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LOCUS COERULEUS NEURONAL AND BEHAVIORAL ACTIVITY FOLLOWING ACUTE AND CHRONIC METHYLPHENIDATE

Bin Tang[1] --- Nachum Dafny[2]†

[1,2]Dept. of Neurobiology and Anatomy University of Texas Medical School at Houston, USA

ABSTRACT

Methylphenidate (MPD) is one of the choices to treat attention-deficit / hyperactivity disorder (ADHD), and its mechanism of action is not clear. Concomitant behavioral and locus coeruleus (LC) neuronal activity were recorded following acute and chronic (0.6, 2.5 and 10 mg/kg) MPD in freely moving rats. The experiment last for 10 days. (1) The behavioral recording showed that acute MPD increases in locomotor activity in a dose dependent manner. (2) The same dose of chronic MPD administration elicits in some animals behavioral sensitization and in others behavioral tolerance. (3) The majority of the LC unit responded to acute MPD exposure by increase their firing rate. (4) The baseline activity on experimental day 10 (ED 10) after six daily repetitive MPD exposure was modulated in most of the LC units. (5) More than 90% of the LC unit respond to chronic MPD exposure and the majority of them by decrease their firing rate compared to the initial MPD effect. (6) The neuronal response to acute and chronic MPD recorded from animals expressing behavioral sensitization was significant difference from the LC units recorded from animals that expressed behavioral tolerance. Results indicated that the LC neuronal activities may contribute to the expression of behavioral sensitization and tolerance induced by chronic MPD administration and suggested that it is essential to record the animal's behavioral responses concomitantly with the LC neuronal activity events.

Keywords: Attention-deficit / hyperactivity disorder, Behavior, Neurophysiology norepinephrine, Neurotransmitters, Tolerance, Sensitization.

Contribution/ Originality

This paper is the first study which reported the acute and chronic dose response property of MPD on LC neuronal activity recorded concomitant with animal behavior. The study show neuronal activity recorded from behavioral sensitized animal response to MPD differently compare to those LC units recorded from behavioral tolerance animals.

† Corresponding author

1. INTRODUCTION

Methylphenidate (Ritalin, MPD) is widely used as a pharmacotherapy for ADHD [1-3]. ADHD is a complex and heterogeneous disorder and its etiology is not yet understood. A dysregulation of the central noradrenergic system has been hypothesized as one of the underlying pathophysiology characteristics of ADHD [4-6]. Indeed, MPD enhances noradrenergic transmission by binding to norepinephrine (NE) transporter (NET), thus blocking NE reuptake from the synaptic cleft to presynaptic terminals [7]. NE is mainly synthesized in the locus coeruleus (LC) and it is known that LC discharge correlates with forebrain NE concentration [8, 9]. LC-noradrenergic neurons project broadly throughout the central nervous system [10, 11]. Considerable evidence suggests that the LC-NE system modulates both attention and arousal related processes [12]. LC neurons are electrophysiologically quiet during low vigilance states such as sleep or in the lack of sensory input, while the LC neurons markedly increase their firing rate during active walking or when exposed to a salient stimulus [13-16]. It has been reported that acute MPD administration increases the locomotor activity in a dose dependent manner [17-27] as well the LC neuronal activity [28] in freely moving rats. Considering the function of LC in vigilance and attention, we hypothesize that the LC neurons may play an important role in the behaviors induced by chronic MPD administration.

It has been reported that the same dose of chronic MPD exposure can elicit behavioral sensitization in some animals and behavioral tolerance in other animals [22, 26, 29, 30]. Behavioral sensitization and tolerance are considered as the expression of the adverse effect [31, 32]. Behavioral sensitization is opposite from tolerance and defined as a further increase in behavioral response following repetitive psychostimulant exposure compared to the initial response [22, 26, 33, 34]. Behavioral sensitization or tolerance provides an experimental model for the induction of persistent changes in the neural circuitry of motivation and reward as a result of chronic exposure to psychostimulants.

