Cerebellar Upregulation of Cell Surface Death Receptor–Mediated Apoptotic Factors in Harmaline-Induced Tremor: An Immunohistochemistry Study

Journal of Cell Death
Volume 11: 1–6

DOI: 10.1177/1179066018809091

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ABSTRACT: Active caspase-3-mediated apoptosis has been implicated in the pathogenesis of harmaline-induced tremor. The aim of this study is to illustrate the impact of tremor induction on the expression of factors mediating the cell surface death receptor–dependent apoptosis. A total of 20 normal Wistar rats were randomly selected and equally divided into control and experimental groups. Tremor was induced in the experimental group by injecting the rats with a single dose of harmaline (50 mg/kg). After that, cerebellar tissues were evaluated by immunohistochemistry to examine the expression of tumor necrosis factor α (TNF-α) and active caspase-8 in the 2 groups of animals. TNF-α and active caspase-8 expression was significantly higher in cerebella from experimental rats compared with that in those from the control rats (P value $< .01$). Thus, our present data suggest the association of tremor induction with the cerebellar overexpression of TNF-α and active caspase-8, correlative with Purkinje cell (PC) loss indicated by loss of calbindin immunoreactivity, indicating the induction of the cell surface death receptor–mediated apoptosis.

KEYWORDS: TNF-α, active caspase-8, cerebellum, Purkinje cells, tremor, harmaline

RECEIVED: September 9, 2018. **ACCEPTED:** September 30, 2018.

TYPE: Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was financially supported by the Deanship of Research at Jordan University of Science and Technology (Grant Number: 20150306).

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Tremor is an involuntary movement that is commonly seen in movement disorders, such as essential tremor, Parkinson disease (PD), and spinocerebellar ataxias.[1–3] It is induced in rodents by harmaline, which is a beta-carboline derivative, by overactivating the olivocerebellar system, which is the same mechanism that contributes to generating tremor in humans.[4] Harmaline causes excitotoxicity and subsequent tremor due to facilitating rhythm generation in the inferior olivary (IO) neurons, which project their axons to the cerebellar Purkinje cells (PCs).[5] Consequently, harmaline selectively induces cerebellar PC death by trans-synaptic excitotoxicity, which can be prevented by IO chemoablation using 3-acetylpyridine.[6] However, chronic harmaline administration will induce tolerance in rats.[7]

Apoptosis causes PC neurodegeneration in many neurodegenerative diseases.[8,9] Increased caspase activity following harmaline administration suggested the implication of apoptosis in the pathogenesis of harmaline-induced tremor causing the degeneration of cerebellar PCs.[5,6] Apoptosis is a programmed cell death that is mediated by caspases, which constitute a family of cysteine proteases.[10,11] Caspases are synthesized in the cell as inactive zymogens that become activated upon apoptotic stimuli.[12,13] Caspases are categorized into initiator caspases and executioner caspases.[14] Initiator caspases are activated subsequent to their cleavage that is induced by the apoptotic stimuli.[12,15] Once activated, initiator caspases cleave and consequently activate executioner caspases, which mediate the apoptotic events that lead to the morphological features of apoptosis.[16,17] Active caspase-3, which is an executioner caspase that plays a key role in apoptosis,[9,18] has been shown in cerebellar PCs and granule cells in harmaline-induced tremor.[19]

Apoptotic pathways can be either mitochondria-mediated or cell surface death receptor–mediated.[20,21] Active caspase-8, which is an initiator caspase, mediates cell death receptor–dependent apoptosis, which is also known as extrinsic apoptotic pathway.[22] Tumor necrosis factor α (TNF-α) is involved in the cell surface death receptor–mediated apoptosis as the cell death receptor ligand that initiates and stimulates the apoptotic pathway.[22]

We hypothesize that the proapoptotic factors TNF-α and active caspase-8, indicative of cell surface death receptor–mediated apoptosis, play a pathological role in the cerebellar PC and granule cell death observed in harmaline-induced tremor. Therefore, using immunohistochemistry and light microscopy, our study has investigated the expression of TNF-α and active caspase-8 in the cerebella of rats with harmaline-induced tremor.

