学位論文

# Role of Tumor Necrosis Factor Receptor 1---Reactive Oxygen Species---Caspase 11 Pathway in Neuropathic Pain Mediated by HIV gp120 with Morphine in Rats

（HIV gp120 とモルヒネにより誘発された神経障害性疼痛モデルラット
における TNFR1-活性酸素種-カスペース 11 経路の役割）

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ORIGINAL LABORATORY RESEARCH REPORT

# Role of Tumor Necrosis Factor Receptor 1—Reactive Oxygen Species—Caspase 11 Pathway in Neuropathic Pain Mediated by HIV gp120 With Morphine in Rats

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**BACKGROUND:** Recent clinical research suggests that repeated use of opioid pain medications can increase neuropathic pain in people living with human immunodeficiency virus (HIV; PLWH). Therefore, it is significant to elucidate the exact mechanisms of HIV-related chronic pain. HIV infection and chronic morphine induce proinflammatory factors, such as tumor necrosis factor (TNF)α acting through tumor necrosis factor receptor I (TNFRI). HIV coat proteins and/or chronic morphine increase mitochondrial superoxide in the spinal cord dorsal horn (SCDH). Recently, emerging cytoplasmic caspase-11 is defined as a noncanonical inflammasome and can be activated by reactive oxygen species (ROS). Here, we tested our hypothesis that HIV coat glycoprotein gp120 with chronic morphine activates a TNFRI-mtROS-caspase-11 pathway in rats, which increases neuroinflammation and neuropathic pain.

**METHODS:** Neuropathic pain was induced by repeated administration of recombinant gp120 with morphine (gp120/M) in rats. Mechanical allodynia was assessed using von Frey filaments, and thermal latency using hotplate test. Protein expression of spinal TNFRI and cleaved caspase-11 was examined using western blots. The image of spinal mitochondrial superoxide was examined using MitoSox Red (mitochondrial superoxide indicator) image assay. Immunohistochemistry was used to examine the location of TNFRI and caspase-11 in the SCDH. Intrathecal administration of antisense oligodeoxynucleotide (AS-ODN) against TNFRI, caspase-11 siRNA, or a scavenger of mitochondrial superoxide was given for antinociceptive effects. Statistical tests were done using analysis of variance (1- or 2-way), or 2-tailed *t* test.

**RESULTS:** Intrathecal gp120/M induced mechanical allodynia and thermal hyperalgesia lasting for 3 weeks ($P < .001$). Gp120/M increased the expression of spinal TNFRI, mitochondrial superoxide, and cleaved caspase-11. Immunohistochemistry showed that TNFRI and caspase-11 were mainly expressed in the neurons of the SCDH. Intrathecal administration of antisense oligonucleotides against TNFRI, Mito-Tempol (a scavenger of mitochondrial superoxide), or caspase-11 siRNA reduced mechanical allodynia and thermal hyperalgesia in the gp120/M neuropathic pain model. Spinal knockdown of TNFRI reduced MitoSox profile cell number in the SCDH; intrathecal Mito-T decreased spinal caspase-11 expression in gp120/M rats. In the cultured B35 neurons treated with TNFα, pretreatment with Mito-Tempol reduced active caspase-11 in the neurons.

**CONCLUSIONS:** These results suggest that spinal TNFRI-mtROS-caspase 11 signal pathway plays a critical role in the HIV-associated neuropathic pain state, providing a novel approach to treating chronic pain in PLWH with opioids. (Anesth Analg 2023;XXX:00–00)

## KEY POINTS

- **Question:** What are the molecular factors involved in the human immunodeficiency virus (HIV)-associated neuropathic pain state?
- **Findings:** HIV-associated neuropathic pain increased spinal tumor necrosis factor receptor I (TNFRI), reactive oxygen species (ROS), and caspase-11.
- **Meaning:** Inhibition of TNFRI, ROS, or caspase-11 can produce antinociceptive effect.

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## GLOSSARY

**ANOVA** = analysis of variance; **ART** = antiretroviral therapies; **AS-ODN** = antisense oligodeoxynucleotide; **AUC** = area under the effect-time curves; **BMDM** = bone marrow–derived macrophages; **casp-11 siR** = siRNA of rat caspase-11; **CNS** = central nervous system; **Drp1** = dynamin-related protein 1; **GFAP** = glial fibrillary acidic protein; **HIV** = human immunodeficiency virus; **HIV-NP** = HIV-neuropathic pain; **IL** = interleukin; **IR** = immunoreactivity; **JNK** = c-Jun N-terminal kinase; **LSD** = least significant difference; **mm-siR** = mismatch siRNA; **mmODN** = mismatch oligodeoxynucleotide; **PLWH** = people living with human immunodeficiency virus; **ROS** = reactive oxygen species; **RSA** = rat serum albumin; **SCDH** = spinal cord dorsal horn; **TLR4** = toll-like receptor 4; **TNFRI** = tumor necrosis factor receptor I

In people living with human immunodeficiency virus (HIV; PLWH), despite effective antiretroviral therapies (ART), chronic pain remains an often-reported issue.[1] Opioid use disorder in PLWH is prevalent. Repeated use of opioids enhances chronic pain in HIV patients.[2] Recent evidence showed that perioperative morphine prolong postoperative pain in rats.[3] Chronic morphine potentiates HIV glycoprotein gp120-induced pain in rodents.[4] The exact molecular mechanisms of interaction of HIV with opioids are still elusive, to which no effective treatment currently exists.

Road and Hoke[5] reported that HIV infection is a risk factor for developing peripheral neuropathy because of the resultant toxic viral products. With the current less or non-neurotoxic ART in PLWH, low levels of HIV and gp120 persist in viral repositories such as the central nervous system (CNS) glia and/or lymphoid tissues,[6] contributing to development of HIV neuropathy.[7] There are remarkable reaction of astrocytes and increase in tumor necrosis factor-alpha (TNFα) in the spinal cord dorsal horn (SCDH) of the "pain-positive" HIV patients, suggesting that TNFα play a role in the maintenance of HIV-associated chronic pain.[8] We have reported that gp120 application onto the sciatic nerve induces spinal glial activity releasing TNFα in the SCDH; blocking tumor necrosis factor receptor I (TNFRI) signal reduces gp120-induced mechanical allodynia.[9] Chronic morphine induces neuroinflammation and release of proinflammatory factors (such as TNFα, etc) through activating glia.[10–12] Evidence demonstrated that morphine activates glia by nonstereoselectively binding to glial toll-like receptor 4 (TLR4) accessory proteins,[11] leading to subsequent TLR4 signaling activation and release of inflammatory factors.

Neuronal mitochondrial superoxide ($mtO_2^{\bullet-}$) in the spinal cord is the major source of reactive oxygen species (ROS) in pain.[13] HIV gp120 increased $mtO_2^{\bullet-}$ in the SCDH, and intrathecal administration of mitochondrial-targeted superoxide scavenger reduced HIV-neuropathic pain (HIV-NP) behavior.[14] HIV gp120 induces ROS signaling through TNFα and its receptors.[15] Our recent study showed that chronic morphine induced mitochondrial ROS in the SCDH.[16] The exact mechanism of mitochondrial ROS induced by TNFRI signal is not clearly understood. It is possible that TNFRI signal trigger ROS through dynamin-related protein 1 (Drp1, a mitochondrial fission factor).[17]

It is known that inflammasomes are cytosolic multiprotein complexes in response to infection and stress or pain.[18,19] Caspase-11 is an activator and regulator of inflammatory responses. Emerging evidence indicated that caspase-11 noncanonical inflammasome played a vital role in many diseases.[20] Recent evidence showed that increased macrophage ROS enhances caspase-11 expression and activation.[21] However, it is not clear about the role of caspase-11 in neuropathic pain and the upstream factor of caspase-11. Here, we hypothesize that the spinal TNFRI—mitochondrial superoxide—caspase-11 pathway plays an important role in neuropathic pain evoked by the combination of spinal gp120 and morphine (gp120/M).

