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ORIGINAL ARTICLE

5,7-Dimethoxycoumarin prevents chronic mild stress induced depression in rats through increase in the expression of heat shock protein-70 and inhibition of monoamine oxidase-A levels

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KEYWORDS

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Abstract The current study was aimed to investigate the role of 5,7-dimethoxycoumarin in the prevention of chronic mild stress induced depression in rats. The chronic mild stress rat model was prepared using the known protocols. The results from open-field test showed that rats in the chronic mild stress group scored very low in terms of crossings and rearings than those of the normal rats. However, pre-treatment of the rats with 10 mg/kg doses of 5,7-dimethoxycoumarin prevented decline in the locomotor activity by chronic mild stress. The level of monoamine oxidase-A in the chronic mild stress rat hippocampus was markedly higher. Chronic mild stress induced increase in the monoamine oxidase-A level was inhibited by pre-treatment with 10 mg/kg doses of 5,7-dimethoxycoumarin in the rats. Chronic mild stress caused a marked increase in the level of caspase-3 mRNA and proteins in rat hippocampus tissues. The increased level of caspase-3 mRNA and protein level was inhibited by treatment of rats with 5,7-dimethoxycoumarin (10 mg/kg). 5,7-Dimethoxycoumarin administration into the rats caused a marked increase in the levels of heat shock protein-70 mRNA and protein. The levels of heat shock protein-70 were markedly lower both

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in normal and chronic mild stress groups of rats compared to the 5,7-dimethoxycoumarin treated groups. Thus 5,7-dimethoxycoumarin prevented the chronic mild stress induced depression in rats through an increase in the expression of heat shock protein-70 and inhibition of monoamine oxidase-A levels.

1. Introduction

Depression is characterized by constant alteration in mood, negative thoughts, lack of interest in commonly encountered activities, loss of hope and attempts of suicide (Kessler et al., 2003). Several studies have been performed over past few decades but the mechanism behind depression pathogenesis is not understood fully yet. Currently the medicines used for treatment of depression are any compound which exhibit antidepressant properties (Nemeroff, 2007). The use of common antidepressant compounds causes loss of sleep, loss of memory, addiction and affects reproductive system (Rosen and Marin, 2003). Therefore, discovery of new and effective chemotherapeutic agents free from harmful effect for depression is needed.

Coumarins, are obtained during the phytochemical investigation of several classes of plants distributed throughout the world (Egan et al., 1990). Biological evaluation led to identification of a broad spectrum of activities of coumarins, including anti-oxidant and anti-cancer properties (Maucher et al., 1993; Sharma et al., 1994; Egan et al., 1997; Hayes et al., 1998). The modified coumarin analogs as well as their parent compounds have reached clinical trial stage against several types of tumors (Lake, 1999). 5,7-Dimethoxycoumarin is isolated from the species of vegetables *Citrus limon* L. and *Carica papaya* L. (Salvatore et al., 2004; Canini et al., 2007). 5,7-Dimethoxycoumarin has been shown to inhibit the rate of proliferation of B16 and A375 melanoma cells (Alesiani et al., 2008). The 5,7-dimethoxycoumarin mediated inhibition of cell proliferation has been shown to involve cell cycle arrest in G0/G1 phase. 5,7-Dimethoxycoumarin treatment inhibits the activation of mitogen-activated protein kinase extracellular signal-related kinase 1/2 (MAPK Erk 1/2) present at a higher level in the carcinoma tissues (Fang and Richardson, 2005). The current study was aimed to investigate the role of 5,7-dimethoxycoumarin in the prevention of chronic mild stress induced depression in rats. The results demonstrated that 5,7-dimethoxycoumarin prevented the chronic mild stress induced depression through an increase in the expression of heat shock protein-70 and inhibition of monoamine oxidase-A levels.

2. Materials and methods

2.1. Reagents

5,7-Dimethoxycoumarin was obtained from Sigma–Aldrich (St. Louis, MO, USA). All the required antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) western blotting kit was supplied by Thermo Scientific (Waltham, MA, USA).

