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懸浮微粒對中樞神經系統的毒性研究

Central Nervous System Toxicity Induced by Particulate Matter

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中文摘要

流行病學與毒理學研究皆有發現懸浮微粒不僅對心血管系統有影響，亦可能會對中樞神經系統產生負面的影響。多數的毒理學研究顯示其可能的機制為神經發炎反應進而造成行為改變。在本研究中，我們利用柴油引擎微粒 (DEPs) 探討急性暴露對中樞神經系統的毒性，也利用大氣細懸浮微粒 ($PM_{2.5}$) 探討亞慢性暴露對中樞神經系統的影響。

本研究分為兩個部分，第一部分，C57BL/6 小鼠以氣管灌注的暴露方式暴露柴油引擎微粒，一周後進行莫式水迷津測試，再分別以學習期的逃脫時間、移動距離、累積相對距離和測試期的區域停留時間、區域經過次數、平均相對距離檢驗小鼠的空間學習與記憶能力。動物犧牲後以 H&E 染色進行腦組織病理檢驗。第二部分，C57BL/6 小鼠則以呼吸暴露的方式暴露大氣細懸浮微粒 12 周 (3 個月)，暴露完後一周進行莫式水迷津測試，同樣計算小鼠的空間學習與記憶能力。另外，小鼠藉由 H&E 染色進行腦組織病理檢驗。

第一部分研究結果顯示急性暴露於柴油引擎微粒會使小鼠於莫式水迷津學習期的表現較差，需要較長的逃脫時間與移動距離才能找到平台，累積相對距離也較長。腦組織病理檢驗未在暴露組與控制組間發現顯著差異且在正常範圍內。第二部分研究中，12 周大氣細懸浮微粒暴露的平均質量濃度為 11.9 $\mu g/m^3$。低濃度亞慢性呼吸暴露於大氣細懸浮微粒後，同樣在莫式水迷津學習期中發現其對小鼠的表現有所影響。小鼠的腦組織病理檢驗未在暴露組與控制組間發現顯著差異且在正常範圍內。

過去的研究發現懸浮微粒暴露後的行為變化可能與神經發炎有關，我們發現急性暴露於柴油引擎微粒或低濃度亞慢性呼吸暴露於細懸浮微粒都可以在莫式水迷津的學習期發現小鼠的表現有所變化，未來需要進一步的生化檢驗、腦部發炎細胞染色與組織病理檢驗去探討相關機制並驗證行為實驗的結果。

關鍵字： 柴油引擎微粒 (DEPs)，氣管灌注 (I.T.)，大氣細懸浮微粒 ($PM_{2.5}$)，呼吸暴露，莫式水迷津，空間學習與記憶

Abstract

Epidemiological and toxicological studies have shown that particulate matter may not only have adverse effects in the cardiovascular system but also in the central nervous system (CNS). Most toxicological studies suggested that particulate matter may cause neuroinflammation and behavioral changes. Here, we used diesel exhaust particles (DEPs) to explore its acute CNS toxicity and also used ambient fine particles ($PM_{2.5}$) to discuss sub-chronic exposure induced CNS toxicity.

There are two parts in this study. In the first part of study, C57BL/6 mice were given DEPs by intratracheal instillation. One week after exposure, Morris water maze was conducted. Escape latency, distanced moved and cumulative distance from the center of platform quadrant or platform in acquisition phase, percentage of time spent in target area, area crossing and average proximity from the center of platform quadrant or platform were calculated to examine spatial learning and memory. Histopathological examination was then conducted in the brain using H&E stain. In the second part of study, C57BL/6 mice were exposed to ambient $PM_{2.5}$ by inhalation for 12 weeks (3 months). Morris water maze was then conducted one week after the end of exposure. Spatial learning and memory ability were tested. Histopathological examination was also conducted in the brain using H&E stain.

In the first part of study, results in Morris water maze test showed that acute exposure to DEPs may impair performance in acquisition phase. Mice required longer escape latency and distance moved to find the platform. Cumulative distance from the center of platform quadrant or platform was also longer. Mice histopathological examination found no significant difference between exposure and control group and was within normal limit. In the second part of study, the mean mass concentration for

exposed ambient $PM_{2.5}$ was 11.9 μg/ m^3 during the exposure duration. Sub-chronic exposure to low concentration ambient $PM_{2.5}$ may also impair performance in acquisition phase in Morris water maze test. Histopathological examination found no significant difference between exposure and control group and was within normal limit.

Previous studies found that behavioral changes after PM exposure may associated with neuroinflammation. We found that both acute exposure to DEPs and low concentration sub-chronic exposure to ambient $PM_{2.5}$ may affect performance in acquisition phase in Morris water maze test in mice. Further biochemical examination, inflammatory cells staining in the brain and detailed histopathological were required to explore the mechanism and support current findings in behavioral changes.

Keywords: Diesel exhaust particles (DEPs), Intratracheal instillation (I.T.), ambient fine particles ($PM_{2.5}$), Inhalation, Morris water maze, Spatial learning and memory

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Chapter 1 Introduction

1.1 Background

Particular matter (PM) is getting increasing concern in recent years. PM has been proved cardiovascular toxicity with many studies. PM may affect the cardiovascular system by causing systemic effects. Besides, few epidemiological and toxicological studies have shown that PM may have adverse effects on the central nervous system (CNS). It is suggested that systemic inflammation may also induce inflammation in the CNS.

Few studies have discussed CNS toxicity induced by PM. Many of those studies were conducted using diesel exhaust, diesel exhaust particles and concentrated ambient particles. However, lack of studies addressed whether acute exposure to diesel exhaust particles have CNS toxicity. Also, even less studies discussed non-concentrated $PM_{2.5}$ exposure induced CNS effects.

Most toxicological studies have targeted at the hippocampus which is responsible for learning and memory. Morris water maze is one of the behavioral test which has been widely used to explore spatial learning and memory impairment in rodents. The results in Morris water maze test may provide information on the behavioral changes related to impairment in the CNS, especially in the hippocampus.

1.2 Objectives

The objective of this study was to explore PM induced central nervous system toxicity in animal model. This study was divided into two parts:

(1) Study of central nervous toxicity induced by diesel exhaust particles by acute (intratracheal instillation) exposure

(2) Study of central nervous toxicity induced by ambient fine particles ($PM_{2.5}$) by sub-chronic inhalation exposure

Chapter 2 Literature review

2.1 Particulate matter

Air pollution includes particulate matter, gases, organic compounds and metals. Particulate matter (PM) is defined as solids or liquids suspended in the air. PM may be attached by other substances and compose by various components. Because of the complexity of PM, it raises more concern about its toxicity than other air pollutants.[1] Moreover, the size of PM could be further characterized into subgroups: PM_{10} (thoracic particles, with aerodynamic size < 10 μm), $PM_{2.5}$ (fine particles, with aerodynamic size < 2.5 μm), $PM_{0.1}$ (ultrafine particles, with aerodynamic size < 0.1 μm) [2]. The toxicity of PM may associate with different sizes.

$PM_{2.5}$ may reach deeper respiratory system including terminal bronchioles and alveoli[3]. Combined with complex components and penetrability, $PM_{2.5}$ raised concerns in its health effects. $PM_{2.5}$ has been linked to pulmonary and cardiovascular effects [4, 5]. Studies using concentrated $PM_{2.5}$ also showed that $PM_{2.5}$ may have potential CNS effects [6-9].

Diesel exhaust particles (DEPs) are important components in ambient PM. The contribution of DEPs to ambient PM is higher in the urban than nonurban area.[10, 11] DEPs were considered to have impact on respiratory system, cardiovascular system and allergy [12, 13]. Studies also found that exposure to diesel exhaust by inhalation may cause effects in the CNS [14-19].

2.2 Particulate matter induced cardiovascular effects and mechanism

In the statement published by American Heart Association [2, 5], $PM_{2.5}$ has been associated with cardiovascular morbidity and mortality. Short-term exposure to $PM_{2.5}$ may increase cardiovascular hospitalization and alter heart rate variability. Both short-term and long-term $PM_{2.5}$ exposure may increase cardiovascular mortality and ischemic heart diseases. Moreover, with longer exposure duration, mortality may increase.

There are three possible pathways between PM and cardiovascular diseases. First, inhaled PM may cause pulmonary oxidative stress and inflammation, and then pro-inflammatory markers may transfer from lung to circulation. This may cause systemic inflammation, accelerate atherosclerosis progression, interferes with the metabolism, and induce thrombosis. Second, PM may perturb autonomic nervous system and then decrease heart rate variability and increase heart rate. Third, PM may transfer into circulation directly, and may impair vascular function as well. Upon these three pathways, the cause of pulmonary and systemic pro-inflammatory responses, the first pathway, is considered with strong evidences. [5]

With an even smaller particle size[10], DEPs induced health effects may share the mechanism described above. Studies suggested that DEPs also cause both pulmonary[13, 20, 21] and systemic inflammation [21]. This may also result in cardiovascular effects[20].

2.3 Possible mechanism in particulate matter induced CNS effects

PM may affect the CNS in two pathways, through the peripheral or the direct way[1]. First, in the peripheral pathway, systemic inflammation has been proved to be the key mechanism induced by PM [5]. A study using lipopolysaccharide has shown that systemic inflammation may cause neuroinflammation [22]. Consistent with this finding, a study found that exposure to DEPs may increase TNF-α in both serum and the brain in parallel[19]. Neuroinflammation was found after ambient PM exposure [6-9] and diesel exhaust exposure [14-19] as well.

It has reviewed that cytokines may pass Blood-Brain-Barrier (BBB) [23, 24]. Furthermore, DEPs may cause damage on BBB through oxidative stress and pro-inflammatory cytokines[25]. BBB permeability may also alter after exposure to air pollutants[26]. This may increase the penetration of peripheral cytokines and cause subsequent effects.

In the second pathway, PM may transfer into the CNS through olfactory bulb directly. Presence of inhaled particles were found in the olfactory bulb after zinc oxide nanoparticles exposure [27] and after ultrafine carbon exposure[28]. Iron content increased in olfactory bulb after intranasal exposure to fine ferric oxide and the hippocampus was affected as well[29]. Higher manganese was observed in the olfactory bulb after manganese oxide particles and the expression of cytokines were also higher in this brain region. Increasing manganese only in left olfactory bulb when right olfactory bulb was occluded further proved that particles may translocate into the CNS through olfactory bulb [30]. This findings suggested that particles may enter the CNS directly and cause neuroinflammation.

In addition, microglia may also play an important role in neuroinflammation. Cytokines and PM may activate microglia [19, 22]. Activated microglia may also

release pro-inflammatory cytokines and reactive oxygen species (ROS) to either cause neuronal damage or activate more microglia (reactive microgliosis) [31]. *In vitro* study suggested that microglia may mediate PM effects to neurons[32]. *In vivo* studies also found that exposure to diesel exhaust may lead to microglial activation [14, 19]. It is suggested that pro-inflammatory cytokines may cause neuron death through apoptosis, excitotoxity, immune activation and cytotoxicity. These changes may lead to neurodegeneration.[33] Thus, it is suspected that PM might contribute to neurodegenerative diseases, like Alzheimer's disease and Parkinson's disease.

2.4 Morris water maze and related neuroanatomy

Base on the discovery of place cells and spatial map[34, 35], Morris developed water maze test to spatial learning and memory in rats[36]. The test was later modified to test in mice. Rodents learned to find the hidden platform which was below water in acquisition phase (memory acquisition). The information then consolidated as memory. Recall of the memory was then tested in probe test.[37] Performance in acquisition phase and in probe test was then analyzed to evaluate spatial learning and memory ability.

Many brain regions are involved in spatial learning and memory. First, senses enter prefrontal cortex. Then, information transfers to entorhinal cortex which is near the hippocampus. Next, information transfer to the hippocampus and pass through multiple regions including dentate gyrus (DG), CA3 and CA1. Finally, this information return to prefrontal cortex. [38] Thus, the frontal cortex and hippocampus are involved in different phases in spatial learning and memory. Studies have found that lesions in the hippocampus were associated with poor performance in Morris water maze [37, 39], whereas whether lesions in frontal cortex may affect spatial learning and memory was controversial[40, 41]. However, reviews still suggested that prefrontal cortex was essential in relative long-term memory[42].

Neuroinflammation is considered to be associated with cognitive impairment, including spatial learning and memory. Lipopolysaccharide exposure may resulted in elevated pro-inflammatory cytokines and impairment in Morris water maze [43]. Likewise, exposure to $PM_{2.5}$[9] or diesel exhaust[15, 16] may cause both neuroinflammation and changes in spatial learning and memory. Neuroinflammation was found in the hippocampus which was a spatial learning and memory dependent brain region.

2.5 Epidemiological studies in particulate matter related CNS effects

Epidemiological studies have linked PM to CNS effects. A recent epidemiological study in the US showed that ambient fine particles ($PM_{2.5}$) may alter cerebral hemodynamics in the elderly, including increasing resting cerebrovascular resistance and decreasing cerebral blood flow velocity. [44] Moreover, elevated fine particles ($PM_{2.5}$) may associated with stroke mortality in a study conducted in Finland [45].

A study in china showed that increasing air pollution index was associated with cognitive impairment[46]. Chen and Schwartz conducted an epidemiological study in US adults aged 20 to 59 and the result showed that higher PM_{10} exposure may not impair cognitive performance after adjusting sociodemographic factors[47]. Nevertheless, another study found that elevated traffic-related black carbon may decrease cognition using intelligence test and assessment of memory and learning in US children (mean age = 9.7 years old) [48]. Air pollution of NO_2 and PM_{10} may associate with poor cognition for children aged 8 to 10 in another study in China[49]. This showed that children may susceptible to PM. In addition, exposure to traffic-related particles also impair cognitive function of older German women aged 68 to 79 [50] and of older US men aged 50 to 99 [51]. $PM_{2.5}$ and $PM_{2.5\text{-}10}$ may attribute to cognitive decline in older US women aged 70 to 81 as well [52].

As for dementia epidemiological studies, exposure to O_3 and $PM_{2.5}$ was associated with newly diagnosed Alzheimer's disease[53]. Another study also found an association in PM_{10} and O_3 exposure and Alzheimer's disease and vascular dementia[54].

Occupational health study also found that railroad workers and electricians in the US who exposed to diesel exhaust showed neurobehavioral impairment comparing to no known chemical exposure referents[55].

2.6 Toxicological studies in particulate matter induced CNS effects

Toxicological studies summarized in Table 1 to 3 showed that particulate matter may induce CNS effects, including neuroinflammation and behavioral changes. A study showed that inhaled diesel engine exhaust may change TNF-α and IL-1α levels in the stratum in the brain of rats [17]. Another study also showed that exposure to diesel exhaust may increase neuroinflammation-related cytokines like TNF-α, IL-6 and MIP-1α in the olfactory bulb, cortex and midbrain. Increasing IL-1β and IBA 1 was found in the cortex and midbrain.[19] In an acute experiment, TNF-α in the serum and brain was increased 20 hours after acute exposure to DEPs by intratracheal instillation. Microglia also show mild activation 20 hours after the exposure.[19] A long-term study found that exposure to diesel exhaust by inhalation may cause elevated pro-inflammatory markers in multiple brain regions including midbrain, olfactory bulb, frontal lobe and temporal lobe. AD-related Tau [ps199] in the temporal lobe and the frontal lobe, AD-related Aβ42 in the frontal lobe and PD-related α-synuclein in the midbrain were also elevated.[18]

Many other studies also showed that concentrated ambient particles may induce neuroinflammation in the brain of OVA sensitized BALB/c mice[7], in the brain[8] and the cortex[56] of Apo E-/- mice and in the striatum and hippocampus of healthy rats [6]. Concentrated ambient particles also induced oxidative stress and unfolded protein response in the striatum and hippocampus of healthy rats [6]. Another study found that 4-month exposure to concentrated ambient PM may decrease dopaminergic neurons and increase astrocytes[57]. Intratracheally instilled PM_{10} may also increase pro-inflammatory cytokines in the cortex of Wistar rats and cause endothelial dysfunction in the cortex and neuronal apoptosis [58]. This neuroinflammation is similar to the early mechanism for neurodegenerative diseases like Alzheimer’s disease and Parkinson's disease.

Studies of human also revealed similar results. People exposed to air pollution resulted in elevated levels of pro-inflammatory markers and showed accumulation of Aβ42. Aβ42 is associated with cognitive decline and Alzheimer's disease.[59] In a study focused in children and young adults aged 2 to 24 years old, air pollution also associated with neuroinflammation and accumulation of Aβ42 [60]. In addition, volunteers who exposed to diesel exhaust for one hour may result in changes in electroencephalography (EEG)[61].

Considering studies in human, exposure to particles showed the same or relative outcomes of neurodegenerative diseases like Alzheimer's disease which is suspected to be the results of long-term air pollution exposure [59]. Since cognitive function impairment may be a marker for neurodegenerative diseases, many study focused on the relationship between particles and cognitive function.

In animal studies, exposure to diesel exhaust and lipoteichoic acid (LTA, known to induce inflammation) may affect Morris water maze performance in acquisition phase, increase spatial learning and memory related NMDA receptor subunits (leading to neural damage) and pro-inflammatory cytokines mRNA levels in the hippocampus[15]. Another similar study which exposure to only diesel exhaust for a longer duration (three month by inhalation) may also found poor performance in acquisition phase of Morris water maze[16] and novel object recognition ability[14]. NMDA receptor subunits, neurotrophins, pro-inflammatory chemokines and glutamate metabolism related mRNA levels also changed in the hippocampus after diesel exhaust exposure [14, 16]. Microglial activation in the hippocampus was observed after diesel exhaust exposure as well[14]. Another study, however, showed that exposure of resuspended DEPs by inhalation may impair locomotor activity but not spatial learning and memory in Morris water maze[62].

Exposure to concentrate ambient $PM_{2.5}$ also showed similar results. With a long-term (10 months) concentrated ambient $PM_{2.5}$ exposure, pro-inflammatory cytokines increased in the hippocampus of mice. Hippocampal morphology also changed in CA1 and CA3 region. This study also found spatial learning and memory impairment using Barnes maze. The mice also showed depressive-like responses in forced swim test and anxiety-like behavior in open field test. [9]

2.7 Susceptibility to particulate matter in CNS effects

The CNS is relatively vulnerable in fetus, postnatal period and even childhood because, in these periods, the CNS forms, grows, matures and develops [63]. Particles may harm the CNS more in these time because the development is still initiating [64].

Prenatal diesel exhaust exposure may impair motor function, decrease spontaneous motor activity, show impulsive behavior and change neurochemical levels in offspring [65-67]. Another study also presented that prenatal and first-week exposure to diesel exhaust particles may change locomotors activity and autism related behavior in mice[68].

Studies focused on postnatal particles exposure (just few days after birth) showed that early exposure to concentrated ambient particles may cause neuroinflammation and neurotransmitters alternations in multiple brain regions including the hippocampus, cortex, midbrain and striatum [69, 70]. Expressions of astrocyte and microglia also changed in the hippocampus. Pathological changes in lateral ventricle were observed.[69] Behavioral tests also showed that postnatal particles exposure may affect neurodevelopment which may be a risk factor for CNS disorders and diseases [70, 71].

Limited studies addressed PM effects in childhood. Intranasal instilled DEPs during childhood of rats (2- to 5-week old) may result in slight changes in motor activity and learning[72].

In addition to the early life of animals, aging is another susceptible factor. Aged brains may associate with increasing cytokines and microglial activation and also susceptible to environmental stress [73, 74]. Additional particulate matter exposure may increase impairments in aged brains.

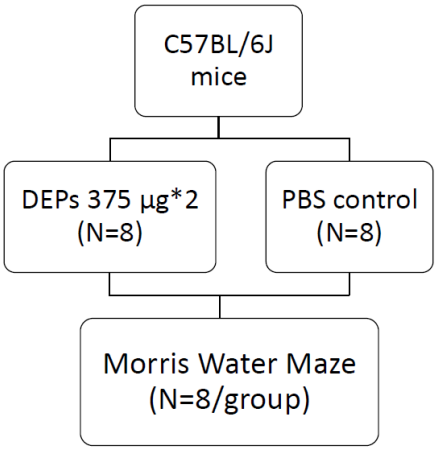
Part 1. Study of central nervous toxicity induced by diesel exhaust particles (DEPs)

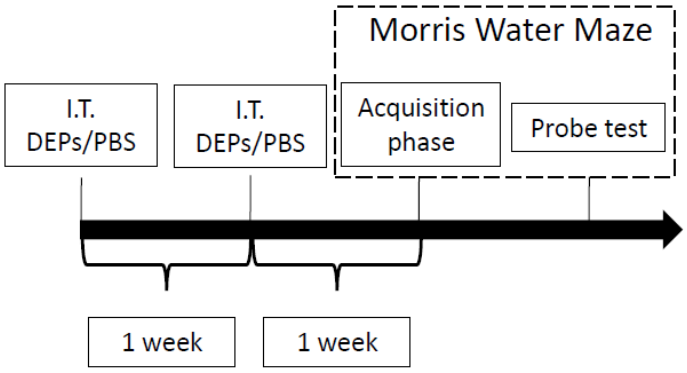
Chapter 3 Materials and Methods

3.1 Study protocol

This part of study was divided into two parts, the experiment 1 and the experiment 2.

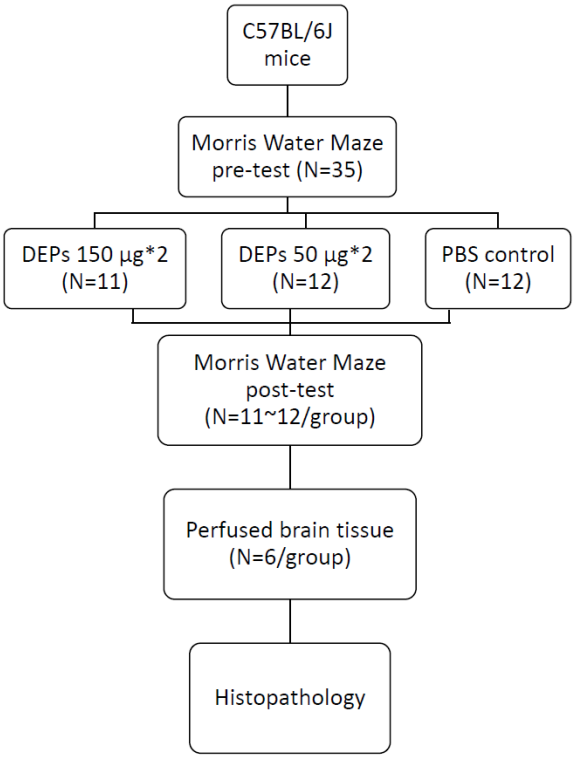
The experiment 1:

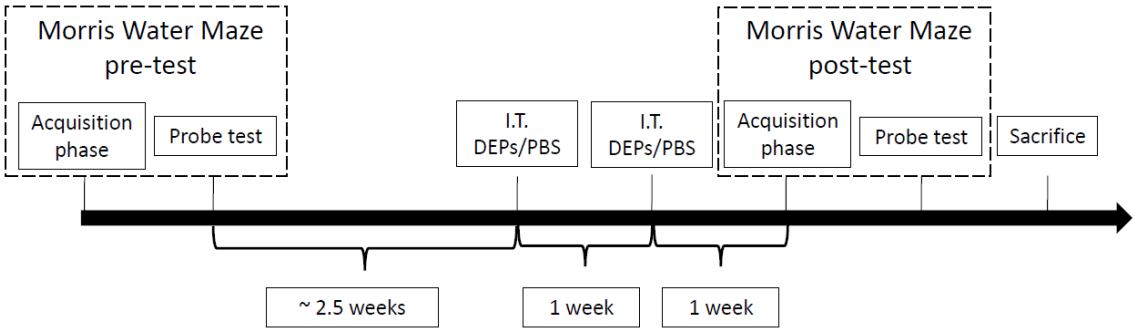




In the experiment 1, male C57BL/6 mice were given either DEPs 375 μg/mice (total 750 μg DEPs/mice) or PBS by intratracheal instillation (I.T.) once in a week for two weeks (two times). After one week after last exposure, Morris water maze was conducted.

The experiment 2:





In the experiment 2, male C57BL/6 mice were first given a Morris water maze pre-test to adjust possible differences of individuals. Mice were then given either DEPs 150 μg/mice (total 300 μg DEPs/mice), 50μg/mice (total 100 μg DEPs/mice) or PBS by intratracheal instillation (I.T.) once in a week for two weeks (two times). After one week after last exposure, Morris water maze (post-test) was conducted. The interval between Morris water maze pre-test and post-test was more than four weeks. The interval may be sufficient to eliminate the possible remaining effects in the pre-test[75].

3.2 Animals

7-week old male C57BL/6 mice were purchased from National Laboratory Animal Center, Taipei, Taiwan. The animals were acclimatized for one week and then were divided into experimental or control group in the experiment 1. In the experiment 2, the animals were acclimatized for one week and then underwent Morris water maze pre-test. Mice were divided into experimental or control group according to their performance in the pre-test. During the acclimation and experiment in both experiment 1 and 2, animals were kept in the animal room in the Laboratory Animal Center, College of Medicine, National Taiwan University. The animals were kept in an individually ventilated cage with normal diet and filtered water. The procedure in this study was approved by Institutional Animal Care and Use Committee (IACUC), College of Medicine and College of Public Health., National Taiwan University (Approval number: 20140340).

3.3 Acute diesel exhaust particles (DEPs) exposure methods

Diesel exhaust particles (DEPs) were purchased from National Institute of Standards and Technology (SRM 2975, NIST, USA). DEPs were suspended in 1% phosphate buffer saline (PBS) with concentration of 375 μg/ 100 μL, 150 μg/ 100 μL and 50μg/ 100 μL. To obtain better dispersing suspensions, DEPs suspensions were put into ultrasonication prior to exposure [76].

