行政院國家科學委員會補助專題研究計畫成果報告

以病理認知行為及海馬回電生理變化來評估新生鼠缺氧缺血腦病變後之連續抽搐發作是否造成更厲害腦傷害

計畫類別：□個別型計畫 □整合型計畫
計畫編號：NSC89 - 2314 - B - 006 - 198 -
執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

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□ 赴國外出差或研習心得報告一份
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□ 國際合作研究計畫國外研究報告書一份
執行單位：國立成功大學醫學院小兒科

中華民國 年 月 日
Can repetitive seizures after neonatal hypoxic-ischemic encephalopathy cause more brain damage in rats?

Abstract
Neonatal asphyxia can cause mortality and long-term neurological sequelae. This study was to test the hypothesis that repetitive seizures following hypoxic-ischemic cerebral injury can worsen the hypoxic-ischemic brain damage, and caused further deficits in hippocampus-related learning and memory. Neonatal hypoxic-ischemic brain injury results in spontaneous epileptiform activity, neuronal damage and deficit in formation of long-term potentiation in the CA1 hippocampus. Repetitive seizures following the hypoxic-ischemic cerebral injury could further significantly increase spontaneous epileptiform activity and caused more deficit in formation of long-term potentiation in the CA1 hippocampus, and also worsened pathological scores, neuronal damage and Timm stain scores as compared with those with hypoxic-ischemic injury alone. In addition, hypoxic-ischemic rats with repetitive seizures had more deficits in spatial memory on the Water maze test, and in inhibitory avoidance test, which may be due to the deficit in the ability of consolidation the long-term memory. The spontaneous epileptiform induced by hypoxic-ischemic injury followed by repetitive seizures could be reduced by administration of serotonin or uptake blocker, fluoxetine. This inhibition effect was mediated by 5-HT{sub 1A} or 5-HT{sub 2} receptor activation, and this inhibition effect...
of serotonin reached its peak at four weeks after hypoxic-ischemic injury followed by repetitive seizures. Co-treatment of 5-HT1A receptor agonist attenuated the neuronal damage induced by hypoxic-ischemia followed by repetitive seizures and improved the spatial memory deficits. These results suggest that neonatal hypoxic-ischemia followed by repetitive seizures may have long-lasting effects in cognitive development and epileptiform, and these effects can be reduced by 5-HT1A receptor agonist.

**Keywords**: asphyxia, repetitive seizures, newborn rats

二、缘由与目的

Seizures continue to be one of the most common yet ominous neurological signs in young infant period (1,2). Seizures may be the first and only sign of a central nervous system disorder; and most seizures in the infantile period are reactive, i.e., secondary to an acute event such as hypoxia, ischemia, trauma, infections, or metabolic disturbance (2,3). The clinical features of the seizures in the young infantile period differ considerably from those seen in older children and adults, since immature brain differs considerably from mature brain in the development and propagation of seizures, the electroencephalographic (EEG) features of the seizures and the consequences of the seizures (1,3,4). Animal studies have demonstrated that immature brain is more prone to seizures than adult brain, presumably secondary to a development imbalance between maturation of excitatory and inhibitory circuits. In addition, immature rats have rapid generalization of seizures, with a quick progression through the early stage of kindling than mature rats (3,4,5). Clinically, there are controversies regarding whether seizures occurring during early development are detrimental to the developing brain (6,7). There are three clinical evidences suggesting that recurrent seizures do not harm the developing brain, which include epidemiological study of febrile convulsions, natural history of childhood epilepsy, and those with status epilepticus (7). A number of animal studies suggest that the immature brain is less vulnerable to the long-term effects of prolonged seizures than the mature brain (3,5,8). It is well known that hippocampal CA1 and CA3 is selectively vulnerable to seizures or hypoxia/ischemia due to the extremely high density of glutamate receptors on pyramidal neurons (1,3). In the mature animal, status epilepticus causes neuronal loss in hippocampal CA1 and CA3 areas and dentate hilus, leads to aberrant sprouting of granule cells axons ( mossy fibers) in the supragranular zone of the fascia dentata, and stratum infrapyramidal of CA3, and results in long-term deficits in learning, memory and behavior. On the other hand, several studies also have demonstrated that a single prolonged seizure in the immature animal results in less cell loss, and sprouting, and fewer deficits in learning, memory, and behavior than similar seizures in adults (3,5,8,9). However, in addition to the increased risk for epilepsy in children, there are some suggestions that seizures during early development may be more detrimental than those occur in the mature brain. While many experimental studies have demonstrated the long-term age-related adverse effects of a single prolonged seizure (10), it is still less clear whether there are cognitive and pathologic sequelae and/or hippocampal electrophysiological changes after repetitive seizures (3,5,8,10,11). Studies are necessary to provide more evidences that repetitive seizures in the developing brain are harmful.

