Morphine produces better thermal analgesia in young Huntington mice and are associated with less neuroinflammation in spinal cord

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Abstract

Background: Huntington's disease (HD) is an inherited disease characterized by both mental and motor dysfunctions. Our previous studies showed that HD mice demonstrate a diminished pain response. However, few studies have focused on the relationship between HD and morphine analgesia. The purpose of this study is to investigate and compare the analgesic effects of morphine in HD and wild-type (WT) mice.

Methods: We used clinically similar transgenic HD mice (7-10 weeks of age with motor dysfunction at 8-9 mo of age) carrying a mutant Huntington CAG trinucleotide repeats to evaluate morphine analgesia. The morphine (10 mg/kg subcutaneously) analgesia was evaluated with a tail-flick in hot water (52°C). Mice spinal cords were harvested at the end of the analgesia studies. An immunofluorescence assay and western blotting were used to identify changes in the cells and cytokines.

Results: Our data demonstrate that preonset young HD mice exhibited a better analgesic response to morphine than the WT mice. Western blotting and an immunohistological examination of the lumbar spinal cord tissue indicated less activation of glial cells and astrocytes in the HD mice compared with the WT mice. The production levels of tumor necrosis factor α and interleukine-1β were also lower in the young HD mice.

Conclusion: Our data demonstrate better morphine analgesic and less pain-related cytokine responses at the spinal cord level for HD mice. Further studies are needed to determine the morphine analgesia mechanism in HD.

Keywords: Astrocytes; Cytokines; Huntington disease; Microglia; Morphine

1. INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by a mutation of the huntingtin (HTT) protein translated from the huntingtin gene.[1] The gene mutation contains polymorphic trinucleotide CAG repeat expansion in the HTT. This results in disease if the expansion >36 repeats.[2,3] Mutant HTT with longer expanded CAG trinucleotides also cause extreme HD expression.[4] Meanwhile, in clinical context, it was found that the pain burden was diminished in HD patients and that HD patients suffer from upper gastrointestinal inflammation but do not complain.[5,6] HD patients have also been reported to drink tea when it is still too hot for consumption without discomfort or pain.[7] Researchers have demonstrated the dysfunction of pain signal transmission and abnormal spinal pain processing in HD patients.[8,9] Functional magnetic resonance images have also revealed an altered pattern in responses to sensory stimulation.[10] Furthermore, our previous results also revealed that HD mice demonstrate a more diminished pain response than wild-type (WT) mice in terms of inflammatory pain.[11] However, very few studies have investigated the analgesic effect of drugs on animals with HD. Our previous findings also indicated morphine-induced gliosis and inflammation within the spinal cord.[12] In this research, we focus on the analgesic effects of morphine and hypothesized that HD mice would experience better analgesic effects after administration of morphine.

2. METHODS

2.1. Animals

Transgenic HD mice (obtained from Dr. S.H. Yang, National Cheng Kung University [NCKU], Tainan, Taiwan) carrying a green fluorescent protein (GFP) fused with a mutant HTT exon 1 containing 84 CAG trinucleotide repeats and age-matched FVB WT mice were used. Mice genotyping expression were checked as described previously.[11] Briefly, mice with a green GFP signal were considered HD mice and WT mice had no green GFP signal. Transgene Ubiquitin-GFP-HTT84Q (Ubi-G-HTT84Q) mice only exhibited significant neuropathological characteristics of HD at the age of 8 to 9 months.[13] All mice were housed in cages with a standard environment, and mixed-gender animals were used. All animal procedures were approved by the Institutional

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Animal Care and Use Committee of NCKU (IACUC approval No: 106096).