Materials and Methods

Animals

A total of 20 normal 3- to 4-month-old Wistar rats were randomly selected and equally divided into control and tremor groups. Same conditions (22°C ± 1°C, free access to standard chow and water, 12 hours dark/light cycle) were used to house the rats in individual cages. Animal-related protocols were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Jordan University of

Science and Technology. Tremor was induced according to the protocol previously described.[5] To summarize, tremor rats were intraperitoneally injected with a single dose of harmaline hydrochloride (50 mg/kg; Sigma Aldrich, Saint Louis, MO, USA). Control rats were simultaneously injected intraperitoneally with same volume of normal saline. Animals were sacrificed by cervical dislocation 24 hours post harmaline treatment, which is the time when the neurodegeneration and active caspase-3 expression in cerebellar PCs and granule cells were shown.[19]

Immunohistochemistry of TNF-α and active caspase-8 in the cerebellum

After sacrificing the animals, cerebella were dissected, fixed in 10% buffered neutral formaline (10% BNF), and processed. After that, midsagittal 4-μm-thick paraffin-embedded sections were prepared. Previously described protocols were used to perform the immunohistochemistry for calbindin, TNF-α, and active caspase-8.[8,9,18,23–27] Briefly, the 4-μm-thick sections were processed via immunohistochemistry using an antibody to TNF-α (ab6671, Abcam, Cambridge, MA, USA) and an antibody to active caspase-8 (sc-5263, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequent to their deparaffinization and rehydration, the sections were processed for antigen retrieval. Then, after their incubation in 3% hydrogen peroxidase for 5 minutes, the sections were washed with phosphate-buffered saline (PBS). After that, some sections were incubated with anti-calbindin antibody, others were incubated with anti-TNF-α antibody, while the rest of the sections were incubated with anti-active caspase-8 antibody, using the dilutions recommended by the vendor, at room temperature for 1 hour. Next, the sections were washed in PBS and subsequently incubated in biotinylated secondary antibody (LSAB kit, Dako, Carpinteria, CA, USA) for 15 minutes at room temperature. Next, the sections were washed in PBS before and after their incubation with streptavidin horse radish peroxidase (LSAB kit, Dako) for 15 minutes at room temperature. Subsequently, 3′-diaminobenzidine (0.05% DAB) was applied for 2 minutes or longer, until the desired intensity was established. Then, the sections were washed with tap water to stop the reaction. Eventually, the sections were counterstained with hematoxylin and examined under the light microscope. Primary antibodies were omitted in the negative control slides. Human lymphoma slides (ab5146, Abcam, Cambridge, MA, USA) were used as positive control slides for active caspase-8 and TNF-α. Five sections of the cerebellum from each animal group were evaluated for TNF-α and active caspase-8 expression.

Data collection and analysis

Five slides from each animal in each of the 2 groups were examined microscopically. A total of 10 random areas from each section were analyzed for TNF-α and active caspase-8 expression in the cerebella. The sections were photographed using digital camera. Adobe Photoshop software was used to count the total pixels area occupied by positive staining in each area relative to the total pixels area as described previously.[18,23,24,28–30] Then, the average of the pixels area occupied by positive staining relative to the total pixels area was computed for each animal in each group.

Statistical analysis

TNF-α and active caspase-8 expression was analyzed, in different cerebella, and statistically compared between the 2 different groups (n = 10 animals per group) using independent samples *t* test using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). Differences in TNF-α and active caspase-8 expression were considered statistically significant at P value $<.05$.

Results

Calbindin-immunoreactive cell bodies and dendrites of the PCs were homogeneously arranged in continuous PC and molecular layers, respectively (Figure 1A). However, unstained patches were viewed in the PC and the molecular layers disrupting their continuity in the cerebella from experimental rats (Figure 1B), indicating PC loss following the administration of harmaline.

