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Toll-Like Receptors in Chronic Pain

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Abstract

Proinflammatory central immune signaling contributes significantly to the initiation and maintenance of heightened pain states. Recent discoveries have implicated the innate immune system, pattern recognition Toll-like receptors in triggering these proinflammatory central immune signaling events. These exciting developments have been complemented by the discovery of neuronal expression of Toll-like receptors, suggesting pain pathways can be activated directly by the detection of pathogen associated molecular patterns or danger associated molecular patterns. This review will examine the evidence to date implicating Toll-like receptors and their associated signaling components in heightened pain states. In addition, insights into the impact Toll-like receptors have on priming central immune signaling systems for heightened pain states will be discussed. The influence possible sex differences in Toll-like receptor signaling have for female pain and the recognition of small molecule xenobiotics by Toll-like receptors will also be reviewed.

Keywords

Chronic pain; nociception; glia; Toll-like receptors; proinflammatory; sex; xenobiotics

Introduction

Chronic pain is an extremely complex disease process affecting approximately 1.5 billion people worldwide, placing large economic and health burdens on the global community (Jacobs, 2005). Until recently, only neuronal mechanisms were recognized to contribute to pathological pain. However, our understanding has since developed, and aberrant signaling and activity of non-neuronal immune cells such as glia and peripheral immune cell trafficking through the central nervous system (CNS), are now recognized to play a

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substantial role in initiating and maintaining the chronic pain process (Grace et al., 2011 “in press”; Haydon, 2001; Milligan and Watkins, 2009).

Communication between neuronal and non neuronal immunocompetent cells is critical to the maintenance of homeostasis within both the peripheral and central nervous systems, but under certain conditions this bi-directional communication can contribute to numerous neuropathologies (Ren and Dubner, 2010). Within the CNS this interaction between neurons and immunocompetent cells can be referred to as “central immune signaling” (Hutchinson et al., 2011). Critical to the central immune signaling process are specialized immune receptors, called Toll-like receptors (TLRs) (Austin and Moalem-Taylor, 2010; Ren and Dubner, 2010). TLRs are pattern recognition receptors which play an important innate immune role in sensing the presence of damage or danger originating both endogenously and exogenously, and translating this into a central immune signal that can be interpreted, and responded to, by neurons and other immunocompetent cells within the CNS.

TLR-induced central immune signaling is now appreciated to contribute to a myriad of CNS pathologies (e.g., CNS consequences of sepsis (Hoshino et al., 1999), Alzheimer's disease (Akiyama et al., 2000; Minoretti et al., 2006; Tan et al., 2008 “in press”) and ischemic stroke (Abate et al., 2010; Caso et al., 2007; Tasaki et al., 1997)), with chronic pain being of specific interest here. Chronic pain associated TLR activation contributes to the pain pathology via the dysregulation of several cellular functions that are critical to the maintenance of homeostasis as well as via the release of numerous proinflammatory mediators, that all sum to enhance the neuroexcitatory tone (Austin and Moalem-Taylor, 2010; Ren and Dubner, 2010). Currently in the literature, excellent reviews and original data articles on pain that have included discussions of TLR involvement in nociception have each provided their own specific focus and expertise (Austin and Moalem-Taylor, 2010; Bokhari et al., 2009; Buchanan et al., 2010; Carty and Bowie, 2011; Combrinck et al., 2002; DeLeo et al., 2004; Frank et al., 2007; Guo and Schluesener, 2007; Inoue and Tsuda, 2009; Kim et al., 2009; Kohli et al., 2010; Loram et al., 2010; Marriott and Wilkin, 1993; Milligan and Watkins, 2009; Perry, 2004; Qi et al., 2011; Tasaki, et al., 1997; Thorlin et al., 1998). This review will provide a distinct perspective by specifically focusing on the evolving role of TLRs in the induction and maintenance of chronic pain through the initiation of central immune signaling, and the implications that TLR-induced central immune signaling may have for pain processing.

Recent evidence has also identified sex differences in TLR-induced proinflammation, adding an additional layer of complexity and insight into central immune signaling (Calippe et al., 2010; Calippe et al., 2008). This newly identified interaction between TLRs and sex steroids may provide a tantalizing contribution to sex differences seen in pain sensitivity. It has additionally become evident that TLRs are able to detect small molecule xenobiotics; that is, chemicals that are found in an organism but which are not normally produced or expected to be present in it. In this context, xenobiotics include compounds such as opioids and other tricyclic compounds. The impact of drugs such as these on TLR-induced central immune signaling results in a modification of drug response and constrains their efficacy, as in the case of opioids, and may perhaps even contribute to their therapeutic indication, as in the case of amitriptyline for example (Hutchinson et al., 2010b; Hutchinson et al., 2009b; Hutchinson et al., 2008c; Snyder, 2004; Watkins et al., 2005). Accordingly, a review of the preclinical research on the role of TLRs in sex- and xenobiotic-induced central immune signaling and their corresponding role in the production and maintenance of chronic pain is included. It is important to recognize that this burgeoning area of TLR-pain research is just as fraught with experimental design and data interpretation flaws as any other field. Hence, comments on experimental issues commonly encountered in this field will also be included. An appreciation of the topics outlined here and discussed in detail below will provide for a

more complete understanding and appreciation of chronic pain mechanisms, which may in turn hopefully translate to improved analgesics and pave the way to improve pain relief.

Who contributes to central immune signaling?

Glia are the main non-neuronal cells in the CNS, outnumbering neurons by ten to one and form greater than 70 percent of the total brain and spinal cord cells (Milligan and Watkins, 2009). Traditionally, glia have been seen as merely structural support for neuronal cells (Haydon, 2001). However, they have now been acknowledged as key mediators of the CNS innate immune response, playing major roles in immune surveillance and the clearance of cellular debris (Faulkner et al., 2004; Milligan and Watkins, 2009). Importantly, glia are recognized as important modulators of pain (Ben Achour and Pascual, 2010; Milligan and Watkins, 2009) and research now indicates a major role for glia in sex differences in both pain sensitivity (Loram, et al., 2010) as well as the analgesic efficacy of opioids (Hutchinson, et al., 2011). Consequently, in order to more fully understand the mechanisms of chronic pain, and develop new and improved therapies especially for the female chronic pain population, this novel immune aspect of pathological pain must be considered.

The most abundant glial cell type involved in central immune signaling are astrocytes (Romero-Sandoval et al., 2008). Astrocytes are derived from neuronal stem cells (Mujtaba et al., 1998) and play numerous functional and structural roles. These roles include formation of the blood brain barrier, regulation of cerebral blood flow (Araque et al., 1999) and their participation in the tripartite synapse, where their complete encapsulation of neuronal synapses allows for neuronal communication (Araque, et al., 1999). Consequently, astrocytes are ideally positioned within the CNS to enable them to play a major role in pathological pain.

Astrocyte activation plays a key role in central immune signaling by specifically contributing to the release of proinflammatory factors involved in the induction and maintenance of chronic pain and by altering glutamate transporter function (Beattie et al., 2002; Milligan and Watkins, 2009; Petrenko et al., 2003; Viviani et al., 2003). Upon astrocyte activation, the extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) pathways become activated, resulting in the release of numerous proinflammatory molecules such as interleukin 1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) (Ben Achour and Pascual, 2010; Milligan and Watkins, 2009; Sung et al., 2003; Tawfik et al., 2006). Moreover, following nerve injury, chronic astrocytic activation results in downregulation of the excitatory amino acid transporters glutamate transporter 1 (GLT1) and the glutamate–aspartate transporter (GLAST), ultimately resulting in decreased glutamate uptake and increased excitatory transmission (Sung, et al., 2003; Tawfik, et al., 2006). Consequently, astrocytic activation plays a major role in pain facilitation and is a fundamental contributor to the chronic pain process.

Microglial cells are derivatives of primitive myeloid precursors (Ginhoux et al., 2010) and comprise approximately 15% of the total cells within the CNS (Graeber and Streit, 2010). Like astrocytes, microglia are now known to form an additional, integral part of the tripartite synapse so to now form a tetrapartite synapse and contribute to the plastic changes which occur post nerve injury known as central sensitization (DeLeo et al., 2006; Milligan and Watkins, 2009). Proinflammatory mediators released from microglial cells under pathological conditions are responsible for activation of other nearby glial cells as well as neighboring neurons, potentiating the proinflammatory response (Ben Achour and Pascual, 2010; Milligan et al., 2005; Milligan and Watkins, 2009; Milligan et al., 2004; Sung, et al., 2003; Tawfik, et al., 2006).

It has further been demonstrated that microglial cells can in fact change phenotype according to their microenvironment. Interestingly, once activated following peripheral nerve injury for example, microglial cells may not return to their original inactivated state. Rather, it has been demonstrated that once activated, microglial cells can remain in a "primed" or "sensitized" state (Perry et al., 1985). Glial priming however, has not only been demonstrated after trauma, but also as a consequence of exposure to environmental stressors (Frank et al., 2011). Consequently, in the event of further tissue injury or damage, an exaggerated pro-inflammatory glial response substantially contributes to neuronal hyperexcitation. These malleable changes within the CNS involving glial cells are now known to majorly contribute to pathological pain.

