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A Wnt5a signaling pathway in the pathogenesis of HIV-1 gp120-induced pain

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Abstract

Pathological pain is one of the most common neurological complications in HIV-1/AIDS patients. However, the pathogenic process is unclear. Our recent studies show that Wnt5a is up-regulated in the spinal cord dorsal horn of the HIV patients who develop pain and that HIV-1 gp120, a potential causal factor of the HIV-associated pain, rapidly up-regulates Wnt5a in the mouse SDH. Using a mouse model, we show here that a specific Wnt5a antagonist, Box-5, attenuated gp120-induced mechanical allodynia. Conversely, a Wnt5a agonist, Foxy5, facilitated the allodynia. To elucidate the molecular mechanism by which Wnt5a regulates gp120-induced allodynia, we tested the role of the JNK/TNF- α pathway. We observed that the JNK-specific inhibitor SP600125 blocked either gp120- or Foxy5-induced allodynia. Similarly, the TNF- α -specific antagonist Enbrel also reversed either gp120- or Foxy5-induced allodynia. These data suggest that JNK and TNF- α mediate the biological effects of Wnt5a in regulating gp120-induced allodynia. To investigate the cellular mechanism, we performed extracellular single-unit recording from SDH neurons in anesthetized mice. Both Box5 and SP600125 negated gp120-induced potentiation of SDH neuron spiking evoked by mechanical stimulation of the hindpaw. Furthermore, while Foxy5 potentiated spike frequency of SDH neurons, either SP600125 or Enbrel blocked the potentiation. The data indicate that Wnt5a potentiates the activity of SDH neurons via the JNK-TNF- α pathway. Collectively, our findings suggest that Wnt5a regulates the pathogenesis of gp120-induced pain, likely by sensitizing pain-processing SDH neurons via JNK/TNF- α signaling.

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INTRODUCTION

Pathological pain is a common neurological complication in HIV-1/AIDS patients [1; 9; 14], and is one of the main detrimental factors that impairs the quality of life of HIV patients [39]. The cost of pain management weighs heavily in patient care [8]. However, the underlying pathogenic mechanism of HIV-associated pain is poorly understood.

HIV-1 gp120 is a viral coat protein that binds its receptor CD4 and co-receptors CCR5 or CXCR4. Although neurons do not express the CD4 receptor, they are stimulated by gp120 via CCR5 and CXCR4, which are expressed there [29]. Gp120 is significantly higher in the spinal cord dorsal horn (SDH) of HIV patients who develop chronic pain ('pain-positive' HIV patients), compared to 'pain-negative' HIV patients [44], although the viral load or replication does not seem to correlate with the manifestation of pain [33; 44]. The pathogenic role of gp120 is also suggested by that intrathecal injection (i.t.) of gp120 (to mimic its spinal increase in 'pain-positive' HIV patients) in mice causes pathologies similar to that of the 'pain-positive' patients [44]. The activity of gp120 in inducing pain has been reported in various animal models [13; 15; 20; 25; 26; 28; 41; 47]. However, the mechanism by which gp120 causes pain remains elusive.

Wnts are secreted signaling proteins that play important roles in various developmental and carcinogenic processes [6; 17; 24]. Wnt proteins signal through either the β -catenin-dependent canonical pathway or β -catenin-independent non-canonical pathways (e.g. the Wnt/ Ca^{2+} or the Wnt/JNK pathway). In the mammalian CNS, specific Wnt ligands such as Wnt3a (the prototypic Wnt ligand for the canonical pathway) and Wnt5a (the prototypic Wnt ligand that activates the non-canonical pathways) are predominantly expressed in neurons [4; 22; 36; 37]. The secretion and synthesis of Wnt protein in neurons are stimulated by synaptic activity [4; 23; 42]. Wnt proteins are up-regulated in the spinal cord of various pain models [37; 45; 46]. Recent studies suggest a critical role of Wnt signaling in the spinal cord [18; 45; 46], cerebrospinal fluid-contacting nucleus [43] and primary sensory neurons [38] in the development of pathological pain. Consistent with a role in the pathogenesis of HIV-associated pain, Wnt ligands (e.g. Wnt5a) are specifically up-regulated in the SDH of 'pain-positive' HIV patients [36]. However, the role and pathogenic mechanism of Wnt signaling in HIV-associated pain remains to be determined.

