行政院國家科學委員會專題研究計畫成果報告

Non-NMDA 麩胺酸受體在發育中大腦皮質神經元之神經營養功能研究

Studies of non-NMDA glutamate receptor-mediated neurotrophic functions in developing cortical neurons

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一、　中文摘要

麩胺酸(Glutamate)為哺乳類動物中樞神經系統的主要興奮性神經傳導物質。過度興奮其受體通常會引起突觸傳導過盛，進而導致神經細胞的死亡。其中，紅藻胺酸 (Kainate) 之神經毒性即為活化 non-NMDA 麩胺酸受體亞型所致，為一致癲癇性藥物。然而在發育期神經系統，紅藻胺酸未顯現其神經毒性，卻可引發多磷酸纖維糖代謝及細胞內鈣濃度升高等現象。我們初步的研究結果發現，阻斷酥胺酸磷酸化脢或磷脂脢 C 活性即可顯現紅藻胺酸之神經毒性，並可抑制其所引起的細胞內鈣升高。而神經營養因子的受體 Trk family 蛋白質，其不但對特定部位的神經發育有決定性影響，而且其表現程度常受紅藻酸刺激而上升。因此紅藻酸對發育期的腦神經細胞可能有神經營養的作用。本計畫中，我們利用初代培養之胚胎鼠腦神經細胞為實驗系統，施予低壓低氧的環境壓力，研究紅藻酸刺激對發育初期神經細胞存活之影響。結果發現紅藻酸可有效的降低皮質神經元的死亡程度。再者，紅藻酸受體之拮抗劑 CNQX 及 NBQX 會使神經細胞在低氧環境下之死亡程度增加。本計畫中亦使用 pluripotent P19 cell-derived neurons 來觀察紅藻酸在其受低氧環境 challenge 時之存活調控。本研究所獲得的資訊，不但有助於瞭解 non-NMDA 受體中在腦神經發育中神經營養作用機轉所扮演的角色，更能提供發育期腦組織對癲癇發作的感受性及突觸興奮性調控的重要證據，對於幼年期癲癇症及成人腦神經退化性疾病之臨床治療提供更完整的學理依據。

關鍵詞：麩胺酸，神經營養，紅藻酸，P19 細胞, 低壓低氧

Abstract

In our previous study, we have shown that non-NMDA glutamate receptors mediate neurotrophic effect through activation of phospholipase C and nerve growth factor-TrkA signaling pathway. In this project, we use cultured cortical neurons and stem cell-derived neurons to investigate whether non-NMDA receptors play a protective role in survival of developing neurons subjected to hypobaric hypoxia. Results obtained from this project is as following. (1) Kainic acid (KA), a specific agonist for non-NMDA receptor, promote neuronal survival of developing cortical neurons under hypobaric hypoxia. (2) AMPA/KA receptor antagonists CNQX and NBQX caused more cell death of hypoxia-insulted cortical neurons. (3) Differentiation of pluripotent P19 cells into neurons was successfully established. (4) Kainic acid, CNQX, and NBQX had similar effects to the survival of P19-derived neurons as they did in the primary cortical neurons.

Keywords: glutamate, neurotrophic, kainic acid, P19 cells, hypobaric hypoxia

二、緣由與目的

In the mammalian brain, excitatory amino acids (EAAs), mainly glutamate and aspartate, function as neurotransmitters to mediate the vast majority of excitatory synaptic transmission and also as neurotrophic factors for neuronal development (Review see McDonald, 1993). KA, a specific glutamate receptor agonist, is a potent neurotoxin commonly used to induce excitotoxicity in adult CNS (Lothman and Collins, 1981; Nadler, 1987). In developing brains, however, administration of KA in vitro or in vivo failed to produce neurotoxicity in various experimental systems (Holmes, 1988; Sperber, 1991). The reason for this phenomenon could be due to various neurotrophic activities occur at the developing stage that may counteract with deleterious events triggered by external insults. Recent studies have shown ample evidences suggesting that the AMPA receptor may have neurotrophic function as that fact that it was found present in non-synaptic regions of neonatal rat brain (Loturco et al., 1995), and it mediates a development-specific PLC activity in cortical neurons and oligodendrocyte progenitor cells (Lee et al, 1994; Liu et al., 1997).

