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PI3K/Akt AND MAPK PATHWAYS EVOKE ACTIVATION OF FOXO TRANSCRIPTION FACTOR TO UNDERGO NEURONAL APOPTOSIS IN BRAIN OF THE SILKWORM *Bombyx mori* (LEPIDOPTERA: BOMBYCIDAE)

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Abstract

The Forkhead box O (FoxO) transcription factors, including FoxO1, FoxO3a, FoxO4, and FoxO6, are implicated in the regulation of cell apoptosis and survival. Here, we examined the role of FoxO transcription factors and the involvement of the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways in neuronal apoptosis in the brain of the silkworm *Bombyx mori* following starvation. Starvation inhibited cell proliferation and induced apoptosis through caspase-3 activation. The level of phosphorylated kinase Akt increased when the animals ceased feeding. Starvation conditions reduced extracellular-signal-regulated kinase phosphorylation but increased both c-Jun N-terminal kinase and p38 (MAPK) phosphorylation. FoxO1 and FoxO3a were simultaneously localized in the nuclei. These results provide new insights into the process of apoptosis of brain neurons through the involvement of FoxO transcription factors following starvation of insect species.

Key words: FoxO transcription factor, PI3K/Akt, MAPK, apoptosis, *Bombyx mori.*

Article information

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INTRODUCTION

Forkhead transcription factors constitute a protein superfamily. Since the identification of the *forkhead* gene in *Drosophila melanogaster*, the founding member of this family whose mutation results in the development of a forkhead-like appearance (38), more than 100-related forkhead transcription factors have been identified.

Forkhead box O (FoxO) transcription factors belong to the "O" class of the Fox superfamily (3, 22), which reflects the fact that FoxO transcription factors form the most divergent subfamily of the Fox family due to sequence differences within their DNA-binding domains. FoxO transcription factors play a critical role in regulating cell growth, survival, apoptosis, differentiation, proliferation, metabolism, and protection from oxidative stress in various cell types (1). The FoxO transcription factors are largely controlled by posttranslational regulation (11, 34), and they play an important role in insulin signaling (13). In the absence of insulin, FoxO transcription factors translocate to the nucleus and then participate in the transcriptional activation of various genes involved in metabolism.

Akt is a serine-threonine protein kinase regulated by phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is implicated in survival signaling in a wide variety of cells, including neuronal and epithelial cells (28). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), also known as MMAC1 or TEP1, increases sensitivity to cell death in response to several apoptotic stimuli by negatively regulating the PI3K/Akt pathway (33). Additionally, PTEN inhibits growth factor-induced Shc phosphorylation and suppresses the MAP kinase signaling pathway (18).

Three main groups of mitogen-activated protein kinases (MAPKs) are found in mammalian cells. MAPKs, which include the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK), and p38 subfamilies, are important regulatory proteins through which various extracellular stimuli and stresses can be transduced into intracellular events (21, 24).

Little is known about neuronal apoptosis evoked by activation of FoxO transcription factors in insect brains following starvation; therefore, we investigated the mechanism by which FoxO transcription factors induce apoptosis in brain neurons, as well as how they regulate the PI3K/Akt and MAPK signaling pathways following starvation during the silkworm larval stage.

MATERIALS AND METHODS

Animals

Cold-treated eggs of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), were obtained from the National Institute of Agricultural Science and Technology (Suweon, Korea). The eggs were incubated in plastic containers for approximately 10 days at 26.5°C following hatching; the time and date of hatching were recorded. The larvae were reared on the Silkmate™ artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) and incubated at 25°C under 12:12 h light/dark cycle, at 70% humidity in a growth chamber (Doori, Seoul, Korea). Fifth-instar larvae were used in starvation experiments, and larvae reared on leaves without starvation were used as controls. Starved larvae were placed in Tupperware plastic containers containing a moist paper towel for 3 days. Control larvae (n = 50), larvae starved for 1 day (n= 50), and larvae starved for 3 days (n = 50) were used for each experiment.

Cell proliferation and MTT assay

A cell survival analysis was performed according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Cell Titer 96 Aqueous Cell Proliferation Assay kit, Promega, Madison, WI) assay method.