In this study, we have focused on analyzing the potential contribution of Wnt5a to gp120-induced pain. Our results reveal a Wnt5a-JNK-TNF- α signaling axis in the pathogenesis of gp120-induced mechanical allodynia.

MATERIALS AND METHODS

Animals

All animals were adult C57BL/6J mice (8-10 weeks old and weighing 18-22 g) purchased from Harlan Labs. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (Protocol 0904031). Pain testing was performed following the guidelines of the International Association for the

Study of Pain. Animals were housed in cages (5 animals/cage) with standard bedding and free access to food and water, in a room maintained at $23\pm3^{\circ}\text{C}$ and a 12/12 light-dark cycle.

Reagents

Recombinant envelop glycoprotein gp120 from the HIV-1bal strain was provided by the NIH AIDS Reagent Program (cat#: 4961, lot: 38 11003). Gp120 was aliquoted and stored at -80°C . Ten minutes before injection, the stock solution of gp120 was diluted to 15 ng/ μl with ice-cold 0.1% bovine serum albumin (BSA, Sigma-Aldrich) in phosphate-buffered saline (PBS, pH7.4; sterilized by 0.2 μm filters) and kept on ice.

Recombinant Wnt5a (R&D, cat#: 645-WN/CF) was stored at -20°C , and diluted with ice cold PBS to a final concentration of 4 ng/ μl before injection (20 ng/animal). Box-5 (Calbiochem, cat #: 681673) and Foxy5 (synthesized by Storkbio) were dissolved in sterilized PBS at a concentration of 2 $\mu\text{g}/\mu\text{l}$ (10 $\mu\text{g}/\text{animal}$). SP600125 (Calbiochem, cat #: 420119) was dissolved (1.25 $\mu\text{g}/\mu\text{l}$; 10 $\mu\text{g}/\text{animal}$) in dimethyl sulfoxide (DMSO) (Sigma Aldrich, cat #: D2650). Enbrel (Amgen, NDC#: 5840644504). The specificity at the used dosages of the agonist (Foxy5) and antagonists (Box-5, SP600125 and Enbrel) have been suggested in many published studies on neural plasticity and/or pain (e.g. Foxy5 [40], Box-5 [22; 43; 45], SP600125 [11], Enbrel (a.k.a. Etanercept) [7]; see Results section for more information about the individual agents).

Intrathecal injection

Intrathecal injection (i.t.) was performed as described previously [16; 34]. C57BL/6 mice were anesthetized with 3% isoflurane for induction and 2.5% for maintenance. After clipping the fur on the mouse lower-back, the skin was sterilized with 70% alcohol. A 30-gauge needle connected to a 10 μl Hamilton syringe (8000, Model 1701) was inserted into the L5-L6 intervertebral space. A sudden tail twist or leg kick was used as an indicator of successful penetration of the needle tip into the vertebral canal at the cauda equina level. The drug was slowly delivered for 30 s and the needle was left in situ for additional 15 s before withdrawal.

Von Frey filament test

The changes of either response rate (%) or threshold (g) revealed by von Frey testing have been commonly used to monitor the development of mechanical allodynia. We used both measurements to confirm the manifestation of gp120-induced mechanical allodynia in the early studies (Fig. 1 &2), and stay with threshold measure afterward to save time and animals. The response rate was measured as described [34], with the von Frey filament 3.61. To measure mechanical pain thresholds, Von Frey testing was conducted as described [45]. Briefly, mice were first habituated to the testing environment (a 5.25" $\times$ 2.125" $\times$ 2" plexi-glass box on a metal mesh floor) for three consecutive days (1 hr/day). On the test day, animals were placed in the plexi-glass box for 15 to 20 minutes until their initial exploring activity phased out. Testing was performed when animals were in a state of resting, alertness or light sleep, avoiding the states of grooming or deep sleeping [3]. The mechanical threshold was measured according to Dixon's up and down method, with von Frey monofilaments (Stoelting, Wood Dale, IL) to stimulate the hindpaw. The filament were

