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# Antagonistic action of caffeine against LY294002-induced apoptosis in cerebellar granule neurons[1]

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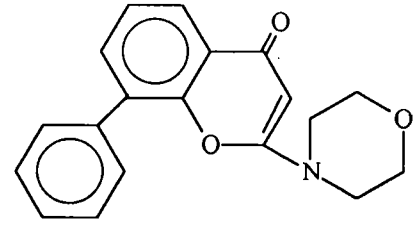
**KEY WORDS** caffeine; apoptosis; LY294002; cultured cells; calcium; cyclic AMP; cerebral cortex; 1-phosphatidylinositol 3-kinase; agar gel electrophoresis; protein kinases

## ABSTRACT

**AIM**: To study the effect of caffeine on apoptosis induced by inhibition of 1-phosphatidylinositol 3-kinase in cerebellar granule neurons. **METHODS**: Cerebellar granule neurons culture, agar gel eletrophoresis, and stress-activated protein kinase (SAPK)/c-*Jun N*-terminal protein kinase (JNK) assay kit to measure SAPK/JNK activity. **RESULTS**: LY294002 evoked apoptosis concentration-dependently in cerebellar granule neurons. But death resulting from LY294002 was prevented by caffeine in a concentration-dependent manner. The survival effect of caffeine was not affected by inhibitors of ryanodine-sensitive $Ca^{2+}$ release, nor was it inhibited by L-type channel blockers and *N*-methyl-*D*-aspartate (NMDA) receptor blocker. In addition, RP-cAMP, H89, and KN62 were not able to inhibit the protective effect of caffeine. Phosphorylation of c-*Jun* was necessary for the induction of apoptosis induced by LY294002 in cerebellar granule neurons. But caffeine directly inhibited the activation of JNK and decreased phospho-c-*Jun* in granule neurons. **CONCLUSION**: Caffeine inhibited the activation of JNK and decreased the phosphorylation of c-*Jun* to protect granule neurons from LY294002-induced apoptosis.

## INTRODUCTION

Depolarizing concentrations of $K^+$ promotes the survival of cultured cerebellar granule neurons by blocking apoptosis[1]. And sustained levels of $[Ca^{2+}]_i$ appear to be pertinent to the survival-promoting effect of high $K^+$[2,3]. But the survival-promoting effect of high potassium was negated by the addition of LY294002 (off-white solid, purity ≥98 %) 30 $\mu mol \cdot L^{-1}$ and wortmannin 1 $\mu mol \cdot L^{-1}$, two distinct inhibitors of 1-phosphatidylinositol 3-kinase (PI-3-K)[4].



**LY294002**

cAMP is implicated as physiologically relevant modulator of neuronal survival; cAMP analogs, or forskolin, a compound raising cAMP levels, prevent the apoptosis of lowering $K^+$-induced cerebellar granule neurons[5].

Caffeine can induce a rapid and transient release of $Ca^{2+}$ from internal store sites of cerebellar granule neurons and elevate cAMP levels by inhibiting the phosphodiesterase activity[6]. Thus, we studied the effect of caffeine on apoptosis induced by inhibiting PI-3-K in cerebellar granule neurons.

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## MATERIALS AND METHODS

**Materials** Caffeine, LY294002, Hoechst 33258, ryanodine, nifedipine and so on, unless otherwise stated, were all obtained from Sigma Chemical Co. Sprague-Dawley rats (7 or 8-day-old, 15 g ± 2 g, Grade Ⅱ, Certificate № 98a 005) of either sex were obtained from Sun Yat-sen University of Medical Sciences.

**Cell culture and cell viability assay** Cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat pups as previously described[7]. Cell viability assays and morphological methods were as the same as before[8].

**Detection of DNA fragmentation** Fragmentation of DNA was analyzed as previously described[9].

**Immunoblotting** Cells were fixed in 3 % paraformaldehyde at 4 ℃ for 20 min, permeabilized with phosphate-buffered saline (PBS) solution for three times, and then were blocked using 5 % goat serum in PBS plus 0.05 % Tween 20 (PBST) for 1 h. After washed 3 times with PBST, the polyclonal phospho-c-*Jun* rat antibody was used at a dilution of 1 : 1000 overnight at 4 ℃. After washed 3 times with PBST, cells were incubated in goat-anti-rat monoclonal antibody. Primary and secondary antibodies were diluted to 2 %.

