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Research Paper

Attenuation of mechanical pain hypersensitivity by treatment with Peptide5, a connexin-43 mimetic peptide, involves inhibition of NLRP3 inflammasome in nerve-injured mice

Ryan S. Tonkin[a], Callum Bowles[a], Chamini J. Perera[a], Brooke A. Keating[a], Preet G.S. Makker[a], Samuel S. Duffy[a], Justin G. Lees[a], Collin Tran[e], Anthony S. Don[e,f], Thomas Fath[a], Lu Liu[a], Simon J. O'Carroll[c], Louise F.B. Nicholson[c], Colin R. Green[d], Catherine Gorrie[b], Gila Moalem-Taylor[a,*]

[a] School of Medical Sciences, Faculty of Medicine, University of New South Wales, UNSW Sydney, NSW 2052, Australia
[b] School of Life Sciences, Faculty of Science, University of Technology, Sydney, NSW 2007, Australia
[c] Department of Anatomy and Medical Imaging and the Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand
[d] Department of Ophthalmology and New Zealand National Eye Centre, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand
[e] Centenary Institute, University of Sydney Medical School, Sydney, NSW 2006, Australia
[f] NHMRC Clinical Trials Centre, Charles Perkins Centre, University of Sydney, Sydney, NSW 2006, Australia

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ABSTRACT

Connexin43 (Cx43) hemichannels in spinal cord astrocytes are implicated in the maintenance of neuropathic pain following peripheral nerve injury. Peptide5 is a Cx43 mimetic peptide that blocks hemichannels. In this study, we investigated the effects of spinal delivery of Peptide5 on mechanical pain hypersensitivity in two mouse models of neuropathic pain, peripheral nerve injury and chemotherapy-induced peripheral neuropathy (CIPN). We demonstrated that 10 days following a chronic constriction injury (CCI) of the sciatic nerve, Cx43 expression, co-localised predominantly with astrocytes, was increased in the ipsilateral L3–L5 lumbar spinal cord. An intrathecal injection of Peptide5 into nerve-injured mice, on day 10 when pain was well-established, caused significant improvement in mechanical pain hypersensitivity 8 h after injection. Peptide5 treatment resulted in significantly reduced Cx43, and microglial and astrocyte activity in the dorsal horn of the spinal cord, as compared to control saline-treated CCI mice. Further *in vitro* investigations on primary astrocyte cultures showed that 1 h pre-treatment with Peptide5 significantly reduced adenosine triphosphate (ATP) release in response to extracellular calcium depletion. Since ATP is a known activator of the NOD-like receptor protein 3 (NLRP3) inflammasome complex, a key mediator of neuroinflammation, we examined the effects of Peptide5 treatment on NLRP3 inflammasome expression. We found that NLRP3, its adaptor apoptosis-associated spec-like protein (ASC) and caspase-1 protein were increased in the ipsilateral spinal cord of CCI mice and reduced to naïve levels following Peptide5 treatment. In the models of oxaliplatin- and paclitaxel-induced peripheral neuropathy, treatment with Peptide5 had no effect on mechanical pain hypersensitivity. Interestingly, in these CIPN models, although spinal Cx43 expression was significantly increased at day 13 following chemotherapy, NLRP3 expression was not altered. These results suggest that the analgesic effect of Peptide5 is specifically achieved by reducing NLRP3 expression. Together, our findings demonstrate that blocking Cx43 hemichannels with Peptide5 after nerve injury attenuates mechanical pain hypersensitivity by specifically targeting the NLRP3 inflammasome in the spinal cord.

1. Introduction

Neuropathic pain is a debilitating chronic pain condition that can be induced by a range of injuries and ailments including peripheral nerve injury, spinal cord injury (SCI), diseases of immune and metabolic origin, as well as cancer and its main treatment, chemotherapy (Colloca et al., 2017). Symptoms include spontaneous abnormal sensations (paraesthesia and dysesthesia), and stimulus-evoked pain (allodynia

* Corresponding author at: School of Medical Sciences, Wallace Wurth Building, Level 3, room 355B, UNSW Medicine, UNSW Sydney, NSW 2052, Australia.
E-mail address: gila@unsw.edu.au (G. Moalem-Taylor).