2.2. Drugs administration and behavioral tests

Morphine hydrochloride (EDQM, Strasbourg, France) (10 mg/kg, subcutaneously) were used as the analgesic. A von Frey hair aesthesiometer (0.02-2.56 g, Stoelting) plus Dixon's up-down method were used for the basal mechanical testing and a Hargreaves apparatus (7370; Ugo Basile) were used for thermal testing, as discussed previously.[14,15] For morphine analgesia test, mice were tested individually via tail-flick in hot water, as previously described.[12,16] Briefly, the mouse was wrapped with the tail exposed. Then, one third of the length of the tail was immersed into 52°C water, and the response latency of the removal of the entire tail from the water was recorded. A maximum cut-off value of 10 s was set to avoid thermal injury. The area under the curve (AUC) from the time-response curve was calculated as an index for the antinociceptive effect of morphine. Tail-flick latency was recorded 30, 60, 90, 120, and 150 minutes after morphine was injected. The AUC value was obtained by calculating the area under the time-response curve of the antinociceptive effect (test latency – basal latency) from 0 to 150 min after the administration of the drug.[17] After the behavioral tests were completed, the animals were rapidly sacrificed under deep anesthesia (4%-5% isoflurane) in the absence of painful stimulation. The mice were then chest opened, and phosphate-buffered saline perfusate (4°C, 50 mL) was given via a heart puncture. The spinal cords (L3-L5) were quickly removed, placed on ice, and stored for further evaluation.

2.3. Western blot analysis

Tissues were incubated on ice after the addition of protein extraction reagent, a protease inhibitor cocktail, and a phosphatase inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO), and then samples were sonicated, incubated, and centrifuged as discussed previously.[11] The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in precast 4% to 12% mini-gels with buffer system (Invitrogen, Carlsbad, CA) for electrophoretic separation, transferred to polyvinyl denefluoride membranes, and blocked in 5% nonfat dry milk for 1 hour at room temperature.[18] They were then incubated overnight at 4°C with primary antibodies against glial fibrillary acidic protein (GFAP) (marker of astrocytes, 1:10 000, rabbit, Millipore, Burlington, MA, MAB360), Iba-1 (marker of microglia, 1:1000, rabbit, GeneTex, GTX100042), tumor necrosis factor alpha (TNF-α) (1:1000, rabbit, GeneTex, Irvine, CA, GTX110520), interleukin 1 beta (IL-1β) (1:1000, rabbit, Abcam, Cambridge, UK, ab9722), and actin (1:10 000, mouse, Millipore, MAB1501). After the primary antibody incubation, the samples were washed (0.05% PBS-Tween 20), and the respective secondary antibody (anti-mouse or anti-rabbit IgG, GE Healthcare Life Sciences, Chicago, IL), chemiluminescence detection was performed with an HRP substrate (Millipore Corporation) and measured with the BioSpectrum Imaging System (UVP BioSpectrum; Thermo Fisher Scientific Inc.).

2.4. Immunofluorescence analysis

The harvested spinal cords (L3-L5) were immersed in 4% paraformaldehyde for 2 hours and placed in 20% sucrose for 48-72 hours at 4°C, as discussed previously.[11] Briefly, the spinal cord sections (10-μm thickness) were blocked with background-reducing components and left overnight at 4°C with primary antibodies against GFAP (mouse, 1:2000, Millipore, MAB360), Iba-1 (rabbit, 1:1000, Wako, 019-19741), TNF-α (1:1500, rabbit, Abcam, ab66579), and IL-1β (mouse, 1:1000, BD Biosciences, Franklin Lakes, NJ, 550605). The sections were then incubated with appropriate secondary antibodies (1:1200, AlexaFluor 594, Invitrogen or 1:1500 AlexaFluor 488, Invitrogen) and were examined with fluorescence microscopy. The staining intensities were measured with a computer-assisted imaging analysis system (Image J; National Institutes of Health, Bethesda, MD).

2.5. Quantification of western blotting and the immunofluorescence signal

Quantification of the western blots was also measured by scanning the films of the protein bands and calculating the intensity and measuring using ImageJ software as previously discussed.[16] The target protein bands were normalized using a specific amount of β-actin. The immunofluorescent staining quantification was performed as described in another study.[16] Briefly, the brightness values of the fluorescent staining in the spinal cord dorsal horn were measured from images captured under a microscope. Five slices of the L3-L5 spinal cord segments were randomly selected, the background was subtracted, and the brightness was measured. For the quantification experiment, all images were taken using identical exposure times and illumination intensities.