Although primary neuronal culture has been limited to the functional studies without genetic manipulations, its complete feature beyond all the neuronal cell lines makes it the method of choice over the decade. Recent studies use pluripotent stem cells such as P19, an embryonic carcinoma cell line derived from a teratocarcinoma in C3H/He mice (McBurney and Rogers, 1982), to undergo various differentiations. Retinoic acid - induced differentiation of P19 results in neuron-like cells, consisting of neuronal property morphologically and electrically (McBurney, 1988; Morassutti, 1994). The P19-differentiated neurons also express all the ionotropic and metabotropic glutamate receptor subtypes and their subunits, and transient expressions were found during development (Ray WJ, 1993; Minakami, 1995). Well-differentiated P19 neurons can also be killed by exposure to NMDA or kainate (Turetsky, 1993). Compared to the primary cultured neurons, differentiation of P19 provides an earlier and wider time span for neuronal development, in which genetic manipulation or chronic antagonist treatment can be applied to evaluate the specific effect of the non-NMDA receptor for neuronal development.

(1) The central aim of this project is to elucidate the molecular and physiological mechanism regarding the neurotrophic functions of non-NMDA glutamate receptors in developing neurons. One of the major findings in our previous studies indicated that the low neurotoxicity of kainate (KA) in developing neurons depends on the extracellular nerve growth factor (NGF) accompanied by activation of NGF receptor Trk A. Non-NMDA receptors therefore may coordinate with neurotrophic factor activities to direct neuronal development in addition to mediating rapid synaptic transmission in adult CNS. In this project, we demonstrated the protective function of kainate-stimulated non-NMDA receptor in developing neurons, which will set the physiological importance of this excitatory amino acid receptor family beyond its well-known excitatory transmission and synaptic plasticity .

三 結果與討論

Protective Effect of Kainic Acid in Developing Cortical Neurons Subjected to Hypobaric Insult

To understand if KA has detrimental or beneficial effect on the survival of developing neurons, 4 DIV cortical neurons were treated with various concentration of KA for 5 min, followed by 5 min hypobaric insult (200 mmHg). LDH activity released from the cells was assayed as the index of cell death. Figure 1 shows that LDH activity present in the medium of cultured neurons subjected to hypobaric insult is reduced in the KA-treated condition as compared with the vehicle-treated group in a dose-dependent manner. To know that if the neuroprotective effect of KA is mediated by AMPA/KA receptor activity, receptor antagonists CNQX and NBQX were included in the preparation. Figure 2 shows that CNQX but not NBQX significantly increased the death rate of hypobaric-insulted cortical neurons. This result suggests that AMPA/KA receptors are indeed mediating a neurotrophic function to protect neurons from neurotoxic insult.

Differentiation of P19 cells into neurons

In this project, we successfully established a P19-derived neuronal culture to study the neurotrophic function of glutamate receptors in early stages of development. Figure 3 shows that P19 cells (A.) subjected to retinoic acid treatment for 4 days (B.) form floating aggregates. After dissociation of aggregates and plated onto poly-L-lysin-coated culture dishes, neuron-like morphology began to develop and form neuronal network by the end of day 7 (C and D). The neuronal population of P19-derived neurons is approximately 70% as immunostained by neuronal markers NFP and PGP (Fig 4), and 30% of neurons expressed the AMPA receptor. Expressions of neuronal marker PGP and AMPA receptor subunits GluR2/3 were not apparent until 7 days after retinoic acid treatment (DAR) as analyzed by western blot experiment (Fig 5). Therefore we chose 7 DAR to evaluate the effect of KA on the survival of early developing P19 neurons under hypobaric condition.

KA can elevate $[Ca^{2+}]i$ in P19-derived neurons

We first examined if KA can increase $[Ca^{2+}]i$ in P19-derived neurons. Fig 6 shows that 500 uM KA cause mild but yet significant increase of $[Ca^{2+}]i$, which was completely blocked by non-NMDA receptor antagonists CNQX and NBQX.

