ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Dec; 21 (12): 1174 – 1178
http://www.chinaphar.com

Stimulatory effect of trans-cinnamaldehyde on norepinephrine secretion in cultured pheochromocytoma (PC-12) cells

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KEY WORDS PC12 cells; cinnamaldehyde; bisoxonol; saxitoxin; 4-aminopyridine; norepinephrine

ABSTRACT

AIM: The effect of trans-cinnamaldehyde (CNMA) on the release of norepinephrine (NE) from nerve terminal was investigated using rat pheochromocytoma 12 (PC-12) cells. **METHODS**: The amount of NA released from PC-12 cells incubated with CNMA or related substances was quantified by high performance liquid chromatography (HPLC)-electrochemical detection. The lipophilic anion bisoxonol was used to monitor the effect of CNMA on the membrane potential. **RESULTS**: CNMA stimulated the secretion of NE in a concentration-dependent manner from 5 μmol/L to 50 μmol/L, while the value of lactate dehydrogenase in the incubated medium was not influenced by CNMA. However, acetaldehyde, cinnamic acid, cinnamoyl chloride and cinnamamide failed to produce similar effect. The action of CNMA can thus be considered specific. The depolarizing effect of CNMA on the membrane potential was also illustrated by a concentration-dependent increase in the fluorescence of bisoxonol, a potential-sensitive dye. Saxitoxin attenuated the depolarizing action of CNMA at concentrations sufficient to block sodium channels. Besides, the effect of CNMA to depolarize the membrane potential in PC-12 cells is greater than that of 4-aminopyridine (4-AP). The action of CNMA on NE releasing depends on extracellular Ca^{2+} and is attenuated by 8-bromo-cAMP at concentrations sufficient to inhibit the action of cyclic AMP. **CONCLUSION**: These findings suggest that CNMA can depolarize the membrane to result in a Ca^{2+}-dependent and cyclic AMP-related release of NE from PC-12 cells.

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Received 2000-09-05 Accepted 2000-10-10

INTRODUCTION

Cortex *Cinnamomi*, commonly referred to as Kuei-Pi or Rou-Gui in China, has been widely used for warming the body interior according to Chinese traditional medicine writing[1]. Patients with soreness and weakness of the lumbar region in addition to frequent urination and/or impotence are diagnosed as having kidney insufficiences (in Chinese medicine, this includes urosexual organs as well) and are treated by this herb[1]. Also, stomach ache and abdominal pain due to hepatic imbalance and/or dysmenorrhea are indicative for treatment with this herb[1]. Trans-cinnamaldehyde (CNMA) was identified as the main component of this herb[2]. Release of norepinephrine (NE) by this principle has been mentioned in isolated heart of guinea-pigs[3]. Recent study showed that CNMA stimulated NE release from the enteric nerve terminals of guinea-pig via depolarization of membranes[4].

Pheochromocytoma (PC-12) clonal cells are derived from rat adrenal medullary tumors. They synthesize dopamine and NE[5,6] and release them in response to a variety of pharmacological agents[7,8]. The mechanisms underlying the secretion of catecholamines from PC-12 cells have been extensively investigated. The mechanisms involve membrane depolarization[5,6] and elevation of cytosolic Ca^{2+} concentration[8,9] upon activation of various membrane-bound receptors. The aim of this study was to analyze further the effects of CNMA on NE secretion in PC-12 cells.

MATERIALS AND METHODS

Cell culture PC-12 cells were obtained from Professor CHENG Min-Hsiung (Chung-Shan Medical and Dental College, Taichung City, Taiwan). Cells were grown in a humidified chamber circulated with 5 % CO_2 and 21 % O_2. The growth medium (RPMI-1640) (Gibco BRL) was supplemented with 10 % horse serum,

5 % fetal calf serum, 100 kU/L of sodium penicillin G and 100 mg/L of streptomycin sulfate. The medium was changed every 2 days. Cells grown to ~80 % confluence were used in the present study.

