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Long-term challenge of methylphenidate changes the neuronal population and membrane property of dopaminergic neuron in rats

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ABSTRACT

Attention deficit hyperactivity disorder (ADHD) has a prevalence of 7.5% in school-age children in Taiwan. A number of ADHD patients start taking medications in elementary school and continue their treatment until they are in college or adulthood. Methylphenidate is the most frequently used medication prescribed for ADHD treatment. The influence of long-term treatment of methylphenidate on neuro-development, especially dopaminergic neurons, in rats would be explored. This study investigated the impact of long-term treatment of methylphenidate on different neurons. Rats aged 1 month were divided into three groups: Normal group receiving only sucrose solution, Low-dose group receiving 2 mg/kg methylphenidate, and High-dose group receiving 10 mg/kg methylphenidate; for each group, the drug was administrated twice per day. After 7 months of the treatment period, then the alterations in number of norepinephrine, serotonergic, cholinergic and dopaminergic neurons were quantified. The number of dopaminergic neurons in the substantia nigra (SN), the serotonergic neurons in the dorsal raphe nucleus, and the cholinergic neurons in the tegmental nucleus significantly decreased as compared with Normal group, whereas the noradrenergic neurons in the locus coeruleus substantially increased. The whole-cell recording was made from dopaminergic neurons residing in the SN for examination of their firing activity. The recorded dopaminergic neurons in SN were categorized into slow and fast firing using 10 Hz as a classified index. The results displayed that the ratio of dopaminergic neurons with fast firing in the High-dose group was less as compared with those in the Normal and the Low-dose group. Furthermore, the amplitude of action potential of the dopaminergic neurons with slow firing was higher in the High-dose group than those in the Normal and Low-dose groups. The firing behavior of dopaminergic neurons and dopamine concentration in the brain is affected by the long-term challenge of methylphenidate.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is the most prevalent neurobehavioral disorders in children. ADHD patients display three symptoms: inattention, hyperactivity, and impulsivity (Berridge et al., 2006; Ferguson, 2000). Evidence exists for the association between ADHD and possible structural (Nakao et al., 2011; Shaw et al., 2015), functional (Makris et al., 2006; Purper-Ouakil et al., 2011) and neurotransmitter (Volkow et al., 2007) alterations in various regions of the brain with ADHD.

Accumulative studies worldwide on ADHD prevalence in children aged 18 and under found a rough approximation of 7.2%. The US Census Bureau estimates 1,795,734,009 people were aged 5–19 worldwide in 2013. Thus, 7.2% of this total population is 129 million - a rough estimate of the number of children worldwide who have ADHD (Thomas et al., 2015) (Web from Children and Adults with attention-Deficit/Hyperactivity Disorder). In order to increase learning effectiveness and decrease disturbing teachers or classmates, school and physicians generally recommend pharmaceutical treatment for ADHD patients. Currently, some students who are not ADHD patients also take

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methylphenidate simply for the purpose to increase learning effectiveness in pursuing improvements in academic performance. Due to tremendous academic stress, most ADHD children begin taking medicine at elementary school and continue until they are in college. Thus, about one-third of their life relies on medications to get through their highly-stressful school years.

Methylphenidate (brand name: Ritalin) is the most common clinically used medication with fewer side effects for treating ADHD (Accardo and Blondis, 2001; Goldman et al., 1998; Markowitz et al., 1999; Meyers et al., 1998; Solanto, 1998; Volkow et al., 2002). Two possible mechanisms of methylphenidate may be involved in treating ADHD. First, methylphenidate could enhance the synthesis and release of dopamine (Cook and Schanberg, 1975). Second, methylphenidate could bind to dopamine transporter and inhibit reuptake of dopamine, which in turn increases dopamine concentration (Bush, 2010; Volkow et al., 1998).

Accumulative evidences show that long-term intake of methylphenidate resulted in the apoptosis of neurons in the hippocampus, damages induced by reactive oxygen species, and DNA fragmentation in young and adult rats (Andreazza et al., 2007; Banihabib et al., 2016; Motaghinejad et al., 2016; Stopper et al., 2008). In addition, neurogenesis significantly decreased in the hippocampus (Lagace et al., 2006) and microglial activity elevated in several brain areas (Gonçalves et al., 2010; Sekine et al., 2008). Since methylphenidate could specifically regulate dopaminergic synapse and alter the synthesis and metabolism pathways of dopamine, we would like to elucidate whether the long-term challenge of methylphenidate would impact dopaminergic neurons.

In this study, we investigate long-term effect of metylphenidate on the dopaminergic, norepinephrine, serotonergic, cholinergic neurons in the substantia nigra, locus coeruleus, dorsal raphe nucleus, and tegmental nucleus respectively. It showed that the number and electrophysiological property of dopaminergic neurons displayed significant alterations. Our investigation may provide a novel estimation for clinical medicine to track the changes of physiology and learning and memory induced by long-term use of metylphenidate.

2. Materials and methods

Male Sprague-Dawley rats were obtained from the Animal Center of National Yang-Ming University. The Animal Research Committee of National Yang-Ming University approved the study in accordance with the guidelines for the care and use of laboratory animals.

2.1. Experimental groups

Rats aged one month with 100–150 g body weight were divided into three groups. Normal group: rats were fed with vehicle solution (3% sucrose in water), n = 20. Low-dose group: rats started to received 2 mg/kg body weight methylphenidate, n = 24. High-dose group: rats were given with 10 mg/kg body weight methylphenidate, n = 24. All groups were orally given with study medication when they were one month of age. The medication was administered two times a day, 9 a.m. and 5 p.m., for a continuous period of 7 months. Oral gavages were applied to feed methylphenidate. To help the feeding process, methylphenidate was dissolved in sucrose solution. All rats in the experimental groups received a 300 μl-sucrose solution containing the medication, whereas the control group received an only 300 μl-sucrose solution. The three groups were treated for 7 months and a subsequent withdrawal for two weeks. The animals were then sacrificed and perfused, or were subjected to electrophysiological analysis. Most ADHD children rely on medications for one-third of their life in order to increase learning effectiveness. Because of the reason, the rats were treated methylphenidate for about one-third of their lifespan, seven months. Average dosage of methylphenidate is 30 mg–60 mg daily for children with body weight is about 20 kg–30 kg (FDA web). In this study, Low-dose, 2 mg/kg body weight methylphenidate, is within the recommended dosage of FDA. High-Dose, 10 mg/kg body weight methylphenidate used in this study, is higher than that of the usual administration.