## METHODS

### Animals

Sprague-Dawley rats (male and female around 8 weeks old, Charles River Laboratories, Wilmington, MA) were housed 1 to 3 per cage approximately 7 days before the beginning of all studies. The study was approved by the University of Miami Animal Care and Use Committee. All animals were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This article adheres to the Animal Research: Reporting of in vivo Experiments guidelines.

### Intrathecal Catheter Implantation and Neuropathic Pain Produced by gp120 With Morphine

For intrathecal catheter implantation, under isoflurane anesthesia, a polyethylene 10 catheter was implanted.[16] The catheter was advanced 8.5 cm caudally through an incision in the atlanto-occipital membrane to position its tip at the level of the lumbar enlargement. The catheter rostral tip was passed subcutaneously and externalized on top of the skull. Rats showing neurological deficits after implantation were excluded. Rats were allowed to recover after surgery during 7 days before treatment started. Neuropathic pain was induced by intrathecal recombinant gp120MN (0.2 μg) with morphine (3 μg) daily for 5 days. In other 3 groups, rats received 0.1% rat serum albumin (RSA, vehicle) plus

saline as control (veh + sal), vehicle plus morphine (veh + M), or gp120 plus saline (gp120 + sal).

### Mechanical Threshold and Thermal Latency

For determining mechanical threshold, we used a series of von Frey filaments (Stoelting, Wood Dale, and IL). Rats were put in nontransparent cubicles on a mesh floor to habituate for at least 30 minutes in the test day. The fibers in ascending order of strength were applied serially to the hind-paw, and mechanical threshold was determined using the up-and-down method as described previously.[16] For thermal latency, animals were placed on the hotplate maintained at a temperature of 51.5°C ± 0.3°C. The latency from the placement of animals on the plate to the positive response of their hind paw (rapid withdrawal, licking, or jumping) was measured. Cutoff time 30 seconds was set to prevent potential tissue damage. Behavioral testing in drug treatments, was performed by a blinded examiner. Animals were randomly assigned to groups.

Immunohistochemistry, Western blots, and detection of $mtO_2^{\bullet-}$ production; cultured neuronal cells with rTNFα treatment; drugs and data; and statistical analysis are shown in Supplemental Digital Content 1, Supplemental Materials, http://links.lww.com/AA/E165.

## RESULTS

### Intrathecal gp120 With Morphine Induced Mechanical Allodynia and Thermal Hyperalgesia

To examine if spinal gp120/M induced neuropathic pain, we injected intrathecally recombinant gp120 and morphine (gp120 + M) for 5 days, and sham group of rats received intrathecal 0.1% rabbit serum albumin (vehicle)/saline (sal) (veh + sal) (see Supplemental Digital Content 1, Supplemental Material, http://links.lww.com/AA/E165, for details). In 2 separate groups, rats received vehicle plus morphine (veh + M), or gp120 plus saline (gp120 + sal). HIV-1 gp120/M caused a persistent lowered mechanical threshold compared to other groups (veh + sal, veh + M, and gp120 + sal) ($P < .001$, 2-way analysis of variance [ANOVA] repeated-measures, n = 8, Supplemental Digital Content 1, Supplemental Material, Figure S1A, http://links.lww.com/AA/E165). Mechanical threshold in gp120/M rats was markedly lower than that in sham rats at day 5, 7, 10, 14, and 21 ($P < .001$, 2-way ANOVA repeated measure, n = 8 rats). Using the trapezoidal rule to express the overall magnitude and duration of effect, we calculated the area under the effect-time curves (AUC) depicting the mechanical threshold/thermal latency over time as difference or change among groups. The AUC in the gp120 + M group was significantly smaller than that in other groups ($P < .01$, 1-way ANOVA, n = 8 rats, Supplemental Digital Content 1, Supplemental Material, Figure S1B, http://links.lww.com/AA/E165). Similarly, intrathecal gp120/M decreased thermal latency (thermal hyperalgesia) compared to other groups in hot plate test ($P < .001$, 2-way ANOVA repeated measures, n = 8; Supplemental Digital Content 1, Supplemental Material, Figure S2A, http://links.lww.com/AA/E165). Thermal latency in gp120/M animals was markedly lower than that in sham at day 5, 7, 10, 14, and 21, 2-way ANOVA, n = 8. The AUC of gp120/M rats was significantly smaller than that of other groups ($P < .001$, 1-way ANOVA, n = 8; Supplemental Digital Content 1, Supplemental Material, Figure S2B, http://links.lww.com/AA/E165).

### Role of TNFRI in the gp120/M Model

To identify the role of TNFRI in spinal gp120/M pain model, the SCDH was collected at day 10 after gp120/M for western blots. Gp120/M increased the expression of TNFRI compared to sham (veh + sal) group ($P < .05$, 2-tailed $t$ test, n = 5; Figure 1A). We examine the distribution of TNFRI in the SCDH using immunohistochemistry. At day 10 since gp120/M, animals were perfused and the SCDH was harvested for immunohistochemistry. Figure 1B shows that immunoreactivity (IR) of TNFRI at the SCDH with low and high magnification images at sham and gp120/M groups. Double-immunostaining showed that IR of TNFRI mainly colocalized with NeuN (a selective marker for neuron, Figure 1C), but neither GFAP (a selective marker for astrocytes) nor OX42 (a selective marker for microglia) (Figure 1C), suggesting that TNFRI was mainly expressed in the neurons of SCDH, but not glia in gp120/M-treated rats. Then, we examined the antinociceptive effect of knockdown of TNFRI using intrathecal administration of antisense oligodeoxynucleotide against rat TNFRI (AS-TNFRI) at day 7 of gp120/M. Increased mechanical threshold in the von Frey test after spinal AS-TNFRI was dose-dependent in contrast to mismatch oligodeoxynucleotide (mmODN; $P < .001$, 2-way ANOVA repeated measure, n = 6, Figure 1D). Increased AUC of mechanical threshold was also dose-dependent after AS-TNFRI (1-way ANOVA, Fisher least significant difference [LSD] test, n = 6, Figure 1E). Similarly, spinal AS-TNFRI increased thermal latency in a dose-dependent way as compared to mmODN in the hot plate test ($P < .001$, 2-way ANOVA repeated measure, n = 6, Figure 1F). AS-TNFRI also increased the AUC of thermal latency in a dose-dependent fashion (1-way ANOVA, n = 6, Figure 1G).

