行政院國家科學委員會補助專題研究計畫成果報告

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行政院國家科學委員會專題研究計畫成果報告

AMPA/KA 受體神經營養功能之分子信號路徑研究

Studies of Molecular Signalings of AMPA/Kainate Receptors-Mediated Neurotrophic Functions

計畫編號：NSC 89-2320-B-038-011
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一、中文摘要

本計畫旨在探討興奮性胺基酸受體在發育期大腦皮質神經元所調控的神經營養功能是透過何種信號路徑所達成。在先前的研究中，吾人已發現興奮性胺基酸中的紅藻胺酸(Kainic acid, 簡稱 KA)能夠誘發神經生長因子受體 TrkA 的表現與磷酸化增加，且此一作用具有鈣離子依存性，因此本計畫中乃針對 KA 是否為藉由活化鈣離子依存性激脢 calcium/calmodulin-dependent protein kinase(CaMK)調控 TrkA 表現加以研究，並探討此一調控為透過影響 TrkA 的 transcription 或是 cytosol 與 membrane 之間的 translocation 所致。

關鍵詞：AMPA/KA 受體，紅藻胺酸，神經營養因子，鈣/調鈣素依賴性蛋白激脢

Abstract

In this project, we proposed that excitatory amino acids receptors, with specific focus on the AMPA/KA receptors, may exert its neurotrophic functions in developing neurons via induction of release of neurotrophic factors and activation of neurotrophic factor receptors through EAA-mediated activation of calcium-dependent signal transduction cascades. We used primary neuronal cultures isolated from embryonic rat brain neocortex to apply various concentrations of AMPA and KA for the time-dependent and concentration-dependent inductions of TrkA and TrkB expressions. Furthermore, as we previously showed that the induction of TrkA expression is calcium dependent, the involvement of a calcium-dependent kinase calcium / calmodulin-dependent protein kinase (CaMK) was also examined. CaMK inhibitor KN93 at 20 μM significantly reduced the KA-increased TrkA expression. This result coincided with the finding that CaMKII-activated transcription factor cAMP-response element binding protein (CREB) can indeed increase its phosphorylation upon KA treatment. Therefore, our results leads to the hypothesis that the AMPA/KA receptors mediated increase of TrkA expression may come from the transcriptional activation of trkA gene promoted by CREB in a calcium-dependent manner.

Keywords: AMPA/KA receptors, kainic acid, neurotrophin, calcium / calmodulin - dependent protein kinase

二、緣由與目的

The central aim of this project is to elucidate the molecular and physiological mechanism regarding the neurotrophic

functions of excitatory amino acid (EAA) receptors in developing neurons. In developing mammalian brain, the EAA receptor subtypes AMPA/KA receptors and the NMDA receptor display a sequential participation in neuronal excitation (Ben-Ari, 1997). When reaching to the adult stage, these ionotropic receptors mediate the majority of rapid synaptic transmission, and excessive degree of the receptor activation may lead to neuronal cells death, known as the glutamate excitotoxicity (Olney, 1986). In developing brains, however, a critical level of EAA receptor activation is required for normal development (McDonald, 1993).

On the other hand, neuronal development requires neurotrophic factors, such as neurotrophins, to transduce specific signals into the developing cells. Receptors for neurotrophins, such as TrkA for nerve growth factor (NGF), TrkB for brain-derived neurotrophic factor, and TrkC for neurotrophin 3 (NT-3) and NT-4/5, consist of a tyrosine kinase activity in the intracellular domain, which is activated upon NT binding and results in autophosphorylation (Bothwell, 1991; Kalaplan and Stephens, 1994). Ample evidence has supported that EAA receptors may work with neurotrophin receptors to maintain activities required for neuronal development. For example, glutamate and NGF were found synergistically promoting survival of cerebellar purkinje cells (Cohen-Corey, 1991). KA was found effective in increasing BDNF and NGF mRNA levels in developing neurons (Zafra, 1990). In our previous studies, KA was found initiating a neuroprotective effect by a calcium-dependent activation of TrkA in developing cortical neurons (Lee, 2000). These information provides a solid connection of the function of EAA receptors to the activation of the neurotrophin system during development.