2.2. Open field test

All groups of rats were evaluated with open field test before drug administration, which served as a baseline (100%) of total travel distance and total travel time for each rat. The data obtained after drug administration was normalized with its baseline and a relative percentage was then displayed. The behavioral test was performed every half month until the animal was sacrificed. Every test was conducted between 7 and 8 a.m. before the light was turned on. Because it was suggested that the methylphenidate concentration declined to 1% at four hours after intravenous injection 10 mg/kg methylphenidate (Aoyama et al., 1997), all behavioral test was performed at least 14 h after the previous drug administration to ensure that the experiments were not affected by the medication itself.

Rats were placed in a transparent acrylic box with a bottom area of 60 cm × 60 cm. The software EthoVision was applied to trace the movement patterns of the rats within the box during 30 min, and to analyze their total travel time and total travel distance (Aga-Mizrachi et al., 2014; Li et al., 2007). During the process of the behavioral test, all procedures were performed in the dark until the end of the test.

2.3. Sacrifice and perfusion fixation of the experimental animals

After drug treatment for 7 months and subsequent withdrawal for two weeks, rats were deeply anesthetized and perfused with normal saline, and 4% paraformaldehyde and 7.5% picric acid as fixation solution. The perfused whole brains were then obtained, and the volume and weight were estimated. The brains were subsequently placed in the fixation solution for 24 h at 4 °C.

2.4. Cryosectioning procedure

The fixed brain tissue was transferred to 30% sucrose solution until the brain tissue sunk to the bottom; then the frozen section procedure was allowed to perform. The procedure, called cryoprotection, could prevent cells from freezing damage. The brain tissue was sectioned into 20 μm-thick slices with a cryostat, which was then placed on slides, dried at room temperature, and subjected to subsequent immunostaining.

The left hemisphere of the rat was serially sectioned from sagittal future toward lateral side. As shown in supplemental Fig. 1, brain slices were numbered (1, 2, 3, 4, 5, and so on) and placed on different slides for different immunostaining. Taking the Normal group as an example, brain slices numbered 1 to 5 located approximately at bregma lateral 0.18 mm, which were placed on slides A, B, C, D, and E, respectively. Then slides numbered 6, 7, 8, 9, and 10 were then placed on slides A, B, C, D, and E, respectively. The procedure was repeated until the 65th slice was put on slide E. The brain slices on panel a (1, 6, 11, 16, 21 … to 61) were subjected to immunostaining with anti-tyrosine hydroxylase to label the alterations of dopaminergic neurons (supplemental Fig. 1A column). The brain slices on panel b (2, 7, 12, 17, 22 … to 62) were immunostained with anti-dopamine-β-hydroxylase for labeling alterations of norepinephrine neurons (supplemental Fig. 1B column). The brain slices of panel c (3, 8, 13, 18, 23 … to 63) were immunostained with anti-tryptophan hydroxylase to mark the changes of serotonin neurons (supplemental Fig. 1C column). For brain slices of panel d (4, 9, 14, 19, 24 … to 64) were immunostained using anti-choline acetyltransferase to identify cholinergic neurons (supplemental Fig. 1D column). Immunostaining using anti-ED1 was applied to observe the inflammatory response of the brain for panel e (brain slices numbered 5, 10, 15, 20, 25 … to 66; supplemental Fig. 1E column).

2.5. Immunohistochemistry and counter staining

Dried slices were washed three times for five minutes each with 0.1 M PBS. Slices were then immersed in 1% H_2O_2 (in 0.1 M PBS) for 20 min to reduce background noise, washed three times for five minutes each with 0.1 M PBS, and reacted with blocking solution (3% bovine serum albumin, 1% Triton X-100, 5% FBS) for 1 h at room temperature. Primary antibody (anti-tyrosine hydroxylase [Millipore MAB318], anti-dopamine-β-hydroxylase [Millipore AB1538], anti-tryptophan hydroxylase [Millipore AB1541], anti-choline acetyltransferase [Millipore AB144P], or anti-ED1 [Millipore MAB1435]) was subsequently added and reacted for 2 days at 4 °C. Afterward, slices were washed three times for five minutes each with 0.1 M PBS, and corresponding secondary antibody (goat anti-mouse IgG conjugated biotin [Millipore AP124B], goat anti-rabbit IgG conjugated biotin [KPL 16-15-06], or rabbit anti-sheep IgG biotin [SIGMA SAB3700922]) was added and reacted for 1 h at room temperature, washed three times for five minutes each with 0.1 M PBS, and reacted with avidin-biotin complex for 1 h at room temperature. After washing, signals were developed using DAB. Following the staining procedure, slices were air dried and subjected to counter staining with cresyl violet. Sections were placed in 1% cresyl violet for 3 min, dehydrated through a series of increasing concentrations of alcohol (50%, 70%, 80%, 90%, 95%, and 100% twice; 2 min each), and subsequently immersed in xylene twice for 5 min each. Finally, slides were mounted with permount and photographed with an optical microscope for observation and quantification purposes.