2.2. Animals and approval

Twenty healthy male adult Sprague–Dawley rats (around190 g in weight) were obtained from The Experimental Animal Center of Wenzhou Medical College, Wenzhou, China. Approval for the present study was obtained from the ethics committee of the Medical University of Wenzhou (Wenzhou, Zhejiang, China). All the experimental protocols involving animals were performed according to the guidelines of the Chinese laws for animal protection. One week before the start of experiment animals were acclimatized to the laboratory atmosphere. The animals were housed under 12 h dark and light cycles with easy access to water and food.

2.3. Preparation of chronic mild stress animal model

For inducing chronic mild stress, the animals were subjected to sequence of events mentioned below: deprivation of food and water for 24 h, movement were restrained for half minute in iron cages, the cages were rotated for 10 h, encounter of the rats with new rats for 24 h and day and night durations were altered. The sequence of events was altered each time starting with new step. The rats subjected to stress were assigned into three groups of 5 each: stress group, stress group treated with 5 mg/kg and stress group treated with 10 mg/kg doses of 5,7-dimethoxycoumarin. Rats in the treatment groups were intraperitoneally injected with 5 and 10 mg/kg doses of 5,7-dimethoxycoumarin before subjecting to chronic mild stress. The animals in normal control and stress groups were given normal saline.

2.4. Open field test

A box with the dimensions of 100 × 100 × 50 cm was used for determination of locomotory activity of rats. Into the central square of the box bearing 25 squares, animals were put and its movements were recorded over a period of 10 min. The number of times animal crossed was calculated and the results were compared among the groups.

2.5. Immuno histochemical examination

After completion of the test animals were anesthetized and sacrificed to extract entire brain which was dissected on ice into right and left halves. Right hippocampus half of the brain was frozen and stored at −80 °C under liquid nitrogen atmosphere for analysis using reverse transcription polymerase chain reaction (RT-PCR). Second hippocampus half was treated with 4% paraformaldehyde at 4 °C for a period of 24 h. After the treatment, samples were embedded in paraffin and subsequently sliced into 2-μm thin sections. The sections were deparaffined, treated with H_2O_2, and then washed with PBS.

Incubation of the slices was performed with polyclonal rabbit anti-mouse Hsp70 for 24 h and subsequently with horseradish peroxidase conjugated secondary antibody for 1 h. The sections were then treated with a solution of DAB followed by staining with hematoxylin.

2.6. Reverse transcription–polymerase chain reaction (RT–PCR)

After completion of the test animals were anesthetized and sacrificed to extract entire brain which was dissected on ice into right and left halves. Right hippocampus half of the brain was frozen and stored at −80 °C under liquid nitrogen atmosphere for analysis using reverse transcription–polymerase chain reaction (RT–PCR). TRIzol reagent (Tiangen Biotech, Co., Ltd., Beijing, China) was used for the isolation of total RNA sample from the rat hippocampus according to manual protocol. The RNA samples were reverse transcribed into cDNA and then subjected to PCR amplification. Separation of the PCR products was performed by electrophoresis using 2% agarose gel. For visualization and quantification of RNA, ethidium bromide and ImageJ software were used, respectively.

2.7. Western blot analysis

The extracted hippocampus samples were treated with RIPA buffer containing 1% PMSF on ice for lysis. TRIzol reagent (Tiangen Biotech, Co., Ltd., Beijing, China) was used for the isolation of total RNA sample from the rat hippocampus according to manual protocol. Homogenization of the brain samples was performed by centrifugation at 1000×*g* for 15 min. The supernatant obtained was centrifuged at 12,000×*g* for a period of 20 min. Following determination of the protein concentration, the isolated proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and subsequently put onto PVDF membranes (Millipore Corp., Bedford, MA, USA). The membrane incubation was performed at 4 °C for overnight with primary antibodies against caspase-3, MAO-A and Hsp70 using β-actin as internal control. The membrane washing was performed with PBS before 1 h incubation with HRP-conjugated secondary antibodies (Rockland, Gilbertsville, PA, USA). The protein bands were analyzed using the system (Odyssey; LI-COR, Inc., Lincoln, NE, USA).

