Thiotaurine Protects Mouse Cerebellar Granule Neurons from Potassium Deprivation-Induced Apoptosis by Inhibiting the Activation of Caspase-3

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Abbreviations

Ara-C	Cytosine-α-D arabinofuranoside
CGNs	Cerebellar granule neurons
cyt-c	Cytochrome complex
DIV	Days *in vitro*
PI	Propidium iodide
TAU	Taurine
TTAU	Thiotaurine

1 Introduction

Death by apoptosis is a normal phenomenon in animal development, essential to eliminate supernumerary, misplaced or damaged cells with high specificity (Meier et al. 2000).

It is known that taurine (TAU), an endogenous amino acid found at high concentrations in the brain, is a neuroprotective molecule acting as osmoregulator, modulating ionic movements, regulating intracellular level of free calcium and increasing mitochondrial buffering of these ions (Kumari et al. 2013). In particular, TAU protects neurons against glutamate-induced neurotoxicity by modulating glutamate-

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induced membrane depolarization, elevation of $[Ca^{2+}]_i$, activation of calpain, reduction of Bcl-2 and apoptosis (Wu et al. 2009). Moreover, TAU prevents the amyloid-β peptide neurotoxicity in chick cultured retinal neurons by binding to $GABA_A$ receptors and increasing Cl^- conductance (Louzada et al. 2004). Several studies have recently reported novel protective effects of TAU against ischemic stroke (Menzie et al. 2013), age-related impairment of the GABAergic system (El Idrissi et al. 2013) and ethanol-induced apoptosis in the mouse cerebellum during postnatal life (Taranukhin et al. 2010).

Hypotaurine, an intermediate in the biosynthesis of TAU, reacts with sulfide to produce thiotaurine (TTAU), a thiosulfonate. Compared to TAU, the anti-apoptotic effect of TTAU has been poorly investigated so far. For instance, it is only know that addition of 100 μM TTAU to incubation medium determines a 55 % inhibition of Caspase-3 activity during the spontaneous apoptosis of human leukocytes (Capuozzo et al. 2013).

We have expanded present knowledge in the field by comparing the neuroprotective efficacy of TTAU and TAU in a well-established experimental system for studying cell survival and apoptosis, such as isolated cerebellar granule neurons (CGNs).

Since CGNs represent the largest homogeneous neuronal population of mammalian brain and are mostly generated postnatally, *in vitro* cultures of these cells can be easily obtained. Most CGNs die after 6 days in culture unless they are maintained under a chronic depolarizing condition that is obtained in a medium containing a high potassium concentration (25 mM K^+). The 25 mM K^+ condition mimics synaptic excitatory inputs these cells receive from mossy fibers *in vivo*. When CGNs are cultured in 25 mM K^+ for 6 days and shifted to a medium containing a lower potassium concentration (5 mM K^+) they undergo apoptosis within 30 min (Canterini et al. 2009) and die during the following 24–48 h, due to the hyperpolarization of plasma membrane. On the other hand, as originally performed by de Luca et al. (1996), CGNs continuously cultured in 5 mM K^+-containing medium undergo spontaneous apoptosis and show progressive accumulation of DNA breaks, chromatin condensation and nuclear fragmentation.

Besides confirming the ability of TAU to prevent neuronal death of CGNs cultured in 5 mM K^+-containing medium, our results enlighten a novel and more remarkable anti-apoptotic effect of TTAU in CGNs committed to apoptosis in the acute paradigm. In conclusion, TTAU may represent a novel class of TAU derivatives playing a key role in counteracting neuronal apoptosis.

2 Methods

2.1 Chemicals

TTAU was prepared from hypotaurine and elemental sulfur and their purity confirmed by HPLC as previously described (Capuozzo et al. 2013).

TAU was purchased from Fluka BioChemica (Sigma Aldrich, St. Louis, MO). *In vitro* culture media and related reagents were purchased from InVitrogen GIBCO (Invitrogen/GIBCO, Cralsbad, California). Chemicals were from Sigma Aldrich, unless otherwise specified.

2.2 Animals

CD1 mice of the Swiss-Webster strain were purchased from Charles River Italia (Calco, Italy) and raised in our colony. Pups were killed by decapitation without anaesthesia. Experimental protocols and procedures were approved by the Italian Ministry of Public Health and animals were raised in accordance with Sapienza University guidelines for the care and use of laboratory animals. All efforts were made to reduce the number of animals used.