applied perpendicularly to the center area of hindpaw with suitable force until the filament was slightly bent. Abrupt withdrawal (sometimes horizontal movement), shaking, lifting, licking or biting of the stimulated paw was considered to indicate a noxious response. The threshold for noxious responses was expressed as mean $\pm$ SE (in grams of pressure exerted) and plotted with GraphPad Prism 5. Statistical significance was determined by two-way ANOVA with Bonferroni post hoc test.

Hindpaw relaxation measurement

We observed pain-related postures of the hindpaw after gp120 administration. The expression of mechanical allodynia was associated with a decrease of hindpaw relaxation. To measure the hindpaw relaxation, we took five photos for each animal at intervals of 15 s. The relaxation of photographed hindpaws was scored by counting the number of curled-up toes in both hindpaws: 0, no curled-up toes were observed (i.e. all ten toes fully extended on the mesh floor, indicating the most relaxed state); 1, one toe curled up or bent; 2, two toes curled up or bent, etc. Zero was the lowest score, while 10 was the highest. The naïve animals without gp120 administration usually scored less than 2.5. A higher score was a postural indicator of more severe pain.

Electrophysiology

As described previously for the rat [30], adult (26-33 g) mice were anesthetized with sodium pentobarbital (60-70 mg/kg, i.p.) and mounted in a stereotaxic frame (David Kopf Instruments). A small laminectomy was performed at vertebral levels T12–L1 to expose the lumbar spinal segments. Subsequently, constant levels of anesthesia were maintained with isoflurane (1.5% in oxygen) delivered by a precision vaporizer throughout the experiment. Depth of anesthesia was monitored closely by testing the corneal blink, hindpaw withdrawal and tail-pinch reflexes, which had to be absent. The dura mater was opened and a small trough (15 μ l volume) was formed with agar for topical drug application.

Individual neurons were recorded extracellularly in the deep dorsal horn (300-1000 μ m) of the lumbar enlargement of the spinal cord (L5/6) using glass-insulated carbon filament electrodes (3–5 M Ω). The recorded signals were amplified, displayed on an analog storage oscilloscope, fed into a window discriminator, digitized by an interface (CED 1401+; Cambridge Electronics Design), and recorded on a computer (Pentium PC). Spike2 software (Cambridge Electronics Design) was used for on-line and off-line analysis of single-unit activity. An individual neuron was identified by the configuration, shape, and height of the recorded action potentials (spikes) in response to innocuous and noxious mechanical stimulation of the skin of the hindpaw. Throughout the experiment, spike size and configuration were continuously monitored on the oscilloscope with the use of Spike-2 software to confirm that the same neuron was recorded and that the relationship of the recording electrode to the neuron remained constant.

Neurons were characterized by their responses to the following stimuli applied to the most responsive sites of the receptive field in the hind paw [30]: innocuous BRUSH (brushing the skin with a soft-hair artist's brush in a stereotyped manner; 1 stroke), innocuous PRESS (100 g/6 mm², which is not painful when applied to the skin in humans) and noxious PINCH

(300 g/6 mm²), which is painful without causing overt damage to the skin). PRESS and PINCH were applied by means of forceps equipped with a force transducer, whose calibrated output was amplified and displayed in grams on a liquid crystal display screen. The output signal was also fed into the CED interface and recorded on the computer for on-line and off-line analysis. All cells included in this study were wide-dynamic-range (WDR) neurons, which responded consistently to innocuous stimuli but more strongly to noxious stimuli.