**Materials and Methods**

**Induction and Observation of Seizures**

Young infantile rats at age before P20 is chosen because animals receiving the convulsants before P20 have less cell loss, mossy fiber sprouting, and spontaneous seizures. It will be better to delineate whether the long-term effects are secondary to repetitive seizures, to spontaneous seizures, to convulsants associated structural damage, or to anti-epileptic drugs themselves (3,10,11,12,13).

1. **Kainate acid (KA) model**: KA has been used to investigate the chronic cognitive and behavior effects of epilepsy on the developing brain. It is a potent analog of the
excitatory amino acid, glutamate, the most prevalent excitatory neurotransmitter in the CNS. In the adult, KA-induced seizures result in widespread structural damage in the hippocampus. The pattern of hippocampal damage induced by KA seizures is similar to that seen in human with temporal lobe epilepsy. The lesion consists of a loss of pyramidal cells in areas CA1 and CA3 of the hippocampus and a loss of interneurons in the hilar region of the dentate. The cell loss is accompanied by a synaptic rearrangement of mossy fibers of dentate granule cells in the supragranular cell layer. Long-term behavior deficits are also observed in these animals. However, in the immature brain before postnatal day 20 (P20), a prolonged seizure causes no major cell loss or sprouting, and fewer spontaneous seizures and behavioral deficits than observed in adults. In this study, repetitive seizures induced by KA, intraperitoneal injection, 2-3 mg/kg/day will be used for rats at age P10, P11, and P12. Immediately after an injection, rats are placed into individual plastic cages and their behavior are scored every 15 minutes for 2 hours, noting the latency-to-onset of seizures. Following the observation period, animals are returned to their home cages until the next KA injection. The mortality rate after KA injection will be recorded. To assess the long-term effects of repetitive KA seizures, the behavioral tests and hippocampal electrophysiological examination will be performed at age P70.

2. **Seizure record:** The animal behaviors and seizures are observed by video-camera for 4 hours, noting the patency-to-onset seizures, the severity, duration, and character of the seizures (3,10,21).

3. **Gross Neuropathologic Grading.** After recovery, animals (P70) are killed with lethal intraperitoneal injection of sodium pentobarbital (150 mg/kg). The rats were perfused transcardially with 200 ml of sodium sulfide perfusion medium (2.93 g Na₂S, 2.98 g NaH₂PO₄·H₂O in 500 ml of distilled H₂O) followed by 200 ml of paraformaldehyde (PFA) 4%. The brains are postfixed in PFA 4% for 24 hours and then placed in a 30% sucrose solution until the brain sink to the bottom of the chamber. Coronal sections through the entire extent of right hippocampus are cut at 30 um on a freezing microtome and sections are stored in phosphate-buffered saline. Every fourth section is stained for mossy fibers by using Timm stain and alternate sections are stained with cresyl violet for cell counting (4,21).