2.8. Statistical analysis

The presented data are mean ± SEM. Comparison of the data among groups was performed using a one-way ANOVA and then by the least significant difference (LSD) or Student–Newman–Keuls (SNK) post hoc tests for multiple pair-wise comparisons. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical tests and *P*-values <0.05 were taken to indicate statistically significant differences.

3. Results

3.1. Locomotor activity of 5,7-dimethoxycoumarin

5,7-Dimethoxycoumarin prevents the chronic mild stress induced damage to locomotor activity in rats. The results from open-field test showed that rats in the chronic mild stress group scored very low in terms of crossings and rearings than those of the normal rats (Fig. 1). However, the rats treated with 10 mg/kg doses of 5,7-dimethoxycoumarin showed no marked change in the locomotor activity after chronic mild stress relative to normal group. Treatment with 5 mg/kg doses of 5,7-dimethoxycoumarin could not completely prevent the chronic mild stress induced damage to the locomotor activity.

3.2. 5,7-Dimethoxycoumarin inhibits chronic mild stress induced increase in level of monoamine oxidase-A

Analysis of the level of monoamine oxidase-A in the hippocampus showed a markedly higher level in chronic mild stress group than those of normal rats. In the rats treated with 10 mg/kg doses of 5,7-dimethoxycoumarin prior to chronic mild stress no change was observed in monoamine oxidase-A level compared to the normal group (Fig. 2).

3.3. 5,7-Dimethoxycoumarin inhibits the chronic mild stress induced increase in level ofcaspase-3

Chronic mild stress caused a marked increase in the level of caspase-3 mRNA and proteins in rat hippocampus tissues compared to the normal group (Fig. 3). However, the caspase-3 mRNA and protein level in 5,7-dimethoxycoumarin (10 mg/kg) treated rat hippocampus was marked lower compared to the chronic mild stress rats. The levels of caspase-3 mRNA and proteins were almost similar in the hippocampus of 5,7-dimethoxycoumarin (10 mg/kg) treated and normal rats (Fig. 3).

3.4. 5,7-Dimethoxycoumarin increases the level of heat shock protein-70 in the rat hippocampus

Chronic mild stress group of rats showed a markedly lower level of heat shock protein-70 mRNA and protein compared to the rats treated with 10 mg/kg doses of 5,7-dimethoxycoumarin (Figs. 4 and 5). The levels of heat shock protein-70 were markedly lower both in normal and chronic mild stress groups of rats compared to the 5,7-dimethoxycoumarin treated groups.

4. Discussion

In the current study protective effect of 5,7-dimethoxycoumarin on chronic mild stress induced depression in rats was investigated. Depression is caused by reduction in the expression level of neurotransmitters which play a vital role in transmission of neuronal signals (Manji et al., 2001; Mathew et al., 2008). Studies have shown that the level of most common neurotransmitter, monoamine oxidase is markedly reduced during depression (Elhwuegi, 2004; Meyer et al., 2006). Catabolism of monoamines is a vital process for transmission of neuronal signals and its up-regulation leads to onset of depression (Du et al., 2002). Current study revealed that rats in the chronic mild stress group showed poor locomotory activity than those of the normal rats. The rats treated with 10 mg/kg doses of 5,7-dimethoxycoumarin prior to chronic mild stress showed no marked change in the locomotor activ-