**Protein kinase assays** JNK activity was measured using SAPK/JNK assay kit[10].

**Statistical analysis** Results were presented as $\bar{x} \pm s$. Statistical analysis was performed with $t$ test.

## RESULTS

**LY294002-induced apoptosis in cerebellar granule neurons** Cerebellar granule neurons cultured from 8-day-old SD rats were switched to depolarizing medium (containing KCl 25 mmol·L$^{-1}$, no serum) either with or without LY294002, and cells survival was assayed 48 h later by counting the number of cells in photomicrographs of fluorescein diacetate (FDA)-stained cultures. LY294002 induced a concentration-dependent cell death of granule cells with an $EC_{50}$ of (20 ± 4) μmol·L$^{-1}$ (Tab 1).

Cell death caused by LY294002 is indistinguishable from that caused by $K^+$ removal, including cytoplasmic blebbing, condensation and aggregation of nuclear chromation (Fig 1), and internucleosomal DNA fragmentation.

**Tab 1. LY294002-induced apoptosis in a concentration-dependent manner in cerebellar granule neurons. $n = 3$ independent experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* caffeine 0 μmol·L$^{-1}$.**

| LY294002/μmol·L$^{-1}$ | Neuronal survival/% control |
|---|---|
| 0 | 98 ± 8 |
| 1 | 93 ± 8$^a$ |
| 5 | 79 ± 7$^c$ |
| 10 | 65 ± 6$^c$ |
| 30 | 46 ± 4$^c$ |
| 50 | 34 ± 5$^c$ |
| 100 | 13 ± 5$^c$ |

**Protective effect of caffeine on LY294002-induced apoptosis** Caffeine maintained survival of granule neurons in the presence of LY294002 in a concentration-dependent manner (Tab 2).

**Tab 2. Protective effect of caffeine on cerebellar granule neurons from apoptosis induced by LY294002. $n = 3$ independent experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* caffeine 0 mmol·L$^{-1}$.**

| Caffeine/mmol·L$^{-1}$ | Neuronal survival/% control |
|---|---|
| 0 | 4.1 ± 1.0 |
| 1 | 3.7 ± 0.9$^a$ |
| 3 | 13.8 ± 4.4$^a$ |
| 5 | 35.3 ± 6.3$^c$ |
| 10 | 81.7 ± 8.5$^c$ |
| 15 | 92.1 ± 9.2$^c$ |
| 20 | 98.6 ± 7.8$^c$ |

The survival effect was observed at concentrations ≥5 mmol·L$^{-1}$, and a maximal effect was reached at 20 mmol·L$^{-1}$. Not surprisingly, DNA fragmentation observed at 24 h after treatment with LY294002 was prevented by caffeine (Fig 2).

**Protective effect of caffeine is not affected by inhibitors of ryanodine-sensitive $Ca^{2+}$ release, nor by inhibitors of $Ca^{2+}$ influx channel** The protective effect of caffeine was not abolished by treating the neurons with dantrolene 20 μmol·L$^{-1}$ or ryanodine 30 μmol·L$^{-1}$, both of which are inhibitors of ryanodine receptor. Moreover, none of the three L-type channel blockers used (nifedipine, nimodipine,