Hematoxylin was used to stain and subsequently identify the nuclei of intact cells in the different layers of the cerebellar cortex (Figures 1 to 3). TNF-α-immunoreactive neurons were linearly aligned along the molecular layer and granule cell layer interface in experimental cerebella (Figure 2B). TNF-α-immunoreactive neurons were also observed in the granule cell layer in experimental cerebella (Figure 2B). However, TNF-α immunoreactivity could be hardly detected in the molecular layer, which contains stellate cells in its superficial part and basket cells in its deep part. Thus, both PCs and granule neurons were immunoreactive to TNF-α in cerebella from harmaline-treated rats. On the other hand, TNF-α expression could be hardly observed in the control cerebellar sections (Figure 2A). In contrast, TNF-α immunoreactivity was very strongly apparent in cerebellar sections from the experimental group (Figure 2B). TNF-α expression is statistically significantly ($P<.01$) upregulated in the cerebella following the induction of tremor by harmaline treatment (Figure 2C).

Similarly, active caspase-8 expression could be barely observed in the control cerebellar sections (Figure 3A). However, active caspase-8 immunoreactivity was strongly obvious in the PC and granule cell layers in cerebellar sections from the experimental group (Figure 3B). In addition, active caspase-8 expression has statistically significantly ($P<.01$) increased following the induction of tremor by harmaline treatment (Figure 3C).

Discussion

This study is the first to illustrate the impact of tremor induction on the expression of the cell surface death receptor–mediated apoptotic factors, namely, TNF-α and active caspase-8, in

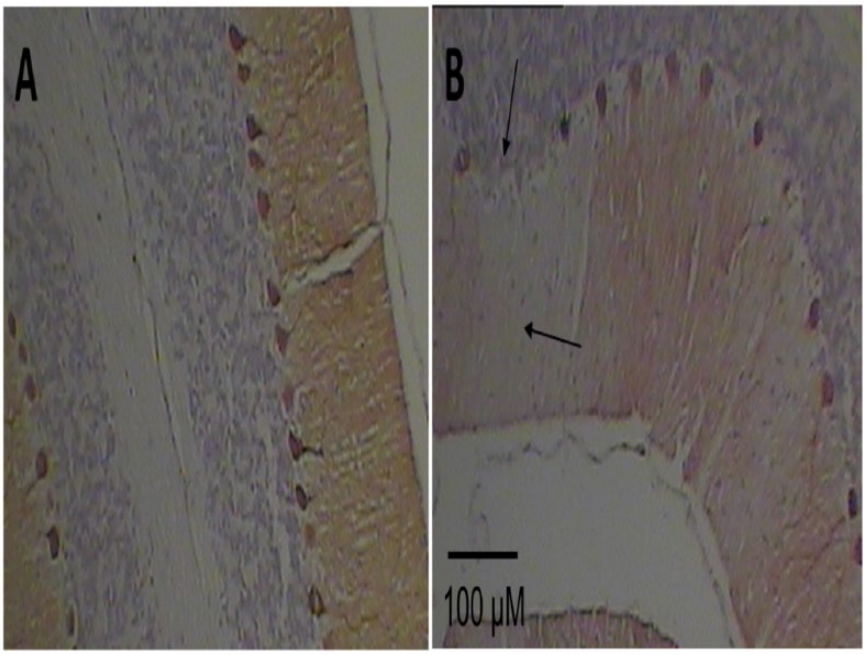

Figure 1. Immunohistochemical staining of calbindin in 4-μm-thick paraffin-embedded cerebellar sections. (A) From control. (B) From experimental. Scale bar shown in (B) applies to both images in the figure. Hematoxylin-stained nuclei helped to differentiate the molecular (ML), Purkinje cell (PC), and granule cell (GL) layers.
Calbindin immunoreactivity was strong in PC cell bodies and dendrites in both control (A) and experimental (B) rats. Nevertheless, in harmaline-treated rats, multiple unstained patches disrupting the continuity of both PCs and molecular layers were present (at the tips of the arrows), indicating a loss of PCs.