2.3 In Vitro *Cultures of CGNs*

In vitro cultures of isolated CGNs were prepared from cerebella of PN5–6-day-old mice as previously described (Canterini et al. 2012). Briefly, cerebella were rapidly dissected from the brain, minced into small pieces, incubated at RT for 15 min in digestion buffer (containing 0.1 % trypsin and 100 μg/mL DNase in PBS) and repeatedly passed through a flame-polished glass pipette until a single-cell suspension was obtained. Cells were then recovered by centrifugation and suspended in DMEM culture medium containing 25 mM K^+, 2 mM glutamine, 2 % B27 (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin, and 5 % fetal bovine serum (FBS), then plated at a density of 1×10^5 cells/well culture multiwell (previously coated with 0.1 μg/mL poly-L-lysine). CGNs were either continuously cultured for 6 days in a medium containing a standard potassium concentration (5 mM K^+) in the presence/absence of 1 mM TAU or TTAU (chronic paradigm) or in a medium containing an high concentration of potassium (25 mM K^+). Under the 25 mM K^+ condition, CGNs differentiate and increase neurite sprouting and elongation. Cell shift from 25 to 5 mM K^+ and treatment with 1 mM of TAU or TTAU for 16 h were performed after 6 days of *in vitro* culture (DIV6) and were preceded by a 12 h incubation in serum-free medium (acute paradigm).

Routinely, glial cell proliferation was inhibited by supplementing the culture medium with 10 μM cytosine-α-D arabinofuranoside (Ara-C; Sigma Aldrich) 18–22 h after plating.

2.4 Detection of Cellular Viability

In vitro cell viability was estimated by staining nuclei with Hoechst 33258 (Sigma Aldrich) and propidium iodide (PI) (Sigma Aldrich). Because plasma membranes of live cells are not permeant to PI, it was used to detect dead cells, whereas Hoechst staining allowed to count the total number of nuclei (Iyer et al. 1998).

Following treatments, cells were incubated with Hoechst 33258 (10 μM) and PI (10 μM) for 15 min at 37 °C and observed under an epifluorescence microscope (Leica Microsystem, Milan, Italy) to determine the number of Hoechst 33258- and PI-positive cells, respectively. The number of cells counted was approximately 1,000 cells for each independent observation (3 randomly acquired microscopic fields). Images were acquired and analyzed using the Metamorph 5.5 software.

2.5 Detection of Caspase-3 Protein Expression by Western Blotting Assays

To analyze the level of Caspase-3, CGNs were quickly rinsed with chilled PBS, detached/collected using a plastic scraper and homogenized in RIPA buffer (Sigma Aldrich) supplemented with protease-phosphatase inhibitors (Protease and Phosphatase Inhibitor Cocktails, Roche Diagnostics, Milan, Italy). Homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C and supernatants were analysed by Western Blotting (WB). Protein concentration was routinely determined by DC Protein Assay (Bio-Rad, Milan, Italy) loading equal amounts of total protein/lane on 4–20 % gradient Mini-Protean TGX precast gel for electrophoresis (Bio-Rad, Milan, Italy). Fractionated proteins were transferred to PVDF membranes (Roche, Milan, Italy) and then processed for WB using an anti-Caspase-3 rabbit polyclonal (Sigma Aldrich; 1:400 dilution), anti-cleaved Caspase-3 rabbit polyclonal (Cell Signaling Technology, Danvas, MA, USA; 1:600 dilution), and anti-β III Tubulin mouse polyclonal (AbCam, Cambridge, UK; 1:400 dilution) antibodies. Secondary antibodies used were anti-rabbit HRP-conjugated goat polyclonal (Pierce, Rockford, IL, USA; 1:650 dilution) and anti-mouse HRP-conjugated horse polyclonal (Pierce; 1:650 dilution) antibodies. Chemiluminescent protein bands were revealed using SuperSignal West Dura reagents (Pierce). Caspase-3 protein was normalized to β III Tubulin levels.

2.6 Statistics

In all figures, histograms represent the mean ± SEM of data obtained in three independent experiments and having at least three replicates for each data point. Graphics and statistical significance was determined using SPSS software.