The development of either behavioral tolerance or sensitization is thought to contribute to the establishment of drug dependence [31]. However, most of previous neurophysiological studies on the properties of MPD have been done *in-vitro* or *in-vivo* under anesthesia condition without monitoring the behavior of the animals. Thus, the aim of this study was to record the LC neuronal activity concomitant with the animal's behavioral activity from non-anesthetized, freely behaving rats previously implanted bilaterally with permanent semi microelectrodes using MPD dose response protocol. To our knowledge, this is the first report targeting the LC to study the effect of dose response MPD exposure using electrophysiological telemetric (wireless) recording technique combined with an animal behavioral assay to evaluate the LC neuronal activity based on the animal's behavioral response to acute and chronic MPD exposure.

2. METHODS

2.1. Animals

Thirty seven male 7- to 8-week-old Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 150-175g upon arrival were housed with free access to food and water for 3 to 5 days prior to electrode implantation. The room temperature was maintained at 21± 2°C with a relative humidity of 55-62% under 12 h:12 h alternating light-dark cycle (light on at 06:00). After electrode implantation, animals were individually housed in clear acrylic cages which were used as both home cage and test cage for the duration of the electrophysiological and the behavioral recordings.

2.2. Surgery

Animals were anesthetized with 50 mg/kg pentobarbital (i.p.). The head of animal was shaved and lidocaine hydrochloride topical gel was applied to the shaved area for local anesthetic. The animal was then placed into a stereotaxic instrument. A one inch incision was made on the scalp, the muscle was removed and the skull was exposed. Holes were drilled above the LC in both hemispheres at 9.3 mm posterior to bregma and 1 mm lateral to midline using the Paxinos and Watson [35] and an additional hole was drilled in the frontal sinus for the reference electrode. Two pair of low impedance 80 Ω ohm Nickel-Chromium electrodes (60 μm in diameter) Teflon insulated except at tips were twisted and each secured to 1 cm copper connector pins. These 2 pair of electrodes were placed into the LC in each hemisphere (i.e. each animal had four recording electrodes). Six anchor screws were inserted in the vacant spots to secure the skull cap with dental acrylic cement. During the placement of electrodes, the LC unit activity was monitored using a grass P511 amplifier with its cathode follower connected to an audiomonitor and oscilloscope. Electrodes were fixed to the skull only when spike activity exhibited at least a 3:1 signal to noise ratio; Otherwise, the electrode was lowered in the steps of 5 to 10 μm increments until a 3:1 ratio spike activity was observed, to maximum depth of 6.6 mm below the skull [28, 36-43]. The copper pins were inserted into an Amphenol plug which was fixed onto the skull with dental acrylic cement. Rats were allowed to recover from the surgical procedure for 4 to 7 days. During this recovery period, rats were daily placed with their home cage in the experimental room and connected to the wireless head stage (Triangle BioSystems Inc, TBSI, Durham, NC, USA) for about two hours to adapt and acclimate to the recording system. On the first recording day, the animals' weight was about 200 to 220g. All experimental procedures were approved by University of Texas health science center Animal Welfare Committee and in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.3. Drug

Methylphenidate hydrochloride (MPD) was obtained from Mallinckrot (Hazelwood, MO U.S.A.). MPD was dissolved in a 0.9% isotonic saline solution and the 0.6, 2.5 and 10.0 mg/Kg MPD doses were calculated as a free base. All MPD injections were equalized to a volume of 0.8

ml with 0.9% saline to keep all the injection volumes the same for all animals. Vehicle injections consisted of 0.8 ml isotonic saline solution (0.9% NaCl). MPD and vehicle were given by **intraperitoneal** injection (i.p.).

2.4. Experimental Protocol and Data Acquisition

The LC neuronal activity and the locomotive activity of the animal were recorded concomitantly using a wireless recording system (Triangle BioSystems Inc, TBSI, Durham, NC, USA) and an open field computerized animal activity system (Opto-M3, Columbus Instruments, Columbus, OH), respectively. The TBSI system is consisting of a wireless head stage (weight less than 5 g) and a remote receiver. The TBSI head stage was connected to the rat head cap containing the electrode pins that sent electrical signal (sampling rates up to 200 KHz) through a transmitter to the receiver that connected to an analog-to-digital converter (Micro1401-3; Cambridge Electronic Design (CED)).The neuronal activities from each electrode were collected and stored on a PC using the CED Spike2.7 software. The open field system is consisting of 40X20 cm cage with 16X8 infrared beam and their sensors which located 5 cm above the floor of the cage. Movement crosses any of the infrared beams result in a beam break and was subsequently recorded as total counts (TC) of locomotion. TC were compiled and downloaded to a computer in 10 minute bin increments.