In each experiment, the background activity and responses of one WDR dorsal horn neuron to graded mechanical stimuli were recorded before and during spinal drug application. Each mechanical stimulus was applied for 10 s followed by a 10-15 s pause before the next stimulus. The entire sequence of mechanical stimuli (BRUSH, PRESS, and PINCH) was repeated three times before and during spinal drug application. Known concentrations of drugs (gp120: 20 µg/µl, 5 µl; Box-5: 2 µg/µl, 5 µl; SP600125: 1.25 µg/µl, 8 µl; Foxy5: 2 µg/µl, 5 µl; Enbrel: 25 µg/µl, 5 µl) were administered into the trough on the dorsal surface of the spinal cord around the recording electrode. The trough was filled with oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (ACSF) equilibrated to pH 7.4. ACSF contained: NaCl 117mM, KCl 4.7mM, NaH₂PO₄ 1.2mM, CaCl₂ 2.5mM, MgCl₂ 1.2mM, NaHCO₃ 25mM, and glucose 11mM. The neurons' responses were measured at 15 min of drug application. Drugs dissolved in ACSF on the day of the experiment were administered for 15-30 min before they were removed with a pipetter and ACSF was reapplied (washout). Responses to the mechanical stimuli were measured before and during drug application.

Recorded activity (spikes per seconds) was analyzed off-line from peristimulus rate histograms using Spike2 software. Background activity was subtracted from the total activity during stimulation to calculate "net" evoked responses. All averaged values are given as the means ± SE. A one-way repeated measures analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was used to compare the neurons' responses (raw data) during drug application to predrug control values. SigmaStat (Systat Software Inc., Richmond, CA) was used for all statistical analysis. Statistical significance was accepted at the level $P < 0.05$.

RESULTS

Wnt5a antagonist attenuated gp120-induced mechanical allodynia

Our recent studies revealed that Wnt5a is expressed in mouse SDH neurons and is rapidly up-regulated after i.t. administration of gp120 [22; 37]. These findings suggest a potential role of Wnt5a in the development of gp120-induced pain. To test this hypothesis, we measured the effect of Wnt5a antagonists on the expression of i.t. gp120-induced mechanical allodynia assessed with von Frey filaments. As shown in Fig. 1A, i.t. gp120 (gp120Bal, 100 ng/5 µl/animal) rapidly increased withdrawal response rate to mechanical stimuli. This dosage was chosen based on our estimation of gp120 in the SDH of 'pain-positive' HIV patients and the findings that this gp120 dose induced extensive pathological phenotypes that resemble to that in the 'pain-positive' HIV patients [44]. Control animals receiving heat-inactivated gp120 did not show a significant increase in response rate, indicating that i.t. gp120 induced mechanical allodynia. The i.t. gp120-induced increase in

response rate peaked at around 50% within 6 hours following i.t. injection and lasted for at least 4.5 days (Fig. 1A, 1B). When coapplied (i.t.) with the Wnt5a antagonist Box-5, gp120 caused a much milder increase in the response rate, which peaked around 30% and returned to baseline by day 4.5 (Fig. 1A, 1B). Box-5 is a chemically modified hexapeptide that is from Wnt5a and thus can specifically antagonizes Wnt5a activity [19]. Concomitant with the development of allodynia, we observed changes in color and posture of the hindpaw (Fig. 1C). The hindpaw where allodynia developed after i.t. gp120 turned red. Administration of Box-5 attenuated the gp120-induced color change (Fig. 1C1-C3). In addition, the toes in the hindpaw with i.t. gp120-induced allodynia tended to curl and did not touch the mesh floor. Again, Box-5 significantly attenuated the toe-curling (Fig. 1C4). These color and posture changes in the hindpaw may reflect the presence of a pain state induced by i.t. gp120.

To further test the role of Wnt5a, we employed neutralizing anti-Wnt5a antibody and measured the threshold of responses to mechanical stimulation using von Frey filaments. Similar to our previous findings [44], i.t. gp120 drastically decreased the threshold (Fig. 1D). Administration (i.t.) of anti-Wnt5a antibody completely blocked the expression of the early-phase allodynia but not allodynia after 20 hours post i.t. gp120 (Fig. 1D). In summary, both Box-5 and anti-Wnt5a antibody impaired the expression of i.t. gp120-induced allodynia.