Toll-like receptors: origins and the basics

The original link between Toll-like receptors and pain was made by the illness response field, a research area which arose before substances such as gram-negative bacterial lipopolysaccharide (LPS) were even discovered to be TLR ligands. Here, systemic live infection or pseudo-infection using LPS resulted in heightened pain states of allodynia or hyperalgesia and was associated with the presentation of the illness/sickness response (reviewed by Watkins et al. (2000)). However, in these cases it is likely that the enhanced nociception resulted from engagement of peripheral immune TLRs that translated into proinflammatory central immune signaling, rather than direct CNS TLR activation by LPS itself.

The Toll pathway was first discovered in Drosophila melanogaster by Christiane Nüsslein-Volhard and Eric F. Wieschaus, which won them the Nobel Prize in medicine in 1995 (Valanne et al., 2011). It soon became evident that the Toll signaling pathways in Drosophila and the immune system were in fact linked (Valanne, et al., 2011) and in 1995 it was first shown that Toll (Toll-1) was an activator of the immune response in a Drosophila cell line (Rosetto et al., 1995).

The first mammalian Toll-like receptor (TLR) was discovered in 1997 (Medzhitov et al., 1997) and currently, 13 TLRs have been identified (Bowie and O'Neill, 2000). TLRs are transmembrane pattern-recognition receptors, which respond to diverse invading pathogens and utilize receptor dimerization in order to have specific agonist identification (Okun et al., 2011). For example, the exogenous ligand that TLR4 predominately recognizes is a component of gram-negative bacteria, namely, endotoxin (Lipopolysaccharide, LPS) (Tsan and Gao, 2004), while TLR2 recognizes lipopeptides from gram-positive bacteria (Kawai and Akira, 2007). Furthermore, TLRs are ideally positioned where they can become activated by their various ligands. TLRs are located both intracellularly and extraceullarly, with TLRs 3, 7, 8 and 9 specifically localized in intracellular membranes (e.g. endosomal and endolysosomal compartments), where they are activated by nucleic acids of bacterial and viral origin (Okun, et al., 2011).

Upon TLR ligand binding, receptor dimerization and conformational changes occur. These molecular changes are essential in order to attract adaptor molecules to TLRs via their Toll Interleukin-1 receptor (TIR) domains. Such adaptor molecules include MyD88, MAL (MyD88 adaptor-like), TIRAP (TIR-domain-containing adaptor protein), TRIF (Toll-receptor-associated activator of interferon), TRAM (TRIF-related adaptor molecule) and SARM (sterile ∝ and Armadillo motifs). Furthermore in order for certain TLRs to produce a signal upon ligand recognition, many utilize certain adaptor proteins and co-receptors (Buchanan, et al., 2010). For instance, TLR4 classically utilizes myeloid differentiation factor 2 (MD-2), forming the TLR4-MD-2 complex, as well as the co-receptor CD14 (Wright et al., 1990), allowing for appropriate signaling to occur upon LPS binding (Nagai

et al., 2002; Shimazu et al., 1999). However, it is now apparent that, dependent on cell type, different combinations of TLR4, TLR4 "surrogates" and associated co-receptors can interplay to elicit proinflammatory signaling events (Acosta and Davies, 2008) (see section below "Toll-like receptors and pain").

Following TLR binding, the signaling pathways activated by these receptors include the MyD88-dependent and independent (the TRIF-dependent) pathways (Kaisho and Akira, 2006; Kawai and Akira, 2007; Okun, et al., 2011). The MyD88 pathway is utilized by all TLRs except TLR3 and results in the activation of nuclear factor kappaB (NF-κB) and activating protein-1 (AP-1). (For an in-depth analysis of TLR signaling cascades, please see the following reviews: (Buchanan, et al., 2010; Kawai and Akira, 2007; Okun, et al., 2011)). As a consequence of NF-κB and AP-1 activation, numerous proinflammatory cytokines are produced such as IL-6, IL-1β and TNF-α (Kaisho and Akira, 2006; Kawai and Akira, 2007; Okun, et al., 2011). Importantly, such TLR-induced proinflammatory cytokines are well established to contribute to initiating and maintaining chronic pain states (Milligan and Watkins, 2009).

Importantly, endogenous signals are also capable of activating these pattern recognition receptors, which is hypothesized to be the source of TLR activation under neuropathic pain conditions (Hutchinson, et al., 2010b; Hutchinson, et al., 2008c; Kim et al., 2007; Tanga et al., 2005). Endogenous danger signals capable of TLR activation are referred to as damage- (or danger-) associated molecular patterns (DAMPs) or 'alarmins' and are released from activated, stressed, or necrotic cells and extracellular matrix molecules upon injury or tissue damage (Kono and Rock, 2008; Piccinini and Midwood, 2010 "in press"). DAMPs that have been demonstrated to induce activation of TLRs include fibronectin, β-defensins, high mobility group box-1 (HMGB1), and heat shock proteins (Asea et al., 2002; Costigan et al., 1998; Ohashi et al., 2000; Piccinini and Midwood, 2010 "in press"; Vabulas et al., 2002a). The activation of TLR receptors via such DAMPs has been demonstrated to lead to further activation of numerous cell types, such as glial cells, which have a well-established role in pathological pain (Piccinini and Midwood, 2010 "in press"; Scholz and Woolf, 2007). The generation of DAMPs associated with pain has been hypothesized to originate from the long term Wallerian degeneration of nociceptive fibres, that can last for years after the original trigger (Kim, et al., 2009; Vargas and Barres, 2007). This finding that TLRs are capable of activation via endogenous ligands is extremely exciting as it demonstrates that TLRs are not solely responsible for pathogen recognition, but highlights that TLRs are involved in pathophysiological roles other than infection (Okun, et al., 2011).

Furthermore, a new line of evidence is accruing that TLRs, found on both microglia and astorocytes, are not only involved in deleterious events, but can also be beneficial in neuronal repair and resolution of inflammation (Milligan and Watkins, 2009; Rivest, 2009). As discussed previously, microglia and astrocytes respond to pathogens, cellular debris and proinflammatory cytokines following injury. However, it is now recognized that the phenotype of macrophages is dynamic ranging from the classical activation resulting in pro-inflammatory mediators, mostly activated by activated by TLR signaling as described above. Macrophages also now have the ability to display an alternatively activated anti-inflammatory state when stimulated by IL-4, IL-13 and IL-10 (Colton et al., 2006; Gordon, 2003; Ponomarev et al., 2007). While most of the information on phenotypic shift of macrophages has been obtained from peripheral macrophages, microglia are showing similar phenotypic changes depending on the microenvironment (Colton, 2009). The alternatively activated state is anti-inflammatory and may contribute to the resolution of inflammation and provide neuroprotection and potentially remyelination (Rivest, 2009).

There is some indication that TLR signaling still plays a role in M2-type microglia where TLR signalling still persists but an anti-inflammatory cytokine profile is expressed instead of the classically pro-inflammatory cytokine expression. Microglial TLR4 signalling specifically, has been demonstrated to clear myelin debris following neuronal injury, resulting in the recruitment of oligodendrocyte progenitor cells, remyelination and neuroprotection (Glezer et al., 2006). TLR4 however is not the only TLR responsible for neuroprotection. It has also been demonstrated that astrocytic TLR3 activation can induce the expression of certain mediators and molecules such as IL-9, IL-10 and IL-11 which promote cellular development, differentiation and migration (Bsibsi et al., 2006). Moreover, microglial TLR9 activation has also been shown to be neuroprotective (Stevens et al., 2008). Despite TLRs playing a role in neuroprotection, their detrimental roles are well documented, with chronic pain being at the forefront. The understanding of alternatively activated phenotypes of glia is still in its infancy. The impact of alternatively activated states on pain remains to be explored.

Central immune signaling and pain: the basics

Early in the 1990's pain researchers discovered that glial cells were important mediators in the pathological pain process. Utilization of peripheral neuropathy models demonstrated increased glial activation markers. For instance, glial fibrillary acidic protein (GFAP) expression, a marker for astrocytic activation, is increased in the grey matter of the spinal cord in the chronic constriction injury model of neuropathic pain (Garrison et al., 1991). Further investigation showed this increased astrocytic activation could be modified, with the specific N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 blocking astrocytic activation (Garrison et al., 1994). Subsequently, it was demonstrated that both microglia and astrocytes were upregulated in numerous models of neuropathic pain such as during subcutaneous inflammation (Fu et al., 1999; Sweitzer et al., 1999) and spinal cord trauma (Popovich et al., 1997).