3 Results

3.1 TAU and TTAU Likewise Rescue the Death of CGNs Cultured in 5 mM K^+-Containing Medium (Chronic Paradigm)

We have first investigated the neuroprotective effect of TAU and TTAU on CGNs continuously cultured in 5 mM K^+ up to 6 days (chronic paradigm).

To this end, CGNs were cultured for 6 days either in the absence (5 mM K^+) or in the presence of 1 mM of TAU (5 mM K^+ + TAU) or 1 mM of TTAU (5 mM K^+ + TTAU). In parallel, CGNs were also continuously cultured in high potassium-containing medium (25 mM K^+).

Cell viability was then assessed by determining the number of apoptotic nuclei after staining unfixed cells with Hoechst (to stain all nuclei) and PI dye (to stain dead cells). The fraction of dead cells was then determined by visualization under an epifluorescence microscope.

Figure 1 shows that CGNs cultured in 25 mM K^+ were mostly alive with only a small fraction (8 %) of PI-positive cells, whereas CGN cultures maintained in 5 mM K^+ displayed a 70 % of dead cells. The presence of 1 mM either TAU (5 mM K^+ + TAU) or TTAU (5 mM K^+ + TTAU) reduced the fraction of dead cells to 20 %. Together, these data indicate that TTAU is a potent protective molecule in this apoptotic system having an efficacy similar to that of TAU, a well-established neuroprotector (Louzada et al. 2004).

3.2 Compared to TAU, TTAU Rescues More Efficiently CGNs from Potassium Deprivation-Induced Apoptosis (Acute Paradigm)

To gain more insights on the mechanisms underlying TAU and TTAU efficacy in counteracting apoptotic death, we next exploited the acute paradigm to induce apoptosis of CGNs. Since, as mentioned in the introduction, in this paradigm CGNs are triggered to apoptosis after they are fully differentiated, these experiments were aimed at investigating the rescuing ability of TAU and TTAU in a condition mimicking the physiological context in which these cells usually are. To this end, DIV6 CGNs continuously maintained in 25 mM K^+ were triggered to apoptosis by lowering potassium in the culture medium in the absence of TAU or TTAU (5 mM K^+) or in the presence of 1 mM either TAU (5 mM K^+ + TAU) or TTAU (5 mM K^+ + TTAU). After 24 h cell viability was assessed by Hoechst and PI staining, as described in the previous paragraph. In parallel, CGNs were also continuously cultured in high potassium-containing medium (25 mM K^+).

Exposure to 5 mM K^+ resulted in approximately 70 % cell death (Fig. 2), whereas 5 mM K^+ medium supplementation with TAU partially protected CGNs from death,

