A critical role of astrocyte-mediated nuclear factor-κB-dependent inflammation in Huntington’s disease

Han-Yun Hsiao1,2, Yu-Chen Chen1,2, Hui-Mei Chen2, Pang-Hsien Tu2 and Yijuang Chern1,2,*

1Institute of Neuroscience, National Yang-Ming University, Taipei 112, Taiwan and 2Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan

Received November 2, 2012; Revised and Accepted January 28, 2013

Huntington’s disease (HD) is an autosomal disease caused by a CAG repeat expansion in the huntingtin (HTT) gene. The resultant mutant HTT protein (mHTT) forms aggregates in various types of cells, including neurons and glial cells and preferentially affects brain function. We found that two HD mouse models (Hdh150Q and R6/2) were more susceptible than wild-type (WT) mice to lipopolysaccharide-evoked systemic inflammation and produced more proinflammatory cytokines in the brain. Such an enhanced inflammatory response in the brain was not observed in N171-82Q mice that express mHTT only in neurons, but not in glial cells. Thus, HD glia might play an important role in chronic inflammation that accelerates disease progression in HD mice. Intriguingly, enhanced activation of nuclear factor (NF)-κB-p65 (p65), a transcriptional mediator of inflammatory responses, was observed in astrocytes of patients and mice with HD. Results obtained using primary R6/2 astrocytes suggest that these cells exhibited higher IkB kinase (IKK) activity that caused prolongation of NF-κB activation, thus upregulating proinflammatory factors during inflammation. R6/2 astrocytes also produced a more-damaging effect on primary R6/2 neurons than did WT astrocytes during inflammation. Blockage of IKK reduced the neuronal toxicity caused by R6/2 astrocytes and ameliorated several HD symptoms of R6/2 mice (e.g. decreased neuronal density, impaired motor coordination and poor cognitive function). Collectively, our results indicate that enhancement of the p65-mediated inflammatory response in astrocytes contributes to HD pathogenesis. Therapeutic interventions aimed at preventing neuronal inflammation may be an important strategy for treating HD.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease characterized by chorea, dementia and psychiatric symptoms. The causative mutation is a CAG trinucleotide expansion in exon 1 of the huntingtin (HTT) gene. The resultant mutant HTT protein (mHTT) forms aggregates in the brain and several peripheral tissues. In neurons, mHTT forms aggregates in nuclei, hijacks numerous proteins, including transcriptional factors, causes transcriptional dysfunction, impairs vesicle transport, disturbs the secretion of brain-derived neurotrophic factor (1–3) and damages mitochondrial fission and fusion (4).

In previous studies, mHTT aggregates were found in various types of cells, including neurons, glial cells, muscle cells and liver hepatocytes (5–7). Astrocytes are the major cell type in the brain and provide structural and metabolic support to neurons. In several neurodegenerative diseases, astrocytes respond to proinflammatory mediators by undergoing astrogliosis that is characterized by increased proliferation and cell hypertrophy, and the induction of astrogial markers [e.g. glial fibrillary acidic protein (GFAP), vimentin, S-100 and glutamine synthetase] (7–9). Previous studies demonstrated that the expression of glutamate transporter-1 in astrocytes harvested from HD mice (R6/2) is impaired, degrading astrocytes’ ability to protect neurons against glutamate-mediated excitotoxicity (7). Moreover, mHTT reduces the production and secretion of CCL5/RANTES by astrocytes and accounts, at least in part, for the neuronal dysfunction associated with HD (10). Expressing mHTT in astrocytes using

*To whom correspondence should be addressed at: Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei 115, Taiwan. Tel: +886 226523913; Fax: +886 227829143; Email: bmychern@ibms.sinica.edu.tw

© The Author 2013. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
an astrocytic promoter (GFAP) exacerbated neuronal degeneration and neurological symptoms of HD mice (N171-82Q) (11,12), suggesting a critical role of glial dysfunction in HD pathogenesis.

Another major function of glial cells is mediation of the immune response to stresses in the brain. In particular, microglia were implicated in several neurodegenerative diseases, including Alzheimer’s disease (AD), amyotrophic lateral sclerosis and HD (13–17). Activation of microglia was shown to be correlated with disease severity in mice and patients with HD (16–18), whereas the suppression of microglial activation lengthened the lifespan of HD mice (R6/2) (19). Microglial activation, thus, might play a pathogenic role in HD. Furthermore, HD patients have higher levels of proinflammatory cytokines, including interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF), in their plasma and striatal tissues (20), prompting further investigation of inflammation in the progression of HD.

In the present study, we report the aberrant activation of nuclear factor (NF-κB in astrocytes of mice and patients with HD, suggesting a potentially authentic mechanism of astrocytic inflammation in HD. Such abnormal upregulation of NF-κB in glial cells was caused by higher κB kinase (IKK) activity and enhanced astrocytic inflammation evoked by proinflammatory mediators [such as lipopolysaccharide (LPS) and cytokines] while contributing to neuronal apoptosis and worsening the disease progression in HD mice.

RESULTS

Aberrant activation of NF-κB signaling in HD astrocytes is associated with an enhanced inflammatory response in the brain

To assess the inflammatory response in brains of HD mice after a pathogenic challenge, we employed an LPS-induced sepsis model for the following experiments. When compared with wild-type (WT) mice, acute stimulation with LPS [2.5 mg/kg, by an intraperitoneal (i.p.) injection] induced higher levels of proinflammatory cytokines (TNF-α and IL-1β) in brains of two HD mouse models (Hdh<sup>150Q</sup>, Fig. 1A, B, E and F and R6/2, Fig. 1I). Similarly, LPS-induced TNF-α and IL-1β levels in the serum and liver of HD mice were also higher than those observed in WT mice (Hdh<sup>150Q</sup>, Fig. 1C, D, G and H and R6/2, Fig. 1J and K). These findings suggest that HD mice reacted much more strongly to systemic inflammatory stimuli than did WT mice.