### Effect of Antioxidative Mito-Tempol in the gp120/M Model

We examined antinociceptive effect of intrathecal administration of antioxidative Mito-Tempol (Mito-T) in the chronic gp120/M pain model. Spinal Mito-T increased mechanical threshold compared to saline (vehicle) in a dose-dependent fashion ($P < .01$, 2-way

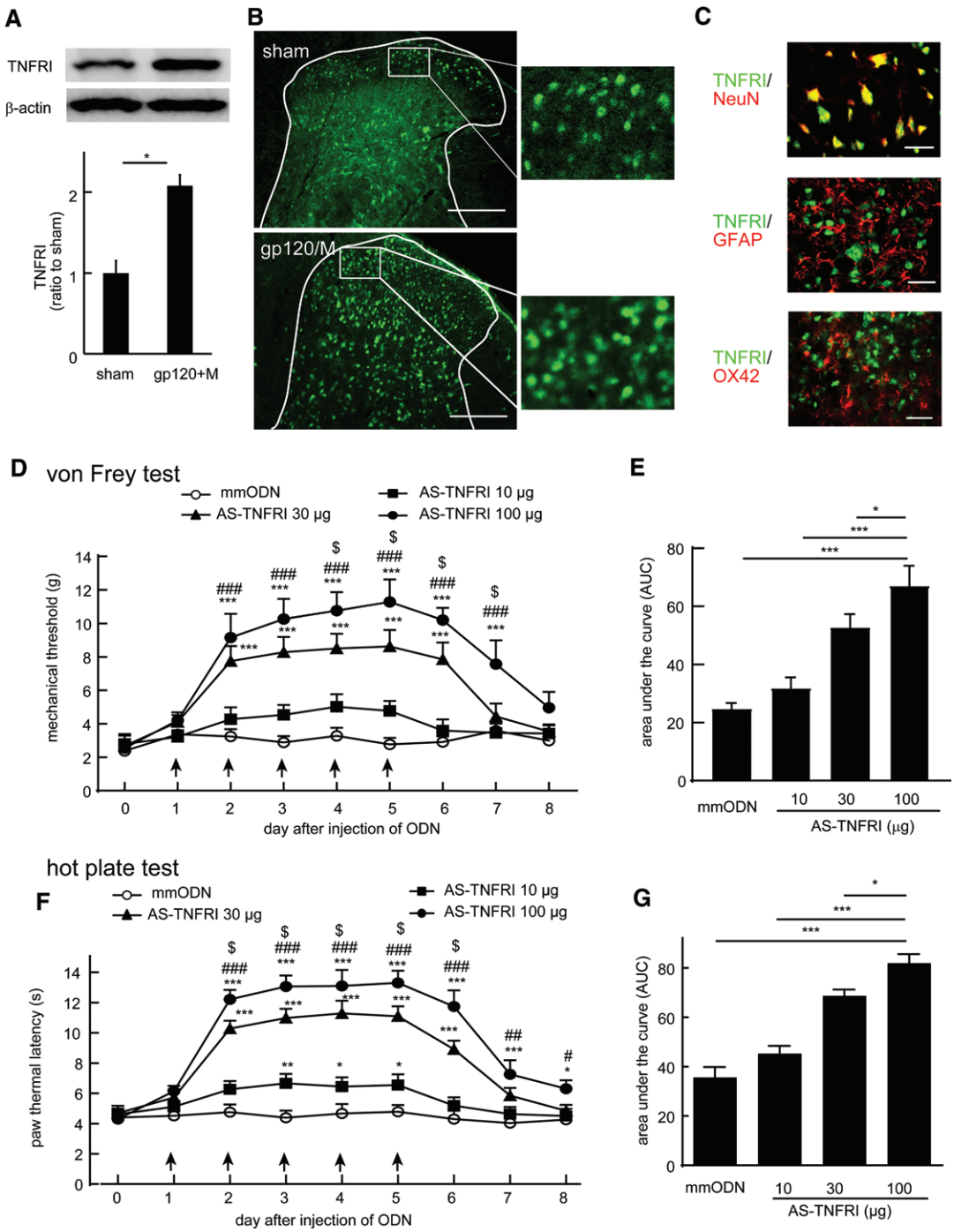


**Figure 1.** Role of TNFRI in the spinal gp120 and morphine model. A, Western blots showed that intrathecal gp120/M significantly increased the expression of TNFRI compared to sham group, $^{*}P < .05$, 2-tailed *t* test, n = 5. B, Representative images of IR of TNFRI at the SCDH with low and high magnification images of sham and gp120/M groups, scale bar, 200 μm. C, Double-immunostaining showed that TNFRI mainly colocalized with NeuN (a marker of neurons), but neither GFAP (a marker of astrocytes), nor OX42 (a marker of microglia), scale bar, 50 μm. D–G, Antinociceptive effect of intrathecal administration of antisense oligodeoxynucleotide against rat TNFRI (AS-TNFRI) at day 7 after gp120/M. D, Spinal AS-TNFRI dose-dependently increased mechanical threshold compared to mmODN, $F_{(24,160)\ \text{interaction}} = 7.32$, $P < .0001$; $F_{(8,160)\ \text{main effect time}} = 32.04$, $P < .0001$; $F_{(3,20)\ \text{main effect treatment}} = 16.41$, $P < .0001$; 2-way ANOVA repeated measure, n = 6. $^{***}P < .001$ vs mmODN; $^{\#\#\#}P < .001$ vs AS-TNFRI 10 μg, $^{\$}P < .05$ vs AS-TNFRI 30 μg; 2-way ANOVA multiple comparisons, Fisher LSD test. n = 6. Arrows shows ODN injection. E, AS-TNFRI dose-dependently increased AUC, $^{*}P < .05$, $^{***}P < .001$, 1-way ANOVA Fisher LSD test, n = 6. F, Spinal AS-TNFRI dose-dependently increased thermal latency compared to mmODN, $F_{(24,160)\ \text{interaction}} = 11.60$, $P < .0001$; $F_{(8,160)}$main effect time = 58.49, $P < .0001$; $F_{(3,20)\text{main effect treatment}} = 36.81$, $P < .0001$; 2-way ANOVA repeated measure, n = 6. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs mmODN; $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs AS-TNFRI 10 μg; $^{\$}P < .05$ vs AS-TNFRI 30 μg; 2-way ANOVA multiple comparisons, Fisher LSD test. n = 6. Arrows show ODN injection. G, AS-TNFRI dose-dependently increased the AUC in the hot plate test, $^{*}P < .05$, $^{***}P < .001$, 1-way ANOVA Fisher LSD test, n = 6. ANOVA indicates analysis of variance; AUC, area under the effect-time curves; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LSD, least significant difference; mmODN, mismatch oligodeoxynucleotide; SCDH, spinal cord dorsal horn; TNFRI, tumor necrosis factor receptor I.

