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Inhibition of the potassium channel $K_{Ca}3.1$ by senicapoc reverses tactile allodynia in rats with peripheral nerve injury

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ABSTRACT

Neuropathic pain is a debilitating, chronic condition with a significant unmet need for effective treatment options. Recent studies have demonstrated that in addition to neurons, non-neuronal cells such as microglia contribute to the initiation and maintenance of allodynia in rodent models of neuropathic pain. The Ca^{2+}-activated K^+ channel, $K_{Ca}3.1$ is critical for the activation of immune cells, including the CNS-resident microglia. In order to evaluate the role of $K_{Ca}3.1$ in the maintenance of mechanical allodynia following peripheral nerve injury, we used senicapoc, a stable and highly potent $K_{Ca}3.1$ inhibitor. In primary cultured microglia, senicapoc inhibited microglial nitric oxide and IL-1β release. *In vivo*, senicapoc showed high CNS penetrance and when administered to rats with peripheral nerve injury, it significantly reversed tactile allodynia similar to the standard of care, gabapentin. In contrast to gabapentin, senicapoc achieved efficacy without any overt impact on locomotor activity. Together, the data demonstrate that the $K_{Ca}3.1$ inhibitor senicapoc is effective at reducing mechanical hypersensitivity in a rodent model of peripheral nerve injury.

1. Introduction

Existing treatments for neuropathic pain provide effective relief to only 1 in 4 patients (Attal et al., 2010; Finnerup et al., 2015). The majority of these treatments are aimed at targets expressed by neurons of the somatosensory system (*e.g.* opioid receptors and α2δ subunit of calcium channels). Expression of these targets in other areas of the CNS are believed to underlie their propensity to cause side effects such as sedation, euphoria and addiction (Finnerup et al., 2015). Recent studies have shown that non-neuronal targets expressed by activated immune cells also contribute to the establishment and maintenance of tactile allodynia in rodent models of neuropathic pain (Ren and Dubner, 2010; Scholz and Woolf, 2007; Zhuo et al., 2011). The clinical relevance of these findings was demonstrated by a recent PET study that showed an increase in PBR28 binding in the thalamus of patients with chronic back pain (Loggia et al., 2015). PBR28 is a ligand for the translocator protein (TSPO) that increased expression in activated microglia, the CNS resident immune cells (Rupprecht et al., 2010). Collectively, the studies suggest that microglial activation may be a mechanism common to neuropathic pain models as well as patients with chronic or neuropathic pain. Targeting microglia might provide novel therapeutic options for patients with these debilitating diseases.

Mechanistically, immune cell receptor activation results in elevations of intracellular Ca^{2+} concentrations which can subsequently stimulate diverse physiological responses including migration, proliferation, phagocytosis as well as production and release of cytokines, chemokines, prostanoids and reactive oxygen and nitrogen species (Hanisch, 2013). Many studies in models of neuropathic pain have demonstrated that inhibition of these immune cell receptors in the CNS (presumably on microglia) blocks the physiological sequelae of immune

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cell activation as well as pain behaviors (Abbadie et al., 2009; Grace et al., 2014; Marchand et al., 2005). In microglia, one important regulator of intracellular Ca^{2+} concentration is the intermediate conductance calcium-activated potassium channel $K_{Ca}3.1$ (Dale et al., 2016). The K^+ efflux resulting from the opening of this channel leads to the hyperpolarization of microglia which in turn facilitates Ca^{2+} influx resulting in the increased and sustained stimulation of various physiological responses *in vitro* (Kaushal et al., 2007; Khanna et al., 2001; Schilling et al., 2004).

The contribution of $K_{Ca}3.1$ to neuropathological processes *in vivo* has been investigated in models of multiple sclerosis and spinal cord injury using $K_{Ca}3.1$ knock out animals as well as the $K_{Ca}3.1$ inhibitor, TRAM-34. The studies demonstrated that inhibition or loss of $K_{Ca}3.1$ function led to a reduction in lesion size as well as a reduction in cytokine levels in the CNS (Bouhy et al., 2011; Reich et al., 2005). Earlier studies in mouse models of traumatic brain injury also demonstrated that $K_{Ca}3.1$ inhibitors were neuroprotective although cytokines and other markers were not measured (Mauler et al., 2004; Urbahns et al., 2005, 2003).

In the current study we evaluate whether inhibition of $K_{Ca}3.1$ alleviates pain behaviors of rats with peripheral nerve injury using senicapoc, a potent, CNS penetrant inhibitor with improved stability and selectivity *vs* TRAM-34 (Dale et al., 2016; Schilling and Eder, 2004, 2007).