Experimental protocols Cells (5×10^6) were washed twice with HEPES-buffered serum-free growth medium and placed in a glass vial containing 5 mL of the same medium equilibrated with 21 % O_2. Release was initiated by incubating the PC-12 cells with CNMA, which was supplied by Professor Feng-Lin HSU (Taipei Medical College, Taipei City, Taiwan), at the desired concentration in a continuous shaking water bath (65 strokes per min) at 37 ± 1 ℃ for 30 min, the time required to induce maximal response as observed from preliminary experiments. Then, the reaction was terminated by chilling the tubes in an ice bath. Following centrifugation of the tubes at 5000 × *g* for 10 min, supernatants were collected for determination of NA. Release was calculated as the amount of NA from cells incubated with CNMA minus the parallel blank treated with the same volume of vehicle. Treatments with saxitoxin (RBI, Natick, MA, USA) and 8-bromo-cAMP (RBI, Natick, MA, USA) were started 30 min before incubation with CNMA. Cells treated with the same volume of vehicle in parallel were taken as control. Incubation in the absence of calcium chloride was carried out in calcium-free growth medium with 1 mmol/L edetic acid.

Measurement of released NE Concentration of NE in the supernatants was estimated using high performance liquid chromatography (HPLC) with an electrochemical detector (BAS 200) according to our previous study[10]. Samples spiked with 20 ng of dihydroxybenzylamine (DHBA) (Sigma Chem Co, St Louis, MO, USA), the internal standard, were adsorbed onto activated alumina by continuous shaking for 30 min. The alumina was then washed three times with 1 mL of distilled water. The catechols were eluted by 0.1 mol/L perchloric acid by shaking for 10 min, lyophilized and dissolved in 0.03 mL of 0.1 mol/L perchloric acid for injection into the HPLC through an autoinjector. All values were corrected for recovery (78 % – 82 %) and expressed as pmol/L per mg protein determined as previously reported[10]. Incubation of standard NE with various compounds tested showed that they did not interfere with the measurement of NE.

Determination of lactate dehydrogenase (LDH) activity Lactate dehydrogenase (LDH) activity in the medium was determined using a commercial kit (Besteck Biotech, USA) as an index of cell viability. As described previously[1,7], after exposure of the cells to CNMA or vehicle, the supernatant was separated by centrifugation (500 g for 5 min). The obtained supernatant (0.02 mL) was mixed with 1 mL of working solution containing 0.35 mmol/L NADH and 0.63 mmol/L sodium pyruvate in 0.1 mol/L phosphate buffer (pH 7.6). After incubation at 37 ℃ for 1 min, kinetic measurement at 340 nm for 2 min was carried out in duplicate using a uv pectrophotometer (Hitachi U-3210, Tokyo, Japan).

Determination of membrane potential of PC-12 cells The lipophilic anion bisoxonol (Molecular Probes Inc, Eugene, OR, USA), a reported dye for the change of membrane potential[11,12], was added at 300 nmol/L to a quartz cuvette containing 2 mL of the prewarmed medium. One min after adding bisoxonol, protein (0.3 – 0.5 mg) of PC-12 cells was added. CNMA or 4-aminopyridine (4-AP) (Sigma Chem Co, St Louis, MO, USA) at the desired concentration was then added into the cuvette during the stable state of fluorescence recorded in Hiuachi F-2000 spectrophotometer; an excitation and emission wavelength of 485 and 515 nm was used, respectively[11]. Similar to the previous report[8], bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values using the valinomycin null-point method[13]. Data of intensity variation were expressed as the arbitrary unit of F/Fo where F means the peak of intensity increase by CNMA or 4-AP and Fo is the basal fluorescence, as previously described[14]. In preliminary experiments, CNMA or 4-AP at the concentrations tested had no effect on the bisoxonol fluorescence in the absence of PC-12 cell proteins.

Data analysis Values of $x \pm s$ for each group were obtained from number (n) of samples. The n in the text refers to the number of separate experiments. Statistical analysis of the differences between two mean values was assessed using t-test; a P value of 0.05 or less was considered significant. Where two or more of the obtained means were compared to one control mean, analysis for significance ($P < 0.05$) was carried out by use of Dunnett's multiple comparisons test.