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and hyperalgesia). A growing body of evidence suggests a key role for spinal astrocytes in the development and maintenance of neuropathic pain, particularly following peripheral nerve injury and in chemotherapy-induced peripheral neuropathy (CIPN) (Austin and Moalem-Taylor, 2010; Chen et al., 2014; Jiang et al., 2016; Yoon et al., 2013). In nerve injury models, spinal cord astrocytes have been shown to undergo proliferation within the first week, with increased glial fibrillary acidic protein (GFAP) positive astrocytes presenting in the ipsilateral spinal cord as early as 3 days post-injury (Echeverry et al., 2008; Tsuda et al., 2011). Under these conditions, astrocytes produce various proinflammatory cytokines such as interleukin (IL)-1β, growth factors such as fibroblast growth factor-2 (FGF-2), as well as the chemokine (C-X-C motif) ligand 1 (CXCL1), that cause neuropathic pain by increasing the activity of spinal cord nociceptive neurons (Chiang et al., 2012; Gao and Ji, 2010; Guo et al., 2007; Kawasaki et al., 2008a; Zhang et al., 2013). Additionally, the development of mechanical pain hypersensitivity in oxaliplatin (OXA)- and paclitaxel (PAC)-induced peripheral neuropathy is associated with hyper-activation of astrocytes and increased levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF)-α and IL-1β in the spinal cord (Janes et al., 2015; Lees et al., 2017; Zhang et al., 2012). Thus, it is apparent that although astrocytes play a pivotal role in the maintenance of neuronal homeostasis, they may be harmful in peripheral neuropathies by contributing to the spinal neuroinflammatory response and neuropathic pain symptoms.

The gap junction protein connexin43 (Cx43) is strongly expressed in astrocytes and has recently been shown to contribute to the maintenance of neuropathic pain (Chen et al., 2014). Six connexin subunits assemble to form molecular pores, called connexons or hemichannels, that align between adjacent cells to form intercellular gap junction channels. These channels allow direct electrical and metabolic signaling between connected cells via the passage of ions and small molecules (Bennett et al., 2003; Hertzberg et al., 1981; Tonkin et al., 2015). It has been reported, however, that connexin function extends beyond their role in gap junction communication to also include extracellular exchanges mediated by undocked hemichannels (Contreras et al., 2002; Thompson et al., 2006). Indeed, astrocytic Cx43 hemichannels have recently been implicated in enhancing spinal cord synaptic transmission and mediating neuropathic pain via the release of chemokines following a chronic constriction injury (CCI) of the sciatic nerve (Chen et al., 2014). In a weight drop model of SCI in mice, both wild type and Cx30 knockout mice developed a persistent neuropathic pain that lasted from 4 to 8 weeks, while double knockout Cx43/Cx30 mice had reduced heat hyperalgesia and mechanical allodynia (Chen et al., 2012). Additionally, RNAi knockout of Cx43 was reported to reduce trigeminal pain behaviours in rats with a CCI of the infraorbital nerve (Ohara et al., 2008).

Of the molecules released by un-apposed hemichannels into the extracellular environment, particular attention has been focused on the excitatory gliotransmitter, adenosine triphosphate (ATP). ATP is a key initiator of the inflammasome via the activation of cell surface purinergic $P2X_4$, $P2X_7$ and $P2Y_{12}$ receptors (Jo et al., 2015; Rathinam and Fitzgerald, 2016). The inflammasome is a large intracellular multiprotein complex that consists of three key proteins, a central scaffold protein named nucleotide-binding oligomerization domain-like receptor (NLR), of which NLRP3 is the most widely studied, the adaptor apoptosis-associated spec-like protein (ASC) and pro-caspase-1. Assembly of these proteins results in the maturation and release of two key pro-inflammatory cytokines: IL-1β and IL-18 (Broz and Dixit, 2016; Zhou et al., 2011). This novel molecular pathway has only recently begun to gain attention as a key regulator of neuroinflammation in central nervous system pathologies, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Halle et al., 2008; Chakraborty et al., 2010).