Cultures were prepared as previously described (19). In

brief, larvae were anesthetized by keeping them at 4°C for 15–30 min and then dissected in a cold room. Larvae brains were isolated under a stereoscope in 0.1 M phosphate buffer (PB, pH 7.4), The isolated brains were incubated for 40 min at room temperature in 2 ml of Hanks Ca^{2+}- and Mg^{2+}-free balanced salt solution (Gibco-BRL) containing 0.5 mg/ml collagenase (Invitrogen, Carlsbad, CA, USA) and 2 mg/ml dispase (Invitrogen). The enzyme-treated brains were dispersed by trituration with a fire-polished Pasteur pipette. The enzyme reaction was stopped at the first wash with 1 ml of culture saline and at the second wash with 1 ml of conditioned Leibovitz's L-15 medium followed by centrifugation at 1000 rpm for 10 min. After the removal of the supernatant, the pellet was resuspended in a fresh Leibovitz's L-15 medium by gentle flicking of the tube bottom and gentle aspiration with a 1-ml pipette.

Neuronal cells (3×10^4/well) prepared from larval brains were seeded in a 96-well plate with 100 µl culture media and incubated for 1 day, and 10 µl of 4 mg/ml MTT solution was added to each well of the plate. The cells were then incubated for 4 h in the dark. Absorbance was measured using a microplate reader at 490 nm, and the results were described as a % of control.

Caspase-3 activity

Cells (3×10^4/well) from larvae brains were seeded in a 96-well plate with 100 µl culture medium and incubated for 1 day. Caspase-3 activity was measured with a fluorometer according to the manufacturer's instructions (EMD Biosciences) (29).

Western blotting

The isolated brains were washed in fresh phosphate buffered saline (PBS), homogenized in lysis buffer, and centrifuged. Protein concentration was determined by Bradford assay. The purified proteins were separated by sodium dodecyl-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to a nitrocellulose membrane.

After blockage in 5% nonfat dry milk in Tris buffered saline, the membranes were incubated in the buffered saline with primary antibodies at 1: 200 and 1:500 dilution, overnight at 4°C. Antibodies against PTEN, phospho-Akt, Akt, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, cleaved caspase-3, p21, p27, cyclinD1, and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies against phospho-FoxO1 and phospho-FoxO3a were also purchased from Abcam (Cambridge, MA, USA). The membranes were treated with a 1:1000 dilution of peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies for 2 h. The proteins were detected using the enhanced chemiluminescence western blotting method (Amersham Biosciences, Piscataway, NJ, USA).

Whole-mount immunohistochemistry and confocal microscopy analysis

Tissue preparation and whole-mount immunohistochemistry were performed, as previously described by Na et al. (2004) (30). After administration of anesthesia to the larvae at 4°C for 1 h, brains were isolated in 0.1 M PB (pH 7.4) and fixed in 4% paraformaldehyde (in 0.1 M PB) for 6 h at 4°C.

The fixed tissues were immersed in 0.1 M PBS with 1% Triton X-100 (PBST) at 4°C overnight. The fixed tissues were immersed in 10% methanol in 3% H_2O_2 for 25 min to block peroxidase activity. The tissues were then washed in 0.1 M Tris-HCL buffer (pH 7.6–8.6) containing 1% PBST and 4% NaCl followed by incubation with gentle shaking with phospho-FoxO1 (Abcam) and phospho-FoxO3a (Abcam), which were diluted to 1:200 with the dilution buffer (0.1 M PBST and 10% normal goat serum) for 1 day.

The tissues were washed with 0.1 M PBS containing 1% PBST and incubated with goat anti-rabbit Cy3-conjugated antibodies diluted to 1:500 for 1 day at 4°C. The tissues were rinsed with 0.005 M Tris-HCl buffer and mounted in glycerin, examined, and finally photographed using a model LSM 310 microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

Data were obtained from 3 separate cultures and expressed as means ± SEM. Statistical comparison was performed using an ANOVA test with Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

RESULTS

The effects of starvation on neuronal viability, including apoptosis, were investigated by western blotting. To estimate starvation-induced neuronal apoptosis through caspase-3 activation, the viability, cleaved caspase-3 levels, and levels of selected proteins implicated in neuronal apoptosis were analyzed with primary-cultured neuronal cells.