4. **Hippocampal Cell Counts.** Cell counts are performed in 5 randomly selected controls and 5 each from the groups of experimental rats by an observer blind to treatment group. All hippocampi are assessed both qualitatively and quantitatively. The hippocampal region analyzed is identical to the region with Timm stain scores. Counting begins with the random selection of the one of first 3 sections of the anterior portion of the hippocampus and continued until the posterior portion. Neurons are counted in the dentate granule cell layer, hilus, CA3, and CA1 (4,21).

5. **Hippocampus Timm Stains.** Rats are killed on P40. After deep anesthesia, rats are perfused transcardially with 200 ml of sodium sulfide perfusion medium (2.93 g Na₂S, 2.98 g NaH₂PO₄·H₂O in 500 ml of distilled H₂O) followed by 200 ml of paraformaldehyde (PFA) 4%. The brains are postfixed in PFA 4% for 24 hours and then placed in a 30% sucrose solution until the brain sink to the bottom of the chamber. Coronal sections through the entire extent of right hippocampus are cut at 30 um on a freezing microtome and sections are stored in phosphate-buffered saline. Every fourth section is stained for mossy fibers by using Timm stain and alternate sections are stained with cresyl violet for cell counting (4,21).

6. **Electrophysiological Study on Hippocampus**

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之變化
大鼠腦切片的製備 (brain slice preparation)
使用 9-10 過大的雄性 Sprague-Dawley (SD) 種系大鼠，重量約在 150 ~ 200 g，斷頭犧牲後，
剪開頭皮移除頭蓋骨，並用鑷子剝開腦膜，用小平匙將大腦與視神經及腦殼分開，把大腦放
入持續以 95% O₂ 及 5% CO₂ 混合氣體灌注的
0~4℃人工腦脊液中 (artificial cerebrospinal
fluid, ACSF) 約 30 秒後，將大腦倒著插入有濾
紙的培養皿中，用手術刀切除小腦及大腦前葉
的一部份，再沿中線將左右大腦分離。將後方
切除齊整後，另用快乾膠固定於切片機的承載
台上，再放入切片機水槽中，(內承以 95% O₂
及 5% CO₂ 混合氣體灌注的 0~4℃ AC SF)
利
用切片機上的刀片，以橫向切片方式把腦切成
厚約 400 m 的腦薄片 (brain slice)。再將這些
腦薄片置於 95% O₂ 及 5% CO₂ 混合氣體灌注
的 0~4℃ ACSF 中，室溫下靜置一小時後再實
驗。

胞外電氣生理活性記錄法 (Extracellular
recording)
將一 brain slice 放入記錄槽 (recording
chamber) 中以尼龍網固定之，並浸潤於
ACSF 中，利用循環式加熱器把記錄槽中
溫度維持在 32 ± 2℃，以每分鐘 2~3 ml 的速度灌流。而後將刺激電極放於而記錄
電極則置於 striatum radiatum 及 striatum
pyramidale 中。記錄電極為利用 Brown
Flaming electrode puller (Sutter lnstrumert)
將毛細管拉成外徑約 1µm 的微細電極後，
再充填 3M NaCl，電阻約在 3~8 MΩ。記
錄所得的訊號由 Axoclamp 2A amplifier 加
以放大。透過 Digidata 1200 的介面與個人
電腦相連，利用 Axon Instrumert s 所設計的
pClamp6 軟體儲存資料並進行數位化分
析。