2.6. Sample size and statistical analysis

As previously discussed,[11] the animal size determination was 11 based on a 30% between-group difference with 25% SD and 80% power plus a 5% type I error. All the data was presented as mean ± standard error mean. All behavioral data were analyzed with an analysis of variance (ANOVA) followed by Bonferroni tests for the post hoc analyses and a Student's *t* test. The western blot and immunohistochemical data and other data for the two groups were analyzed using a Student's *t* test. The criterion for statistical significance was $p < 0.05$.

3. RESULTS

3.1. Young HD mice presenting normal sensory test and carrying HTT exhibited better morphine analgesic effects at 2 to 4 months of age

As discussed previously, we first confirmed the genotypes of the transgenic HD mice. According to a previous study, these mice had a mutant HTT gene that was ligated into the 30 end of the GFP gene.[11] The expression of the GFP-HTT signal was determined under a fluorescence microscope. Obvious GFP green fluorescence signals were found for the transgenic HD mice but not for the WT mice (Fig. 1A).

We then confirmed the basal mechanical sensory phenotypes of the HD and WT mice (2-4 mo of age). For the basal nociceptive sensation, von Frey hair testing was conducted for mechanical sensitivity (Fig. 1B, mechanical: 2.19 ± 0.15 [WT], 2.26 ± 0.13 [HD]; Fig. 1C, thermal: 9.47 ± 0.16 [WT], 9.49 ± 0.15 [HD]), which revealed no significant between-group differences.

We compared the morphine analgesia in the HD and WT mice. The repeated-measures ANOVA and Student's *t* test showed significance in the thermal behavior (Fig. 1D, [1.5 h] 6.69 ± 0.29 [WT], 10.00 ± 0.00 [HD]; [2.0 h] 3.93 ± 0.19 [WT], 6.02 ± 0.44 [HD]; [2.5 h] 2.43 ± 0.05 [WT], 3.09 ± 0.10 [HD], $^*p < 0.01$ vs WT) (Fig. 1E, AUC: 8.88 ± 0.32 [WT], 10.97 ± 0.57 [HD]; $^*p = 0.01$ vs WT).

3.2. Morphine induces astrocyte and microglia overexpression in WT mice, but the effect was less in HD mice in the case of young mice (2-4 mo of age)

The expression of astrocytes and microglia in the spinal cord dorsal horn after morphine injection was investigated. The western blot analysis of the spinal cord dorsal horn tissues (n = 5-6