NF-κB is a major downstream transcription factor that is responsible for turning on the transcription of many inflammatory mediators [e.g. inducible nitric oxide (NO) synthase (h), TNFα, and IL-1β] during inflammatory stimulation. We next measured the nuclear localization of p65 (a subunit of NF-κB) in brain cells. Double immunostaining for p65 and cell-type markers (GFAP, an astrocytic marker, Fig. 2A; Iba1, a microglial marker, Fig. 2C and neuN, a neuronal marker, Fig. 2E) revealed no significant activation of NF-κB in microglia or neurons of WT or R6/2 mice at the basal level (Fig. 2D and F), whereas that in R6/2 astrocytes was markedly enhanced (Fig. 2B). These data suggest that aberrant activation of NF-κB occurs in astrocytes (but not in microglia or neurons) of R6/2 mice under basal conditions. Similarly, nuclear localization of p65 (red) was evident in GFAP-positive astrocytes (dark blue) in the caudate nucleus (Fig. 3) of HD patients. We observed no nuclear localization of p65 in five age-matched non-HD individuals (Fig. 3, Table 1). Thus, abnormal activation of the NF-κB pathway occurred in astrocytes of HD patients.

An acute inflammatory stimulus causes chronic inflammation in brains of HD mice and exacerbates HD progression

Earlier studies showed that acute systemic inflammation evoked by a single administration of LPS in mice led to chronic inflammatory responses (21). Acute LPS-induced inflammatory stimulation induced higher production of proinflammatory cytokines in brains of R6/2 mice when compared with WT mice (Fig. 1I) and also caused greater nuclear localization of p65-NF-κB in both astrocytes and microglia of R6/2 mice when compared with WT mice (Fig. 2B and D). To evaluate whether abnormal upregulation of NF-κB signaling in R6/2 glial cells is important for HD progression, R6/2 mice (at 7 weeks of age) were subjected to acute systemic inflammation triggered by a sublethal dose of LPS (2.5 mg/kg, by an i.p. injection). We reasoned that because of elevated NF-κB signaling, an acute LPS insult might trigger chronic inflammation in brains of R6/2, but not WT, mice. Consistent with this hypothesis, 8 weeks after systemic inflammation was triggered by a single stimulation of LPS as described above, R6/2 mice exhibited higher levels of TNF-α than did WT mice in the cortex (Fig. 4A) and two peripheral systems (the serum and liver; Fig. 4B and C). These samples were assayed on the same enzyme-linked immunosorbent assay (ELISA) plates at the same time so that they could be presented on the same scale. Collectively, the TNFα level in HD mice peaked at 1 h after administration of LPS and gradually dropped to a steady level at 6 h. Even up to 56 days after administration of LPS, levels of TNFα in LPS-treated R6/2 mice remained higher than those of phosphate-buffered saline (PBS)-treated R6/2 mice (Fig. 4).

Similarly, higher levels of IL-1 were also found in the cortex, striatum, serum and liver of R6/2 mice than those in WT mice (Supplementary Material, Fig. S1). These data suggest that acute systemic inflammation induced more significant chronic inflammation in HD than in WT mice.

Because the acute LPS insult induced NF-κB activation only in glia and not in neurons (Fig. 2), we hypothesized that abnormal NF-κB signaling in HD glia might mediate chronic inflammatory responses. To test this hypothesis, we subjected N171-82Q mice (22), an HD mouse model that expresses mHTT only in neurons, but not in glial cells, to the same acute LPS stimulation (2.5 mg/kg, by an i.p. injection). Although LPS caused an acute and transiently elevated brain level of TNF-α, no difference in this acute LPS-elevated TNF-α level was found between brains of N171-82Q mice and their littermate controls (Data not shown), supporting an important role of HD glia in chronic inflammation triggered by acute systemic inflammation.

To determine whether chronic inflammation results in toxicity, R6/2 mice (15 weeks old) were intravenously injected...
with sulforhodamine (SR)-conjugated-FLIVO that preferentially forms covalent bonds with active caspases, to evaluate neuronal apoptosis in vivo. Compared with WT mice treated with PBS (the control vehicle), LPS-treated R6/2 mice exhibited greater caspase activation in the cortex, striatum and substantia nigra (red, Fig. 5A–C), suggesting that chronic inflammation induced more severe toxicity in multiple brain areas of R6/2 mice.

Next, we evaluated the effects of chronic inflammation on disease progression in mice by measuring the rotarod performance, locomotor activity and beam walking that were used as indicators of motor coordination (Fig. 6B–D) and by measuring their performance in a T maze that was used as an indicator of cognitive function (Fig. 6E). An acute LPS injection at the age of 7 weeks did not affect the body weight of R6/2 mice in the subsequent 8 weeks (Fig. 6A). Acute LPS treatment significantly accelerated the progressive deterioration of rotarod performance in R6/2 mice (Fig. 6B). No effect of LPS treatment on the rotarod performance of WT mice was observed. In addition, R6/2 mice showed

