行政院國家科學委員會專題研究計畫 期末報告

藥理性調節麩胺酸神經訊遞在 MPTP 所誘發之巴金森氏症動物神經退化及行為缺陷之效果：進階研究

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中 華 民 國 103 年 01 月 05 日

中文摘要： 麩胺酸神經系統過度活化（glutamatergic hyper-activity）會導致興奮性毒性（excitotoxicity）並且參與巴金森氏症（Parkinson's disease）的神經退化，因此以藥物調節麩胺酸神經系統之活性，將有利於治療巴金森氏症。目前已知頭孢曲松（ceftriaxone）可以促進麩胺酸轉運子（glutamate transporter）的表現，因此可以增加麩胺酸再回收。本研究之目的在測量頭孢曲松對 MPTP 所誘發之巴金森氏症動物之工作記憶、物件辨識及神經退化之效果。將 MPTP 局部注射到雄性 Wistar 大鼠中腦黑質體緻密區（substantia nigra pars compacta）以誘發巴金森氏症大鼠，隔天起連續 14 天每天給予大鼠腹腔注射頭孢曲松（200 mg/kg/day）或生理食鹽水（1 ml/kg/day）；在第 8-9 天施予 T-型迷宮試驗，在第 12-14 天施予物件辨識試驗。行為實驗顯示，巴金森氏症動物會出現工作記憶及物件辨識功能之缺陷，但是上述兩項缺陷都可以經由投予頭孢曲松治療而被抑制。另外，頭孢曲松也會抑制 MPTP 所誘發之黑質紋狀體多巴胺神經系統退化，也會減少黑質體內微膠細胞之活化，並防止海馬迴 CA1 區錐狀神經細胞之退化。以上結果顯示：麩胺酸神經系統過度活化參與巴金森氏症之生理病理機轉，頭孢曲松可能有利於治療巴金森氏症失智。

中文關鍵詞： 巴金森氏症、麩胺酸神經系統過度活化、頭孢曲松、失智症、神經保護、認知功能

英文摘要： Hyperactivity of the glutamatergic system is involved in excitotoxicity and neurodegeneration in Parkinson's disease (PD) and treatment with drugs modulating glutamatergic activity may have beneficial effects. Ceftriaxone has been reported to increase glutamate uptake by increasing glutamate transporter expression. The aim of this study was to determine the effects of ceftriaxone on working memory, object recognition, and neurodegeneration in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD rat model. MPTP was stereotaxically injected into the substantia nigra pars compacta (SNc) of male Wistar rats. Then, starting the next day (day 1), the rats were injected daily with either ceftriaxone (200 mg/kg/day, i.p.) or saline for 14 days and underwent a T-maze test on days 8-10 and an object recognition test on days 12-14. MPTP-lesioned rats showed impairments of working memory in the T-maze test and

of recognition function in the object recognition test, and both impairments were prevented by ceftriaxone treatment. Furthermore, this study provides evidence that ceftriaxone inhibits MPTP lesion-induced dopaminergic degeneration in the nigrostriatal system, microglial activation in the SNc, and cell loss in the hippocampal CA1 area. In conclusion, these data support the idea that hyperactivity of the glutamatergic system is involved in the pathophysiology of PD and suggest that ceftriaxone may be a promising pharmacological tool for the development of new treatments for the dementia associated with PD.

行政院國家科學委員會補助專題研究計畫　　　□期中進度報告
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中　　華　　民　　國 103 年 01 月 05 日

目錄

中文摘要

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英文摘要

　　Hyperactivity of the glutamatergic system is involved in excitotoxicity and neurodegeneration in Parkinson's disease (PD) and treatment with drugs modulating glutamatergic activity may have beneficial effects. Ceftriaxone has been reported to increase glutamate uptake by increasing glutamate transporter expression. The aim of this study was to determine the effects of ceftriaxone on working memory, object recognition, and neurodegeneration in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD rat model. MPTP was stereotaxically injected into the substantia nigra pars compacta (SNc) of male Wistar rats. Then, starting the next day (day 1), the rats were injected daily with either ceftriaxone (200 mg/kg/day, i.p.) or saline for 14 days and underwent a T-maze test on days 8-10 and an object recognition test on days 12-14. MPTP-lesioned rats showed impairments of working memory in the T-maze test and of recognition function in the object recognition test, and both impairments were prevented by ceftriaxone treatment. Furthermore, this study provides evidence that ceftriaxone inhibits MPTP lesion-induced dopaminergic degeneration in the nigrostriatal system, microglial activation in the SNc, and cell loss in the hippocampal CA1 area. In conclusion, these data support the idea that hyperactivity of the glutamatergic system is involved in the pathophysiology of PD and suggest that ceftriaxone may be a promising pharmacological tool for the development of new treatments for the dementia associated with PD.

關鍵字：Parkinson's disease, glutamatergic hyperactivity, ceftriaxone, dementia, neuroprotection, cognition

報告內容

1. 前言與文獻探討

　　Glutamate, an excitatory neurotransmitter in the mammalian central nervous system, plays a role in excitotoxicity in oxidative stress and neurodegeneration (Johnston, 2001). Excessive synthesis and release of glutamate can overstimulate N-methyl-D-aspartate (NMDA) receptors, causing calcium overload in neurons and triggering apoptotic cell death (Chihab et al., 1998; Luetjens et al., 2000; Perez Velazquez et al., 1997). A recent study using magnetic resonance spectroscopy found dysregulation of glutamatergic neurotransmission in several brain regions of patients with Parkinson's disease (PD) (Martin, 2007). Nigrostriatal dopaminergic (DAergic) depletion causes overactivity of the glutamatergic projections from the subthalamic nucleus to the basal ganglia output nuclei (Marino et al., 2003). Moreover, DAergic degeneration in PD leads to hyperactivity of the corticostriatal glutamatergic pathway (Blandini et al., 1996; Centonze et al., 2005). Thus, in addition to DAergic degeneration, hyperactivity of the glutamatergic system also plays a role in the pathophysiology of PD.