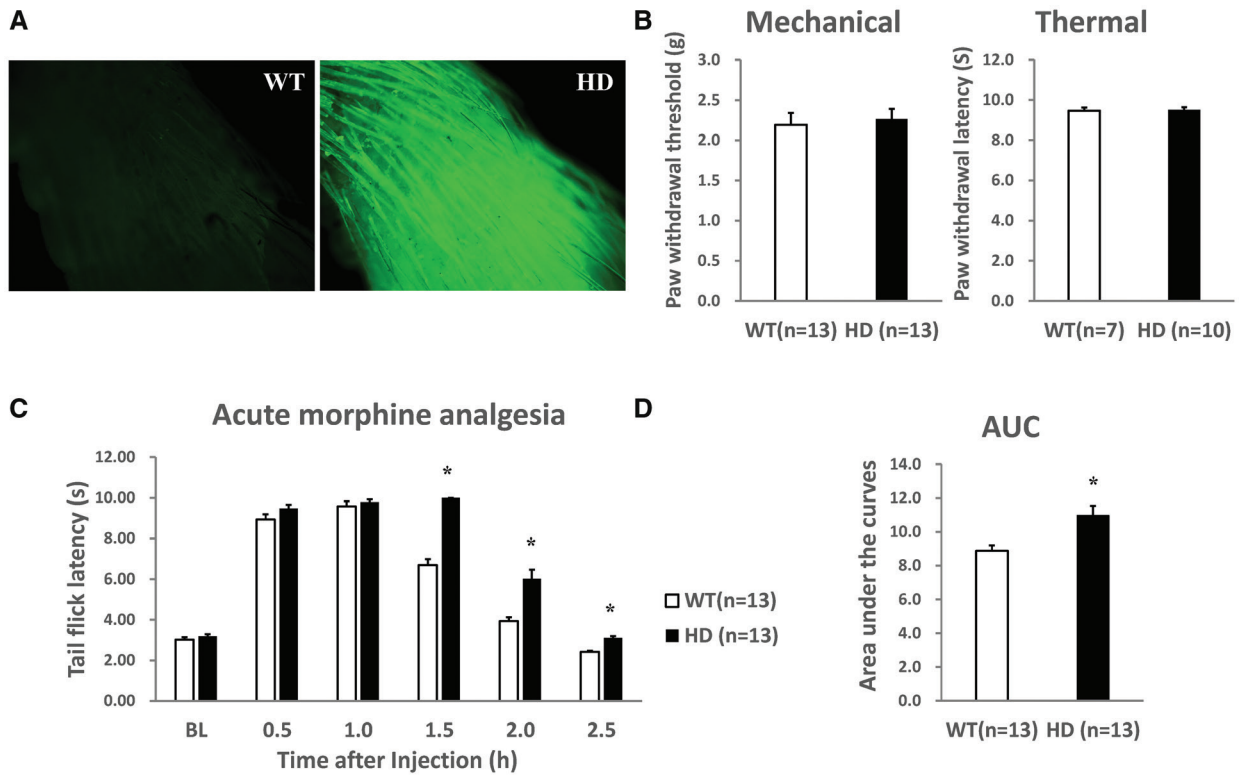

Fig. 1 Characterization of Ubi-G-HTT84Q transgenic mice. Young Huntington's disease (HD) mice presented normal basal nociceptive sensation but better morphine analgesia at 2 to 4 mo of age. A, Characterization of green fluorescent protein fluorescence signals in mouse tails of HD mice and wild-type (WT) mice. Behavioral phenotypes of mechanical (B) and thermal (C) sensitivity in the WT and HD mice. Mechanical and thermal pain sensitivity was measured by von Frey hairs testing and radiant heat (Hargreaves; n = 7 to 13). D and E, Presented better morphine analgesia and total analgesia area under the curve (AUC) (n = 13). *$p < 0.05$ vs WT. Data are presented as means ± SEM. BL = Basal level.