Figure 1. Aberrant neuroinflammatory responses in HD mice. (A–H) Hdh150Q mice and their littermate controls (3 months of age) were given PBS or LPS 2.5 mg/kg for 1 h via an i.p. injection (n = 3 in each group). Levels of TNF-α (A–D) and IL-1β (E–H) in the cortex, striatum, serum and liver were assessed by an ELISA. Data are presented as the mean ± SEM of three independent experiments. a, Specific comparison between PBS-treated mice and LPS-treated mice of the same group; b, specific comparison between WT and Hdh150Q mice of the same treatment. (I–K) R6/2 and their littermate control mice (10.5 weeks of age) were given PBS or LPS (2.5 mg/kg) via an i.p. injection. Levels of TNF-α in the cortex, serum and liver were assessed using an ELISA (n = 5 in each group) at 1, 3, 6, 24 and 48 h after the injection. Data are presented as the mean ± SEM of three independent experiments. c, Specific comparison between LPS-treated WT and LPS-treated R6/2 mice. P < 0.01.
significantly decreased horizontal activity (Fig. 6C) and were slower in completing the beam walk (Fig. 6D) after the LPS injection. LPS treatment also exacerbated the decline in cognitive function, as assessed using a T maze, in R6/2 mice, but not in WT mice (Fig. 6E). Collectively, our data suggest that an elevated inflammatory response in HD glia renders HD mice prone to acute inflammatory stimulus-evoked chronic inflammation that accelerates disease progression. Because aberrant activation of NF-κB occurred only in HD astrocytes (but not in HD microglia) under basal conditions

Figure 2. Aberrant NF-κB-p65 signaling in HD mice. R6/2 and their littermate control mice (10.5 weeks of age) were given PBS or LPS:2.5 mg/kg via an i.p. injection. (A, C and E) After 3 h, brain tissues were harvested to assess activation of NF-κB by determining the nuclear distribution of p65 in the cortex using double immunostaining of p65 (red) and GFAP (an astrocytic marker, green; A), Iba1 (a microglial marker, green; C) or NeuN (a neuronal marker; E). Nuclei were stained with Hoechst 33258 (blue). Arrowheads mark cells with nuclear p65. (B, D and F) Percentages of GFAP-, Iba1- or NeuN-positive cells with nuclear p65 were quantified from at least 50 cells in each experiment. Data are presented as the mean ± SEM of three independent experiments. Scale bar indicates 50 μm. a, Specific comparison between PBS-treated mice and LPS treated mice of the same group; b, specific comparison between WT and R6/2 mice of the same treatment. P < 0.01.
The specificity of p65 binding was confirmed by R6/2 astrocytes at 2 h when compared with WT astrocytes (Fig. 8D). The amount of an unlabeled DNA oligomer harboring the NF-κB-binding site. Next, we examined the activity of an NF-κB upstream kinase (the IkB kinase, IKKα/β) by measuring the phosphorylation level of IKKα/β. As shown in Figure 8E, LPS-induced phosphorylation of IKKα/β at Ser176 in R6/2 astrocytes was higher than that observed in WT astrocytes. Note that activated IKK phosphorylates IκB and causes the degradation of IκB via the ubiquitin-proteasome pathway (23). Consistent with the increased IKK activity observed in R6/2 astrocytes, the level of IκB in R6/2 astrocytes after LPS treatment was lower than that observed in LPS-treated WT astrocytes (Fig. 8E). It is likely that a greater reduction in the expression of IκB during inflammation mediated the prolonged activation of NF-κB signaling by R6/2 astrocytes.

To evaluate the effect of inflammatory activation of R6/2 astrocytes on neurons during inflammation, primary cortical neurons were isolated from E18 WT or R6/2 mice and directly co-cultured with WT or R6/2 astrocytes (days in vitro (DIV) 30) for 4 days. To specifically determine the susceptibility of neurons, these co-cultures were stimulated with LPS (0.5 μg/ml) for 72 h and double immunostained for βIII-tubulin (red) and activated caspase 3 (green) (24). The total intensity of activated caspase 3 was measured and normalized to the number of neurons in each co-culture (Fig. 9A and B). Specific neuronal survival was also measured using a MAP2 assay (Fig. 9C and D) (25). Results showed no detrimental effect when WT neurons were co-cultured with WT astrocytes under basal conditions (Fig. 9A and C). R6/2 astrocytes produced a more damaging effect on neurons than did WT astrocytes (Fig. 9B and D). LPS induced the most severe harmful effect on R6/2 neurons (Fig. 9B and D). Levels of NO in the conditioned medium of these co-cultures were determined. Neurons alone do not react to LPS due to the lack of toll-like receptor 4 (26). Therefore, no production of NO was detected in pure neuronal cultures (Fig. 9E). LPS induced the highest NO production when R6/2 neurons were co-cultured with R6/2 astrocytes (Fig. 9F). These results support our hypothesis that R6/2 astrocytes play a critical role in inflammation-evoked neurotoxicity.

Blockage of IKK activity delayed disease progression in R6/2 mice and reduced the neuronal toxicity caused by astrocytic inflammation

To assess whether elevated IKK activity is functionally important in HD pathogenesis, N-terminally truncated HA-DN-IKKγ (27) was expressed using a lentiviral infection in the striatum and cortex of R6/2 mice. Mice were infected at 7 weeks and sacrificed at 15 weeks of age. The body weight and behavior (rotarod performance, beam walking and T maze) were evaluated at 7–15 weeks of age. Tissues (cortex, striatum, serum and liver) were collected at 15 weeks of age to determine levels of TNF-α and IL-1β. Exogenous expressions of HA-DN-IKKγ in the striatum and cortex were confirmed by immunostaining of HA-tag and GFAP (an astrocytic marker), βIII-tubulin (a neuronal marker) or Iba1 (a microglial marker) (Data not shown). Intrastriatal expression of HA-DN-IKKγ significantly reduced levels of TNF-α and IL-1β in the cortex and striatum of
effectively reversed the decrease in neuronal density (Fig. 11A). Inhibitor IV, In; 10 μM, using the performance in the T maze (Fig. 12D). These findings suggest that abnormal activation of IKK and downstream signaling in R6/2 astrocytes led to a detrimental effect on neurons in an inflammatory context. Therefore, we reasoned that TNF-α and 10 ng/ml LPS (Fig. 1A and B). Thus, NF-κB occurs only with HD astrocytes, and not with HD microglia or HD neurons, under basal conditions (Fig. 2). Such an enhanced NF-κB signaling in R6/2 astrocytes plays a detrimental role in neuronal survival after inflammation.