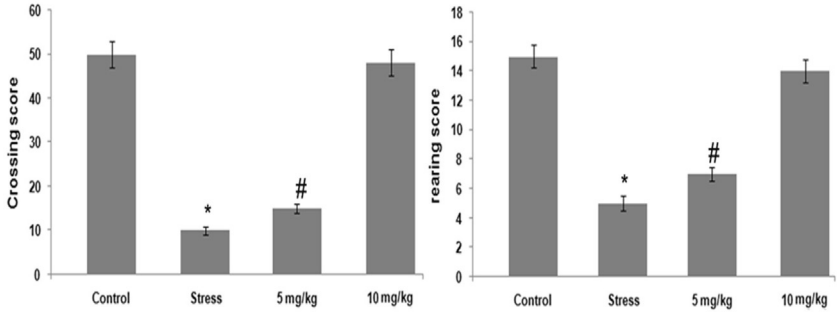

Figure 1 5,7-Dimethoxycoumarin prevents chronic mild stress induced damage to locomotor activity in rats. The rats were treated with 5 and 10 mg/kg doses of 5,7-dimethoxycoumarin and then subjected to chronic mild stress. Following chronic mild stress induction number of crossing (A) and rearings (B) in the box by each rat was calculated over a 10 min period. The expressed values are mean ± SD. $^{*}p < 0.02$ vs control and $^{**}p < 0.02$ vs stress group.

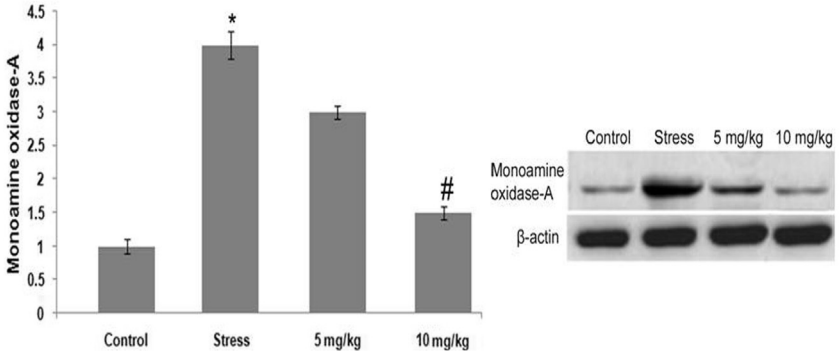

Figure 2 5,7-Dimethoxycoumarin inhibits chronic mild stress induced increase in monoamine oxidase-A. Rats after pre-treatment with 5,7-dimethoxycoumarin were subjected to chronic mild stress and then sacrificed to extract the brain samples. The brain samples were subjected to RT-PCR and western blot assay for analysis of monoamine oxidase-A expression. $^{*}p < 0.02$ vs control, $^{\#}p < 0.02$ vs stress group.

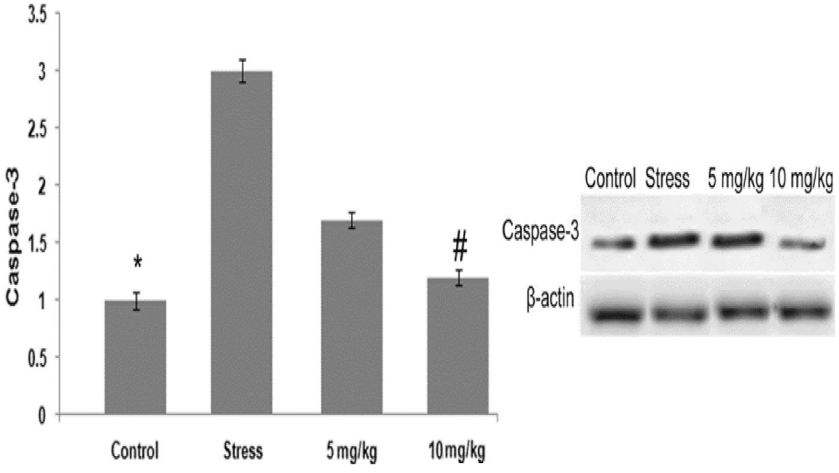

Figure 3 5,7-Dimethoxycoumarin inhibits the chronic mild stress induced increase in level of caspase-3. Rats after pre-treatment with 5,7-dimethoxycoumarin were subjected to chronic mild stress and then sacrificed to extract the brain samples. The brain samples were subjected to RT-PCR and western blot assay for analysis of caspase-3 expression. $^{*}p < 0.02$ vs control, $^{\#}p < 0.02$ vs stress group.

ity. In the current study, pre-treatment of rats with 10 mg/kg doses of 5,7-dimethoxycoumarin prevented the chronic mild stress induced increase in monoamine oxidase-A level. The level of monoamine oxidase-A mRNA and proteins was found to be markedly higher in the chronic mild stress rat hippocampus. Studies have shown that antidepressants also interfere with the expression level of heat shock protein-70 which plays an important role in cell protection (Tanito et al., 2005). Our