　　Functional interaction between the DAergic and glutamatergic systems in the brain has been shown to regulate motor function, positive reinforcement, attention, and working memory (Cepeda and Levine, 1998). Degeneration of the nigrostriatal DAergic system results in increased striatal release of glutamate (Meshul et al., 1999) and blockade of glutamatergic activity therefore attenuates parkinsonian motor symptoms and improves DAergic therapy (Hill et al., 2004). Several studies in the last decade have demonstrated beneficial effects of NMDA receptor antagonists in animal models of PD (Loschmann et al., 2004; Nash et al., 2000; St-Pierre and Bedard, 1995). Furthermore, the NMDA receptor antagonists amantadine and memantine (Kornhuber et al., 1994) have been found to produce antiparkinsonian effects in monoamine-depleted rodents (Skuza et al., 1994) and are currently used clinically for the treatment of PD. Although antagonism of the glutamatergic system is effective in treating motor dysfunction in PD patients, NMDA receptors are critical for cognitive function (Santini et al., 2001) and agents blocking NMDA receptors are not well tolerated in primates due to a high number of unwanted side effects (Kornhuber and Weller, 1997).

　　Glutamate released at the synapse is taken up by glial cells via glutamate transporter-1 (GLT-1) and is then converted to glutamine, terminating glutamate function at the synapse (Nicholls and Attwell, 1990). Since glutamatergic hyperactivity contributes to excitotoxicity, neurodegeneration, and memory loss, increasing glutamate uptake from the synapse could be effective in preventing excitotoxic cell death. Ceftriaxone, a β–lactam antibiotic, is an FDA-approved antibiotic for treating respiratory tract infection, urinary tract infection, bacterial septicemia, and meningitis (Congeni, 1984). In 2005, Rothstein et al. (Rothstein et al., 2005) reported that ceftriaxone upregulated expression of GLT-1, and several subsequent studies demonstrated the antiexcitotoxic potential of this compound (Chu et al., 2007). Neurohistological and molecular changes have been demonstrated following 5 days of pretreatment with ceftriaxone (200 mg/kg/day) in ischemia and stroke (Lipski et al., 2007). Treatment with ceftriaxone (200 mg/kg/day) for 7 or 14 days during hypoxic exposure was found to increase GLT-1 expression, resulting in sequestration of excess glutamate into glial

cells, protection of neurons from excitotoxicity, and improved spatial memory retrieval (Hota et al., 2008). However, nothing is known about the effects of ceftriaxone on cognitive behavior and neurodegeneration in PD.

Since it increases GLT-1expression and reuptake of released glutamate and may thus reduce excitotoxicity, ceftriaxone may be useful for treating PD symptoms. In addition to motor dysfunction, dementia is seen in 25-30% of patients with PD and is referred to as PD dementia (PDD) (Aarsland et al., 2001; Brown and Marsden, 1984), the symptoms of which include deficits of working memory and object recognition (Barnes et al., 2003; Laatu et al., 2004; Ramirez-Ruiz et al., 2006). The effects of increasing GLT-1 expression on cognitive function in PDD have not yet been examined. Our previous studies demonstrated that MPTP-lesioned rats show cognition deficits accompanied by neurodegeneration in the nigrostriatal system and hippocampus and thereby can act as a model for the symptoms and pathophysiology of PDD (Ho et al., 2011; Hsieh et al., 2012a; Hsieh et al., 2012b; Wang et al., 2010; Wang et al., 2009).

研究目的

The aim of the present study was to elucidate the effects of ceftriaxone on working memory, object recognition, and neurodegeneration in this MPTP-induced PD rat model.

2. 研究方法

2.1. Animals

Male Wistar rats (430 ± 6.0 g; National Laboratory Animal Center, ROC) were housed in groups of four in acrylic cages (35 cm × 56 cm × 19 cm) in an animal room on a 12 h light-dark cycle (lights on at 07:00 h) with food and water available *ad libitum*. Each animal was handled for 5 min/day on 3 consecutive days, starting one day after arrival, before being used in the study. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Chung Shan Medical University (IACUC approval No. 1001). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.2. General procedure

All animals underwent stereotaxic surgery on day 0. Brain surgery was performed as described in our previous reports (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009). Briefly, the rats were anesthetized by intraperitoneal injection (i.p.) of Zoletil (20 mg/kg; Virbac, Carros, France), then two groups underwent bilateral infusion of MPTP-HCl (1 μmol in 2 μl of saline; Rocephine, USA) into the SNc using the following coordinates adapted from the rat brain atlas (Paxinos G, 1986): AP: -5.0 mm, ML: ±2.0 mm, DV: -7.7 mm from the bregma, midline, and skull surface, respectively, while a third group was infused with 2 μl of saline (sham-operated group). Immediately after surgery, the rats were injected intramuscularly with penicillin-G procaine (0.2 ml, 20,000 IU), then housed individually in acrylic cages for a week before being returned to their initial home cages (rats from the same home cage underwent the same treatment). During the first 5 post-operative days, 10% sucrose solution was provided *ad libitum* to prevent weight loss

after surgery and reduce mortality (Da Cunha et al., 2001; Ferro et al., 2005).