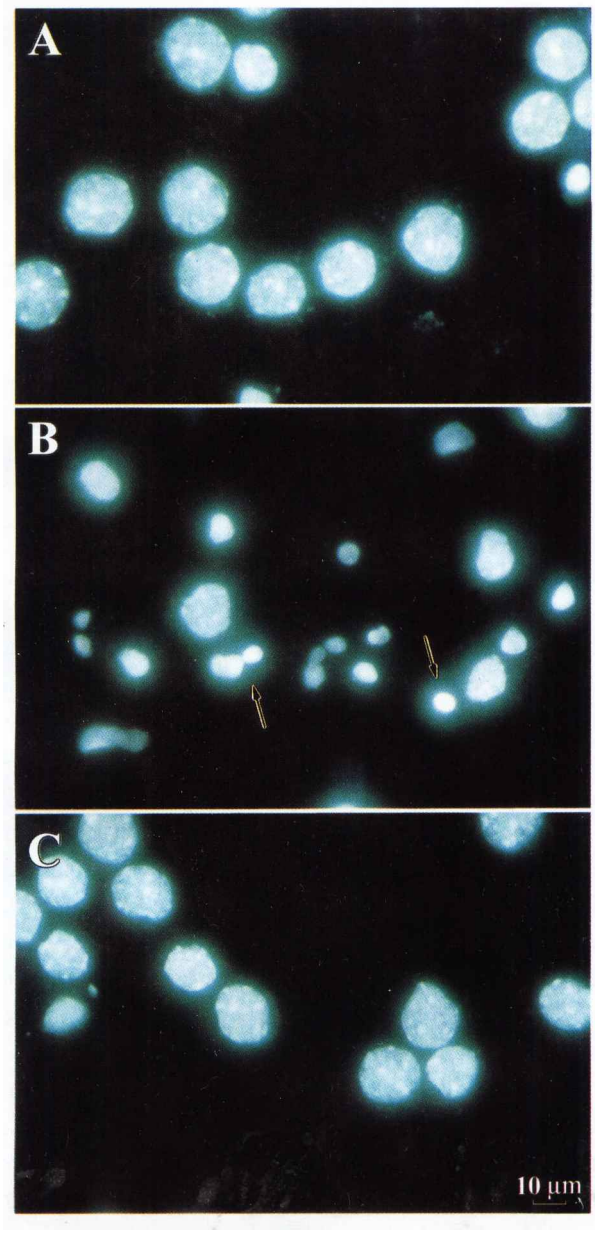


**Fig 1. Phase-contrast micrographs of granule neurons after PI-3-K inhibition. Note the typical apoptotic morphology in neurons in B, but not in A and C. Arrows indicated apoptotic neurons. Hoechst 33258 stain, ×600.**

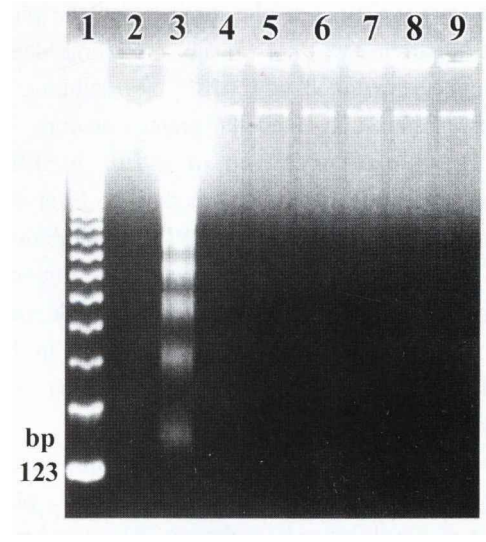


**Fig 2. Agarose gel electrophoresis for detecting DNA fragmentation induced by LY294002 and inhibitory effect of caffeine on apoptosis. *Lane* 1, DNA size marker ladder; *Lane* 2, KCl 25 mmol·L$^{-1}$ medium control; *Lane* 3, LY294002 50 μmol·L$^{-1}$ in K25 medium; *Lane* 4, caffeine 20 mmol·L$^{-1}$ with LY294002 50 μmol·L$^{-1}$ in K25 medium. Note that 185-base pair ladder characteristic of the DNA degradation that occurs in apoptotic cells is detected in LY294002-treated but not in other treated neurons.**