In this project, we used primary cultured cortical neurons to further examine the signal transduction pathway and transcription factors involved in KA-increased TrkA expression. Invovement of calcium-dependent signal transduction pathways, such as calcium/calmodulin-dependent protein kinase (CaMK)-triggered protein phosphorylation cascade and subsequent transcriptional activation, was elucidated.

三、結果與討論

AMPA 及 Kainate 對大腦皮質神經元細胞內鈣離子濃度之影響

在先前研究所發現麩胺酸受體 Kainate 導致神經生長因子受體 TrkA 表現增加的現象，作者所採用之 Kainate 濃度為 500 μM (Lee et al., 2000)。在本次實驗中，為避免造成非專一性的麩胺酸受體活化，遂將促效劑作用濃度降低為 50 μM。首先為證明在此濃度下，麩胺酸受體亞型仍然可被活化，而造成 ligand-gated 鈣離子通道開啟，是故將大腦皮質神經元細胞以 50 μM AMPA 或 Kainate 處理，並測量其細胞內鈣離子濃度受促效劑作用而產生的變化。

由實驗結果可看到，當加入 AMPA 後，瞬間產生一波峰極為顯著的波形；亦即細胞內鈣於 50 μM 的 AMPA 作用下，立刻明顯地增加約 40nM (***Fig. 1A***)。而在 Kainate 作用的作用下，也造成約 80nM 左右的內鈣濃度升高(***Fig. 1B***)。由此可證實在較低的濃度下，Kainate 或 AMPA 促效劑仍能有效活化麩胺酸受體亞型，而開啟鈣離子通道，造成細胞內鈣增加，並且進一步啟動細胞內相關的訊息傳遞。

AMPA 及 Kainate 對神經生長因子受體 TrkA 表現之影響

5DIV 之大腦皮質神經元細胞，在 50 μM 濃度的 AMPA 或 Kainate 作用，控制組則以 EBSS 處理，再分成以下兩種處理方式：***(ⅰ)***立刻收細胞，***(ⅱ)***以 BME 洗去促效劑之後，經 30 分鐘 Incubation 再收細胞。隨即以 Western Blot Analysis 評估 TrkA 蛋白質表現量。細胞膜蛋白上 TrkA 表現的增減，相較在麩胺酸受體亞型促效劑 AMPA 與 Kainate 的處理，有相似的趨勢(***Fig. 2, Fig. 4***)。在 10 分鐘促效劑處理後立刻收細胞的情況下，細胞膜上 TrkA 的表現與控制組相較有減少的現象；而將作用時間延長為 30 分鐘，或經過 Wash→Incubation 過程後才收細胞時，則可觀察到

TrkA 的表現大量增加。若藥物作用時間為 30 分鐘，且又經過 Wash→Incubation 過程後，TrkA 的表現便又回到 Baseline。由此推測，AMPA 或 Kainate 所造成 TrkA 表現的增加，與藥物處理時間的長短無關，而可能是自加藥的開始，便已啟動細胞內相關的反應，而經過 30~45 分鐘後，不論培養液中是否有促效劑的存在，都會造成 TrkA 表現達到最高。此外，控制組 EBSS 作用再經 Wash→Incubation 的過程後，也會使細胞膜上 TrkA 的表現略為增加，造成此情形的原因，據推測乃是由於 wash 並更換新鮮(Fresh)培養液的步驟，對細胞而言，屬於外在環境的改變，故需要神經營養因子的作用，以幫助其適應新環境。

為探討更進一步的機轉，在此根據上述結果，選取 Kainate 造成 TrkA 表現增加最為明顯之兩組：***(ｉ)***組 30 分鐘處理(Prolonged)與***(ii)***組 10 分鐘處理(Brief)，進行後續的實驗。