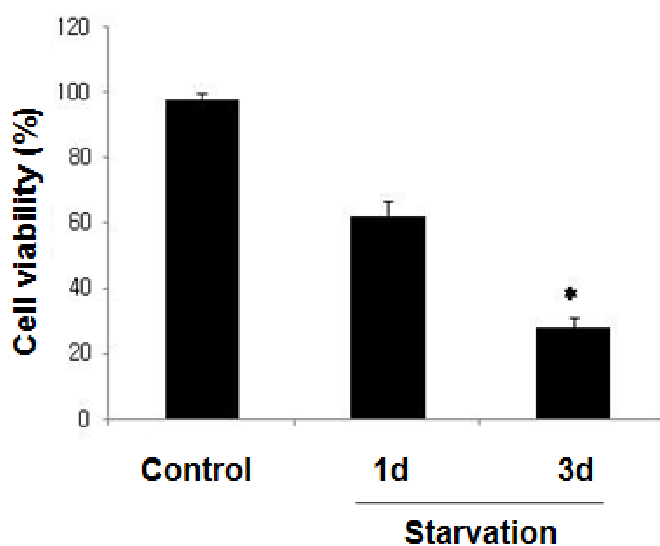

Figure 1. Effect of starvation on viability of neuronal cells. Neuronal cells from the brains of starved larvae were cultured, and the viability was estimated 24 h after initiation of the MTT assay. Values represent the mean ± standard error of the mean (SEM) for 3 different cultures, with n = 3 dishes/culture. *$p< 0.05$, compared with control group.

As shown in Figure 1, neuronal cell viability decreased gradually from 1 day following initiation of starvation and was maximally reduced (by 30%) by 3 days following starvation, compared with the control. Cleaved (or active) caspase-3 levels began to increase at 1 day following initiation of starvation and markedly increased at 3 days after starvation, compared with the control (Fig. 2).

