgesia following nerve injury (De Leo et al., 2006). One example of this is activation of TLRs by HSPs, intracellular chaperones whose function is to improve protein folding into native/active conformations. When the cell is subjected to stress of some kind, they are upregulated in the attempt of maintaining normal cell functioning. However, in the case of severe damage, as happens with neurons after injury, they are released and activate glia either through TLRs or by inducing increased production of glial activators from stressed glia or neurons (Milligan and Watkins, 2009). Some HSPs such as HSP60, HSP70 and HSP96 are confirmed as potencial TLRs activators (Milligan and Watkins, 2009).

When TLRs function is inhibited (either by antagonists or molecular and genetic manipulation) there is reduced allodynia after nerve injury, as well as diminished expression of microglial markers and production of inflammatory cytokines (Inoue and Tsuda, 2009).

4.1.2 Microglial activation (Figure 2)

4.1.2.1 *Intracellular mechanisms*

p38 are one class of MAPK thought to have a pivotal role in the development of neuropathic pain (Milligan and Watkins, 2009). p38 are involved in Ca^{2+} sensitive

intracellular signaling cascades that respond to various different extracellular stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are implicated in cell differentiation and apoptosis (Roux and Blenis, 2004; Schieven, 2009). Their levels increase after nerve injury, and their expression is very restricted to spinal microglia cells. Inhibiting p38 action pharmacologically prevents installation of allodynia or suppresses it, if already installed. Several studies show that p38 are as necessary for the development as for the sustaining of allodynia (Inoue and Tsuda, 2009). It is known that MMP-9 is one of the molecules that work activating p38. MMP-9 is upregulated in DRG neurons at an early phase, day 1 after neuropathy induction. It has also been observed that inhibition of p38 before an MMP-9 stimulus prevented allodynia from installing. MMP-9 regulates the cleavage of pro-IL-1β, TNFα and fractalkine, and it is probably through these molecules that it exerts its action as activator of p38 (Inoue and Tsuda, 2009).

Other MAPK implicated in neuropathic pain processing are MAPK1, also known as the extracellular signal-regulated kinases (ERK) (Cruz and Cruz, 2007; Ji et al., 2009). Expression of ERK5, one of the elements of the family, is highly restricted to microglia, and its knockdown decreases microglia activation, as well as neuropathic pain. One of the molecules that likely activate ERK belongs to the Src-family kinases (SFK), namely Lyn, the most frequent in spinal microglia. In fact its level is increased after nerve injury, and when Lyn is not present there is a reduction of the $P2X_4R$ upregulation that is normally observed under neuropathic conditions (Inoue and Tsuda, 2009). Several data indicate that it occurs first an activation of ERK in microglia and then in astrocytes, and that this sequential activation is important for the induction and maintenance of neuropathic pain, respectively. Indeed, ERK seems to be crucial for the intracellular signaling events taking

place in glial cells after nerve injury, that will ultimately lead to the production of proinflammatory and pronociceptive mediators (for review see (Ji et al., 2009)).

Another intracellular mechanism occurring in microglial activation is the enhanced expression of proteins belonging to the complement cascade. Indeed this is one of the most common transcriptional changes found in neuropathic pain models. Its importance can be inferred from the data supporting that its blockade abolishes manifestations of allodynia and hypersensitivity (Inoue and Tsuda, 2009).

4.1.2.2. *Actions provoked by microglial activation*

Production of cytokines, like IL-1β, TNFα or IL-6, contributes to the induction of cell adhesion molecules expression, recruitment of T-leukocytes into the lesion site and astrocytic activation (De Leo et al., 2006; Romero-Sandoval et al., 2008). The sequential activation of microglia and then astrocytes is suggested by the different timing in the expression of each cell group markers. Thus microglial CR3/CD11b mRNA and protein expression precedes sustained increase in the expression of GFAP following nerve injury (De Leo et al., 2006). Sequential activation does not mean that microglia is only important for the initiation of chronic pain, since increases in spinal CR3/CD11b protein can be detected at days 28 and 42 after nerve injury, when the pain abnormal processing is already fully installed (Romero-Sandoval et al., 2008).