Animals were exposed to DEPs by intratracheal instillation (I.T.) under anesthesia. First, animals were anesthetized by inhaled 4% isoflurane for 4 minutes. Animals were then put on an equipment to maintain vertical to the table and the trachea of animals was kept vertical throughout the exposure procedure. 100 μL DEPs suspension in exposure group or PBS in control group was injected directly and slowly into trachea. Animals

were then kept upright for 1 minute to make sure that the suspension reached deeper airway. After the animals awoke form anesthesia, the exposure procedure was complete.

3.4 DEPs exposure schedule

Acute DEPs exposure experiment was divided into two experiment, the experiment 1 and the experiment 2. In the experiment 1, C57BL/6 mice (n=8) were exposed to 375 μg DEPs /mice by intratracheal instillation (I.T.) once in a week for two weeks (two times) and mice control group (n=8) were given 1%PBS. Total does of 750 μg DEPs were given to each mice in the exposure group. As for the experiment 2, C57BL/6 mice (n=11~12) were exposed to 150 or 50 μg DEPs /mice by intratracheal instillation (I.T.) once in a week for two weeks (two times) and mice in control group (n=12) were given 1%PBS. Total does of 300 or 100 μg DEPs were given to each mice in the exposure group. The exposure schedule was the same in both experiment except different doses.

3.5 Morris water maze test

Morris water maze test (MWM) was then conducted one week after last exposure in both experiment 1 and 2 or before exposure in the experiment 2. MWM is a widely used method designed for rodents [36, 77]. It is used to test spatial learning and memory function of animals [78]. Since spatial learning and memory function is part of cognitive function which may be an indicator of CNS injuries, we used this test to evaluate whether DEPs exposure may impair cognitive function in mice. MWM test included two parts, a four-consecutive-day acquisition phase and a one-day probe test.

In the acquisition phase, the test was conducted in a water pool with a hidden platform. Glutinous rice flour (SUNRIGHT, Taiwan) was added in the water and mixed well so the platform was not visible directly by the mice. The pool was divided into four

quadrants and the platform was placed in the center of one quadrant (platform quadrant) every day. On the edge of four quadrants around the pool, there were four kinds of shapes of cues which were provided for mice to recognize their own place in the pool. In each day, each mouse was placed in the pool for four trials. Swimming pattern and time which each mouse escaped form the water and found the hidden platform (escape latency) were recorded using a timer and a camera coupled with behavioral tracking software (EthoVision 3.1, The Netherlands). The escape latency which was less than 5 seconds was considered as a lucky trial and the trial was excluded from further analysis. Mean data from trials in each day and each mouse were calculated. Once the mouse found the platform, the mouse was allowed to stay on the platform for 15 seconds. If the mouse couldn't find the hidden platform within 60 seconds, the mouse was put onto the platform carefully and allowed to stay on the platform for 15 seconds. Escape latency, distance moved, cumulative distance from the center of platform quadrant in the experiment 1 or from the center of platform in the experiment 2 (searching error [79]) and swimming velocity were analyzed.

In the probe test, the test was conducted in the same condition except the hidden platform was removed. Swimming pattern in the probe test was recorded using the same equipment and software. Percentage of time spent in platform quadrant, percentage of time spent in platform area (platform area plus 5 cm around the platform), quadrant area (the quadrant previously with platform) crossing, platform area (platform area plus 5 cm around the platform) crossing, average distance from the center of platform quadrant in the experiment 1 or from the center of platform in the experiment 2 (average proximity [79]) and swimming velocity were analyzed.

3.6 Histopathology

In the experiment 2, upon accomplishment of Morris water maze, six mice in either total 300 μg DEPs group, total 100 μg DEPs group and control group were sacrificed on the second day. Mice were anesthetized with CO_2 and then perfused with lactated Ringer's solution with 10 IU heparin and bouin's solution (Muto pure chemicals, Tokyo, Japan). Brain tissue were collected and fixed in 10% formalin for at least 24 hours. Brain tissue was embedded in paraffin wax and the sections were stained with haematoxylin and eosin (H&E) for histopathological examination.

3.7 Statistics

Because of small sample size, in the experiment 1 (n=8/group), comparison between total 750 μg DEPs group and control group was conducted using non-parametric methods, Wilcoxon rank sum test. Student's t-test was also conducted to confirm similar trend in parametric methods. In the experiment 2 (n=11~12/group), comparisons between total 300 μg DEPs group, total 100 μg DEPs group and control group was conducted using non-parametric methods, Kruskal-Wallis test and post hoc test, Dunn's test. ANOVA and post hoc test, Sheffe test, was also conducted to confirm similar trend in parametric methods. Significance level was set to be 0.05.

Chapter 4 Results

4.1 Mice body weight

Body weight of mice in the experiment 1 was shown in Figure 1. Through the experimental duration of the experiment 1, although there were differences between total 750 μg DEPs group and control group, the differences were below 10%.

Body weight of mice in the experiment 2 was shown in Figure 2. There were no difference between total 300 μg DEPs group, total 100 μg DEPs group and control group through the experimental duration.

4.2 Acquisition phase of Morris water maze test

In the experiment 1, escape latency, distance moved, cumulative distance and swimming velocity in acquisition phase were shown in Table 4 and Figure 3 to 4.

The median escape latency in total 750 μg DEPs group was longer than that in the control group in the fourth day of acquisition phase which was close to significance (45.1 seconds vs. 17.7 seconds, P=0.059). Median distance mice moved in total 750 μg DEPs group was also longer than that in the control group in the fourth day of acquisition phase (464 cm vs. 325 cm, P=0.156). Median cumulative distance from the center of platform quadrant was significant longer in total 750 μg DEPs group than in control group in the fourth day of acquisition phase (11.5 m vs. 4.02 m, $P<0.05$). These differences were under no clear difference in median swimming velocity of mice between total 750 μg DEPs group and the control group (10.4 cm/s vs. 16.6 cm/s, P=0.227).

In the experiment 2, escape latency, distance moved, cumulative distance and

swimming velocity in acquisition phase were shown in Table 5 and Figure 5 to 6.

The median escape latency in total 300 μg DEPs group was longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase but without significance (40.7 seconds, 31.8 seconds vs. 30.4 seconds, P=0.550). Median distance mice moved in total 300 μg DEPs group was also longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase (370 cm, 268 cm vs. 304 cm, P=0.088). Median cumulative distance from the center of platform in total 300 μg DEPs group was also longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase (9.05 m, 6.88 m vs. 5.94 m, P=0.600). These were under no clear difference in median swimming velocity of mice between total 300 μg DEPs group, total 100 μg DEPs group and control group (11.5 cm/s, 9.16 cm/s vs. 12.2 cm/s, P=0.361).

We observed that swimming velocity in near half of mice was below 10 cm/s in the experiment 2 in all groups. Mice with swimming velocity below 10 cm/s may consider as failure in the Morris water maze test[75]. We then excluded mice with mean swimming velocity below 10 cm/s in acquisition phase and the results were presented in Table 6 and Figure 7 to 8.

The exclusion revealed significant differences in the escape latency and cumulative distance. The median escape latency in total 300 μg DEPs group was significantly longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase (35.4 seconds, 23.2 seconds vs. 14.2 seconds, $P<0.05$). Post hoc test showed that the significant difference was between total 300 μg DEPs group and control group. Median distance mice moved in total 300 μg DEPs group was still longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase (440 cm, 237 cm vs. 252 cm, P=0.081). Median cumulative distance from the center of

platform in total 300 μg DEPs group was also significant longer than that in total 100 μg DEPs group and control group in the fourth day of acquisition phase (8.64 m, 4.29 m vs. 2.18 m, P<0.05). Swimming velocity was still comparable with higher mean and median after exclusion in the fourth day of acquisition phase (13.2 cm/s, 10.9 cm/s vs. 15.6 cm/s, P=0.201).

4.3 Probe test of Morris water maze test

In the experiment 1, percentage of time spent in target area, area crossing, average proximity and swimming velocity in probe test were shown in Table 7 and Figure 9 to 10.

The median percentage of time spent in platform quadrant for total 750 μg DEPs group was 25.4%. The median percentage of that for the control group was 26.1%. (P=0.958). The difference was not obvious. Median times mice crossed the quadrant area previously with platform in total 750 μg DEPs group was slightly lower than that in the control group (3.25 times vs. 4.88 times, P=0.343). Median average distance from the center of platform quadrant (average proximity) was almost the same in total 750 μg DEPs group and control group (41.8 cm vs. 43.5 cm, P=0.564).The median swimming velocity of total 750 μg DEPs group and control group were comparable in probe test (12.4 cm/s vs. 15.0 cm/s, P=0.189).

In the experiment 2, percentage of time spent in target area, area crossing, average proximity and swimming velocity in probe test were shown in Table 8 and Figure 11 to 12.

The median percentage of time spent in platform quadrant for total 300 and 100 μg DEPs group were 40.9% and 31.4% and the median percentage of that for control group was 24.2% (P=0.437). The median percentage of time spent in platform area (platform

area plus 5 cm around the platform) for total 300 μg DEPs group, total 100 μg DEPs group and control group were also showed no difference (3.22%, 1.52% vs. 2.03%, P=0.724). Median times mice crossed the quadrant previously with platform (quadrant area crossing) and the area previously around platform (platform area plus 5 cm around the platform, platform area crossing) were not significantly different between total 300 μg DEPs group, total 100 μg DEPs group and control group (3.00 times, 2.63 times vs. 3.00 times, P=0.981 and 2.25 times, 0.88 times vs. 1.00 times, P=0.520). Median average distance from the center of platform (average proximity) showed no difference in total 300 μg DEPs group, total 100 μg DEPs group and control group (36.7 cm, 39.0 cm vs. 41.9 cm, P=0.652). The median swimming velocity of total 300 μg DEPs group, total 100 μg DEPs group and control group were comparable in probe test (10.4 cm/s, 8.82 cm/s vs. 10.0 cm/s, P=0.839)..

As mentioned above, we also tried to exclude mice with swimming velocity below 10 cm/s in probe test and the results were presented in Table 9 and Figure 13 to 14. However, most results showed similar pattern after exclusion except slight difference in median times mice crossed the area previously around platform (platform area plus 5 cm around the platform, platform area crossing) with P=0.098 (total 300 μg DEPs group: 4.13 times, total 100 μg DEPs group: 5.00 times and control group: 3.50 times).

4.4 Histopathology

In the experiment 2, we performed a histopathological examination in multiple brain regions, including olfactory bulb, frontal cortex, hippocampus and cerebellum, and the results were shown in Figure 15 to 20. However, we found no significant difference in these regions. Each mice in total 300 μg DEPs group, in total 100 μg DEPs group and in control group were within normal limit.

Chapter 5 Discussion

Our results showed that acute exposure to diesel exhaust particles (DEPs) may impair performance in acquisition phase of Morris water maze test in mice. Both experiment 1 and 2 in this part of study revealed that mice needed more time and distanced moved to reach the platform in acquisition phase after exposure to DEPs. Cumulative distance from the center of platform quadrant in the experiment 1 or from the center of platform in the experiment 2 (searching error) was also longer in acquisition phase after exposure to DEPs. Particularly in the experiment 2 with exclusion of mice with low motivation to swim, mice in total 300 μg DEPs exposure group may significantly require more time to find the platform and make more searching error. However, performance in total 100 μg DEPs exposure group was comparable with control group. These results in the experiment 2 showed a possible dose response on acute DEPs exposure. Distance mice moved in acquisition phase also showed a possible dose response manner which was close to significance. Histopathological examination found no significant difference between total 300 μg DEPs group, total 100 μg DEPs group and control group.

5.1 DEPs dose and effects in neurofunctions

We observed acquisition impairment in both total 750 μg and 300 μg DEPs exposure group and no clear effects in total 100 μg DEPs exposure group. A total 300 μg DEPs were given to mice in two weeks. This was equivalent to exposure to near 200 $\mu g/m^3$ DEPs for two weeks. Considering deposition rate of inhaled particles, mass concentration of exposed DEPs maybe even higher. US EPA reviewed that mass concentration of DEPs in the air was about 1 to 4 $\mu g/m^3$ in the environmental which was

a way lower than equivalent mass concentration in current study[10].

DEPs concentration in occupational environment, on the other hand, was higher. Railroad workers may expose to DEPs with 37 to 191 μg/m^3 and firefighters may expose to DEPs with 4 to 478 μg/m^3[10]. Equivalent mass concentration using in current study was close to these occupational environment and found behavioral changes in acquisition. It was reported that railroad works with long-term exposure and electricians with relatively short-term exposure to diesel exhaust may have neurobehavioral effects[55]. Human volunteers who acute exposure to diesel exhaust with 300 μg/m^3 for one hour also showed changes in EEG[61]. Although the exposure route and duration were not exactly the same, both previous and our results showed that exposure to higher DEPs may have effects in neurofunctions.

5.2 DEPs characterization and DEPs effects

Although we didn't measure the characterization of DEPs suspension using in this study, other study measured DEPs size in a similar condition revealed that DEPs size in suspensions was 402 nm[80]. Small particles with relative higher surface area may attach organic chemicals and metals. A study showed that particle core of DEPs and extractable chemicals of DEPs may have different toxicological effects[81]. Whether DEPs with different sizes and whether particle core or chemical extract of DEPs cause different effects in the CNS required more studies to verify.

Different sources of DEPs may result in different chemical composition. We used SRM 2975 in this study which was generated from forklifts with diesel engines. A study compared the difference between forklift DEPs and automobile DEPs and found variations in chemical composition. This study also conducted a toxicological experiment using these two DEPs but found differences in biological effects. Chemical

composition may play roles in this findings. [82] CNS effects cause by different sources of DEPs were worth further studies.

5.3 CNS effects induced by DEPs

As reviewed in Table 1, most studies used diesel exhaust to evaluate behavioral changes and other CNS effects in mature mice. These studies were conducted in a relative long-term exposure [14-16]. Sub-chronic exposure to diesel exhaust has showed impairment in acquisition using the same behavioral test, Morris water maze[16]. Here, we found that acute exposure to DEPs may cause similar effects in the CNS with changes in acquisition ability.

In the study conducted by Win-Shwe et al, mice were exposed to diesel exhaust with DEPs and gases (whole DE) or filtered diesel exhaust with only gaseous portion of diesel exhaust (F-DE). In acquisition phase of Morris water maze test, they found differences between whole DE and control group, but there were no difference between F-DE and control. Moreover, they found differences between whole DE group and F-DE group. This showed that particles part rather than gaseous part in diesel exhaust may cause poor performance in acquisition phase of Morris water maze.[16] Our results were consistent with their findings.

Limited studies used the same commercial DEPs (SRM 2975) in our experiment to study CNS effects induced by DEPs. In a study conducted by Hougaard et al., mice were exposed to resuspended DEPs (SRM 2975) 90 minutes/day for four days and found no changes in Morris water maze test[62]. The mass concentration of resuspended DEPs using in that study was 71.5 mg/ m^3 which was higher than our study, but there were differences in exposure schedule and exposure route between these two studies. As mentioned by Hougaard et al, volatile and semivolatile chemicals were

eliminated in resuspended DEPs. With many attached volatile and semivolatile chemicals, it may be the reason that there were differences between previous study and current study [83].

Whether acute effects we found in this study may recover was unknown under current study design. Recent studies targeted at postnatal exposure to concentrated ambient particles found that early exposure may cause neurobehavioral changes and lateral ventricle dilation in the adulthood [70, 71, 84]. This results indicated that articles exposure in developmental period may have persistent effects in the CNS. In reality, organisms exposed to particles air pollution through their whole life, so whether exposure in adulthood may persist was worth further research.

5.4 Mechanism involved in DEPs induced CNS effects

Possible mechanism involved in DEPs induced changes in acquisition phase of Morris water maze was neuroinflammation. A study found that, twenty hours after 20mg/kg DEPs (SRM 2975) exposure in rats, TNF-α in both serum and the brain was elevated. Microglial expression was also increased. This implicated that DEPs may cause systemic effects and may further induced neuroinflammation.[19] Since the same DEPs were used in previous study and current study, our results may share similar mechanism. Previous studies using diesel exhaust also found that pro-inflammatory markers were elevated in multiple brain regions, including the hippocampus, midbrain, olfactory bulb, frontal lobe, temporal lobe and striatum[16-19]. Microglial activation was also observed in the hippocampus[14]. We should then conduct other tests in pro-inflammatory cytokines and microglial activation in brain regions to support the results of acquisition impairment in this study. Since the hippocampus was the major brain region involved in spatial learning and memory, further studies should put more

emphasis on this part of the brain.

As for histopathology, we didn't observed changes in multiple brain regions after DEPs exposure. Studies showed that maternal exposure to diesel exhaust may resulted in apoptosis in the cortex, hippocampus and cerebellum [85, 86]. On the one hand, these studies observed apoptosis after maternal exposure and this exposure was in a relatively vulnerable period. In our study, DEPs were given to mice in adulthood. On the other hand, other studies found that cognitive changes were related to subtle morphological changes in axons or dendrites in the hippocampus [9, 87, 88]. Further analysis in detailed morphological or histopathological examination was required to verify current results in behavioral test.

5.5 Spatial learning and memory

We observed significant results in acquisition phase and non-significant results in probe test in current study. This maybe a result of different regions involved in memory acquisition, memory consolidation and memory recall[38]. A study showed that hippocampus CA3 region may involve in acquisition phase and memory consolidation but not in memory recall[37]. This implied that, in a complicated process of learning and memory, different regions in the brain and even in the hippocampus was involved with different function. Current study found changes in acquisition phase but not in probe test may due to different regions with different impacts to the exposure.

Or, the current study design with one-day probe trial after acquisition phase may not sensitive enough to show the difference in experimental and control groups. A study conducted Morris water maze comparing the different interval between acquisition phase and probe test. It was found that preference for the quadrant previously with the platform (platform quadrant) was decreased when the interval between acquisition

phase and probe test increased. They found no significant preference in probe test 24 hours after acquisition phase which interval was used in current study.[89] Although they conducted the experiment using rats and a different procedure for Morris water maze, our results without differences in probe test may share the same reason.

5.6 Limitations

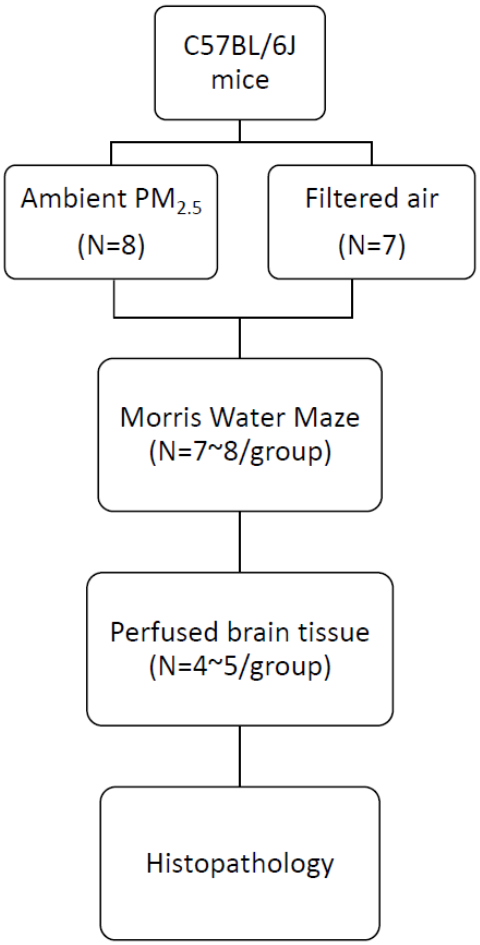
We measured swimming velocity of mice in the Morris water maze to check the swimming ability was the same between groups. Additionally, cued platform training in the Morris water maze test or other physical measurements may provide additional information to make sure that there are no difference in visual acuity and swimming ability between groups. Moreover, mice was more stressful than rats[90]. This may cause a failure in behavioral tests. We found that near half of mice in the experiment 2 swam slower. Stress in the Morris water maze may be the reason. Rats which were less stressful in the Morris water maze may be used to confirm current findings.

Since previous study showed that multiple regions may affect by PM, many other behavioral tests related to different brain regions may conducted to confirm PM induced toxicity. On the other hand, further biochemical examinations like cytokines, inflammatory cells examinations like microglia and detailed histopathological examination were essential to support our findings.

Part 2. Study of central nervous toxicity induced by ambient fine particles ($PM_{2.5}$)

Chapter 6 Materials and Methods

6.1 Study protocol



Time	Week 1 to 12	Week 13	Week 14	
			4-day	1-day
$PM_{2.5}$ exposure group (N=8)	$PM_{2.5}$	Quarantine	Morris water maze Acquisition phase	Morris water maze Probe test
HEPA-filtered air control group (N=7)	Filtered air			

Male C57BL/6 mice were exposed to either ambient $PM_{2.5}$ or filtered-air by inhalation for 12 weeks (about 3 months). After one week after last exposure, Morris water maze was conducted.

6.2 Animals

3-week old male C57BL/6 mice were purchased from National Laboratory Animal Center, Taipei, Taiwan. The animals were acclimatized for one week and then were divided into two groups for either a 12 weeks (about 3 months) ambient $PM_{2.5}$ exposure or filtered-air control. During acclimation and exposure, animals were kept in the animal room in the College of Public Health, National Taiwan University. The animal room was maintain in a controlled environment (lights on 0800-2000, temperature: 22 ± 1℃, relative humidity: 50 ± 5%). The procedure in this study was approved by Institutional Animal Care and Use Committee (IACUC), College of Medicine and College of Public Health., National Taiwan University (Approval number: 20130531).

6.3 Ambient $PM_{2.5}$ exposure

Ambient $PM_{2.5}$ (fine particulate matter) exposure was conducted by using Taipei Air Pollution Exposure System for Health Effects (TAPES) which located in the animal room. The TAPES is a system which directly introduces $PM_{2.5}$ outside the building of the College of Public Health, Zhongzheng district, Taipei city, Taiwan. This system provides a non-concentrated, low concentration and real-world $PM_{2.5}$ exposure form downtown Taipei city. Our previous study using this system showed that exposed particles were mainly fine particles ($PM_{2.5}$) [91]. Animals in control group were exposed to HEPA-filtered-air without most particles in ambient air

6.4 Ambient PM exposure monitoring and characterization

To monitor the ambient $PM_{2.5}$ exposure in the TAPES system, the DustTrak II Aerosol Monitor 8530 (TSI, Shoreview, Minnesota, USA) was used to access the

concentration of ambient $PM_{2.5}$ and PM_1 in the exposure chamber. DustTrak is a direct reading instrument and it may provide a real-time measurement for entire time of the experiment. A 37-mm cassette is also in the DustTrak for manual sampling. Manual sampling results using DustTrak were used to monitor ambient PM exposure. Manual sampling was conducted using Teflon filter papers (Pall Corporation, Port Washington, New York, USA) with 2 litter per minute as actual sampling volume. The manual sampling duration for $PM_{2.5}$ and PM_1 was about one week.

Teflon filter papers used in this study were conditioned in a weighing chamber before and after manual sampling for at least 24 hours. After condition, filter papers were weighed in the weighing chamber by electronic balance. Mass changes after sampling were calculated and used to evaluate PM exposure. Teflon filter papers were stored at -20℃ before further chemical analysis.

In order to understand the composition of exposed $PM_{2.5}$, Teflon filter papers were then analyzed. First, energy-dispersive X-ray fluorescence spectrometry (XRF) was used to quantified elemental components in exposed $PM_{2.5}$, including Magnesium (Mg), Aluminum (Al), Silicon (Si), Sulfur (S), Potassium (K), Calcium (Ca), Titanium (Ti), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Nickel (Ni), Copper (Cu), Zinc (Zn), Barium (Ba), and Lead (Pb)[92]. Mean detection limit (MDL) in XRF was calculated as three times of standard deviation of results in analyzing surface concentration on the blank filter papers for ten times. Samples with surface concentration below MDL were presented as BDL (below detection limit). Samples with surface concentration below sum of surface concentration of blank and MDL were presented as BBK (below blank)**.** Samples which were BDL or BBK were replaced by 1/2 MDL for further calculation. Data of XRF were presented by calculating mean, median, minimum (Min), maximum (Max), percentage of BDL or BBK and percentage

of mean $PM_{2.5}$.

In addition, ion chromatography (IC) was also applied to analyzed soluble ions, including Na^+, K^+, Ammonium (NH^{4+}), Ca^{2+}, Mg^{2+}, Chlorine ion (Cl^-), Nitrate (NO_3^-), and Sulfate ($SO4^{2-}$). Limit of detection (LDL) in IC was calculated as three times of standard deviation of results in analyzing 0.025 ppm of individual ions for ten times. Samples with concentration below LDL were presented as <LDL. Samples which were <LDL were replaced by 1/2 LDL for further calculation. Data of IC were presented by calculating mean, median, minimum (Min), maximum (Max), percentage of <LDL and percentage of mean $PM_{2.5}$.

6.5 Ambient $PM_{2.5}$ exposure schedule

After one week of accumulation, 4-week old healthy C57BL/6 mice were exposed to ambient $PM_{2.5}$ by using the TAPES system for a 12 weeks (about 3 months) exposure. Fifteen C57BL/6 mice were randomly divided into two groups (n=8 for $PM_{2.5}$ exposure group and n=7 for HEPA-filtered air control group). After ambient $PM_{2.5}$ or HEPA-filtered-air exposure, mice were transfer to the Laboratory Animal Center, College of Medicine, National Taiwan University for a one week quarantine. The cognitive function of spatial learning and memory was then tested by using Morris water maze.

6.6 Morris water maze test

Morris water maze test (MWM) was conducted one week after the end of 12 weeks exposure. The experimental procedure was the same as the first part of this study. Cumulative distance from the center of platform and average distance from the center of platform were calculated in this part of study. Other data analysis was also the same as

the first part of this study. Please refer to Chapter 3.5 for other procedures and analysis.

