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$P2Y_{12}$ shRNA treatment relieved HIV gp120-induced neuropathic pain in rats

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ABSTRACT

Human immunodeficiency virus (HIV) envelope glycoprotein (glycoprotein 120, gp120) can induce chronic neuropathic pain by directly stimulating primary sensory afferent neurons. Activation of satellite glial cells (SGCs) in dorsal root ganglia (DRG) plays an important role in the transmission of neuropathic pain. The $P2Y_{12}$ receptor is expressed in SGCs of DRG. In this study, we investigated the role of the $P2Y_{12}$ receptor in HIV gp120-induced neuropathic pain. The results showed that peripheral nerve exposure to HIV gp120 increased mechanical and thermal hyperalgesia in gp120-treated model rats. The gp120 treatment increased the expression of $P2Y_{12}$ mRNA and protein in DRG SGCs. Treatment with $P2Y_{12}$ short hairpin RNA (shRNA) in DRG SGCs decreased the upregulated expression of $P2Y_{12}$ mRNA and protein in DRG SGCs as well as relieved mechanical and thermal hyperalgesia in gp120-treated rats. Reduction of $P2Y_{12}$ receptor decreased co-expression of $P2Y_{12}$ and glial fibrillary acidic protein (GFAP), expression of GFAP, interleukin (IL)-1β, tumor necrosis factor (TNF)-receptor 1 (TNF-R1), and phosphorylation of Akt (*p*-Akt) proteins in DRG of gp120-treated rats. Upregulation of GFAP is a marker of SGC activation. Therefore, $P2Y_{12}$ shRNA treatment decreased HIV gp120-induced mechanical and thermal hyperalgesia in gp120-treated rats.

1. Introduction

Chronic neuropathic pain is a common symptom in patients with human immunodeficiency virus (HIV)-1 infection. Glycoprotein 120 (gp120) is an HIV-1 protein that can cause pain behaviors in animal models (Hao, 2013; Nasirinezhad et al., 2015; Yuan et al., 2014; Zheng et al., 2011). Concomitant with chronic pain manifestation, approximately 30% of HIV-1/AIDS patients develop clinically detectable peripheral neuropathy. Distal symmetrical polyneuropathy (DSP) is a major neurological disorder in HIV/AIDS patients (Schütz and Robinson-Papp, 2013; Maratou et al., 2009). Symptoms of DSP include neuropathic pain, such as allodynia, hyperalgesia, and dysesthesia (Freeman et al., 2014; Verma et al., 2005; Schütz and Robinson-Papp, 2013). Dorsal root ganglion (DRG) afferent fibers are distributed to both central and peripheral terminals as well as transmit noxious stimuli from the periphery to the central nervous system (Basbaum et al., 2009). Thermal hyperalgesia and mechanical allodynia in rats are enhanced by peripheral administration of gp120 (Hao, 2013; Maratou et al., 2009; Herzberg and Sagen, 2001; Kamerman et al., 2012; Milligan et al., 2000; Wallace et al., 2007). At present, clinical interventions provide only symptomatic relief rather than a cure. Because the pathogenic mechanism of HIV-associated chronic pain is not yet clear, the understanding of how HIV-1 infection leads to chronic pain is very important for the development of effective therapies.

Purinergic P2 receptors are activated by extracellular purine (ATP, ADP) and/or pyrimidine (UTP, UDP) nucleotides (Burnstock, 2013, 2014; Idzko et al., 2014; Magni and Ceruti, 2013). P2

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receptors consist of metabotropic (i.e., G-protein-coupled) P2Y receptors and ionotropic P2X receptors (i.e., nucleotide-gated ion channels) (Burnstock, 2013, 2014; Idzko et al., 2014; Magni and Ceruti, 2013). ATP is released from both neurons and glial cells (Fields and Burnstock, 2006; Verderio and Matteoli, 2011; Sperlágh et al., 1995, 1998; Sperlagh et al., 1997). Satellite glial cells (SGCs) are the most abundant cell type in DRG (Costa and Moreira, 2015; Hanani, 2005). After peripheral nerve injury, SGCs undergo structural changes and proliferate (Hanani, 2005; Jasmin et al., 2010). Neuron–glia interactions involve purinergic signaling (Fields and Burnstock, 2006; Rajasekhar et al., 2015; Verderio and Matteoli, 2011). SGCs of DRG express the $P2Y_{12}$ receptor (Katagiri et al., 2012; Kobayashi et al., 2008, 2013). The $P2Y_{12}$ receptor participates in the transmission of nociceptive signals (Burnstock, 2013; Horváth et al., 2014; Katagiri et al., 2012; Magni and Ceruti, 2013). However, it is uncertain whether the $P2Y_{12}$ receptor is involved in the pathogenic mechanisms underlying HIV neuropathic pain. In this experiment, our aim was to investigate the role of the $P2Y_{12}$ receptor in HIV gp120-induced neuropathic pain.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (200–250 g) were housed for at least one week before the start of the experiment and maintained under temperature-controlled conditions at 22 °C and 60% humidity with freely available food and water. All experiments conformed to the ethical regulations of the International Association for the Study of Pain and were approved by the Nanchang University Animal Care and Use Committee.