Starting on the day after surgery (day 1), the MPTP-treated rats received 14 daily injections (1 ml/kg, i.p.) at 15:00 h of either ceftriaxone (200 mg/kg/day; Hoffmann La Roche, Switzerland) (MPTP+ceftriaxone group, n = 12) or saline (MPTP+saline group; n = 15), while the sham-operated rats received saline injections (1 ml/kg) (sham+saline group; n = 15). This dosage of ceftriaxone was chosen because of a previous report that treatment with ceftriaxone (200 mg/kg/day i.p.) for 5 days increases GLT-1 expression in the forebrain and has protective effects on hippocampal CA1 neurons in an ischemia animal model (Ouyang et al., 2007). In addition, ceftriaxone injection (200 mg/kg/day i.p.) for 7 or 14 days during exposure to hypobaric hypoxia was shown to improve the spatial memory of rats in the water maze and enhance neuronal survival (Hota et al., 2008).

The rats were then subjected to a battery of behavioral tests performed as in our previous studies (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009), namely a bar test on days 1 and 7, a T-maze test on days 8-10, and an object recognition test on days 12-14. All behavioral tests were started at least 2 h after the beginning of the light phase (7:00 h) and were performed in a dim observation room (28 lx red light) with sound isolation reinforced by a masking white noise of 70 db. The test equipment and objects used in this study were cleaned using 20% ethanol and thoroughly dried before each trial. On day 15 after MPTP lesioning, the rats were euthanized by exposure to CO_2, transcardially perfused with phosphate-buffered saline (PBS), and the brain immediately removed for histological examination.

2.3. Behavioral tests

Bar test: The bar test was performed on days 1 and 7 after MPTP lesioning. Catalepsy was evaluated by measuring the mean time taken for a rat to climb over a 9 cm high bar after being laid across it with its hind limbs on the floor (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009). Randomly selected animals from each group were tested in 3 consecutive trials on each trial day.

T-maze test: The construction of the T-maze and the test procedures were identical to those described in our previous studies (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009). Briefly, in the training sessions performed on 2 consecutive days (days 8 and 9), the rats learned to find food rewards (chocolate pellets; Kellogg's, Taiwan) in the T-maze using their working memory, then, on day 10, a test session was performed and the percentage of correct responses recorded. Each training session consisted of 9 trials, each composed of two parts, a forced run and a choice run. In the forced run, one of the arms (left or right in a random order) was closed by a sliding door and the reward was located at the end of the open arm. In the choice run, which was carried out 30 sec after the forced run, both arms were open and the correct response for obtaining a reward was to choose the newly opened arm, the opposite to that used in the forced run. On the test day, 3 forced-choice-choice run trials were carried out, in which the rats made 2 choices following a single forced run, and correct responses in the 6 choice runs were recorded. On the day before T-maze training, the rats were partially food restricted, the diet only being provided for 1 h, while, on the 2 training days, the diet was provided for only 1 h after the behavioral observation on that day and, on the test day, food was not provided before testing, but was freely available afterwards.

Object recognition test: The apparatus, an open box (60 cm long × 60 cm wide × 60 cm high), and the test procedure for the object recognition test were identical to those in our

previous reports (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009). Each rat was subjected to 3 exposure sessions at 24 h intervals (days 12-14), then, 5 min after the last exposure session on day 14, a test session was performed. Four different objects that were unfamiliar to the rats before the experiment were used for each rat. Three of the objects ("A", "B", and "C") were fixed to the floor 27 cm from three corners of the arena. Starting on day 12 after MPTP lesioning, the rat was allowed to explore the objects in the open box for 5 min on 3 consecutive days, then, 5 min after the last exposure session, object "B" was replaced by a novel object, "D", and the animal was returned to the open box for a 5 min test session. The time spent exploring the objects during the exposure sessions and test session was recorded, exploration of an object being defined as the rat approaching it and making physical contact with it with its snout and/or forepaws. The difference in the percentage of time spent exploring object "B" in exposure session 3 and the novel object "D" in the test session served as a measure of recognition memory for the familiar object. In addition, rearing number in the test was also recorded; rearing was recorded when the rat stood on its hind legs, raised both forepaws off the ground, and stretched its back, and was considered to end when at least one forepaw had been returned to the floor.

2.4. Histological assessment and image analysis

For histological assessment, 4 randomly selected rats per group were perfused intracardially with 4% paraformaldehyde in PBS, then the brains were rapidly removed and post-fixed in PBS containing 30% sucrose and 4% paraformaldehyde at 4°C until use. To detect DAergic degeneration and microglial activation, frozen coronal brain sections (30 μm) were cut and immunostained overnight at 4°C with mouse monoclonal antibodies against rat tyrosine hydroxylase (TH) (1:2000; Zymade, USA) or rat MHC class II (OX-6; 1:200; BD Biosciences Pharmingen, CA, USA), as in our previous reports (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009). In sections containing the hippocampus, Nissl staining was used to identify neurons.

