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Nucleoside Reverse Transcriptase Inhibitors (NRTIs) Induce Pathological Pain through Wnt5a-Mediated Neuroinflammation in Aging Mice

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Abstract

Highly Active Antiretroviral Therapy (HAART) has significantly contributed to the increase of HIV-infected survivors over 50 years of age. Unfortunately, patients are required to stay on long-term HAART, which may be causally related to the development of neurological problems such as chronic pain. Little is known about the contribution of HAART or its therapeutic agents to the pathogenesis of pain during aging. In this study, we determined the effect of nucleoside reverse transcriptase inhibitors (NRTIs) on the development of mechanical allodynia and the potential underlying mechanism in aging mice (15.5 months). We found that systemic administration of individual NRTIs, including ddC (2'-3'-dideoxycytidine), ddI (didanosine), AZT (3'-azido-3'-deoxythymidine) and d4T (2', 3'-didehydro-2', 3'-dideoxythymidine), induced allodynia in similar magnitudes and temporal profiles. We used ddC as a representative to investigate cellular and molecular processes induced by NRTIs in the spinal cord that probably underlie the development of allodynia. The results showed that ddC caused evident neuroinflammation in the spinal cord, suggested by the up-regulation of proinflammatory cytokines TNF- α and IL-1 β and the reactions of microglia and astrocytes. In addition, we found that Wnt5a, a critical regulator of neuroinflammation, was also up-regulated. Pharmacological inhibition of Wnt5a blocked ddC-induced up-regulation of TNF- α and astrocyte reaction, while activation of Wnt5a signaling potentiated these processes. Furthermore, our data showed that inhibition of Wnt5a significantly reversed ddC-induced mechanical allodynia in aging mice. The results collectively suggest that NRTIs may contribute to the development of chronic pain in aging patients by inducing Wnt5a-regulated

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Compliance with Ethical Standards

All animal procedures were performed in accordance with the ethical standards of the University of Texas Medical Branch, Galveston, Texas, USA

Competing Interests

The authors declare that they have no competing interests.

Keywords

NRTI; pain; Wnt; neuroinflammation; glia

INTRODUCTION

The life expectancy of patients with HIV-1 infection has increased dramatically since the advent of highly active antiretroviral therapy (HAART). It has been estimated that more than half of the patients living with HIV-1 in the US were in aging populations (over 50 years of age) in 2015 (Myers, 2009). Unfortunately, they need to stay on long-term HAART to suppress HIV replication and transmission. HAART may contribute to the development of neurological disorders in these patients despite decreased HIV loads (Brew et al., 2009a; Kalayjian and Al-harthi, 2009).

One of the most common neurological disorders in HIV patients is pathological pain (Hewitt et al., 1997). Epidemic analysis of patients suggests that HAART medications, especially nucleoside reverse transcriptase inhibitors (NRTIs), are probably causally associated with HIV-associated pain (Zheng et al., 2011). Studies with animal models support this (Weber et al., 2007).

Aging is a potential comorbid factor for the pathogenesis of HIV-associated pain and/or neuropathy (Myers, 2009; Patel and Crane, 2011). Given the expansion of the aging HIV-infected population, it is clinically important to characterize the effect of HAART components on the pathogenesis of pain and elucidate the underlying pathogenic mechanism in the context of aging. However, despite reported effects of specific NRTIs in young adult rodents (Zheng et al., 2011; Munawar et al., 2017), no studies have been carried out on aging animals.

In this paper, we report the results from studies on the effects of four NRTIs on the development of mechanical allodynia in aging mice at an age of 15.5 months (equivalent to 50 y/o in human). We found that administration of all NRTIs at a clinically relevant dose caused the development of mechanical allodynia. Using ddC as an NRTI representative, we also investigated the potential pathogenic processes in the spinal cord. We identified that ddC caused Wnt5a-dependent neuroinflammation, and Wnt5 critically contributes to the development of the allodynia.

MATERIALS AND METHODS

Animals

Aging (15–16 months old) male C57BL/BL6 mice with body weights between 35–40 g were purchased from NIH. This age of mice is equivalent to the age of 50 years in humans. Animals were housed in cages with soft bedding under a 12-hour light/dark cycle. Animal handling procedures were performed following protocols that were reviewed and approved by the University of Texas Medical Branch Animal Care and Use Committee.