CaMKⅡ抑制劑在 Kainate 長時間作用下對神經生長因子受體 TrkA 及轉錄因子 CREB 表現之影響

KN-93 為第二型鈣離子/鈣制素依存型蛋白質激 (CaMKⅡ)的專一性抑制劑。在此利用 10μM 濃度的 KN-93 預先處理 30 分鐘，接著再以 50μM 濃度的 Kainate 作用 30 分鐘後收細胞(***Fig. 6A***)。以 Western Blot Analysis 評估 TrkA 表現量的結果發現，當 KN-93 單獨處理時，TrkA 的表現會增加；而 KN-93 與 Kainate 結合使用時，卻使 TrkA 表現較兩者單獨處理時減少，但仍比控制組為高(***Fig. 6B, C***)。

為更深入研究 KN-93 引發的 TrkA 表現增加，是否與 CaMKⅡ的功能被抑制，進而影響轉錄因子 CREB 的活化有關，於是將相同藥物處理下的細胞，同樣以 Western Blot Analysis 方式，觀察 CREB 及磷酸化態 CREB 的表現，是否有所改變。細胞核中磷酸化態的 CREB，在 Kainate 單獨作用時表現增加；KN-93 單獨處理並不會對其有任何表現量上的影響；而 KN-93 與 Kainate 結合處理時，則 Kainate 所造成細胞核中磷酸化態 CREB 增加的現象，並不會被 KN-93 抑制或增強(***Fig. 7***)。然而在細胞質中，Kainate 的單獨處理，造成 CREB 或磷酸化態 CREB 的表現量都呈現明顯的減少；KN-93 與 Kainate 結合處理時，CREB 減少的現象便被抑制(***Fig. 8, Fig. 9***)。

綜合上述結果可知，Kainate 單獨處理時所造成細胞核中磷酸化 CREB 增加而細胞質中減少的情形，是因細胞質中本來即存在的 CREB 被活化，並 Translocation 送入核中所導致的；KN-93 單獨處理下，細胞質與細胞核內 CREB 表現量與控制組相較並無差異，可能代表在控制組 EBSS 的處理並不會造成 CaMKⅡ。至於 KN-93 與 Kainate 結合處理所造成細胞核中磷酸化 CREB 的增加而細胞質內 CREB 表現量不變，則牽涉到 CREB 的新生成。

CaMKⅡ抑制劑與 Kainate 短時間作用下對神經生長因子受體 TrkA 及轉錄因子 CREB 表現之影響

利用 10μM 濃度的 KN-93 預先處理 30 分鐘，接著以 50μM 濃度的 Kainate 作用 10 分鐘；以 BME 洗去促效劑之後，經 30 分鐘 Incubation 再收細胞(***Fig. 10A***)。接著以 Western Blot Analysis 評估 TrkA 表現量(***Fig. 10B, C***)，發現當 KN-93 單獨處理時，TrkA 的表現會增加；而 KN-93 與 Kainate 結合使用時，卻使 TrkA 表現增加的現象被抑制，此與 Prolonged Kainate 及 KN-93 處理時，TrkA 的表現十分類似。接下來將相同藥物處理下的細胞以 Western Blot Analysis 方式，觀察其細胞核及細胞質中 CREB 及磷酸化態的 CREB 表現，是否有所改變。KN-93 單獨處理同樣並不會對其有任何表現量上的影響；而 KN-93 與 Kainate 結合處理，細胞核內磷酸化態 CREB 仍然會增加；然而與 Prolonged Kainate 處理結果不同的是，在細胞核內磷酸化態的 CREB，於 Kainate 單獨作用時，卻呈現減少的現象(***Fig. 11***)。在細胞質中，Kainate 的單獨處理造成 CREB 或磷酸化態 CREB 的表現量，皆明顯的減少；KN-93 與 Kainate 結合處理時，CREB 減少的現象便被抑制(***Fig. 12, Fig. 13***)。

四、計畫成果自評

本計畫成果，因經費補助尚稱充裕，因此完成程度與原計畫約有 80%的相符程度，而若干實驗為因應研究中途新的發現而將實驗設計加以調整所得，如測定 CREB phosphorylation 等。本計畫成果，為本研究室繼去年發表在 Journal of Neurochemistry 之研究報告後(見參考文獻)，對於興奮性胺基酸受體對神經發育的貢獻，做了更詳細的闡明，對於相關理論在學術及臨床上的建立與應用價值，提供更堅實的學理基礎。本計畫成果並將於近期投稿於 Journal of Neurochemistry，作一系列的發表。