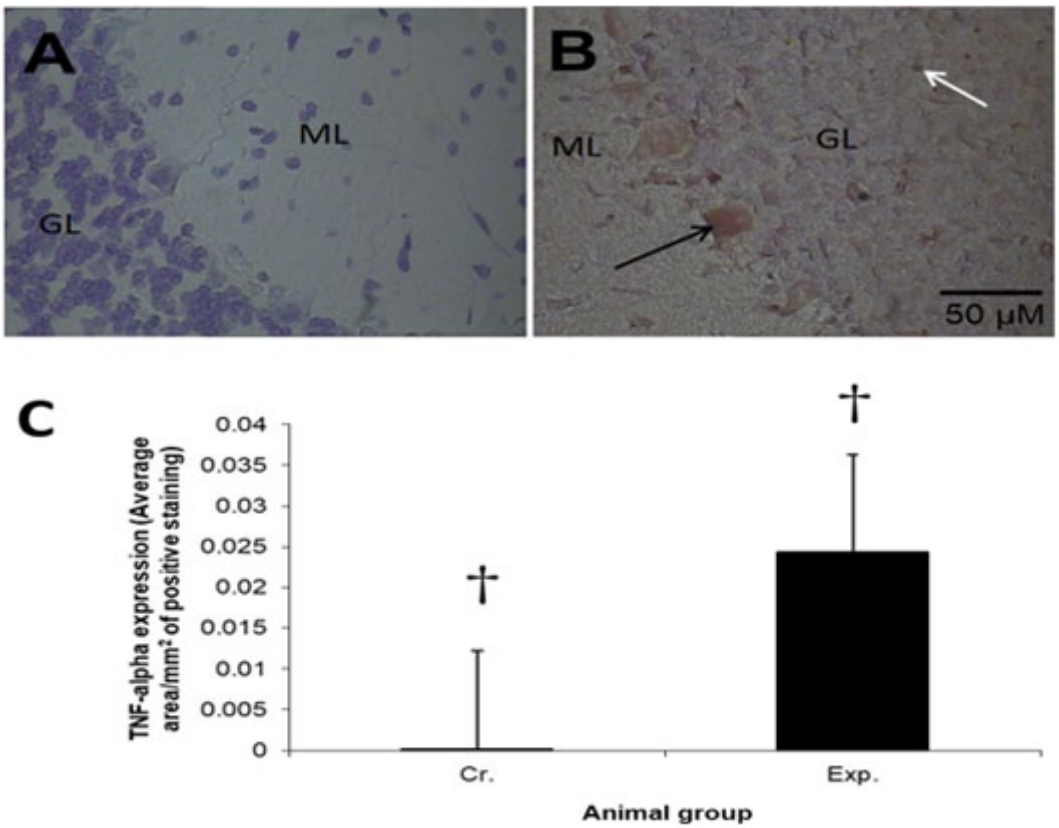

Figure 2. Immunohistochemical staining of TNF-α in 4-μm-thick paraffin-embedded cerebellar sections. (A) From control. (B) From experimental. Scale bar shown in (B) applies to both images (A and B) in the figure. Hematoxylin-stained nuclei help to differentiate the molecular (ML), Purkinje cell (PC), and granule cell (GL) layers. (A) TNF-α immunostaining is hardly observed in the control cerebellum. (B) TNF-α immunoreactivity is strong in PCs (such as that at the tip of the black arrow) and granule neurons (such as that at the tip of the white arrow) from the experimental group. (C) The level of TNF-α expression increased significantly in the experimental cerebella compared to that in the control group ($P < .01^{\dagger}$). Cr., control; Exp., experimental; TNF-α, tumor necrosis factor α.