Reagents

NRTI inhibitors: 2', 3'-dideoxycytidine (ddC; Sigma, D5782-250MG; 15 mg/kg), didanosine (ddI; Fisher, 50-555-1; 25 mg/kg), 2', 3'-didehydro-2', 3'-dideoxythymidine (d4T; Sigma, D1413-50MG; 25 mg/kg) and 3'-azido-3'-deoxythymidine (AZT; Sigma, A2169-250MG; 25 mg/kg) were dissolved in 0.9% saline (1 mg/ml) and injected (i.p.) every other day. These free base forms of NRTIs were dissolved well in saline to preparing the NRTI solution for i.p. injection. Saline was used as the vehicle control.

The antibodies used for 3, 3'-diaminobenzidine (DAB) immunohistochemical staining were rat anti-CD11b (AbD Serotec, MAG74GA, dilution 1:200), rabbit anti-Ib1 (Wako, 019-19741, dilution 1:500), mouse anti-GFAP (Millipore, mab360, dilution 1:1500), rabbit anti-IL1 β (Santa Cruz Technology, sc-7884, dilution 1:200), rabbit anti-Wnt5a (Abcam, ab72583, dilution 1:300), and rabbit anti-TNF α (Santa Cruz Technology, sc-8301, dilution 1:200).

The antibodies used for western blotting were rabbit anti-Wnt5a (CST cat.; 2530, 1 g/ml), goat anti-IL-1 (R&D Systems, cat. AF-401-NA, 0.2 g/ml), goat-anti-TNF- α (R&D Systems cat. AF-410-NA, 0.1 g/ml), and rabbit anti-GFAP (Millipore, cat # 04-1062).

Intrathecal injection (i.t.)

The injection method was described previously (Yuan et al., 2015). Briefly, animals were anesthetized with 2.5% isoflurane mixed in 1.5 L/min oxygen in the induction chamber until they lost consciousness as tested by pinching the hind paw. Anesthetized mice were moved outside the chamber, and anesthesia was maintained via nose cones with 2.5% isoflurane in oxygen. Intrathecal injection (5 μ l) was performed with a 30G $\times$ 1/2 needle attached to a 10 μ L syringe (Hamilton cat No, 80001, Model 1701 LT SYR). The needle tip was placed at the gap between lumbar vertebrae 5 and 6. A successful intrathecal penetration was judged by the observation of tail twists.

Von Frey test

Von Frey testing was performed as described previously (Yuan et al., 2012; Yuan et al., 2015). Briefly, mice were first habituated in a 13.2 $\times$ 5 $\times$ 4 cm Plexiglas box for three continuous days prior to behavioral testing. On the testing day, the mouse was first put in the restricting box for 20 min to minimize their exploring behavior. We used the 3.61 filament (Stoelting, Wood Dale, IL) in the initial measurement. Test was performed by touching the center area of hindpaws perpendicularly with the filament with appropriate forces until the filament was slightly bent and remained bent for one second. Based on the response to the initial filament (3.61/0.4g), we used the next larger filament in the next test if the animal did not withdraw its paw. If the animal did withdraw its paw in response to the initial filament, then the next smaller filament was used in the next test. This process was continued until the animals' pain threshold was determined. Signs of a noxious withdraw response included abrupt withdrawal, lateral moving, shaking, lifting, licking, and/or biting of the touched area of the hind paw. The threshold of the force that evoked a noxious response was computed (mean $\pm$ SE) and plotted with GraphPad Prism 5. Statistical significance was determined by two-way analysis of variance (ANOVA) with post-hoc tests.

Immunohistochemistry (IHC)

Mice were anesthetized with 14% urethane (0.2–0.3 ml; i.p.), followed by transcardial perfusion with saline containing 1 µg/ml heparin. The lumbar enlargement (L1-6) region of the spinal cord was collected for IHC or western blotting analysis. For IHC, the spinal cord was immersed in iced-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight and then transferred to 30% sucrose/PB for another overnight. Cryosections (14 µm) were collected and mounted on poly-L-lysine-coated glass slides (Fisher Scientific, Pittsburg PA) and stored at –80°C before IHC. Diaminobenzidine (DAB) staining was performed as described previously (Yuan et al., 2014). Briefly, the thawed sections were first treated with 0.3% peroxide for 30 min to block endogenous peroxidase. Then, the peroxide-treated sections were incubated for 1 h at room temperature with blocking buffer [0.5% BSA in PBS containing 3% Tween-20 and 4 drops avidin/biotin solution (Vector laboratory, SP2001)]. This was followed by sequential incubation with primary and secondary antibodies diluted with 2.5% BSA in PBS. IHC signals were developed with the ABC system (Vector Laboratories; Vectastain Elite ABC HRP Kit 6105). Sections were counter-stained with hematoxylin and mounted with Acrymount (StatLab Medical Products). Images were captured with an Olympus BX51 microscope.