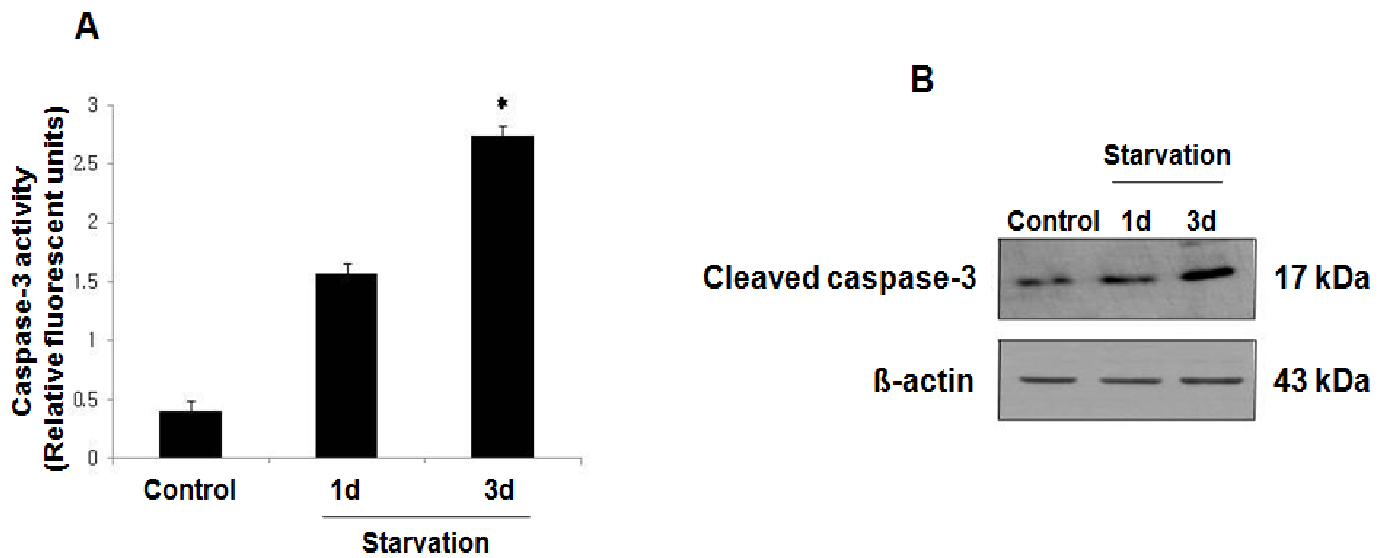

Figure 2. Effect of starvation on caspase-3 activity. (A) Neuronal cells from the brains of starved larvae were cultured, and apoptosis was estimated 24 h after initiation of caspase-3 assay. (B) Pattern of cleaved caspase-3 in the brain of starved larvae obtained from western blotting. Values represent the mean ± standard error of the mean (SEM) for 3 different cultures, with n = 3 dishes/culture. **p* < 0.05 compared with control group.

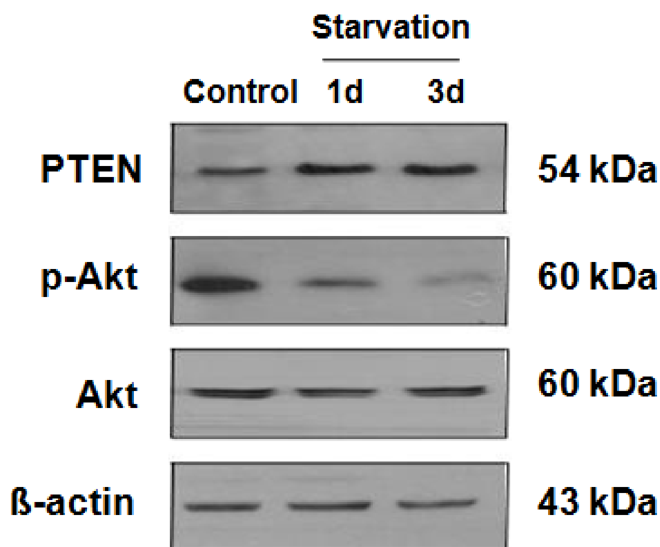

Figure 3. Starvation-induced changes in Akt and PTEN levels. p-Akt, Akt, and PTEN levels were measured by western blotting. The p-Akt level decreased after initiation of starvation, but the PTEN level increased after starvation.

Akt was constitutively active and enhanced cell proliferation. Starvation induced PTEN production and also inhibited Akt phosphorylation (Fig. 3). The MAPK pathway regulated cellular activities, which ranged from gene expression to metabolism and apoptosis. The levels of ERK, JNK, and p38 MAPKs in neuronal cells were changed in a starvation-dependent manner (Fig. 4). Starvation decreased ERK phosphorylation and increased phosphorylation of both JNK and p38.

The role of phosphorylation in relation to FoxO1 and FoxO3a were investigated by performing immunohistochemical analysis. Brain tissues obtained from larvae starved for 3 days exhibited increased nuclear translocation of FoxO1 and FoxO3a in the labeled neurons, compared with that of the control (Fig. 5).

The PI3K/Akt signaling pathway is implicated in cell cycle control, most likely through mechanisms involving the activation of the FoxO transcription factor. Therefore, effects of starvation on cell cycle regulatory genes were examined with neuronal cells. Starvation induced the production of the cell cycle inhibitors p21 and p27, and it inhibited the expression of cyclinD1, as shown in Fig. 6.