6.7 Histopathology

Upon accomplishment of Morris water maze, five mice in $PM_{2.5}$ exposure group and four mice in filtered-air control group were sacrificed on the second day. Mice were anesthetized with CO_2 and then perfused with lactated Ringer's solution with 10 IU heparin and bouin's solution (Muto pure chemicals, Tokyo, Japan). Brain tissue were collected and fixed in 10% formalin for at least 24 hours. Brain tissue was embedded in paraffin wax and the sections were stained with haematoxylin and eosin (H&E) for histopathological examination.

6.8 Statistics

Because of small sample size, comparison between $PM_{2.5}$ exposure group (n=8) and filtered-air control group (n=7) was conducted using non-parametric methods, Wilcoxon rank sum test. Student's t-test was also conducted to confirm similar trend in parametric methods. Significance level was set to be 0.05.

Chapter 7 Results

7.1 Mice body weight

Body weight of mice in this part of study was shown in Figure 21. Mostly, there were no difference between $PM_{2.5}$ exposure group and control group except the second week of exposure. Nevertheless, the difference was below 10%.

7.2 $PM_{2.5}$ and PM_1 exposure monitoring and characterization

Mice were exposed to ambient $PM_{2.5}$ for 12 weeks. The mean mass concentration for $PM_{2.5}$ was 11.9 $\mu g/m^3$ and ranged from 4.7 to 16.8 $\mu g/m^3$ during the 12-week exposure duration using manual sampling. The mean mass concentration for PM_1 was 9.9 $\mu g/m^3$ and ranged from 3.8 to 14.6 $\mu g/m^3$ during the 12-week exposure duration using manual sampling. (Table 10 and Figure 22)

Elemental analysis of chemical composition of $PM_{2.5}$ using XRF was shown in Table 11 and Figure 23 (A). Sulfur (S) with mean concentration of 1935.81 ng/m^3 accounted for 16.29% of mean mass concentration of $PM_{2.5}$. Potassium (K) and silicon (Si) compromised near 1 % of mean mass concentration of $PM_{2.5}$ with mean concentration of 198.84 and 106.39 ng/m^3 individually.

Soluble ions analysis of chemical composition of $PM_{2.5}$ using IC was shown in Table 12 and Figure 23 (B). SO_4^{2-} was the major component of ambient $PM_{2.5}$, accounting for near 40% of mean mass concentration of $PM_{2.5}$ with the concentration of 4673.66 ng/m^3. NH_4^+ also accounted 15.4% of mean mass concentration of $PM_{2.5}$ with the concentration of 1830.17 ng/m^3.

7.3 Acquisition phase of Morris water maze test

Escape latency, distance moved, cumulative distance and swimming velocity in acquisition phase were shown in Table 13 and Figure 24 to 25.

The median escape latency in $PM_{2.5}$ exposure group was significantly longer than that in filtered-air control group in the third day of acquisition phase (45.1 seconds vs. 27.6 seconds, $P<0.05$). Median distance mice moved in $PM_{2.5}$ exposure group was also longer than that in filtered-air control group in the third day of acquisition phase (564 cm vs. 296 cm, $P=0.073$). Median cumulative distance from the center of platform was significant longer in $PM_{2.5}$ exposure group than in filtered-air control group in the third day of acquisition phase (11.5 m vs. 8.17 m, $P<0.05$).This difference was under no clear difference in median swimming velocity of mice between $PM_{2.5}$ exposure group and filtered-air control group in the third day of acquisition phase (12.1 cm/s vs. 16.2 cm/s, $P=0.272$).

7.4 Probe test of Morris water maze test

Percentage of Time spent in target area, area crossing, average proximity and swimming velocity in probe test were shown in Table 14 and Figure 26 to 27.

The median percentage of time spent in platform quadrant for the $PM_{2.5}$ exposure group was 26.1%. The median percentage of that for the control group was 27.0%. ($P=0.954$). The difference was not obvious. However, although without significance, the median percentage of time spent in platform area (platform area plus 5 cm around the platform) for the $PM_{2.5}$ exposure group was less than that in filtered-air control group (0.96% vs. 2.70%, $P=0.272$. Median times mice crossed the quadrant previously with platform (quadrant area crossing) and the area previously around platform (platform area plus 5 cm around the platform, platform area crossing) were not significantly

different between $PM_{2.5}$ exposure group and filtered-air control group (3.88 times vs. 3.33 times, P=0.908 and 0.88 times vs. 1.50 times, P=0.816). Median average distance from the center of platform (average proximity) was almost the same in $PM_{2.5}$ exposure group and filtered-air control group (42.8 cm vs. 41.1 cm, P=0.385). The median swimming velocity of $PM_{2.5}$ exposure group and filtered-air control group was comparable in probe test (12.5 cm/s vs. 10.8 cm/s, P=1.00).

7.5 Histopathology

We performed a histopathological examination in multiple brain regions, including olfactory bulb, frontal cortex, hippocampus and cerebellum, and the results were shown in Figure 28 to 32. However, we found no significant difference in these regions. Both mice in $PM_{2.5}$ exposure and in filtered-air group were within normal limit.

Chapter 8 Discussion

Our results suggested that exposure to $PM_{2.5}$ in ambient level for 12 weeks (3 months) may impair performance in acquisition phase of Morris water maze test in mice. In acquisition phase, both escape latency and distance moved which mice needed to find the platform were longer in the $PM_{2.5}$ exposure group. Mice in control group, on the other hand, may reached the platform with less time and distance. Cumulative distance from the center of platform was also longer in acquisition phase after exposure. This showed that non-concentrated, low-concentration ambient $PM_{2.5}$ exposure for 12 weeks (3 months) may impair acquisition in mice. Impairment in probe test, in contrast, was relative unobvious, although mice in $PM_{2.5}$ exposure group spent less time in the platform quadrant and platform area. Histopathological examination found no significant difference between $PM_{2.5}$ exposure group and filtered-air control group.

8.1 Ambient PM exposure concentration and characterization

The mean $PM_{2.5}$ mass concentration in the exposure duration was 11.9 $\mu g/m^3$ which was close to WHO air quality guidelines ($PM_{2.5}$, annual mean, 10 $\mu g/m^3$)[93] and below standard of environment protection agency, Taiwan ($PM_{2.5}$, annual mean, 15 $\mu g/m^3$).

Analysis of chemical composition of $PM_{2.5}$ using XRF found the major components were S, K and Si. Sources of S may be mobile emission (gasoline and diesel vehicles). K may come from road dust, windblown dust, construction and demolition and biomass burning. Sources of Si may be road dust, windblown dust, construction and demolition, and industrial activities. As for soluble ions analyzing by IC, SO_4^{2-} and NH_4^+ were major components in $PM_{2.5}$. Sources of SO_4^{2-} may be

industrial fuel combustion and secondary aerosol. NH_4^+ may come from secondary aerosol. [94, 95] These showed an urban $PM_{2.5}$ air pollution characterization which was similar to our previous study [91].

Studies showed that there were different chemical composition of PM in different cities[96], in urban and rural area[97, 98] and in different seasons[98]. Moreover, different $PM_{2.5}$ composition may result in different lung toxicity *in vivo* [98] and DNA damage *in vitro* [99]. There is a possibility that different composition may also have different effects in the CNS. For example, metals like Mn may translocate to the CNS through olfactory bulb and increase Mn levels in multiple brain regions[30]. Exposure to Mn may change behavior in the animal study [100]. Ambient air exposure with higher Mn may thus have CNS effects due to excess Mn. However, whether specific component in ambient $PM_{2.5}$ may affect the CNS required more studies. Future study may also comparing CNS toxicity induced by ambient PM from different origins or areas with a variety of composition.

8.2 CNS effects induced by PM

Our previous study found that chronic exposure to ambient $PM_{2.5}$ using the same exposure system may change lung morphology and increase inflammatory cells. Lipidomics also revealed biochemical changes related to lung injuries. [101] Besides lung effects, we also found that subchronic ambient $PM_{2.5}$ exposure using the same exposure system may affect glucose homeostasis and cause systemic inflammation in diabetes mellitus (DM) rat model. Inflammation in heart, thickness in aorta and injuries in kidney were also found in histopathological examination after $PM_{2.5}$ exposure. [91] Current study using the same exposure system, in addition to injuries in lung, heart and kidney, we also found possible effects in the central nervous system.

Previous study conducted by Fonken et al. showed that long-term exposure to concentrated ambient particles may impair spatial learning and memory using Barnes maze[9]. Our results showed similar results in acquisition phase using Morris water maze with a relatively lower concentration and a shorter exposure duration. Mean $PM_{2.5}$ mass concentration in current study was 11.9 $\mu g/m^3$ without concentrators. However, in the study conducted by Fonken, equivalent mean concentrated $PM_{2.5}$ mass concentration was 16.85$\mu g/m^3$. The exposure in our study was 24 hours a day, 7 days a week for 12 weeks instead of 6 hours a day, 5 days a week for 10 months in Fonken et al. On the other hand, Fonken et al found differences in both acquisition phase and probe test after exposure. Different tests used in previous and current studies may be the reason that we didn't find differences in probe test. A study which compared Morris water maze and Barnes maze indicated that corticosterone levels of mice were elevated after behavioral test more in Morris water maze than in Barnes maze. The study also found a correlation between poor performance in Morris water maze and increasing stress levels.[102] Since probe test was conducted on the fifth day, elevated stress level may surpass the effects of $PM_{2.5}$ exposure in Morris water maze.

There were more studies using diesel exhaust as a surrogate to air pollution also showed similar results. Co-exposure to diesel exhaust with abundant nanoparticles and lipoteichoic acid for one month may affect performance in Morris water maze[15]. Another study with a longer exposure duration (3 month) conducted by the same authors showed that diesel exhaust alone may affect acquisition as well. Results of this study also showed that the particles part in diesel exhaust may have contribution to the impairment. Same results were also found in this study and current study that there were no difference in the probe test. [16]

Our results also support previous epidemiological studies regarding CNS toxicity

induced by PM. Suglia et al. suggested that children's cognition may decrease with increasing exposure to black carbon[48]. In our study, mice with the age of 4-week were exposed to ambient $PM_{2.5}$ and found impairment in acquisition phase. The study of Fonken et al. mentioned above also found behavioral impairment using 4-week old mice[9]. However, our study design may not be able to suggest that whether PM exposure in childhood has more impact in cognition or other CNS effects than PM exposure in other life period. Most studies which found behavioral changes after diesel exhaust exposure used mature mice. A study found minor changes in behavior after DEPs exposure in the childhood[72]. More study should be made to elaborate the effects of $PM_{2.5}$ CNS toxicity in children and compare the effects of $PM_{2.5}$ between exposure in childhood and exposure in adulthood.

8.3 Mechanism involved in PM induced CNS effects

The possible mechanism of spatial learning and memory impairment may be through systemic inflammation and neuroinflammation[1]. Levesque et al. suggest that exposure to diesel exhaust particles may increase systemic pro-inflammatory marker and cause neuroinflammation by elevating pro-inflammatory cytokines and microglial expression[19]. Exposure to concentrated PM for 2 to 8 weeks in healthy rats, Apo E knockout mice and sensitized BALB/c mice may all cause neuroinflammation in multiple brain regions, including the olfactory bulb, striatum, frontal cortex and hippocampus[6-8, 56]. Also, exposure to diesel exhaust may also result in neuroinflammation in multiple brain regions, including the midbrain, olfactory bulb, frontal lobe, temporal lobe and striatum [17-19]. Microglial activation was also observed after diesel exhaust particles exposure[14]. The study conducted by Fonken et al. for a longer exposure duration also suggested that the toxicity caused by ambient PM

may be related to elevated pro-inflammatory cytokines level in the hippocampus.

Studies showed that exposure to PM resulted in morphological or pathological changes in many brain regions [9, 85, 86]. Therefore, we used a standard H&E stain to examine histopathological changes. However, animals were within normal in both $PM_{2.5}$ exposure group and filtered-air control group. Morphological changes in the hippocampus CA1 and CA3 region was observed in a long-term $PM_{2.5}$ exposure study. Spine density in hippocampus CA1 region and dendritic length in hippocampus CA3 region were decreased.[9] These changes were rather subtle and couldn't detect in H&E stain. Another study regarding age-related changes in morphology of the hippocampus also showed that subtle anatomical changes rather than neuron loss may associate with spatial memory impairment [87, 88].

Further tests in pro-inflammatory cytokines, microglial activation and detailed histopathological examination should be conducted to support the results of acquisition impairment in this study. Because multiple brain regions has been linked with PM induced neuroinflammation, tests should be conduct in those regions individually. The hippocampus which involved in spatial learning and memory was particularly interested in.

8.4 Spatial learning and memory

We observed different results in acquisition phase and probe test in current study. This maybe a result of different regions involved in spatial learning and memory or the interval between acquisition phase and probe trial. For detailed discussion, please refer to Chapter 5.5.

8.5 Limitations

We used TAPES to conduct a sub-chronic ambient $PM_{2.5}$ exposure to mice. However, the TAPES itself may not provide different concentration or dose of ambient $PM_{2.5}$, so we may not be able to confirm that whether the CNS effects induced by ambient $PM_{2.5}$ was in a dose response manner. Additionally, influence of different composition of ambient $PM_{2.5}$ may not address under current exposure system and study design. Other limitations were discussed previously. Please refer to Chapter 5.6.

Chapter 9 Conclusions

Both acute exposure to DEPs by intratracheal instillation and sub-chronic (12 weeks or 3 months) exposure to non-concentrated, low concentration ambient $PM_{2.5}$ may affect performance in acquisition phase in Morris water maze test.

The possible mechanism may be DEPs or $PM_{2.5}$ induced neuroinflammation. Pro-inflammatory cytokines and microglial activation should be tested to support current findings in behavioral changes. Although we didn't find difference in histopathological examination using H&E stain, further detailed histopathological examination should also be conducted to explore mechanism in behavioral changes induced by DEPs or $PM_{2.5}$.

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Table 1. Studies of particulate matter induced behavioral and other CNS effects

Animals	Exposure	Behavioral effects	Other CNS effects	References
4-week old C57BL/6 mice	Concentrated ambient $PM_{2.5}$ by inhalation for 10 months	1. Impair spatial learning and memory (Barnes maze) 2. Showing depressive-like (forced swim) and anxiety-like behavior (Open field)	1. Increasing TNF-α, IL-1β and HO1 mRNA level in the hippocampus 2. Morphological changes in the hippocampus CA1 and CA3 region	Fonken et al., 2011[9]
7-week old BALB/c mice	Diesel exhaust by inhalation for 1 month (with or without LTA injection)	Impair acquisition (Morris water maze) with co-exposure to diesel exhaust and LTA	Increasing NMDA receptor subunits, TNFα, IL-1β mRNA levels in the hippocampus with co-exposure to diesel exhaust and LTA	Win-Shwe et al., 2008[15]
7-week old BALB/c mice	Diesel exhaust by inhalation for 3 months	Impair acquisition (Morris water maze)	Increasing NMDA receptor subunits, BDNF (neurotrophins), CCL3 (pro-inflammatory chemokines) mRNA levels in the hippocampus	Win-Shwe et al., 2012[16]
7-week old BALB/c mice	Diesel exhaust by inhalation for 3 months	Impair the novel object recognition ability (hippocampus related)	1. Change glutamate metabolism related mRNA expression in the hippocampus 2. Microglial activation in the hippocampus	Win-Shwe et al., 2012[14]
3-week old C57BL/6 mice	Resuspended DEPs by inhalation	Impair locomotor activity		Hougaard et al., 2009[62]

Table 2. Studies of diesel exhaust or diesel exhaust particles induced CNS effects

Animals	Exposure	CNS effects	References
10- to 12-week old Fischer 344 rat	Diesel exhaust by inhalation for 6 months	1. Increasing TNF-α in the midbrain, olfactory bulb, frontal lobe and temporal lobe 2. Increasing AD-related Tau [ps199] in the temporal lobe and frontal lobe, AD-related Aβ42 in the frontal lobe and PD-related α synuclein in the midbrain	Levesque et al., 2011[18]
15- to 16-week old Fischer 344 rat (with ozone pretreatment)	Diesel exhaust by nose-only exposure for 1 month	Increasing TNF-α and IL-1α in the striatum	Gerlofs-Nijland et al., 2010[17]
12- to 14-week-old WKY rats	Diesel exhaust by inhalation for 1 months	1. Increasing IL-6, IBA-1, nitrosylated protein in the whole brain homogenate 2. Increasing TNF-α, IL-6, MIP-1α in the OB, cortex and midbrain 3. Increasing IL-1β, IBA-1 in the cortex and midbrain 4. Increasing RAGE in the midbrain	Levesque et al., 2011[19]
SD rats	DEPs by I.T.	1. Increasing TNF-α in the serum and brain 2. Mild microglial activation	Levesque et al., 2011[19]

Table 3. Studies of ambient particulate matter induced CNS effects

Animals	Exposure	CNS effects	References
Apo E-/- mice	Concentrated ambient PM by inhalation for 6 weeks	Increasing NF-κB, AP-1, GFAP and JNK (a MAP kinase) in the cortex	Kleinman et al., 2008[56]
6-week old SD rats	Concentrated ambient PM by inhalation for 8 weeks	1. Increasing HO-1 in the olfactory bulb, striatum, frontal cortex and hippocampus 2. Increasing Nrf-2 and Sod2 in the striatum and hippocampus 3. Increasing TNF-α, IL-1β and NF-κB in the striatum and hippocampus 4. Increasing BiP and XBP-1S (unfolded protein response markers) in the striatum and hippocampus	Guerra et al., 2013[6]
6-week old OVA sensitized BALB/c mice	Concentrated ambient $PM_{2.5}$ by inhalation for 2 weeks	Increasing TNF-α, IL-1α and NF-κB in the brain	Campbell et al., 2005[7]
Apo E-/- mice	Concentrated ambient $PM_{2.5}$ by inhalation for 5 weeks	Increasing TNF-α, IL-1α in the brain	Campbell et al., 2009[8]
Apo E-/- mice	Concentrated ambient PM by inhalation for 4 months	1. Decreasing dopaminergic neurons 2. Increasing GFAP-stained astrocytes	Veronesi et al., 2005[57]
Wistar rat	Ambient PM_{10} by I.T.	1. Increasing ET-1 and eNOS (endothelial mediators) in the cortex 2. Increasing IL-1β, TNF-α, COX-2, iNOS and ICAM-1 in the cortex 3. Neuronal apoptosis	Guo et al., 2012[58]

Table 4. Morris water maze performance in acquisition phase in the experiment 1 in the first part of study

		Day 1			Day 2		
		Total 750 μg DEPs	Control	P-value	Total 750 μg DEPs	Control	P-value
	N	8	8		8	8	
Escape latency (s)	Mean ± SEM	50.6 ± 4.95	47.7 ± 4.08	0.661 [b]	44.6 ± 5.92	47.2 ± 5.78	0.753 [b]
	Median	60.0	46.3	0.653 [c]	48.9	54.6	0.829 [c]
Distance moved (cm)	Mean ± SEM	719 ± 65.0	603 ± 61.6	0.217 [b]	504 ± 59.6	610 ± 69.8	0.269 [b]
	Median	742	597	0.270 [c]	466	576	0.270 [c]
Cumulative distance [a] (m)	Mean ± SEM	14.6 ± 1.61	13.3 ± 1.34	0.532 [b]	12.5 ± 2.08	12.9 ± 2.11	0.910 [b]
	Median	16.1	13.2	0.564 [c]	11.4	13.0	0.875 [c]
Swimming velocity (cm/s)	Mean ± SEM	14.4 ± 0.67	12.9 ± 1.41	0.356 [b]	11.9 ± 1.15	13.5 ± 1.40	0.388 [b]
	Median	13.9	13.0	0.270 [c]	12.5	14.1	0.318 [c]

		Day 3			Day 4		
		Total 750 μg DEPs	Control	P-value	Total 750 μg DEPs	Control	P-value
	N	8	8		8	8	
Escape latency (s)	Mean ± SEM	47.0 ± 5.04	38.9 ± 6.83	0.354 [b]	43.9 ± 4.30	27.4 ± 7.03	0.064 [b]
	Median	51.7	36.8	0.311 [c]	45.1	17.7	0.059 [c]
Distance moved (cm)	Mean ± SEM	515 ± 56.0	499 ± 59.3	0.851 [b]	491 ± 67.4	367 ± 61.0	0.194 [b]
	Median	484	463	0.637 [c]	464	325	0.156 [c]
Cumulative distance [a] (m)	Mean ± SEM	13.0 ± 1.98	10.6 ± 2.19	0.434 [b]	11.6 ± 1.70	6.63 ± 2.06	0.081 [b]
	Median	12.1	9.56	0.318 [c]	11.5 *	4.02	0.041 [c]
Swimming velocity (cm/s)	Mean ± SEM	11.9 ± 1.69	14.3 ± 1.41	0.300 [b]	11.8 ± 1.72	15.3 ± 1.26	0.118 [b]
	Median	12.7	15.5	0.270 [c]	10.4	16.6	0.227 [c]

[a] Cumulative distance was calculated as total distance from the center of platform quadrant.
[b] Comparisons were conducted using Student's t-test. [c] Comparisons were conducted using Wilcoxon rank sum test.
* Significant differences from control group in Wilcoxon rank sum test

Table 5. Morris water maze performance in acquisition phase in the experiment 2 in the first part of study

		Day 1				Day 2			
		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value	Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	11	12	12		11	12	12	
Escape latency (s)	Mean ± SEM	43.0 ± 3.93	39.9 ± 3.90	45.1 ± 3.36	0.609 [b]	38.9 ± 4.17	41.7 ± 5.04	39.4 ± 4.93	0.907 [b]
	Median	43.9	36.7	45.8	0.563 [c]	35.0	41.8	39.7	0.889 [c]
Distance moved (cm)	Mean ± SEM	387 ± 37.4	403 ± 33.8	380 ± 33.0	0.890 [b]	373 ± 34.5	396 ± 45.0	362 ± 41.6	0.836 [b]
	Median	359	344	331	0.760 [c]	341	376	344	0.793 [c]
Cumulative distance [a] (m)	Mean ± SEM	10.8 ± 1.61	10.2 ± 1.32	11.3 ± 1.15	0.843 [b]	8.78 ± 1.40	10.3 ± 1.49	9.89 ± 1.67	0.783 [b]
	Median	10.6	9.09	10.8	0.762 [c]	7.14	11.7	9.27	0.840 [c]
Swimming velocity (cm/s)	Mean ± SEM	9.72 ± 1.11	10.9 ± 1.13	9.11 ± 1.21	0.524 [b]	10.5 ± 1.19	10.4 ± 0.95	10.5 ± 1.29	0.997 [b]
	Median	10.2	10.4	7.21	0.570 [c]	9.88	11.0	10.7	0.988 [c]

		Day 3				Day 4			
		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value	Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	11	12	12		11	12	12	
Escape latency (s)	Mean ± SEM	47.9 ± 4.17	46.8 ± 4.03	41.2 ± 5.93	0.579 [b]	38.3 ± 4.88	36.7 ± 5.50	31.4 ± 5.46	0.634 [b]
	Median	51.7	50.2	49.3	0.923 [c]	40.7	31.8	30.4	0.550 [c]
Distance moved (cm)	Mean ± SEM	520 ± 57.8	448 ± 40.5	412 ± 65.6	0.392 [b]	400 ± 37.6	304 ± 40.5	290 ± 34.7	0.105 [b]
	Median	515	419	346	0.256 [c]	370	268	304	0.088 [c]
Cumulative distance [a] (m)	Mean ± SEM	11.6 ± 1.27	10.9 ± 1.06	10.6 ± 1.88	0.875 [b]	9.03 ± 1.35	9.03 ± 1.80	7.36 ± 1.58	0.695 [b]
	Median	11.5	11.8	11.0	0.897 [c]	9.05	6.88	5.94	0.600 [c]
Swimming velocity (cm/s)	Mean ± SEM	11.8 ± 1.58	10.5 ± 1.28	11.7 ± 1.54	0.807 [b]	11.8 ± 1.33	9.54 ± 1.22	11.6 ± 1.47	0.426 [b]
	Median	12.0	10.1	11.9	0.839 [c]	11.5	9.16	12.2	0.361 [c]

[a] Cumulative distance was calculated as total distance from the center of platform.
[b] Comparisons were conducted using ANOVA. [c] Comparisons were conducted using Kruskal-Wallis test.