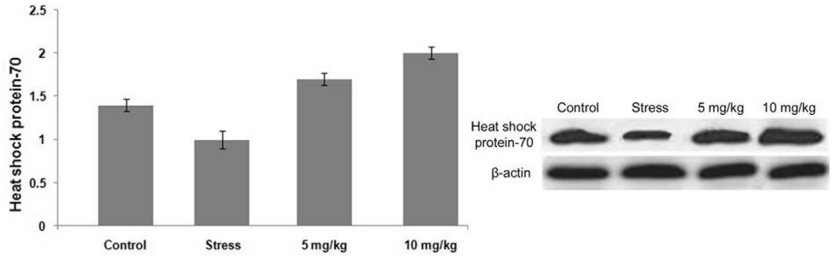

Figure 4 5,7-Dimethoxycoumarin increases the level of heat shock protein-70 in the rat hippocampus. Rats after pre-treatment with 5,7-dimethoxycoumarin were subjected to chronic mild stress and then sacrificed to extract the brain samples. The brain samples were subjected to RT-PCR and western blot assay for analysis of heat shock protein-70 expression. $^{*}p < 0.02$ vs control, $^{\#}p < 0.02$ vs stress group.

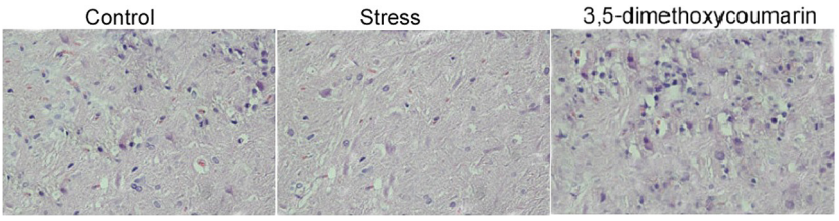

Figure 5 Effect of 5,7-dimethoxycoumarin on heat shock protein-70 expression using immunohistochemical staining. Rats after pre-treatment with 5,7-dimethoxycoumarin were subjected to chronic mild stress and then sacrificed to extract the brain samples. The brain samples were subjected to RT-PCR and western blot assay for analysis of heat shock protein-70 expression.

results from current study showed a marked increase in the level of heat shock protein-70 in rats on treatment with 5,7-dimethoxycoumarin. The heat shock protein-70 level in chronic mild stress and normal control rats was found to be markedly lower.

It has been observed in patients with depression that neurons and glial cells have undergone apoptosis (Lenze et al., 1999; Eastwood and Harrison, 2001; Nolan et al., 1999). The density of neurons in hippocampus of patients with depression has been found to be low (Gould and Tanapat, 1999; McEwen 2007). The cell apoptosis is induced by an increase in the expression level of caspase-3 (Woo et al., 1998). Our results showed a markedly higher level of caspase-3 in chronic mild stress induced rats. In the rats pre-treated with 5,7-dimethoxycoumarin the chronic mild stress induced increase in the level of caspase-3 was prevented. Thus, 5,7-dimethoxycoumarin treatment prevents induction of apoptosis in rat hippocampal neurons by inhibiting expression of caspase-3.

5. Conclusion

In summary, 5 mg/kg doses of 5,7-dimethoxycoumarin prevents chronic mild stress induced depression in rats through reduction in the expression of monoamine oxidase-A and increase in the level of heat shock protein-70 as well as inhibition of apoptosis induction. Thus, 5,7-dimethoxycoumarin has a scope to be evaluated further for the treatment of depression.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Alesiani, D., Cicconi, R., Mattei, M., Montesano, C., Bei, R., Canini, A., 2008. Cell cycle arrest and differentiation induction by 5,7dimethoxycoumarin on melanoma cell lines. Int. J. Oncol. 32, 425–434.