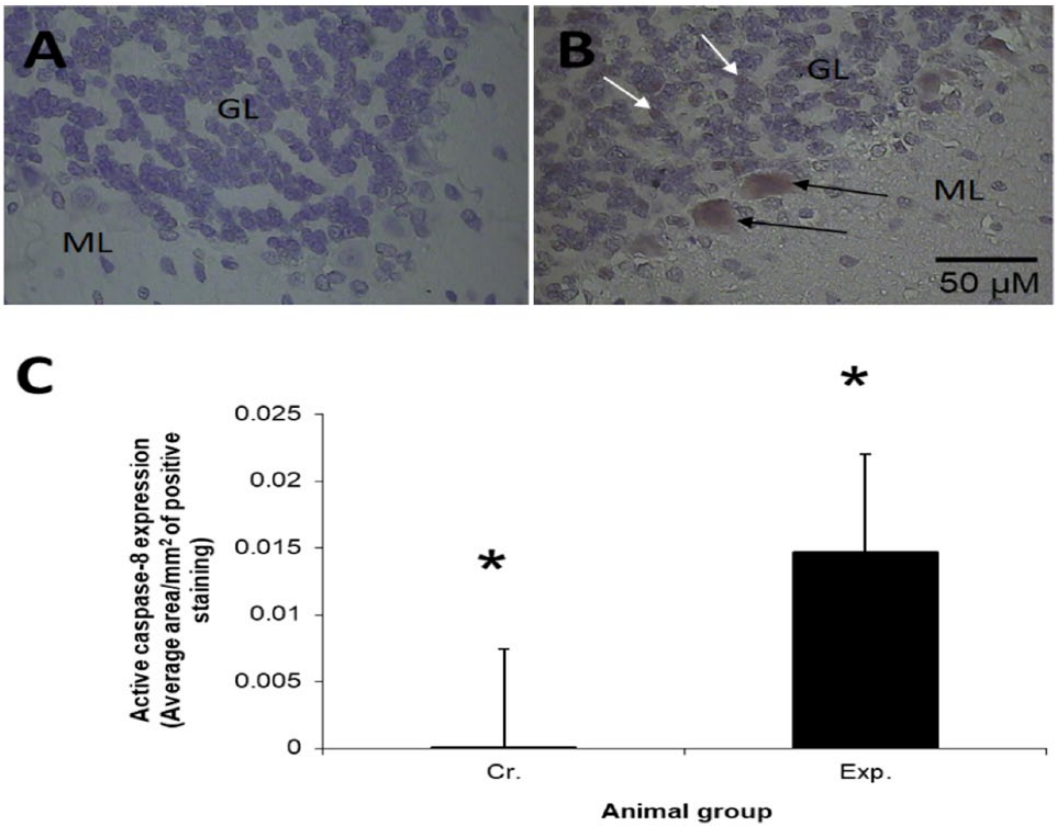

Figure 3. Immunohistochemical staining of active caspase-8 in 4-μm-thick paraffin-embedded cerebellar sections. (A) From control. (B) From experimental. Scale bar shown in (B) applies to both images (A and B) in the figure. Hematoxylin-stained nuclei help to differentiate the molecular (ML), Purkinje cell (PC), and granule cell (GL) layers. (A) Active caspase-8 immunostaining is hardly observed in the control cerebellum. (B) Active caspase-8 immunoreactivity is strong in PCs (such as those at the tips of the black arrows) and granule neurons (such as those at the tips of the white arrows) from the experimental group. (C) The level of active caspase-8 expression increased significantly in the experimental cerebella compared to that in the control group ($P < .01$*). Cr., control; Exp., experimental.

the cerebellum. The consequent analysis reveals TNF-α and active caspase-8 upregulation in the cerebellar PCs and granule cells subsequent to the induction of tremor by harmaline treatment.