2. Materials and methods

2.1. Compounds and formulations

Senicapoc (ICA-17043, MedChem Express, Monmouth Junction, NJ) was dissolved at 100 mM in DMSO and diluted in media for *in vitro* studies. For *in vivo* studies, senicapoc was formulated with 20% 2-Hydroxypropyl-β-cyclodextrin (Kleptose®, Roquette, Lestrem, France) as a suspension at 10, 30 and 100 mg/kg. Gabapentin (Toronto Research Chemicals, Toronto, ON, Canada) was dissolved in saline at 100 mg/mg. All drug solutions were prepared the day of experiments. Acetonitrile (ACN), dimethyl sulfoxide (DMSO), isopropyl alcohol (IPA) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. CHO-K1 cells expressing recombinant human $K_{Ca}3.1$

CHO-K1 cells stably expressing human $K_{Ca}3.1$ (Chantest, Cleveland, OH) were grown in T75 tissue culture flasks to 70–80% confluence (Ham's F12K and 10% Fetal Bovine Serum, Thermo Fisher). On the day of the experiment, the cells were washed with Dulbeco's Phosphate buffered saline, lifted with 2 ml of Detachin™ (Genlantis, San Diego, CA) and centrifuged at 250×*g* for 2 min. The supernatant was removed and the cells were washed and re-suspended in Qpatch extracellular solution to achieve a final cell density of ~3×10^6 cells/ml.

2.3. QPatch electrophysiology

Whole-cell patch-clamp experiments were carried out on a QPatch-16 automated electrophysiology platform (Sophion Biosciences, Paramus, NJ). (Jenkins et al., 2013). $K_{Ca}3.1$ channels were activated by including 10 μM free Ca^{2+} in the internal patch pipette solution. Following establishment of the whole-cell configuration, cells were held at −80 mV. $K_{Ca}3.1$ current was elicited by a voltage protocol that held at −80 mV for 100 ms then stepped from −90 mV to +90 mV for 600 ms in 20 mV increments. For dose response experiments, $K_{Ca}3.1$ current was measured at 0 mV. The external solution contained (in mM): 140 NaCl, 10 HEPES, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$ 10 Glucose (pH 7.4). The internal patch pipette solution contained (in mM): 110 K-gluconate, 34 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES, 4.86 $CaCl_2$ to achieve free Ca^{2+} concentration of 10 μM (pH 7.2). $[Ca^{2+}]_i$ was calculated according to WEBMAX STANDARD software, http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm. Currents were measured using the Sophion QPatch software and exported to Microsoft Excel and Prism (GraphPad, San Diego, CA) for further analysis.

2.4. Animals

All studies were conducted in accordance with the recommendations set forth in the Guide for the Care and Use of Laboratory Animals (2011) and with approval of Lundbeck Research USA's Institutional Animal Care and Use Committee. All rats received food and water*ad libitum*and were maintained on a 12 h light/dark cycle. For microglial cultures, E14 timed-pregnant Sprague Dawley rats were ordered (Crl: SD, Charles River, Kingston, NY). For pharmacokinetic and locomotor studies, 150–175 g male Sprague Dawley rats (Crl: SD, Charles River) were purchased. For the chronic constriction injury model (CCI), twenty-five ~150 g male Sprague Dawley rats were used (Envigo, Indianapolis, IN). Rats were allowed to acclimate for 6 - 7 days prior to any *in vivo* procedure.

2.5. Primary microglial cultures

Rat primary microglia were prepared and cultured as described by Möller et al. (2000). Mixed glial cultures were maintained in T150 flask (Falcon - Corning, Glendale, AZ) in Dulbecco's modified Eagle medium (DMEM)-GlutaMax (Gibco - Thermo Fischer Scientific, Waltham, MA) containing 4.5g/l of D-glucose and supplemented with 10% low endotoxin (0.06 EU/ml) heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (P/S) (Gibco). Cultures were grown in a humidified incubator at 37 °C under 5% CO_2 for 10–14 days at which time they were harvested by tapping the flasks and collecting the microglia-containing medium. Microglia were pelleted by centrifugation at 276×*g* for 5 min, re-suspended in DMEM/10%FBS/PS medium and plated at desired density in poly-D-lysine (PDL)-coated plates (BioCoat - Corning). Purity was assessed by labeling with the microglial maker CD11b, which identified > 95% of cells as microglia.

2.6. Microglial IL-1β release

Rat primary microglia were primed with 3 EU/ml of ultra-pure Lipopolysaccharide (control standard endotoxin (CSE) Associates of Cape Cod, Falmouth, MA). After 3.5 h, vehicle or senicapoc were added and allowed to incubate for 30 min. Finally, BzATP (1 mM; Sigma-Aldrich, St. Louis, MO) was added to activate P2×7 receptors and trigger release of IL-1β (BzATP also inhibits other receptors such as P2×4 receptors). Cell free supernatants were assayed for IL-1β using a custom rat cytokine assay (N45IA-1; MesoScale Discovery, Rockville, MD). IC_{50}s were determined using Prism (Graphpad, San Diego, CA).

2.7. Microglial NO release (measurement of nitrite)

Rat primary microglia were pre-incubated with vehicle or senicapoc for 30 min. Next, 3 EU/ml of ultra-pure Lipopolysaccharide (CSE, Associates of Cape Cod) was added to induce iNOS expression and NO synthesis. After a total incubation time of 24 h, media was collected, spun down to remove cells and assayed for nitrite (the stable breakdown product of NO) using Griess Reagent (Promega, Madison WI).

2.8. Electrophysiology recordings in primary microglia

Primary rat microglia were plated at a density of 300,000/35 mm dish for 24 h. Whole cell patch clamp recordings were performed using an EPC9 patch-clamp amplifier and PatchMaster software (HEKA Instruments Inc.). The cell's membrane potential was held at