Four groups of rats were randomly assigned: saline (control), 0.6, 2.5 and 10mg/kg MPD treatment groups. On experimental day one (ED1), rat was placed with their home cage in a Faraday testing box. The wireless head stage (TBSI) was connected to the electrode pins of the skull cap, and animal was allowed to acclimate for an additional 30 min prior to the recording session. After a saline injection, neuronal and locomotive activities were recorded concomitantly for 1 hour, this recording serves as baseline activity. Then, the animal receives saline or 0.6 or 2.5 or 10.0 mg/kg MPD injection (depending on the group, Table 1) and recordings were resumed for an additional hour. On ED2 through ED6 animals received daily injection of either saline (the control group) or a single dose of MPD (0.6, 2.5 or 10.0 mg/kg) in their home cage to initiate the MPD chronic effect [17, 19, 30, 44, 45]. On ED7 to ED9 the animals underwent a washout period, in those days no injections were given. On ED10, injection and recording was carried out identically as on ED1 (Table 1).

2.5. Data Analysis

2.5.1. Behavioral Data

Locomotor activity recorded from ED1 and ED10 was summed into 10 min bins for a total of 60 min after saline injection and another 60 min following MPD injection (i.e. 12 bins total on each experimental day). Three comparisons were made in each dose group as follows: (1) Locomotor activity after MPD administration on ED1 was compared to ED 1 saline baseline activity, which indicated the acute effect of MPD; (2) Locomotor activity after saline injection on ED10 was compared with that after saline injection on ED1, which indicated whether the baseline

change after chronic MPD administration; and (3) Locomotor activity after MPD administration on ED10 was compared to that following the initial MPD treatment on ED1, which could indicate whether sensitization or tolerance was expressed. Significant difference in individual animals was determined by the critical ratio test (C.R. = (E−C)/ ($\sqrt{E}$+C) = ±1.96 = $p < 0.05$) where C is the control and E is the drug activity. Significant difference among the groups (treatment days and drug dose) was determined by two-way ANOVA. $p<0.05$ was accepted as the minimal level of significance.

2.5.2. Neuronal Data

The recorded neuronal activity was replayed offline for spike sorting and statistical analysis using the CED Spike 2.7 software fixed template matching system. The data are captured by the program and processed using low and high pass filters (0.3-3.0 KHz). There are two window discriminator levels, one for positive-going spikes and one for negative-going spikes. The spikes were extracted when the input signal enters the previously determined amplitude window. Selected spikes with peak amplitude that enter the window were used to create a template. One thousand waveform data points were used to define the selected spike. The algorithm used to capture a spike allows the extraction of templates that provide high-dimensional reference points that can be used to discriminate consistent and accurate spike sorting. All temporal templates are compared with the selected spike event to find the best fitting template that yields the minimum residue variance. Secondly, a template matching procedure is then performed; when the distance between the template and waveform exceeds some threshold (80%) the waveforms are rejected. That means that the spike sorting accuracy in the reconstructed data is about 95%. The templates that were used to analyze the ED1 file were then loaded onto the ED10 file of the same electrode and the same animal to evaluate the neuronal activity on ED10. This ensured that the spike amplitude and pattern sorted from ED1 was the same on ED10. Once spike sorting was completed, the data were exported into a spread sheet which calculated the average neuronal firing rate and produced a sequential firing rate graph [40-42]. Statistical comparisons were made for each LC unit as follows: (1) LC unit firing rate after the initial MPD exposure was compared to LC unit firing rate following saline administration (saline baseline activity) on ED1 to determine the MPD acute effect; (2) LC unit firing rate after saline exposure on ED10 was compared with the LC unit firing rate after saline exposure on ED1 to find out whether six daily MPD exposure and three washout days modulated the ED10 baseline; (3) LC unit firing rate after rechallenge of MPD administration on ED10 was compared to LC unit firing rate after MPD exposure on ED10 to determine the chronic effect of the drugs as compared to the initial MPD effect. Significant change and direction of the change (decrease or increase) for each LC unit was determined by the critical ratio test (C.R. = (E−C)/ ($\sqrt{E}$+C) = ±1.96 = $p < 0.05$) where C is the control and E is the drug activity [22, 28, 37, 40]. In addition, the above data analysis was summarized into three subgroups matched on the basis of the animal's behavioral response to chronic MPD treatment as follows: (1) Electrophysiological data of all the LC units; (2) Electrophysiological data recorded from animals