Wnt5a agonist potentiated gp120-induced mechanical allodynia

The effect of Box-5 shown above indicates a critical role of Wnt5a in gp120-induced allodynia. Next, we sought to determine if purified Wnt5a or its agonist, Foxy5, can promote gp120-induced allodynia. Wnt5a (20 ng; i.t.), but not heat-inactivated Wnt5a, potentiated the gp120-induced increase of response rate to mechanical stimulation (0.4 g; von Frey filament 3.61) (Fig. 2A, 2B). Wnt5a on its own induced a small increase in the response rate (Fig. 2A). We further tested the effect of Foxy5, a Wnt5a-derived hexapeptide same as Box-5 but with different chemical modifications, that can specifically evoke Wnt5a signaling [32]. We observed that co-administration of Foxy5 (10 µg; i.t) with gp120 caused a further decrease in the response threshold to mechanical stimulation, compared to the gp120-only group (Fig. 2C). The Foxy5-potentiated threshold decrease was confirmed in a separate set of experiments (Fig. 4B), although the Foxy5 potentiation in Fig.2C appeared to be bigger than that in Fig. 4B. This discrepancy was probably caused by the fact that the batch of gp120 used in Fig. 4B led to a much bigger decrease of the threshold, and thus left little room for Foxy5 to further develop its potentiation effect. Taken together, these data indicate that stimulation of Wnt5a signaling can enhance gp120-induced allodynia.

JNK inhibitor impaired gp120- and Foxy5-induced allodynia

The above results suggested that Wnt5a is necessary for gp120-induced allodynia and able to enhance the pain behavior. Wnt5a activates JNK signaling, which plays important roles in the development of pain pathologies in animal models [10; 11], in the spinal cord [22]. Wnt5a is up-regulated in the SDH of HIV-infected patients who developed pathological pain [35; 44]. Therefore, we reasoned that JNK signaling is a downstream effector through which Wnt5a regulates gp120-induced allodynia. To test this hypothesis, we determined the effect of SP600125, a widely used specific inhibitor of JNKs [2], on the activity of Wnt5a in the

regulation of gp120-induced allodynia. We first show that SP600125 deminished the effect of gp120 in decreasing the mechanical threshold (Fig. 3A). Next, we observed that i.t. Foxy5 induced a significant decrease in the mechanical threshold assessed with von Frey filaments (Fig. 3B), indicating that activation of Wnt5a signaling indeed causes mechanical allodynia. It is interesting to note that while Wnt5a recombinant protein by itself only caused a small increase of mechanical responses (Fig. 2A), Foxy5 induced a rather robust effect (Fig. 3B). This discrepancy is presumably due to different dosages of Wnt5a (20ng, i.t.) and Foxy5 (10µg, i.t.) used in these experiments. Then, we tested if SP600125 can impair Wnt5a-induced allodynia. When administered (i.t.) 30 minutes before Wnt5a (i.t.), SP600125 prevented the Wnt5a-induced decrease of the mechanical response threshold (Fig. 3B). Next, we tested if SP600125 would be able to block the activity of Wnt5a in potentiating gp120-induced allodynia. As shown in Fig. 3C, SP600125 inhibited the effect of gp120+Foxy5 on mechanical response thresholds. These data suggest that Wnt5a modulates gp120-induced allodynia via JNK signaling.