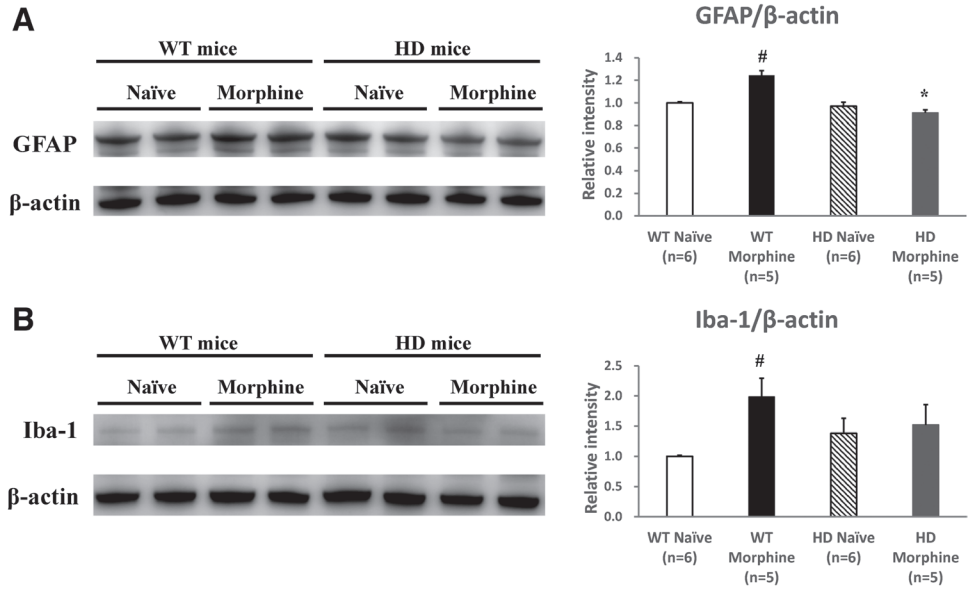

Fig. 2 Western blot analysis of astrocyte (glial fibrillary acidic protein [GFAP]) and microglia (Iba-1) expression in the spinal dorsal horn of the wild-type (WT) and Huntington's disease (HD) mice after morphine injection. Morphine significantly increased the GFAP (A) and Iba-1 (B) expressions in the WT but not in the HD mice (n = 5 to 6). #$p < 0.01$ vs naive; *$p < 0.01$ vs WT mice. Data are presented as means ± SEM.

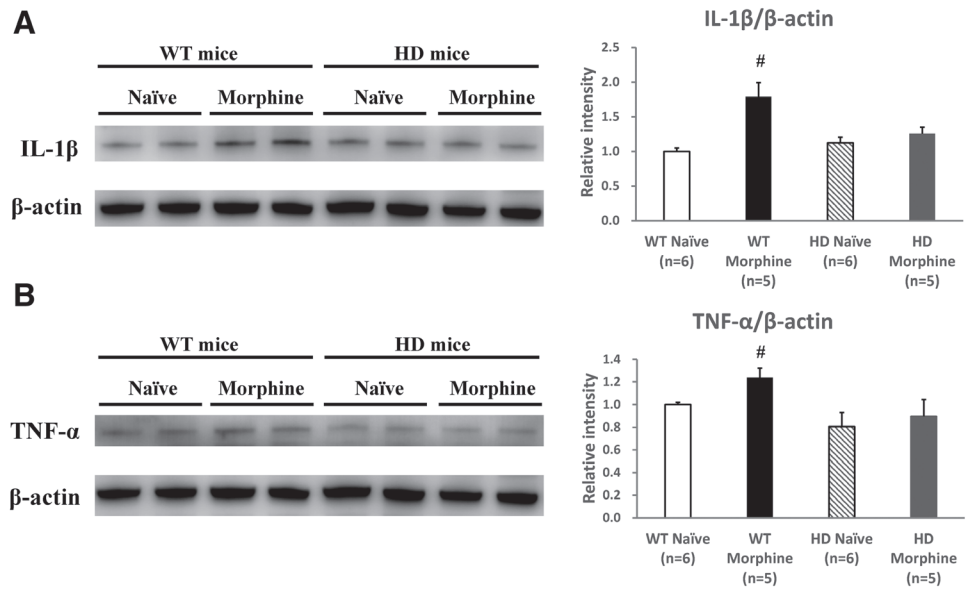

Fig. 3 Western blot analysis of interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) expression in the spinal dorsal horn of the wild-type (WT) and Huntington's disease (HD) mice after morphine injection. Morphine significantly increased IL-1β (A) and TNF-α (B) expression in WT but not in HD mice (n = 5 to 6). #$p < 0.01$ vs naive. Data are presented as means ± SEM.