Subsequent research has consolidated the role for proinflammatory central immune signaling in heightened pain states. It has been demonstrated that upon tissue injury, numerous mediators are released which activate and perhaps also sensitize immunocompetent cells within the CNS, such as glia. Once activated by local damage, the CNS immunocompetent cells stimulate nearby neurons and other receptors and are responsible for the release of proinflammatory mediators, contributing to pathological pain (Ben Achour and Pascual, 2010; Scholz and Woolf, 2007). In addition, early observations found that numerous neurotransmitters and neuromodulators were responsible for the activation of neuron-to-glia signaling. Such neurotransmitters and neuromodulators include Substance P (Marriott and Wilkin, 1993; Svensson et al., 2003), excitatory amino acids (Takuma et al., 1996), adenosine triphosphate (ATP) (Nakajima and Kohsaka, 2001) and nitric oxide (Mollace et al., 1998). Furthermore, other neuronally derived glial activators were also demonstrated such as CX3CL1 also known as fractalkine (Chapman et al., 2000; Clark et al., 2009; Maciejewski-Lenoir et al., 1999; Zhuang et al., 2007). The precise role of neuronal immune signaling remains to be fully elucidated. However, it is evident that neurons can either indirectly or directly activate nearby glia. The neuronal chemokine CX3CL1 for example is one such neuron-to-glia signal. CX3CL1 binds to the CX3CR1 receptor, producing the proinflammatory cytokines IL-1ß and IL-6, both known to play a role in pathological pain (Abbadie et al., 2009; Clark, et al., 2009; Zhuang, et al., 2007). Furthermore, other neuronal mediators such as ATP have also been demonstrated to activate glial cells, binding to purinergic ionotropic receptors (e.g., P2X4 and P2X7) on microglia, resulting in the release of proinflammatory cytokines such as TNF-α (Suzuki et al., 2004) as well as further microglial ATP and brain-derived neurotrophic factor (BDNF) release, again known to increase the excitability of spinal lamina neurons (Coull et al., 2005). Importantly,

it became evident that glial activation was not merely just correlated with pain, but that it was critically involved in inducing pathological pain. This was firmly demonstrated with glial modulating agents, which were found to decrease elevated pain states (Hashizume et al., 2000) (Meller et al., 1994; Sweitzer et al., 2001).

The proinflammatory cytokines released as part of the pain-central immune signaling response play a substantial role in pathological pain. Numerous cytokines released upon astrocytic/microglial activation for example increase neuronal excitability and synaptic strength by specifically increasing the conductivity of alpha amino 3-hydroxy-5 methyl-4-isoxazole-propionic acid (AMPA) and NMDA receptors and by increasing the number of these receptors on the surface of neurons (Beattie, et al., 2002; Kawasaki et al., 2008; Ozaktay et al., 2006; Stellwagen and Malenka, 2006). Moreover, this increased neuronal excitability, the hallmark of neuropathic pain, is further brought about by proinflammatory cytokine effects on potassium channels, where their release is known to increase sodium and decrease potassium channel activity (Manning, 2004). Furthermore, proinflammatory central immune signaling has also been shown to directly affect the inhibitory neurotransmitter GABA (γ-aminobutyric acid), by inverting the polarity of currents activated by GABA receptors in spinal lamina neurons (Coull, et al., 2005). Consequently, proinflammatory cytokines are known to increase excitatory synaptic transmission and/or decrease inhibitory synaptic transmission, contributing to the central sensitization and pain sensitization (For a more detailed discussion of pain sensitization: (Hulsebosch et al., 2009; Kawasaki, et al., 2008; Latremoliere and Woolf, 2009; Meeus and Nijs, 2007; Petrenko, et al., 2003; Woolf and Salter, 2000)).

In addition to the above nociceptive regulators and signals, TLRs are now known to contribute to CNS immunocompetent cell activation and the resulting proinflammatory cascade producing pathological pain (Buchanan, et al., 2010; Scholz and Woolf, 2007). TLRs are widely distributed throughout the human and non-human CNS and form the essential link between the innate immune system and the CNS. TLRs are expressed in a variety of immune cell types including B cells (Gerondakis et al., 2007), mast cells (Iwamura and Nakayama, 2008), natural killer cells (Eriksson et al., 2006), macrophages, dendritic cells (Kaisho and Akira, 2006) and importantly glia, including microglia (Olson and Miller, 2004) (Fig. 2), astrocytes (Bowman et al., 2003) (Fig. 1) and oligodendrocytes (Aravalli et al., 2007). Furthermore, recent studies now indicate TLR expression on a variety of cells not classically thought of as immunocompetent including endothelial cells (Wittebole et al., 2010 “in press”) as well as neurons (Ochoa-Cortes et al., 2010) (Fig. 3). The abundance of TLRs in pain responsive regions makes them a critical potential component of pain signaling.

TLR activation in these specific cell locations has been demonstrated to release numerous proinflammatory mediators known to play a role in pathological pain including but not limited to monocyte chemotactic protein-1 (MCP-1), reactive oxygen species (ROS), nitric oxide (NO), interleukin-6 (IL-6), interleukin-1alpha (IL-1α), tumor necrosis factor-alpha (TNF-α), IL-5, IL-13, interferon-beta (IFN-β), CXCL10, CCL5, inducible nitric oxide synthase (iNOS), prostaglandin E_2 (PGE_2) and calcitonin gene related peptide (CGRP) (Aravalli et al., 2008; Bowman, et al., 2003; Bsibsi et al., 2007; Bsibsi, et al., 2006; Butchi et al., 2010; Carpentier et al., 2005; Diogenes et al., 2011; El-Hage et al., 2011; Hou and Wang, 2001; Jack et al., 2005; Kakimura et al., 2002; Krasowska-Zoladek et al., 2007; Li et al., 2004; Mayer et al., 2011; Ochoa-Cortes, et al., 2010; Qi, et al., 2011; Suzuki, et al., 2004; Town et al., 2006; Tsuda et al., 2003; Wadachi and Hargreaves, 2006; Wang et al., 2000).

Toll-like receptors and pain

It was not until recent studies from the DeLeo group (DeLeo, et al., 2004; Raghavendra et al., 2003; Raghavendra et al., 2004; Tanga, et al., 2005; Tanga et al., 2004) that up regulation of TLR detection systems and requirement of, in this case TLR4, was shown in preclinical models for the initiation of pathological pain. Raghavendra et al. (2003) first demonstrated, following L5 nerve transection, that TLR4 mRNA increased significantly in a minocycline sensitive fashion, regardless of whether minocycline was administered preemptively or as an intervention. This finding led Raghavendra et al. (2004) to establish that TLR4 transcriptional up regulation also occurred after an inflammatory preclinical pain model, in this case intraplantar Complete Freunds adjuvant (CFA) induced peripheral inflammation. Interestingly, these TLR4 transcriptional events occurred rapidly, within 4 hours, in disparate CNS regions including the lumbar spinal cord, brain stem and forebrain and remained elevated for more than 14 days, when CFA induced allodynia resolves. Similarly, DeLeo et al. (2004) established that spinal lumbar TLR4 and CD14 mRNA expression was up regulated within 4 hours of L5 nerve transection, with CD14 mRNA expression peaking at 4 days and TLR4 continuing to increase until day 14 after injury, correlating with the time course of allodynia.

These association studies were then capitalized upon by Tanga et al. (2005), who established for the first time a causal role for TLR4 in initiating a preclinical model of neuropathic pain. Using two mouse lines genetically deficient in functional TLR4, and with intrathecal administration of TLR4 antisense oligodeoxynucleotide in L5 nerve transected rats, they found significantly attenuated behavioral hypersensitivity and decreased expression of spinal microglial markers and proinflammatory cytokines. Moreover, DeLeo then further demonstrated that the LPS-TLR4 signaling pathway accessory molecule CD14 majorly contributes to TLR4-dependent nerve injury induced neuropathic pain (Cao et al., 2009). Here it was found that CD14 knockout mice displayed significantly decreased behavioral sensitivity at day 1 post L5 spinal nerve transection (Cao, et al., 2009).

These foundational studies led on to others documenting that the pharmacological blockade of TLR4 is also able to prevent (Bettoni et al., 2008) and rapidly reverse preclinical models of neuropathic pain (Hutchinson et al., 2007; Hutchinson, et al., 2010b; Hutchinson, et al., 2008c). Hutchinson et al. (2007; 2008b) and Bettoni et al. (2008) specifically demonstrated this, each using novel small molecule TLR4 antagonists: (-)- and (+)- naloxone, and (-)- and (+)- naltrexone (Hutchinson, et al., 2007; Hutchinson, et al., 2008c); and FP-1 (Bettoni, et al., 2008). Importantly, the non-stereoselective action of naloxone and naltrexone observed by Hutchinson et al. (2007; 2008c), contrast these compound's well established neuronal opioid receptor antagonist properties which result only from the (-)- isomer. The action of these novel TLR4 inhibitors was validated by Hutchinson et al. (2008c), who also observed reversal of allodynia by intrathecal administration of competitive TLR4 antagonists (mutant forms of LPS).

Interestingly, the range of small molecule ligands that Hutchinson et al. (2007; 2010a; 2010b) found to modify TLR4 signaling is extensive, suggesting at minimum TLR4 may be capable of recognizing and being manipulated by a plethora of small molecule xenobiotics. Importantly, they demonstrated that some tricyclic compounds that are commonly used for clinical neuropathic pain treatment, such as amitriptyline, possess significant TLR4 inhibitory activity (Hutchinson, et al., 2010b). Therefore, these data demonstrating xenobiotic activation of TLR receptors indicate that TLR4 engagement under pathological conditions contribute to central immune signaling, rather than such receptors playing a passive role being permissively required for proinflammatory events to ensue. Moreover, it

also suggests at least some existing utilized pharmacotherapies have beneficial TLR4 activity that may contribute to their therapeutic indication.