2.6. Quantification of immunohistochemistry

To quantify the number of each kind of neurons, the number of neurons was counted individually after each kind of immunostaining performed. Taking immunostaining using anti-tyrosine hydroxylase as an example, there were 4 brain tissue slides of the panel a in supplemental Fig. 1, each contained 13 brain slices from bregma lateral 0.18–3.30 mm. The substantia nigra was located at bregma lateral 0.70–2.78 mm. A total of 9 slices were found with substantia nigra, as magnified in supplemental Fig. 2A and B. Subsequently, all cells labeled with DAB in the substantia nigra on these slices were counted and summed, representing the number of dopaminergic neurons of the rat. Similarly, the total number of cells stained with anti-dopamine-β-hydroxylase in the locus coeruleus was counted from bregma lateral 0.98–1.24 mm (there were 2 slices containing locus coeruleus), which represented the number of norepinephrine neurons in the locus coeruleus of the rat (supplemental Fig. 3A). The serotonin neurons were also counted in three brain slices containing dorsal raphe nucleus, from bregma lateral 0.48–1.00 mm. The numbers of cells labeled with anti-tryptophan hydroxylase were summed (supplemental Fig. 3B). Finally, after immunostaining with anti-choline acetyltransferase, the number of cholinergic neurons on six slices containing the tegmental nucleus (from bregma lateral 0.76–2.06 mm) was counted (supplemental Fig. 3C). Anti-ED1 was applied to label microglial cells (supplemental Fig. 1E). Because no significant changes were found in every brain area as compared with the Normal group, the images were not displayed at high magnification.

2.7. Whole-cell patch recording

After treatment of study medications for 7 months and withdrawal for 2 weeks, the animals were anesthetized with 5% isoflurane in O_2 and decapitated, then their brains were rapidly exposed and chilled with ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM): 119 N-Methyl-D-Glucamine (NMDG), 2.5 KCl, 1.3 $MgSO_4$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. The brain was blocked and imbedded in 3% agar. Sagittal brain slices of 300 μm thickness was sectioned in ice-cold aCSF using a vibroslicer (D.S.K. Super Microslicer Zero 1, Dosaka EM, Kyoto, Japan). The slices containing SN were collected and maintained in a moist air-aCSF interface chamber and allowed to recover for at least 90 min before being transferred to an immersion-type chamber mounted on an upright microscope (BX51WI, Olympus Optical Co., Ltd., Tokyo, Japan) for recordings. During recovery period, ice-cold aCSF was gradually replaced with standard aCSF for perfusion of the slices. The compositions of standard aCSF were same as ice-cold aCSF except for substitution of 119 mM NaCl to NMDG and addition of 2.5 mM $CaCl_2$.

Neurons were viewed using Nomarski optics. Patch pipettes were pulled from borosilicate glass tubing (1.5 mm outer diameter, 0.32 mm wall thickness; Warner Instruments Corp., Hamden, CT, USA) and had a resistance of about 5–8 MΩ when filled with the pipette solution consisting of (in mM) 131 K-gluconate, 20 KCl, 10 HEPES, 2 EGTA, 8 NaCl, 2 ATP, and 0.3 GTP, pH adjusted to 7.2 with KOH. Recordings were made in the whole-cell configuration and current-clamp mode with a patch amplifier (Multiclamp 700B; Molecular Device, Sunnyvale, CA, USA) at room temperature (~25 °C). The bridge was balanced and recordings were only accepted if the recorded neuron had a membrane potential (Vm) of at least −45 mV without applying a holding current and if the action potential was able to overshoot 0 mV. Signals were low-pass filtered at a corner frequency of 2 kHz and digitized at 10 kHz using a Micro 1401 interface running Signal software (Cambridge Electronic Design, Cambridge, UK). In some experiments, 6.7 mM biocytin was included in the internal solution used to fill the recorded neurons for *post hoc* confirmation of the morphology of the recorded neuron. After recording, the slices were fixed overnight at 4 °C in 4% paraformaldehyde in 0.1 M PB, then were subjected to biocytin histochemistry without further sectioning. The slices were incubated for 1 h at room temperature in TPBS containing 2% bovine serum albumin and 10% normal goat serum, then were incubated overnight at 4 °C in PBST containing avidin-biotinylated horseradish peroxidase (Vector Lab; 1:200 dilution in PBST). After PBST rinses, they were incubated for 2 h with DAB and H_2O_2, then observed under microscope.

2.8. Statistical analysis

The experimental results were expressed as mean ± standard error (SE). SPSS was applied to perform one-way ANOVA and least significant difference (LSD) analyses. The result was considered to be statistically different when $p < 0.05$.

3. Results

3.1. Long-term intake of high-dose methylphenidate causes reduction of rat body weight

The recording of body weights showed increases in all three groups. Nevertheless, there was no statistical difference between the Normal group and the Low-dose group. The gained body weight of the High-dose group was lower than those in the Normal and Low-dose groups from four months of age and maintained until eight months of age ($p < 0.05$; Fig. 1A).

3.2. Long-term intake of methylphenidate does not affect the appearance of rat's whole brain

By observing the appearance of the whole brain, there was no significant difference among the three groups (data not shown). In addition, the brain volumes and brain weights of the three groups were in range of 2.01 ± 0.05 to 2.23 ± 0.09 cm^3 and 2.09 ± 0.10 to 2.22 ± 0.04 g, respectively. No statistical differences in brain volumes or in brain weights were found among the three groups ($p > 0.05$).