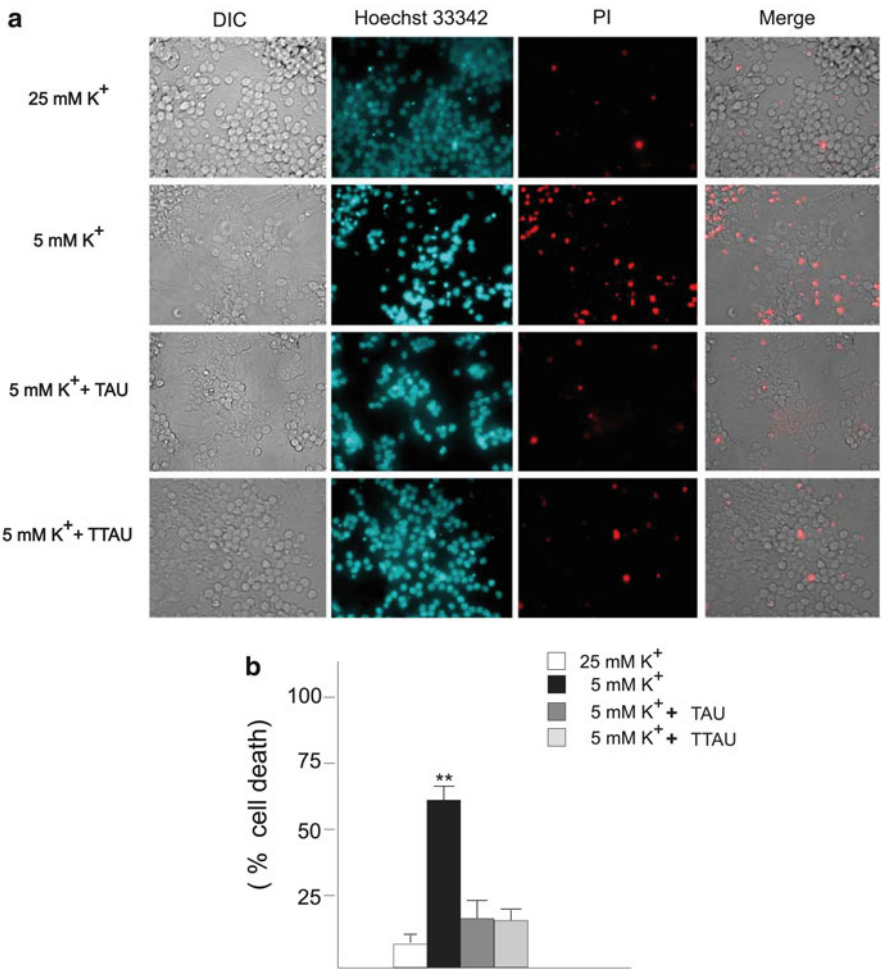

Fig. 1 (**a**) Hoechst (nuclei) and propidium iodide (PI, apoptotic nuclei) double staining of CGNs that were cultured in $K5^+$-containing medium for 6 days (chronic paradigm) either in the absence (5 mM K^+) or in the presence of 1 mM of TAU (5 mM K^++TAU) or 1 mM of TTAU (5 mM K^++TTAU). Scale bar indicates 30 μm. (**b**) Histograms represent the fraction of dead cells, determined by PI staining (*red*)

reducing the fraction of death cells to 35 % of total cells. Interestingly, the supplementation of 5 mM K^+ medium with TTAU more efficiently improved cell survival further reducing the fraction of dead cells to 10 % of total cells.

Given that molecular events downstream from potassium dyshomeostasis in CGNs include the activation of Caspase-3 by proteolysis of the pro Caspase-3 (Mora et al. 2001), we decided to evaluate whether TTAU prevented the proteolytic cleavage of this pro-enzyme.