Western blotting

Collected mice spinal cord lumbar enlargement segments were homogenized in radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, pH 7.4) containing protease inhibitor cocktail (Sigma, St Louis, MO) and incubated for 30 min at 4°C with mild shaking. Then, the homogenates were centrifuged (12000 g) for 10 min at 4°C. The supernatants were collected and protein concentrations were determined with the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL). Total loading protein (20 µg) was resolved by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by transferred to polyvinylidene fluoride membranes. Membranes after protein transfer were first washed briefly with Tris-buffered saline with Tween (TBST) buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) and then incubated in blocking buffer (TBST with 5% of nonfat milk powder). After blocking, membranes were sequentially incubated with primary and secondary antibodies. Protein bands were visualized with the Enhanced Chemiluminescence kit (Pierce) and quantified with NIH Image J. β -actin was used as the loading control.

Statistics

Statistical significance of results from von Frey tests was determined by Two-Way ANOVA followed by Bonferroni Multiple Comparisons Test, and the significance of western blotting results was determined by One-Way ANOVA followed by Tukey's Multiple Comparisons Test.

RESULTS

NRTIs induce pathological pain in aging mice

To test the effect of NRTIs on pain development in aging mice (15.5 months old), we first determined the influence of ddC, the first NRTI used in anti-HIV therapy. We observed that administration of ddC (50 mg/kg; i.p./2 days) readily induced a significant decrease in the threshold of mechanical response even one day after beginning the administration, indicating the expression of mechanical allodynia (Fig. 1a). To determine the generality of this pathogenic effect of ddC, we expanded the behavioral analysis to other NRTIs, including AZT, d4T and ddI, using a dose relevant to patient treatment (25 mg/kg; i.p./2 days). The different NRTIs showed similar effects to ddC at inducing mechanical allodynia (Fig. 1b). All of the NRTIs tested showed similar temporal dynamics of allodynia expression. These observations suggest that the development of pain is a common pathogenic effect of NRTIs in aging animals.

ddC activates astrocytes and microglia in the spinal cord of aging mice

The observation of NRTI-induced mechanical allodynia indicates the development of central sensitization in the dorsal spinal cord. Since neuroinflammation is commonly associated with the development of pathological pain in various animal models and patients (Chiu et al., 2013; Hao, 2013; Cao et al., 2014; Yuan et al., 2014; Zheng et al., 2014), we tested if microglia and astrocytes were activated in the spinal cord of mice with NRTI-induced pain. Using both immunoblotting and immunocytochemistry staining, we observed that ddC (50 mg/kg; i.p. /2 days; 2 weeks) caused a marked upregulation of the astrocyte marker GFAP in spinal cords (Fig. 2a, b). In addition, GFAP-marked spinal astrocytes in ddC-treated mice displayed a hypertrophic morphology, a structural characteristics of reactive astrocytes (Fig. 2c, d). Furthermore, IbaI, a microglia/macrophage marker was also up-regulated in the spinal cord dorsal horn (SCDH) of ddC-treated mice, and the IbaI-labelled microglia also displayed hypertrophic morphologies (Fig. 2e, f, g, h). These findings indicate that astrocytes and microglia are reactive in the spinal cords of aging mice following ddC administration.

ddC induces cytokine up-regulation in the spinal cord of aging mice

Because reactive glia are a major source of pro-inflammatory cytokines, we determined the expression of specific pro-inflammatory cytokines in spinal cords from the ageing mice on ddC described above. Immunoblotting analysis showed that TNF- α increased ~3.5 fold in the spinal cords after ddC administration (Fig. 3a, b). Immunocytochemistry staining confirmed the increase of TNF- α in the SCDH (Fig. 3c, d, e, f.), likely in astrocytes as suggested by a recent study on adult rodents (Zheng et al., 2011). Similarly, we also observed the spinal up-regulation of IL-1 β induced by ddC, as revealed by immunoblotting (Fig. 3g, h,) and immunocytochemistry staining (Fig. 3i, j, k, l).