3.3. Long-term intake of methylphenidate affects rats' locomotion

The relative percentage of travel distance showed that there was no

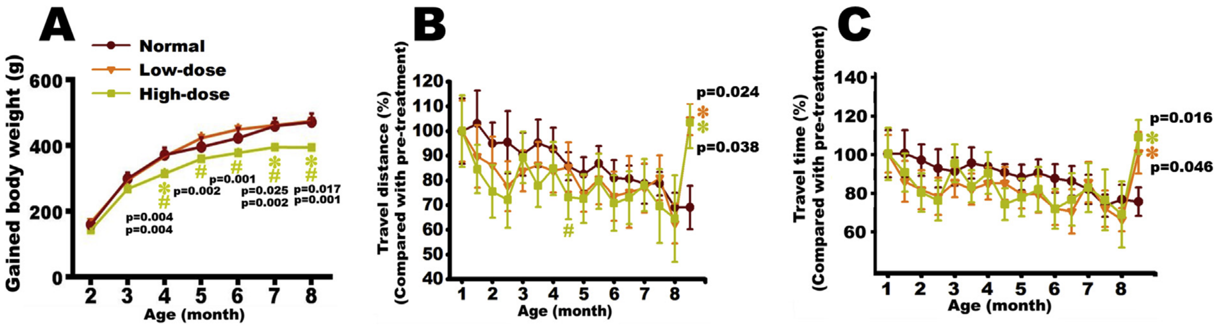

Fig. 1. Long-term intake of methylphenidate alters body weight and locomotion of rats. The gained body weight of the High-dose group was significantly lower than that of the Normal group when a long-term treatment of methylphenidate was started from four months of age. This trend sustained until withdrawal when the rats were at eight months of age (A). When the travel distance and travel time were normalized with each rat's baseline, it showed that, at the age of eight and a half months, both the total travel distance and total travel time were significantly higher in the Low-dose and High-dose groups as compared with the Normal group (B and C). ∗ $p < 0.05$ High-dose group or Low-dose group *vs* the Normal group at the same age. # $p < 0.05$ High-dose group *vs* the Low-dose group at the same age.

statistical difference among the Normal, Low-dose, and High-dose groups in the locomotion during the 7-month treatment. However, after withdrawal, the travel distance and travel time in Low-dose and High-dose groups were significantly higher than those in the Normal group (Fig. 1B and C).

3.4. Long-term intake of methylphenidate results in the reduction of number of dopaminergic neurons in rats' substantia nigra

Dopaminergic neurons were labeled using immunostaining with anti-tyrosine hydroxylase (TH). Nine brain slices containing substantia nigra were obtained laterally from bregma 0.7–2.78 mm. The sum of dopaminergic neurons in the substantia nigra compacta part was counted (Supplemental Fig. 2A and B). The results showed that the number of dopaminergic neurons was significantly lower in the Low-dose and High-dose groups as compared with the Normal group (Fig. 2A and E).

3.5. Long-term intake of high-dose methylphenidate leads to an increase in norepinephrine neurons in the locus coeruleus

Norepinephrine neurons were labeled using immunostaining with anti-dopamine-β−hydroxylase antibody. Two brain slices were obtained laterally from bregma 0.98–1.24 mm, and the number of norepinephrine neurons in the locus coeruleus were summed up (Supplemental Fig 3A). The results indicated that the number of norepinephrine neurons was significantly higher in the High-dose group than those in the Normal and Low-dose groups (Fig. 2B and F).

3.6. Long-term intake of methylphenidate causes a decline in serotonin neurons in the dorsal raphe nucleus of rats

Via immunostaining with anti-tryptophan hydroxylase, serotonin neurons in the dorsal raphe nucleus were marked. The number of cells labeling with anti-tryptophan hydroxylase were summed in the three slices containing dorsal raphe nucleus, which was obtained laterally from bregma 0.48–1.00 mm (Supplemental Fig 3B). The results showed that the number of serotonin neurons was significantly lower in the Low-dose and High-dose groups when comparing to the Normal group (Fig. 2C and G).

3.7. Long-term intake of high-dose methylphenidate reduces the number of cholinergic neurons in the tegmental nucleus in rats

Cholinergic neurons were labeled by immunostaining using anti-choline acetyltransferase. Six slices containing tegmental nucleus were obtained laterally from bregma 0.76–2.06 mm. The total cell number of cholinergic neurons of the six slices was counted (Supplemental Fig 3C) and the results indicated that the cholinergic neurons were significantly fewer in the High-dose group than the Normal and Low-dose groups (Fig. 2D and H).

3.8. Long-term intake of methylphenidate does not induce robust inflammatory responses in rats' brain

Macrophage or activated microglia were labeled with anti-ED1 to elucidate brain inflammation. The results showed that there were no strong inflammatory responses found in each brain area (data not shown).

3.9. Long-term intake of high-dose methylphenidate results in the reduced ratio of dopaminergic neurons with high-frequency firing in the substantia nigra

The whole-cell patch recording was employed to record electrophysiological properties of dopaminergic neurons. In the brain slices containing substantia nigra, compact parts were localized and neurons in this area were selected for recording under Normaski optics (Fig. 3A and B). The recorded neurons were filled with biocytin for *post hoc* examination of their morphology and locations in slices, with a typical example shown in Fig. 3C. For investigation of the excitation features of the recorded neurons, recordings were performed in the I-clamp mode and the membrane potential was hold at about −50 mV. A depolarizing current pulse with duration and intensity of, respectively, 1 s and 50 pA was applied and the responses of recorded neurons were recorded.

The distribution of firing rate upon depolarizing current injection of all recorded neurons was plotted in Fig. 3D. It showed that the majority of the recorded neurons in the High-dose groups had firing rate lower than 10 Hz; nevertheless, those in the Normal and Low-dose groups were more evenly distributed (Fig. 3D). When grouping the recorded neurons according to their firing rate, the spike frequency < 10 Hz defined as cells with slow firing, on the other hand, the spike frequency > 10 Hz defined as cells with fast firing. The results showed that the percentages of cells with slow firing in the Normal and Low-dose groups were 54.16% and 54.54%, respectively. Moreover, 45.53% and 45.45% of the cells had fast firing, respectively (Fig. 3E). Nevertheless, in the High-dose group, 64.28% of the cells had slow firing, whereas only 35.71% belonged to fast firing cells (Fig. 3E). Thus, it was speculated that long-term intake of methylphenidate leads to a significant reduction in the ratio of dopaminergic neurons with fast firing in the substantia nigra.