Table 6. Morris water maze performance in acquisition phase in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

		Day 1				**Day 2**			
		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value	Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	8	6	6		8	6	6	
Escape latency (s)	Mean ± SEM	39.5 ± 4.74	32.8 ± 2.87	37.2 ± 3.21	0.494 [b]	34.5 ± 3.98	34.1 ± 8.46	26.5 ± 4.85	0.566 [b]
	Median	39.8	34.0	36.2	0.495 [c]	34.9	29.4	24.8	0.429 [c]
Distance moved (cm)	Mean ± SEM	431 ± 38.5	445 ± 54.9	443 ± 45.9	0.972 [b]	397 ± 44.2	416 ± 89.7	380 ± 71.7	0.939 [b]
	Median	398	448	443	0.996 [c]	361	369	344	0.945 [c]
Cumulative distance [a] (m)	Mean ± SEM	8.93 ± 1.65	7.61 ± 0.82	8.33 ± 0.96	0.780 [b]	7.47 ± 1.30	8.69 ± 2.74	5.69 ± 1.40	0.551 [b]
	Median	7.45	7.64	9.14	0.905 [c]	6.70	5.92	4.57	0.465 [c]
Swimming velocity (cm/s)	Mean ± SEM	11.4 ± 0.89	13.6 ± 1.22	12.3 ± 1.50	0.428 [b]	12.1 ± 1.20	13.0 ± 0.75	14.4 ± 0.75	0.281 [b]
	Median	10.9	13.5	12.2	0.443 [c]	11.1	12.3	14.2	0.136 [c]

		Day 3				**Day 4**			
		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value	Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	8	6	6		8	6	6	
Escape latency (s)	Mean ± SEM	44.4 ± 5.19	38.1 ± 6.08	24.7 ± 6.35	0.076 [b]	36.0 ± 6.08	26.7 ± 7.08	14.7 ± 2.69	0.054 [b]
	Median	46.2	42.8	20.2	0.099 [c]	35.4 *	23.2	14.2	0.036 [c]
Distance moved (cm)	Mean ± SEM	598 ± 58.6	517 ± 64.6	416 ± 124	0.315 [b]	425 ± 48.0	324 ± 80.7	229 ± 41.3	0.076 [b]
	Median	603	566	297	0.259 [c]	440	237	252	0.081 [c]
Cumulative distance [a] (m)	Mean ± SEM	10.5 ± 1.57	9.00 ± 1.48	5.60 ± 1.72	0.118 [b]	8.74 ± 1.79 #	5.66 ± 1.78	2.69 ± 0.72	0.046 [b]
	Median	10.6	10.3	4.35	0.131 [c]	8.64 *	4.29	2.18	0.029 [c]
Swimming velocity (cm/s)	Mean ± SEM	14.1 ± 1.41	14.2 ± 1.15	16.2 ± 0.93	0.432 [b]	13.2 ± 1.44	12.5 ± 1.56	15.7 ± 1.10	0.299 [b]
	Median	13.7	13.8	16.9	0.256 [c]	13.2	10.9	15.6	0.201 [c]

[a] Cumulative distance was calculated as total distance from the center of platform.

[b] Comparisons were conducted using ANOVA. [c] Comparisons were conducted using Kruskal-Wallis test.

Significant differences from control group in post hoc test, Scheffe test

* Significant differences from control group in post hoc test, Dunn's test

Table 7. Morris water maze performance in probe test in the experiment 1 in the first part of study

		Total 750 μg DEPs	Control	P-value
	N	8	8	
Time spent in platform quadrant (%)	Mean ± SEM	27.4 ± 4.78	24.6 ± 2.84	0.627 [b]
	Median	25.4	26.1	0.958 [c]
Quadrant area crossing (times)	Mean ± SEM	3.47 ± 0.53	4.28 ± 0.53	0.230 [b]
	Median	3.25	4.88	0.343 [c]
Average proximity [a] (cm)	Mean ± SEM	41.9 ± 2.07	43.9 ± 2.11	0.509 [b]
	Median	41.8	43.5	0.564 [c]
Swimming velocity (cm/s)	Mean ± SEM	11.9 ± 1.08	14.3 ± 1.34	0.179 [b]
	Median	12.4	15.0	0.189 [c]

[a] Average proximity was calculated as average distance from the center of platform quadrant.
[b] Comparisons were conducted using Student's t-test. [c] Comparisons were conducted using Wilcoxon rank sum test.

Table 8. Morris water maze performance in probe test in the experiment 2 in the first part of study

		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	11	12	12	
Time spent in platform quadrant (%)	Mean ± SEM	37.0 ± 5.22	33.5 ± 4.45	28.6 ± 3.87	0.433 [b]
	Median	40.9	31.4	24.2	0.437 [c]
Time spent in platform area (%)	Mean ± SEM	3.88 ± 0.99	3.46 ± 1.19	3.49 ± 0.99	0.956 [b]
	Median	3.22	1.52	2.03	0.724 [c]
Quadrant area crossing (times)	Mean ± SEM	3.11 ± 0.53	3.15 ± 0.63	2.94 ± 0.38	0.954 [b]
	Median	3.00	2.63	3.00	0.981 [c]
Platform area crossing (times)	Mean ± SEM	2.41 ± 0.51	1.81 ± 0.51	1.81 ± 0.49	0.637 [b]
	Median	2.25	0.88	1.00	0.520 [c]
Average proximity [a] (cm)	Mean ± SEM	38.2 ± 2.43	39.3 ± 2.40	40.5 ± 1.83	0.759 [b]
	Median	36.7	39.0	41.9	0.652 [c]
Swimming velocity (cm/s)	Mean ± SEM	9.13 ± 1.36	9.78 ± 1.47	10.7 ± 1.57	0.759 [b]
	Median	10.4	8.82	10.0	0.839 [c]

[a] Cumulative distance was calculated as total distance from the center of platform.
[b] Comparisons were conducted using ANOVA. [c] Comparisons were conducted using Kruskal-Wallis test.

Table 9. Morris water maze performance in acquisition phase in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

		Total 300 μg DEPs	Total 100 μg DEPs	Control	P-value
	N	6	5	6	
Time spent in platform quadrant (%)	Mean ± SEM	36.3 ± 3.82	35.7 ± 4.36	29.1 ± 6.53	0.546 [b]
	Median	39.8	30.5	26.8	0.527 [c]
Time spent in platform area (%)	Mean ± SEM	5.83 ± 1.28	6.69 ± 2.18	3.66 ± 1.04	0.363 [b]
	Median	5.64	7.18	4.11	0.273 [c]
Quadrant area crossing (times)	Mean ± SEM	4.29 ± 0.52	5.40 ± 0.47	3.75 ± 0.43	0.086 [b]
	Median	4.13	5.00	3.50	0.098 [c]
Platform area crossing (times)	Mean ± SEM	3.46 ± 0.59	3.50 ± 0.66	2.83 ± 0.76	0.739 [b]
	Median	3.88	3.5	3.13	0.779 [c]
Average proximity [a] (cm)	Mean ± SEM	34.8 ± 1.57	33.9 ± 3.32	40.3 ± 2.88	0.209 [b]
	Median	35.2	37.6	39.3	0.371 [c]
Swimming velocity (cm/s)	Mean ± SEM	12.6 ± 1.04	14.5 ± 1.77	15.2 ± 1.46	0.407 [b]
	Median	11.7	14.8	14.9	0.454 [c]

[a] Cumulative distance was calculated as total distance from the center of platform.
[b] Comparisons were conducted using ANOVA. [c] Comparisons were conducted using Kruskal-Wallis test.

Table 10. $PM_{2.5}$ and PM_1 exposure monitoring

Time (week)	1	2	3	4	5	6	7	8	9	10	11	12	12-week mean
Manual sampling ($PM_{2.5}$, μg/m^3)	15.0	12.7	11.4	14.1	16.6	4.7	10.5	16.8	11.4	9.5	6.2	13.8	11.9
Manual sampling (PM_1, μg/m^3)	13.6	9.9	10.1	11.3	13.5	3.8	8.5	14.6	9.7	7.5	5.2	10.8	9.9

Table 11. Chemical composition of exposed $PM_{2.5}$ analyzed by XRF

	Mean (ng/m^3)	Median (ng/m^3)	Min (ng/m^3)	Max (ng/m^3)	BDL or BBK (%)	Percentage of mean $PM_{2.5}$ (%)
Manual sampling						
$PM_{2.5}$	11886	12063	4685	16850		100%
XRF analysis						
Mg	15.55	BDL or BBK	BDL or BBK	64.26	91.67%	0.13%
Al	41.86	BDL or BBK	BDL or BBK	106.42	66.67%	0.35%
Si	106.39	96.17	16.17	247.55	0.00%	0.90%
S	1935.81	2015.08	860.86	2627.29	0.00%	16.29%
K	198.84	195.67	47.20	409.98	0.00%	1.67%
Ca	22.43	20.83	10.21	42.43	0.00%	0.19%
Ti	3.47	3.11	1.21	6.23	0.00%	0.03%
V	4.15	3.35	1.85	11.30	0.00%	0.03%
Cr	0.66	BDL or BBK	BDL or BBK	1.28	91.67%	0.01%
Mn	6.71	7.30	2.18	11.12	0.00%	0.06%
Fe	68.55	75.78	28.64	93.52	0.00%	0.58%
Ni	1.84	1.75	BDL or BBK	4.88	16.67%	0.02%
Cu	5.30	4.50	BDL or BBK	12.87	8.33%	0.04%
Zn	34.84	38.90	12.33	53.42	0.00%	0.29%
Ba	5.74	BDL or BBK	BDL or BBK	17.12	91.67%	0.05%
Pb	19.28	18.95	BDL or BBK	54.60	8.33%	0.16%

Table 12. Chemical composition of exposed $PM_{2.5}$ analyzed by IC

	Mean (ng/m^3)	Median (ng/m^3)	Min (ng/m^3)	Max (ng/m^3)	<LDL (%)	Percentage of mean $PM_{2.5}$ (%)
Manual sampling						
$PM_{2.5}$	11886	12063	4685	16850		100%
IC analysis						
Na^+	66.50	71.73	44.11	83.33	0.00%	0.56%
NH_4^+	1830.17	1891.71	717.67	2559.16	0.00%	15.40%
K+	217.55	217.55	57.56	407.74	0.00%	1.83%
Mg^{2+}	6.28	5.00	0.62	21.70	0.00%	0.05%
Ca^{2+}	13.05	14.15	< LDL	26.22	8.33%	0.11%
Cl^-	12.04	10.62	7.42	24.68	0.00%	0.10%
NO_3^-	45.31	46.20	18.07	83.74	0.00%	0.38%
SO_4^{2-}	4673.66	4923.04	2129.12	6641.39	0.00%	39.32%

Table 13. Morris water maze performance in acquisition phase in the second part of study

		Day 1			Day 2		
		$PM_{2.5}$ exposure	Filtered-air control	P-value	$PM_{2.5}$ exposure	Filtered-air control	P-value
	N	8	7		8	7	
Escape latency (s)	Mean ± SEM	60.0 ± 0.00	52.2 ± 3.89	0.094 [b]	47.9 ± 4.18	44.6 ± 4.39	0.590 [b]
	Median	60.0	60.0	0.057 [c]	51.8	47.5	0.563 [c]
Distance moved (cm)	Mean ± SEM	684 ± 59.6	747 ± 66.9	0.491 [b]	623 ± 57.1	635 ± 54.2	0.885 [b]
	Median	668	711	0.524 [c]	652	620	0.954 [c]
Cumulative distance [a] (m)	Mean ± SEM	15.1 ± 0.49	13.5 ± 1.15	0.190 [b]	13.2 ± 1.18	11.0 ± 1.35	0.248 [b]
	Median	14.7	13.6	0.272 [c]	13.9	11.8	0.224 [c]
Swimming velocity (cm/s)	Mean ± SEM	11.3 ± 0.99 #	14.4 ± 0.79	0.036 [b]	13.5 ± 1.44	14.5 ± 0.85	0.577 [b]
	Median	11.1	14.9	0.093 [c]	12.9	14.1	0.385 [c]

		Day 3			Day 4		
		$PM_{2.5}$ exposure	Filtered-air control	P-value	$PM_{2.5}$ exposure	Filtered-air control	P-value
	N	8	7		8	7	
Escape latency (s)	Mean ± SEM	45.7 ± 3.63 #	29.0 ± 4.35	0.011 [b]	43.3 ± 4.22	35.0 ± 5.33	0.239 [b]
	Median	45.1 *	27.6	0.018 [c]	46.0	33.0	0.203 [c]
Distance moved (cm)	Mean ± SEM	551 ± 34.8 #	395 ± 63.2	0.044 [b]	531 ± 43.2	541 ± 86.2	0.919 [b]
	Median	564	296	0.073 [c]	539	476	0.685 [c]
Cumulative distance [a] (m)	Mean ± SEM	12.3 ± 1.26 #	7.03 ± 1.30	0.013 [b]	11.1 ± 1.33	8.48 ± 1.62	0.237 [b]
	Median	11.5 *	8.17	0.024 [c]	10.5	7.84	0.183 [c]
Swimming velocity (cm/s)	Mean ± SEM	12.4 ± 1.02	14.6 ± 2.04	0.327 [b]	12.7 ± 1.05	15.9 ± 1.38	0.087 [b]
	Median	12.1	16.2	0.272 [c]	11.9	17.6	0.183 [c]

[a] Cumulative distance was calculated as total distance from the center of platform.

[b] Comparisons were conducted using Student's t-test. [c] Comparisons were conducted using Wilcoxon rank sum test.

Significant differences from control group in Student's t-test

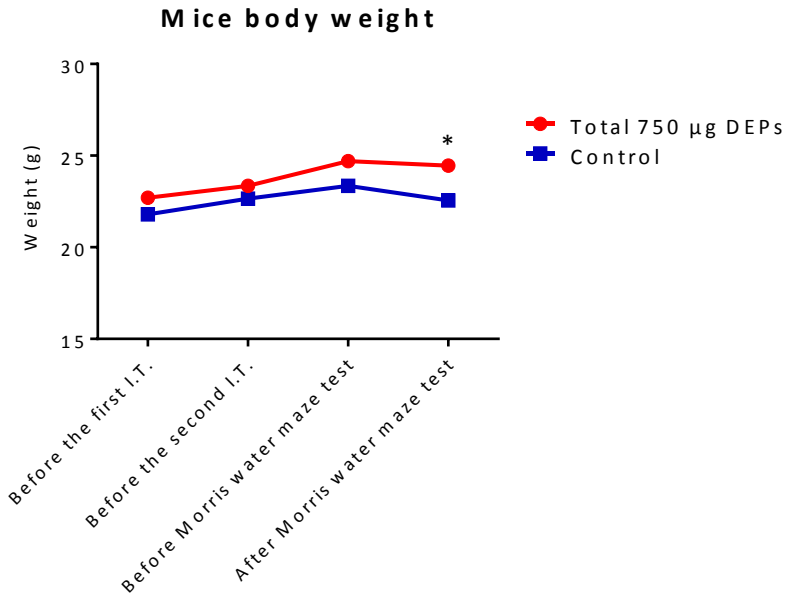
* Significant differences from control group in Wilcoxon rank sum test

Table 14. Morris water maze performance in probe test in the second part of study

		$PM_{2.5}$ exposure	Filtered-air control	P-value
	N	8	7	
Time spent in platform quadrant (%)	Mean ± SEM	26.4 ± 4.99	27.0 ± 2.61	0.929 [b]
	Median	26.1	27.0	0.954 [c]
Time spent in platform area (%)	Mean ± SEM	1.52 ± 0.48	2.56 ± 0.60	0.196 [b]
	Median	0.96	2.70	0.272 [c]
Quadrant area crossing (times)	Mean ± SEM	3.41 ± 0.63	3.58 ± 0.52	0.834 [b]
	Median	3.88	3.33	0.908 [c]
Platform area crossing (times)	Mean ± SEM	1.34 ± 0.38	1.51 ± 0.34	0.749 [b]
	Median	0.88	1.50	0.816 [c]
Average proximity [a] (cm)	Mean ± SEM	45.5 ± 2.93	40.9 ± 2.15	0.242 [b]
	Median	42.8	41.1	0.385 [c]
Swimming velocity (cm/s)	Mean ± SEM	12.0 ± 1.36	11.7 ± 1.91	0.881 [b]
	Median	12.5	10.8	1.000 [c]

[a] Average proximity was calculated as average distance from the center of platform.
[b] Comparisons were conducted using Student's t-test. [c] Comparisons were conducted using Wilcoxon rank sum test.

(A)



(B)

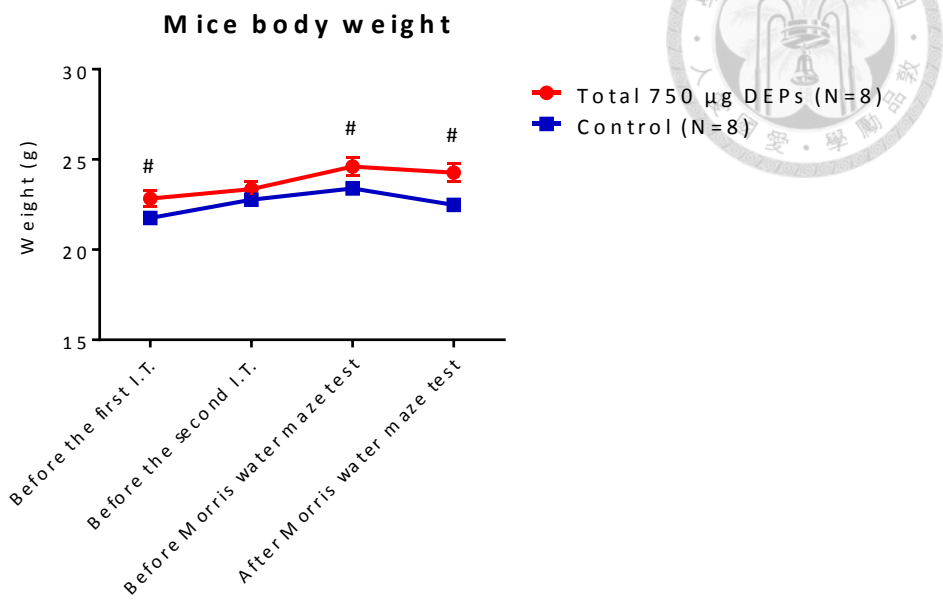
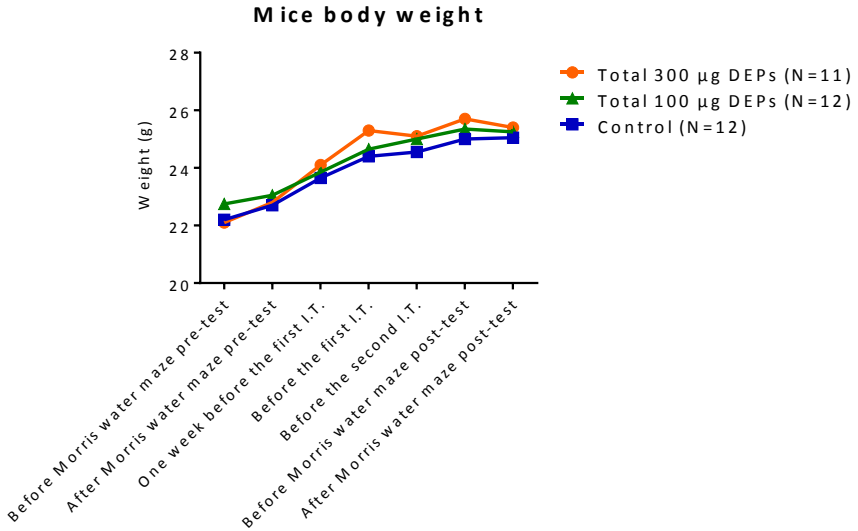


Figure 1. Mice body weight through the experiment 1 in the first part of study

Body weight of mice was presented in median (A) and mean ± SEM (B).
* Significant differences from control group in Wilcoxon rank sum test
# Significant differences from control group in Student's t-test

(A)



(B)

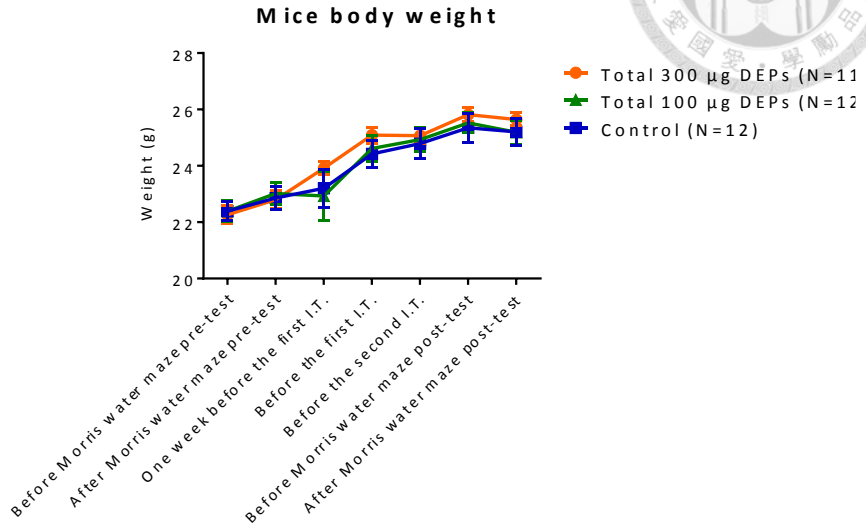


Figure 2. Mice body weight through the experiment 2 in the first part of study

Body weight of mice was presented in median (A) and mean ± SEM (B).

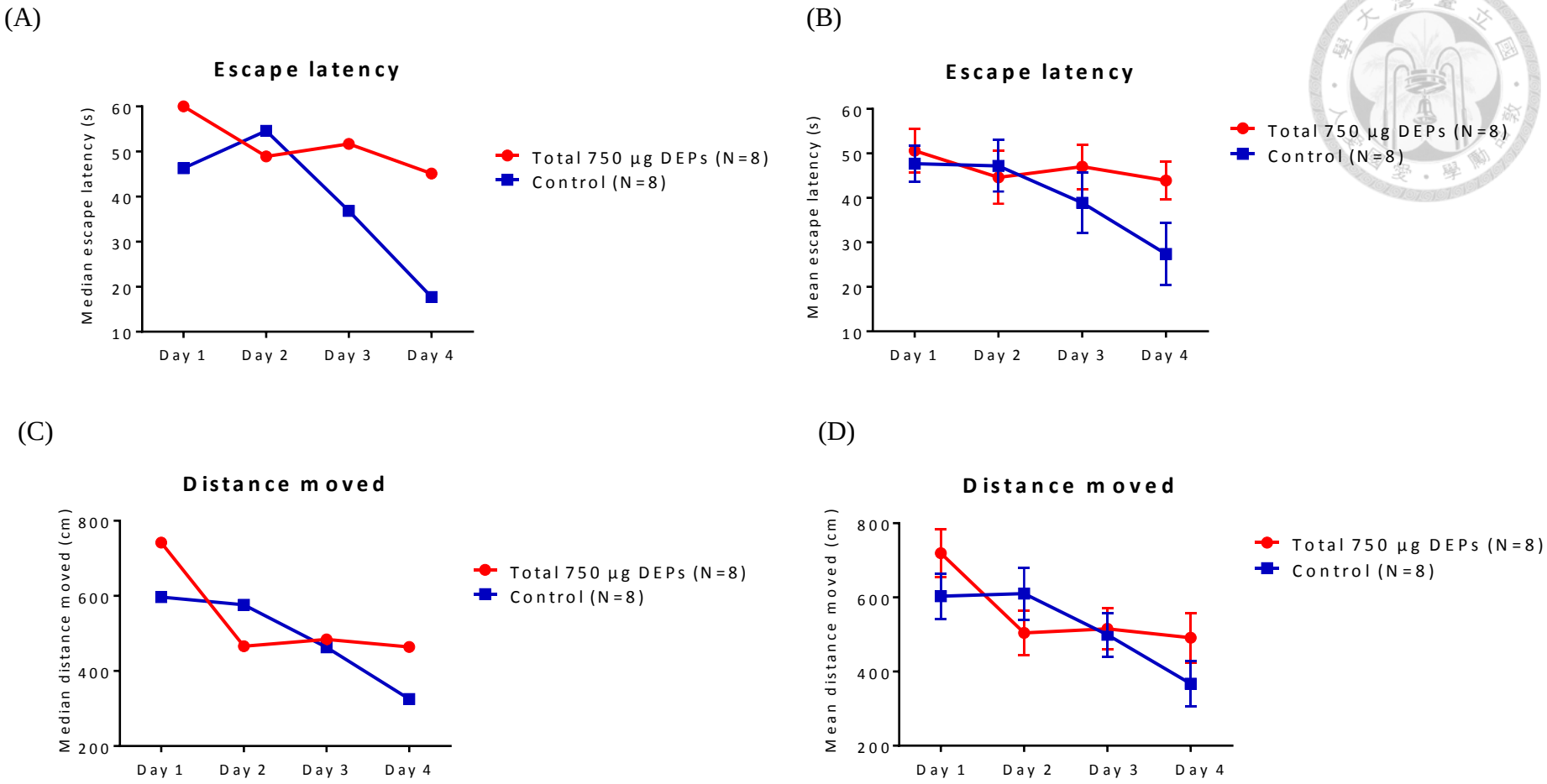


Figure 3. Morris water maze performance (escape latency and distance moved) in acquisition phase in the experiment 1 in the first part of study

Escape latency was presented in in median (A) and mean ± SEM (B); distance moved was presented in median (C) and mean ± SEM (D)