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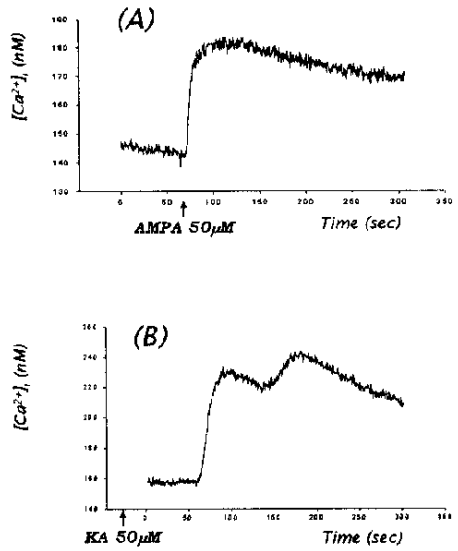

Fig. 1 ***Effects of Glutamate Receptor Subtype Agonists (A)AMPA and (B)KA on Intracellular Calcium Level In Developing Cortical Nuerons.*** Cultured cortical neurons on coverslips were loaded with 5μM fura-2 and fluorescent measurement of intracellular calcium was carrued out in a dual excitation wavelength (340 and 380nM) spectrofluorocytometer. AMPA and KA at 50μM were added at the time indicated (arrow head) and incubated with cells for over 300 seconds.

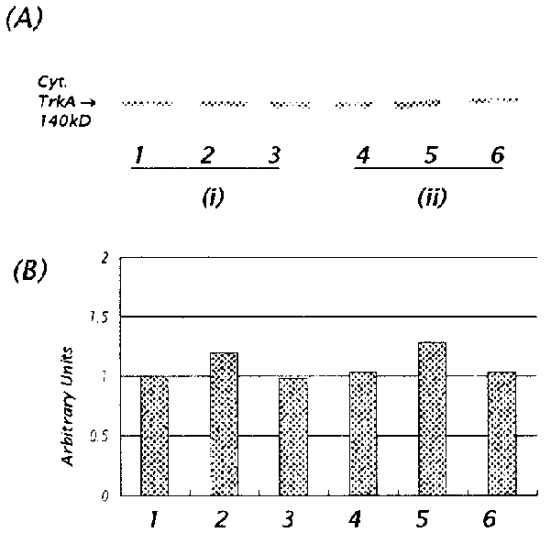

Fig. 3 ***AMPA-Induced Cytosol TrkA Expression.*** (A) Cortical neurons incubated with 50μM of AMPA for 10min (2, 5), 30min (3, 6), and EBSS for 30min (1, 4) were then (i) harvested right away or (ii) washed twice, incubated with BME for 30 min, and harvested. Crude membrane fraction and cytosol protein were subjected to Western blot by which TrkA expression was examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

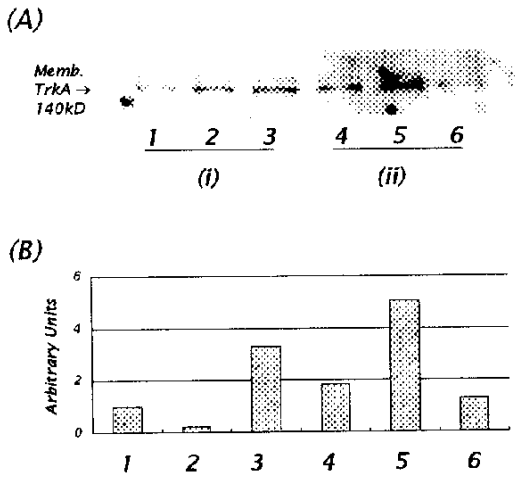

Fig. 2 ***AMPA-Induced Membrane TrkA Expression.*** (A) Cortical neurons incubated with 50μM of AMPA for 10min (2, 5), 30min (3, 6), and EBSS for 30min (1, 4) were then (i) harvested right away or (ii) washed twice, incubated with BME for 30 min, and harvested. Crude membrane fraction and cytosol protein were subjected to Western blot by which TrkA expression was examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