Activation of astrocytes by microglia leads to increased levels of specific chemokines, causing microglia to release harmful substances (proteases, ROS, NO) that have cytotoxic effects in the surrounding environment. All these substances act in a synergic way as pronociceptive molecules, enhancing CNS sensitization and contribute to neuropathic pain. (De Leo et al., 2006)

4.2 Astrocytes

Astrocytes derive from the neuroectoderm, and are the most abundant of glial cells, constituting 40-50% of all glial population (for review see (Sofroniew and Vinters, 2010)). Some of their functions include providing energy and neurotransmitter precursors to neurons, clearing debris, maintaining ion concentrations stable and also uptaking neurotransmitters from the synaptic space, to terminate their actions (Watkins et al., 2009). By possessing voltage-gated channels and neurotransmitter receptors (NMDA, glutamate, purinergic and SP receptors, to name some (Milligan and Watkins, 2009)), and their privileged position (encapsulating synapses and close contact with the neurons' somas (Milligan and Watkins, 2009), dendrites and nodes of Ranvier (Watkins et al., 2007a)) enables them to be activated by, and modulate, neurotransmission. Synaptic "memory" is also part of their actions – prior synaptic activity leads, as later times, to greater astrocyte responses and to subsequent synaptic input (Watkins 2006).

Astrocytes do not constitute a homogenous population, differing in the expression of channels or other substances (i.e. GFAP *versus* proteoglican NG2). Arranged in different microdomains, interaction with microvasculature and synapses is therefore very function-specific (De Leo et al., 2006). Their importance as regulators of ion homeostasis is easily grasped when accounting for the major role that astrocytic Na^+/K^+ ATPase plays in the removal of excess synaptic potassium, storing it and regulating its release back into the synaptic space (De Leo et al., 2006). This will be important since it is known that lipopolysaccharyde (LPS)-activated microglia express outward K^+ channels, accounting for a faster depolarization of neurons and microglia release of neuronal sensitizing mediators (De Leo et al., 2006). Calcium is another ion whose cytosolic concentrations are regulated

by astrocytes. Calcium waves and oscillations cross from cell to cell via gap junctions between astrocytes, thus inducing neuronal responses, such as modulation of neurotransmitter release (De Leo et al., 2006).

Besides regulating levels of ions, astrocytes are also key actors in maintaining glutamate at appropriate low levels, having high affinity transporters such as glutamate transporter 1 (GLT-1, responsible for over 90% of the clearance) and glutamate-aspartate transporter (GLAST) for executing this function. After injury, astrocytes assume their activated phenotype (which includes proliferation, hypertrophy and overexpression of GFAP (Romero-Sandoval et al., 2008)) and the astrocytical GLT-1 levels drop, the same happening in astrocyte cultures upon addiction of TNFα and IL-1β. Excessive glutamate in the synaptic space produces enhanced signaling at neuronal glutamate receptors, probably creating ectopic action potentials (Figure 2). When propentofylline, a glial modulating agent, is used to suppress astrocytic activation, GLT-1 expression is induced (De Leo et al., 2006). In the process of glutamate uptake three Na^+ ions are co-transported with it. The rising of intracellular Na^+ concentrations causes the astrocytes to initiate glycolysis. The lactate released by this process "feeds" the neuron, suppressing its energy needs which are enhanced in virtue of the synaptic activity. After nerve injury, and because GLT-1 decreases, the astrocyte will no longer be able to meet the neuron's requirements, making it at risk for excitotoxic damage. Given these mechanisms, it is proposed that these glutamate transporters function as metabolic sensors (De Leo et al., 2006). In addition to uptake, release of glutamate is also an astrocytical function in response to injury, thus causing its own excitation - a vicious cycle. Microglia also releases glutamate, and given the proximity of both cell types to neurons, glia can directly sensitize and stimulate neurons during initiation and/or maintenance of pathological pain (De Leo et al., 2006).

Similar to microglia, activation of astrocytes involves signaling by MMPs, usually type 2. It results, as in microglia, in cleaving pro-IL-1β. Also common is intracellular signaling via ERK, as well as via c-Jun N-terminal kinase (JNK/MAPK8), leading to increased release of inflammatory factors such as IL-1β, IL-6, TNFα, prostaglandin E_2 (PGE_2) and NO. These contribute to the downregulation of GLAST, as previously discussed (Milligan and Watkins, 2009).