Behavior Testing
1. Morries Water-Maze 用以測量實驗動物
的空間記憶行為，茲簡述方法如下：利
用一直徑約八英尺之圓形水池，注入約
1.5 英呎深的自來水 (水中混入顏料，使之呈
不透明狀) 水中置入一平台 (一英尺平方
英呎) 平台表面沉入水中約 1.5 英呎。水
池上方以膠帶平分為四等分。每一等分稱
為一個象限 (quadrant)。平台所在 的
quadrant 稱為目標象限 target quadrant (TQ)，其他 quadrant 則根據其相對於 target
quadrant 的位置而分別命名為 left,right 及
opposite quadrant。經過六至七天的訓練
後，動物能經由水池四週的環境線索 (environmental cue) 而得知平台的所在位
置。當正式測試時，把平台移除，測量 2
分鐘內實驗動物於各 quadrant 中停留的時
間。動物如建立了對平台位置的空間決記
憶，則會在 T Q 中停留較長的時間。反之
則動物在各 quadrant 中的停留時間將會十
分相近 (動物對各 quadrant 作隨機性的搜
尋 ) (22)。
2. Inhibitory avoidance task 有別於
water-maze inhibitory avoidance task 並不是
測量動物的空間記憶行為，而是一種聯結
性學習。實驗器具為一長度為一公尺的亞
克力材質膠盒，中置一治門。把膠盒分為
等待區 (亮區、因此部的亞克力材質可透
光) 及電擊區 (暗區、因此部位的亞克力
材質為不透光的，且地板部位為金屬材質
並接上電刺激器 )。訓練時，動物被放入
亮區中，待其面對活門時打開活門。正常
情況下，動物會自發性地由亮區進入暗區
中，待其完全進入暗區後，關上活門，馬
上施以一次 0.4mA, 60Hz , 1 sec 之電刺
激。電擊後，把動物放回飼養箱中，靜待
24 小時後，再予以正式測試。正式測試與
訓練時相彷 (但不予以電擊) 主要計量的
是動物停留於亮區中的時間 (latency) 如
動物已建立了暗區中存有電擊的記憶，則
動物會主動留在亮區中較長的時間，反之
則動物留在亮區中的時間則較短。

Statistics
實驗結果皆以平均數 ± 標準誤 (Mean ± SEM) 表示，而組間差異先以單向變異數分
析 (one-way ANOVA) 評估，變量為藥物處
理，再使用 student’s t-test 計算，並且以
p<0.05 代表具有統計上的意義

Results and Discussion
At P30, the rats with hypoxic-ischemic
cerebral injury at P7 followed by
repetitive seizures at P10 showed
significantly higher pathological scores
as compared with those with
hypoxic-ischemic cerebral injury at P7
only (2.6±0.5 vs 1.8±0.9, P<0.05). Timm stain scores at CA3 were also significantly higher in the rats with hypoxic-ischemic cerebral injury followed by repetitive seizures as compared with those with hypoxic-ischemic cerebral injury only (3.7±1.6 vs 2.5±1.8, P<0.05). Timm stain scores of dentate gyrus were also significantly higher in the rats with hypoxic-ischemic cerebral injury followed by repetitive seizures (2.9±1.7 vs 1.8±1.1, P<0.05).

Of the Water maze test P40, the rats with prior hypoxic-ischemic injury followed by repetitive seizures had significantly longer time in their escape latency to the hidden platform and also spent significantly less time in the target quadrant (19±4 % vs 27±5%, P<0.05) as compared with those with hypoxic-ischemic injury only. To further demonstrate the difference of water maze learning, extended training of water maze for 7 days (four sections a day for 7 days) showed that the rats with prior hypoxic-ischemic injury achieved best performance in 5 days, however, the hypoxic-ischemic rats followed by repetitive seizures showed best performance only after 7 days of training. In addition, there was significant difference of best escape latency between the two groups (8.5±2.4 seconds for hypoxic-ischemic rats, and 45.4±8.6 for hypoxic-ischemic rats with repetitive seizures, P<0.05). Regarding the inhibitory avoidance test at P50, there was no significant difference between the two groups in the retention time during training. However at testing 24 hours after training, the rats with hypoxic-ischemic cerebral injury at P7 followed by repetitive seizures at P10 showed significantly less retention time as compared with those with hypoxic-ischemic cerebral injury only (210±59 vs 410±84 seconds, P<0.05). There was no difference in the two groups in the pain susceptibility test.