This work has been further replicated by laboratories world wide, demonstrating the key role of TLR4 in several preclinical models of neuropathic pain (Lan et al., 2010; Wu et al., 2010). Lan et al. (2010) showed that TLR4 small interfering RNA (siRNA) administered intrathecally in rats prior to the development of allodynia induced with a bone cancer pain model displayed significantly attenuated behavioral hypersensitivity and decreased expression of spinal microglial markers and proinflammatory cytokines. Similarly, Wu et al. (2010a) demonstrated blockade of chronic constriction injury-induced allodynia with a similar TLR4 siRNA preventative dosing regimen.

The evidence for CNS TLR involvement in preclinical pain models has now also extended to the extracellular TLR2 (Kim, et al., 2007; Shi et al., 2011) and intracellular TLR3 (Obata et al., 2008). TLR2 activation in both astrocytes and microglia and TLR3 activation in microglia have also been shown to mediate neuropathic pain (Fig. 1 & 2). Kim and colleagues (Kim, et al., 2007) specifically demonstrated that damaged sensory neurons activate glial cells via TLR2 and the resulting pain hypersensitivity is a direct consequence of TLR2 activation. Furthermore, TLR2 knockout animals have been shown to suppress the development and maintenance of nerve injury-induced allodynia (Kim, et al., 2007). Similarly, Obata (Obata, et al., 2008) established that TLR3 is essential in the activation of spinal cord glia in addition to the resulting tactile allodynia following peripheral nerve injury, albeit via intracellular signaling events that are as yet undefined.

The cross validation of the role of TLRs in chronic pain has occurred with the establishment of the requirement of TLR accessory proteins in the establishment of allodynia. As noted previously, DeLeo et al. (2004) demonstrated that spinal lumbar CD14 mRNA was elevated following nerve injury. Cao et al. (2009) established the causal role CD14 played by demonstrating that genetic ablation of CD14, an accessory molecule for both TLR2 (Yoshimura et al., 1999) and LPS-TLR4 signaling (Wright, et al., 1990), significantly reduced behavioral sensitivity, decreasing both mechanical allodynia and thermal hyperalgesia induced in response to nerve injury. Given the expansion of the TLRs now shown to be involved in pain sensitization, discussed above, the CD14 dependency of each of these other TLR signaling pathways is an intriguing question yet to be tested.

The examination of TLR involvement in chronic pain has taken a further step forward with the discovery of nociceptive fiber expression of TLRs. Wadachi et al. (2006) first conducted an immunohistochemical analysis of trigeminal neurons (human and rat) and demonstrated that a transient receptor potential cation channel, subfamily V, member 1 (TRPV1) (capsaicin receptor) positive population of cells expressed both TLR4 and CD14. They extended this finding to discover the co-localization of TLR4 and CD14 with N52, a marker of peripheral sensory neurons, in human dental pulp collected from patients with tooth decay (Wadachi and Hargreaves, 2006). The extraordinary implications of these data are that nociceptive fibers can possibly detect the presence of pathogen associated molecular patterns (PAMPs) and DAMPs independent of classic immunocompetent cells, albeit via intracellular signaling events that are as yet undefined.

These results have been extended by Diogenes et al. (2011) and Ferraz et al. (2011). Diogenes et al. (2011) found that LPS competitive binding to receptors in trigeminal neurons, is sufficient to elicit intracellular calcium accumulation and inward currents, and sensitizes TRPV1 to its ligand (capsaicin-induced release of calcitonin gene-related peptide, and inward currents. Similarly, Ferraz et al. (2011) found that LPS from a classic oral pathogen *Porphyromonas gingivalis* was able to increase the TRPV1-dependent release of

calcitonin gene related peptide and that human tooth pulp tissue showed the co-localization of TLR4 with calcitonin gene-related peptide containing nerve fibers.

This discovery of innate immune competency of the neuronal system has spurred additional findings that have established that human and murine dorsal root ganglion (DRG) neurons express TLR3, TLR7 and TLR9 (Qi, et al., 2011) (Fig. 3). Furthermore, recent studies indicate that mouse colonic nociceptive DRG neurons also express TLRs including TLR1, 2, 3, 4, 5, 6 and 9 (Ochoa-Cortes, et al., 2010) as well as TLR4 expression in mouse DRG sensory neurons (Acosta and Davies, 2008) (Fig. 3). Importantly, challenge of murine DRG neurons with TLR ligands induced expression and production of proinflammatory chemokines CCL5, CXCL10, and cytokines interleukin 1 alpha (IL-1α), IL-1β, and inflammatory mediator prostaglandin E_2, all of which have previously been shown to augment pain (Milligan and Watkins, 2009) (Fig. 3). Interestingly, as observed in trigeminal tissues, this TLR3, TLR7 and TLR9 stimulation also appears to produce pro-nociceptive feed forward effects (Diogenes, et al., 2011; Ferraz, et al., 2011) with up regulation of the expression of TRPV1, and enhanced calcium flux by TRPV1-expressing DRG neurons (Qi, et al., 2011). In contrast, TLR7 was expressed in C-fiber primary sensory neurons and was important for inducing itch (pruritus), but was not necessary for eliciting mechanical, thermal, inflammatory and neuropathic pain in mice (Liu et al., 2010). However, these neuronal TLR discoveries cannot be immediately drawn together with the established wealth of knowledge of “classical” TLR data. Murine DRG neurons require MD-1 and CD14 for functional TLR4 signaling, but not MD-2, as classical Toll-like receptor innate immune signaling requires (Acosta and Davies, 2008) (Fig. 3). Moreover, under the experimental conditions Acosta et al. (2008) employed, neurons lacked significant expression of MD-2 mRNA. Despite these results however, in mouse colonic nociceptive DRG neurons, MD 1 and MD-2 mRNA expression has been observed (Ochoa-Cortes, et al., 2010). Parallels can be drawn between this clear distinction with the TLR2 dependent microglial response to group b *streptococcus*, which produces polar apoptotic responses in peripheral monocytes (Lehnardt, 2010). Hence, these disparities require further examination as it may enable dissecting of the neuronal innate immune signaling from the classical innate immune responses. As mentioned above, the intracellular signaling events downstream from neuronal TLR activation have yet to be characterized and may be distinct from those observed in classical immunocompetent cells. Moreover, it remains to be established if the same intracellular cascades are engaged in central immunocompetent cells versus the peripheral immune cells that were used to originally establish these cascades.

It is important to recognize that the area of TLR pain research is fraught with experimental issues. Despite the above studies utilizing TLR4 antibodies in order to detect TLR4 expression, our extensive experience suggests these antibodies have both poor specificity and sensitivity. Attempts to use fluorescently tagged LPS to identify and validate TLR4 expression are applauded (Diogenes, et al., 2011), however without appropriate controls fail to demonstrate TLR4 specificity. Moreover, it is also important to highlight that the effects of LPS are not always significantly different in TLR4 knockout animals, compared with wild type animals and that the effects of ultra pure LPS administration does not always replicate that seen with standard grade LPS (Ochoa-Cortes, et al., 2010). Consequently, further studies are needed to validate the use of TLR4 antibodies and caution is required when interpreting current literature utilizing TLR4 antibodies and the effects of standard grade LPS.

Indirect evidence of Toll like receptors in pain

In addition to the growing literature of direct assessment of TLR-induced central immune signaling and pain, there is also a parallel literature of TLR changes associated with pain.

For example, in the transcriptional examination of differences between wild type and cholecystokinin B2 receptor knockout mice, reductions in supraspinal TLR4 were also identified (Koks et al. (2008). Interestingly, these cholecystokinin B2 receptor knockout mice are protected against allodynia induced by chronic constriction injury (Koks, et al., 2008). Transgenic mouse models also provide insightful evidence for the role of TLRs in pain. Using a transgenic line expressing mutated superoxide dismutase 1 (Berger et al., 2011) or transgenic mice expressing human sickle hemoglobin (Kohli, et al., 2010), both transgenic states result in heightened nociception and elevated TLR4 expression, which was observed in the spinal cord. Once again, these data provide associative evidence of TLR4 involvement in these heighted pain states.

Whilst the endogenous DAMPs are hypothesized to mediate the TLR driven central immune signaling that results in heighted pain states following inflammation and nerve injury, exogenous TLR ligands have been administered experimentally directly into the spinal cord to produce proinflammatory cytokine production. However, studies failed to produce rapid (less than 2 hr) alterations in nociception following direct central administration of TLR4 ligands (Hutchinson, et al., 2009b; Meller, et al., 1994) and in some cases hyperalgesia was not induced. The studies that have succeeded in producing this exogenous TLR ligand-induced responses either required large doses (Cao, et al., 2009; Clark et al., 2010; Kehl et al., 2004; Loram et al., 2011 “in press”; Meller, et al., 1994; Reeve et al., 2000; Saito et al., 2010), chronic indwelling intrathecal injection catheters (Saito, et al., 2010) or repeated administrations (Cahill et al., 2003; Clark et al., 2006).