Table 1. Summary of demographic data, neuropathology and experimental results of human subjects

<table>
<thead>
<tr>
<th>Publication number</th>
<th>Race (gender)</th>
<th>Brain area</th>
<th>Age (years)</th>
<th>HD Vonsattel grade</th>
<th>Other pathology</th>
<th>Cause of death</th>
<th>PMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-1</td>
<td>Caucasian (M)</td>
<td>Caudate nucleus</td>
<td>43</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Complications</td>
<td>10 h</td>
</tr>
<tr>
<td>HD-3</td>
<td>Caucasian (M)</td>
<td>Caudate nucleus</td>
<td>57</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ASCVD</td>
<td>15 h</td>
</tr>
<tr>
<td>HD-4</td>
<td>Caucasian (M)</td>
<td>Substantia nigra</td>
<td>58</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ASCVD</td>
<td>17 h</td>
</tr>
<tr>
<td>HD-5</td>
<td>Caucasian (F)</td>
<td>Substantia nigra</td>
<td>69</td>
<td>II~III</td>
<td>Acute thalamic hemorrhage</td>
<td>COPD</td>
<td>10 h</td>
</tr>
<tr>
<td>Non-HD-1</td>
<td>Caucasian (F)</td>
<td>Caudate nucleus</td>
<td>42</td>
<td>n.a.</td>
<td>HBP/DM</td>
<td>HASCVD</td>
<td>4 h</td>
</tr>
<tr>
<td>Non-HD-2</td>
<td>Caucasian (F)</td>
<td>Caudate nucleus</td>
<td>53</td>
<td>n.a.</td>
<td>HBP/Asthma</td>
<td>HASCVD</td>
<td>15 h</td>
</tr>
<tr>
<td>Non-HD-3</td>
<td>Caucasian (M)</td>
<td>Caudate nucleus</td>
<td>58</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>9 h</td>
</tr>
<tr>
<td>Non-HD-4</td>
<td>Caucasian (F)</td>
<td>Substantia nigra</td>
<td>72</td>
<td>n.a.</td>
<td>NHL/DM/heart disease</td>
<td>Exsanguination (accident)</td>
<td>19 h</td>
</tr>
<tr>
<td>Non-HD-5</td>
<td>Caucasian (M)</td>
<td>Substantia nigra</td>
<td>75</td>
<td>n.a.</td>
<td>n.d.</td>
<td>HASCVD</td>
<td>16 h</td>
</tr>
</tbody>
</table>

Brain sections were analyzed by immunofluorescence staining of NF-κB (red) and GFAP (blue) as shown in Figure 1 and Supplementary Material, Figure S1. Brain sections were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, College Park, MD, USA. Male; F, female; n.d., not determined; PMI, post-mortem interval; HBP, high blood pressure; DM, diabetes mellitus; NHL, non-Hodgkin’s lymphoma; ASCVD, arteriosclerotic cardiovascular disease; HASCVD, hypertensive arteriosclerotic cardiovascular disease; COPD, chronic obstructive pulmonary disease; Complications, the detailed cause of death was unknown.

R6/2 mice when compared with those observed in R6/2 mice infected with a lentivirus expressing green fluorescent protein (GFP) (Fig. 10A–D). A histological examination using Nissl staining revealed that intrastriatal expression of HA-DN-IKKγ effectively reversed the decrease in neuronal density (Fig. 11A and B), but not the neuronal volume (Fig. 11A and B), observed in the striatum of R6/2 mice. No effect on the decline in body weight was found in mice infected with HA-DN-IKKγ (Fig. 12A). Treatment with HA-DN-IKKγ also ameliorated the impaired motor function, as determined by the rotarod performance and beam walking (Fig. 12B and C) and improved the deficient cognitive function, as assessed using the performance in the T maze (Fig. 12D). These findings suggest that abnormal activation of IKK and downstream NF-κB-mediated signaling in brains of HD mice contributed to the pathogenesis of HD. Double immunohistochemical staining for the transgene (HA-DN-IKKγ) and cell-type markers showed that HA-DN-IKKγ was expressed in GFAP-positive astrocytes, βIII-tubulin-positive neurons and Iba1-positive microglia (Data not shown). The beneficial effect of HA-DN-IKKγ, thus, might be mediated by multiple cell types. Nonetheless, we noted that among the cell types tested, only astrocytes in brains of unchallenged R6/2 mice exhibited detectable nuclear localization of p65 (a hallmark of NF-κB activation; Fig. 2A and B). Therefore, we reasoned that HA-DN-IKKγ might exert its beneficial effects on HD progression, at least in part, via normalization of IKK-NF-κB signaling in R6/2 astrocytes.