The stained brain sections were used to measure histological changes as described previously(Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009) using a microscope (ZEISS AXioskop2, Germany) coupled to a CCD (Optronics, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, CA, USA). In this study, we created three square areas of interest, one of 32,037 μm^2 in the striatum to determine the background-corrected optical density of TH immunoreactivity, and one of 2,817,932 μm^2 in the SNc and another of 147,410 μm^2 in the hippocampal CA1 area to determine neuronal density in these regions. In the striatum, we measured the density of DAergic projections by converting the TH-stained images to gray-scale, then measuring the gray level of the area of interest and subtracting background staining measured in the non-immunoreactive corpus callosum, giving the background-corrected optical density of the TH-reactive tissue. In the SNc, we measured the density of DAergic neurons and activated microglia by capturing images, overlaying an area of interest in this region, and counting the somas of TH-immunoreactive neurons and activated microglia in these areas. In the hippocampal CA1 area, as the neurons are tightly packed, it is difficult to directly count the number of pyramidal neurons from a 30 μm thick brain section, so we measured the density of pyramidal neurons by estimating neuronal density using a semi-quantitative method involving calculating the percentage of an area of interest in the CA1 area occupied by Nissl-stained neurons. Although a stereological approach involving the counting of cells in a complete series of sections would provide additional data (Ferro et al., 2005), calculating the cell number in representative brain sections yielded similar histological results to those reported in the literature (Da Cunha et al., 2001).

2.5. Data analysis

Analysis of variance (ANOVA), followed by the least-significant difference (LSD) post hoc test, was used to analyze the bar test, T-maze test, and histological results, while the paired-samples t-test was used to analyze the object recognition test data. All results are expressed as the mean ± SEM. The level of significance was defined as $P < 0.05$ (two-tailed).

3. 結果

Fig. 1 shows changes in motor function after MPTP lesioning. ANOVA followed by the LSD test showed that, at one day after MPTP lesioning, the crossing latency in the bar test was significantly longer in rats that had undergone MPTP lesioning (the MPTP+saline and MPTP+ceftriaxone groups) ($F(2,24) = 5.94$, both P values < 0.05) than in the sham-operated group, indicating that MPTP lesioning induced motor impairment. However, on day 7 after MPTP lesioning, no difference was observed between the groups, indicating spontaneous recovery of motor function, as in our previous reports (Hsieh et al., 2012a; Hsieh et al., 2012b; Sy et al., 2010; Wang et al., 2010; Wang et al., 2009).

As shown in Fig. 2, MPTP lesioning significantly decreased the percentage of correct responses in the T-maze test performed on day 10 compared to the sham-operated group ($F(2, 36) = 7.88$, $P < 0.001$), indicating a deficit of working memory, and this was prevented by ceftriaxone treatment.

The procedure used in the object recognition test is shown in Fig. 3A. ANOVA revealed that there were no differences between the groups in total exploration time or the percentage of time exploring object "B" in the 3 exposure sessions (data not shown). As shown in Fig. 3B, ANOVA followed by the LSD post hoc test showed that MPTP+saline group spent a smaller percentage of time exploring object "D" ($F(2,41) = 4.73$, $P < 0.01$) than sham+saline group. Analysis using the paired-samples t-test showed that rats in the sham-operated group (df = 11, $t = 4.57$, $P < 0.001$) and the MPTP+ceftriaxone group (df = 14, $t = 3.00$, $P < 0.01$), but not the MPTP+saline group, spent a higher percentage of time exploring object "D" than exploring object "B".

Representative photomicrographs of immunostained and Nissl-stained brain sections are shown in Figs. 4-7. TH immunoreactivity was observed in the cell bodies of DAergic neurons in the SNc and in DAergic processes in the striatum. ANOVA showed that rats in the MPTP+saline group exhibited a decreased density of DAergic neurons in the SNc ($F(2,11) = 15.58$, $P < 0.001$) (Fig. 4B and E) and a lower background-corrected TH immunoreactivity optical density in the striatum (F(2,11) = 70.97, $P < 0.001$) (Fig. 5B and E) compared to the sham-operated group. The MPTP-induced decrease in the density of DAergic neurons in the SNc was totally prevented by ceftriaxone treatment (Fig. 4C and E), while the MPTP-induced decrease in TH immunoreactivity in the striatum was ameliorated by ceftriaxone treatment ($P < 0.05$) (Fig. 5C and E).

An increase in the number of activated microglia was indicated by an increase in OX-6-positive cells. In the SNc, the density of activated microglia in the MPTP+saline group

was much higher than that in the sham-operated control ($F_{(2,11)} = 100.18$, $P < 0.001$) (Fig. 6B and E) and this effect was ameliorated by ceftriaxone treatment ($P < 0.001$ compared to the MPTP+saline group) (Fig. 6C and E).

Figure 7D shows a schematic drawing of the hippocampal area. Neuronal density in the pyramidal cell layer in the hippocampal CA1 area was decreased in the MPTP+saline group compared to the sham-operated group ($F_{(2,11)} = 14.77$, $P < 0.001$) (Fig. 7B and E) and this effect was prevented by ceftriaxone treatment (Fig. 7C and E).

4. 討論

In the present study, MPTP lesioning caused behavioral deficits in working memory and object recognition which were prevented or ameliorated by two weeks of treatment with ceftriaxone at a dosage of 200 mg/kg/day. MPTP lesioning also decreased the density of DAergic neurons in the SNc and of pyramidal neurons in the hippocampal CA1 area and induced microglia activation in the SNc and all of these neurohistological and neuroinflammatory changes were inhibited by ceftriaxone treatment. To our knowledge, this is the first evidence that ceftriaxone can prevent hippocampal cell loss and improve cognitive function in a PD rat model. These results suggest that treatment with ceftriaxone may have beneficial effects on neuronal and behavioral impairments in PDD.