Intrathecal LPS induces mechanical allodynia by enhancing the activity of dorsal horn neurons, including facilitation of neuronal post-discharge (Reeve, et al., 2000). In addition, intrathecal LPS increases caspase-1 and IL-1 (Clark, et al., 2006), all of which are produced predominantly by glia. In addition, intrathecal LPS-induced allodynia is attenuated by pretreatment of pentoxifylline, minocycline, etanercept (Saito, et al., 2010) and interleukin-1 receptor antagonist (Loram et al, 2011) but not ketorolac (Saito, et al., 2010). These data suggest that TLR4 activation within the spinal cord induces allodynia via an induction of proinflammatory mediators and that the origin is likely to be on immunocompetent cells such as glia.

Perhaps more clinically relevant work pertains to work investigating “priming” or “two hit” dosing regimens where glial cells are sensitized from the initial immune challenge resulting in potentiated responses to a subsequent TLR4 challenge (Loram, et al., 2011 “in press”) (Cahill, et al., 2003; Clark, et al., 2006; Hains et al., 2010). Induction of accessory proteins, such as heat shock proteins, results in allodynia with lower doses of intrathecal LPS (Hutchinson et al. (2009b). An *in vitro* example of this theoretical accessory protein requirement for TLR4 signaling can also be drawn from Hutchinson et al. (2010b), where a significant increase in potency in TLR4 dependent activation of IP3/Akt signaling in RAW264.7 cells (a macrophage cell line) of more than 500 fold was observed, by using imaging media that had been “conditioned” by 24 hours exposure to RAW264.7 cells, rather than using fresh “naïve” imaging media (Evans and Falke, 2007; Hutchinson et al., 2010c). The identity of the factors released into the “conditioned” media during the 24 hours of conditioning remain to be determined, but given the results of Cao et al. (2009), CD14 and other TLR accessory proteins are likely candidates. Therefore, it is possible that in chronic pain, the accessory proteins required for optimal TLR signaling are upregulated and thus sensitizing the TLR pathway. Disparities have also been reported between the hypernociception induced by peripherally and centrally administered LPS. The Kehl et al. (2004) assessment of LPS-induced decreases in grip strength were found to tolerate following systemic LPS administration, but these responses did not diminish following

intrathecal administration. The role of chronic indwelling catheter implantation as a priming event in such models is also worthy of future examination.

Sensitization of TLR signaling

Normal nociceptive pain is necessary and important for survival. However, acute protective pain transitions, in some cases, to a pathological chronic pain state. The underlying mechanism for the transition from acute to chronic pain is now beginning to be explored. While microglia can exhibit many activation states, the sensitized or "primed" state is of particular interest and has been shown to contribute to other potentiated neuroinflammatory diseases. Much of what is known about microglial priming is based on prion disease, where persistent neuroinflammation is due to primed microglia (Perry, 2004). With infection, microglia undergo changes consistent with activation (Kato et al., 1995). Despite the anatomical appearance of microglial activation with persistent infection, levels of proinflammatory cytokines (e.g., IL-1β) in the brains of these rodents do not differ from controls (Walsh et al., 2001). However, in response to peripheral or intracerebral injection of LPS, prion infected rodents show a greatly exaggerated magnitude and duration of brain IL-1β induction (Combrinck, et al., 2002). "Primed" microglial responses are not restricted to prion disease. Potentiated microglial responses occur in rats following peripheral or central trauma/inflammation, tailshock stress, physiologic stress levels of adrenal corticosteroids and opioids, all resulting in potentiated IL 1β responses (Frank, et al., 2007; Hains, et al., 2010). While the understanding of the "primed" microglia phenotype is in its infancy, an upregulation of TLR4 mRNA has been identified, resulting in exaggerated microglial response to new challenges.

The concept of rebound proinflammatory effects of glucocorticoid administration (Frank, et al., 2007; Sorrells et al., 2009; Sorrells and Sapolsky, 2007) has recently been extended to pain, where prior glucocorticoids potentiated mechanical allodynia induced by LPS (Loram, et al., 2011 "in press"). In addition, others have shown that prior stress and prior glucocorticoids potentiated neuropathic pain and epinephrine-induced allodynia (Alexander et al., 2009, Khasar, 2008). We (Frank et al., 2010) and others (Lan, et al., 2010; Tanga, et al., 2004) have shown that spinal TLR4 mRNA is robustly upregulated by nerve injury, bone cancer, opioids, and by both tailshock stress and stress levels of corticosteroid (after a delay beyond the initial anti-inflammatory effects). Also, spinal TLR4 and accessory protein mRNA are potentiated by prior glucocorticoids and stress (Alexander et al., 2009; Loram, et al., 2011 "in press"). Therefore, TLR4 upregulation may be a marker for "primed" or sensitized microglia and may present an exciting avenue of exploration for understanding the transition from acute pain to chronic pain.

The inflammasome: a crucial translator of CNS TLR activation

It is evident that TLRs play a substantial role in the engagement of central immune cells, which are now known to play a role in pathological pain. The production of proinflammatory mediators released via TLR signaling however cannot produce pathological pain without a molecular scaffold complex, termed the inflammasome (Hoffman and Wanderer, 2010). The innate immune system not only includes TLRs but another class of pattern recognition receptors termed NOD-like receptors (NLRs). Like TLRs, NLRs respond to microbial PAMPs as well as endogenous danger signals and include: NODs (nucleotide-binding oligomerization domain-1), NALPs (NACHT-, LRR- and pyrin-domain-containing proteins), IPAF (ICE-protease activating factor), NAIPs (neuronal apoptosis inhibitor factors), and CIITA (class II transactivator) (Ting et al., 2006).

It is currently believed that NLRs are localized in the cytosol and numerous NLRs including NALP1, NALP2 and NALP3 form cytosolic protein complexes called inflammasomes. The

NALP3 inflammasome specifically is activated by ATP and the central effector of the inflammasome is caspase-1 which, when activated, is responsible for cleaving and activating pro-IL-1β and pro-IL-18 (Hoffman and Wanderer, 2010) (Becker and O'Neill, 2007), proinflammatory cytokines centrally involved in neuropathic pain (Ben Achour and Pascual, 2010; Sung, et al., 2003; Tawfik, et al., 2006). ATP is the main endogenous ligand for the purinergic receptor P2X7. Upon activation, a selective potassium channel is opened, leading to a rapid efflux of potassium and plasma membrane depolarization. By a currently unknown mechanism, this activation results in activation of the NALP3 inflammasome and caspase-1.

Considering that pro-IL-1β is released as a consequence of TLR4 activation, the inflammasome is essential in central immune signaling as it allows for the maturation of TLR signaling molecules (i.e. pro-il-1 to mature il-1) (Becker and O'Neill, 2007). Additionally, since the inflammasome is now recognized to be involved in the pathogenesis of numerous diseases such as Cryopyrinopathies (Hoffman et al., 2001), gout (Terkeltaub et al., 2009) and Type 2 Diabetes Mellitus (Maedler et al., 2009) amongst others, this regulatory central immune signaling component is an attractive target for not only investigating the role of TLRs in chronic pain, but is also a biomarker for numerous diseases. Interestingly, whilst not referring to the inflammasome by this recently coined term, research by several groups discussed above have touched upon this by identifying the key role of caspase-1 (Clark, et al., 2006; Kahlenberg et al., 2005), of IL-18 similar to IL-1β (Miyoshi et al., 2008) and of ATP activation of P2X7 receptor (Arulkumaran et al., 2011; Clark, et al., 2010) as important in pain processing. It is tempting to hypothesize that a potential reason for the lack of pain enhancement in studies in response to low doses of intrathecal LPS may be due to the lack of a sufficient second signal that facilitates the activation of the inflammasome. Future studies are warranted to examine such a hypothesis.

A role for TLR4 in enhanced female nociception?

Current research indicates that chronic pain preferentially affects females, with enhanced female nociception in both clinically and experimentally-induced pain (Fillingim et al., 2009). Epidemiological and experimental evidence demonstrates that women are overrepresented in numerous chronic pain conditions and are at greater risk for developing several chronic pain conditions (Fillingim, et al., 2009; Riley et al., 1998). Furthermore, both women and female rodents display heightened sensitivity to noxious stimuli compared with men and male rodents (Fillingim, et al., 2009; Hurley and Adams, 2008; Riley, et al., 1998). Despite the immense amount of experimental and clinical evidence demonstrating this enhanced female pain sensitivity, there is inconsistency within the literature, with some studies finding no sex difference between their male and female healthy volunteers when using thermal challenges as the painful stimulus (Chung, 2004; Riley, et al., 1998). However, the majority of studies using mechanical stimuli reflect heightened pain sensitivity in healthy female volunteers compared to male counterparts (Chesterton et al., 2003; Hurley and Adams, 2008; Sarlani and Greenspan, 2002). Notwithstanding these inconsistencies, the majority of studies do reflect what is seen with clinical pain, in that females report greater pain responses than males.