To further evaluate whether expression of mHTT in astrocytes led to a detrimental effect on neurons in an IKK-NFκB-mediated pathway, primary astrocytes were isolated from the cortex of WT and R6/2 mice and treated with proinflammatory cytokines (10 ng/ml TNF-α and 10 ng/ml IL-1β) in the absence or presence of an IKK inhibitor (IKK inhibitor IV, In; 10 μM) for 72 h. We next prepared primary WT neurons and treated these neurons for 72 h with the indicated astrocyte-conditioned medium (ACM) prepared as described above. Immunocytochemical staining was conducted to analyze the expression of an apoptosis marker (activated caspase 3). Neuronal survival was also assessed using a sensitive MAP2 assay. As shown in Figure 13A–C, ACM collected from R6/2 astrocytes treated with cytokines caused much greater elevation of activated caspase 3 and lower neuronal survival than ACM collected from WT astrocytes treated with cytokines. Inclusion of an IKK inhibitor when preparing the ACM markedly reduced neuronal toxicity (Fig. 13A–C) and reduced NO production (Fig. 13D). These findings support our hypothesis that enhanced IKK-NF-κB signaling in R6/2 astrocytes plays a detrimental role in neuronal survival after inflammation.

**DISCUSSION**

Neuroinflammation is a common feature of many neurodegenerative diseases, including Parkinson’s disease (PD), AD and HD (28–31). The LPS-induced sepsis model was previously used to demonstrate that induction of neuroinflammation exacerbated the progression of PD and AD (21,32,33). We showed herein, for the first time, that an acute inflammatory stimulus caused chronic inflammation that accelerated disease progression in HD mice. Several earlier reports suggested that microglial activation might play a pathogenic role in HD (16–19). It is important to point out that aberrant activation of NF-κB occurred only with HD astrocytes, and not with HD microglia or HD neurons, under basal conditions (Fig. 2). Such an enhanced NF-κB-mediated inflammatory response in HD astrocytes might play a crucial role in regulating the initial response of HD brains to inflammatory stimuli. We also showed in the present study that enhanced NF-κB activation in astrocytes was observed in brains of mice and humans with HD (Figs 2 and 3). In addition, we demonstrated that mHTT enhanced IKK activity and subsequently caused prolonged NF-κB activation in astrocytes during inflammation (Fig. 8). Due to the increased sensitivity of R6/2 astrocytes to inflammatory stimuli, acute inflammatory stimulation promoted chronic inflammation in brains of R6/2 mice and exacerbated disease progression in R6/2 mice (Figs 4–6). Blocking endogenous IKK by exogenous expression of HA-DN-IKKγ in R6/2 mice decreased cytokine levels, rescued the reduced neuronal density and improved the impaired motor and cognitive functions (Figs 10–12).
Because many infected cells were GFAP-positive astrocytes (Data not shown), suppression of IKK-NF-κB signaling in R6/2 astrocytes might, at least partially, have mediated the beneficial effects of HA-DN-IKKγ. In line with the in vivo findings, incubation of neurons purified from R6/2 and WT mice with ACM revealed that proinflammatory mediators (e.g., NO, TNFα and IL-1β) released by astrocytes caused detrimental effects on neurons as assessed by caspase 3 activation and a MAP2 assay (Figs 9 and 13). Collectively, our findings demonstrated that abnormal regulation of NF-κB signaling in astrocytes and neuronal inflammation play critical roles in HD pathogenesis. Besides R6/2 mice that show robust phenotypes and rapid disease progression, it would also be of interest to further evaluate the role of astrocytes in disease progression using other HD mouse models. Understanding the mechanism underlying prolonged activation of the IKK/IκB/NF-κB pathway in R6/2 astrocytes could provide a means to manipulate neuronal inflammation during HD progression and might improve HD therapies.

Our finding showing that inflammatory stimuli induced higher IKK activity in R6/2 astrocytes (Fig. 8E) is consistent with a previous report which demonstrated that higher IKK activities were found in the striatum and cortex of R6/2 mice (27). In that study, Khoshnan and colleagues reported that higher IKK activities resulted from a direct interaction between polyQ-expanded mHTT and the IKK complex, thus causing a neurotoxic effect. Because mHTT also exists in astrocytes (7,10), it is plausible that mHTT might interact with and potentiate activation of the IKK complex in R6/2 astrocytes and thereby lead to prolonged NF-κB activation and overactivation of astrocytes during inflammation. Alternatively, IKK might be regulated by other pathways. For example, AMP-activated protein kinase (AMPK)-α1 was shown to mediate phosphorylation of IKK and activation of NF-κB signaling in kainic acid-treated C6 glioma cells (34). This possibility is interesting because aberrant activation of AMPK-α1 was found in brains of R6/2 mice and patients (35). The role of AMPK in aberrantly potentiated NF-κB signaling in R6/2 astrocytes during inflammation is currently unknown and requires further investigation. In addition, the potential involvement of other upstream regulators (e.g., transforming growth factor-β-activated kinase 1; TAK1) of the IKK complex in the enhanced inflammatory response of R6/2 glia is also of great interest.

Besides infection, many other events (such as surgery) might also induce inflammatory responses in the brain. An intrastratial injection of lentiviruses, as was performed in the present study, requires an invasive surgical procedure that might, by itself, cause inflammation. This could explain why the level of TNFα protein in brains of HD mice infected with a control virus (GFP; Fig. 10A) was higher than that of PBS-treated (by an i.p. injection) HD mice (Fig. 4A). This observation indicates that the surgical procedure needed for the intrastratial injection itself was sufficient to trigger neuroinflammation. Consistent with the importance of neuroinflammation in HD pathogenesis, disease progression of R6/2 mice that had a surgical operation for the intrastratial injection was much faster than those that did not (Figs 6B and E, 12B and D). The beneficial effect of HA-DN-IKKγ (Figs 10–12)
suggests that the IKK/IκB/NF-κB pathway is critical for neuroinflammation triggered by brain injury. Therefore, it might be beneficial to protect HD patients from potential causes of systemic inflammation (such as non-essential surgery, infections and falls).