To test if epileptiform discharges can be recorded from the hippocampus, the hippocampal brain slice preparations for CA1 recording were used. More spontaneous epileptiform discharges were found in the hippocampus from rats with hypoxic-ischemic cerebral injury at P7 followed by repetitive seizures at P10 as compared with those with hypoxic-ischemic cerebral injury at P7 only. Although spontaneous epileptiform discharge was frequently observed in the hypoxic-ischemic rats, the long-term potentiation (LTP) in the CA1 hippocampus was significantly decreased in the hypoxic-ischemic rats followed by repetitive seizures as compared with those with hypoxic-ischemic injury alone (10±5% vs 75±24%, P<0.01). The cell numbers of the pyramidal neurons in the CA1 hippocampus from rats with hypoxic-ischemic cerebral injury at P7 followed by repetitive seizures as compared with that from those with hypoxic-ischemic cerebral injury at P7 only (125±65 vs 274±97/mm, P<0.05). There was no difference in the resting membrane potential, input resistance and paired-pulse facilitation in the hippocampus between the two groups. Input-out relation showed increased fEPSP in the hippocampus of hypoxic-ischemic rats followed by repetitive seizures, suggesting postsynaptic mechanism for the hippocampal epileptiform discharges. D-APV (500 uM) could partially decrease the hippocampal epileptiform discharges, and combined use of CNQX and D-APV completely inhibited the epileptiform discharges from the hippocampus of the rats with hypoxic-ischemic cerebral injury followed by repetitive seizures.

Serotonin is an inhibitor of excitatory neurotransmission. In the CA1 of hippocampal brain slice preparation, fEPSP was significantly inhibited by serotonin in the hippocampus from rats with hypoxic-ischemia followed by repetitive seizures compared with those from hypoxic-ischemia alone (P<0.01). Meterogoline (5-HT1/2 antagonist) completely blocked the inhibition induced by serotonin and fluoxetine, but 8-OH-DPAT (5-HT1A receptor agonist) or NAN-190 (5-HT1A antagonist)
receptor antagonist) could only partially block the inhibition induced by serotonin. Furthermore, receptor-binding assay showed significantly decreased 5-HT$_{1A}$ and increased 5-HT$_2$ receptor in the rats with hypoxic-ischemia followed by repetitive seizures than those hypoxic-ischemia only. Treatment with 8-OH-DPAT (5-HT$_{1A}$ receptor agonist) at 2 hours, 12 hours, 24 hours after hypoxic-ischemic injury before repetitive seizures was performed to test if serotonin agonist has the neural protective effect against hypoxic-ischemia and repetitive seizures. Water maze test showed that rats with hypoxic-ischemia and repetitive seizures treated with 8-OH-DPAT had better escape latency performance than the hypoxic-ischemic rats only (20.07±9.89 vs 58.76±11.73 seconds, P<0.05). In addition, the neuronal damage in the hippocampus was decrease by post-treatment of hypoxic-ischemia with 8-OH-DPAT.

The spontaneous epileptiform induced by hypoxic-ischemic injury followed by repetitive seizures could be reduced by administration of serotonin or uptake blocker, fluoxetine. This inhibition effect was mediated by 5-HT$_{1A}$ or 5-HT$_2$ receptor activation, and this inhibition effect of serotonin reached its peak at four weeks after hypoxic-ischemic injury followed by repetitive seizures. Co-treatment of 5-HT$_{1A}$ receptor agonist could attenuate the neuronal damage induced by hypoxic-ischemia followed by repetitive seizures and improved the spatial memory deficits. These results suggest that neonatal hypoxic-ischemia followed by repetitive seizures may have long-lasting effects in cognitive development and epileptiform, and these effects can be reduced by 5-HT$_{1A}$ receptor agonist in the early stage.

References
5. Camfield PR. Recurrent seizures in the developing brain are not harmful. Epilepsia 1997;38:735-737.