In glutamatergic hyperactivity, glutamate acts as an excitotoxic agent and is involved in the degeneration of DAergic neurons seen in PD (Albin and Greenamyre, 1992). DAergic degeneration induced by MPTP lesioning in the SNc results in disturbances of motor function and cognitive behavior, for example, learning (Ferro et al., 2005; Gevaerd et al., 2001), working memory (Bellissimo et al., 2004; Braga et al., 2005; Ho et al., 2011), episodic-like memory (Wang et al., 2010), and object recognition (Sy et al., 2010; Wang et al., 2009). Blockade of NMDA receptors has been found to be effective in the treatment of PD. Administration of MK-801, both by systematic injection and direct injection into the medial pallidus, has antiparkinsonian activity in an MPTP-induced PD rat model (Graham et al., 1990; Hsieh et al., 2012a). Clinically, NMDA receptor antagonists, for example, amantadine and memantine, have been used for decades in the treatment of motor dysfunction in PD (Goetz, 1998). In agreement with a previous report (Turski et al., 1991; Zuddas et al., 1992), our recent studies demonstrated that suppressing hyperactivity of the glutamatergic system using either MK-801 (Hsieh et al., 2012a) or 2-methyl-6-(phenylethylnyl)-pyridine (Hsieh et al., 2012b), a metabotropic glutamate receptor antagonist, reduces DAergic degeneration in the SNc and improves cognitive behaviors in an MPTP-induced PD rat model, suggesting that excessive glutamatergic activity is involved in the neuronal and behavioral deficits in PD. Reduction of glutamatergic hyperactivity has therefore been suggested as an effective therapeutic intervention for neurodegeneration and cognitive deficits in PD (Pittenger et al., 2006).

Removal of synaptically released glutamate ameliorates glutamate excitotoxic cell death. GLT-1, which is present in the membrane of glial cells, is one of the main glutamate transporters and is essential for recycling glutamate from the synaptic space and maintaining functional levels of glutamate in the synapse (Mao, 2005). The glutamate that is taken up is then converted to glutamine and shuttled back to the neurons for synthesis of glutamate (Beart and O'Shea, 2007). Increased clearance of glutamate from the synapse helps prevent

glutamate excitotoxicity (Huang et al., 2010; Kanai and Hediger, 2003; Rothstein et al., 1996) and could be an alternative strategy for protecting neurons from excitotoxic cell death.

The neurological symptoms of patients suffering from neurodegenerative diseases, such as PD, often worsen during infection (Perry et al., 2007). Interestingly, Ebert et al. (Ebert et al., 2010) reported that the onset and course of PD in an α-synuclein transgenic PD mice model were not influenced by repeated systematic infections with *Streptococcus pneumonia* and that no signs of microglial activation were observed in the mouse brain; however, the *Streptococcus pneumonia* was co-administered with ceftriaxone (100 mg/kg, twice per day for 3 days) which may have had not only antibiotic activity, but also other effects, for example, a direct neuroprotective effect. Ceftriaxone can pass freely through the blood brain barrier (Lipski et al., 2007) and can be found in the cerebrospinal fluid (Nau et al., 1993). In an animal model of cerebral ischemia, i.p. injection of ceftriaxone (200 mg/kg/day for 5 days) was reported to reduce brain damage (Chu et al., 2007). Systemic injection of ceftriaxone (200 mg/kg/day for 7 days) increased the expression and function of GLT-1 on glia and neurons, potentiated glutamate uptake, and acted as a neuroprotection agent in a mouse model of amyotrophic lateral sclerosis (Rothstein et al., 2005) and in neurological disorders associated with glutamate excitotoxicity (Verma et al., 2010). A ceftriaxone-induced decrease in glutamatergic hyperactivity might explain the neuroprotective effects of ceftriaxone in the striatum, SNc, and hippocampus seen in the present study.

A previous study demonstrated the presence of activated microglia in PD brains and suggested that these cells are involved in the neurodegenerative process (McGeer et al., 1988). Animal studies showed that activated microglia are also seen in the SNc after MPTP lesioning (Ho et al., 2011; Sy et al., 2010; Wang et al., 2010), indicating that neurodegeneration leads to microglia activation. In addition, activated microglia release inflammatory cytokines (Yasuda et al., 2008), which may lead to cell death (Nakajima and Kohsaka, 2004) and aggravate neuroinflammation, and thus play an important role in the pathophysiology of PD (McGeer and McGeer, 2004). In the present study, MPTP lesioning-induced microglial activation in the SNc was prevented by ceftriaxone treatment. In parallel, the MPTP-induced DAergic degeneration in the SNc and striatum was also abolished by ceftriaxone treatment. This result provides support for a correlation between microglial activation and neurodegeneration, as suggested previously (McGeer and McGeer, 2004).

The hippocampus is involved in many processes, such as working memory, long-term memory, memory retrieval, declarative memory, and spatial navigation. Excessive release of glutamate and excitotoxicity-induced neurodegeneration in the hippocampus may be responsible for the memory impairment observed in neurodegenerative animal models. Intraperitoneal injection of ceftriaxone (200 mg/kg/day for 7 or 14 days) increases GLT-1 expression and ameliorates hypoxia-induced memory impairment and cell loss in the hippocampus (Hota et al., 2008), indicating reduced glutamate-induced excitotoxicity in the hippocampus. The hippocampal CA1 area is rich in glutamatergic synapses and is particularly vulnerable to excitotoxic damage. As CA1 neurons play a crucial role in memory consolidation and retrieval, excitotoxic damage to these neurons could contribute to the impairments of working memory and recognition seen in PD. Increased GLT-1 expression and glutamate uptake may explain our present results that ceftriaxone protects neurons in the hippocampal CA1 area and improves cognitive behaviors in our MPTP-induced rat PD model.