**Tab 3. Effects of caffeine and several drugs on neuronal survival in cerebellar granule neurons. $n = 4$ independent experiments. $x \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* KCl 25 mmol·L$^{-1}$.**

| Group/mmol·L$^{-1}$ | | Neuronal survival/% control |
|---|---|---|
| KCl | 25 | 100.0 ± 9.8 |
| LY294002 | 0.05 | 18.6 ± 4.4$^c$ |
| Caffeine | 20 | 97.6 ± 7.8$^a$ |
| Ryanodine | 0.03 | 95.9 ± 8.2$^a$ |
| Dantrolene | 0.02 | 96.3 ± 7.9$^a$ |
| Nifedipine | 0.01 | 96.8 ± 7.6$^a$ |
| MK801 | 0.01 | 98.2 ± 8.9$^a$ |
| RP-cAMP | 1 | 97.5 ± 8.1$^a$ |
| KN62 | 0.005 | 96.3 ± 9.0$^a$ |
| H89 | 0.03 | 97.9 ± 8.5$^a$ |

and verapamil) inhibited the ability of caffeine to maintain survival. While, the saving action of caffeine was not affected in the presence of the NMDA receptor blocker, MK801 (Tab 3).

**Protective effect of caffeine is not due to the increased accumulation of cAMP by phosphodiesterase inhibition** The protective effect of caffeine was not abolished by a competitive cAMP antagonist Rp-cAMP, PKA antagonist H89 and KN62, a $Ca^{2+}$/calcium-dependent protein kinase Ⅱ ($Ca^{2+}$/CaM-dependent PK Ⅱ) inhibitor (Tab 3), nor by a combination of both or three of these compounds (data

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not shown). These results implied that the protective effect of caffeine was not, if any, in whole due to increasing accumulation of cAMP by inhibiting cAMP phosphodiesterase in cerebellar granule neurons.

**The phosphorylation of c-*Jun* is inhibited by caffeine** To determine whether the level of phospho-c-*Jun* was changed after PI-3-K inhibition, we used the phospho-c-*Jun* antibody in immunofluorescence experiments with cerebellar granule neurons. Control cultures that had been maintained in KCl 25 mmol·$L^{-1}$ showed little, if any, staining with the phospho-c-*Jun* antibody.

After the cells had been switched into serum-free medium containing LY294002 50 μmol·$L^{-1}$, phosphorylated c-*Jun* localized to the nucleus was observed. However, when the cells were maintained with caffeine 20 mmol·$L^{-1}$ and LY294002 50 μmol·$L^{-1}$, neurons were hardly stained with the phospho-c-*Jun* antibody.

Previous results showed that c-*Jun* phosphorylation was increased after PI-3-K inhibition in cerabellar granule neurons, but caffeine inhibited the phosphorylation of c-*Jun*.

**Activation of JNK decreases in the presence of caffeine** To determine whether the activity of JNK decreased in parallel with the decreases in c-*Jun* protein and phosphorylation levels that occurred after treatment with caffeine and/or LY294002, we performed immune complex kinase assays with extracts from granule neurons. Cerebellar granule neurons contain a high level of Jun kinase activity, which does not increased after LY294002 treatment. As the same, caffeine, which inhibited apoptosis induced by LY294002, did not affected the level of Jun kinase. It was through inhibiting the activity of Jun kinase that caffeine protected neurons from apoptosis induced by inhibition of PI-3-K (Fig 3).

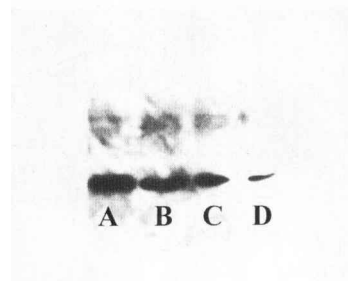


**Fig 3. Analysis of JNK activity of LY294002- and/or caffeine-treated cerebellar granule neurons by Western blot using Phospho-c-*Jun* (Ser63) Antibody. A) Control, in K25 medium with LY294002 50 μmol·$L^{-1}$; B) plus caffeine 5 mmol·$L^{-1}$; C) plus caffeine 10 mmol·$L^{-1}$; D) plus caffeine 20 mmol·$L^{-1}$.**