Numerous mechanisms have been posited to underlie this sex/pain difference. The majority of evidence indicates that the sex steroids play a major role. Literature indicates for example that prepubescent boys and girls display equal prevalence in the majority of chronic pain conditions. However, after the approximate age of 12 years, a female predominance is established up until menopause (characterized by gradually declining estrogen levels), where this prevalence thereafter declines (Abu-Arefeh and Russell, 1994; Bigal et al., 2007; Lipton et al., 2001; Ogura et al., 1985; Sonmez et al., 2001). Furthermore, pain sensitivity

differences throughout the female menstrual cycle (Crawford et al., 2009; LeResche et al., 2003; Pamuk and Cakir, 2005) and the rodent estrous cycle (Frye et al., 1992; Giamberardino et al., 1997; Kayser et al., 1996) also indicate a role for gonadal hormones in chronic pain states, with higher levels of estrogens correlating with higher pain scores. Exogenous estrogen administration, as occurs with oral contraceptives in human studies and estrogen supplementation in female rodents, also further demonstrates a correlation between estradiol and enhanced female nociception (Brynhildsen et al., 1998; Musgrave et al., 2001; Wise et al., 2000). Interestingly, in a study of hormone replacement in transsexuals, a correlation was found between hormone treatment and pain; with approximately one third of the male-to-female subjects developing chronic pain concurrently with combined estrogen and anti-androgen treatment (Aloisi et al., 2007). Despite the growing amount of evidence indicating a role for 17β-estradiol in enhanced female pain, there are inconsistencies within the literature and further investigation is required.

One possible mechanism behind this sex difference seen in pain sensitivity is the strong association between 17β-estradiol and TLR4 (Calippe, et al., 2010; Loram, et al., 2010). As described previously, chronic pain is maintained in part by an upregulation and increased signaling of TLR4, which are found abundantly on microglia and other glial cells. TLR4 activation results in the induction of pro inflammatory cytokines and other pro inflammatory mediators, maintaining the pro inflammatory environment within the spinal cord and neuronal central sensitization.

Both the estrogen receptor alpha and beta (ERα, ERβ), and the recently identified GPR30 membrane receptors are present on most cells within the CNS including microglia and astrocytes (Azcoitia et al., 1999; Baker et al., 2004; Blurton-Jones and Tuszynski, 2001; Tapia-Gonzalez et al., 2008). The downstream signaling of these estrogen receptors may interact with the TLR4 signaling cascade resulting in a changes in NFκB activation. Chronic *in vivo* estrogen treatment in rats potentiates proinflammatory cytokine gene expression in microglia, following an inflammatory challenge, including when challenged by the classic TLR4 agonist LPS (Calippe, et al., 2010; Calippe, et al., 2008). The peripheral immune literature have identified comparable results where chronic, *in vivo*, estrogen, via activation of estrogen receptors, potentiates NFκB activation, resulting in heightened pro inflammatory responses (Calippe, et al., 2010; Rettew et al., 2009; Soucy et al., 2005). Recent data have suggested that ovariectomized female rats supplemented with chronic estrogen have an increased TLR4 responsivity on microglia, which may be partially explained by the action of 17β-estradiol on TLR4 signaling (Loram, et al., 2010). Considering the role TLR4 signaling plays in the initiation and maintenance of chronic pain, this difference in neuroinflammation may partly explain sex differences in pain sensitivity.

Estradiol however, is not the only sex hormone to influence pain responses. Progesterone has been identified to have anti-inflammatory properties in various diseases including multiple sclerosis and spinal cord injury in rodent models (Labombarda et al., 2011 “in press”). In addition, it is found to have anti-inflammatory properties on glia (Garcia-Ovejero et al., 2005; Labombarda, et al., 2011 “in press”; Muller and Kerschbaum, 2006). However, the complexity of gonadal steroids is such that seemingly contradictory results have also been found with both progesterone and 17β-estradiol. 17β-estradiol does possess anti-inflammatory properties when administered *in vitro* and after inflammation or injury (Cuzzocrea et al., 2007; Ghisletti et al., 2005; Vegeto et al., 2003; Vegeto et al., 2001; Vegeto et al., 2000). Thus, whilst more work is required to investigate the underlying mechanisms of sex steroids and pain, there does appear to be a complex interaction between neuroimmune function and the sex steroids.

Sexual dimorphism not only occurs in chronic pain states, but the efficacy of morphine analgesia is also recognized to be greater in males compared to females. The commonalities in mechanism between neuropathic pain and morphine tolerance are becoming increasingly recognized and understood (Hutchinson, et al., 2011). One such common link lies in pain enhancement induced by proinflammatory activation of TLR4 by opioids, counter-acting the analgesic properties of opioids on opioid receptor activation. Thus it is a reasonable hypothesis that estrogens may potentiate TLR4 activity in response to opioid administration, thereby reducing analgesia. Indeed, work by ourselves and others have demonstrated that chronic *in vivo* supplementation of 17β-estradiol in ovariectomized female rats reduces the analgesia induced by (-)- morphine (Craft, 2003) and increased the thermal hyperalgesia induced by (+)- morphine, which has no opioid activity but is a putative TLR4 agonist (Loram, et al., 2010). While reduced efficacy of morphine analgesia does appear to occur in intact females where estrogens are elevated, some studies show little to no change throughout the estrus cycle (Craft et al., 2004).

Other sexual dimorphic differences have been identified that may contribute to the reduced analgesic properties of opioids, including decreased opioid receptor density within the periaquaductal gray of females (Loyd et al., 2008). Despite the evidence that sex differences exist in neuropathic pain and following opioid administration, most chronic pain studies are conducted in male animal models. Evidently, further investigation into altered neuroimmune responses in the female population are warranted.

TLR4 and opioid-induced pain and analgesia

Opioid analgesics are considered one of the first line therapies for neuropathic pain sufferers and are used to treat both acute and chronic pain (Park and Moon, 2010). Despite their continued use, the precise mechanisms of opioid pharmacological action, such as their action on immune signaling within the CNS, has not yet been fully appreciated. Furthermore, there are many complications associated with opioid exposure including but not limited to tolerance and hyperalgesia (Chang et al., 2007; Watkins et al., 2007b). It has been demonstrated, however, that direct opioid-induced receptor activation is not the only mechanism behind such unwanted effects, but that central immune signaling plays a role (Hutchinson, et al., 2011). For example, triple opioid receptor knockout mice still experience opioid-induced hyperalgesia (Juni et al., 2007) (Waxman et al., 2009) and utilization of the opioid-inactive TLR4 agonist (+)- isomer of morphine, or interestingly LPS, still produces tolerance/antianalgesia (Hutchinson et al., 2010a; Johnston and Westbrook, 2005; Wu et al., 2006a; Wu et al., 2006b; Wu et al., 2006c; Wu et al., 2005). Importantly, proinflammatory central immune signaling events have been implicated in some of these behavioral consequences following opioid engagement of non-classical opioid systems.

Nonetheless, immunocompentent cells within the CNS, including but not limited to, microglia (Bokhari, et al., 2009; Chao et al., 1996; Chao et al., 1997; Horvath et al., 2010) and astrocytes (Bunn et al., 1985; Burbassi et al., 2010; Dobrenis et al., 1995; Festa et al., 2002; Hauser et al., 1996; Maderspach et al., 1995; Ruzicka et al., 1996; Thorlin, et al., 1998) express opioid receptors. However, the role of the opioid receptor in initiating central immune signaling is unclear as it has been demonstrated that some TLRs (eg TLR2 and TLR4) are also capable of recognizing opioids. Morphine administration for example, has been demonstrated to specifically activate TLR4 signaling, and the blockade of TLR4, TLR4 accessory molecules, or genetic knockout of TLR4 or its signaling components, have all been shown to potentiate acute morphine analgesia (Hutchinson, et al., 2010b; Hutchinson, et al., 2009b; Hutchinson, et al., 2010c), thereby implicating at minimum TLR4 in the pronociceptive opposition of acute morphine analgesia. Moreover, it has been demonstrated that co administration of (-)- morphine along with the inactive isomer (+)-

naloxone or (+)- naltrexone, both which have been demonstrated to inhibit both (-)-morphine and LPS-induced activation of TLR4 signaling, protects against the development of opioid dependence and hyperalgesia (Hutchinson, et al., 2010c) and reverses chronic constriction-induced allodynia (Hutchinson, et al., 2008c). Consequently, it has become apparent that central immune signaling via specific TLRs is heavily involved in the pharmacodynamics of opioids. As a result, compounds which have been found to block TLR and/or central immune signaling and have been demonstrated to increase analgesic potency such as the general glial attenuators minocycline (Hutchinson et al., 2008a; Hutchinson et al., 2008b) and ibudilast (Hutchinson et al., 2009a), may in the future may serve as new treatments to enhance patient analgesia with the use of opioids (Hutchinson, et al., 2011).

The wider implications of these opioid-TLR discoveries are that perhaps xenobiotics more broadly may act as ligands for TLRs, thereby causing either sensitization or full activation of TLR-dependent central immune signaling events that may have both acute and chronic implications for nociception. Moreover, metabolism of xenobiotics and endogenous compounds (e.g. hormones), previously acknowledged to reduce the primary biological activity and enhance elimination of the compound, may also act to “haptenate” small molecules making them now appear on the innate immune radar. The conversion of morphine to morphine-3-glucuronide is an excellent example of such a process, with clear nociceptive consequences (Lewis et al., 2010). Therefore, characterization of metabolites is also vital to understanding small molecule activation of TLR-dependent central immune signaling.