Since the upregulation of GLT-1 expression by ceftriaxone is short-lived (Rothstein et al., 2005), long-term administration has been suggested so as to potentiate and prolong its beneficial effects. In clinical application, the dosage of ceftriaxone used to treat bacterial

infections and meningitis in a human adult has been reported to be 2 g/day for 2 months, with no side-effects being reported (Roelcke et al., 1992). Based on dose translation from animal to human studies (Reagan-Shaw et al., 2007), a daily dose of ceftriaxone of 200 mg/kg was used in the present study and no adverse side-effects were observed. Similarly, no side-effects were reported when ceftriaxone at the dose of 200 mg/kg per day was tested in a Huntington's disease mouse model (Miller et al., 2008).

In summary, the present study shows that sub-chronic administration of ceftriaxone inhibits MPTP-induced deficits in working memory and object recognition and suppresses neuroinflammation and neurodegeneration in the DAergic system and hippocampal CA1 area. These data provide support for a role for glutamatergic hyperactivity in the pathophysiology of PD and suggest that ceftriaxone is a promising pharmacological tool for the development of new treatments for PDD.

5. 參考文獻

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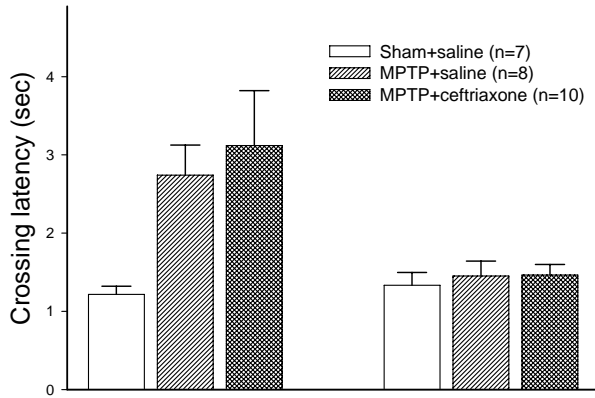
Fig. 1. Effect of ceftriaxone on the catalepsy of MPTP-lesioned rats in the bar test. MPTP (1 μmol) was bilaterally infused into the substantia nigra pars compacta, then ceftriaxone (200 mg/kg/day, i.p.) or saline (1 ml/kg/day, i.p.) was administered from day 1 after MPTP lesioning for 14 days. The bar test was performed on days 1 and 7 after MPTP lesioning. The data are expressed as the mean ± SEM for the indicated number of rats. * $P < 0.05$, ** $P < 0.001$ compared to the sham+saline group.
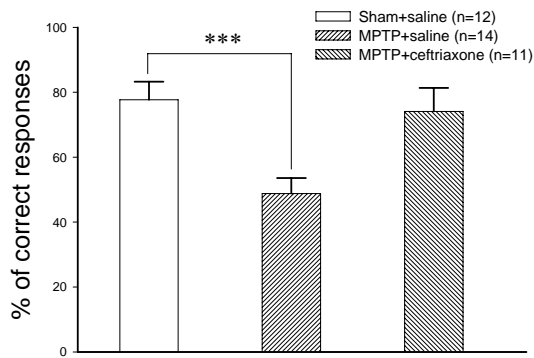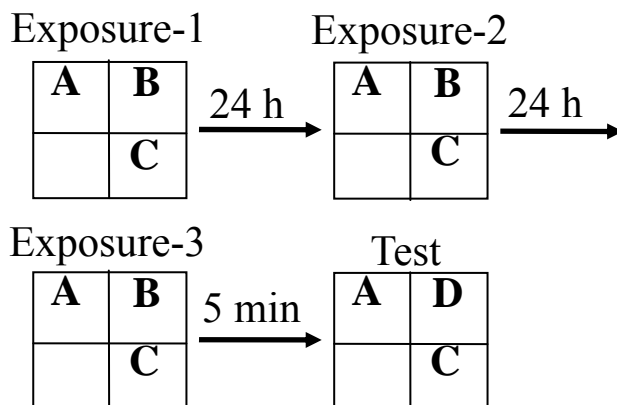

Fig. 2. Effect of ceftriaxone on the behavior of MPTP-lesioned rats in the T-maze test. Animals were treated as in Fig. 1, then the T-maze test was performed on day 10. The data are expressed as the mean ± SEM. *** $P < 0.001$ compared to the sham+saline group.
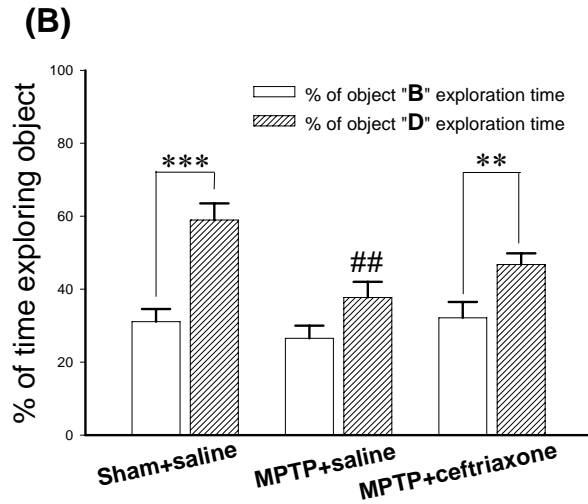
(A)