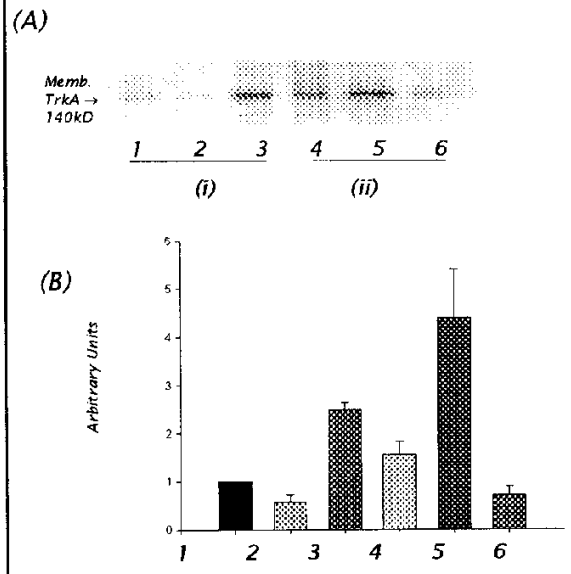

Fig. 4 ***KA-Induced Membrane TrkA Expression.*** (A) Cortical neurons incubated with 50μM of KA for 10min (2, 5), 30min (3, 6), and EBSS for 30min (1, 4) were then (i) harvested right away or (ii) washed twice, incubated with BME for 30 min, and harvested. Crude membrane fraction and cytosol protein were subjected to Western blot by which TrkA expression was examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

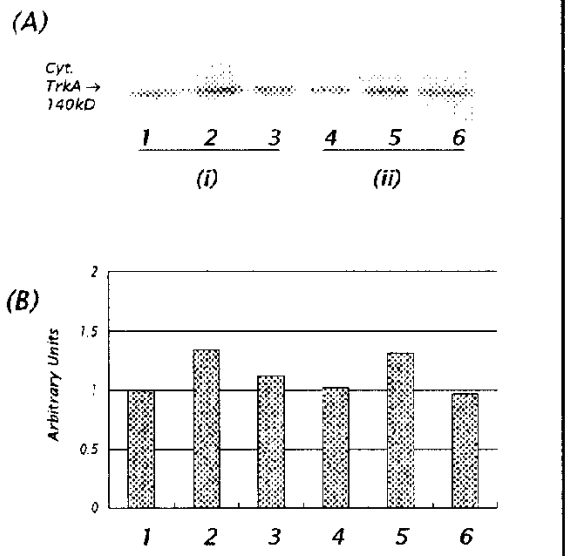

Fig. 5 ***KA-Induced Cytosol TrkA Expression.*** (A) Cortical neurons incubated with 50μM of KA for 10min (2, 5), 30min (3, 6), and EBSS for 30min (1, 4) were then (i) harvested right away or (ii) washed twice, incubated with BME for 30 min, and harvested. Crude membrane fraction and cytosol protein were subjected to Western blot by which TrkA expression was examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

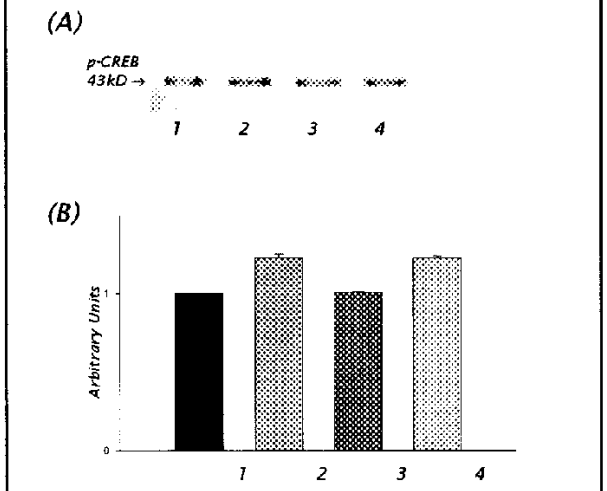

Fig. 7 ***Nucleic p-CREB Expression in Prolonged KA Treatment.*** (A) nuclei protein obtained after drug treatment as Fig.7(A) shows was subjected to Western blot by which phospho-CREB expression was examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