## DISCUSSION

We have shown that inhibition of the activation of PI-3-K induces apoptosis in granule neurons. But caffeine prevents cerebellar granule neurons from apoptosis induced by LY294002. Caffeine has been known to increase intracellular $Ca^{2+}$ levels by causing $Ca^{2+}$ release from an intracellular ryanodine-sensitive $Ca^{2+}$ store in cerebellar granule cells. However, this release occurs only transiently because of a limited capacity of internal storage. Whether the saving effect of caffeine originates from its short-term activity to release $Ca^{2+}$ from internal storage sites? We found that the saving effect of caffeine was not abolished by treating the neurons with dantrolene 20 μmol·$L^{-1}$ or ryanodine 30 μmol·$L^{-1}$, both have been proved to block the release of internal $Ca^{2+}$ induced by caffeine.

Thus, it is rather safe to conclude that a transient rise of $[Ca^{2+}]_i$ caused by caffeine treatment is not sufficient for neuronal survival. In some cases, however, depletion of internal stores is followed by increase influx of $Ca^{2+}$ through L-type voltage-gated $Ca^{2+}$ channels. To determine whether an influx of $Ca^{2+}$ was involved in the survival effect of caffeine, granule neurons were co-treated with caffeine and various L-type $Ca^{2+}$ channel blockers. It was found that all of the three L-type channel blockers were not capable of inhibiting the saving effect of caffeine. $Ca^{2+}$ entry into cerebellar granule neurons has also been shown to occur by activation of the NMDA receptors. In fact, exposure of cerebellar granule neurons to subtoxic concentrations of NMDA or glutamate promotes survival of these neurons by preventing their apoptosis, a phenomenon accompanied by increased influx of $Ca^{2+}$ through NMDA channels as previously described. But the results showed that the survival promoting effect of caffeine was not affected in the presence of the NMDA receptor blocker, MK801 10 μmol·$L^{-1}$. These results suggest that the survival effect of caffeine is not due to the release of $Ca^{2+}$ from internal stores or $Ca^{2+}$ entry through L-type $Ca^{2+}$ channels and NMDA receptor. It is to say increasing $[Ca^{2+}]_i$ is not required for survival-pro-

moting effect of caffeine.

Another major biological activities of caffeine is mediated through an increase in cAMP levels by inhibiting of cAMP phosphdiesterase. The saving effect of caffeine may be mediated through increasing accumulation of cAMP by inhibiting cAMP phosphodiesterase in cerebellar granule neurons and then the elevated cAMP increases cAMP-dependent protein kinase activity. However, the protective effect of caffeine was not abolished by a competitive cAMP antagonist (RP-cAMP 1 mmol·$L^{-1}$), an inhibitor of PKA (H89 30 $\mu$mol·$L^{-1}$), and $Ca^{2+}$/CaM-dependent PK Ⅱ inhibitor (KN62 5 $\mu$mol·$L^{-1}$), nor by the combination of both or three of these compounds (data not shown). These results implied that the protective effect of caffeine was not, if any, in whole due to increasing accumulation of cAMP by inhibiting cAMP phosphodiesterase in cerebellar granule neurons. This is inconsistent with the result of Tanaka S[11].

Recent studies found that phosphorylation of c-*Jun* was necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons[12]. Phosphorylation of serines 63 and 73 in the c-*Jun* transactivation domain is known to increase c-*Jun* activity[13]. By using an antibody specific for c-*Jun* phosphorylation on serine 63, it was found that the site was phosphorylation soon after LY294002 treatment. But caffeine decreased the levels of phospho-c-*Jun*. This indicated that the caffeine protected neurons from apoptosis by acting upstream of a c-*Jun*-dependent pathway. It was JNK that activated c-*Jun* phosphorylation. And JNK has been thought to mediate apoptosis processes elicited by some stimuli[10]. We found that caffeine did not affect the level of JNK, but inhibited its activity directly.

Taken together, caffeine protected granule neurons from apoptosis induced by LY294002. Increasing calcium and cAMP was not required for saving action of caffeine. We first showed that inhibiting the activity of JNK and decreasing the levels of phospho-c-*Jun* made caffeine have save-promoting effect.