RESULTS

Effect of CNMA on the release of NE from PC-12 cells Incubation of PC-12 cells with CNMA induced an elevation of NE release in a time-related manner reaching a plateau within 25 min in 6 preliminary experiments. Therefore, in all subsequent experiments, CNMA was incubated for 30 min. The release of NE in-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Dec; 21 (12)
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duced by CNMA increased in a concentration-dependent manner, reaching the maximum at 50 μmol/L CNMA (Fig 1). In order to ensure a marked secretion of NE, 50 μmol/L of CNMA was thus used.

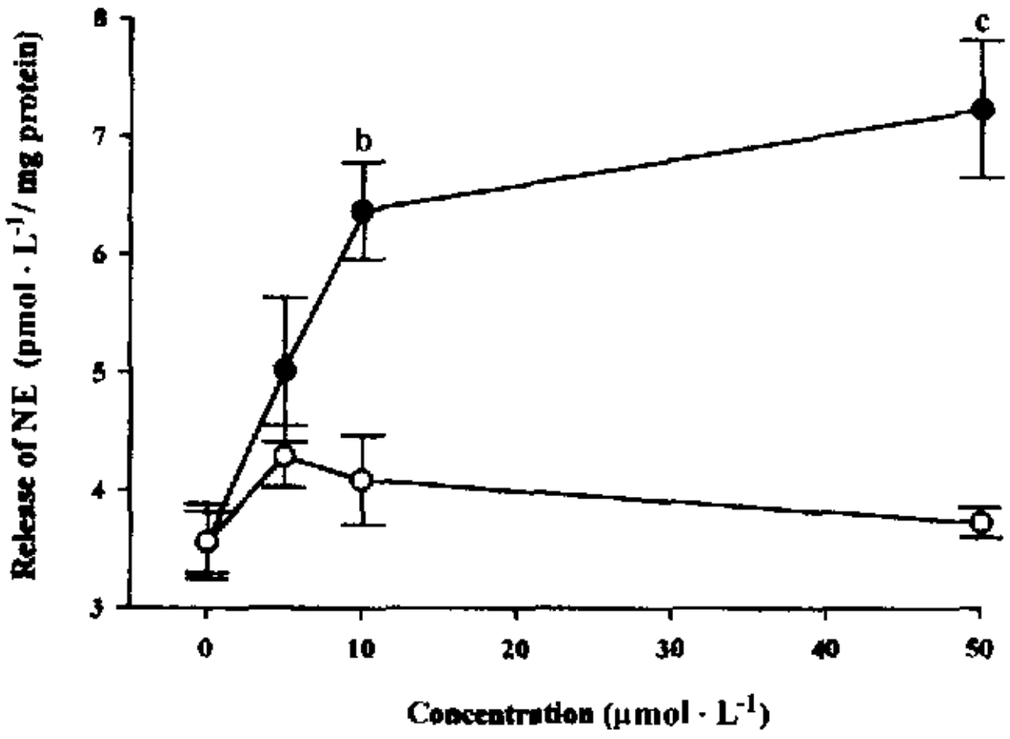

Fig 1. Concentration-response effect of trans-cinnamaldehyde (CNMA) on norepinephrine (NE) secretion from PC-12 cells. The NE secretion was measured in samples incubated for 30 min with CNMA at the indicated concentration; 0 indicates the spontaneous secretion of NE. $n = 8$ separate experiments in normal medium (●) or in calcium-free medium (○). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* corresponding value in the absence (0) of CNMA via unpaired comparison, respectively.

Effect of CNMA on lactate dehydrogenase (LDH) from PC-12 cells Lactate dehydrogenase (LDH) activity was not influenced in samples incubated with CNMA. The activity in samples incubated with 50 μmol/L of CNMA was (0.112 ± 0.01) unit per mL ($n = 8$), which was not different from the control [(0.13 ± 0.03) unit per mL; $n = 8$].

Effect of CNMA-analogues on the release of NE from PC-12 cells Incubation of PC-12 cells with CNMA-like compounds failed to induce the secretion of NE. Tab 1 shows that acetaldehyde, cinnamic acid, cinnamoyl chloride and cinnamamide had no influence on the secretion of NE even at a concentration higher than that of CNMA (Tab 1). Spontaneous secretion of NE was also not different ($P > 0.05$) from the vehicle-treated control.