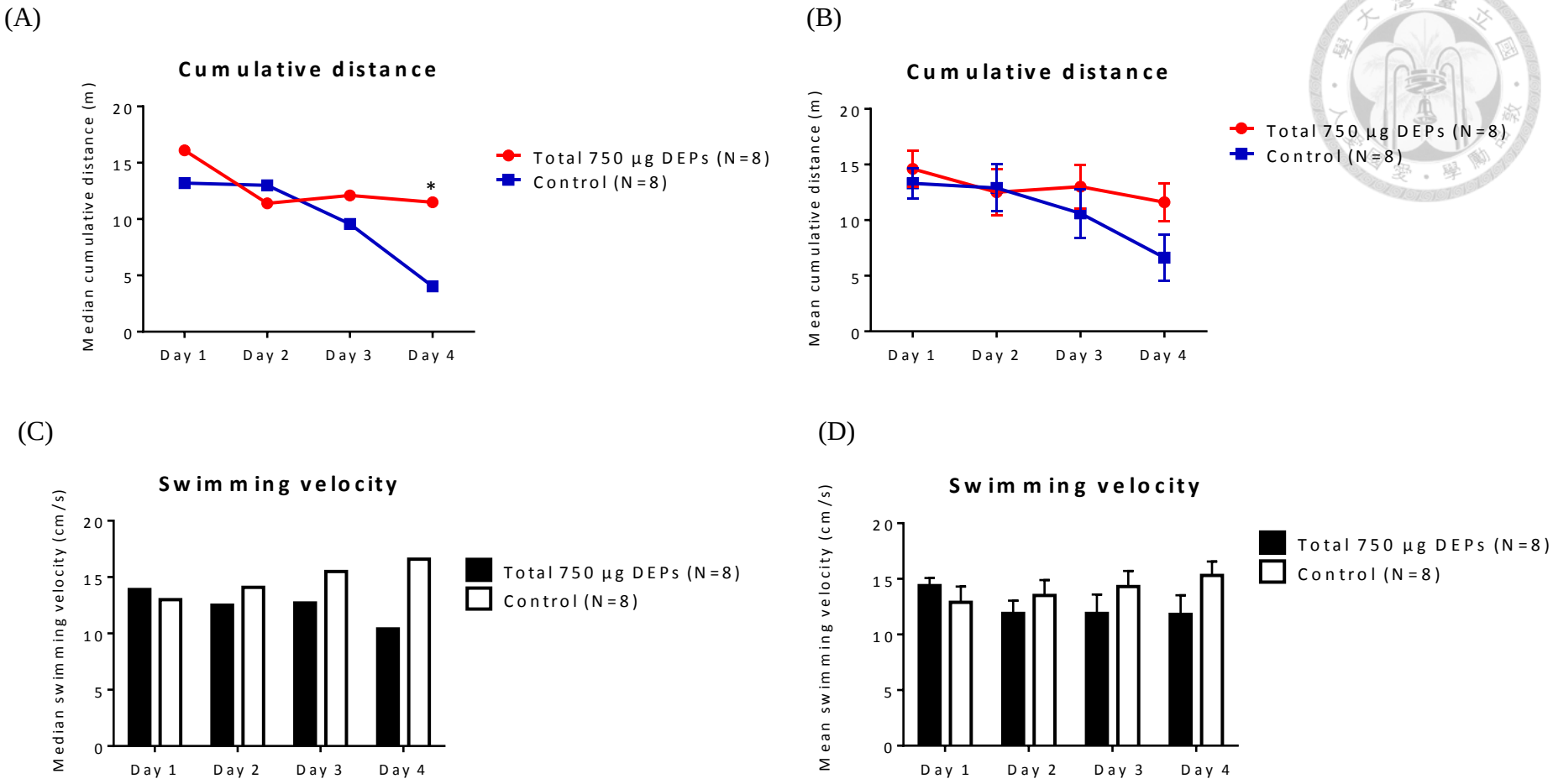


Figure 4. Morris water maze performance (cumulative distance and swimming velocity) in acquisition phase in the experiment 1 in the first part of study

Cumulative distance was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)
* Significant differences from control group in Wilcoxon rank sum test

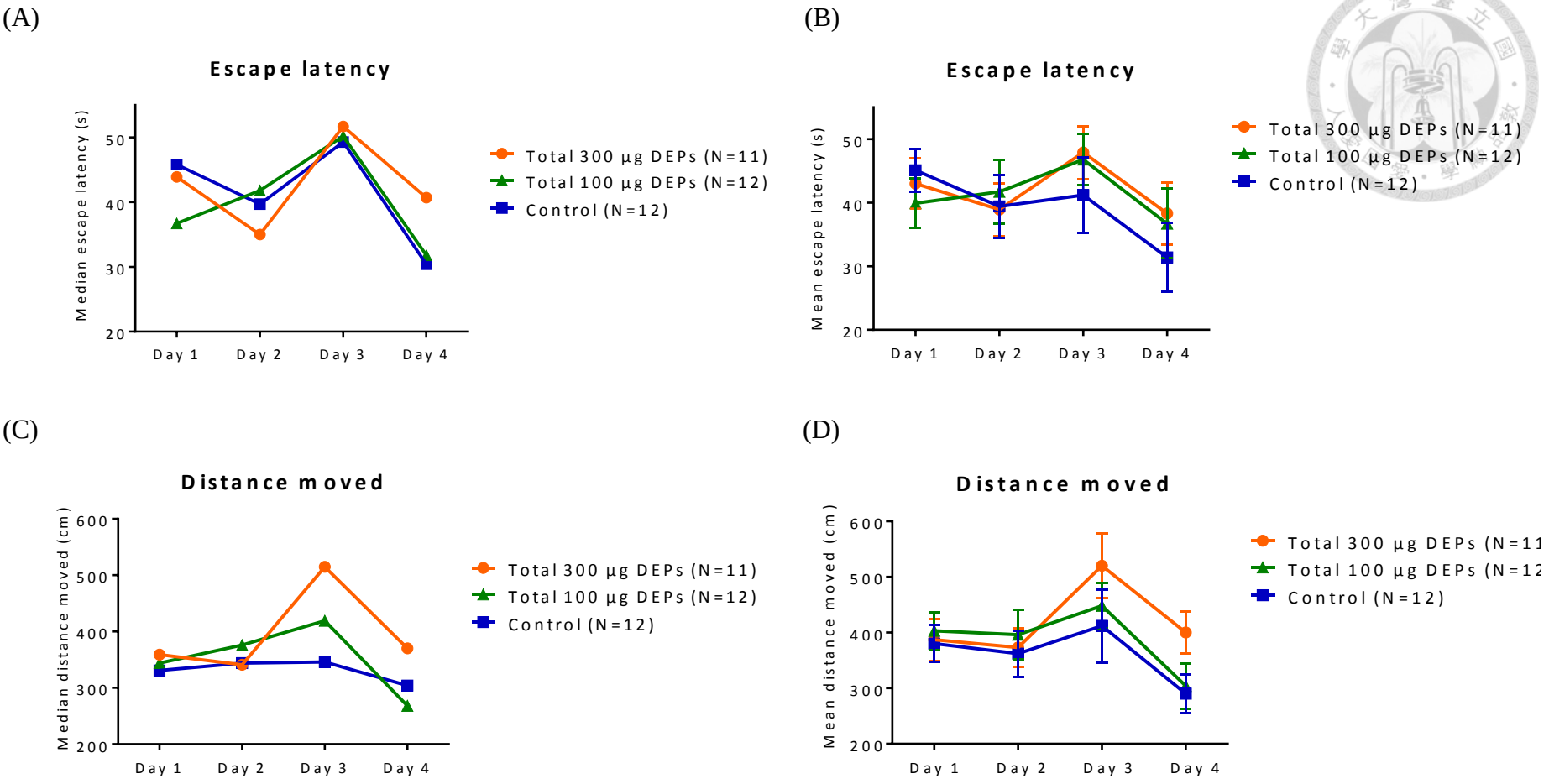


Figure 5. Morris water maze performance (escape latency and distance moved) in acquisition phase in the experiment 2 in the first part of study

Escape latency was presented in median (A) and mean ± SEM (B); distance moved was presented in median (C) and mean ± SEM (D)

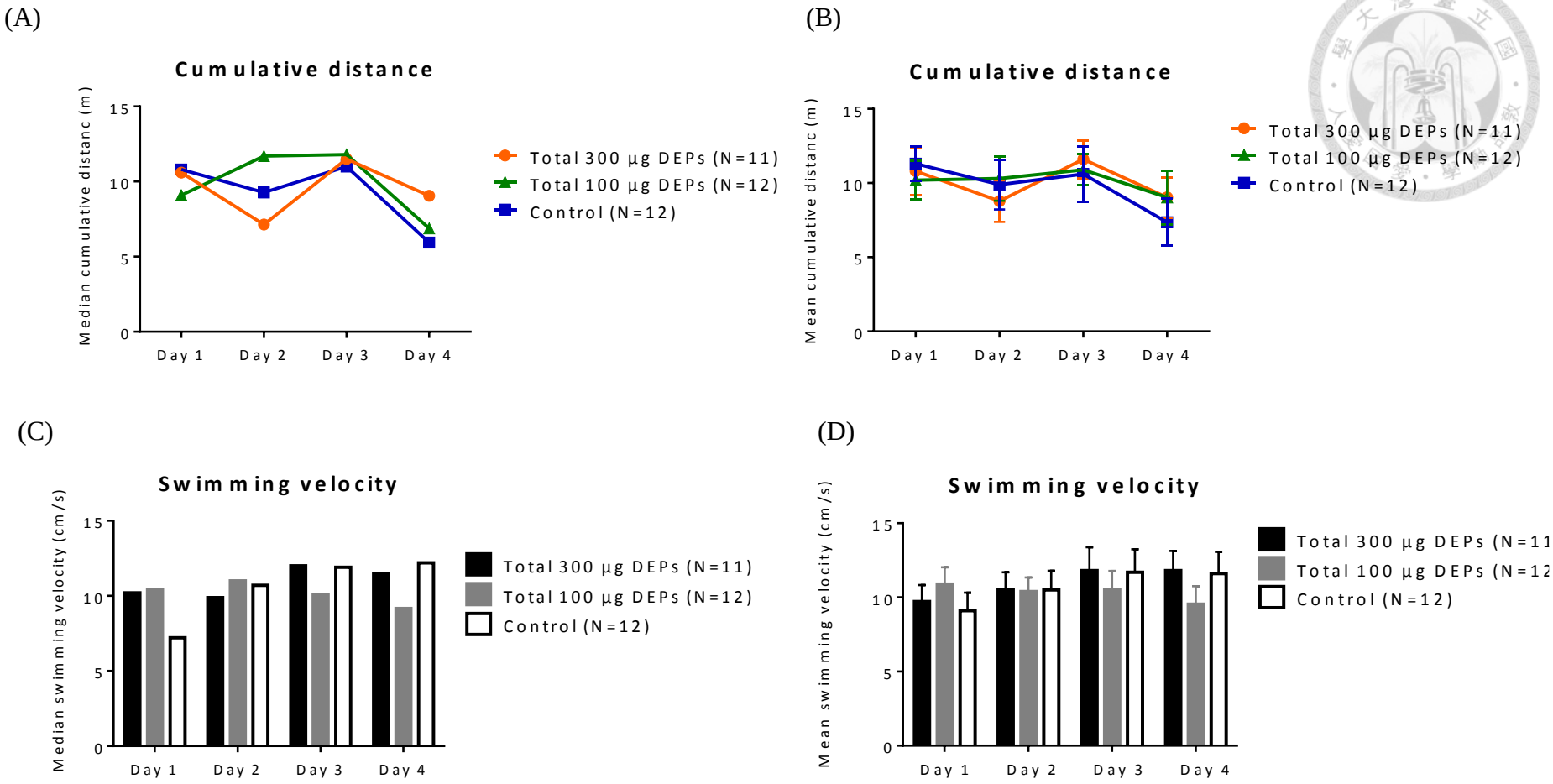


Figure 6. Morris water maze performance (cumulative distance and swimming velocity) in acquisition phase in the experiment 2 in the first part of study

Cumulative distance was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

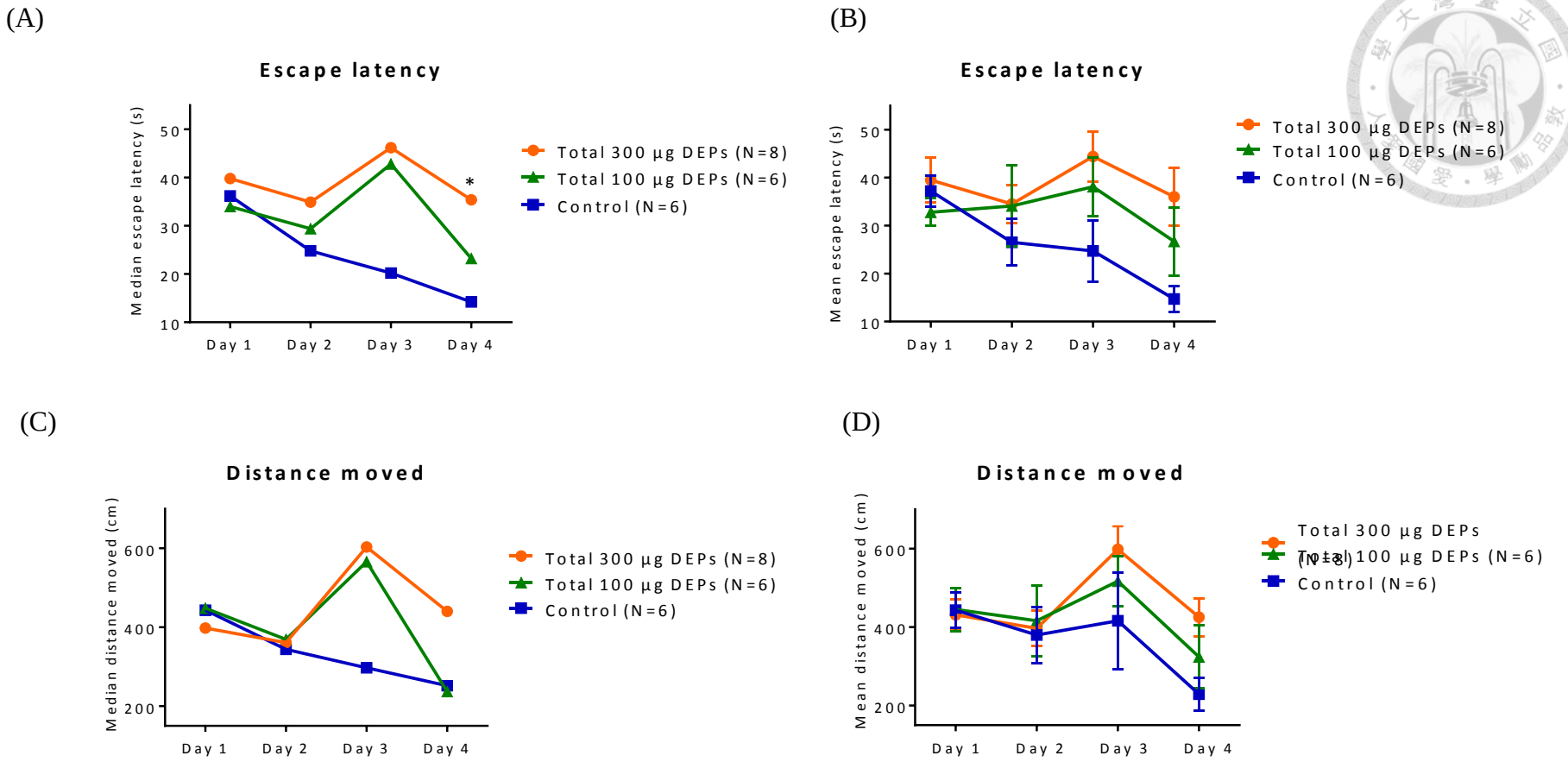


Figure 7. Morris water maze performance (escape latency and distance moved) in acquisition phase in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

Escape latency was presented in median (A) and mean ± SEM (B); distance moved was presented in median (C) and mean ± SEM (D)
* Significant differences from control group in post hoc test, Dunn's test

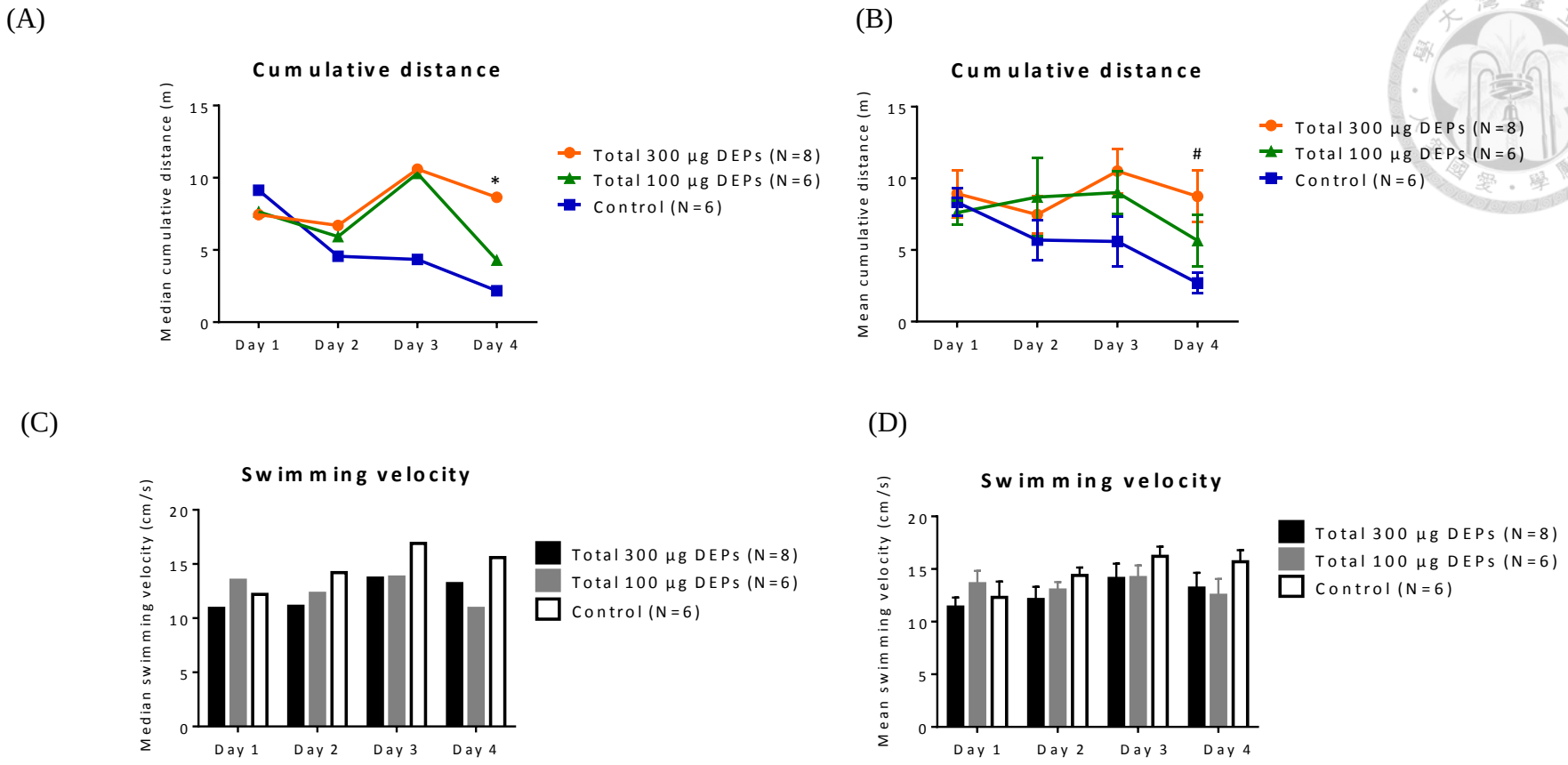


Figure 8. Morris water maze performance (cumulative distance and swimming velocity) in acquisition phase in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

Cumulative distance was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)
*Significant differences from control group in post hoc test, Dunn's test # Significant differences from control group in post hoc test, Scheffe test

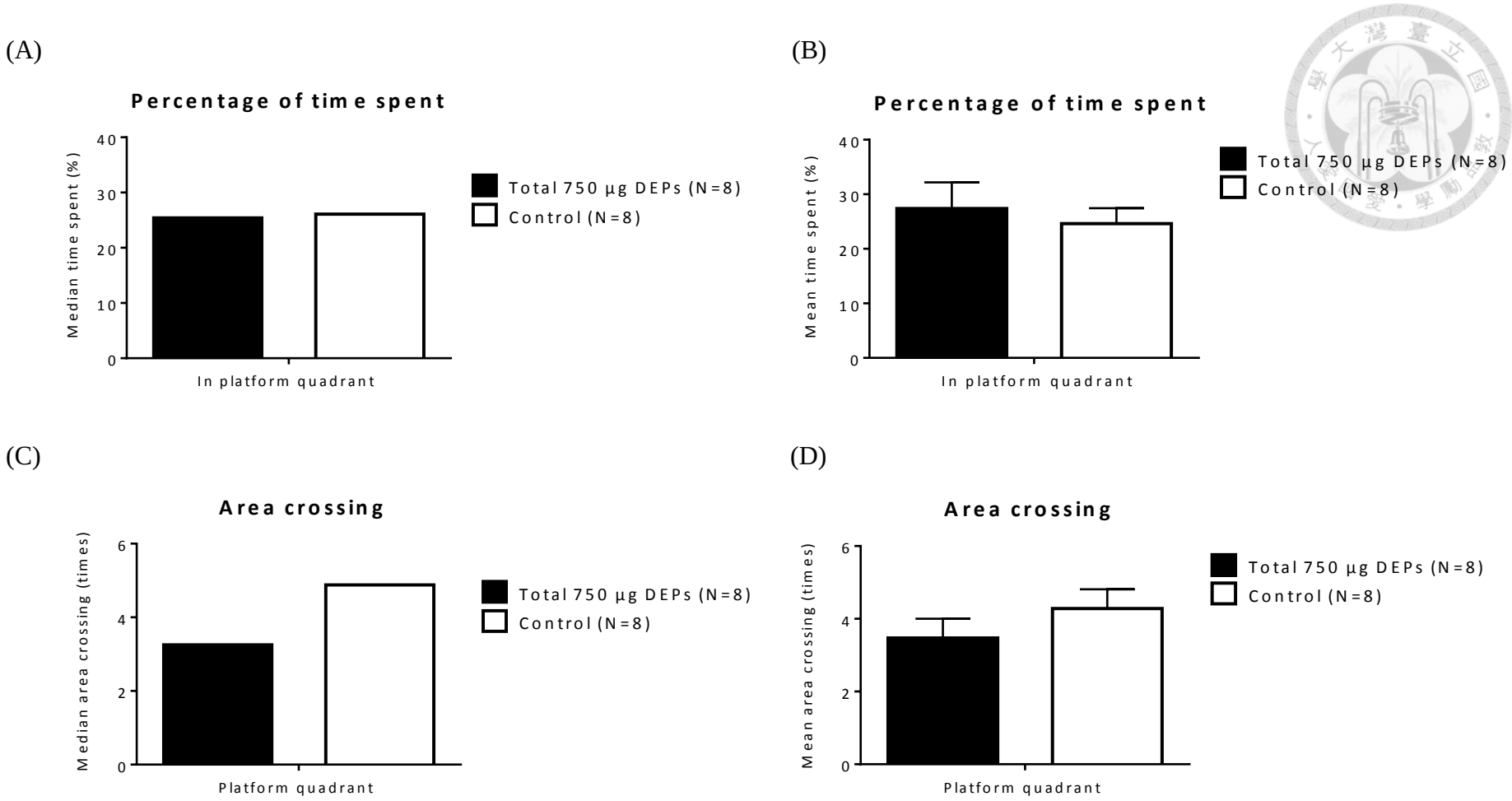


Figure 9. Morris water maze performance (percentage of time spent and area crossing) in probe test in the experiment 1 in the first part of study

Percentage of time spent was presented in median (A) and mean ± SEM (B); area crossing was presented in median (C) and mean ± SEM (D)

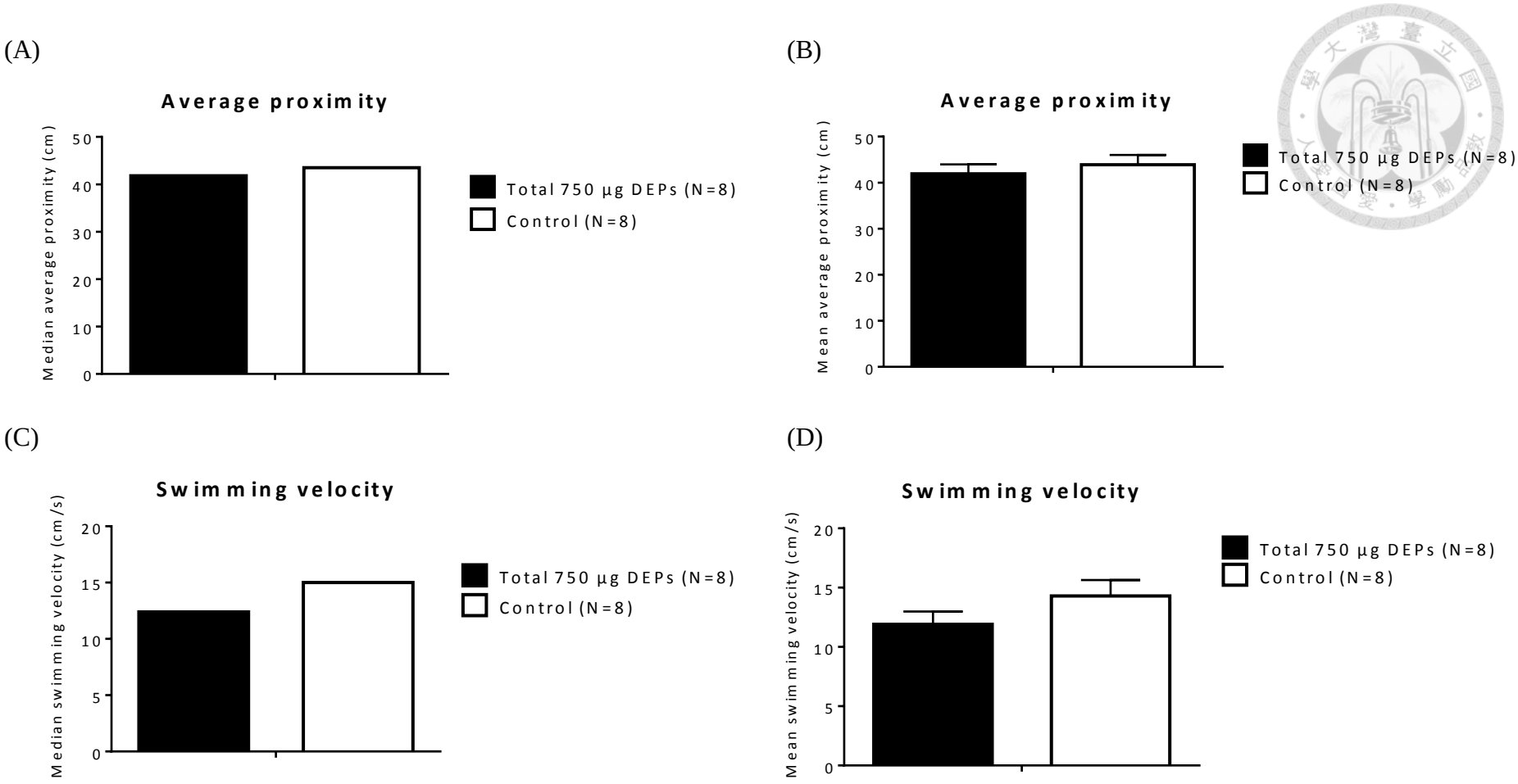


Figure 10. Morris water maze performance (average proximity and swimming velocity) in probe test in the experiment 1 in the first part of study Average proximity was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