4.3 Summarizing

Glia influence neuronal transduction of abnormal pain signaling by enhancing it. First, they recognize a pattern of numerous mediators that relay the message from the site of peripheral nerve or tissue injury (Watkins et al., 2007a). Then, producing proinflammatory cytokines, they increase the number of AMPA and NMDA receptors in the surface of neurons, as well as their conductivity (Milligan and Watkins, 2009), and simultaneously downregulate expression of GABA receptors (Watkins et al., 2007a). IL-1β is an example, as it leads to phosphorylation of a subunit of NMDA, activating it and increasing its conductivity (Watkins et al., 2007a). TNFα increases AMPA conductivity and increases spontaneous neurotransmitter release from pre-synaptic terminals. Ultimately this leads to overall increase in neuronal excitatory tone (Watkins et al., 2007a) (Figure 2).

NMDA activation ultimately results in production of NO and PGE_2, that directly affect neurons, once again amplifying excitability of pain-projecting neurons (Milligan and Watkins, 2009).

Calcium influx also leads to p38 and ERK signaling, resulting in the increased activity of transcription factors (like NFκB) and subsequent secretion of IL-1β, TNFα, IL-

6,, PGE_2 and BDNF. All these contribute to infiltration and phagocytosis and thereby to the pathological effects of glia (Milligan and Watkins, 2009).

5. Opioids

Opioids are a very well known pharmacological class of potent analgesics, often constituting the first choice treatment to minimize suffering of patients with acute, post-surgical and neoplasic-derived pain (Romero-Sandoval et al., 2008). Neuropathic pain, however, is very difficult to control, and evidence is arising that this lack of efficacy might be related with glial cells intervention. In this section we will discuss the recent scientific data related to glial modulation of opioid actions.

Opioids are used in the clinical practice because of their analgesic action, but their applicability is frequently undermined by undesired effects, like tolerance (need to escalate the dose administered to get the same analgesic effect), dependence and reward (which may generate drug addictions). These adverse effects derive from activation of counter-regulatory mechanisms by which pain suppression is diminished (Watkins et al., 2009). Some of these mechanisms have a neuronal origin, like release of endogenous anti-opioid peptides (like cholecystokinin), downregulation and/or diminished action of opioid receptors and alterations in signaling cascades that follow receptor activation, to name a few (Watkins et al., 2009). One such example is the opioid-induced release of proinflammatory cytokines, with subsequent enhancement of neuronal excitability – caused, among others, by increase of conductivity and expression of NMDA and AMPA receptors and downregulation of GABA receptors (Watkins et al., 2007a). Chemokine release may also be induced, such as CCL1/MCP-1, CCL5 and CCL12. These can bind to

their correspondent receptors (CCR1, CCR5 and CXCR4) and cause desensitization/inactivation of opioid receptor in that same cell (Adler et al., 2005; Zhang et al., 2004).

Modulation systems involved in chronic pain and opioid actions were once though of as totally different phenomenon. However, researchers were recognizing more and more similarities between these two apparently separate systems, and eventually begun to question if glia, already proved to be of major importance in chronic pain modulation, would play a similar role in opioid actions. In fact, mechanisms involved in neuropathic pain were surprisingly similar to those of morphine tolerance (Mayer et al., 1999). A decade has passed since the first finding that brought glia and opioids toghether, and it now seems that they may even share some common mechanisms (Watkins et al., 2009).

The first report that proved existence of a link between glia and diminished effectiveness of opioids, namely morphine tolerance, was on a study by Song and Zao, in 2001 (Song and Zhao, 2001). It testified that chronic administration of morphine (systemically) increased astrocyte activation in the spinal cord, and that both glial activation and tolerance were attenuated by fluorocitrate, an inhibitor of glial Krebs cycle (Watkins et al., 2007a). Since this first breakthrough, several evidence arose sustaining the glial involvement in opioid effectiveness: chronic morphine induced upregulation of microglial and astrocytic activation markers (like CR3CD11b and GFAP) (Cui et al., 2006); morphine-induced enhancement and release of proinflammatory substances (IL-1β, IL-6, TNFα) (Raghavendra et al., 2002); an upregulation of proinflammatory cytokines in microglia but not in neurons was observed upon morphine administration (Tai et al., 2006) as well as selective activation of microglial p38 and improved analgesic efficacy of morphine when p38 MAPK inhibitors are used (Cui et al., 2006); and also its was detected

a temporal correlation between increasing glial activation and cytokine production with progressive tolerance effects to morphine (Raghavendra et al., 2004).