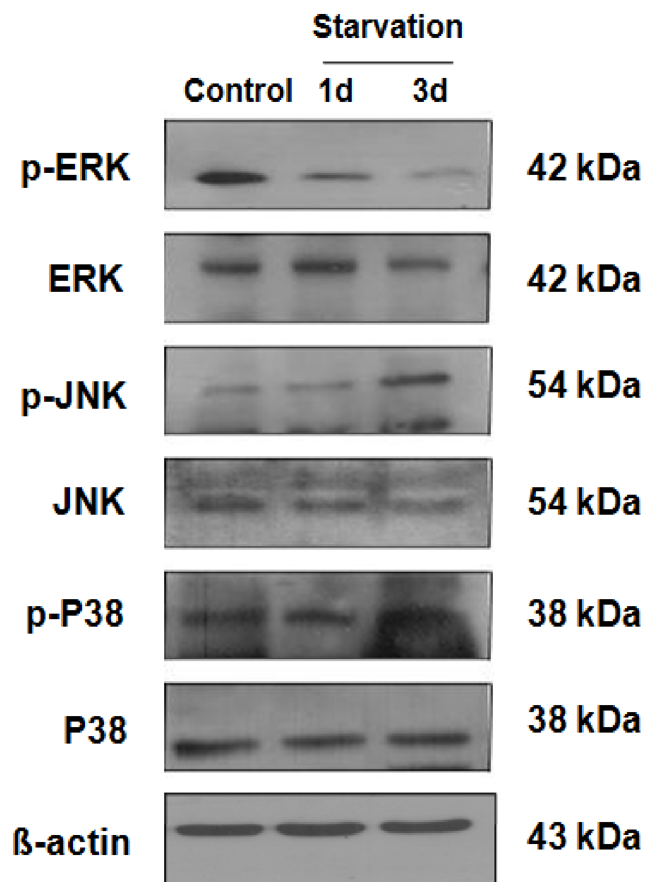

Figure 4. Changes in the MAPK pathway upon starvation. p-ERK, ERK, p-JNK, JNK, p-p38, and p38 levels were measured by western blotting. The p-ERK level decreased following starvation, but the p-JNK and p-p38 levels increased after starvation.

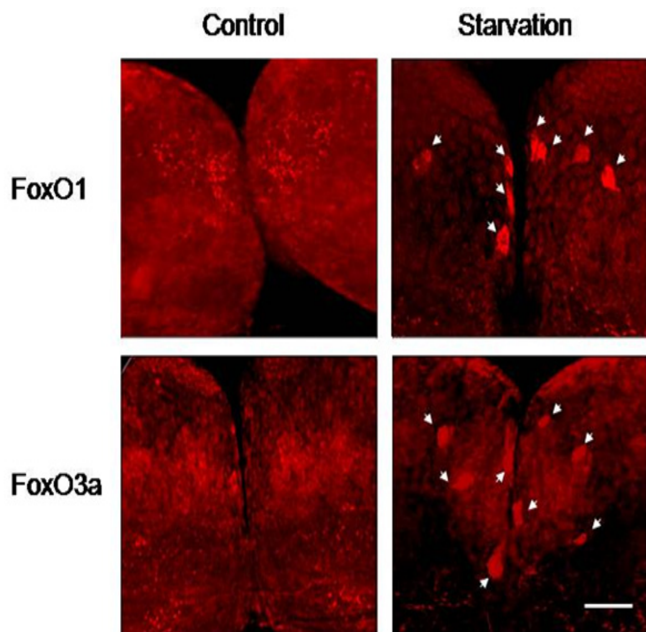

Figure 5. Immunohistochemistry of the silkworm brains revealed localization of p-FoxO1 and p-FoxO3a in several neurons. The brains isolated from the silkworm under starvation condition (starved for 3 days) were treated and then analyzed with p-FoxO1 and p-FoxO3a antibodies (red). They showed the location in the nuclei (white arrows). Scale bar indicates 50 μm. The other 3 photos without scale bar have the same magnification as in the photo with a scale bar.