2.2. Reagents

Recombinant envelope glycoprotein 120 (gp120) Fragment 421–438, which was derived from the CD4 attachment region of HIV gp120, was obtained from Sigma-Aldrich. Gp120 was kept in stock solution at a concentration of 50 μM and temperature of −80 °C. Fifteen minutes before testing, the stock solution was diluted to the desired concentrations with 0.1% rat serum albumin (RSA, Sigma-Aldrich) on ice.

2.3. Neuropathic pain model of perineural gp120 application

Rats were randomly divided into four groups: sham operation group (sham group), HIV-gp120 group (gp120 group), gp120 group treated with $P2Y_{12}$ short hairpin RNA (shRNA) (gp120 + $P2Y_{12}$ shRNA) and gp120 group treated with scrambled shRNA (gp120 + NC shRNA). Each group contained 8 rats. For perineural HIV-gp120 application, previously described methods were used (Herzberg and Sagen, 2001; Yi et al., 2017). Briefly, rats were anesthetized by injecting them intraperitoneally with 10% chloral hydrate, and the left sciatic nerve was exposed in the popliteal fossa without perineurium damage under aseptic surgical conditions. A 2 × 6 mm strip of oxidized regenerated cellulose that had previously been soaked in 250 μl of 0.1% RSA in saline containing 400 ng of gp120 (or in 0.1% RSA in saline for the sham group) was loosely wrapped around the sciatic nerve. Then, the nerve was gently put back into place and the incisions were closed with 4/0 sutures. Per the manufacturer's instructions for the Entranster™-in vivo transfection reagent (Engreen Biosystem Company of Beijing), 20 μl of a transfection complex consisting of shRNA ($P2Y_{12}$ or NC shRNA) and transfection reagent at a ratio of 1:2 was intrathecally injected into rats of the gp120 + $P2Y_{12}$ shRNA and gp120 + NC shRNA groups on day 7. Rats in the sham and gp120 groups received an equal amount of saline. Three different shRNAs targeting sequences of $P2Y_{12}$ (NM_022800.1) and a negative control scrambled shRNA (not homologous to any gene) were synthesized by RiboBio Co. Ltd. (Guangzhou, China). The optimal $P2Y_{12}$ shRNA (data not shown) was selected by real-time RT-PCR. The sequences were as follows: sense 5′-CACCGCTTCGTTCCCTTCCACTTTGCGAACAAAGTGGAAGGGAACGAAGC -3′, antisense 3′-CGAAGCAAGGGAAGGTGAAACGCTTGTTTCACCTTC CCTTGCTTCGAAAA -5'. Rats' behavior was assessed by the same investigator at 0, 1, 4, 7, 10, 12 and 14 days after the start of the experiment. The tester was blind to the group designation. At day 14, the animals were sacrificed with CO_2, and the left L4–6 DRGs were removed.

2.4. Measurement of the mechanical withdrawal threshold (MWT)

A BME-404 electronic mechanical stimulator, which was provided by Institute of Biomedical Engineering of Chinese Academy of Medical Sciences, was used to evaluate the MWT between 10:00–12:00. The end face diameter of the test needle, pressure measurement range, and pressure measurement resolution of this equipment were 0.6 mm, 0.1–50 g, and 0.05 g, respectively. Animals were placed in a clean glass box that was positioned on the sieve of the metal frame for an acclimation period of at least 1 h. The test needle touched the left hind paws until the animal withdrew its paws. The computer recorded pressure values automatically. This procedure was performed 5 times for each rat at intervals ≥5 min, and the MWT was calculated as the mean value of these measurements (Lin et al., 2010; Nasirinezhad et al., 2015). The baseline was calculated from an average of 5 consecutive withdrawal responses of the left hindpaw.

2.5. Measurement of the thermal withdrawal latency (TWL)

Determination of the TWL was performed using a BME-410C Thermal Paw Stimulation System. The animals were left in a transparent, bottomless box on a glass plate for an acclimation period of at least 1 h. A beam of radiant heat oriented the plantar surface of their paws. TWL was taken as an index of the nociceptive threshold. The light beam was switched off when the animal lifted its paw. The time on the screen was designated as the paw withdrawal latency. The hind paws were tested alternately at 5-min intervals. The cutoff time for heat stimulation was 30 s.

2.6. Quantitative real-time PCR

DRGs were isolated immediately and flushed with ice-cold PBS. Total RNA samples were prepared using the TRIzol Total RNA Reagent (Beijing Tiangen Biotech Co.). cDNA synthesis was performed with 2 μg of total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). The primers were designed with Primer Express 3.0 software (Applied Biosystems), and the sequences were as follows: for $P2Y_{12}$, forward 5′-CTTCGTTCCCTTCCACTTTG-3′ and reverse 5′-AGGGTGCTCTCCTTCACGTA-3'; for β-actin, forward 5′-TAAAGACCTCTATGCCAACACAGT-3′ and reverse 5′-CACGATGGAGGGGCCGGACTCATC-3'. Quantitative PCR was performed using SYBR® Green MasterMix in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Inc.: Foster City, CA). Gene expression was quantified using the ΔΔCT method with CT as the threshold cycle. The relative levels of target genes, normalized to the sample with the lowest CT, were reported as $2^{-\Delta\Delta\ CT}$.

2.7. Double-labeled immunofluorescence

Double-labeled immunofluorescence was performed as

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