A targeted approach to preventing the cascade of neuroinflammatory events following injury is blocking connexin hemichannels. Mimetic peptides, short peptide sequences that directly target the extracellular EL1 and EL2 loops of connexin channels, can block the pore to prevent hemichannel opening (Chaytor et al., 1997; Martin et al., 2005). Using an EL1 Cx43 mimetic peptide, Peptide5, we previously demonstrated that both topical and systemic administration improves functional recovery following traumatic SCI in rats by reducing neuroinflammation and secondary tissue damage (Mao et al., 2016; O'Carroll et al., 2013). Furthermore, Peptide5 treatment also resulted in reduced at-level mechanical allodynia after SCI (Mao et al., 2016). While several studies have previously examined blocking Cx43 channels in neuropathic pain, there are conflicting reports regarding their role, and the underlying mechanisms remain elusive (Chen et al., 2014; Cronin et al., 2008; Mao et al., 2016; O'Carroll et al., 2008; O'Carroll et al., 2013). We therefore investigated the effects of spinal delivery of Peptide5 on mechanical pain hypersensitivity in two mouse models of neuropathic pain, peripheral nerve injury and CIPN. In addition, we examined whether the effects on pain are dependent on the assembly of the spinal NLRP3 inflammasome, a key mediator of neuroinflammation.

2. Materials and methods

2.1. Animals

Male C57BL6/J mice (Australian BioResources, NSW, Australia) of approximately 8 weeks of age were used in this study. They were housed under a controlled 12 h light/dark cycle environment at 23 °C. Food and water was provided ad libitum. All procedures and tests were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney NSW Australia.

2.2. Neuropathic pain models

a) *Chronic constriction injury* – A CCI of the left sciatic nerve (SN) was performed as previously described by (Bennett and Xie, 1988). In brief, mice were firstly anesthetised with 5% isoflurane (Delvet Pty Ltd., NSW, Australia), and maintained with 2% isoflurane in oxygen during the surgical procedure. The surgical site was cleaned with 70% ethanol and iodine (Orion Laboratories, WA, Australia). Along the left hind leg, a small incision was made dorsal to the pelvis. The left SN was then exposed and freed by cutting the connective tissue between the gluteus superficialis and the biceps femoris muscles. Two loose ligatures (1 mm apart) were then tied around the exposed nerve using chromic gut sutures (6–0 Ethicon, NJ, USA), with enough tightness as to slightly constrict the nerve, but not arrest the epineural blood flow. In sham operated mice, the sciatic nerve was exposed but not ligated. Following surgery, the exposed muscle layers and subcutaneous tissue were sutured with 5–0 silk (Mersilk, Ethicon, NJ, USA) and the incision was enclosed with staples (9 mm, Autoclips, BD Diagnostic, NSW, Australia). Mice were post surgically monitored daily for 7 days after surgery.

b) *CIPN* – Chemotherapy was administered as previously described (Makker et al., 2017). In brief, paclitaxel (PAC) was obtained as a stock solution (Tocris Bioscience, BRS, England; 15 mg/ml) and mixed with Cremaphor (Sigma Aldrich, MO, USA) and ethanol in a 1:1 ratio, then further diluted in 0.9% sterile saline. Oxaliplatin (OXA) was obtained as a stock solution (Sigma Aldrich, MO, USA; 5 mg/ml) and diluted with 5% dextrose or with saline. Mice were injected on every other day (days 0, 2, 4, 6) intraperitoneally (i.p.) over 4 injection cycles to a cumulative dose of 20 mg/kg (equal to 740 mg/m^2 in humans). Control mice were injected with the vehicle only (5% dextrose or saline).

2.3. Peptide5

Peptide5 was custom synthesised (sequence VDCFLSRPTEKT;