TNF α inhibitor attenuated gp120- and Foxy5-induced allodynia

Next we addressed the mechanism by which the Wnt5a/JNK signaling pathway could facilitate gp120-induced allodynia. We recently showed that this signaling pathway mediates gp120-induced up-regulation of TNF- α [22]. TNF- α is a well-established pro-inflammatory factor that plays a key role in the pathogenesis of chronic pain [21]. Our recent studies revealed that TNF- α is up-regulated in the SDH of HIV patients who developed chronic pain [35; 44] and in the spinal cord in the mouse model of HIV-associated pain [44]. Therefore, we tested the hypothesis that TNF- α is an important downstream effector that mediates Wnt5a activity in promoting gp120-induced allodynia. We used the TNF- α antagonist Enbrel (a.k.a. Etanercept), which is a dimeric fusion protein of TNF- α receptor and IgG and thus acts as a specific TNF- α scavenger. Enbrel is a FDA-approved drug that is used to treat chronic pain in patients with diseases such as rheumatoid arthritis [5], although its efficacy in treating different types of pain remains controversial [31]. Since previous studies revealed that TNF- α plays a critical role in maintaining chronic pain, we tested the effect of Enbrel on the maintenance of allodynia caused by Foxy5. Enbrel was administered 20 hours after Foxy5 injection, when the allodynia had fully developed. We found that Enbrel significantly reversed Foxy5-induced allodynia (Fig. 4A), suggesting that TNF- α is critical for Foxy5-induced mechanical allodynia. Enbrel also transiently reversed the allodynia induced by gp120+Foxy5 (Fig. 4B).

Inhibition of Wnt5a, JNK or TNF- α blocks the gp120- or Foxy5-induced potentiation of activity in SDH neurons

The results so far suggest that spinal administration of HIV-1 gp120 causes mechanical allodynia via the Wnt5a/JNK/TNF- α pathway. Next, we sought to determine the neuronal mechanisms through which the Wnt5a/JNK/TNF- α pathway regulates gp120-induced allodynia. We hypothesized that HIV gp120 protein would enhance nociceptive processing in SDH neurons through a mechanism that depends on the Wnt5a/JNK/TNF- α pathway and that inhibiting Wnt5a or JNK function would normalize activity in these neurons. To test this hypothesis, we performed extracellular single-unit recordings of 19 SDH wide dynamic range (WDR) neurons, which were defined as neurons that responded more strongly to

noxious than to innocuous stimuli applied to their receptive field in the hindpaw [27]. We recorded the responses of WDR neurons to innocuous (Brush or Press) and noxious (Pinch) mechanical stimuli applied to the hindpaw of anesthetized mice before and after topically administration of gp120 and inhibitors of Wnt5a, JNKs or TNF- α onto the lumbar spinal cord (Fig. 5). Drugs were applied into a small trough on the dorsal surface of the spinal cord for 15-30 min (see Methods). One neuron was recorded and tested in each animal. Spinal application of gp120 (100 ng) significantly increased the responses of SDH neurons to innocuous (Brush, Press) and noxious (Pinch) mechanical stimuli (Fig. 5A, 5B), which reflects “sensitization”. The gp120-induced sensitization was attenuated by co-application of a Wnt5a antagonist (Box5) (Fig. 5A). This finding indicates that Wnt5a signaling is critical for gp120 to facilitate nociceptive processing in SDH neurons. We further tested the involvement of JNK signaling. The JNK inhibitor, SP600125, significantly impaired the gp120-induced facilitation of neuronal responsiveness (Fig. 5B), indicating that JNK signaling is critical for pro-nociceptive effects of gp120 on SDH neurons.

Since i.t. Foxy5 alone was sufficient to cause allodynia, we also tested for facilitatory effects of spinally administered Foxy5 on SDH neurons. Foxy5 application potentiated the responses of SDH neurons to mechanical stimuli (Fig. 5C), and this effect was blocked by the spinal administration of either SP600125 or Enbrel. The data suggest that the activation of Wnt5a signaling can “sensitize” SDH neurons via a JNK/TNF- α pathway.