Protective effect of KA on survival of P19-derived neurons

To understand if KA also has protective effect on the P19-derived neurons, hypobaric condition was applied to 7DAR neurons to evaluate the degree of cell death. Figure 7 shows that KA reduced hypoxia-induced cell death in a dose dependent manner. On the other hand, non-NMDA receptor antagonist CNQX dose-dependently increase neuronal death in the hypobaric condition, suggesting that non-NMDA receptors are indeed responsible for the survival of early developing neurons. The crucial role of non-NMDA receptors to maintain viability of developing neurons is therein demonstrated.

四、計畫成果自評

1. 研究內容與原計畫相符程度: 80%, 其中有關成熟神經元的作用因為已有較多相關資訊, 故本計畫之執行轉而就發育期神經元做深入研究,

2. 達成預期目標情況: 礙於核定經費與申請經費差距過大，故謹能達成預期目標的 80%，有關 retrograde signaling, 現正

進行中。

3. 是否適合在學術期刊發表或申請專利: 本計畫成果已投稿至 J. of Neuroscience Research。

4. 主要發現或學術或應用價值: 本計畫成果就興奮性氨基酸對發育神經元的保護提出具體的作用機轉, 此一新發現提供發展促進神經保護或神經修復時之理論基礎,

5. 學生畢業論文: 本計畫部份為臺北醫學院醫研所碩士班學生林君樺之碩士論文, 林生之論文已於八十八年六月完成,

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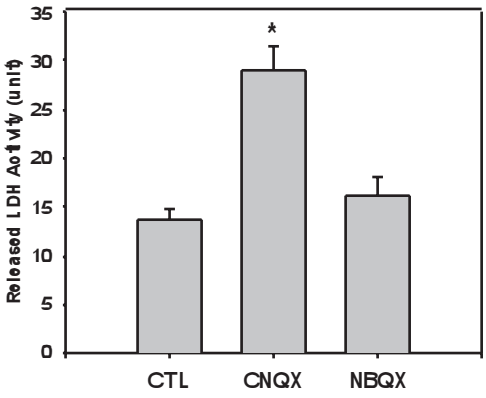

Figure 2. Effect of non-NMDA receptor antagonists on hypobaric hypoxia-induced cell death in 4 DIV cortical neurons

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圖表: Figure 3-6 因會檔案太大而無法上傳，請參閱書面報告

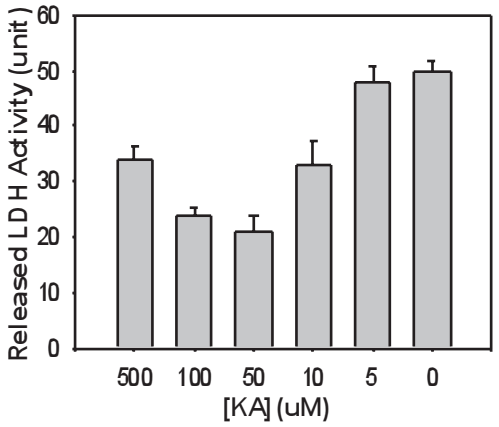
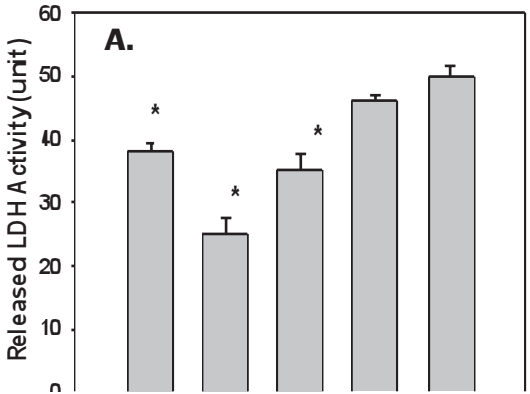
Figure 1. KA protects 4 DIV cortical neurons from hypoxia-induced cell death