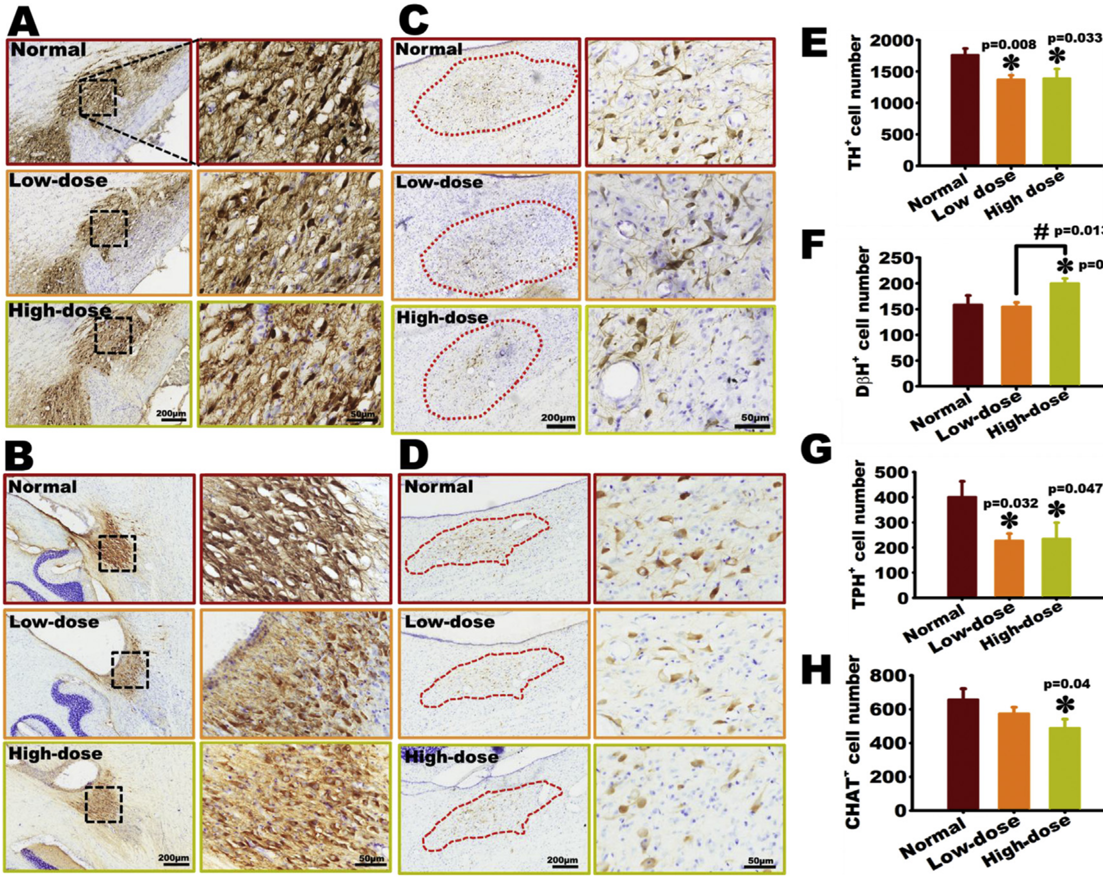

Fig. 2. Long-term intake of methylphenidate causes the alterations in neuron numbers of rat. The brain slices of the three groups were immunostained with anti-tyrosine hydroxylase to label the dopaminergic neurons in the substantia nigra (A). The right panel represents the magnified images of the regions (dashed box) in the left panel. After quantification, it was observed that the dopaminergic neurons within substantia nigra markedly reduced in the Low-dose and High-dose groups (E). The brain slices of the three groups were immunostained with anti-dopamine-β-hydroxylase to label the norepinephrine neurons in the locus coeruleus (B). The right panel was magnified images from the dashed regions of the left panel. The results showed that the number of norepinephrine neurons significantly increased in the High-dose group (F). The brain slices of the three groups were immunostained with anti-tryptophan hydroxylase to label serotonin neurons in the dorsal raphe nucleus (C). The right panel indicates the region (dashed circle) of magnification for the left panel. The quantitative results demonstrated a significant reduction in the number of serotonin neurons located in the dorsal raphe nucleus for the Low-dose and High-dose groups (G). The brain slices of the three groups were immunostained with anti-choline acetyltransferase to label the cholinergic neurons located in the tegmental nucleus (D). The right panel was magnified images from the dashed regions of the left panel. After quantification, a significant decrease of cholinergic neurons was found in the tegmental nucleus of the High-dose group (H). $*p < 0.05$ High-dose group or Low-dose group *vs* the Normal group. $\#p < 0.05$ High-dose group *vs* the Low-dose group.