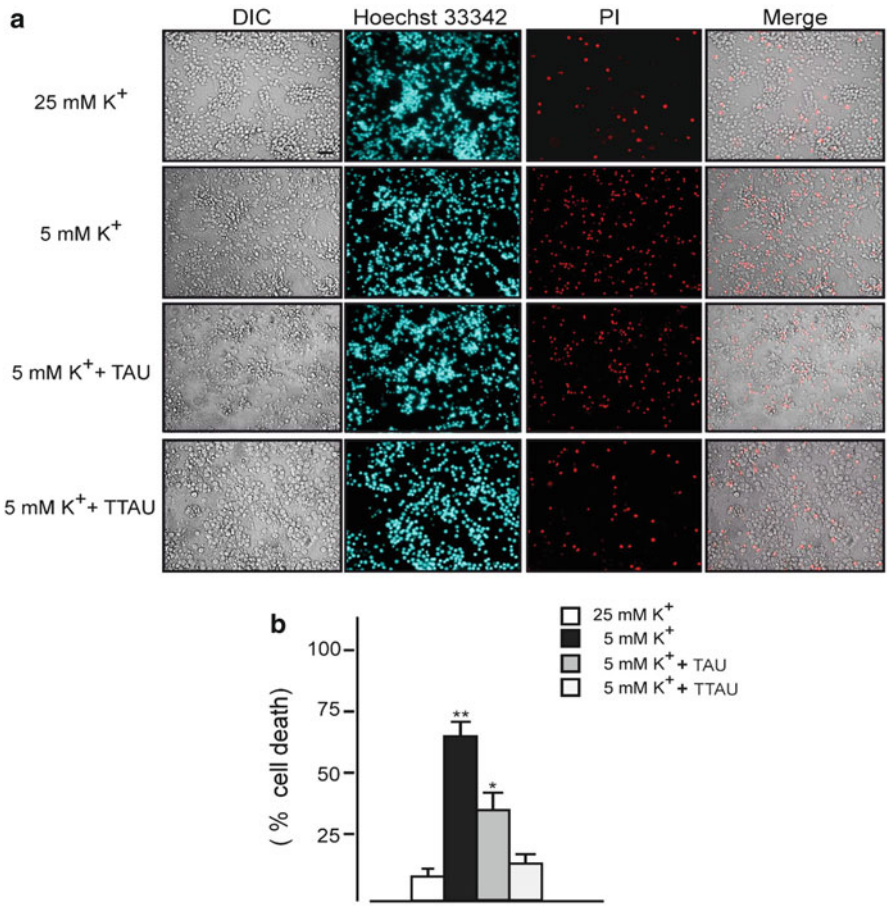

Fig. 2 (**a**) Hoechst (nuclei) and propidium iodide (PI, apoptotic nuclei) double staining of CGNs shifted to 5 mM K^+ medium for 24 h (acute paradigm) after 6 days of culture in 25 mM K^+ medium (depolarizing, pro-survival condition) either alone (5 mM K^+, apoptotic condition) or in the presence of 1 mM TAU (5 mM K^+ + TAU) or 1 mM TTAU (5 mM K^+ + TTAU). Scale bar indicates 30 μm. (**b**) Histogram represents the fraction of dead cells, detected using propidium iodide (*red* staining)

To this end, we determined the amount of activated Caspase-3 by performing WB assays of total proteins extracted from CGNs 16 h after the shifting to 5 mM K^+ medium. Both the uncleaved and the cleaved form of Caspase-3 were detected using specific antibodies and their relative abundance was expressed as ratio between cleaved Caspase-3 and β III Tubulin standard.

While the expression levels of pro Caspase-3 were not changed under the various conditions, K^+ deprivation was marked by a sevenfold increase of activated Caspase-3 compared to the 25 mM K^+ condition. The supplementation of 5 mM K^+ medium with TAU or TTAU significantly reduced and precluded Caspase-3 activation, respectively (Fig. 3).

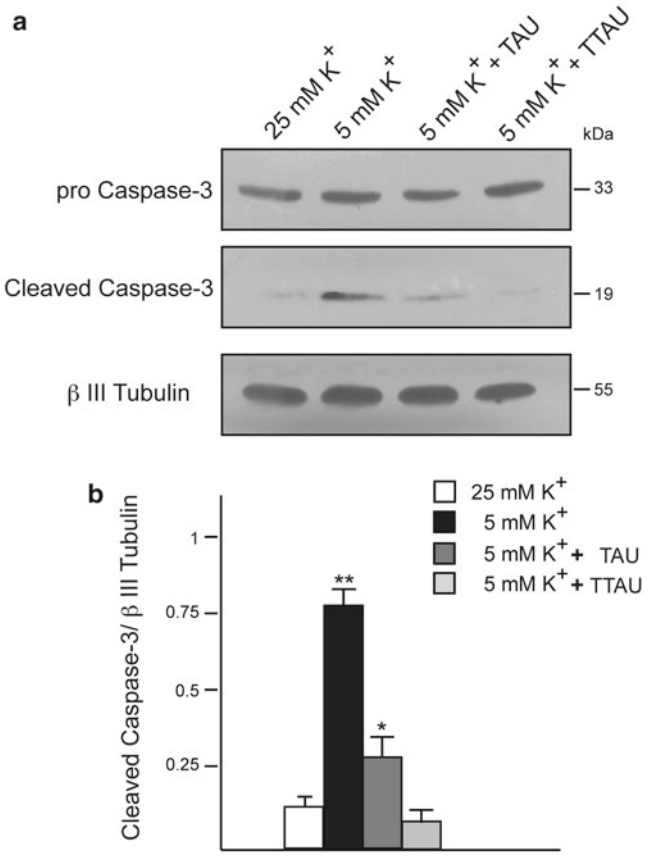

Fig. 3 (**a**) Total protein extracts of CGNs in acute paradigm were processed for WB with anti-pro Caspase-3 and anti-cleaved Caspase-3. (**b**) The relative abundance of cleaved Caspase-3 protein bands was expressed as ratio between pixels of cleaved Caspase-3 and β III Tubulin bands. Histograms represent the mean ± SEM of the ratios obtained in three independent experiments

Taken together these results strongly suggest that when apoptosis is induced by potassium-deprivation in the acute paradigm the TTAU directly prevents the activation of Caspase-3.