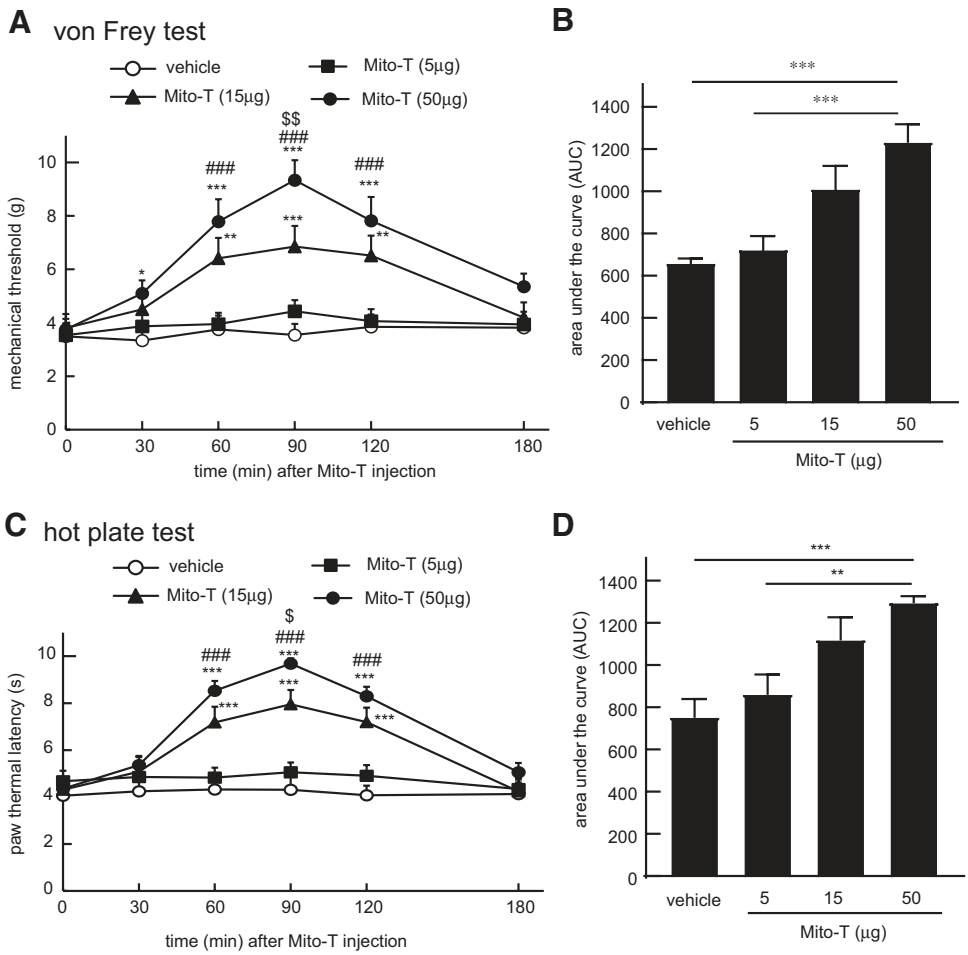


**Figure 2.** The antinociceptive effect of intrathecal administration of antioxidative Mito-Tempol (Mito-T) on gp120/M. A, Spinal Mito-T dose-dependently increased mechanical threshold compared to vehicle, $F_{(15,100)\ \text{interaction}} = 5.389$, $P < .001$; $F_{(5,100)\ \text{main effect time}} = 20.20$, $P < .001$; $F_{(3,20)\ \text{main effect treatment}} = 10.35$, $P < .001$; 2-way ANOVA repeated measure, n = 6. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs vehicle; $^{\#\#\#}P < .001$ vs Mito-T (5 μg); $^{\$\$}P < .01$ vs Mito-T (15 μg), 2-way ANOVA multiple comparisons, Fisher LSD test, n = 6. B, Mito-T dose-dependently increased AUC, $^{***}P < .001$ vs vehicle, 1-way ANOVA Fisher LSD test, n = 6. C, Spinal Mito-T dose-dependently increased thermal latency compared to vehicle, $F_{(15,100)\ \text{interaction}} = 21.03$, $P < .001$; $F_{(5,100)\ \text{main effect time}} = 76.81$, $P < .001$; $F_{(3,20)\ \text{main effect treatment}} = 6.957$, $P < .01$, 2-way ANOVA repeated measure, n = 6. $^{***}P < .001$ vs vehicle, $^{\#\#\#}P < .001$ vs Mito-T (5 μg); $^{\$}P < .05$ vs Mito-T (15 μg), 2-way ANOVA multiple comparisons, Fisher LSD test. n = 6. D, Mito-T dose-dependently increased AUC in hot plate test, $^{**}P < .01$, $^{***}P < .001$ vs vehicle, 1-way ANOVA Fisher LSD test, n = 6. ANOVA indicates analysis of variance; AUC, area under the effect-time curves; LSD, least significant difference; mmODN, mismatch oligodeoxynucleotide.

ANOVA repeated measure, n = 6, Figure 2A). Mito-T elevated the AUC, 1-way ANOVA with Fisher LSD test, n = 6 (Figure 2B). Similarly, increased thermal latency after spinal Mito-T was dose-dependent ($P <$ .05, 2-way ANOVA repeated measure in the hot plate test, Figure 2C). Mito-T also significantly increased the AUC in the hot plate test, 1-way ANOVA Fisher LSD test (Figure 2D).

### Role of Caspase-11 in the gp120/M Model

To determine the role of caspase-11 in the gp120/M model, the SCDH was collected at day 10 after gp120/M for western blots. Gp120/M significantly increased the expression of cleaved caspase-11 compared to sham ($P < .05$, 2-tailed $t$ test, Figure 3A). We examined spinal caspase-11 IR at day 10 after gp120/M using immunohistochemistry. Figure 3B shows the distribution of caspase-11 of the SCDH in sham and gp120/M groups at low and high magnification images. Double-immunostaining showed that caspase-11 IR mainly colocalized with NeuN (Figure 3C), but neither GFAP, nor Iba1 (a marker of microglia) (Figure 3C), suggesting that caspase-11 was mainly expressed in the neurons. We also examined the antinociceptive effect of knockdown of caspase-11 using intrathecal siRNA against caspase-11 (casp-11 siR) on gp120/M. Casp-11 siR dose-dependently increased mechanical threshold, compared to mismatch siRNA (mm-siR) in the von Frey test ($P < .001$, 2-way ANOVA repeated measure, Figure 3D). Casp-11 siR dose-dependently increased the AUC, 1-way ANOVA Fisher's LSD test, n = 7–10 (Figure 3E). Spinal casp-11siR dose-dependently increased thermal