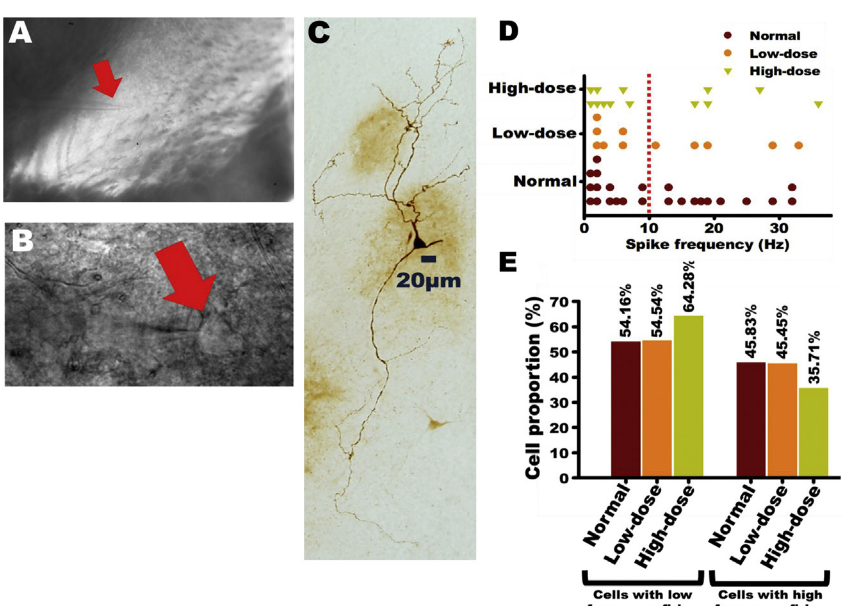

Fig. 3. Long-term intake of methylphenidate modulates membrane physiological properties of dopaminergic neurons. The glass electrode was located in the substantia nigra compacta of the brain slice (A; arrow). The magnified image shows a glass electrode was inserted into the cell body of a single neuron (B; arrow). After recording, the cell was injected with biocytin and labeled with DAB to observe its location and morphology (C). For whole-cell patch-clamp recording, a 50 pA, 1-s inward current was given to the neuron in compacta part of the substantia nigra to generate an action potential. A categorical scatter chart was plotted according to the spike firing frequency (D), in which a dashed line at 10 Hz divided cells into those with spike frequency > 10 Hz (cells with high-frequency firing) and < 10 Hz (cells with low-frequency firing). The percentage of cells with low-frequency firing was 64% for the High-dose group, which was significantly higher than those in the Normal and Low-dose groups (E).

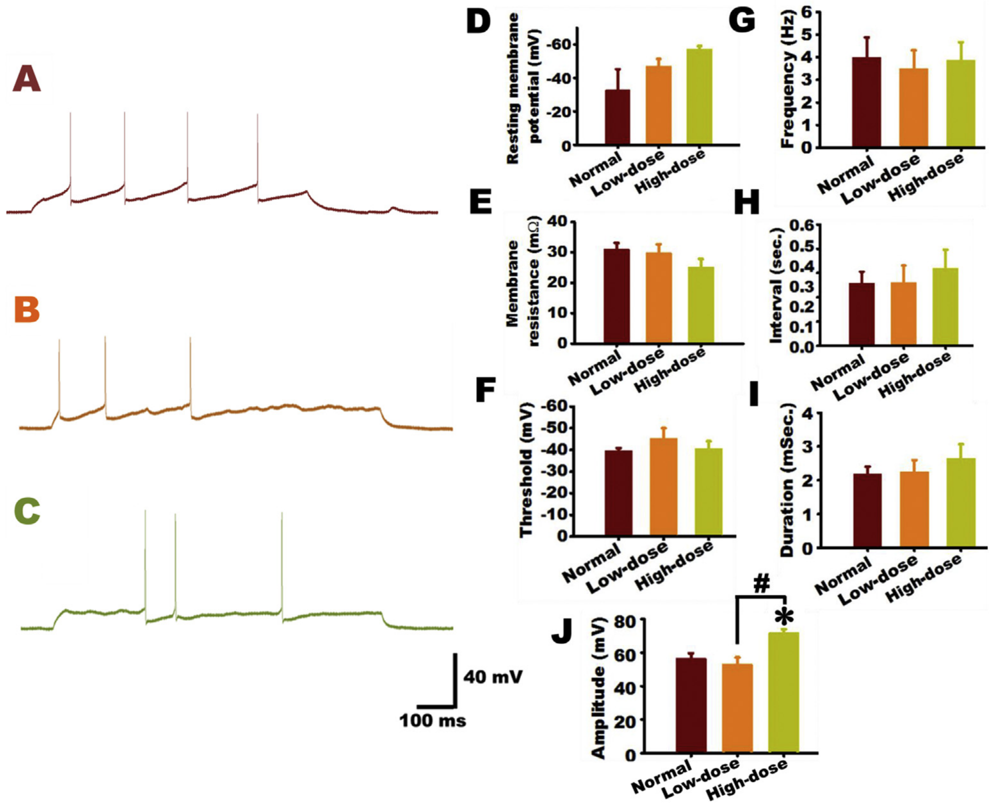

Fig. 4. Long-term intake of high dose methylphenidate increases the amplitude of dopaminergic neurons with low-frequency firing. A 50 pA, 1-s inward current was given to the neurons with low-frequency firing in the substantia nigra compacta to generate action potential. The firing patterns of the neurons with low-frequency firing were similar between the three groups (A, B, and C). There were no statistical differences between the three groups regarding cellular resting membrane potential (D), membrane resistance (E), and threshold (F), frequency (G), interval (H), and duration (I) of the action potential. Nevertheless, long-term intake of high-dose methylphenidate increases the amplitude of neurons with low-frequency firing (J). ∗ High-dose group *vs* the Normal group, $p = 0.0017$. # High-dose group *vs* the Low-dose group, $p = 0.0039$.

3.10. *Long-term intake of high-dose methylphenidate increases the amplitude of action potential in dopaminergic neurons with low-frequency firing*

Since majority of neurons showing slow firing upon 50 pA depolarizing current injection after long-term treatment of methylphenidate, we first further examined and compared other electrophysiological properties of slow firing neurons among the three groups. No significant difference was found among the Normal, Low-dose, and High-dose groups regarding firing patterns (Fig. 4A–C). The resting membrane potential, input resistance, and threshold, frequency, interval, and duration to develop action potential were further quantified. There was no statistical significance among the three groups (Fig. 4D–I). However, the amplitude of action potential of neurons with low-frequency firing increased to 71.34 ± 2.72 mV in the High-dose group, which was significantly higher than the Normal and Low-dose groups (Fig. 4J).