exhibiting behavioral sensitization; (3) Electrophysiological data recorded from animals exhibiting behavioral tolerance. Significant difference among the groups was determined by the Chi-Square test. $p<0.05$ was accepted as the minimal level of significance.

2.6. Histological Verification of Electrode Placement

An overdose of sodium pentobarbital was administrated to rat at the end of the ED10 recording session. The rat was perfused intracardially with 10% formalin solution containing 3% potassium ferrocyanide, and a 50 μA DC current was passed through the electrode connector pin for 30 seconds to produce a small lesion in the recording sites. The brain was excised and stored in 10% formalin for subsequent histological processing. The position of the electrodes was confirmed by the location of the lesion and the Prussian blue spot using the Rat Brain Atlas [35].

3. RESULTS

Three hundred and fifty-seven LC units were recorded from 37 animals with 96 electrodes that were histologically confirmed to be within the LC (Figure 1) and exhibiting similar spike amplitude and pattern on ED1 and ED10. The locomotor activity and LC neuronal activity before and after acute and chronic dose response protocol of MPD exposure were concomitantly recorded. The correlation between the animal's behaviors and LC neuronal activities were evaluated.

3.1. Behavioral Results

3.1. 1. Effect of MPD on Locomotor Activities of all Animals (Fig 2A)

Following acute and repetitive injections of saline (n=6), all animals exhibit similar locomotor activity on ED1 and ED10. Similar observations were obtained previously using single and repetitive saline administration [17, 20, 26, 29, 30, 33, 44, 46]. Therefore the activity after saline injection on ED1 can be used as control for the effect of MPD.

On ED1, after acute 0.6 (n=9), 2.5 (n=11) and 10.0 mg/kg MPD (n=11) administration, the locomotor activities of the animals increased by 38%, 252% and 604% compare to their saline baseline activities (Fig 2A ED1).

The locomotor activity after rechallenge of 0.6 mg/kg and 2.5 mg/kg MPD on ED 10 (i.e. after five additional daily MPD exposures and three washout days) was not significantly different to activity after the initial (acute) 0.6 mg/kg and 2.5 mg/kg MPD on ED 1. The locomotor activity following 10mg/kg MPD rechallenge on ED 10 were significantly decreased by 32 % compared to the locomotor activity after acute 10 mg/kg MPD on ED1 respectively (One way RM AVOVA, $F=6.395$, $p<0.05$; Fig 2A).

In order to find out whether the same dose of chronic MPD can elicit either behavioral sensitization or behavioral tolerance, each animal was evaluated individually and separated into behavioral sensitization and behavioral tolerance subgroups by comparing the locomotor activity post MPD on ED10 to that of ED1 post MPD exposure.

3.1.2. Animals Expressing Behavioral Sensitization (Fig 2B)

Two (2/9, 22 %), 5 (5/11, 45 %) and 4 (4/11, 36 %) rats expressed a significant increase in their locomotor activity following rechallenge MPD on ED 10 compared to acute MPD exposure on ED 1 in the 0.6, 2.5 and 10 mg/kg MPD treatment group respectively (critical ratio test, $p<0.05$). The locomotor activities of these rats on ED10 compared to ED1 were significant different (One way RM ANOVA, $F=22.84$, $p<0.001$, Fig 2B). This increase of locomotor activity is considered to be expression of behavioral sensitization.