Given the complex role of neuroinflammation in HD pathogenesis, the extent and duration of systemic inflammation are likely important. A recent study reported that chronic inflammation triggered by a low level of LPS did not affect the disease progression of another transgenic mouse model (YAC128) of HD. Starting from the age of 5 months, YAC128 mice were subjected to weekly administration of LPS (1 mg/kg, by i.p. injection) for four consecutive weeks. Although no information regarding levels of brain cytokines was available, the dose of LPS used in the above study appeared to be very low because only slight increases in the size and morphology of microglia were detected. No changes in the number of microglia, striatal atrophy or the course of motor deterioration were found in YAC128 mice (36). Because preconditioning with a low dose of LPS was previously shown to exert a neuroprotective role by preventing an inflammatory response during ischemia (37), it would be of great interest to further evaluate whether neuroinflammation functions as a doubled-edged sword and exerts biphasic effects on HD pathogenesis.

MATERIALS AND METHODS

Reagents

LPS prepared from *Escherichia coli* serotype O111:B4 was purchased from Sigma Aldrich (St Louis, MO, USA). One nanogram of endotoxin is equivalent to five endotoxin units (Limulus lysate assay) (38). The Griess reagent, mouse-TNFα and mouse-IL-1β were purchased from Sigma Aldrich. The anti-IKKα/β-pS176 antibody, anti-IKKα/β antibody, anti-IκB antibody and anti-cleaved caspase 3 antibody were purchased from Cell Signaling (Danvers, MA, USA). The anti-

Figure 5. Chronic inflammation induced by an acute inflammatory stimulus evoked more severe caspase activation in brains of R6/2 mice than in WT mice. Mice at the age of 7 weeks (n = 4 in each group) were given a single i.p. injection of PBS or LPS 2.5 mg/kg. Brain sections were collected 8 weeks after the initial injection. Activation of caspases (red) in brain sections [cortex (A), striatum (B) and substantia nigra (C)] was quantified. Brain sections were stained with NeuN (A and B) or tyrosine hydroxylase (C) (green). Scale bar indicates 50 μm. a, Specific comparison between PBS-treated mice and LPS-treated mice of the same group; b, specific comparison between WT and R6/2 mice of the same treatment. P < 0.01.
GFAP antibody and anti-βIII-tubulin antibody were obtained from Merck Millipore (Billerica, MA, USA). The anti-p65 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The anti-Iba1 antibody was purchased from Wako Pure Chemical Industries (Chuo-Ku, Osaka, Japan). The anti-iNOS antibody was purchased from BD Biosciences (San Jose, CA, USA). SR-FLIVO was obtained from Immunochemistry Technologies (Bloomington, MN, USA). The IKK inhibitor was purchased from Calbiochem (San Diego, CA, USA).

Animals and treatments

R6/2 mice and littermate controls were initially obtained from Jackson Laboratory (Bar Harbor, ME, USA) and mated with female control mice (B6CBAFl/J). Offspring were verified by a polymerase chain reaction genotyping technique of genomic DNA extracted from tail tissues using primers (5'-CCGCTCAGTTCTGCTTTTA-3' and 5'-GGCTGAGGA AGCTGAGGAG-3') located in the transgene. The number of CAG repeats of R6/2 mice used in the present studies was...
Human Molecular Genetics, 2013, Vol. 22, No. 9

---

**Figure 7.** Proinflammatory agents induced overactivation of R6/2 astrocytes. Primary astrocytes (DIV 30) were stimulated with LPS; 0.5 μg/ml; A–C or cytokines (10 ng/ml TNF-α and 10 ng/ml IL-1β; D and E) for 72 h. (A and D) Levels of iNOS in the total lysate were assessed by western blot analyses. Results were normalized to those of actin. (B and E) The level of NO in the medium was determined with the Griess reagent. (C) Levels of TNF-α and IL-1β in the medium were determined using an ELISA. Data are presented as the mean ± SEM of three independent experiments. a, Specific comparison between PBS-treated astrocytes and LPS-treated astrocytes of the same group; b, specific comparison between WT and R6/2 astrocytes of the same treatment. P < 0.01.

---

239 ± 7.8 (mean ± SEM, n = 80). The knock-in HD mouse models of Hdh 

---

**Behavioral testing**

Motor coordination was assessed using a rotarod apparatus (UGO BASILE, Comerio, Italy) at a constant speed (12 r.p.m.) over a period of 2 min (5). Latency to falling was automatically recorded. Each mouse was given three trials for a maximum of 2 min for each trial. Locomotor activity was measured for 10 min using an activity chamber (Coulbourn Instruments, Allentown, PA, USA) equipped with 16 × 16 horizontal sensors as described elsewhere (43). The beam walk analysis was conducted as described in a previous study (44). In brief, mice were first trained on a 17 mm-diameter beam one time, followed by a weekly test on an 11 mm-diameter beam. The latency exhibited by each animal to walk across the beam was recorded. The T maze analysis (spontaneous alteration) was used to assess the cognitive function of animals and was conducted as described earlier with minor modifications (45). Briefly, each mouse was placed in the start area of the T-maze and allowed to walk to the intersection, where it chose the right or left arm. When the animal was subjected to the choice again in the T maze, by nature, it should explore the other arm that it did not visit in the previous trial. Each animal was tested for 10 consecutive trials. The number of alternative choices out of 10 trials was recorded and presented as the percentage of correct choices for each animal.