DISCUSSION

The goal of this study was to identify the molecular pathways that play a critical role in the development of HIV-associated pain. Using a gp120 i.t. mouse model that phenocopies many pathologies seen in ‘pain-positive’ HIV patients, we tested the role of Wnt5a, JNK and TNF- α . Our results show that Wnt5a, JNK and TNF- α are essential for the expression of gp120-induced allodynia. Since gp120 stimulated TNF- α expression via the Wnt5a/JNK pathway in the SDH [22], we interpret our data to suggest that a Wnt5a/JNK/TNF- α signaling pathway critically regulates the expression of gp120-induced mechanical allodynia.

To date, little is known about the molecular pathogenic mechanism of HIV-associated pain. Here we show that blockage of Wnt5a signaling impairs gp120-induced allodynia, while activation of Wnt5a signaling facilitates allodynia (Figs. 1 and 2). These findings suggest that Wnt5a signaling plays a critical role in pain pathogenesis. Consistent with this notion, Wnt5a is up-regulated in the SDH of HIV patients with manifested chronic pain [36]. In both mouse and human SDH, Wnt5a is mainly expressed in neurons [22; 36; 37]. Since the secretion and synthesis of Wnt5a are presumably stimulated by synaptic activity [4; 23], we envision a feedback interaction between the pathological pain and Wnt5a signaling: hyper-activation of SDH neurons under pathological pain conditions would stimulate Wnt5a signaling, which in return would promote pain expression. This feedback interaction may provide a mechanism for the maintenance of persistent pain.

We also show here that JNK inhibition impairs the allodynia that is induced by Foxy-5 (Fig. 3). Allodynia induced by gp120 plus Foxy-5 was blocked by the JNK-specific inhibitor

SP600125 (Fig. 3C). This finding indicates that the activity of Wnt5a in promoting gp120-induced allodynia is mediated by JNK signaling. At a molecular level, recent studies have demonstrated that gp120 activates JNK signaling via Wnt5a in the SDH [22]. Thus, gp120-Wnt5a-JNK constitutes a pathway that leads to the pathogenesis of HIV-associated pain. In support of this, gp120, Wnt5a and active JNK protein are found at higher levels in the SDH of 'pain-positive' HIV patients [35; 36; 44]. In animal models, JNK signaling has been shown to play a key role in the development of various types of pain [10]. By targeting JNK signaling, gp120-stimulated Wnt5a is connected to an important molecular process that contributes to pain pathogenesis.

TNF- α is an important pro-inflammatory cytokine that has been implicated in the pathogenesis of chronic pain in humans and animals [21]. TNF- α from glia has been suggested to play a critical role in gp120-induced neuropathic pain [47]. Our recent work revealed that the Wnt5a/JNK signaling pathway is activated by gp120 and evokes TNF- α expression [22]. In this study, we show that the TNF- α antagonist Enbrel is able to reverse mechanical allodynia induced by Foxy-5 as well as by gp120 plus Foxy-5 (Fig. 4). These findings suggest that TNF- α is a downstream effector protein of the gp120-JNK-TNF- α pathway that mediates gp120-induced allodynia. TNF- α has been shown to modulate spinal synaptic plasticity [12] and has pro-inflammatory effects on glial cells. We propose that TNF- α induced by Wnt5a/JNK signaling may contribute to gp120-induced allodynia by stimulating either neurons or glia.

Our results from single unit recordings in anesthetized animals indicate that Wnt5a and JNK signaling are essential for gp120 to cause increased responsiveness ("sensitization") of SDH neuron (Fig. 5). Our data further show that JNK signaling and TNF- α are critical for Wnt5a signaling to sensitize SDH neurons (Fig. 5). These data collectively suggest that gp120 induces central sensitization in the SDH via a Wnt5a/JNK/TNF- α pathway, which may be a neuronal mechanism for the behavioral manifestation of gp120-induced allodynia (Fig. 6). It should be noted that the acute onset (15 min) of electrophysiological effects of gp120 on WDR neurons may not accurately replicate the type of pain experienced by HIV patients, which is more likely of slower onset. Also, the peak of allodynia induced by gp120 in the behavioral testing occurred later than 15 min (2-6 hr). The data would suggest that changes at the level of individual spinal neurons precede the behavioral consequences.