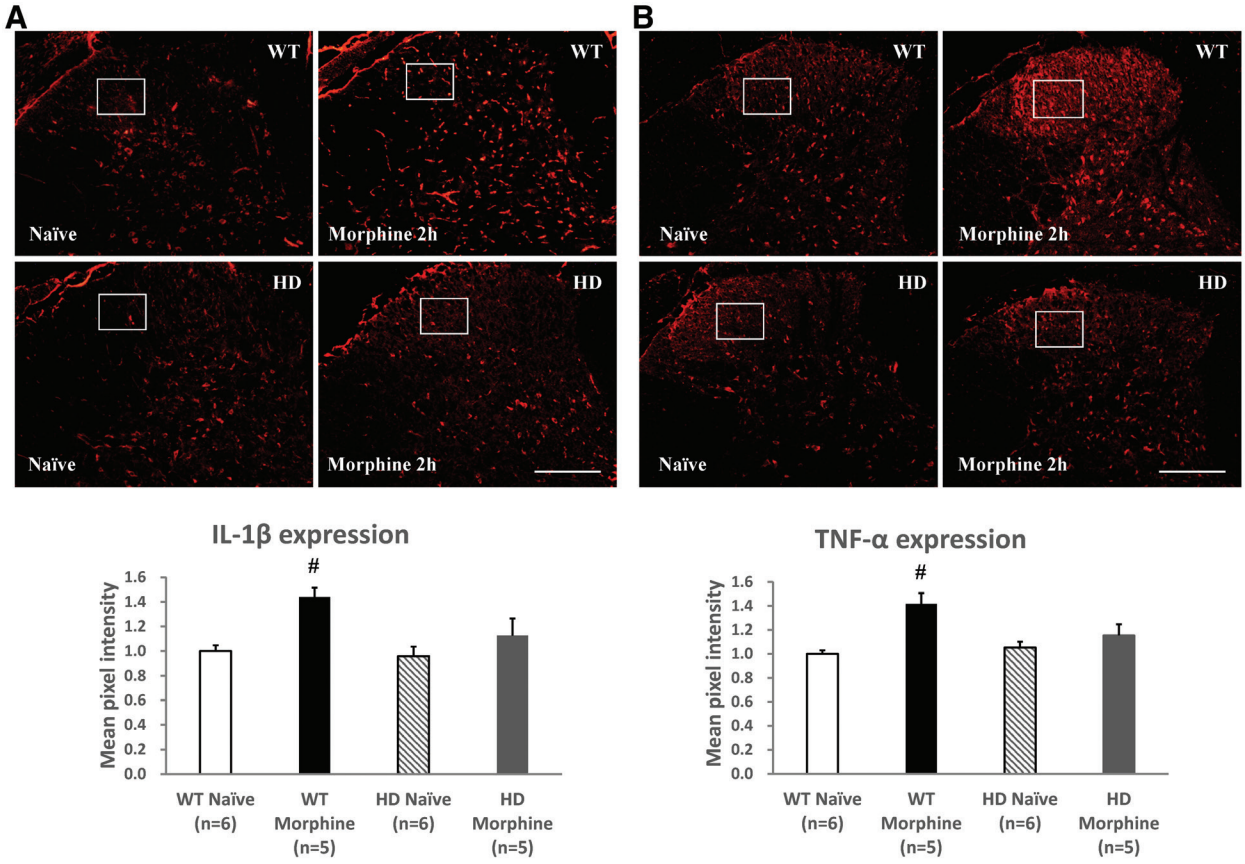

Fig. 4 Immunofluorescence staining intensity analysis of interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) expression in the spinal dorsal horn of the wild-type (WT) and Huntington's disease (HD) mice after morphine injection. Again, morphine significantly increased IL-1β (A) and TNF-α (B) expression in the WT but not in the HD mice. Scale: 200 μm (n = 5 to 6). #$p < 0.01$ vs naive. Data are presented as means ± SEM.

for all mice) showed the expression of GFAP (Fig. 2A, naive: 1.00 ± 0.01 [WT], 0.97 ± 0.04 [HD]; morphine: 1.24 ± 0.05 [WT], 0.91 ± 0.03 [HD]; *p < 0.01 vs WT mice; #p < 0.01 vs naive) and Iba-1 (Fig. 2B, naive: 1.00 ± 0.02 [WT], 1.38 ± 0.24 [HD]; morphine: 1.98 ± 0.34 [WT], 1.52 ± 0.37 [HD] p = 0.38; #p < 0.01 vs naive) to be significantly increased in the WT mice, but the increase was less in the HD mice.