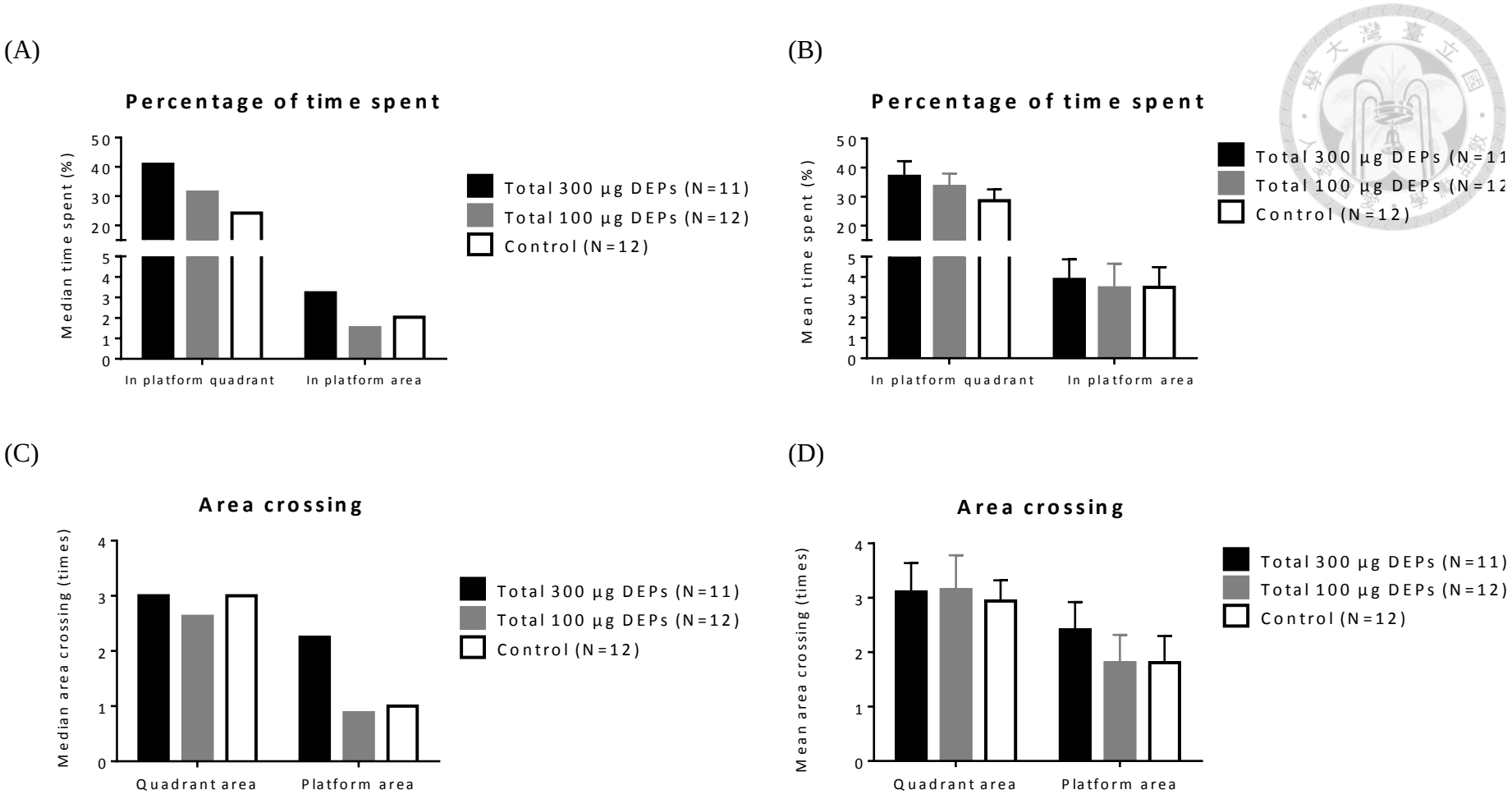


Figure 11. Morris water maze performance (percentage of time spent and area crossing) in probe test in the experiment 2 in the first part of study

Percentage of time spent was presented in median (A) and mean ± SEM (B); area crossing was presented in median (C) and mean ± SEM (D)

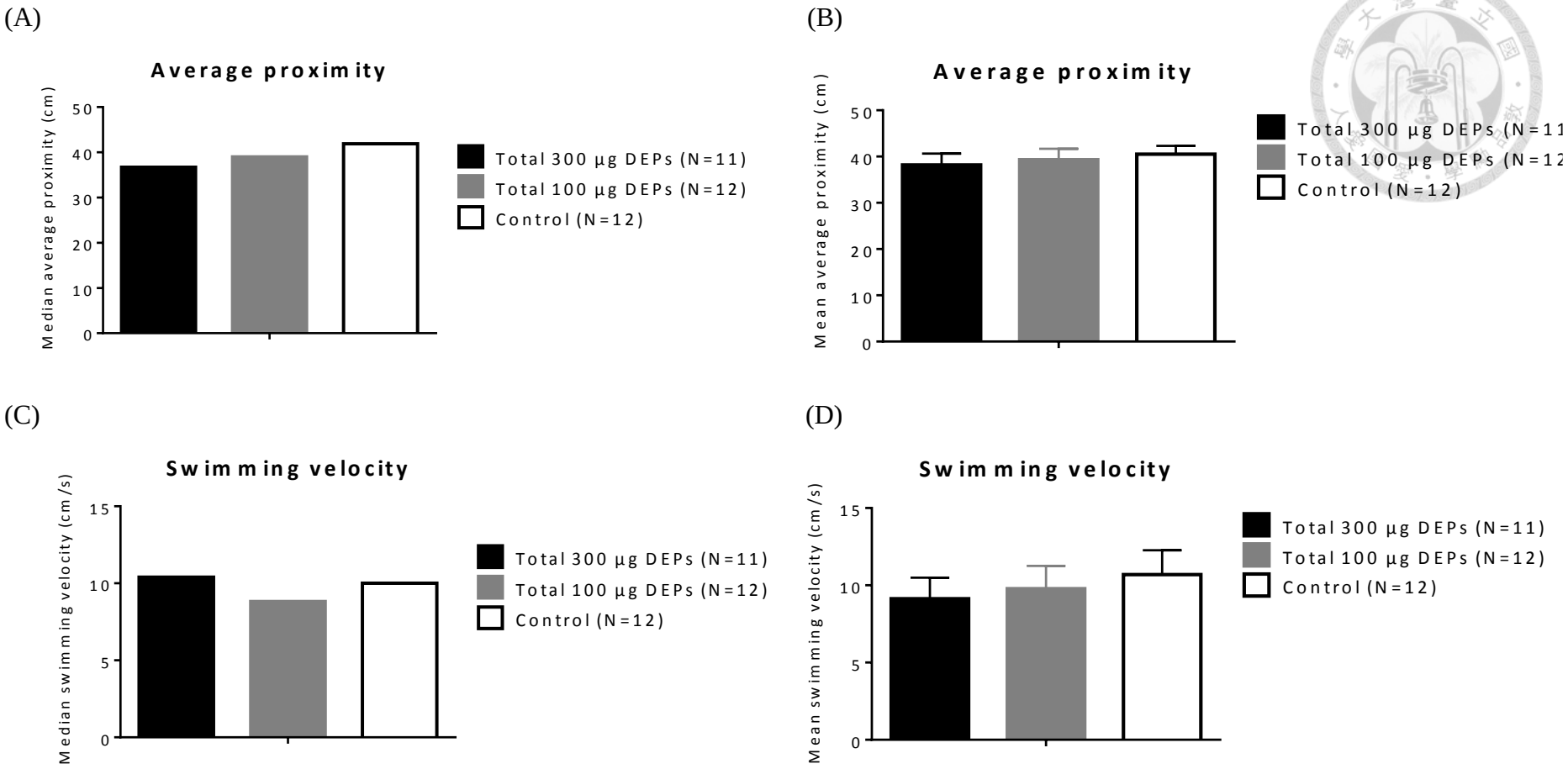
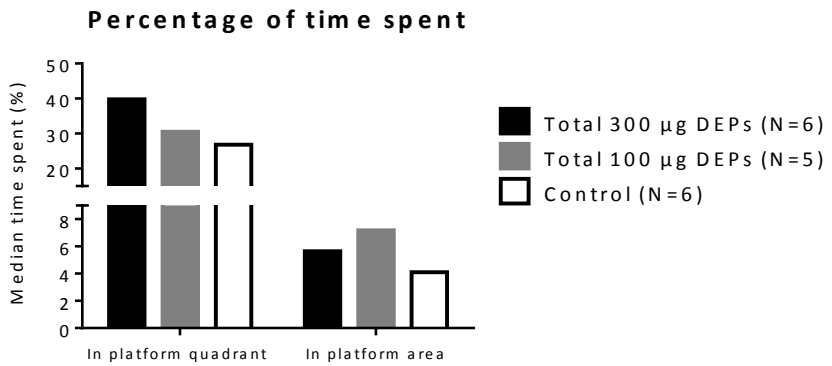
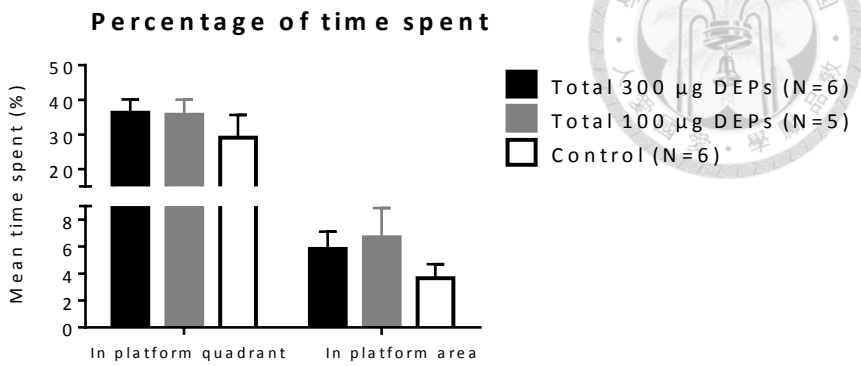


Figure 12. Morris water maze performance (average proximity and swimming velocity) in probe test in the experiment 2 in the first part of study Average proximity was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

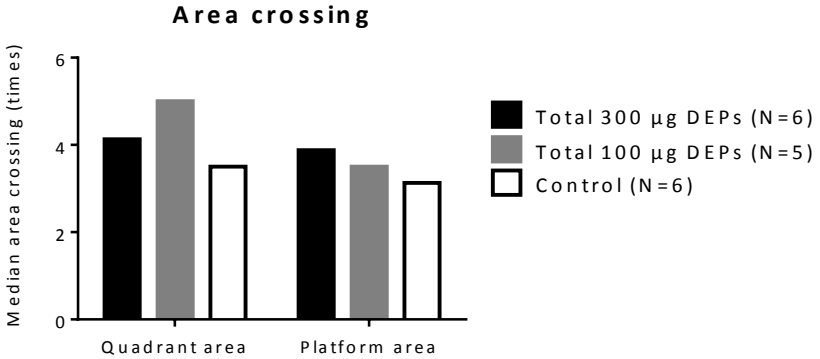
(A)



(B)



(C)



(D)

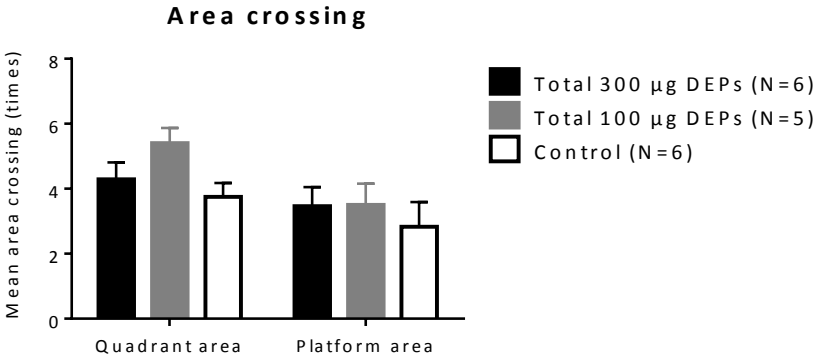


Figure 13. Morris water maze performance (percentage of time spent and area crossing) in probe test in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

Percentage of time spent was presented in median (A) and mean ± SEM (B); area crossing was presented in median (C) and mean ± SEM (D)

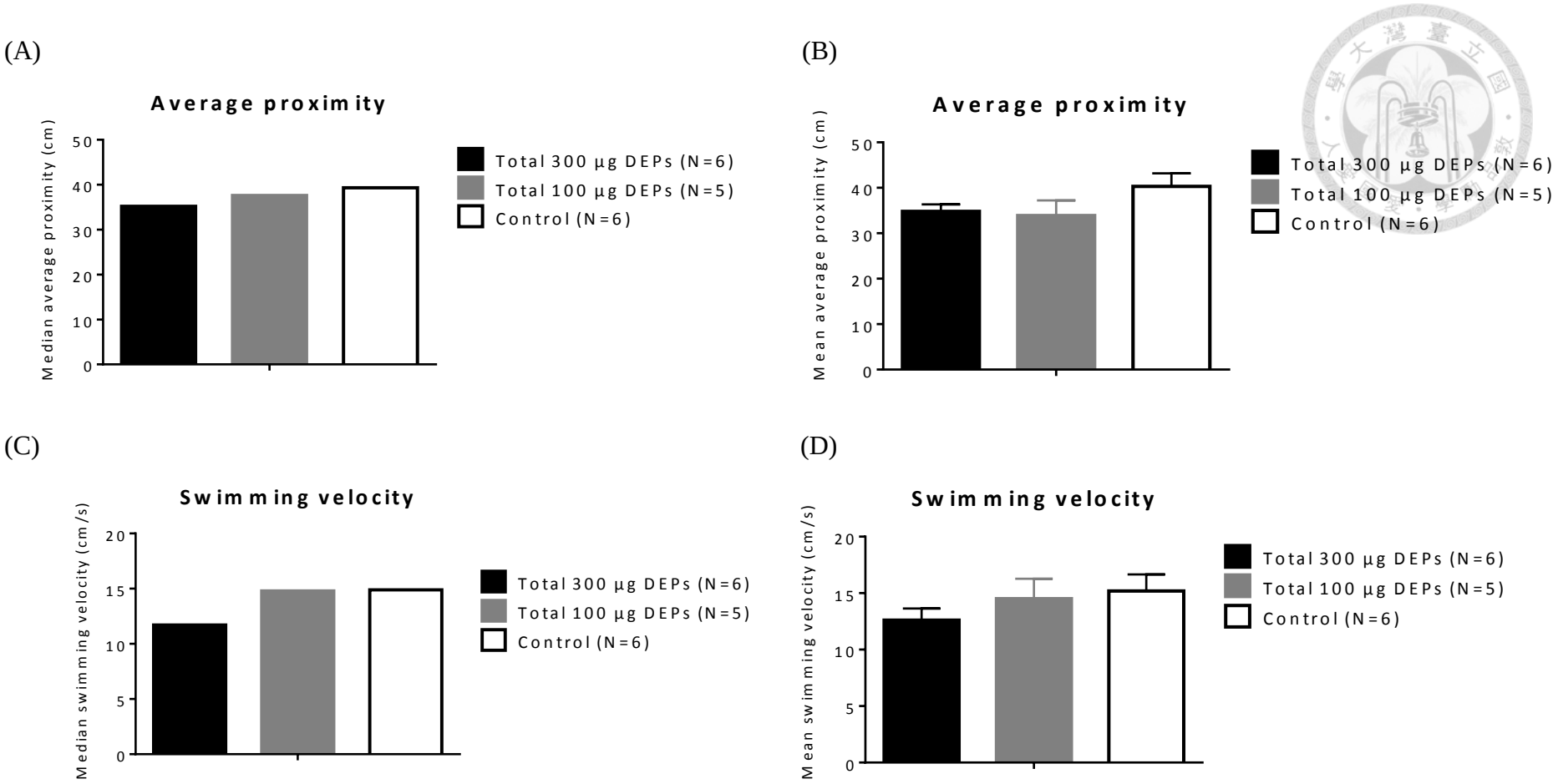
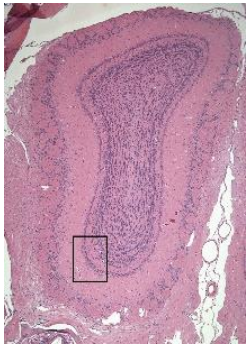
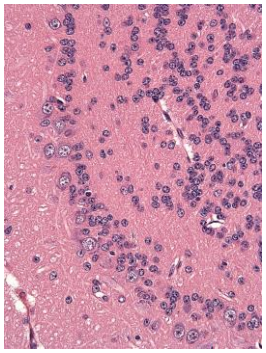
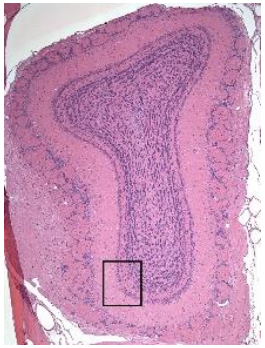
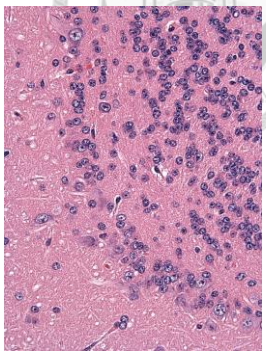


Figure 14. Morris water maze performance (average proximity and swimming velocity) in probe test in the experiment 2 in the first part of study, excluding mice with swimming velocity below 10 cm/s

Average proximity was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

(A) Total 300 μg DEPs		(B) Total 100 μg DEPs	
			
Olfactory bulb (40x)	Olfactory bulb (400x)	Olfactory bulb (40x)	Olfactory bulb (400x)

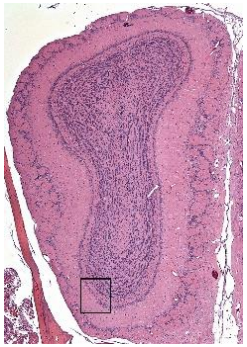
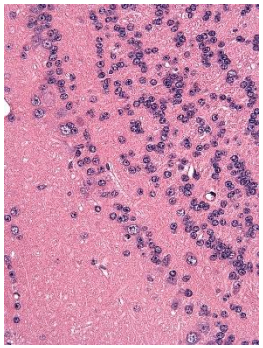
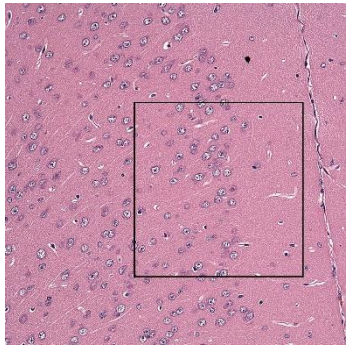
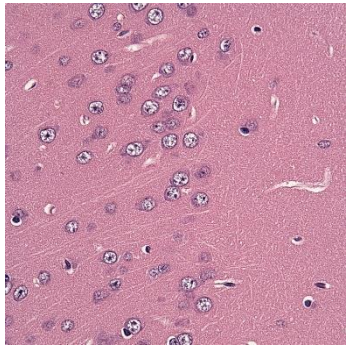
(C) Control	
	
Olfactory bulb (40x)	Olfactory bulb (400x)

Figure 15. Olfactory bulb in total 300 μg DEPs (A), total 100 μg DEPs (B) and control (C) group in the experiment 2 in the first part of study with H&E stain

(A) Total 300 μg DEPs

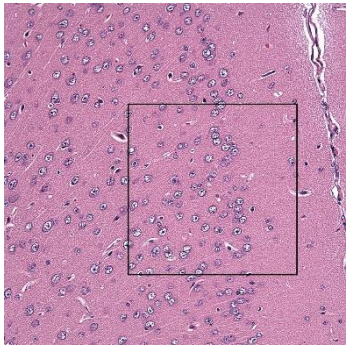


Frontal cortex (200x)

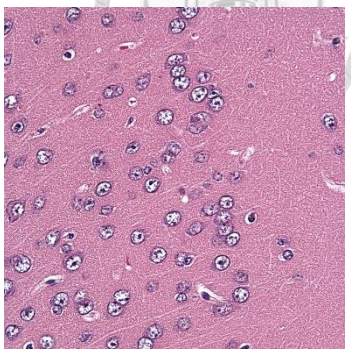


Frontal cortex (400x)

(B) Total 100 μg DEPs

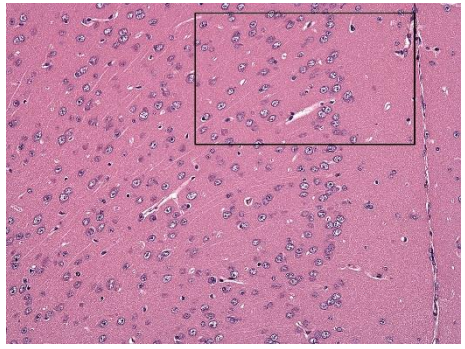


Frontal cortex (200x)

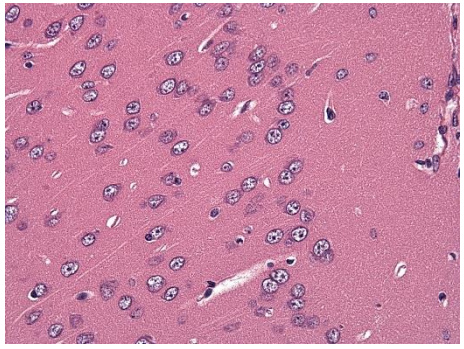


Frontal cortex (400x)

(C) Control



Frontal cortex (200x)



Frontal cortex (400x)

Figure 16. Frontal cortex in total 300 μg DEPs (A), total 100 μg DEPs (B) and control (C) group in the experiment 2 in the first part of study with H&E stain

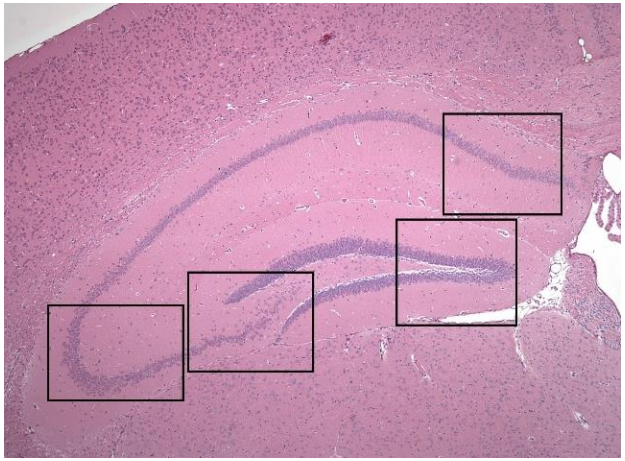
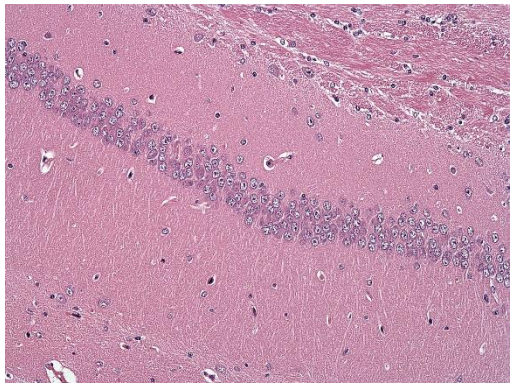
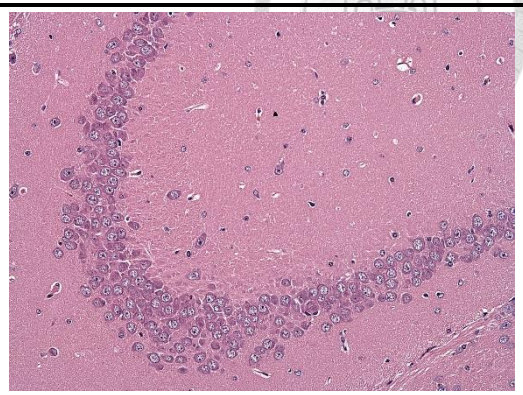
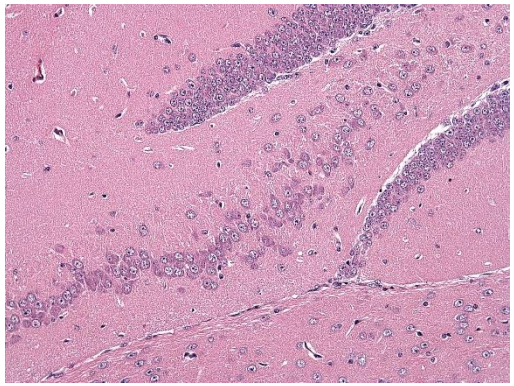
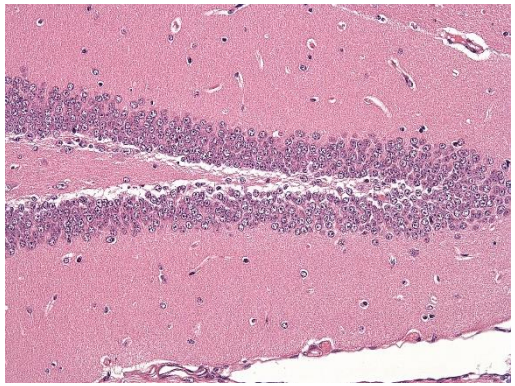
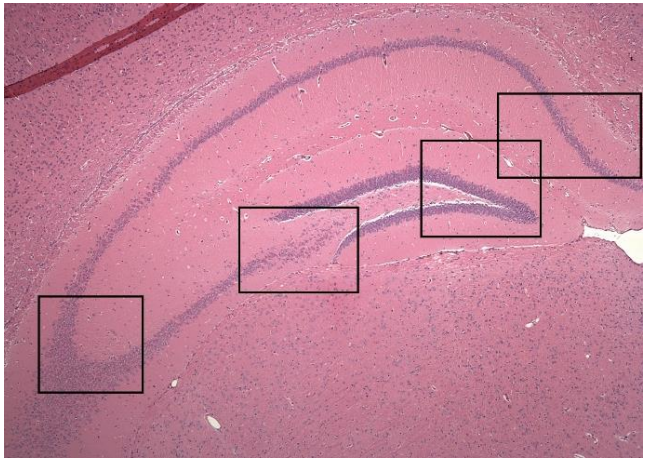
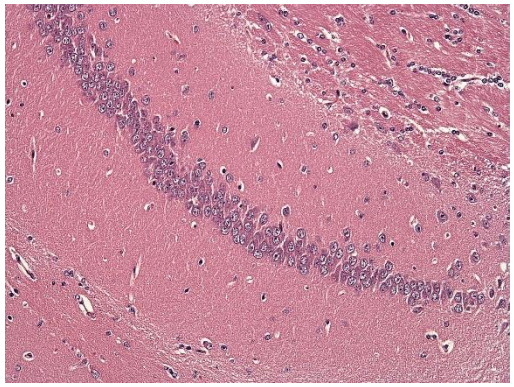
Total 300 μg DEPs		
		