(B)

Fig. 3. Effect of ceftriaxone on object recognition in MPTP-lesioned rats. Animals were treated as in Fig. 1 and the object recognition test was performed on days 12–14. (A) Schematic diagram of the arrangement of the objects in the test. The rats underwent 3 exposure sessions (5 min each) at 24 h intervals, then were tested for 5 min starting 5 min after the end of exposure session 3. In the test session, object "B" was replaced by a novel object "D". (B) Percentage of time spent exploring object "B" or "D". The data are expressed as the mean ± SEM. ## $P < 0.01$ compared to percentage of time exploring object "D" in sham+saline group. ** $P < 0.01$, *** $P < 0.001$ compared to the percentage of time spent exploring object "B" (paired t-test).

Fig. 4. Effect of ceftriaxone (Cef) on the MPTP-induced change in the density of dopaminergic neurons in the SNc on day 15 after MPTP lesioning. Animals were treated as in Fig. 1. (A-C) Dopaminergic neurons stained for tyrosine hydroxylase are shown in representative coronal sections. Magnification, 50×; bar, 200 μm. The rectangle in D indicates the area shown in A-C, and the small black square inside the rectangle indicates the area used for measuring the density of dopaminergic neurons. (E). Quantitative results. *** $P < 0.001$ compared to the sham+saline group. ### $P < 0.001$ compared to the MPTP+saline group.

Fig. 5. Effect of ceftriaxone (Cef) on the MPTP-induced change in tyrosine hydroxylase immunoreactivity in the striatum on day 15 after MPTP lesioning. Animals were treated as in Fig. 1. (A-C) Tyrosine hydroxylase immunoreactivity in representative coronal sections. Magnification, 50×; bar, 200 μm. The rectangle in D indicates the area shown in A-C, and the two small black squares inside the rectangle indicate the areas used for measuring the optical density (OD). (E). Quantitative results. *** $P < 0.001$ compared to the sham+saline group. ### $P < 0.001$ compared to the MPTP+saline group.
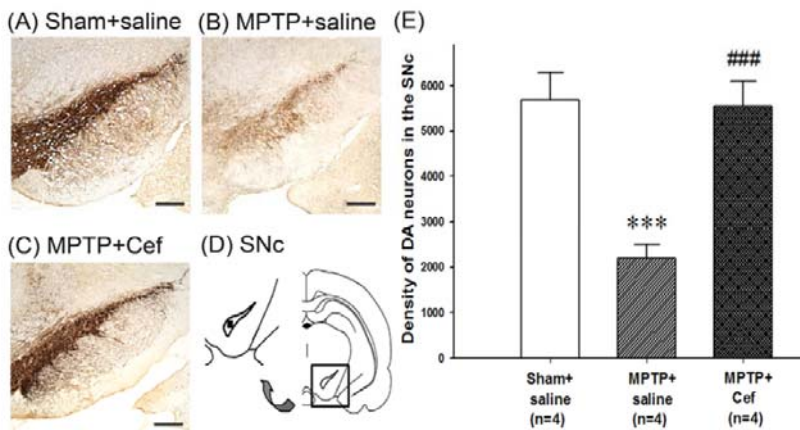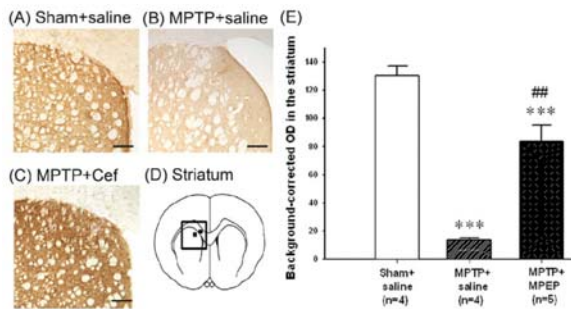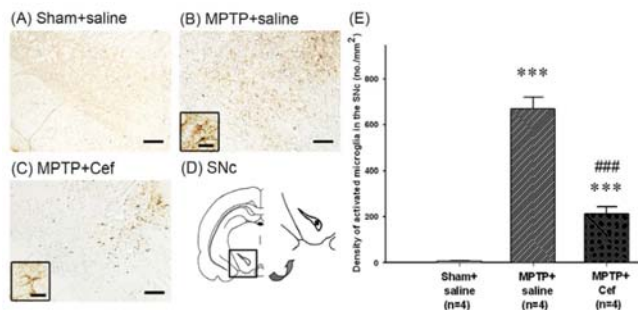