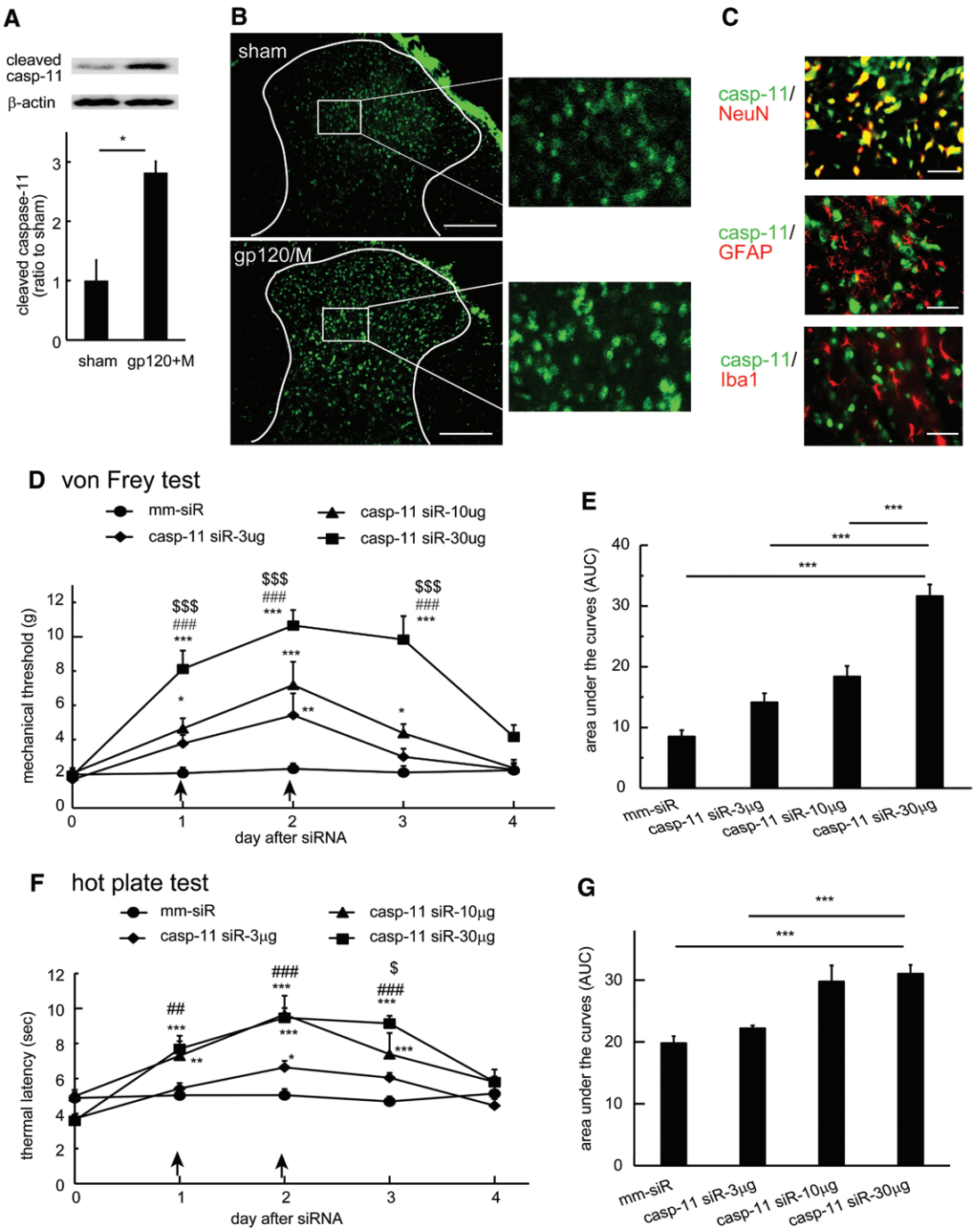


**Figure 3.** Role of caspase-11 in the spinal gp120 and morphine model. A, Western blots shows that gp120/M significantly increased the expression of caspase-11 (cleaved, 25 kDa) compared to sham, $*P < .05$, 2-tailed *t* test, n = 5. B, IR of caspase-11 at the SCDH with low and high magnification images at sham and gp120/M groups, scale bar, 200 μm. C, Double-immunostaining showed that IR of caspase-11 mainly colocalized with NeuN (a marker of neurons), but neither GFAP (a marker of astrocytes), nor Iba1 (a marker of microglia), scale bar, 50 μm. D–G, The antinociceptive effect of intrathecal administration of casp-11 siR on gp120/M. D, Spinal casp-11siR dose-dependently increased mechanical threshold compared to mm-siR, $F_{(12,116)\ \text{interaction}} = 5.355$, $P < .0001$; $F_{(4,116)\ \text{main effect time}} = 23.61$, $P < .0001$; $F_{(3,29)\ \text{main effect treatment}} = 36.64$, $P < .0001$; 2-way ANOVA repeated measure, n = 7–10. $*P < .05$, $**P < .01$, $*** P < .001$ vs mm-siR; $^{\#\#\#}P < .001$ vs casp-11 siR-3 μg; $^{\$\$\$}P < .001$ vs casp-11 siR-10 μg; 2-way ANOVA multiple comparisons, Fisher LSD test, n = 7–10. Arrows show siRNA injection. E, Spinal casp-11siR dose-dependently increased AUC, $***P < .001$ 1-way ANOVA Fisher LSD test, n = 7–10. F, Spinal casp-11siR dose-dependently increased thermal latency compared to mm-siR, $F_{(12,116)\ \text{interaction}} = 6.359$, $P < .001$; $F_{(4,116)\ \text{main effect time}} = 34.93$, $P < .001$; $F_{(3,29)\ \text{main effect treatment}} = 10.11$, $P < .001$; 2-way ANOVA repeated measure, n = 7–10. $*P < .05$, $**P < .01$, $***P < .001$ vs mm-siR; $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs casp-11 siR-3 μg; $^{\$}P < .05$ vs casp-11 siR 10 μg; 2-way ANOVA multiple comparisons, Fisher LSD test. n = 6. Arrows show siRNA injection. G, Spinal casp-11siR dose-dependently increased the AUC in the hot plate test, $***P < .001$, 1-way ANOVA Fisher LSD test, n = 7–10. ANOVA indicates analysis of variance; AUC, area under the effect-time curves; casp-11 siR, siRNA of rat caspase-11; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LSD, least significant difference; mm-siR, mismatch siRNA; SCDH, spinal cord dorsal horn.

latency compared to mm-siR in hot plate test ($P < .001$, 2-way ANOVA repeated measure, n = 7–10, Figure 3F). Casp-11 siR dose-dependently elevated AUC, 1-way ANOVA with Fisher LSD test (Figure 3G).

### Effect of TNFRI Knockdown on Mitochondrial Oxidative Stress in gp120/M

To determine whether oxidative stress was downstream factor of TNFRI in gp120/M model, we tested the effect of knockdown of TNFRI using spinal AS-TNFRI on spinal $mtO_2^{\bullet-}$. At day 10 of gp120/M, MitoSox reagent was given intrathecally 1.5 hours before perfusion. Figure 4A–D shows representative images of MitoSox-positive cell profiles in the laminar III–V in groups of sham/mmODN, sham/AS-TNFRI, gp120-M/mmODN, or gp120-M/AS-TNFRI. We found a significant increase in MitoSox-positive cells at lamina I-II in the gp120-M/mmODN compared to that in the sham/mmODN ($P < .001$, 1-way ANOVA Fisher LSD test, n = 5–6, Figure 4E). The number of MitoSox-positive cells in the gp120-M/AS-TNFRI rats was less than that in the gp120-M/mmODN at SCDH lamina I–II ($P < .001$, 1-way ANOVA Fisher LSD test, Figure 4E). Similarly, MitoSox-positive cells were increased at lamina III–V (Figure 4F) or total amount of the positive cells of lamina I–V (Figure 4G) in gp120-M/mmODN compared to that in sham/mmODN. In gp120-M/AS-TNFRI group, the number of MitoSox-positive cells was less than that in gp120-M/mmODN at lamina III–V (Figure 4F) or in the total number at lamina I–V(Figure 4G).

Double-immunostaining demonstrated that TNFRI was colocalized with MitoSox cell profiles (Figure 5A–C). MitoSox-positive cell profiles were colocalized with caspase-11 (Figure 5D–F). TNFRI was also colocalized with caspase-11 immunostaining (Figure 5G–I).

### Effect of Spinal Mito-Tempol on Active Caspase-11 in gp120/M Model of Rats

Recent evidence from in vitro studies showed that increased macrophage ROS enhances caspase-11 expression and activation.[21] To determine the role of mitochondrial oxidative stress on active caspase-11 in gp120/M model of rats, we investigated the effect of spinal Mito-T on caspase-11 expression. Around 10 days after gp120/M, animals received spinal Mito-T, and SCDH was harvested for expression of caspase-11. Western blots demonstrated no significant difference in caspase-11 expression between sham/vehicle and sham/Mito-T (Figure 6A). However, a significant increase in caspase-11 was seen in gp120-M/vehicle rats compared to sham/vehicle ($P < .001$, 1-way ANOVA Fisher LSD test, Figure 6B), and a decrease in caspase-11 in gp120-M/Mito-T rats as compared to gp120-M/vehicle group ($P < .001$, 1-way ANOVA Fisher LSD test, Figure 6B).

### Suppression of Active Caspase-11 by Antioxidative Stress in the Cultured B35 Neuronal Cells

Our previous data show that rTNFα increased $mtO_2^{\bullet-}$ in cultured B35 cells, and that Mito-T reversed the increased $mtO_2^{\bullet-}$.[15] To further determine the causality between oxidative stress and caspase-11, we used neuronal B35 cells with Mito-T under rTNFα application. Western blots showed no significant difference in caspase-11 between vehicle/sal and Mito-T/sal groups (Figure 6C). However, a significant increase in caspase-11 in vehicle/TNFα group compared to vehicle/saline was seen ($P < .05$, 1-way ANOVA Fisher LSD test, n = 4, Figure 6D), and a decrease in caspase-11 in Mito-T/rTNFα compared to vehicle/rTNFα group ($P < .05$, 1-way ANOVA Fisher LSD test, Figure 6D).