Conclusion

The revelations made in the past decade implicating TLRs in initiating and maintaining heightened pain states begins to complete the circuit of how central immune signaling is initiated and maintained chronically to contribute long term to neuropathic pain processes. However, the discovery of neuronal TLRs and their detection of DAMPs and PAMPs, bypassing classical immunocompetent cells and central immune signaling response systems, and their potential direct link to modification of nociceptive systems, now requires extensive investigation. Clearly the results from *in vitro* systems in isolation need to be integrated with *in vivo* data to appreciate the complex neuron-glia interplay. Moreover, the nociceptive consequences of non-nociceptive fiber TLR expression and hypothetical DAMP/PAMP detection remain to be investigated if such systems are networked to nociceptive pathways.

Acknowledgments

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Abbreviations

TLRs	Toll-like receptors
ERK	extracellular signal-regulated kinase
JNK	c jun N-terminal kinase
IL	interleukin
TNF-α	tumor necrosis factor-α
PGE_2	prostaglandin E_2
GLT1	glutamate transporter 1
GLAST	glutamate aspartate transporter
LPS	lipopolysaccharide
TIR	Toll Interleukin-1 receptor
MAL	MyD88 adaptor like
TIRAP	TIR domain containing adaptor protein
TRIF	Toll-receptor-associated activator of interferon
TRAM	TRIF related adaptor molecule
SARM	sterile ∝ and Armadillo motifs
MD-2	myeloid differentiation factor 2
NFκB	nuclear factor kappaB
AP 1	activating protein 1

DAMPs	damage associated molecular patterns
HMGB1	high mobility group box-1
GFAP	glial fibrillary acidic protein
NMDA	N-methyl D-aspartate
CX3CL1	fractalkine
BDNF	brain-derived neurotrophic factor
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
siRNA	small interfering RNA
TRPV1	transient receptor potential cation channel subfamily V member 1
PAMPs	pathogen-associated molecular patterns
DRG	dorsal root ganglion
MAPK	mitogen activated protein kinase
NOD	nucleotide binding oligomerization domain 1
NLRs	NOD-like receptors
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
LTA	lipoteichoic acid
PGN	peptidoglycan
MCP-1	monocyte chemotactic protein-1
IFN-β	interferon-beta
TIR	Toll/IL1 receptor
HSPs	heat shock proteins
MMP-9	matrix metallopeptidase 9
MIP-2	macrophage inflammatory protein 2

Highlights

- Proinflammatory central immune signaling contributes to chronic pain.
- Toll-like receptors are now recognized to contribute to the chronic pain process.
- Recent evidence shows neuronal Toll-like receptor expression and activation.
- This review examines Toll-like receptors in chronic pain and xenobiotic recognition.

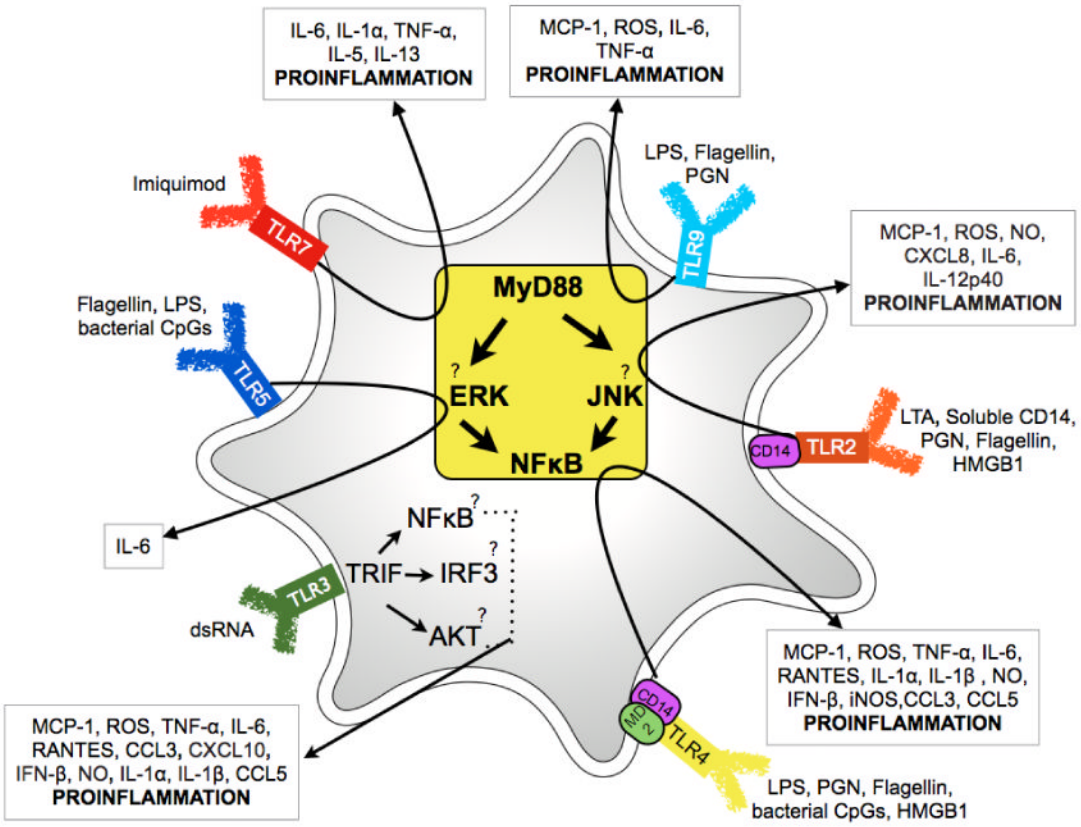

Figure 1. Toll-like receptor activation in astrocytes

Numerous TLRs are expressed and activated in astrocytes including TLRs 2, 3, 4, 5, 7, and 9. Several ligands have been found to activate certain TLRs in astrocytes including, but not limited to: lipoteichoic acid (LTA) (TLR2), soluble CD14 (TLR2), peptidoglycan (PGN) (TLR2, TLR4 TLR9), flagellin (TLR2, TLR4, TLR9), bacterial CpGs (TLR4, TLR5), high mobility group box-1 (HMGB1) (TLR2, TLR4), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharide (LPS) (TLR4, TLR9) and Imiquimod (TLR7). Upon astrocytic TLR activation, numerous mediators are released, resulting in proinflammation. Such mediators include the proinflammatory cytokines: interleukin-6 (IL-6), interleukin-1alpha (IL-1α), tumor necrosis factor-alpha (TNF-α), IL-5, IL-13, IL-12p40, in addition to numerous proinflammatory mediators and chemokines: monocyte chemotactic protein-1 (MCP-1), reactive oxygen species (ROS), RANTES, interferon-beta (IFN-β), CXCL10, CCL5, inducible nitric oxide synthase (iNOS) and nitric oxide (NO). All TLRs, are known to signal via the MyD88 pathway excluding TLR3 which utilises the Toll/IL1 receptor (TIR) adaptor protein TRIF (Toll-receptor-associated activator of interferon), and TLR4 which can signal via both MyD88 and TRIF. Upon astrocytic TLR activation however, certain signalling components have been shown to be upregulated. TLR2 activation in astrocytes for example, has been demonstrated to utilise the co-receptor CD14. Furthermore, upon TLR4 activation, the TRIF-dependent pathway of TLR4 signalling has been shown to be inactive in astrocytes and utilizes the co-receptors MD-2 and CD14. (Bowman, et al., 2003; Bsibsi, et al., 2007; Bsibsi, et al., 2006; Butchi, et al., 2010; Carpentier, et al., 2005; El-Hage, et al., 2011; Jack, et al., 2005; Krasowska-Zoladek, et al., 2007).