3.11. *Long-term intake of methylphenidate does not affect the membrane physiology of dopaminergic neurons with high-frequency firing*

As for dopaminergic neurons with fast firing, there was no significant difference among the Normal, Low-dose, and High-dose groups (Fig. 5A–C). The resting membrane potential, input resistance, and threshold, frequency, interval, duration, and amplitude for developing action potential were further quantified, and no statistical significance was observed among the three groups (Fig. 5D- J).

4. Discussion

Clinical studies have found that children aged 6–11 years who continuously took methylphenidate for six months or five years had a relatively lower body weight than regular children (Benard et al., 2015; Poulton and Cowell, 2003). Animal experiments showed that rats continuously administered with methylphenidate for 90 days had decreased body weights as compared with the Normal groups (Teo et al., 2002). Both clinical studies and animal experiments indicated that administration of methylphenidate leads to reduced body weights during the administration period, which was in line with the results of the present study.

A previous report has demonstrated that mice continuously administered with methylphenidate for 10 days increased travel time in an open-field test (Wooters et al., 2011). When rats were given methylphenidate of 2 mg/kg via intraperitoneal injection or 5 mg/kg through oral gavage, the mobility increased and maintained until two hours after administration (Gerasimov et al., 2000). Nevertheless, the results of the present study did not show increased mobility during the treatment period. It is possible that, in this study, the open field test was performed in the morning before drug administration, and there was a 13-h gap between the time point and the previous treatment on the day before. While the behavioral tests conducted in the two aforementioned studies were performed right after drug administration, it may lead to the different results obtained. Additionally, only 1% of the blood methylphenidate concentration was found left after it was injected intravenously four hours later (Aoyama et al., 1997; Wargin et al., 1983). Thus, the results of the behavioral experiments in this study could exclude the pharmaco-dynamic effects of methylphenidate, but to attribute to its impacts from long-term treatment. The locomotion in the High-dose and Low-dose groups at the age of eight and a half months significantly rose, suggesting that the short-term rebound was responded to the 7 month-treatment and subsequent 2 week-withdrawal. In fact, multiple studies have brought up the same opinion that long-term intake of methylphenidate results in behavioral alterations (Schmitz et al., 2017; Sekine et al., 2001, 2003).

During the first two postnatal weeks, the number of dopaminergic neuron in substantia nigra continued to increase. Adult levels were reached at P28, then, the cell number maintained stable from P28 to P90 (Lieb et al., 1996). In this study, rats were given with methylphenidate when they were one month of age, suggesting that the development of dopaminergic neuron were steady. Previous study showed that rats fed with 10 mg/kg methylphenidate once per day, a prominent

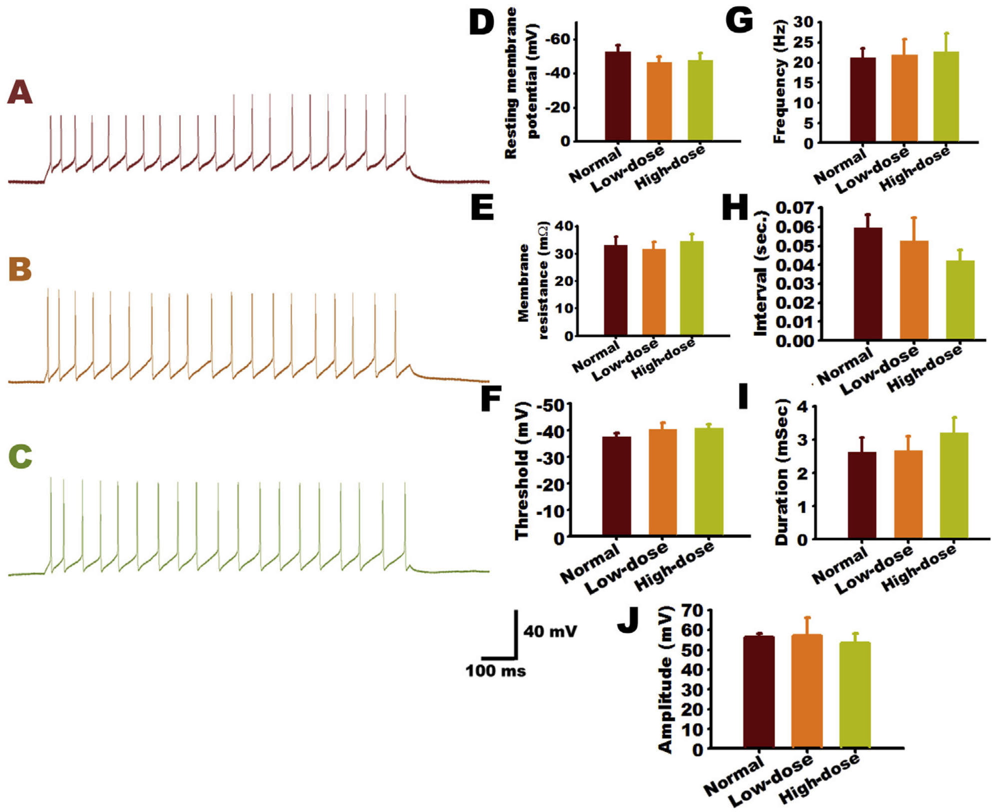

Fig. 5. Long-term intake of methylphenidate does not change the membrane physiological property of dopaminergic neurons with high-frequency firing. A 50 pA, 1-s inward current was given to the neurons with high-frequency firing in the substantia nigra compacta to generate an action potential. The firing patterns of the neurons with high-frequency firing were similar between the three groups (A, B, and C). No statistical differences were found between the three groups regarding cellular resting membrane potential (D), membrane resistance (E), and threshold (F), frequency (G), interval (H), duration (I), and amplitude (J) of the action potential.