## DISCUSSION

In this study, we found that (1) neuropathic pain induced by gp120/M increased TNFRI expression, mitochondrial superoxide, and cleaved caspase-11 in the rat SCDH; (2) spinal knockdown of TNFRI or caspase-11, or scavenger of mitochondrial superoxide reduced mechanical allodynia and thermal hyperalgesia, and (3) knockdown of TNFRI reduced mitochondrial oxidative stress, and (4) Mito-T decreased spinal caspase-11 expression in gp120/M rats and in cultured neuronal cells, suggesting that the TNFRI—ROS—caspase-11 pathway plays a critical role in the neuropathic pain model in rats. As patients with HIV enjoy longer life spans with effective antiretroviral therapy, HIV-neuropathic pain is a common and often disabling neurological complication. Currently opioid use disorder in the United States is a great concern. This serious health issue is further exacerbated by the chronic treatment of HIV-related neuropathic pain, often complicated by increased severity of neurological deficits. One clinic report showed that patients with HIV on daily opioid therapy experienced pain that was significantly worse than those with chronic pain diagnoses who were not on daily opioid therapy.[2] The exact molecular mechanisms/pathway of the interaction of HIV and opioids are poorly understood.

Traditionally, HIV-related sensory polyneuropathy mainly includes the HIV infection-related distal sensory polyneuropathy and antiretroviral toxic neuropathies. In the past 10 years, old neurotoxic antiretroviral therapy (eg, D-drugs, ddC, DDI, d4T) is either no longer used or is rapidly waning in the United States.[22,23] Peripheral neurotoxicity induced by current less toxic or non-neurotoxic ART is minimal.[22,23] Nonetheless, persistence of low-titer of HIV

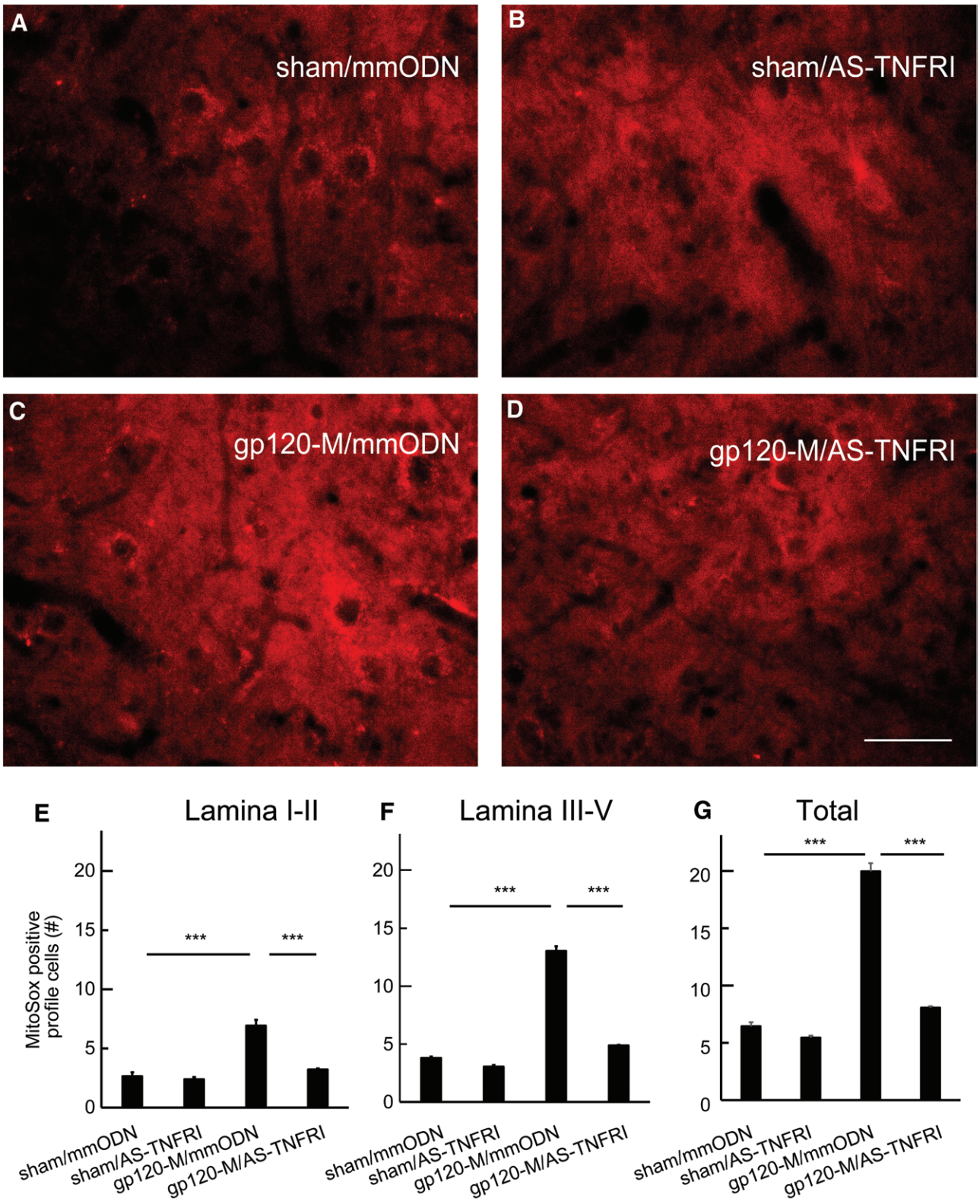


**Figure 4.** Effect of knockdown of TNFRI on mitochondrial ROS in SCDH. A–D, Representative image of MitoSox in the SCDH in (A) sham/mmODN, (B) sham/AS-TNFRI, (C) gp120-M/mmODN, and (D) gp120-M/AS-TNFRI. Scale bar, 50 μm. E, An increase in MitoSox profile cell number at Laminar I–II (E), Lamina III–V (F), or total lamina I–V (G) of the SCDH in the gp120-M/mmODN group compared to the sham/mmODN groups, ***$P < .001$, 1-way ANOVA Fisher LSD test, n = 5–6. The number of MitoSox-positive cells of gp120-M/AS-TNFRI group was lower than that of the gp120-M/mmODN group in Laminar I–II (E), Lamina III–V (F), or total lamina I–V (G), ***$P < .001$, 1-way ANOVA Fisher LSD test, n = 6. ANOVA indicates analysis of variance; LSD, least significant difference; mmODN, mismatch oligodeoxynucleotide; ROS, reactive oxygen species; SCDH, spinal cord dorsal horn; TNFRI, tumor necrosis factor receptor I.