---

**Primary astrocyte, microglia and neuron cultures**

Primary astrocytes were purified from brains of postnatal 1–2-day-old mice as described previously (10). ACM was collected as detailed elsewhere (10). Primary microglia were

---

**Human brain tissues**

Brain sections (5 μm) of human subjects were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD, USA). The demographic data and neuropathology of subjects are summarized in Table 1.

---

**Lentivirus package**

The HA-DN-IKKγ construct was kindly provided by Dr Ali Khoshnan and Dr Paul H. Patterson (California Institute of Technology, Pasadena, CA, USA). Briefly, HA-DN-IKKγ is an IKKγ mutant that is fused to a hemagglutinin epitope tag at its N-terminus and lacks the binding domain (amino acids 1–134) for interaction with IKKα/β. Expression of HA-DN-IKKγ thus interferes with the activity of IKK (40,41). HEK293T cells were used as the host cell to produce lentiviruses. Three constructs, FUGW-HA-DN-IKKγ, pCMV-ΔR8.91 and pMD.G, were co-transfected at the respective molar amounts of one, seven and seven into HEK293T cell using T-Pro NTRII (T-Pro Biotechnology, Taipei, Taiwan, China) for 72 h. Culture medium was collected to harvest and titer the resultant viruses (42).
isolated from the astrocytic monolayer at 14 DIV by gentle agitation based on the distinct adhesive properties of microglia and astrocytes (46) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂-containing atmosphere. The purity of primary microglial cultures was determined by immunocytochemical staining using an antibody against a microglia-specific marker (Iba1, Wako Pure Chemical Industries). Approximately, 99% of primary microglia were Iba1 positive. Primary neuronal cultures were prepared from cortices of indicated mice on embryonic days 17 (E17) and E18 as previously described (10). The purity of the neuronal cultures (≥95%) was determined by immunocytochemical staining for a neuron-specific marker (βIII-tubulin, Promega, Madison, WI, USA).

**Immunocytochemical staining**

Cells or brain sections were fixed with 4% paraformaldehyde plus 4% sucrose in PBS at pH 7.4 and room temperature (RT) for 30 min and then permeabilized with 0.1% Triton X-100 at
Figure 9. Overactivation of R6/2 astrocytes evokes damaging effects on neurons. Primary cortical neurons were isolated from E18 mice and directly co-cultured with primary astrocytes (DIV 30) for 4 days. (A and B) Co-cultures were stimulated with LPS; 0.5 μg/ml for 72 h and double immunostained for βIII-tubulin (red) and activated caspase 3 (green). The total intensity of activated caspase 3 was measured and normalized to the number of neurons. At least, 180 cells from 3 independent mice were quantified. (C and D) Specific neuronal survival in co-cultures was measured using a MAP2 assay. (E and F) The conditioned medium of the indicated co-cultures was collected to NO production using the Griess reagent. Data are presented as the mean ± SEM of three independent experiments. a, Specific comparison between WT neurons/WT astrocytes and R6/2 neurons/R6/2 astrocytes; b, specific comparison between WT astrocytes and R6/2 astrocytes in the same group of neurons; c, specific comparison between WT neurons and R6/2 neurons in the same group of astrocytes., P < 0.01. Neu, neurons; AST, astrocytes.
RT for an additional 30 min. Non-specific antibody binding was blocked by incubating cells with 2% normal goat serum plus 2% bovine serum albumin (BSA) in PBS for 1 h at RT and incubated with the desired primary antibody at 4°C for 18 h, followed by incubation with the corresponding secondary antibody for 2 h at RT. Nuclei were stained with Hoechst 33258. Patterns of immunostaining were analyzed with the aid of MetaMorph software (Universal Imaging, West Chester, PA, USA) and a CCD microscope (Zeiss, Göttingen, Germany) or a confocal microscope LSM 780 (Zeiss).

Figure 10. Blockage of IKK reduced TNFα and IL-1β levels in brains of R6/2 mice. Mice (at 7 weeks of age) were intrastrially injected with a lentivirus carrying a dominant negative mutant of IKKγ (HA-DN-IKKγ) or an irrelevant control protein (GFP). Eight weeks after the injection, the cortex and striatum were collected to assess levels of TNFα and IL-1β by an ELISA (n = 4 in each group). Intrastriatal expression of HA-DN-IKK significantly reduced levels of TNFα (A and B) and IL-1β (C and D) in the cortex and striatum of R6/2 mice, but not in WT mice. Data are presented as the mean ± SEM of three independent experiments. a, Specific comparison between GFP-expressing mice and DN-IKK-expressing mice of the same group; b, specific comparison between WT and R6/2 mice of the same treatment. P < 0.01.

Nissl staining and quantification
Brain sections of 15-week-old mice containing the striatum (AP, +0.98 to 0.14; L, +1.5 to 2.5; DV, −3 to −4 mm relative to the bregma and dural surface) were stained with Nissl substance using cresyl violet as previously described (35). Neurons appeared round and light purple in Nissl-stained brain sections. Three pictures were taken in the striatal area of each brain section using a CCD camera (Axio Imager Z1 Microscope, Zeiss). Nine frames from three sections spaced evenly throughout the striatum were analyzed for each animal by an investigator blinded to the experimental condition. At least, 1500 cells from each animal were counted and measured to determine the size of neurons and the number of neurons in the striatum. Data are presented as the mean ± SEM.