3.3. Morphine induces IL-1β and TNF-α expression in the WT mice, but the effects were less in the HD mice in the case of young mice (2-4 mo of age)

The inflammatory cytokine IL-1β and TNF-α expressions were examined using a western blot (Fig. 3) and an immunofluorescence (Fig. 4) analysis. The data showed that in both spinal cord dorsal horn tissues, the intensity expression of IL-1β and TNF-α was significantly increased in the WT group, but the intensity was less in the HD group after administration of morphine (Fig. 3A, IL-1β, naive: 1.00 ± 0.05 [WT], 1.12 ± 0.08 [HD]; morphine: 1.79 ± 0.22 [WT], 1.26 ± 0.10 [HD] p = 0.059, #p < 0.01 vs naive; Fig. 3B, TNF-α, naive: 1.00 ± 0.02 [WT], 0.81 ± 0.12 [HD]; morphine: 1.24 ± 0.09 [WT], 0.90 ± 0.16 [HD], p = 0.10, #p < 0.05 vs naive). In Fig. 4, it can be seen that the immunofluorescence intensity of IL-1β and TNF-α was significantly increased in the WT group, but this increase was less in the HD group after administration of morphine (Fig. 4A, IL-1β, naive: 1.00 ± 0.05 [WT], 0.96 ± 0.08 [HD]; morphine: 1.44 ± 0.08 [WT], 1.13 ± 0.14 [HD]; p = 0.08, #p < 0.01 vs naive; Fig. 4B, TNF-α, naive: 1.00 ± 0.03 [WT], 1.05 ± 0.05 [HD]; morphine: 1.42 ± 0.09 [WT], 1.15 ± 0.09 [HD]; p = 0.07, #p < 0.01 vs naive).

3.4. Cell and cytokine co-localization

The immunohistological data indicated good colocalization of astrocytes (GFAP) with IL-1β (Fig. 5A) and glia (Iba-1) with TNF-α (Fig. 5B), respectively. The HD mice are not presented because HD mice carry the GFP-HTT gene, and a green signal cannot be used.

4. DISCUSSION

Previous studies of HD have typically focused on cognitive or motor disturbances. This is the first study to demonstrate that transgenic preonset HD mice exhibited better analgesic effects after administration of morphine and a lower inflammatory response in the spinal cord compared with WT mice. The animal results presented in this study suggest pain sensory impairment or different morphine analgesia in HD patients.[6–9]

Astrogliosis or microgliosis releasing inflammatory mediators has been demonstrated as a pathogenesis in HD.[19,20] However, as mentioned earlier, most research has focused on the aged manifested model, and prior researchers did not check pain behavior due to possible motor dysfunction.[11] HTT may lead to both a loss and a gain of function and may modulate neuronal sensitivity to neurodegeneration.[21,22] The mutated form of HTT (mHTT) has the ability to interact with several proteins (huntingtin-associated protein [HAP1] and huntingtin-interacting protein [HIP1]),[23–25] and these proteins also modify mHTT function and