Effect of CNMA on the membrane potential When exposed to CNMA, PC-12 cells developed a concentration-dependent depolarization, monitored as an increase of bisoxonol fluorescence (Fig 2). Depolarization of the membrane potential was observed at concentration of 10 μmol/L CNMA and was maximal at 50 μmol/L. Similar incubation with distilled water at the same volume did not depolarize the PC-12 cells or show marked changes of basal fluorescence. Depolarization of the membrane potential in PC-12 cells at the 50 μmol/L of CNMA ($F/Fo = 1.29 \pm 0.01$) was about 112.52 ± 0.01 % of the depolarization induced by 4-AP at 100 μmol/L ($F/Fo = 1.15 \pm 0.01$) taken as 100 % ($n = 6$)

Tab 1. Effect of analogues of trans-cinnamaldehyde on secretion of NE from PC-12 cells. $n = 8$ separate experiments. $\bar{x} \pm s$.

Groups/μmol·L^{-1}	Secretion of NE (pmol·L^{-1}/mg protein)
Control	3.61 ± 0.3
Acetaldehyde	
1	3.6 ± 0.6
3	4.2 ± 0.3
Cinnamoyl chloride	
50	3.6 ± 0.2
100	3.8 ± 0.3
Cinnamamide	
50	4.2 ± 0.3
100	3.3 ± 0.7
Cinnamic acid	
50	3.9 ± 0.7
100	3.9 ± 0.9

No difference ($P > 0.05$) was found for each value versus vehicle-treated control. Vehicle solutions were made in the same manner without adding the drug to be tested.

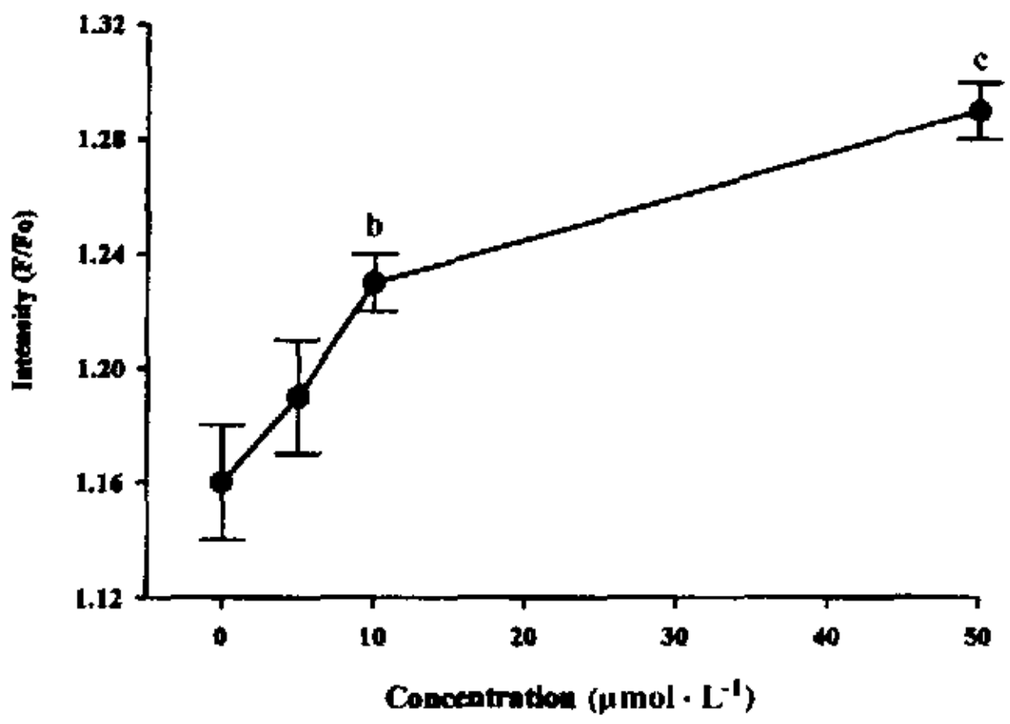

Fig 2. Effect of trans-cinnamaldehyde (CNMA) on membrane potential in PC-12 cells monitored by bisoxonal dye. Values are the arbitrary unit of *F/Fo* where F represents the peak of intensity induced by CNMA and *Fo* the basal fluorescence. $n = 6$ separate determinations. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* vehicle-treated control in the absence (0) of CNMA via unpaired comparison, respectively.

in PC-12 cells. The membrane potential induced by CNMA (50 μmol/L) was markedly reduced in samples pretreated (30 min) with saxitoxin in a concentration-dependent manner (Tab 2).