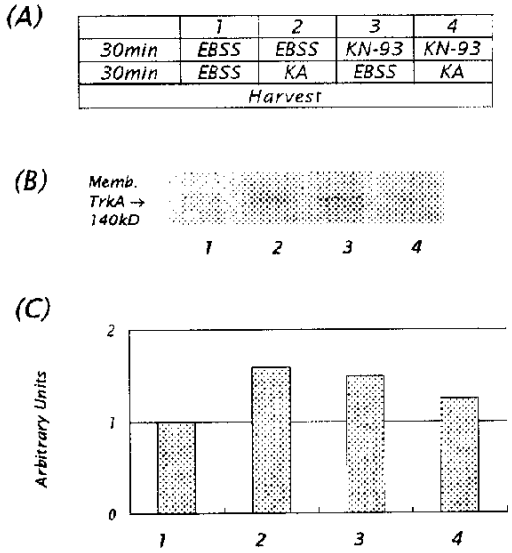

Fig. 6 ***Membrane TrkA Expression in Prolonged KA Treatment.*** (A) Cortical neurons were treated with 10μM of KN-93, 50μM of KA, or EBSS under four conditions as the table shows. (B) Membrane protein obtained after drug treatment was subjected to Western blot by which TrkA Expression were examined. (C) The intensity of the bands shown in (B) was quantitated and plotted as ratio to the intensity of the control group (B1).

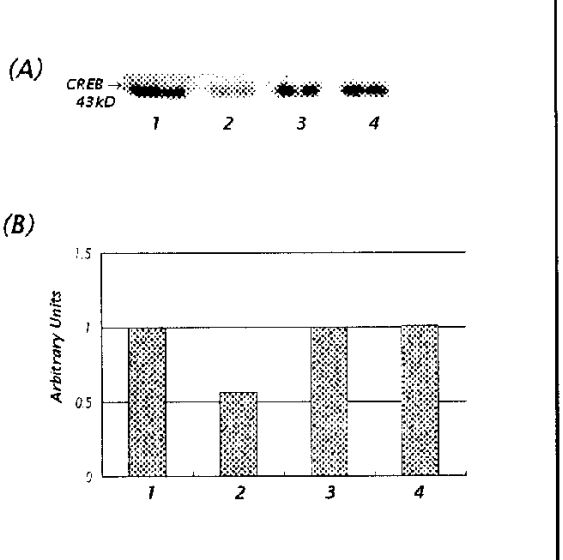

Fig. 8 ***Cytosolic CREB Expression in Prolonged KA Treatment.*** (A) Cytosolic protein obtained after drug treatment as Fig.7(A) shows was subjected to Western blot by which CREB expression were examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

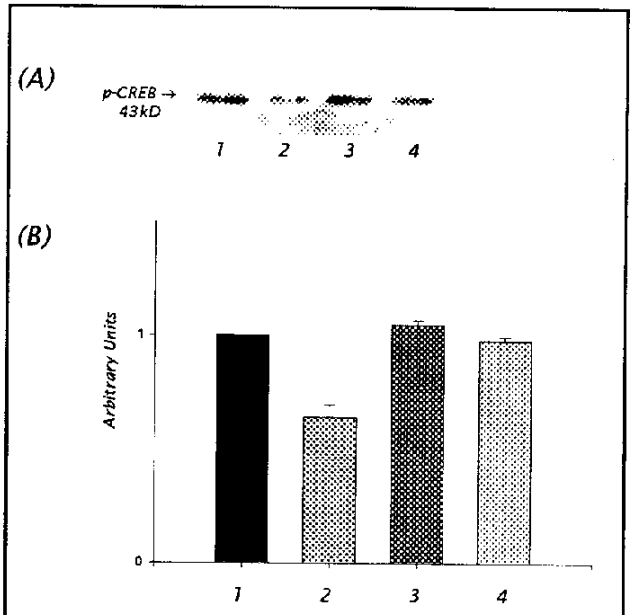

Fig. 9 ***Cytosolic p-CREB Expression in Prolonged KA Treatment.*** (A) Cytosolic protein obtained after drug treatment as Fig.7(A) shows was subjected to Western blot by which phospho-CREB expression were examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