4 Discussion

This study provides novel inside on the anti-apoptotic activities of TAU and TTAU in a well-established model system of neuronal apoptosis (Gallo et al. 1987).

The chronic and acute paradigms exploited in this study allowed us to investigate the neuroprotective effects of TAU and TTAU in neuronal cells that markedly differ

in terms of differentiative traits and spontaneously die or can be triggered to die, respectively. In fact, CGNs that are cultured in 5 mM K^+-containing medium (chronic paradigm) are functionally immature and spontaneously die after DIV6 entering a death program that is not mediated by cytochrome complex (cyt-c) release from mitochondria and Caspase-9 activation (Alavez et al. 2003). By contrast, CGNs that are cultured in 25 mM K^+-containing medium for more than 5–6 days fully differentiate and can be committed to apoptosis by the exposure to a 5 mM K^+-containing medium (acute paradigm). Thereafter, they die within 24–48 h, showing typical feature of apoptotic death, including DNA condensation and Caspase-3 activation (Moran et al. 1999).

The responsiveness of CGNs to K^+ deprivation is acquired during *in vitro* differentiation; DIV3-4 CGNs do not respond to the shift in 5 mM K^+ in terms of commitment to apoptosis because they have not yet developed the dependence on membrane electrical activity for survival (de Luca et al. 1996).

Our findings indicate that under the chronic paradigm TAU and TTAU share a similar efficacy in rescuing the survival of CGNs, indicating that TAU and TTAU promote CGN survival through a similar pathway(s). However, in spite of the wealth of information available on the neuroprotective effect of TAU, the role of TTAU has been poorly investigated so far. Among the pathways responsible for the pro-survival effect of TAU in our chronic paradigm, we favor the hypothesis that a regulation of the homeostasis of intracellular K^+ concentration plays a major role. In fact, it has been reported that TAU inhibits several classes of K^+ channels, including Ca^{2+}-activated K^+ channels (Tricarico et al. 2001) and influences K^+ conductance through the inhibition of inward voltage dependent K^+ channels (Kv) (Bulley et al. 2013).

Under the acute paradigm, TTAU more efficiently improves cell survival compared to the very weak effect of TAU. This difference likely relies on the specific feature of the apoptosis induced by K^+-deprivation in fully differentiated CGNs, as outlined above. Apoptosis triggered by the acute paradigm is marked by an early-phase (0–3 h after the apoptotic stimulus) in which ROS production increases and cyt-c release from mitochondria occurs to begin Caspase-3 activation. Then, during a later phase (3–15 h after the apoptotic stimulus) proteosomes activity decreases, Caspase-3 activity increases, cyt-c is degraded and ROS levels remain still high (Atlante et al. 2003).

Because TTAU is a potent anti-oxidant (Acharya and Lau-Cam 2013) and a biochemical intermediate in the transport, storage, and release of sulfide (Pruski and Fiala-Médioni 2003; Capuozzo et al. 2013), we believe that its strong efficacy in counteracting apoptosis in the acute paradigm mostly relies on the ability to reduce the level of ROS. However, Capuozzo et al. (2013) suggested that TTAU prevents apoptosis of human neutrophils by generating H_2S from sulfane sulfur atom, a gaseous molecule that has a regulatory activity on inflammatory responses (Zanardo et al. 2006). Moreover, it is widely recognized that H_2S promotes the short-term survival of neutrophils by inhibiting of Caspase-3 cleavage (Rinaldi et al. 2006) and exerts its anti-aging effects by directly increasing the inhibitory effects of GSH and SOD on ROS production and the redox enzyme levels improving the resistance of

cell to stress (Zhang et al. 2013). In spite of the different efficacy displayed by TAU and TTAU in the acute paradigm, results of Fig. 3 indicate that their pro-survival effect is dependent on the ability to inhibit Caspase-3 activation, indicating a specific role in the early phase of cell commitment to apoptosis.

5 Conclusion

Our study adds novel inside on the anti-apoptotic activity of TTAU, the biological relevance of which is still a challenge to biochemical research, by showing that it strongly inhibits Caspase-3 activation. This pinpoints an important role of this molecule in the biochemical changes associated with early phases of apoptosis in differentiated and functionally mature neurons.

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