Hippocampus (40x)	Hippocampus CA1 (200x)	Hippocampus CA2 (200x)
		
	Hippocampus CA3 (200x)	Hippocampus dentate gyrus (200x)

Figure 17. Hippocampus in total 300 μg DEPs group in the experiment 2 in the first part of study with H&E stain

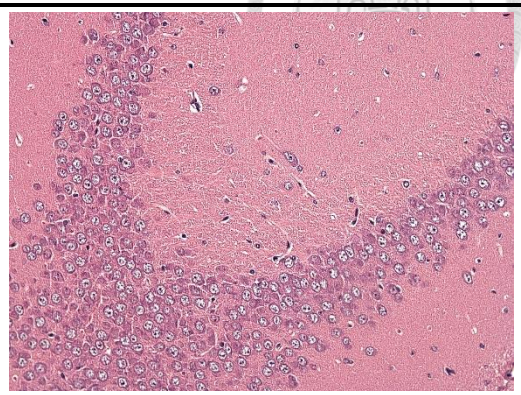
Total 100 μg DEPs



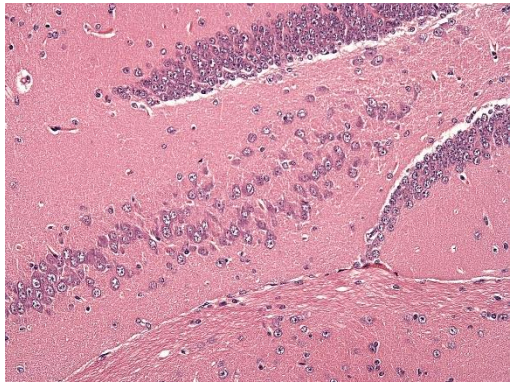
Hippocampus (40x)



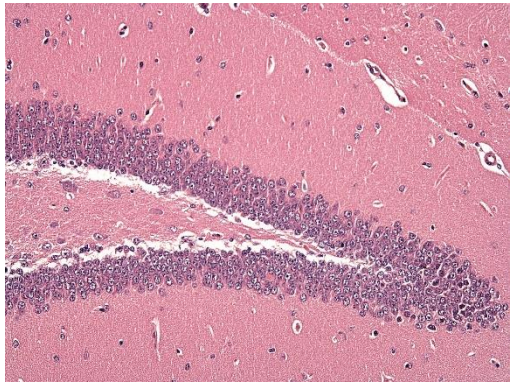
Hippocampus CA1 (200x)



Hippocampus CA2 (200x)



Hippocampus CA3 (200x)



Hippocampus dentate gyrus (200x)

Figure 18. Hippocampus in total 100 μg DEPs group in the experiment 2 in the first part of study with H&E stain

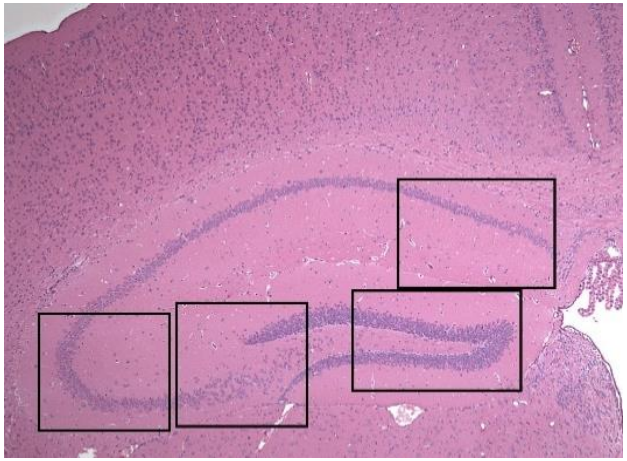
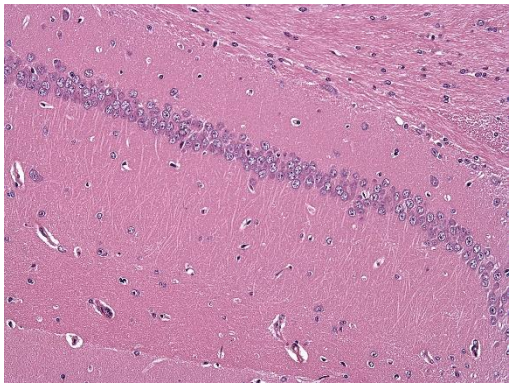
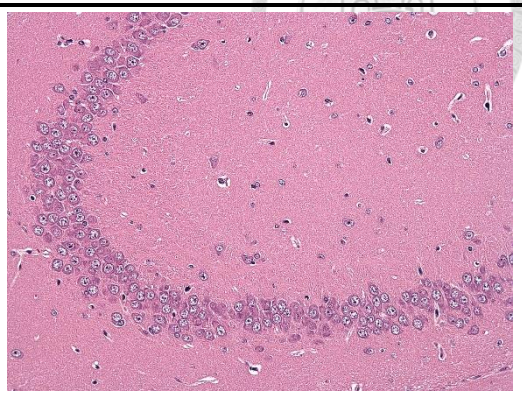
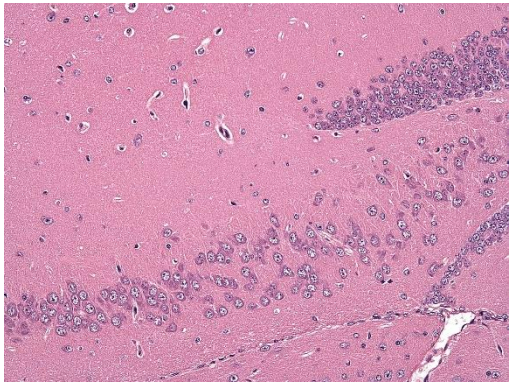
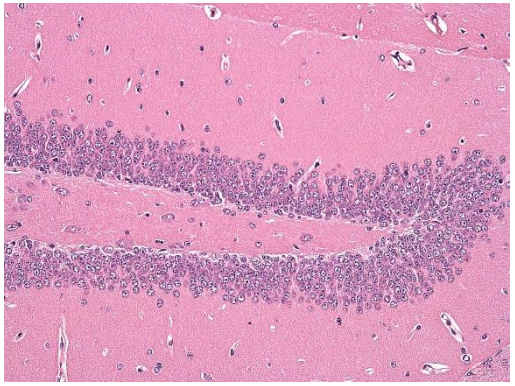
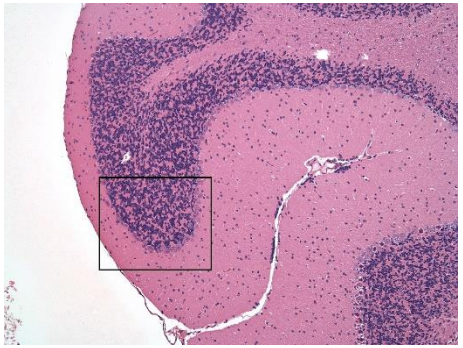
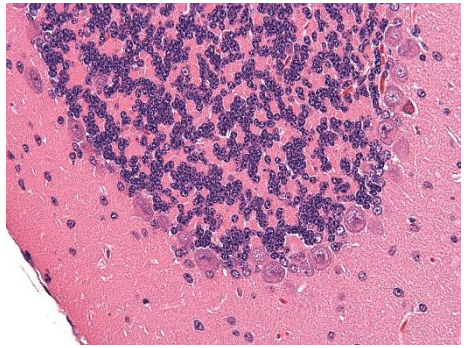
Control		
		
Hippocampus (40x)	Hippocampus CA1 (200x)	Hippocampus CA2 (200x)
		
	Hippocampus CA3 (200x)	Hippocampus dentate gyrus (200x)

Figure 19. Hippocampus in control group in the experiment 2 in the first part of study with H&E stain

(A) Total 300 μg DEPs

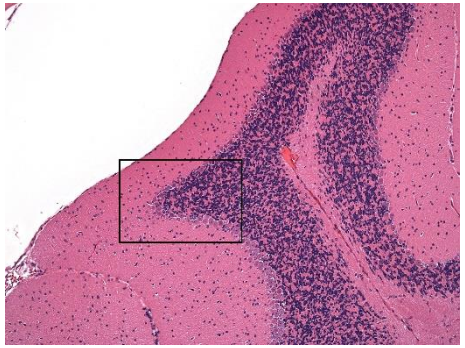


Cerebellum (100x)

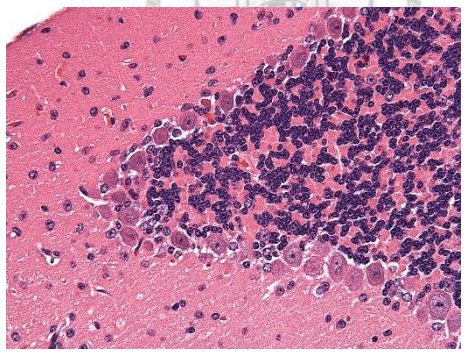


Cerebellum (400x)

(B) Total 100 μg DEPs

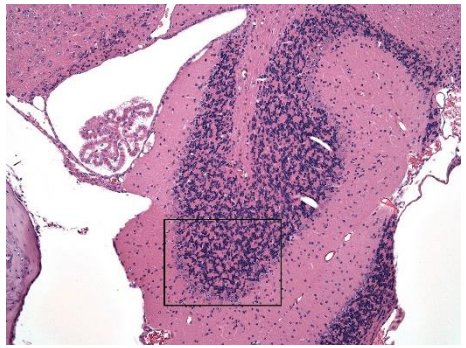


Cerebellum (100x)

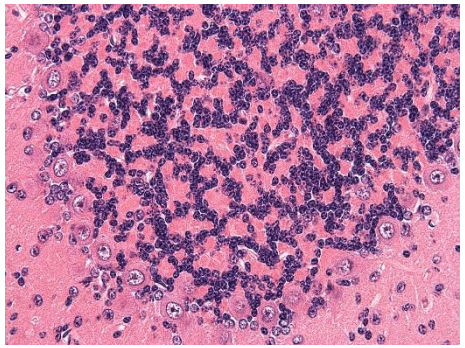


Cerebellum (400x)

(C) Control



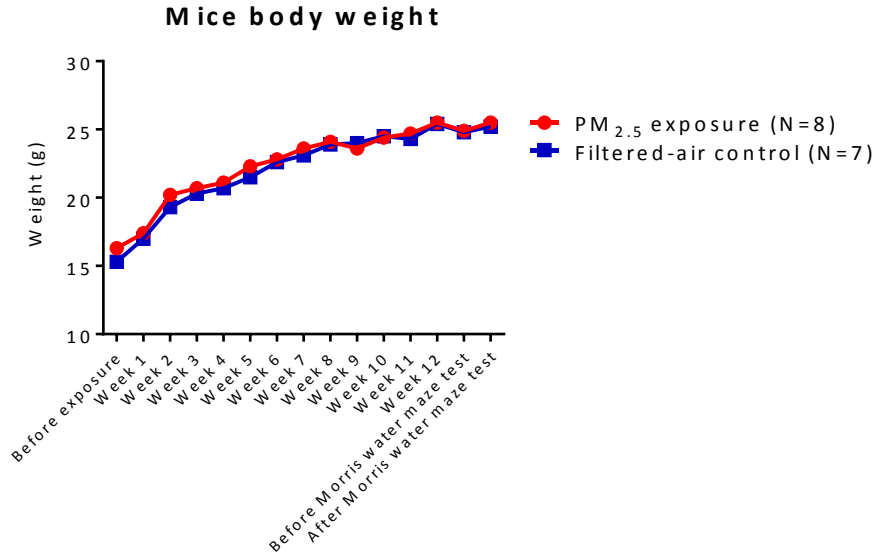
Cerebellum (100x)



Cerebellum (400x)

Figure 20. Cerebellum in total 300 μg DEPs (A), total 100 μg DEPs (B) and control (C) group in the experiment 2 in the first part of study with H&E stain

(A)



(B)

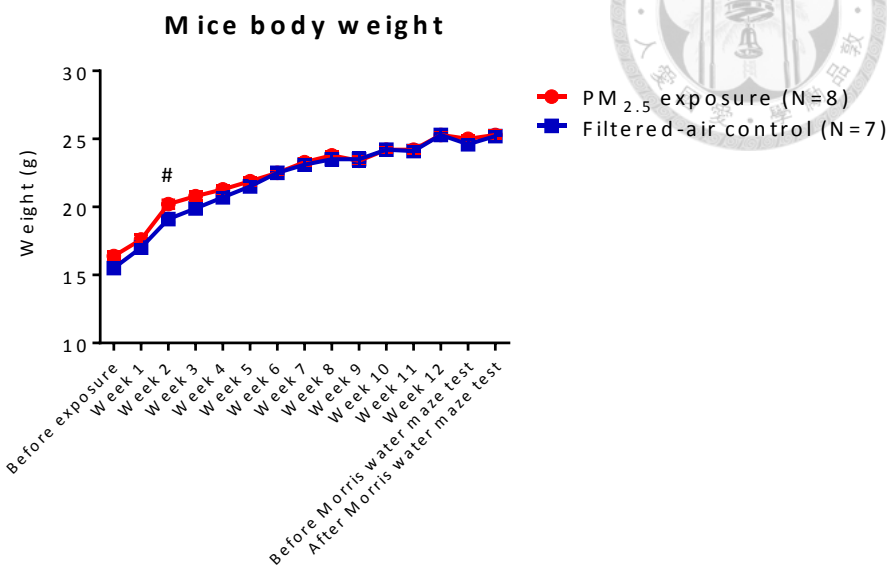


Figure 21. Mice body weight through the experiment in the second part of study

Body weight of mice was presented in median (A) and mean ± SEM (B).
Significant differences from control group in Student's t-test

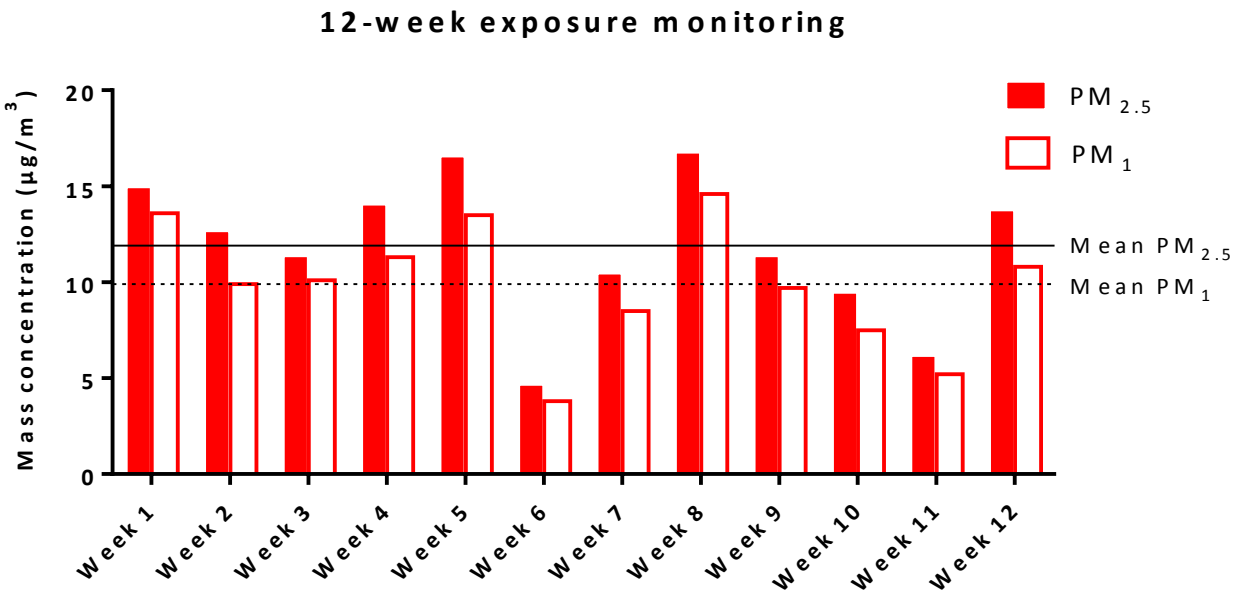
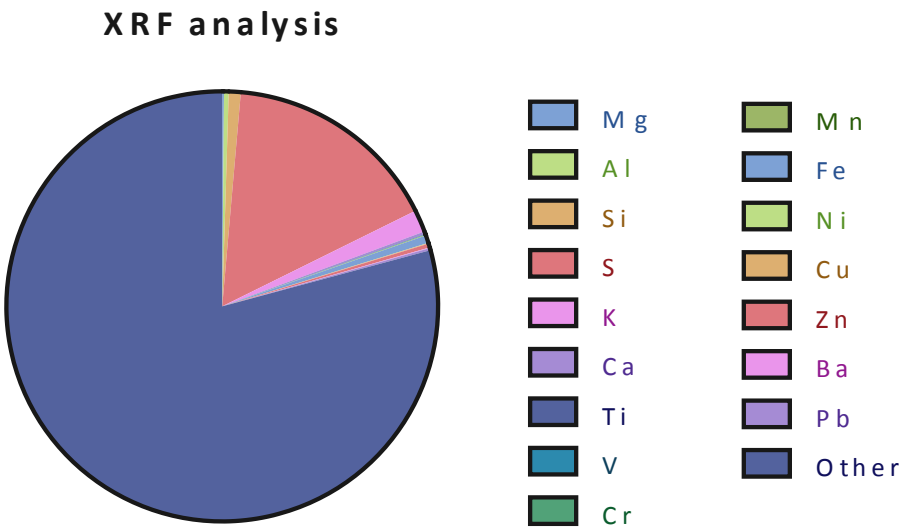


Figure 22. 12-week $PM_{2.5}$ and PM_1 exposure monitoring

(A)



(B)

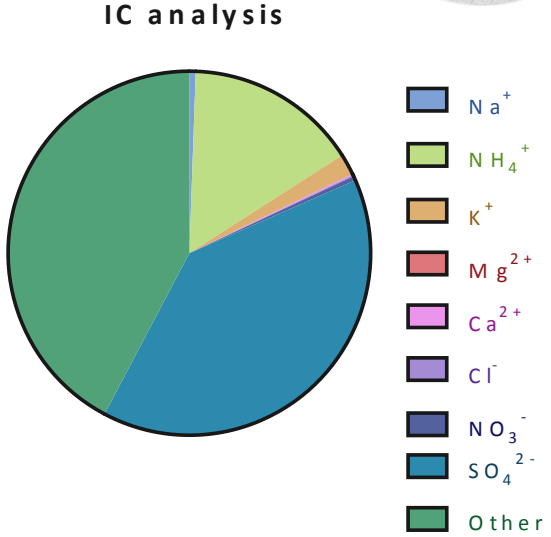


Figure 23. Chemical composition of exposed $PM_{2.5}$ analyzed by XRF (A) and IC (B)

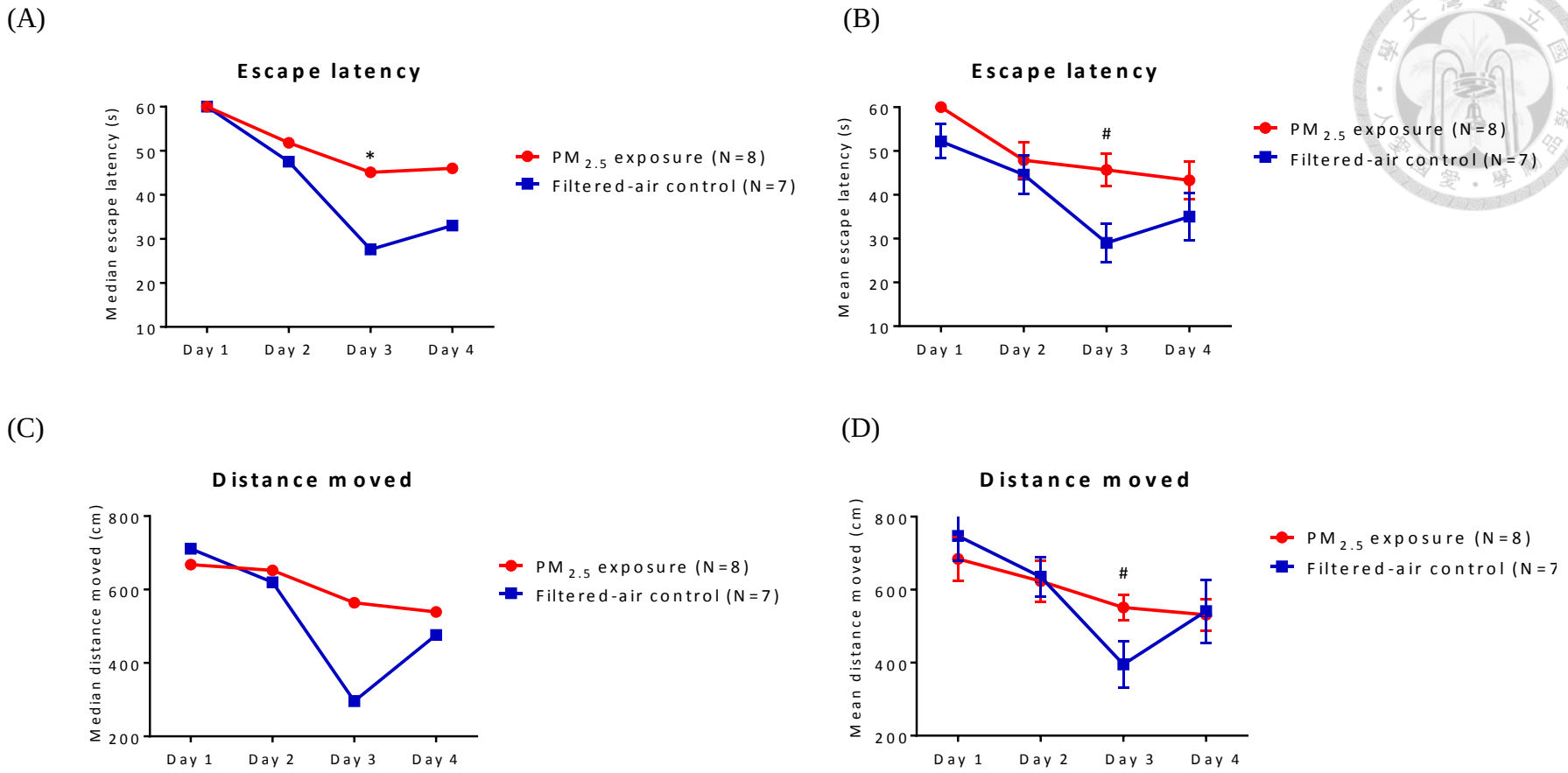


Figure 24. Morris water maze performance (escape latency and distance moved) in acquisition phase in the second part of study

Escape latency was presented in median (A) and mean ± SEM (B); distance moved was presented in median (C) and mean ± SEM (D)