In summary, this study identifies a gp120-Wnt5a-JNK-TNF- α molecular axis that is critical for the development of HIV-associated pain. The Wnt5a-JNK-TNF- α pathway may provide valuable targets for designing relevant therapeutic interventions.

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Figure 1. Wnt5a antagonists impaired gp120-induced allodynia

A. Box5 administration (i.t.) attenuated gp120-induced potentiation of response rate to mechanical stimulation. B. Statistical analysis of data at selected time points in Fig. 1A (n=6 mice). C. Analysis of hindpaw phenotypes. Compared with controls (C1), gp120 (i.t.) caused a toe-curling phenotype in the hindpaws (arrows), which also displayed increased redness (C2). Both toe-curling and increased redness were attenuated by Box5 administration (C3). C4: Quantitative analysis of the toe-curling phenotype. D. Wnt5a antibody also impaired the expression of gp120-induced allodynia. *, p<0.05; **, p<0.01; ***, p<0.001; #, p>0.05; NS, not significant :one-way ANOVA with Tukey's post-test in B, while, two-way ANOVA with Bonferroni post-test in D.

Figure 2. Wnt5a agonists potentiate gp120-induced allodynia

A. Administration of purified Wnt5a (i.t.), but not heat-inactivated Wnt5a, enhanced gp120-induced potentiation of response rate to mechanical stimulation. B. Statistical analysis of data at selected time points in Fig. 2A (n=6 mice). C. Foxy5 potentiated gp120-induced mechanical allodynia. *, p<0.05; **, p<0.01; ***, p<0.001; , p<0.001; ns, p>0.05 (One-way ANOVA with Tukey's posttests).

Figure 3. Inhibition of JNK signaling attenuated allodynia induced by gp120 or Foxy5

A. JNK inhibitor SP600125 blocked the expression of gp120-induced mechanical allodynia.

B. SP600125 attenuated Foxy5-induced allodynia. C. SP600125 blocked allodynia induced by gp120 and Foxy5. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; , $p < 0.01$; , $p < 0.001$; xxx, $p < 0.001$; #, $p > 0.05$ Two-way ANOVA with Bonferroni post-test in A, B and C.

Figure 4. TNF- α antagonist (Enbrel) reversed allodynia induced by Foxy5 and gp120

A. Enbrel administration (i.t.) reversed the Foxy5-induced allodynia. B. Enbrel transiently reversed allodynia induced by gp120 and Foxy5. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Δ, $p < 0.001$; xxx, $p < 0.001$; #, $p > 0.05$; NS, not significant (Two-way ANOVA with Bonferroni post-test).

Figure 5. Antagonists of Wnt5a, JNK or TNF- α blocked gp120- or Foxy5-induced facilitation of nociceptive processing ("sensitization") in SDH neurons

A. gp120 increased the responses (number of action potentials, "spikes") of SDH neurons to innocuous and noxious mechanical stimuli applied to the hindpaw (*, $p < 0.05$; **, $p < 0.01$). Box-5 blocked the effect of gp120 (+, $p < 0.05$). B. SP600125 blocked gp120-induced increase in responsiveness of SDH neurons (+, $p < 0.05$). C. Foxy5 increased the responsiveness of SDH neurons (*, $p < 0.05$; **, $p < 0.01$). The facilitatory effects was blocked by SP600125 (++, $p < 0.01$). D. Enbrel blocked Foxy5-induced increase in responsiveness of SDH neurons (+, $p < 0.05$, ++, $p < 0.01$). One-way ANOVA with Tukey's posttests. '*' is used to indicate the statistical significance of the spike difference induced by the same stimulations between 'pre-drug' and 'gp120' (or 'Foxy5') groups, while '+' between the 'gp120' (or 'Foxy5') groups and the 'gp120' or 'Foxy5' plus individual antagonists (Box-5, SP600125, Enbrel).

Figure 6.
A model of the Wnt5a/JNK/TNF- α pathway in the development of gp120-induced allodynia.