Fig. 6. Effect of ceftriaxone (Cef) on the MPTP-induced density of activated microglia in the SNc on day 15 after MPTP lesioning. Animals were treated as in Fig. 1. (A-C) Staining for activated microglia (anti-OX-6 antibody) in representative coronal sections. Magnification, 50×; bar, 200 μm. A high magnification image (200×, bar, 20 μm) of the activated microglia is shown in the insets. The rectangle in D indicates the area shown in A-C, and the small black square inside the rectangle indicates the area used for measuring the density (no./mm^2) of activated microglia in the SNc. (E). Quantitative results. *** $P < 0.001$ compared to the sham+saline group. ### $P < 0.001$ compared to the MPTP+saline group.
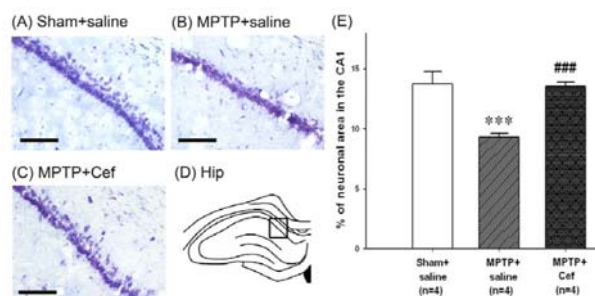
Fig. 7. Effects of ceftriaxone (Cef) on the MPTP-induced pyramidal cell loss in the hippocampal CA1 area on day 15 after MPTP lesioning. Animals were treated as in Fig. 1. The images show Nissl-stained pyramidal neurons in the CA1 area of the hippocampus, as indicated in the square in the schematic drawing. Magnification, 200×; bar, 100 μm. The rectangle in D indicates the area shown in A-C for measuring the density of pyramidal neurons in the hippocampal CA1 area. *** $P < 0.001$ compared to the sham+saline group. ### $P < 0.001$ compared to the MPTP+saline group.

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

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1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標

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2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 ■申請中 □無
   技轉：□已技轉 □洽談中 ■無

   說明：
   1. 已經發表一篇 SCI 論文
   2. 另有 2 篇論文已經投稿

   1. Ho SC, CC Hsu, CH Yu, WN Huang, M A Tikhonova, MC Ho, FY Wu, TG Amstislavskaya*, **YJ Ho*** . Measuring Attention in an MPTP-induced Rat Model of Parkinson's disease using the 5-arm Maze Test. (submitted to an SCI journal, Physiol & Behavior).
   2. SC Ho, CC Hsu, CR Pawlak, MA Tikhonova, TG Amstislavskaya, TJ Lai, **YJ Ho***. Effects of ceftriaxone on the behavioral and neuronal changes in an MPTP-induced Parkinson's disease rat model. (submitted to an SCI journal, Neuropharmacol).

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值：

部分研究成果正在申請台灣、中國、美國之專利。如果獲得通過，預計在生醫製藥領域將具有巨大的推廣潛力。

研究成果已經**提出申請台灣、中國、美國發明專利**

1. 中山醫學大學、<u>**何應瑞**</u>、晉亞化工廠。使用頭孢曲松來治療和/或預防巴金森氏症失智。專利申請號碼：101115464. 申請日期：May. 1, 2012.申請台灣專利

2. 中山醫學大學、<u>**何應瑞**</u>、晉亞化工廠。使用頭孢曲松來治療和/或預防巴金森氏症失智。專利申請號碼：201210154964.2. 申請日期：Oct. 15, 2012.申請中國專利

3. 中山醫學大學、<u>**何應瑞**</u>、晉亞化工廠。Treatment and / or prevention of Parkinson's disease dementia with ceftriaxone cross-reference to related application。申請號：13/801480。申請日期：Mar. 13, 2013　申請美國專利

國科會補助計畫衍生研發成果推廣資料表

國科會補助計畫	計畫名稱: 藥理性調節麩胺酸神經訊遞在MPTP所誘發之巴金森氏症動物神經退化及行為缺陷之效果：進階研究
	計畫主持人: 何應瑞
	計畫編號: 101-2410-H-040-003-　　　學門領域: 生物心理學

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：何應瑞　　　計畫編號：101-2410-H-040-003-

計畫名稱：藥理性調節麩胺酸神經訊遞在 MPTP 所誘發之巴金森氏症動物神經退化及行為缺陷之效果：進階研究

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	9	0	100%		
		專書	0	0	100%		
	專利	申請中件數	1	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	2	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

	其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等,請以文字敘述填列。)	無		

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

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1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以 100 字為限）
   　　　□實驗失敗
   　　　□因故實驗中斷
   　　　□其他原因
   　說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 ■申請中 □無
   技轉：□已技轉 □洽談中 ■無
   其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

   1.部分成果已經發表在 SCI 期刊：Hung YT, MA Tikhonova, SJ Ding, PF Kao, HHC Lan, JM Liao, JH Chen, TG Amstislavskaya, and YJ Ho*. Effects of chronic treatment with diosgenin on bone loss in a D-galactose-induced aging rat model. Chin J Physiol (in press) (SCI).

   2.部分成果已經進行論文投稿：SC Ho #（何詩君）, CC Hsu #(許志全), CR Pawlak, MA Tikhonova, TG Amstislavskaya, TJ Lai, YJ Ho*. Effects of ceftriaxone on the behavioral and neuronal changes in an MPTP-induced Parkinson's disease rat model. (submitted to an SCI journal, 13.12.20).

   3.部分成果正在申請專利：

   (1). 中山醫學大學、何應瑞、晉亞化工廠。使用頭孢曲松來治療和/或預防巴金森氏症失智。專利申請號碼：101115464. 申請日期：May. 1, 2012.申請台灣專利

   (2). 中山醫學大學、何應瑞、晉亞化工廠。使用頭孢曲松來治療和/或預防巴金森氏症失智。專利申請號碼：201210154964.2. 申請日期：Oct. 15, 2012.申請中國專利

   (3). 中山醫學大學、何應瑞、晉亞化工廠。Treatment and / or prevention of Parkinson's disease dementia with ceftriaxone cross-reference to related application。申請號：13/801480。申請日期：Mar. 13, 2013　申請美國專利