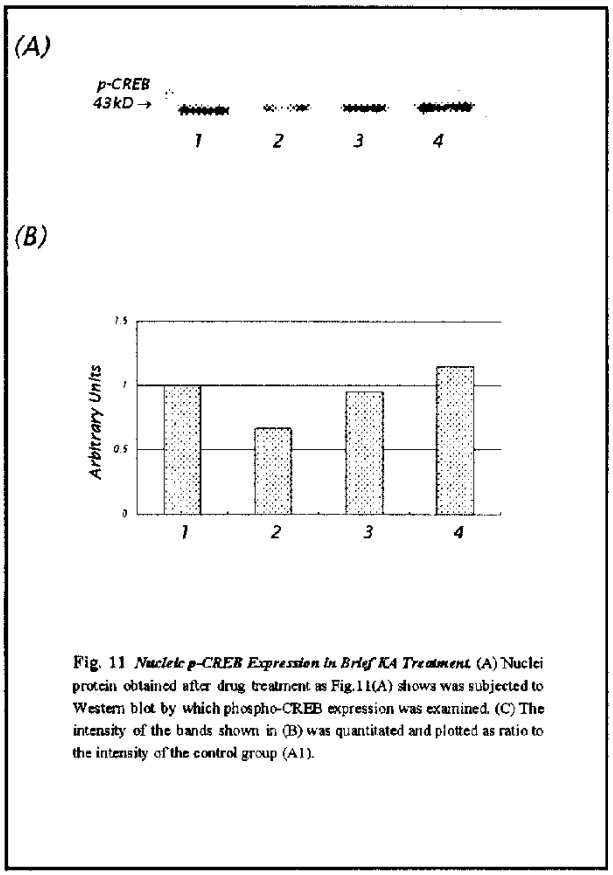

Fig. 11 ***Nucleic p-CREB Expression in Brief KA Treatment.*** (A) Nuclei protein obtained after drug treatment as Fig.11(A) shows was subjected to Western blot by which phospho-CREB expression was examined. (C) The intensity of the bands shown in (B) was quantitated and plotted as ratio to the intensity of the control group (A1).

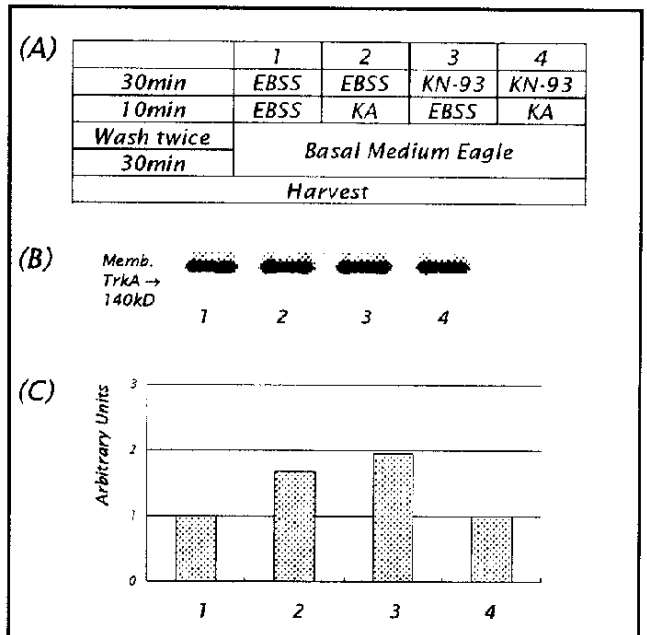

Fig. 10 ***Membrane TrkA Expression in Brief KA Treatment.*** (A) Cortical neurons were treated with 10μM of KN-93, 50μM of KA, or EBSS under four conditions as the table shows. (B) Membrane protein obtained after drug treatment was subjected to Western blot by which TrkA expression were examined. (C) The intensity of the bands shown in (B) was quantitated and plotted as ratio to the intensity of the control group (A1).