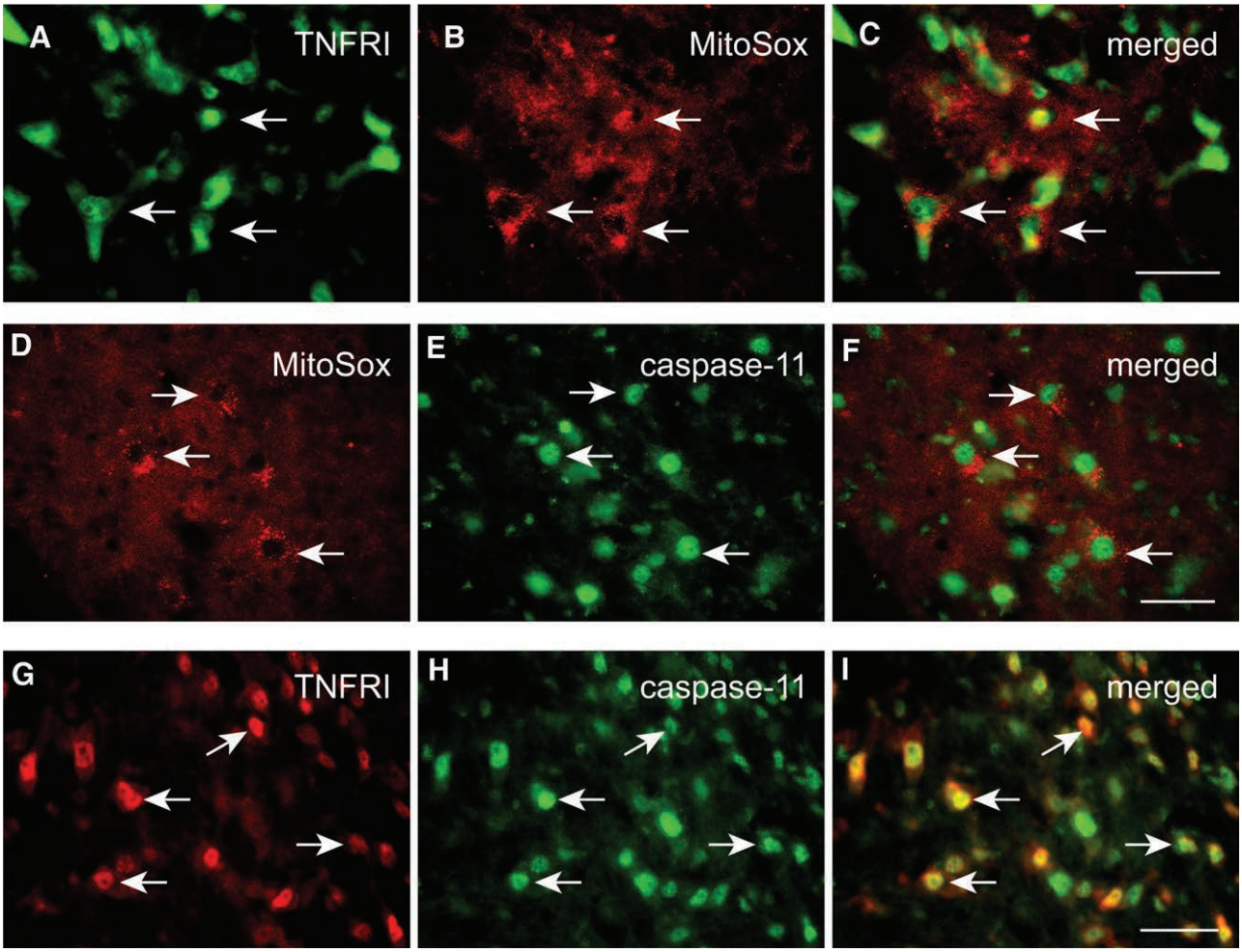


**Figure 5.** Colocalization of TNFRI or MitoSox with caspase-11 in the SCDH in gp120/M rats. A–C, TNFRI immunoreactivity was colocalized with MitoSox-positive cell profiles. D–F, MitoSox cell profiles were colocalized with caspase-11 immunoreactivity. G–I, TNFRI immunoreactivity was colocalized with caspase-11 immunostaining, scale bar, 50 μm. SCDH indicates spinal cord dorsal horn; TNFRI, tumor necrosis factor receptor I.

in reservoir sites despite effective ART still contributes to the development of chronic HIV neuropathy.[5,7] HIV gp120 increases inflammatory products, such as TNFα that induces neurotoxicity in neurons through its receptor TNFRI.[24] Evidence demonstrated that morphine activates glia and induces neuroinflammatory effects by nonstereoselectively binding to glial TLR4 accessory proteins,[11] leading to subsequent TLR4 signal activity and release of proinflammatory factors. Accumulated evidence showed that chronic morphine activates glia to induce neuroinflammation and release of proinflammatory factors (such as TNFα, etc).[10–12] Clinical studies show that repeated use of opioids such as morphine can increase chronic pain in PLWH,[2] and this is likely due to neuroinflammation.[11,25,26] Our previous studies show that blocking TNF receptor signal with soluble TNF receptor reduces HIV gp120-induced mechanical allodynia.[9] Knockdown of TNFRI reduced mechanical allodynia induced by application of gp120 to the sciatic nerve.[15] In the study, we found that TNFRI was located on the neurons of the SCDH, that expression of TNFRI was increased, and that knockdown of TNFRI using antisense ODN decreased mechanical allodynia and thermal hyperalgesia, indicating that TNFRI signal plays an important role in the gp120/M model.

Neuronal $mtO_2^{\bullet-}$ as the major source of ROS plays important roles in pain model.[13] ROS scavengers produce an antinociceptive effect in neuropathic and/or inflammatory pain.[27] Low level of HIV replication persists in HIV cellular reservoirs, secreting gp120, etc, generating ROS which in turn can initiate immune activation/inflammation,[28] disrupting mitochondrial function and biogenesis. Chronic morphine application increases spinal ROS.[16,29] Morphine-induced ROS under different experimental conditions is involved in many pathological processes, including degenerative diseases and organ dysfunction.[30] In this study, we demonstrated that chronic gp120/M application increased spinal $mtO_2^{\bullet-}$, and that $mtO_2^{\bullet-}$ scavenger