reduction in the number of dopaminergic neurons and an increase in the number of microglia were identified in the substantia nigra (Sadasivan et al., 2012). In agreement with their study, rats administered with 2 mg/kg and 10 mg/kg methylphenidate displayed a significant decrease in the number of dopaminergic neuron within the substantia nigra. We suggest that long-term treatment of metylphenidate may induce the cell death of dopaminergic neurons or trigger the negative feedback mechanism to reduce of tyrosine-hydrolyase synthesis in dopaminergic neurons. The reduced cell number or activity of dopaminergic neurons in the groups of 2 mg/kg and 10 mg/kg methylphenidate may eventually lead to the shortage of dopamine concentration in the brain. Accumulative evidences have been provided that dopamine mediates dendritic/axonal growth (Parish et al., 2001; Penrod et al., 2015; Shinohara et al., 2018), facilitates the induction of Hebbian synaptic plasticity, accelerates the process of learning and memory (Gangarossa and Valjent, 2012; Ntamati and Lüscher, 2016; Sariñana et al., 2014).

Dopamine is converted into norepinephrine by dopamine − β − hydroxylase. Thus, as methylphenidate affects dopamine levels, it could also alter the biogenesis of norepinephrine (Agster et al., 2011; Hartman et al., 1972). Previous studies showed that, when dopamine was given locally, it suppressed the firing of norepinephrine neurons in the locus ceruleous (Cedarbaum and Aghajanian, 1977; Elam et al., 1986; Guiard et al., 2008a). In addition, when dopaminergic neurons in the ventral tegmental area (VTA) was depleted partially, the firing activity of the locus ceruleous norepinephrine neurons increased immediately (Guiard et al., 2008b). In the present study, an increase in the number of norepinephrine neurons in the High-dose group was observed. Therefore, it was speculated that the methylphenidate-induced reduction in dopaminergic neurons, which possess the inhibitory effect toward norepinephrine neurons, may, in turn, lead to an increased number of locus ceruleous norepinephrine neurons or increased enzyme activity of dopamine-β − hydroxylase, similar results were reported by the studies of Hashimoto et al. (2007).

In addition to the changes in norepinephrine neurons, Kharas et al. also suggested that long-term intake of methylphenidate modulated physiological properties of dorsal raphe nucleus neurons (Kharas et al., 2017). While in the present study, long-term administration of methylphenidate leads to reduced serotonin neurons significantly in the dorsal raphe nucleus. Previous studies showed that long-term intake of methylphenidate causes increased microglial activity in multiple brain areas, along with elevated inflammatory reactions (Gonçalves et al., 2010; Sekine et al., 2008). Nevertheless, from the results of immunostaining, there was no strong microglial activity observed in the present study, which may attribute to the different treatment periods or that the inflammatory responses may have already ended at the time of sacrifice.

The electrophysiological properties of neurons in each group were examined with whole-cell patch recording. Using firing frequency of 10 Hz as a category boundary, dopaminergic neurons in the substantia nigra pars compacta could be divided into cells with slow firing and those with fast firing. The firing rate of neurons represents the quantity of neurotransmitter that they release. For cells with lower firing frequency (4.00 ± 0.88 Hz), the spike duration was 2.19 ± 0.21 msec, whereas the other group of cells with higher firing frequency (21.27 ± 2.16 Hz) had a spike-duration of 2.63 ± 0.44 msec. The results were in agreement with previous studies (Chiodo, 1988; Grace and Bunney, 1983; Steffensen et al., 1998; Wang, 1981) as the spike duration was longer than 1 msec in cells with either low- or high-frequency spiking. In contrast, the spike duration of GABAergic neurons should be shorter than 1 msec. Therefore, when observed using infrared microscopy or cells' spike duration, the membrane physiological properties recorded exhibited similarity with the dopaminergic neurons in the substantia nigra pars compacta but were different from the GABAergic neurons in the substantia nigra pars reticulate (Grace and Onn, 1989).

When analyzing the electrophysiological properties, around 45% of the neurons in the Normal and Low-dose groups had high-frequency firings. On the contrary, around 35% of the neurons displaying high-frequency firing in the High-dose group, suggesting that long-term treatment with high-dose methylphenidate may cause a reduced release of dopamine in response to a stimulus. Moreover, the quantitative results from immunostaining indicate that long-term, low-dose methylphenidate treatment leads to significant decline in the number of dopaminergic neurons. On the other hand, the reduced total number of dopaminergic neurons and the decreased ratio of dopaminergic neurons with high-frequency firing may cause the shortage of dopamine concentration in the brain. Nevertheless, in rats receiving long-term, high-dose methylphenidate treatment, the dopaminergic neurons with low-frequency firing in the substantia nigra pars compacta raised to 65%. In the meantime, the spike amplitude of the dopaminergic neurons with low-frequency firing increased substantially, suggesting that the dopamine released may increase when excited, in order to complement the shortage of dopamine in the substantia nigra pars compacta after long-term, high-dose methylphenidate intake.

5. Conclusions

The present study showed that although long-term intake of methylphenidate does not cause acute inflammatory responses in rats, the number of neuronal cells and their physiological characteristics has been changed. Our results provide new important message on the change of dopamine after long-term medication of methylphenidate. Therefore, treatment with methylphenidate should be under rigorous monitoring and long-term follow-up for its impacts on physiology, behavior, learning and memory.

Conflicts of interest

The authors have declared that no conflict of interest exists.

CRediT authorship contribution statement

Sanford PC. Hsu: Data curation, Funding acquisition, Investigation. **Dao-Yuan Wang:** Data curation, Investigation, Methodology. **Ming-Yuan Min:** Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Yu-Show Fu:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Min MY and Fu YS designed the experiments, acquired, analyzed and interpreted data, and drafted the manuscript. Hsu SPC and Wang DY supported the materials and acquired data, analyzed, interpreted data, and contributed to manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.11.001.

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