Tab 2. Effect of saxitoxin on membrane potential induced by CNMA from PC-12 cells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **CNMA (50 μmol/L)-induced membrane depolarization via unpaired comparison by Dunnett's multiple comparisons test. Vehicle solutions were made in the same manner without *adding the drug to be tested*.**

	Intensity (F/Fo)
Vehicle	1.16 ± 0.04 ($n=8$)^c
CNMA (50 μmol/L)	
+ vehicle	1.29 ± 0.01 ($n=7$)
+ saxitoxin (μmol/L)	
0.1	1.18 ± 0.03 ($n=7$)^c
0.2	1.13 ± 0.01 ($n=6$)^c
0.3	1.09 ± 0.04 ($n=7$)^c

All values shown are arbitrary unit of F/Fo from 6 to 8 separate experiments. F represents the peak of intensity induced by CNMA and Fo the basal fluorescence.

Role of Ca^{2+} and cyclic AMP in the action of CNMA In the absence of extracellular Ca^{2+} (calcium chloride-free medium), both the spontaneous NE release and the action of CNMA were lowered markedly compared to that in the presence of Ca^{2+} (Fig 1). Also, the analogs of cAMP, 8-bromo-cAMP lowered the CNMA-induced release of NE in a concentration-dependent manner (Fig 3). The spontaneous secretion of NE was slightly but not significantly lowered compared to the vehicle-treated control [(3.6 ± 0.4) pmol·L^{-1}/mg protein; $n=8$, by treatment with 8-bromo-cAMP at 0.05 μmol·L^{-1} (3.3 ± 0.5) pmol·L^{-1}/mg protein; $n=8$].