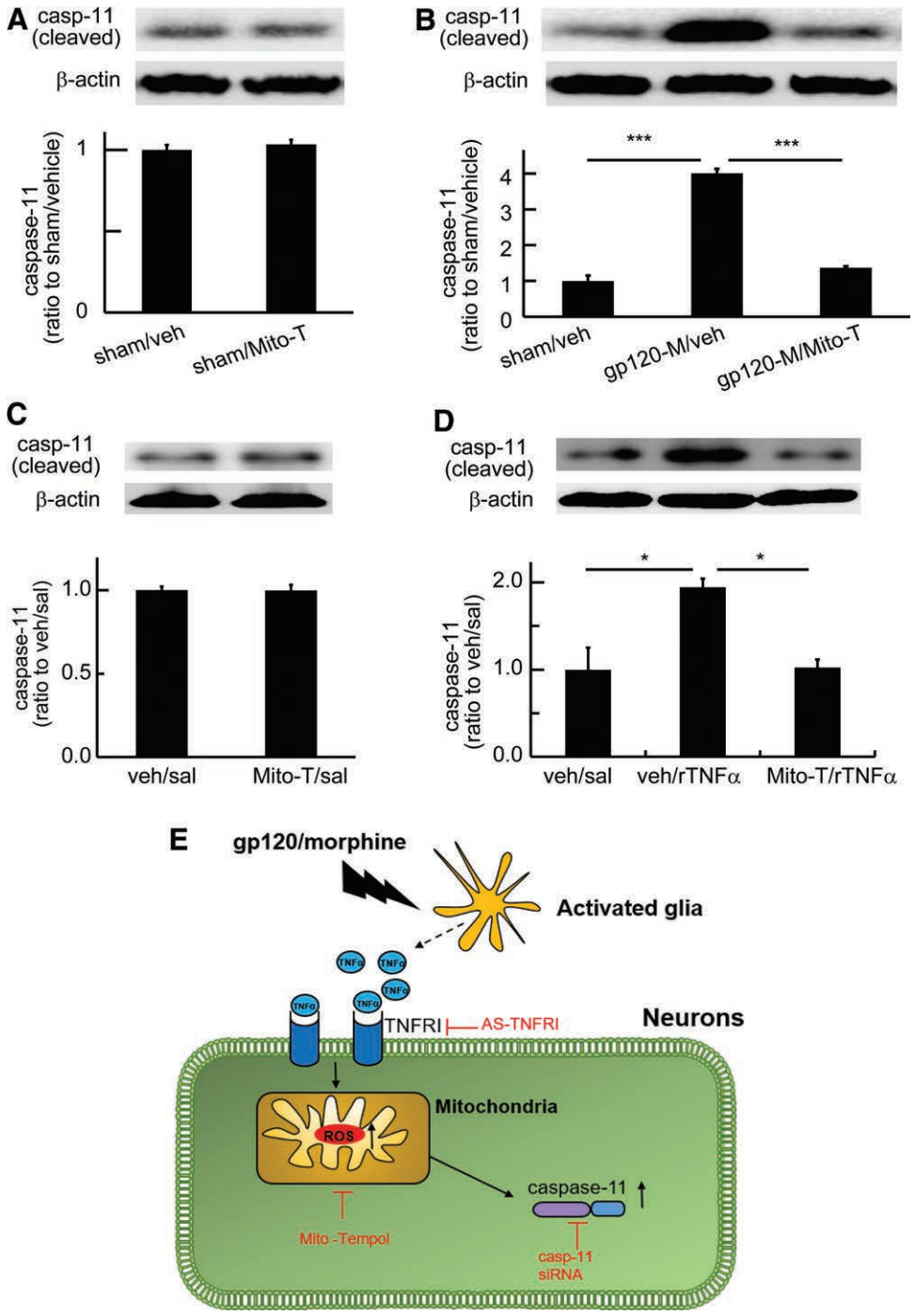


**Figure 6.** Effect of Mito-Tempol (Mito-T) on caspase-11 in gp120/M in rats and in the cultured neuronal cells. At 10 d of gp120/M treatment, animals received spinal Mito-T and SCDH was harvested for expression of casp-11. A, Western blots demonstrated that there was no significant difference in the expression of casp-11 between sham/vehicle and sham/Mito-T groups. B, Western blots show the expression of casp-11 in sham/vehicle, gp120-M/vehicle, or gp120-M/Mito-T group, ***$P < .001$, 1-way ANOVA Fisher LSD test, n = 4. C and D, Suppression of casp-11 by antioxidative stress in the cultured neuronal cells. C, Western blots show no significant difference in expression of casp-11 between vehicle/sal and Mito-T/sal groups. D, A significant increase in casp-11 in vehicle/TNFα group compared to vehicle/saline, *$P < .05$, 1-way ANOVA Fisher LSD test, n = 4, and a decrease in casp-11 in Mito-T/rTNFα compared to vehicle/rTNFα group, *$P < .05$, 1-way ANOVA Fisher LSD test, n = 4. E, Proposed pathway of spinal TNFRI-ROS-casp-11 in gp120/M-mediated neuropathic pain. HIV glycoprotein gp120/M induces releases of proinflammatory factors such as TNFα,[9,11,15] that act on the neurons through TNFRI. Neuronal TNFRI signal increases oxidative stress. Mitochondria are the major locations of ROS production in neurons.[13] Increased mitochondrial ROS evokes the casp-11 products, which is in line with the previous work showing that ROS in macrophages increases inflammatory factor casp-11 expression and activation.[21] ANOVA indicates analysis of variance; HIV, human immunodeficiency virus; LSD, least significant difference; ROS, reactive oxygen species; SCDH, spinal cord dorsal horn; TNF, tumor necrosis factor.

inhibited mechanical allodynia and thermal hyperalgesia, which confirmed that spinal ROS plays critical role in this model.

Inflammatory response contributes the pathogenesis of chronic pain. It is known that inflammasomes are cytosolic multiprotein complexes in response to infection and stress or pain.[18,19] The activation of canonical inflammasomes (without caspase-11) activates caspase-1, maturing interleukin (IL)-1β and/or IL-37 and induces a form of cell pyroptosis.[31] Canonical (caspase-1-dependent) and noncanonical (caspase-1-independent) inflammasomes pathways are 2 main mechanisms implicated in inflammation, pain, and pyroptotic cell death.[32]

Recently, the noncanonical inflammasome pathway involving caspase-11 was shown to act as a sensor and effector protein inducing the secondary activation of the canonical inflammasome.[18,33] Emerging evidence indicated that caspase-11 noncanonical inflammasome played the vital role in inflammatory diseases.[20] Caspase-11 is activator and regulator of inflammatory responses. The role of chronic opioids was further implicated in neuropathic pain development through activation of inflammasomes.[34] This study demonstrated that gp120/M increased caspase-11 expression and knockdown of caspase-11 reduced neuropathic pain, indicating that caspase-11 plays a role in this pain model. To our knowledge, we are the first to report the function of caspase-11 in the HIV-associated neuropathic pain.

While our finding demonstrated the role of caspase-11 in the gp120/M-mediated pain model, we are not clear about the exact upstream pathway of caspase-11. Previous studies show that TNFRI activity leads to ROS generation.[35] Inhibition of TNFRI reduced mechanical allodynia, and decreased $mtO_2^{\bullet-}$ induced by gp120 application to sciatic nerve.[15] In the current study, we also observed that TNFRI knockdown using antisense oligodeoxynucleotide (AS-ODN) against TNFRI reduced mitochondrial oxidative stress in gp120/M rats, indicating that spinal $mtO_2^{\bullet-}$ is downstream factor of TNFRI in gp120/M model. The exact mechanism of $mtO_2^{\bullet-}$ induced by TNFRI signal in the pain model is not clear. Mitochondrial dynamics (fusion and fission) are critical to mitochondrial function. Drp1 is involved in mitochondria fission.[36] In a L929 cell culture study, TNFα induces mitochondrial fission, and inhibition of Drp1 prevented mitochondrial fission.[17] Moreover, evidence showed that in HeLa cells, TNFα induced ROS production through ROS modulator 1 (Romo1) and B-cell lymphoma-extra large (Bcl-X(L)).[37] Thus, it is possible that TNFRI signal triggers ROS through multiapproach, such as Drp1, Romo1, and Bcl-XL. Drp1-mediated mitochondrial fragmentation induces excess ROS generation, which plays essential roles in neuropathic pain development.[38] Indeed, our previous studies showed that gp120 into the sciatic nerve induced neuropathic pain and increased expression of Drp1 and mitochondrial superoxide, and that knockdown of Drp1 and blockage of Drp1 with selective inhibitor mdivi-1 reversed the upregulation of spinal mitochondrial superoxide in the gp120 neuropathic pain state.[39]

ROS as a signaling molecule is involved in various cellular pathways. In the present study, ROS scavenger Mito-Tempol reduced spinal caspase-11 expression in gp120/M neuropathic pain and in cultured neurons. However, that exact molecular mechanisms by which mitochondrial ROS mediated expression of caspase-11 is not fully understood yet. Evidence demonstrated that increased ROS in macrophages enhances c-Jun N-terminal kinase (JNK) signaling resulting in increased caspase-11 expression. Bone marrow–derived macrophages (BMDM) infected with *Citrobacter rodentium* induced ROS and increased mRNA and protein expression of caspase-11.[21] ROS can induce expression of the JNK.[40] JNK inhibitor SP600125 reduced caspase-11 levels, indicating that ROS likely regulates caspases-11 through a JNK-mediated pathway.[21]

In summary, neuropathic pain induced by HIV-gp120/M increased spinal TNFRI, mitochondrial superoxide, and caspase-11. Inhibition of TNFRI reduced oxidative stress, and mitochondrial ROS scavenger decreased spinal caspase-11 expression, suggesting that the pathway of TNFRI—ROS—caspase 11 plays a critical role in the chronic pain model. Our study provides novel insights that is possibly useful in the treatment of HIV-related chronic pain.


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## DISCLOSURES

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