* Significant differences from control group in Wilcoxon rank sum test

Significant differences from control group in Student's t-test

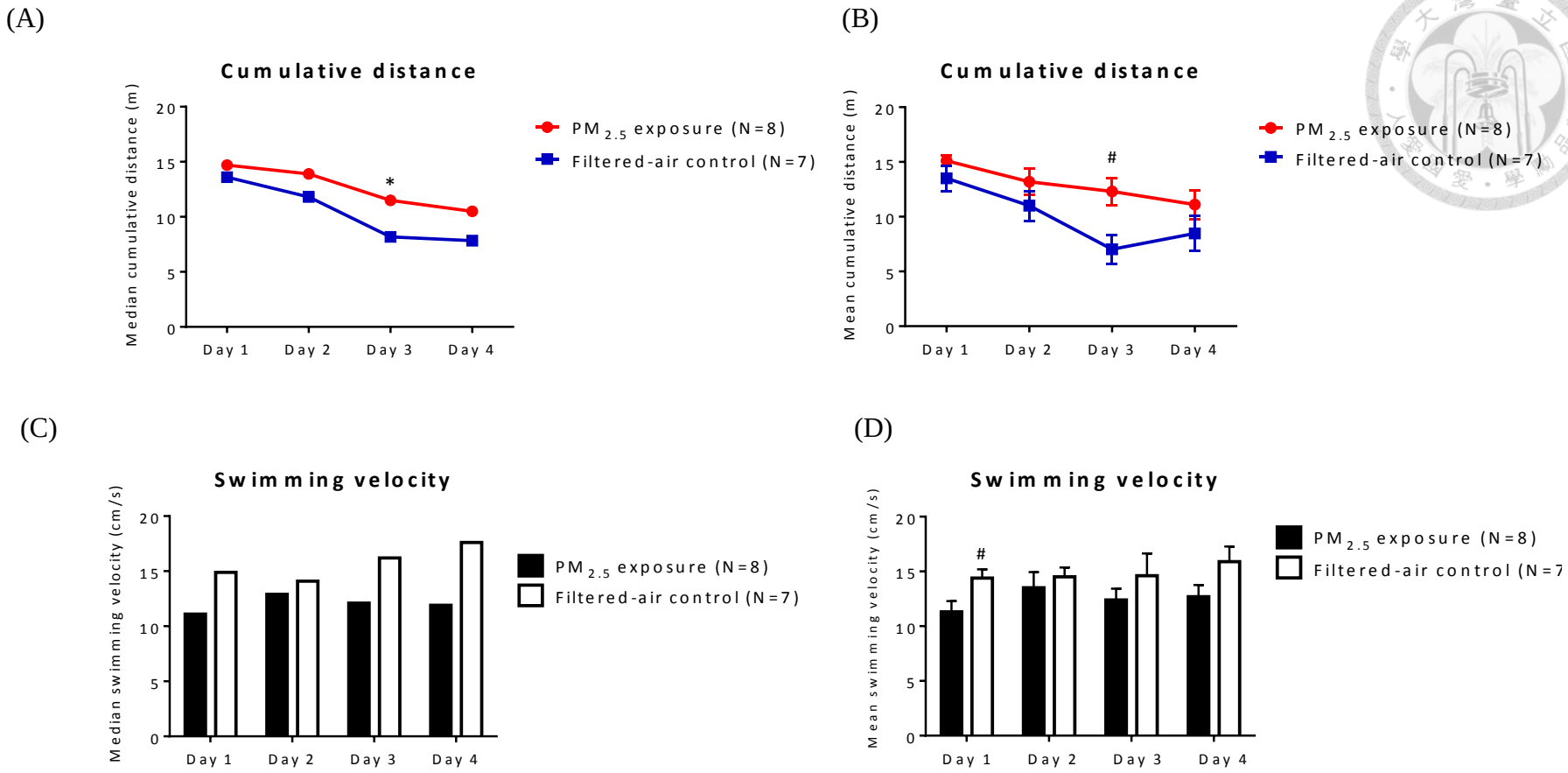


Figure 25. Morris water maze performance (cumulative distance and swimming velocity) in acquisition phase in the second part of study

Cumulative distance was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

* Significant differences from control group in Wilcoxon rank sum test

\# Significant differences from control group in Student's t-test

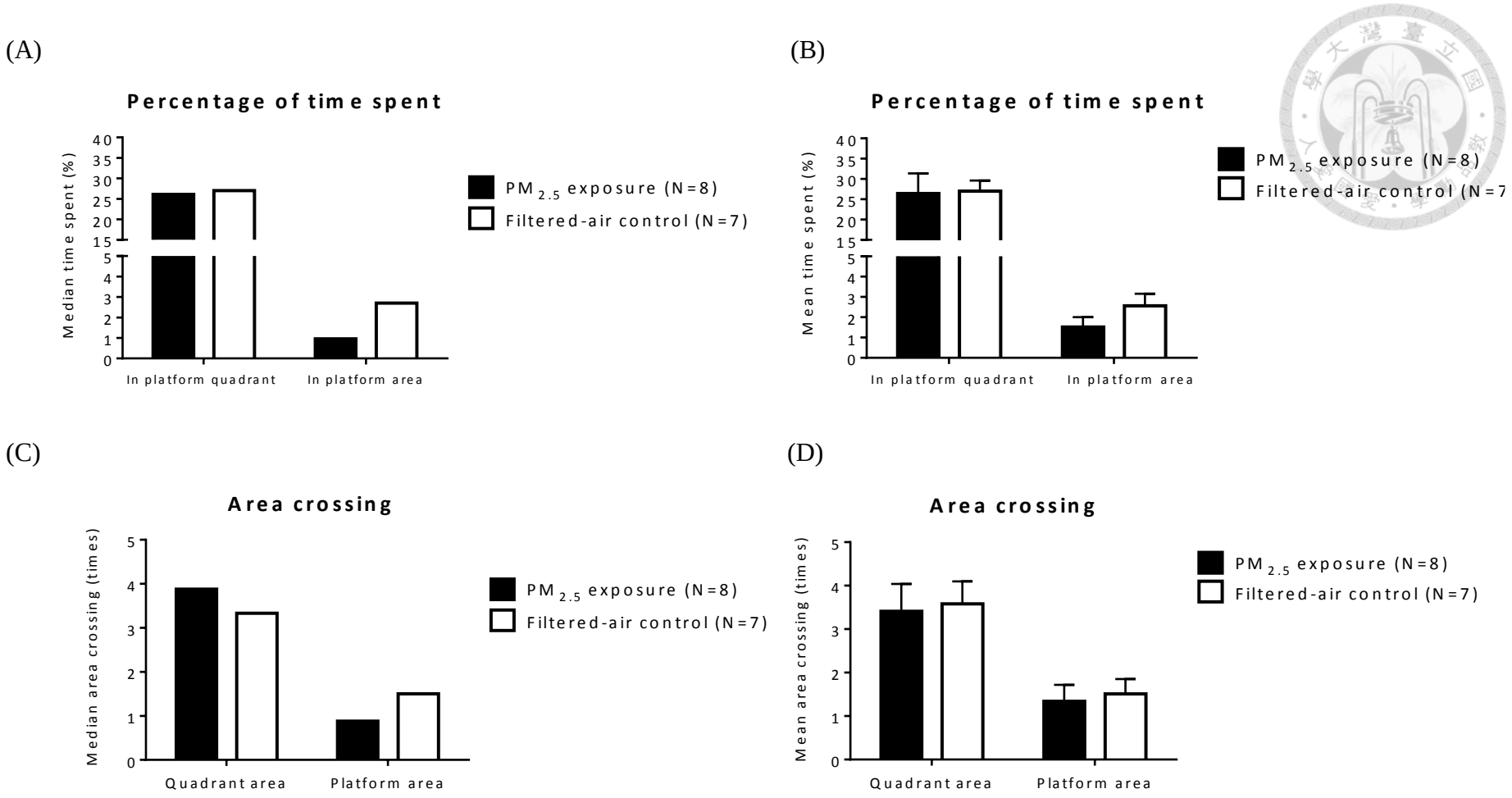


Figure 26. Morris water maze performance (percentage of time spent and area crossing) in probe test in the second part of study

Percentage of time spent was presented in median (A) and mean ± SEM (B); area crossing was presented in median (C) and mean ± SEM (D)

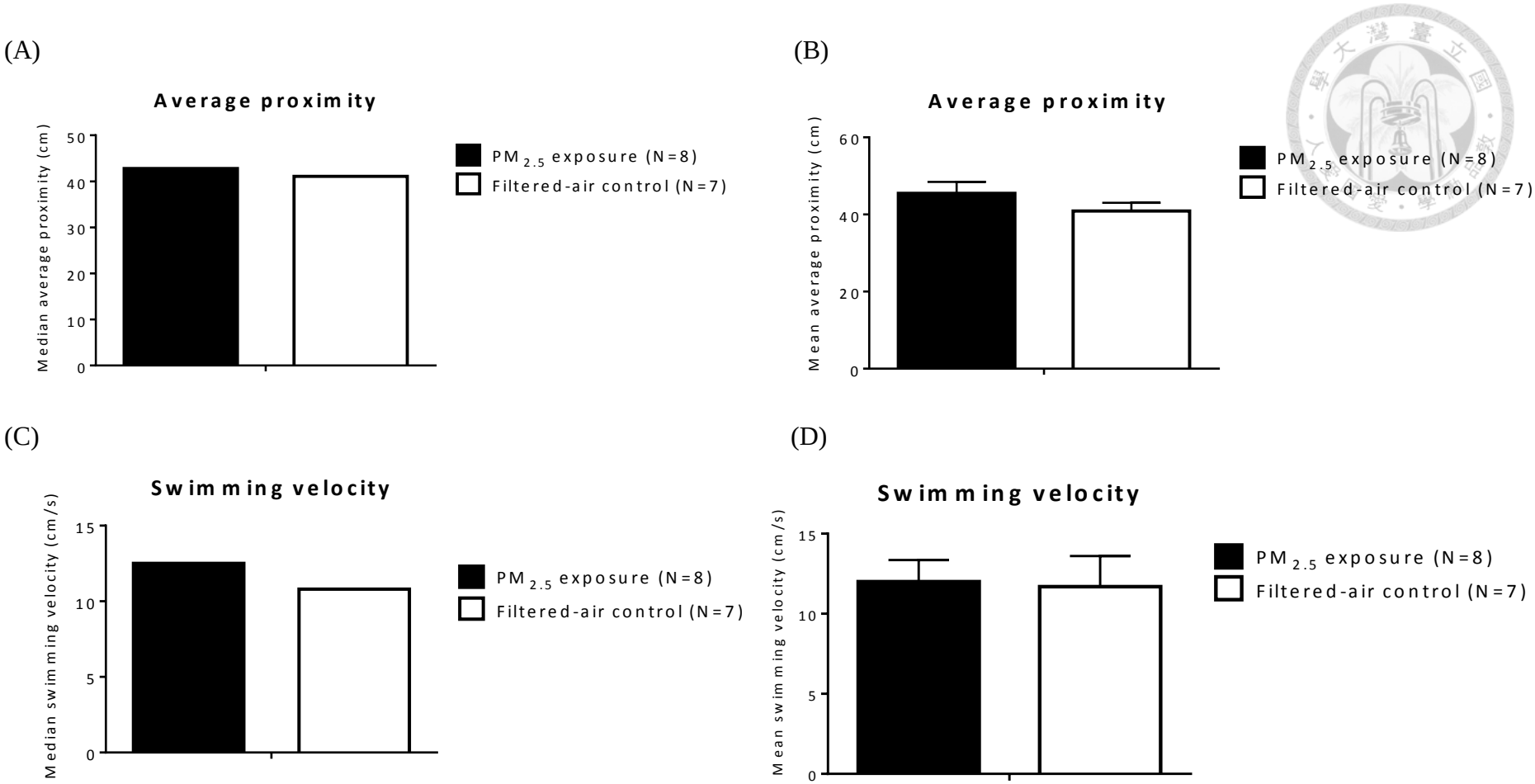
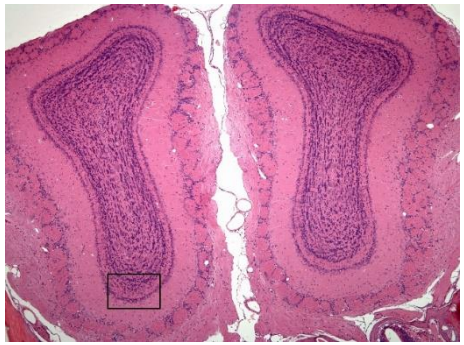


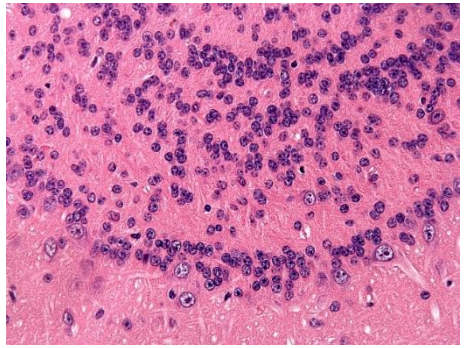
Figure 27. Morris water maze performance (average proximity and swimming velocity) in probe test in the second part of study

Average proximity was presented in median (A) and mean ± SEM (B); swimming velocity was presented in median (C) and mean ± SEM (D)

(A) PM2.5 exposure

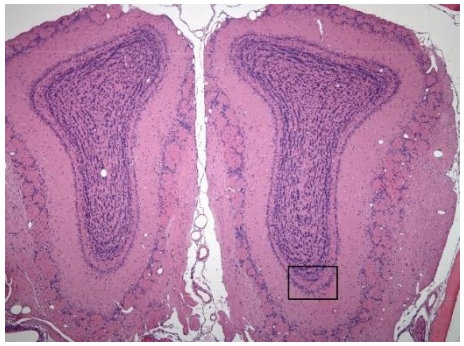


Olfactory bulb (40x)

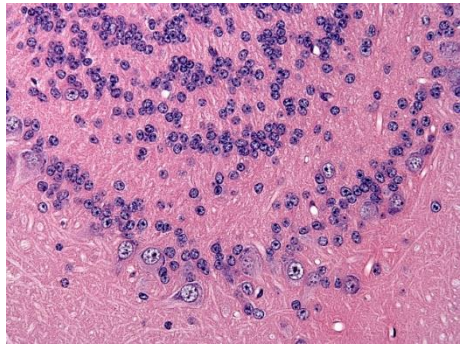


Olfactory bulb (400x)

(B) Filtered-air control



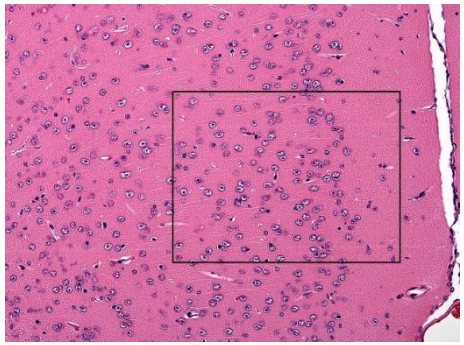
Olfactory bulb (40x)



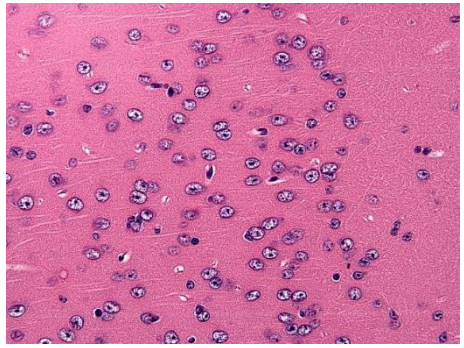
Olfactory bulb (400x)

Figure 28. Olfactory bulb in $PM_{2.5}$ exposure group (A) and filtered-air control (B) in the second part of study with H&E stain

(A) PM2.5 exposure

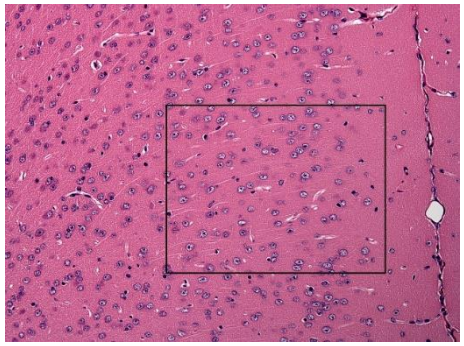


Frontal cortex (200x)

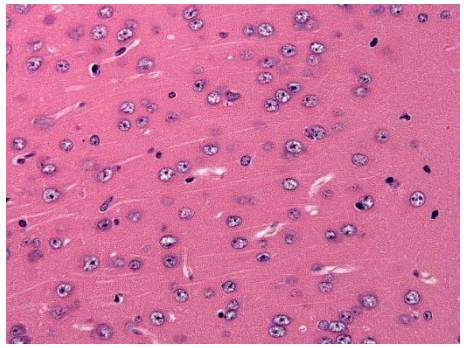


Frontal cortex (400x)

(B) Filtered-air control



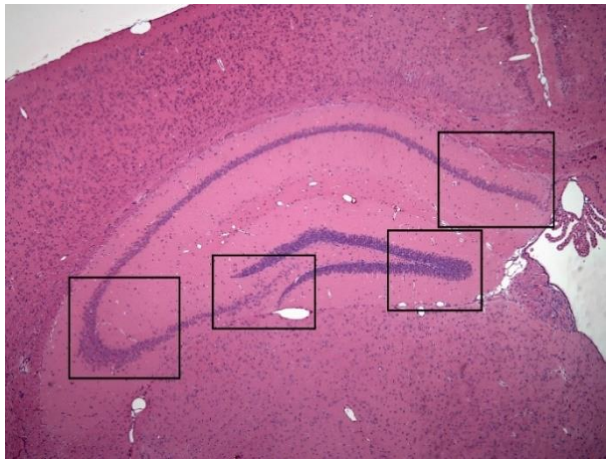
Frontal cortex (200x)



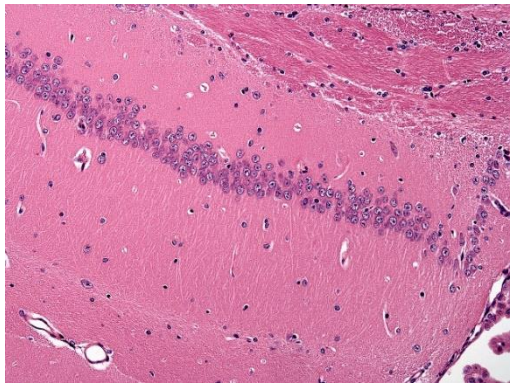
Frontal cortex (400x)

Figure 29. Frontal cortex in $PM_{2.5}$ exposure group (A) and filtered-air control (B) in the second part of study with H&E stain

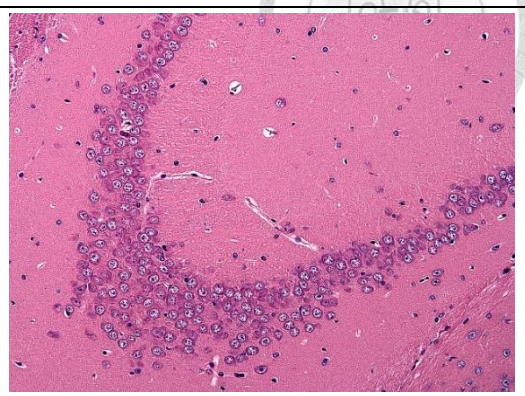
PM2.5 exposure



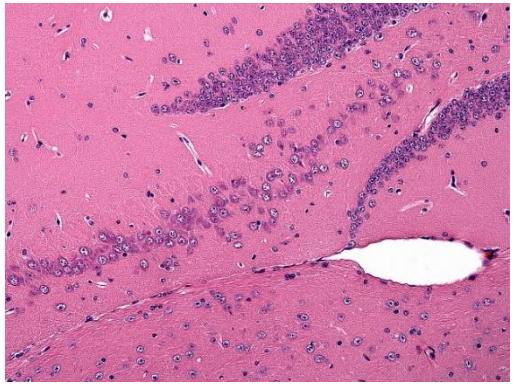
Hippocampus (40x)



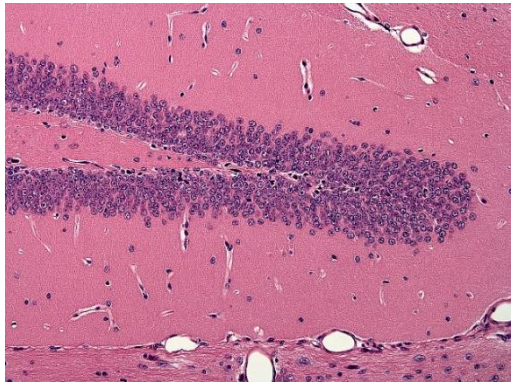
Hippocampus CA1 (200x)



Hippocampus CA2 (200x)



Hippocampus CA3 (200x)



Hippocampus dentate gyrus (200x)

Figure 30. Hippocampus in $PM_{2.5}$ exposure group in the second part of study with H&E stain

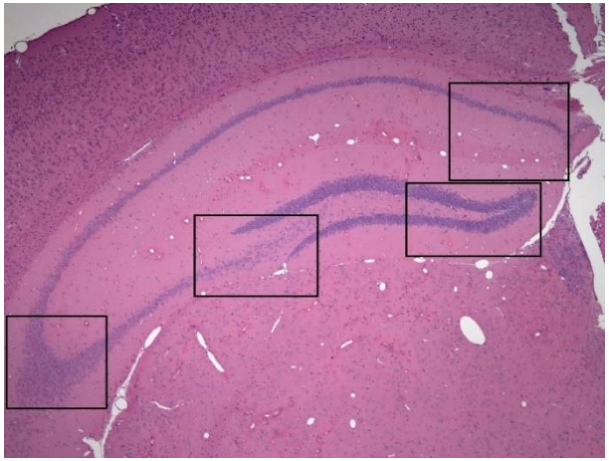
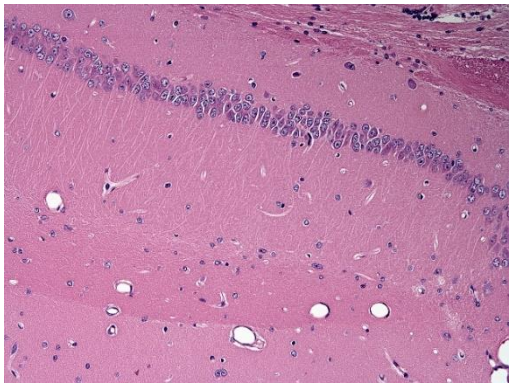
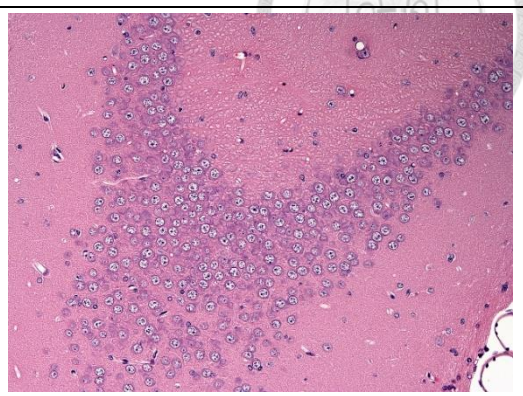
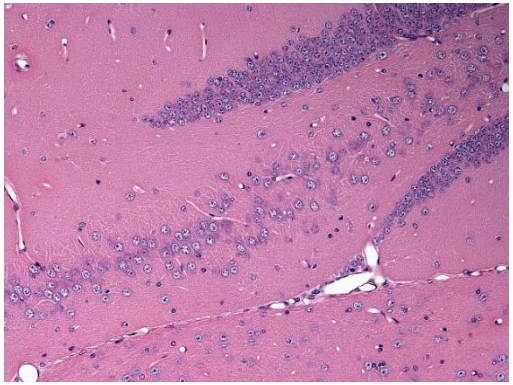
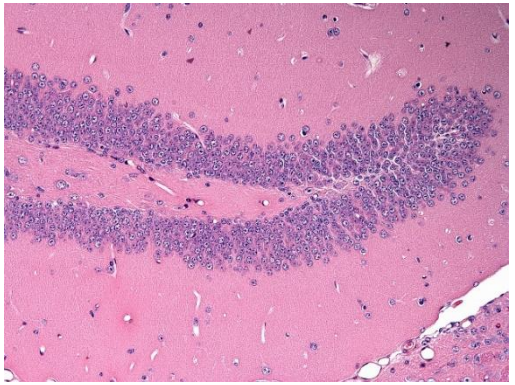
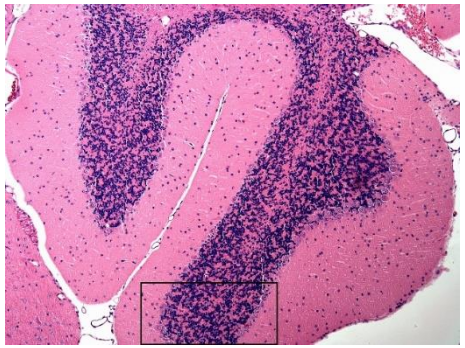
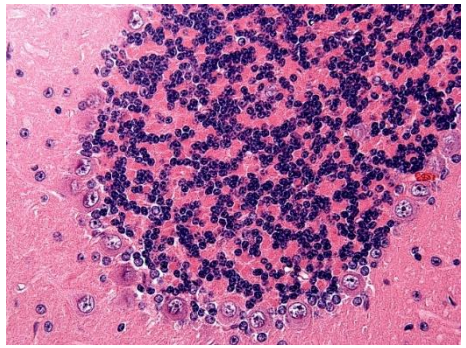
Filtered-air control		
		
Hippocampus (40x)	Hippocampus CA1 (200x)	Hippocampus CA2 (200x)
		
	Hippocampus CA3 (200x)	Hippocampus dentate gyrus (200x)

Figure 31. Hippocampus in filtered-air control in the second part of study with H&E stain

(A) $PM_{2.5}$ exposure

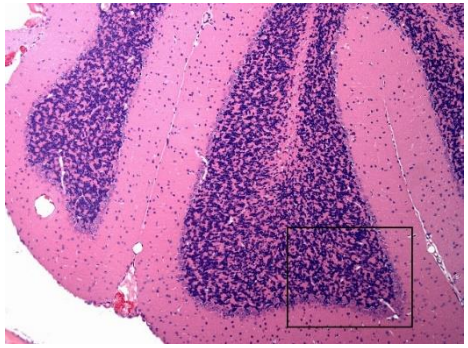


Cerebellum (100x)

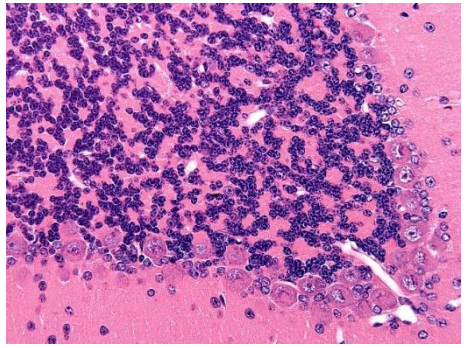


Cerebellum (400x)

(B) Filtered-air control



Cerebellum (100x)



Cerebellum (400x)

Figure 32. Cerebellum in $PM_{2.5}$ exposure group (A) and filtered-air control (B) in the second part of study with H&E stain