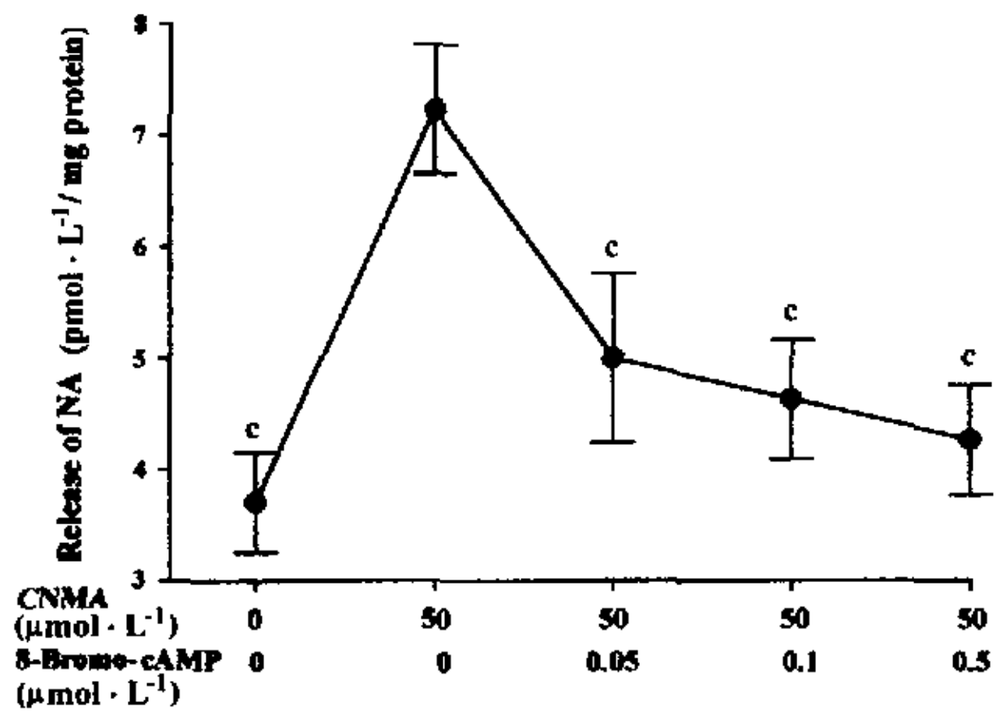

Fig 3. Effect of 8-bromo-cAMP on trans-cinnamaldehyde (CNMA)-induced release of NE from PC-12 cells. The NA secretion was measured in samples incubated for 30 min with CNMA at 50 μmol/L while 8-bromo-cAMP at the indicated concentration was added 30 min before CNMA; 0 indicates the spontaneous secretion of NE. $n=6$ **separate experiments.** $\bar{x} \pm s$. $^cP < 0.01$ *vs* **the value of CNMA at 50 μmol/L with that in the absence (0 μmol/L) of 8-bromo-cAMP via unpaired comparison.**

DISCUSSION

In the present study, we found that CNMA stimulated the secretion of NE from PC-12 cells in a concentration-dependent manner from 5 μmol/L to 50 μmol/L without affecting the release of LDH, one of the cytosolic enzymes[15], suggesting that its action on NE release is not a result of nonspecific damage of cell membrane. Also, the narrow range of the effective concentration (5 to 50 μmol/L) observed in CNMA-treated samples suggests that the activation of endogenous receptors seems unlikely because the agonist is generally effective at a wide range of concentration[16]. The fact that similar action was not observed using CNMA-analogues, such as acetaldehyde, cinnamic acid, cinnamoyl chloride and cinnamamide, even at higher concentrations (Tab 1) suggests that CNMA is specific in causing the release of NE from PC-12 cells. Study with bisoxonol shows that CNMA acts via deolarization of the membrane potential in PC-12 cells (Fig 2). Measurement of change of membrane potential using this lipophilic anion bisoxonol dye has also been depmonstrated in noradrenergic nerve terminals in the guinea-pig ileum[4]. This finding is consistent with the findings using saxitoxin, a specific blocker for sodium channels. Saxitoxin markedly decreased the CNMA-induced membrane depolarization. Like tetrodotoxin (TTX), saxitoxin is introduced as a specific blocker for sodium channel and the saxitoxin-sensitive release of neurotransmitter is usually due to an excitation of nervous cells[17,18]. This follows that sodium channels may participate in stimulus-secretion induced by CNMA in PC-12 cells.

The 4-aminopyridine, a K^+ channel blocker, reduced the delayed potassium current and thereby was able to increase Ca^{2+}-influx during transient Na^+ channel-dependent depolarization to enhance the release of transmitters[19]. The effect of CNMA to depolarize the membrane potential in PC-12 cells is more potent than that of 4-AP. Moreover, the increase of NE secretion by

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Dec; 21 (12)
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CNMA was attenuated by the removal of calcium chloride from the bathing medium indicating a dependence on Ca^{2+}. It is possible that CNMA causes Ca^{2+} influx via membrane depolarization of PC-12 cells. 8-bromo-cAMP, a particularly potent competitive inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinase A[20] suppressed this NE releasing action of CNMA in a concentration-dependent way (Fig 3) within the range effective to block the action of cyclic AMP[17]. It has been reported that cyclic AMP is involved in the secretion of NE from PC-12 cells[21]. Our results are supportive of this view. Future study of electrical stimulated NE release by CNMA would be helpful to further elucidate the pharmacological significance of CNMA in PC-12 cells. Also, the possibility of CNMA being able to open sodium channel also needs more investigation. The results presented in this study suggest that PC-12 cells may serve as a potential model cell line for examining stimulus-secretion coupling during pharmacological manipulation.

In conclusion, our results indicate that CNMA can stimulate NE secretion from PC-12 cells via depolarization of membrane in a saxitoxin-sensitive way.

ACKNOWLEDGMENTS We appreciate Professor FL HUS for the kind supply of trans-cinnamaldehyde. Thanks are also due to Prof CHENG Min-Hsiung (Chung-Shan Medical and Dental College, Taichung City, Taiwan) for the donation of PC-12 cells and Miss YP Lin for her excellent technical assistance.

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