ddC up-regulates spinal Wnt5a in aging mice

We recently reported that Wnt5a is an important regulator of TNF- α and IL-1 β expression in primary cortical cultures (Li et al., 2013). We thus examined if ddC, which increased the

level of TNF- α (Fig. 3), also up-regulated the expression of Wnt5a. Indeed, as shown by western blotting (Fig. 4a, b), Wnt5a increased by ~2 fold in the spinal cords of ageing mice that had been administered with ddC for 2 weeks. The ddC-induced Wnt5a up-regulation was also clearly seen by immunocytochemistry and in the SCDH (Fig. 4c, d, e, f). These observations suggest that ddC causes aberrant activation of Wnt5a signaling.

Wnt5a is critical for ddC-induced cytokine expression and astrocyte activation

We next sought to test the potential role of Wnt5a in ddC-induced neuroinflammation. To this end, we determined the effect of a Wnt5a-specific antagonist, Box5 (Jenei et al., 2009) (Li et al., 2013). We administered Box5 (10 μ g/i.t.) to ageing mice that had been on ddC for 2 weeks. At 12 h after Box5 injection, the L4-L6 spinal cords were collected for immunoblotting analysis. We observed that Box5 attenuated ddC-induced TNF- α up-regulation (Fig. 5a). In addition, Box5 also abolished ddC-up-regulated GFAP expression, indicating an inhibitory effect of Box5 on astrocyte reaction (Fig. 5b). We also determined the effect of a Wnt5a-specific agonist, Foxy5 (Safholm et al., 2006). We found that Foxy5 potentiated ddC-induced up-regulation of TNF- α and GFAP (Fig. 5a, b), indicating that activation of Wnt5a promotes neuroinflammation.

Wnt5a is critical for ddC-induced mechanical allodynia in ageing mice

The results described above suggest that Wnt5a is critical for ddC to induce neuroinflammation in the CNS of ageing mice (Fig. 4). Given the critical role of neuroinflammation in pathological pain, we next tested the potential role of Wnt5a in ddC-induced mechanical allodynia. We observed that Box5 (i.t.) significantly reversed the established mechanical allodynia in ageing mice that had been observed after 16 days of ddC administration (Fig. 6). This effect lasted at least for 24 hours following Box5 injection. These observations indicate that Wnt5a is critical for maintaining ddC-induced chronic pain.

DISCUSSION

To our knowledge, this is the first study designed to determine the effect of HAART on pain pathogenesis in the context of aging. We found that NRTI treatment caused mechanical allodynia in ageing mice and that ddC induced Wnt5a-mediated neuroinflammation in the spinal cord. We also observed that inhibition of Wnt5a signaling led to reversal of ddC-induced mechanical allodynia.

We tested the effect of four different NRTIs (ddC, ddI, AZT and d4T) on the expression of mechanical allodynia in ageing mice. These NRTIs are known to cross the blood brain barrier and can be detected in the cerebrospinal fluid (Eisfeld et al., 2013). The results show that their administrations all caused evident allodynia in similar magnitudes and temporal profiles (Fig. 1). Previous studies indicated that ddC administration also induce pain in young adult rats (Zheng et al., 2014; Iida et al., 2016). These results collectively indicate that pathogenesis of pain is a general side effect of the NRTI components in HAART. It is currently unclear what the potential combinatorial effect of different NRTIs that are commonly used in HAART regimens may be. The potential contribution of other HAART components other than NRTIs remains to be elucidated as well. Although the NRTIs used in

this study has phased out (or been phasing out) in developed countries, they are still used in developing countries. Importantly, because they have similar backbone structures and inhibit viral replication via a similar mechanism, we reasoned that studying the neurotoxic effect of these NRTIs will help the understanding of such effects of this class of anti-retroviral drugs in general.

Mechanistically, we observed that ddC induced spinal neuroinflammation in aging animals, as manifested by the up-regulation of proinflammatory cytokines (Fig. 3) and glial reaction (Fig. 2). Recent studies revealed NRTI-induced neuroinflammation in the spinal cord of young adult rodents (Zheng et al., 2014; Wu et al., 2017). It is likely that neuroinflammation is a common pathogenic consequence in the pain neural pathway in different ages of animals. We currently do not know if aging animals show differences in their sensitivity in the expression of NRTI-induced neuroinflammation. Neuroinflammation is one of the most consistent pathological conditions observed in the HIV-infected CNS (Brew et al., 2009b; Grovit-Ferbas and Harris-White, 2010; Valcour et al., 2011). The results from the current study expand this finding by revealing a contribution of NRTIs to this condition during aging.