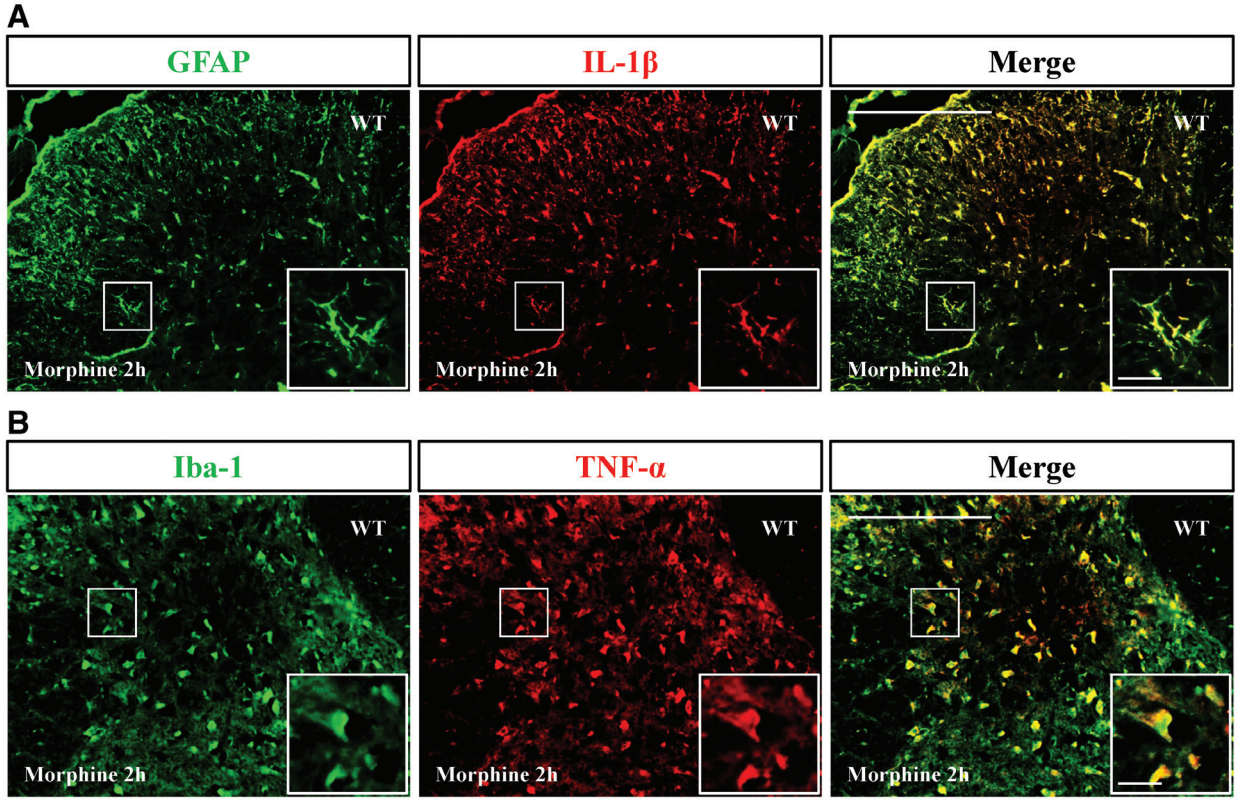

Fig. 5 Double immunofluorescence staining for cell and cytokine colocalization after morphine injection. The data clearly demonstrated that after morphine injection, astrocyte (glial fibrillary acidic protein [GFAP]) presented with interleukin (IL)-1β (A) and microglia (Iba) present with tumor necrosis factor alpha (TNF-α) (B). Scale: 25 μm.

possibly influence pain perceptions. mHTT may thus play an interesting role in pain response or morphine analgesia.

Morphine, which is one of the most commonly used analgesic drugs, induces multiple biological networks by altering protein phosphorylation and impacting cytoskeletal reorganization, neuroplasticity, protein folding and modulation, signal transduction, and biomolecular metabolisms.[26] Morphine administration increases brain-derived neurotrophic factor (BDNF) expression in selected brain areas.[27] However, HTT has also been linked to BDNF. The normal function of HTT is to increase the transcription of BDNF and stimulate BDNF vesicular trafficking in neuronal cells, whereas mHTT represses it.[18] It has been recently shown that microglial cells secrete BDNF and BDNF to promote their own proliferation and survival.[28] Our data demonstrate that the HD mice showed less activation of astrocytes and microglia cells in the morphine analgesia model. BDNF may thus interact with astrocytes and microglia in pain processing. Another possible reason for the activation being less may have been that mHTT impairs fast axonal transport, and this impairment might contribute to less release of substance P or calcitonin gene-related peptide (CGRP) from primary afferent terminals in HD mice.[29] More evidence is needed to support all of the above hypotheses. This study also did not exclude any possibility of a difference in the supra-spinal analgesia mechanism of morphine between HD and WT animals. However, further studies are needed on morphine complexity.

In conclusion, our findings revealed that transgenic premanifested HD mice exhibited better analgesic effects than their non-HD counterparts after morphine administration. The premanifested HD mice had less activation of astrocytes and microglia cells in the spinal cord after injection of morphine. The expressions of inflammatory cytokines TNF-α and IL-1β were also lower in the HD mice than in the WT mice. Our findings may lead to impacts on HD patient care, and certainly, clinical evidence is required to confirm for our animal findings.

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