Canini, A., Alesiani, D., D'Arcangelo, G., Tagliatesta, P., 2007. Gas chromatography-mass spectrometry analysis of phenolic compounds from *Carica papaya* L. leaf. J. Food Compost. Anal. 20, 584–590.

Du, L., Faludi, G., Palkovits, M., Sotonyi, P., Bakish, D., Hrdina, P.D., 2002. High activity-related allele of MAO-A gene associated with depressed suicide in males. NeuroReport 13, 1195–1198.

Eastwood, S.L., Harrison, P.J., 2001. Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a western blot study of synaptophysin, GAP-43 and the complexins. Brain Res. Bull. 55, 569–578.

Egan, D., O'Kennedy, R., Moran, E., Cox, D., Prosser, E., Thornes, R.D., 1990. The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. Drug Metab. Rev. 22, 503–529.

Egan, D., James, P., Cooke, D., O'Kennedy, R., 1997. Studies on the cytostatic and cytotoxic effects and mode of action of 8-nitro-7hydroxycoumarin. Cancer Lett. 118, 201–211.

Elhwuegi, A.S., 2004. Central monoamines and their role in major depression. Prog. Neuropsycho. Pharmacol. Biol. Psychiatry 28, 435–451.

Fang, J.Y., Richardson, B.C., 2005. The MAPK signalling pathways and colorectal cancer. Lancet Oncol. 6, 322–327.

Gould, E., Tanapat, P., 1999. Stress and hippocampal neurogenesis. Biol. Psychiatry 46, 1472–1479.

Hayes, J.D., Pulford, D.J., Ellis, E.M., et al, 1998. Regulation of rat glutathione S-transferase A5 by cancer chemo-preventative agents: mechanisms of inducible resistance to aflatoxin B1. Chem. Biol. Interact. 111–112, 51–67.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., Rush, A.J., Walters, E.E., Wang, P.S., 2003. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). JAMA 289, 3095–3105.

Lake, B.G., 1999. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food Chem. Toxicol. 37, 423–453.

Lenze, E., Cross, D., McKeel, D., Neuman, R.J., Sheline, Y.I., 1999. White matter hyperintensities and gray matter lesions in physically healthy depressed subjects. Am. J. Psychiatry 156, 1602–1607.

Manji, H.K., Drevets, W.C., Charney, D.S., 2001. The cellular neurobiology of depression. Nat. Med. 7, 541–547.

Mathew, S.J., Manji, H.K., Charney, D.S., 2008. Novel drugs and therapeutic targets for severe mood disorders. Neuropsychol. Pharmacol. 33, 2080–2092.

Maucher, A., Kager, M., von Angerer, E., 1993. Evaluation of the antitumour activity of coumarin in prostrate cancer models. J. Cancer Res. Clin. Oncol. 119, 150–154.

McEwen, B.S., 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol. Rev. 87, 873–904.

Meyer, J.H., Ginovart, N., Boovariwala, A., et al, 2006. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Arch. Gen. Psychiatry 63, 1209–1216.

Nemeroff, C.B., 2007. The burden of severe depression: a review of diagnostic challenges and treatment alternatives. J. Psychiatr. Res. 41, 189–206.

Nolan, C.L., Moore, G.J., Madden, R., et al, 1999. Prefrontal cortical volume in childhood-onset major depression: preliminary findings. Arch. Gen. Psychiatry 59, 173–179.

Rosen, R.C., Marin, H., 2003. Prevalence of antidepressant-associated erectile dysfunction. J. Clin. Psychiatry 64 (10), 5–10.

Salvatore, A., Borkosky, S., Willink, E., Bardòn, A., 2004. Toxic effects of lemon peel constituents on *Ceratitis capitata*. J. Chem. Ecol. 30, 323–333.

Sharma, S., Stutzman, D., Kellof, J.G., Steele, V.E., 1994. Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. Cancer Res. 54, 5848–5855.

Tanito, M., Kwon, Y.W., Kondo, N., et al, 2005. Cytoprotective effects of geranylgeranylacetone against retinal photo oxidative damage. J. Neurosci. 25, 2396–2404.

Woo, M., Hakem, R., Soengas, M.S., et al, 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12, 806–819.