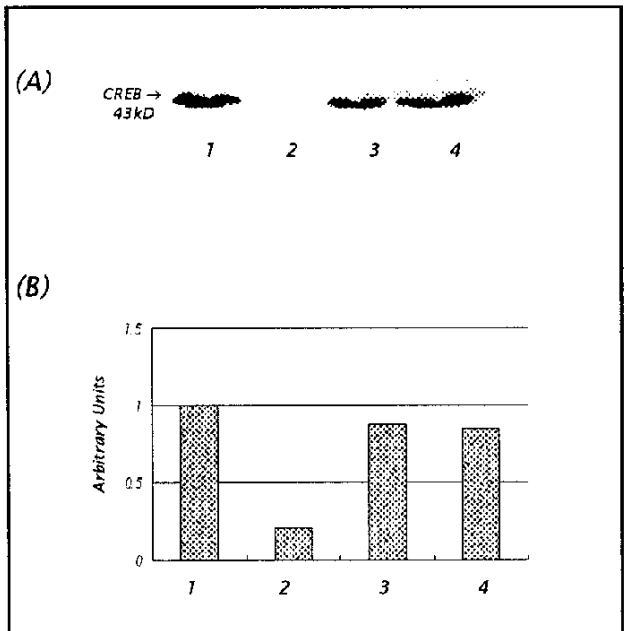

Fig. 12 ***Cytosolic CREB and Expression in Brief KA Treatment.*** (A) Cytosolic protein obtained after drug treatment as Fig.11(A) shows was subjected to Western blot by which CREB expression were examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

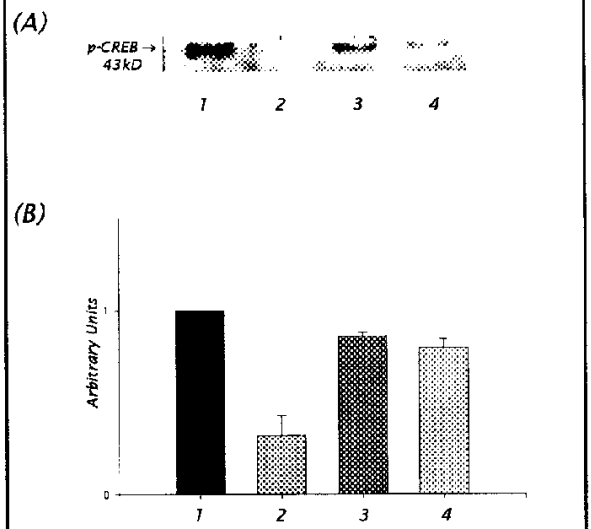

Fig. 13 ***Cytosolic p-CREB Expression in Brief KA Treatment.*** (A) Cytosolic protein obtained after drug treatment as Fig.11(A) shows was subjected to Western blot by which phospho-CREB expression were examined. (B) The intensity of the bands shown in (A) was quantitated and plotted as ratio to the intensity of the control group (A1).

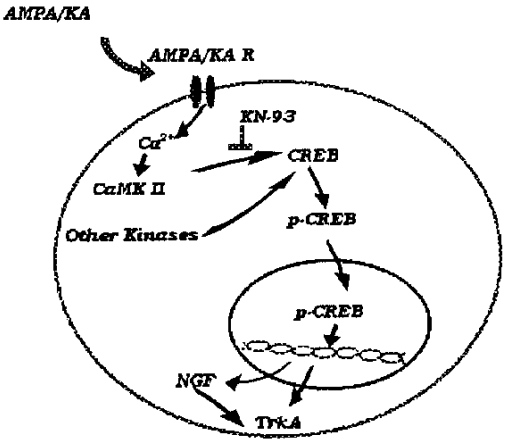

Fig. 14 ***Deduced Mechanism of KA Induced-TrkA Expression in Developing Cortical Neurons.*** The sequence of events is as follows. KA activates AMPA/KA receptors ion channels, resulting in calcium influx. Calcium activates CaMK II followed by CREB phosphorylation and translocation from cytosol to nuclei. AND phospho-CREB activates transcription of TrkA or NGF mRNA which lead to increase of TrkA protein expression.