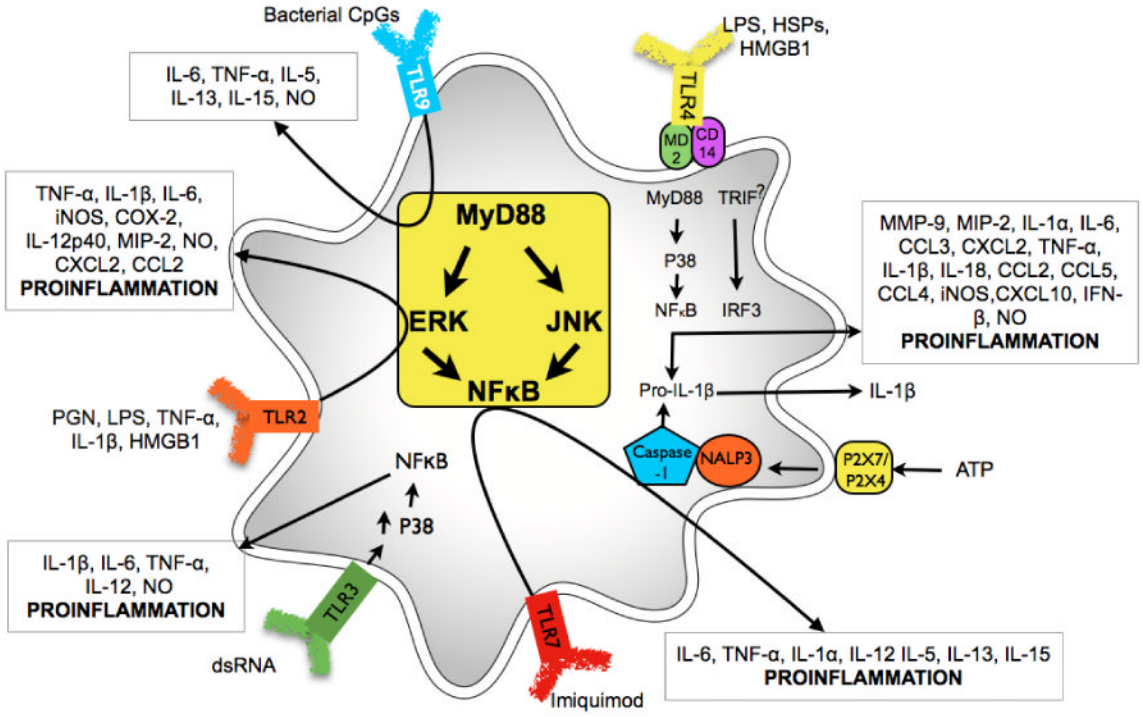

Figure 2. Toll-like receptor activation in microglia

Several TLRs have been identified in microglial cells including TLRs 2, 3, 4, 7 and 9. Upon ligand recognition, these TLRs have been shown to release numerous proinflamamtory mediators, which play a role in chronic pain. Numerous ligands have been identified to activate certain TLRS in microglial cells including, but not limited to: endogenous danger signals such as heat shock proteins (HSPs) (TLR4) and high mobility group box-1 (HMGB1) (TLR4 and TLR2); lipopolysaccharide (LPS) (TLR4 and TLR2), peptidoglycan (PGN) (TLR2), double-stranded RNA (dsRNA) (TLR3), Imiquimod (TLR7) and bacterial CpGs (TLR9). Furthermore, numerous proinflamamtory cytokines have been shown to be released including interleukin-6 (IL-6), IL-18, tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL1β), interleukin-1alpha (IL-1α), IL-12, IL-5, IL-13, IL-15; and proinflamamtory mediators including, but not limited to: superoxide anion ($O2^-$), matrix metallopeptidase 9 (MMP-9), macrophage inflammatory protein-2 (MIP-2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclo-oxygenase 2 (COX-2), CCL3, CXCL2 and CXCL10. Upon microglial TLR activation, specific signalling components are activated. TLR4 activation in microglia for instance, has been shown to increase NFκB activation specifically and TLR3 receptor binding results in increased p38 MAPK activation. Moreover, the ATP receptors P2X4 and P2X7 have been located on microglial cells and ATP administration has demonstrated activity at P2X7 receptors in microglia, known to facilitate the release of IL-1β via the inflammasome complex. Furthermore, TLR2 receptor binding has demonstrated increased MyD88 activation including increased ERK, JNK, and NFκB activation. (Aravalli, et al., 2008; Butchi, et al., 2010; Chan et al., 2003; Kakimura, et al., 2002; Kielian et al., 2005; Mayer, et al., 2011; Suzuki, et al., 2004; Town, et al., 2006; Tsuda, et al., 2003; Wang, et al., 2000; Zhang et al., 2005)

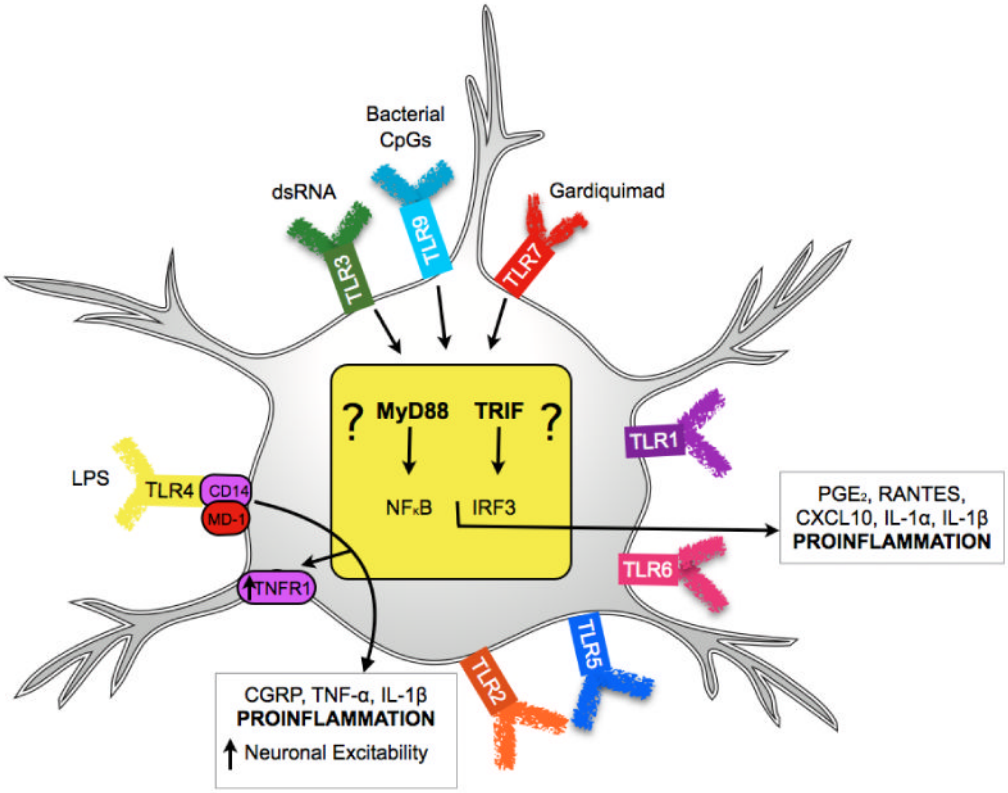

Figure 3. Toll-like receptor expression and activation in dorsal root ganglion (DRG) and trigeminal ganglion neurons

Recent findings have demonstrated Toll-like receptor (TLR) expression and activation in human, murine and rodent DRG and/or trigeminal ganglion neurons. TLR 1, 2, 3, 4, 5, 6, 7 and 9 have been identified in DRG and/or trigeminal neurons. Challenge of certain DRG TLRs with their appropriate ligands including, double-stranded RNA (dsRNA) (TLR3), bacterial CpGs (TLR9), Gardiquimad (TLR7) and lipopolysaccharide (LPS) (TLR4), has resulted in the expression of proinflammatory mediators known to play a role in pathological pain. Moreover, LPS has also been identified to bind to receptors in trigeminal neurons. TLR 3, 7 and 9 stimulation in DRG neurons, results in increased release of prostaglandin E_2 (PGE_2) and calcitonin gene related peptide (CGRP) and the upregulation of CCL5, CXCL10, interleukin-1alpha (IL-1α) and interleukin-1beta (IL-1β) mRNA. In rat and mouse DRG neurons, LPS administration has also been demonstrated to increase the neuronal expression of tumor necrosis factor-alpha (TNF-α)receptors, specifically TNFR1, and also increases neuronal excitability. Furthermore, utilisation of TLR signalling co-receptors has also been identified in DRG neurons. TLR4 signaling in DRG neurons for example has been shown to utilize the diverse co-receptor MD-1. (Diogenes, et al., 2011; Hou and Wang, 2001; Li, et al., 2004; Ochoa-Cortes, et al., 2010; Qi, et al., 2011; Wadachi and Hargreaves, 2006; Xing et al., 2002).

Table 1

Toll-like receptor activation in astrocytes, microglia and dorsal root ganglion (DRG) neurons

Numerous TLRs are expressed and activated in astrocytes, microglia and specific DRG neurons including TLRs 1-7 and 9. Several ligands have been found to activate certain TLRs in these specific cell types including but not limited to lipopolysaccharide (LPS), double stranded RNA, bacterial CpGs and peptidoglycan.

	Proinflammatory	**Mediators released**	**via TLR activation**
	Astrocytes	**Microglia**	**DRG Neurons**
TLR1			Expressed
TLR2	MCP-1, ROS, NO, CXCL8, IL-6, IL-12p40	TNF-α, IL-1β, iNOS, COX-2. NO, CCL2, CXCL2	Expressed
TLR3	MCP-1, ROS, TNF-α, IL-6, CCL3, NO, IL-1α, IL-1β	IL-1β, IL-6, TNF-α, IL-12, NO	PGE_2, RANTES, CXCL10, IL-1α, IL-1β
TLR4	MCP-1, TNF-α, IL-6, IL-1α, IL-1β, NO, IFN-β, iNOS	IL-1α, IL-6, CCL3, CXCL2, TNF-α, IL-18, iNOS, NO	CGRP, TNF-α, IL-1**β**
TLR5	IL-6		Expressed
TLR6			Expressed
TLR7	IL-6, IL-1α, TNF-α, IL-5, IL-13	IL-6, TNF-α, IL-1α, IL-12, IL-13, IL-15	PGE_2, RANTES, CXCL10, IL-1α, IL-1β
TLR9	MCP-1, ROS, IL-6, TNF-α	IL-6, TNF-α, IL-5, IL-13, IL-15, NO	PGE_2, RANTES, CXCL10, IL-1α, IL-1β