Proving the causal relation between glial activation and morphine tolerance is the fact that tolerance effects are either reversed or, at least, attenuated, with the use of spinal proinflammatory cytokine inhibitors (Johnston et al., 2004; Raghavendra et al., 2002; Shavit et al., 2005), or by knocking IL-1 signal (Shavit et al., 2005). The resultant analgesia also occurred when metabolic inhibition of glia (by fluorocitrate, minocycline or ibudilast) was tested (Cui et al., 2008; Hutchinson et al., 2009a; Hutchinson et al., 2008a). Morphine tolerance was also linked to downregulation of dorsal horn glutamate transporters, GLAST and GLT-1, with the concomitant increase in extracellular excitatory amino acids (Mao, 2002; Tai et al., 2006), fact that sustains the already discussed effect of proinflammatory cytokines causing inhibition of glutamate uptake and reduced GLAST and GLT-1 expression (Watkins et al., 2007a). All the referred data suggest that morphine-induced tolerance might be due to activation of glia, and that the consequent production of proinflammatory cytokines and elevations in glutamate cause an enhanced neuronal excitability (Watkins et al., 2007b).

When considering neuropathic pain, tolerance is not the only factor that diminishes opioid efficacy. There is also a certain "naïve opioid tolerance", in which morphine does not accomplish its total analgesic potency, even without prior exposure to opioids (Watkins et al., 2007b). When proinflammatory cytokines are inhibited, morphine restores its full analgesic ability (Raghavendra et al., 2002). In addition to that, the analgesic power of morphine is reduced when prior sub-analgesic doses of morphine are used, and glial involvement is inferred from reinstatement of normal analgesia when the glial modulating agent propentofylline is used (Wu et al., 2005).

In neuropathic pain conditions, opioids not only do not diminish pain sensations as much as they should, but they might also be the agents inducing an abnormal sensitivity to pain (Watkins et al., 2007b). This effect occurs independently of the use of continuous opioid dose (using osmotic minipumps) to make sure pain is not due to repeated minor-scale withdrawals between doses (Mao, 2002). According to this data, opioids may themselves cause neuropathic pain, through reduction of pain thresholds (Watkins et al., 2007b).

Alongside with tolerance, dependence/withdrawal is another unwanted effect of opioids, which is characterized by the physical need of opioid doses to avoid developing a withdrawal syndrome. Glia might also be involved in the mechanisms that generate dependence, as was suggested by studies in which the pain enhancement that usually follows withdrawal of opioids could be attenuated by inhibiting proinflammatory cytokines or IL-1 receptor (Johnston et al., 2004; Raghavendra et al., 2002; Raghavendra et al., 2004). Additionally, spinal glia activation consequent to morphine administration could be maintained at basal levels when ibudilast, an anti-inflammatory drug, was given concomitantly with morphine (Ledeboer et al., 2007).

Modulation of opioid effects through glia does not occur only in prolonged exposition to morphine – it is present even in acute exposure (Watkins et al., 2007a). Once again, the mechanism seems to depend on proinflammatory cytokines, since blocking them enhances and prolongs acute analgesia (Johnston et al., 2004; Shavit et al., 2005), whereas administering IL-1, even in a neutral, non-noxious dose abolishes morphine analgesia (Shavit et al., 2005).

It was first hypothesized that glial activation with opioids, and all the unwanted side effects, must depend on the classic opioid receptors, the only known opioid receptors in

neurons (Watkins et al., 2007b). However, opioid hyperalgesia occurred even when mice were knocked-out for all the 3 known receptors, δ, κ and μ (Watkins et al., 2009). Considering stereoselectivity, only (-)-opioid isomers can activate the classic receptors, whereas (+)-isomers have very low affinity to them. However, both (+) and (-)-isomers, when administered intrathecally, were found to equally induce IL-1, IL-6 and TNFα expression in spinal glia (Watkins et al., 2007a). These data suggested that possible non-classical receptors, indifferent to stereoselectivity, are activated and are the main causers of glial pain enhancing effects, implicating that it could be possible to block those receptors, allowing opioids to exert their full analgesic effect. Experiments with (+)-naloxone were successful. Indeed, although not being active on neuronal opioid receptors, it enhances analgesia, delays development of tolerance, decreases withdrawal syndrome (following (-)-naloxone administration), and also blocks the anti-analgesic effect that is usual of morphine (Wu et al., 2006a; Wu et al., 2006b; Wu et al., 2004; Wu et al., 2005). Reassuring that these actions are due to glial activation, (+)-opioid agonists can minimize normal (-)-opioid analgesia (Wu et al., 2007), an effect that is blocked with use of propentofylline (Wu et al., 2005).