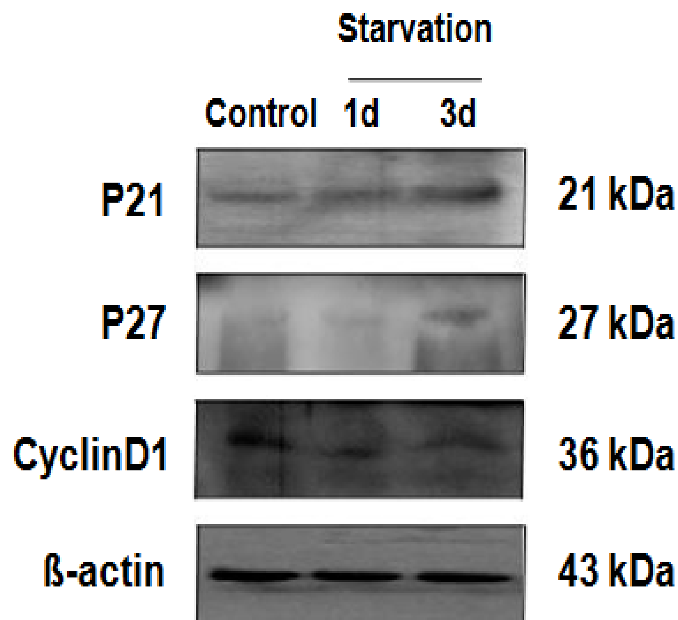

Figure 6. Effects of starvation on cell cycle regulation. p21, p27, and cyclinD1 levels were measured by western blotting. Both p21 and p27 levels increased, but the CyclinD1 level decreased.

DISCUSSION

FoxO transcription factors include orthologs from diverse animal species (e.g., fruit fly, worm, and mammals) that regulate conserved cellular and physiological processes ranging from apoptosis to stress resistance and growth (2). FoxO transcription factors play critical roles in the regulation of tissue homeostasis in organs (8, 26). They are increasingly recognized as critical transcriptional integrators of pathways regulating differentiation, proliferation, survival, and angiogenesis (10, 15).

The FoxO transcription factor is phosphorylated by Akt, inhibited by the insulin signaling pathway, and activated by starvation. Overexpression of FoxO reduces body size and induces starvation in *Drosophila* (27). Therefore, the FoxO transcription factor is an important target of insulin and growth factor activity. Specific signal transduction pathways in the silkworm brain responded to starvation. Apoptosis was induced through caspase-3 activation, and the PI3K/Akt and MAPK pathways were also involved in the apoptotic process.

FoxO1 and FoxO3a were examined in the silkworm brain because they have been known to be abundant in the adult brain (4, 16, 20). FoxO transcription factors contain both nuclear localization and nuclear export signals, which facilitate their shuttling between the cytoplasm and nucleus. Generally, FoxO transcription factors are localized in the cytoplasm, which prevents their transactivation function. Upon activation, the FoxO transcription factors were relocated to the nuclei of the neurons. However, the mechanisms of FoxO transcription factors regulation in insect brains have remained unclear until recently.

The PI3K/Akt pathway phosphorylates each of the FoxO transcription factors (5, 37). Inhibition of the PI3K pathway leads to nuclear translocation of the active FoxO transcription factor, which, in turn, induces apoptosis and cell cycle arrest (31). Loss of PTEN results in increased Akt synthesis, leading to inhibition of the FoxO protein through phosphorylation and cytoplasmic sequestration. FoxO transcriptional activity controls cellular proliferation and apoptosis downstream of PTEN (12). FoxO also regulates cell cycle and apoptotic genes such as the cyclin-dependent kinase inhibitor p27, Bim, and Bcl-2 (6, 17, 35).

FoxO transcription factors act as mediators between the MAPK pathways. Under oxidative stress conditions, FoxO1 changes its intracellular localization from the cytoplasm to the nucleus (23, 25). Furthermore, activation of the JNK pathway decreases the activity of Akt, leading to the phosphorylation of FoxO following nuclear localization. Activated ERK can phosphorylate FoxO, resulting in nuclear exclusion and transcriptional repression. Phosphorylation of FoxO by Akt results in cytoplasmic retention and inactivation, inhibiting the expression of FoxO-regulated genes, which controls the cell cycle, cell death, and oxidative stress (32, 36).

The PI3K pathways regulate the function of the forkhead transcription factor Foxa2, which is expressed in the liver and adipocytes during fasting (39). FoxO and Foxa2 proteins exert distinct and complementary effects on glucose and lipid metabolism (39). Delineating the metabolic functions of FoxO transcription factor in insect brains should be a priority for future studies.

In conclusion, FoxO transcription factors induce neuronal apoptosis in the brain of the silkworm after starvation, through the PI3K/Akt and MAPK pathways.

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