Enzyme-linked immunosorobnt assay
Levels of TNF-α and IL-1β were determined using ELISA kits (DuoSet ELISA Development System, R&D Systems, McKinley Place, MN, USA) following the manufacturer’s protocol. Briefly, 96-well microplates were coated overnight with the capture antibody, blocked with 1% BSA in PBS and extensively washed. Samples or standards (100 μl) were
added to the microplates, incubated for 2 h at RT, washed extensively, followed by a 2 h incubation with the biotin-conjugated detection antibody and a 30 min incubation with streptavidin-horseradish peroxidase (HRP) plus substrate for signal development. The optical density of each well was detected using a microplate reader set to 450 nm, and readings were subtracted from those at 540 nm using an ELISA Reader (SpectraMAX340PC; Molecular Devices, Sunnyvale, CA, USA).

Transfection and luciferase activity assay

Two days before transfection, primary astrocytes were seeded onto a six-well plate at $5 \times 10^5$ cells per well. Cells were transiently transfected with the desired DNAs using Lipofectamine 2000 (Invitrogen) for 48 h. Luciferase activities of 20 µl of lysate were determined following the manufacturer's protocol using a TD-20/20 Luminometer (Promega) and normalized to the amount of proteins in the lysate. Four independent experiments using at least two different preparations of plasmids were performed for each condition. The promoter region of the mouse CCL5/RANTES gene (GenBank accession no. AB051897) containing an NF-κB-responsive element was subcloned into the pGL-3 basic vector (Promega) that harbors a luciferase reporter gene (Promega) as described previously (10).

Sodium dodecylsulfate polyacrylamide gel electrophoresis and western blotting

Protein concentrations were determined using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis using 10% polyacrylamide gels as detailed earlier (47). After electrophoresis, gels were transferred to nitrocellulose membranes, blocked with 5% skimmed milk in tris-buffered saline with Tween 20 (0.2M Tris-base, 1.37 M NaCl and 0.05% Tween 20) and probed with the indicated antibody at 4°C overnight. Membranes were then incubated with a peroxidase-conjugated secondary antibody for 1 h at RT and washed three times with TBST. The immunoreactive bands were stained using a light-emitting non-radioactive method (ECL; PerkinElmer, Waltham, MA, USA).

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Primary astrocytes (28 DIV, $10^7$ cells/dish) were seeded onto 100 mm culture Petri dishes for 48 h and then stimulated with 10 ng/ml TNF-α plus IL-1β for 0.5, 2 and 4 h. Nuclear proteins were extracted using a CNM Compartment Protein Extraction kit (Biochain Institute, Newark, CA, USA).

Figure 11. Blockage of the IKK-mediated activation of NF-κB signaling reversed the decreased neuronal density in R6/2 mice. Mice (7 weeks of age) were intrastriatally injected with a lentivirus carrying a dominant negative mutant of IKKγ (HA-DN-IKKγ) or an irrelevant control protein (GFP). (A) Eight weeks after the injection, brain sections of 15-week-old mice (n = 4 in each group) were harvested and subjected to Nissl staining. The scale bar indicates 20 µm. (B and C) Three pictures were taken of each striatal section. The striatal neuronal volume and density were quantified. The histograms show the striatal neuronal volume (B) and striatal neuronal density (C). Data are presented as the mean ± SEM of three independent experiments. a, Specific comparison between GFP-expressing mice and DN-IKK-expressing mice of the same group; b, specific comparison between WT and R6/2 mice of the same treatment. P < 0.01. Nine frames from three brain sections spaced evenly throughout the striatum were analyzed for each animal by an investigator blinded to the experimental condition. At least, 1500 cells from each animal were, respectively, measured and counted to determine the size and number of neurons in the striatum. Data are presented as the mean ± SEM.
according to the procedures of the manufacturer. The electrophoretic mobility shift assay (EMSA) was performed as previously described (46).

**MAP2 assay**

The MAP2 assay was conducted as described elsewhere (25). Briefly, cells were fixed with 4% paraformaldehyde, incubated with an anti-MAP2 antibody (1: 1000, Sigma) overnight at 4°C, followed by incubation with HRP-conjugated mouse immunoglobulin G (1: 5000, Perkin-Elmer, Boston, MA, USA) in PBS containing 1% BSA for 30 min at RT. The substrate solution containing 10-acetyl-3,7-dihydroxyphenoxazine (50 μM; Molecular Probes, Eugene, OR, USA) and hydrogen peroxide (200 μM) in 50 mM sodium phosphate buffer (pH 7.4) was added to each well for a 30 min incubation at RT. The resultant resorufin fluorescence in the mixture was measured with a fluorescence reader (with excitation at 550 nm and emission at 590 nm; Molecular Devices).

**Statistical analysis**

Data are expressed as the mean ± SEM of triplicate samples. Each experiment was performed at least three times. Statistical significance was determined by Student’s t-test or a one- or two-way analysis of variance followed by the Student–Newman–Keuls test. A P-value of <0.01 was considered significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We thank Dr Ali Khoshnan and Dr Paul H. Patterson for providing the lentivirus-HA-DN-IKKγ construct and for helpful suggestions. We are grateful to Mr Wei-Cheng Chang and Mr Jheng-Jie Kang for technical support. Human brain sections were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD, USA).

Conflict of Interest statement. None declared.
FUNDING
This work was supported by grants from the National Science Council (NSC97-2321-B-001-030, NSC98-2321-B-001-017, NSC99-2321-B-001-012 and NSC100-2321-B-001-009) and Academia Sinica (AS-100-TP2-B02), Taiwan.

REFERENCES