Harmaline administration has been reported to induce tremor in rats.[4,5,19] Indeed, harmaline-induced tremor is considered the best model of tremor, because activation of the olivocerebellar system has been suggested to play a key role in the pathophysiological mechanisms underlying essential tremor occurring in human patients and induced by harmaline in rats.[5,19] Harmaline has been shown to induce neurodegeneration and active caspase-3-mediated apoptosis in cerebellar PCs and granule cells 24 hours following harmaline administration.[5,19] Thus, we sought to examine the expression of mediators of the cell surface death receptor–mediated apoptosis, namely, TNF-α and active caspase-8, as an underlying mechanism of PC neurodegeneration seen in essential tremor, 24 hours post harmaline administration.

TNF-α initiates and promotes the cell surface death receptor–mediated apoptosis.[31–33] Previous studies have demonstrated TNF-α overexpression in many neurodegenerative diseases, such as PD, Alzheimer disease (AD), and amyotrophic lateral sclerosis (ALS).[34–37] In addition, elevated levels of TNF-α have been shown in the cerebellar PCs in many pathological conditions.[36] These previous reports are consistent with our results that reveal TNF-α upregulation in the cerebellar PCs subsequent to tremor induction. To our knowledge, these are the first data to demonstrate the alterations in TNF-α expression in cerebellar PCs and granule cells subsequent to harmaline administration. TNF-α has been reported to induce death receptor–mediated apoptosis by recruiting and subsequently activating caspase-8, which eventually leads to the cleavage and the subsequent activation of caspase-3.[38–41] Thus, to further investigate the occurrence of cell surface death receptor–mediated apoptosis in PCs, we tested the alterations in active caspase-8 expression following the induction of tremor by harmaline. Abundant active caspase-8 has been reported in the developing cerebellum, but its expression gradually decreases and disappears as development proceeds.[42,43] This is in agreement with our finding of hardly detected active caspase-8 in the cerebellum of control rats (Figure 3). Active caspase-8 has been implicated in many neurodegenerative diseases, such as PD, AD, and ALS.[44–47] Caspase-8 activation has been reported to occur upstream of caspase-3 activation.[48,49] However, caspase-8 activation has been shown to occur downstream of caspase-3 activation as well.[50] In addition, harmaline

has been reported to upregulate the expressions of Fas/FasL, activated caspase-8, and caspase-3. Furthermore, harmaline-induced apoptosis could be significantly inhibited by blocking Fas/FasL signaling, suggesting that cell surface death receptor–mediated pathway was involved in harmaline-induced apoptosis.[51] Thus, in agreement with those previous reports,[44–51] our results reveal elevated levels of active caspase-8 following tremor induction by harmaline treatment.

Previous studies have suggested the upregulation of TNF-α and active caspase-8 mediating the cell surface death receptor–mediated apoptosis in neurodegenerative diseases.[36,52,53] In addition, TNF-α and active caspase-8 upregulation revealed by our results (Figures 2 and 3) was correlative with PC neurodegeneration indicated by partial loss of continuity of calbindin immunoreactivity (Figure 1) following tremor induction by harmaline. Thus, our finding of upregulated TNF-α and active caspase-8 (Figures 2 and 3) in cerebellar PCs and granule cells may indicate the occurrence of cell surface death receptor–mediated apoptosis subsequent to the induction of tremor by harmaline.

Thus, to summarize, our study illustrates the association of harmaline-induced tremor with the overexpression of TNF-α and active caspase-8 in the cerebellar PCs and granule cells, indicating the occurrence of cell surface death receptor–mediated apoptosis in cerebellar PCs and granule cells subsequent to the induction of tremor by harmaline. Moreover, our data suggest that inhibition of TNF-α and/or active caspase-8 might be considered as a potential therapeutic approach to ameliorate tremor in patients.

Author Contributions

NSE conceived and designed the experiments, collected, analyzed and interpreted the data, wrote and revised the manuscript, and approved the final manuscript.

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