## REFERENCES

1 Yan GM, Ni B, Weller M, Wood KA, Paul SM. Depolarization or glutamate receptor activation blocks apoptotic cell death of cultured cerebellar granule neurons. Brain Res 1994; 656: 43 - 51.

2 Galli C, Meucci O, Scorziello A, Werge TM, Calissano P, Schettini G. Apoptosis in cerebellar granule cells is blocked by high KCl, forskolin, and IGF-1 through distinct mechanisms of action: the involvement of intracellular calcium and RNA synthesis. J Neurosci 1995; 15: 1172 - 9.

3 Franklin JL, Sanz-Rodriguez G, Juhasz A. Chronic depolarization prevents programmed death of sympathetic neurons *in vitro* but does not support growth: requirment for $Ca^{2+}$ influx but not Trk activation. J Neurosci 1995; 15: 643 - 64.

4 Miller TM, Tansey MG, Johnson EM Jr, Greedon DJ. Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization- and insulin-like growth factor Ⅰ-mediated survival of cerebellar granule cells. J Biol Chem 1997; 272: 9847 - 53.

5 D'Mello SR, Galli C, Cotti T, Colisano P. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor/and cAMP. Proc Natl Acad Sci USA 1993; 90: 10989 - 93.

6 Sawynok J, Yaksh TL. Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. Pharmacol Rev 1993; 45: 43 - 75.

7 Yan GM, Lin SZ, Irwin RP, Paul SM. Activation of muscarinic cholinergic receptors blocks apoptosis of cultured cerebellar granule neurons.
Mol Pharmacol 1995; 47: 248 - 57.

8 Li MT, Su XW, Sun J, Qiu PX, Wu YP, Yan GM. Protection of Bcl-2 protein against cyclopiazonic acid-induced apoptosis in Chinese hamster ovary cells. Chin J Pharmacol Toxicol 1998; 12: 184 - 7.

9 Yan GM, Irwin RP, Lin SZ, Weller M, Wood KA, Paul SM. Diphenylhydantoin induces apoptotic cell death of cultured rat cerebellar granule neurons. J Pharmacol Exp Ther 1995; 274: 983 - 90.

10 Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR. Correlation between sustained c-*Jun* N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-α in rat mesangial cells. J Biol Chem 1998; 273: 4027 - 34.

11 Tanaka S, Koike T. Caffeine promotes survival of cultured sympathetic neurons deprived of nerve growth factor through a cAMP-dependent mechanism, Biochem Biophys Acta 1992; 1175: 114 - 22.

12 Schulz JB, Weller M, Klockgether T. Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirment of new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. J Neurosci 1996; 16: 4696 - 706.

13 Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J. Phosphorylation of c-*Jun* is necessary for apoptosis

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induced by survival signal withdrawal in cerebellar granule neurons. J Neurosci 1998; 18: 751 – 62.

## 咖啡因对 LY294002 诱导的小脑颗粒神经元凋亡的拮抗作用[1]

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**关键词** 咖啡因; 细胞凋亡; LY294002; 培养的细胞; 钙; 环腺苷一磷酸; 大脑皮质; 1-磷酯酰肌醇-3-激酶; 琼酯凝胶电泳; 蛋白激酶类

**目的**: 研究咖啡因对磷酸肌醇-3-激酶抑制剂诱导的小脑颗粒神经元凋亡的作用及机制. **方法**: 神经元体外培养, 凝胶电泳, SAPK/JNK 分析盒测定 JNK 活性. **结果**: LY294002 浓度依赖性地触发小脑颗粒神经元凋亡, 但咖啡因具有浓度依赖性的保护作用. 此作用不受 ryanodine-敏感性钙释放阻断剂、L-型钙通道阻断剂和 NMDA 受体阻断剂的影响. 而且, RP-cAMP, H89 和 KN62 均不能抑制咖啡因的保护作用. c-*Jun* 的磷酸化是 LY294002 诱导神经原凋亡所必需, 咖啡因可直接抑制 JNK 的活性, 降低神经元内磷酸化 c-*Jun* 的含量. **结论**: 咖啡因通过直接抑制 JNK 活性而抑制小脑颗粒神经元的凋亡.

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