Our results indicate that ddC induces spinal neuroinflammation via a Wnt5a-dependent mechanism in aging mice (Fig. 5). Wnt5a is also critical for expression of proinflammatory cytokines by other NRTIs (Wu et al., 2017). Furthermore, our previous studies show that Wnt5a also plays a key role in HIV-1 gp120-induced neuroinflammation in the spinal cord (Li et al., 2013; Yuan et al., 2014). Together, these data suggest that Wnt5a is a general regulator of neuroinflammation.

In summary, the results presented here demonstrate a common side effect of NRTIs, the backbone components in many HAART regimens, to induce pathological pain in aging mice. The underlying pathogenic mechanism may involve the Wnt5a-dependent expression of neuroinflammation in the spinal pain neural circuits. Blocking Wnt5a signaling and/or neuroinflammation is a potential strategy to develop effective approaches to control HAART-related pathological pain.

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Figure 1.

Intraperitoneal injection (i.p.) of NRTIs caused mechanical allodynia in aging mice. Threshold of hind paw withdrawal was measured by von Frey testing. a, ddC dissolved in saline was administered by i.p./2 days at the dose of 50 mg/kg. Aging mice body weight range was 35–40 g. Six animals were used in each group. b, ddI (25 mg/kg), d4T (25 mg/kg), and AZT (25 mg/kg) were administered i.p./2 days, and each group had 5 mice. Threshold was expressed as mean $\pm$ SE (** $p < 0.01$, *** $p < 0.001$) analyzed by two-way ANOVA with Bonferroni multiple comparisons test.

Figure 2.

Administration of ddC caused activation of astrocytes and microglia in aging mice. a, Spinal cord GFAP revealed by immunoblotting. b, Quantification of a ($n=6$, * $p<0.05$). c, d. Immunocytochemistry staining of GFAP. Comparison of astrocytes' morphology in vehicles (c, arrows) and in ddC-treated mice with hypertrophic processes (d, arrows). e–h Spinal microglia revealed by immunocytochemistry staining of Iba1. Comparing the glia in the vehicle-treated mice with fine morphology and glia in ddC-treated mice with hypertrophic cell bodies and processes.

Figure 3.

ddC upregulated TNF- α and IL-1 β expression in mouse SCDH. a. TNF- α immunoblots. b. Quantification of a (n=6, *** p<0.001). c-f. Immunocytochemistry staining of TNF- α , comparing the SCDH in vehicle-treated mice (c and d, see arrows in d) and ddC-treated mice (f, see arrows). g. IL-1 β immunoblots. h. Quantification of g (n=6, * p<0.005) by one-way ANOVA. i-l. Immunocytochemistry staining of IL-1 β . Compare the difference of IL-1 β positive signals in vehicle-treated mice (see arrows in j) and ddC-treated mice (l arrows).

Figure 4. ddC up-regulated spinal Wnt5a expression in the spinal cords of aging mice. a. Immunoblots. b. Quantification of a (n=6, ** p<0.01). c, d. Immunohistochemistry staining of Wnt5a in SCDH of vehicle-treated mice. e, f, SCDH of ddC-treated mice. Compare arrows in d and f.

Figure 5. Role of Wnt5a signaling in regulating spinal neuroinflammation induced by ddC in aging mice. a, b, Box5, a Wnt5a antagonist, attenuates ddC-induced TNF- α (a) and GFAP (b) expression while Foxy5, a Wnt5a agonist potentiates expression of these cytokines. Data shown are based on immunoblots (n=6, * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA).

Figure 6.

Effect of Box5 on ddC-induced mechanical allodynia in aging mice. a. ddC decreased the mechanic threshold, and this decrease was maintained for 16 days. b. Box5 partially reversed the ddC- induced threshold decrease. (n=5, # p<0.05; black*** p<0.001, ddC vs. saline; red *** p<0.001, ddC vs. ddC+Box5). Mean $\pm$ SE; two-way ANOVA with Tukey's multiple comparisons test.