The non-classical opioid receptors, capable of being activated without consideration for stereoselectivity, were linked to TLR4 in 2007 (Hutchinson et al., 2007), when it was realized that all structural classes of opioids activate TLR4 (some in a non-stereoselective way), and that opioid antagonists sometimes block TLR4 signaling also in a non-stereoselective way, like naloxone and naltrexone (Hutchinson et al., 2010). Also, blockade of TLR4 function, by knockout or inhibition of the receptor/signaling cascade, resulted in enhanced and more sustained opioid analgesia. (Watkins et al., 2009)

It appears that the common fact between glial activation and opioid unwanted effects – such as attenuation of acute and chronic analgesic efficacy, opioid-induced hyperalgesia, dependence and reward – is TLR4 receptor (Watkins et al., 2009). In fact, it was proved that both (+) and (-) naloxone and naltrexone inhibit TLR4 signaling after LPS stimulation, causing diminished release of proinflammatory cytokines and blocking microglial activation (Hutchinson et al., 2008b). It is thought that opioids interact directly with the myeloid differentiation factor 2 (MD2)/TLR4 complex, more specifically with the LPS binding pocket of MD2. Major contributions for this hypothesis are the observations that TLR4 signaling by opioids is blocked by a competitive inhibitor of LPS, and that TLR4 knockout mice have potentiated opioid analgesia (Hutchinson et al., 2009b; Watkins et al., 2009). Interaction with other subtypes of TLR has also been observed, namely TLR 2 and TLR 9 (Watkins et al., 2009).

6. Final remarks

Knowledge about glial activity has increased quite considerably in the past years, and its role in modulation of neuropathic pain, including the mechanisms by which it occurs, is progressively becoming clearer. Although not forgetting the crucial part that glia plays in the CNS, namely in answering to homeostatic threatens by putting out pathogen menace or executing neuronal damage repair, its activation and subsequent enhancement of neuronal excitability and noxious-stimuli transmission in chronic pain conditions make it a much desired inhibition target. Considering opioids, it is now clear that glia is to blame for many of their unwanted consequences – not only it reduces opioids' analgesic power, but it

also contributes to their dreaded dependence and withdrawal effects. In fact, fear of dependence is one of the main reasons for under-prescription of opioids.

Currently, and knowing that apparently there is a clean separation between the glial-activating and the neuronal-activating opioid actions, there is hope that new drugs, – like (+)-opioid antagonists that block glial activation, possibly by specifically targeting TLR4 receptor – can enhance opioid analgesia and inhibit development of dependence, currently responsible for drug-abuse return rates of 60-80% (Charles et al., 2003). Controlling the devastating influence of neuropathic pain would be a breakthrough sure to influence millions of people worldwide, diminishing their suffering and majorly improving their quality of life.

Appendix

Figure 1:

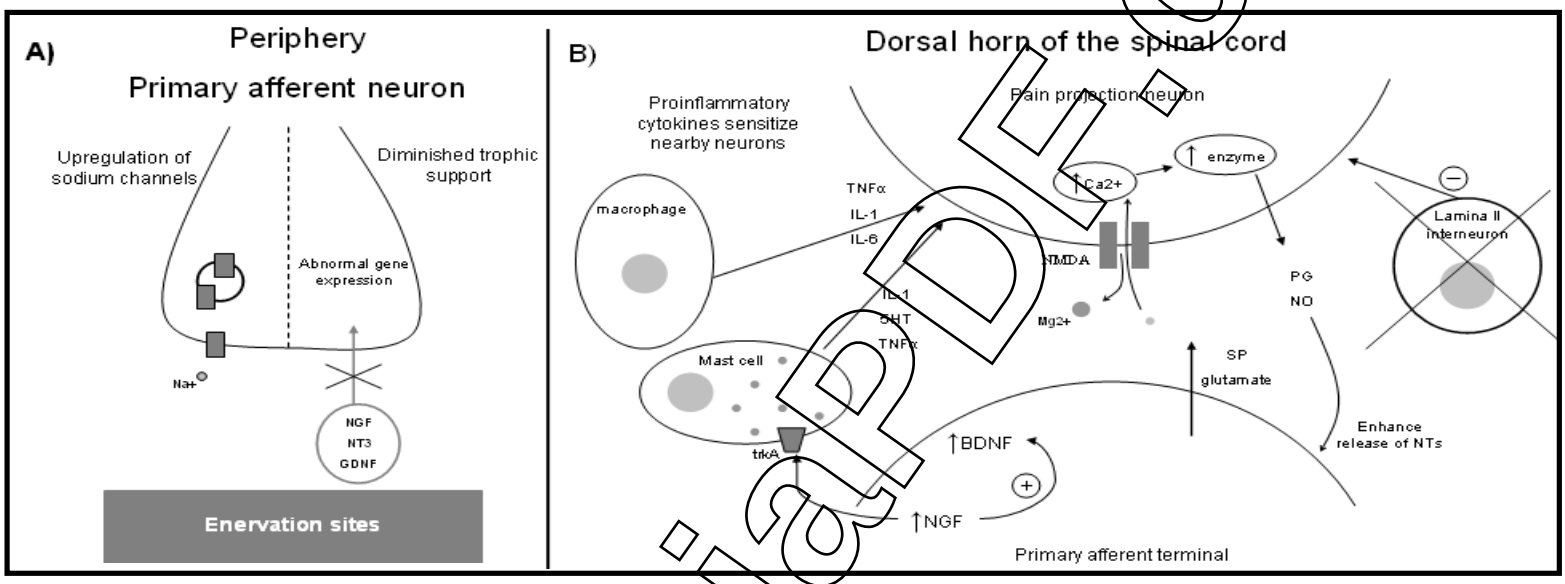

Figure 2:

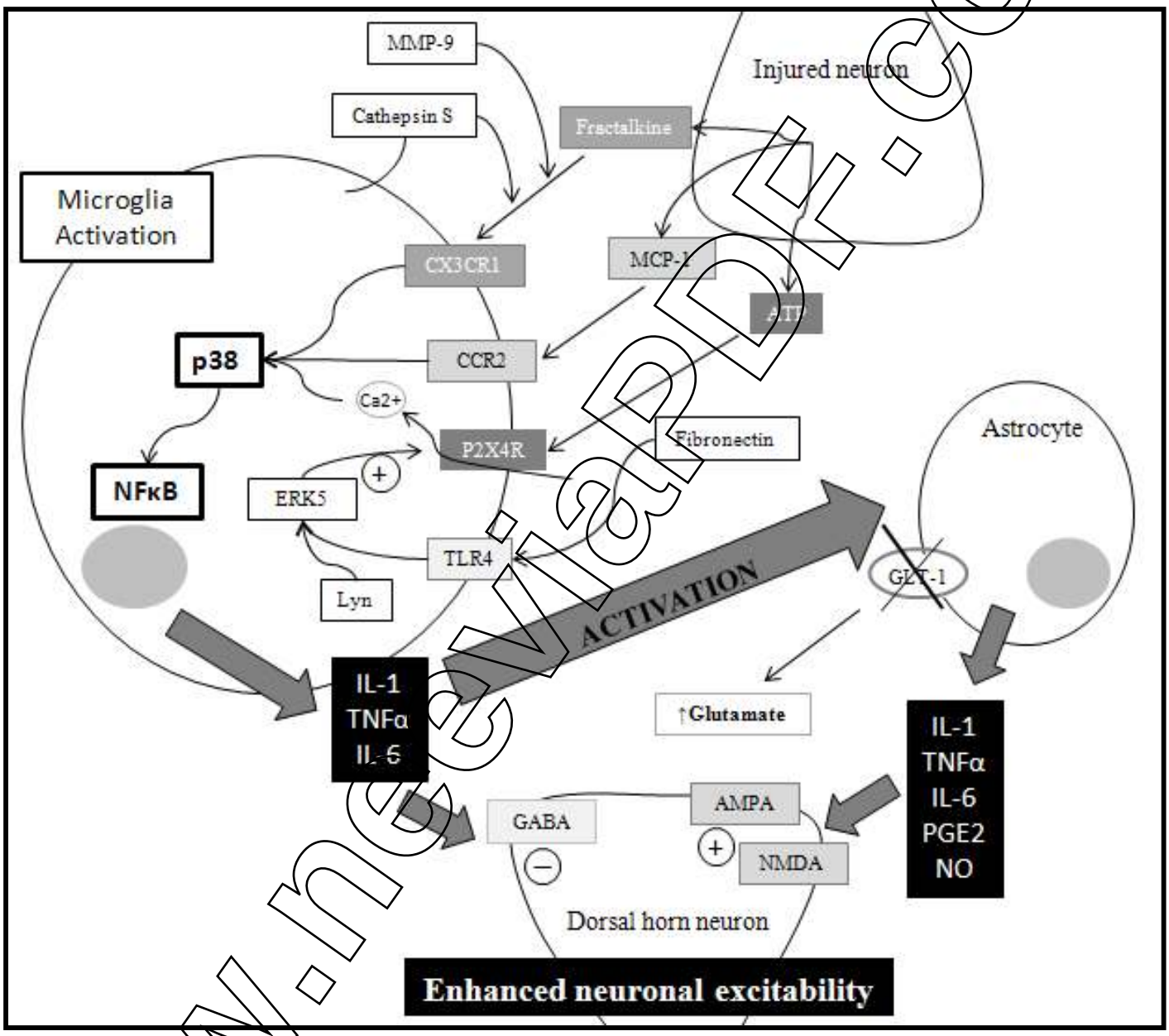

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Figure Captions

Figure 1: Abnormal neuronal functioning following nerve injury.

A) At the primary afferents neurons in DRGs (peripheral nervous system), there is upregulation of sodium channels after peripheral nerve injury, leading to generalized hyper-excitability. In addition, the interruption of retrograde trophic support from the target sites will result in disruption of normal gene expression, resulting in abnormal sensory nerve function.

B) Enhanced excitability in nociceptors, following persistent painful stimuli, implicates increased and continuous release of substance P and glutamate. The resultant influx of calcium and sodium into the spinal cord neuron causes NMDA channels to be unblocked (removal of Mg^{2+} plug) and, thus, glutamate activation induces a stronger calcium entrance wave. Rising Ca^{2+} intracellular concentration stimulates enzymatic activity, and release of prostaglandins and nitric oxide follows. Not only do these mediators increase spinal cord neurons excitability, but also cause an augmented neurotransmitters production and diffusion from the nociceptor pre-synaptic terminal. Lamina II interneurons degenerate some time after nerve injury, and so their inhibitory influence over synaptic transmission is lost. Increased levels of NGF in spared neurons of the injured nerve causes activation of trkA receptors on mast cells, making them proliferate and desgranulate. Their release of inflammatory mediators, together with those produced by macrophages who invaded the dying nerve, is proven to be harmful for nearby neurons, ultimately resulting in behavioral signs of neuropathic pain. BDNF release, stimulated by NGF, has the same effect.

Figure 2: Mechanisms of microglial activation by neurons, and resultant astrocytic activation and enhanced neuronal excitability

Neurons release several substances capable of microglial activation. Fractalkine, cleaved by MMP-9 or Catepsin S, activates CX3CR1 receptors. Likewise, MCP-1 activates CCR2 receptor and ATP may act through $P2X_4R$. $P2X_4R$ receptors are upregulated either by fibronectin influence (with TLR4 mediation) or by Lyn, a Src-family kinase. Both upregulation pathways involve ERK activity. CX3CR1, CCR2 and $P2X_4R$ signaling converge to p38 activity, which concurrently induces NFκB transcription regulation, and ultimately lead to release of inflammatory cytokines. These cause astrocytic activation, from which GLT-1 activity is attenuated and production of IL-1, IL-6, TNFα, PGE_2 and NO also follows. Inflammatory substances in the synaptic space will upregulate NMDA and AMPA glutamate channels (and downregulate GABA), and activation of these by the excess of glutamate present in the synaptic space will result in enhanced neuronal excitability, causing increased noxious and non-noxious stimuli transmission – neuropathic pain.