3.1.3. Animals Expressing Behavioral Tolerance (Fig 2C)

Seven (7/9, 78%), 6 (6/11, 55%) and 7 (7/11, 64%) rats expressed a significant decrease in their locomotor activity following MPD rechallenge on ED 10 compared to acute MPD exposure on ED 1 in 0.6, 2.5 and 10 mg/kg MPD treated groups respectively (critical ratio test, $p<0.05$). The locomotor activities of these rats on ED10 compared to ED1 were significant different (One way RM ANOVA, $F=15.205$, $p<0.001$, Fig 2C). This decrease of locomotor activity is considered to be expression of behavioral tolerance.

In summary, the observed data show that the same dose of MPD can elicit behavioral sensitization in some animals and behavioral tolerance in other animals.

3.2. Electrophysiological Results

Three hundred and fifty seven LC units were evaluated which exhibit identical spike amplitude and pattern on ED 1 and ED 10. The firing rates of 48, 94,106 and 109 LC units were evaluated following acute and repetitive saline, 0.6, 2.5 and 10 mg/kg MPD exposure respectively. .

3.2.1. The Effect of Saline (Control) on LC Units

Effects of saline injection were recorded in 48 LC units from 24 electrodes to obtain the control recording for animal handling and the injection volume. The neuronal activities were not significant changed after acute and repetitive saline injection (Table 2 and Fig 3) indicating that animal handling and the volume of the injection did not alter the LC units. Thus any significant change following MPD exposure compared to control will be considered as the drug effects.

3.2.2. Acute Effect of MPD on all LC Neuronal Activity (Table 2 A)

On ED1, following the initial dose of MPD administration, the firing rate of LC units were recorded and compared with their baseline activity.48% (45/94), 87% (92/106) and 83% (91/109) of the recorded LC units responded significantly ($p < 0.05$) to 0.6, 2.5 and 10 mg/kg MPD respectively. The majority of the responsive LC units exhibited increase in their firing rate following MPD exposure compared to their control baseline (Table 2A, acute effect). The percentage number of the LC units exhibiting an increase, decrease or no change was significantly (Chi-square test, $p < 0.05$) different among the 0.6, 2.5 and 10 mg/kg MPD treated groups (Table

2 A. Acute effect). Figure 4 shows two representative LC units that responded to 10 mg/kg MPD administration by significantly increasing its firing rate in A, and by decreasing its firing rate in B, as compared to their saline baseline firing rate.

3.2.3. Acute effect of MPD on the LC Units Recorded from Behavioral Sensitization and Tolerance Rats (Table 3)

Table 3 summarizes the initial effect of MPD on LC units recorded from the animals exhibiting behavioral sensitization and tolerance following repetitive MPD exposure. The majority of these responsive LC units to acute MPD exposure exhibited an increase in their firing rate. The percentage of LC units responding to acute MPD by increasing or decreasing firing rates showed a significant difference ($p < 0.05$, Chi-square test) between behavioral sensitization subgroups or behavioral tolerance subgroups from all three MPD dose groups.

3.2.4. Comparing ED 10 Baseline Activities to ED 1 Baseline Activity from all Rats (Table 2 B)

The baseline neuronal activity on ED 10 after six daily MPD exposure and three washout days were compared to ED 1 baseline activity. 94%, 100% and 95% of the recorded LC units exhibited a significant change in their baseline activity on ED10 compare to their baseline activity on ED 1 in 0.6, 2.5 and 10 mg/kg MPD treated groups respectively ($p < 0.05$).The majority of them exhibited a significant decrease in their baseline activity on ED10 compared with their baseline activity on ED1 (Table 2,B. ED10 BL vs. ED1 BL).

3.2.5. Comparing ED 10 Baseline Activities to ED 1 Baseline Activity of LC Units Recorded from Behavioral Sensitization and Tolerance Rats (Table 4)

The baseline activity on ED10 and ED 1 recorded from behaviorally sensitized and tolerance rats were compared and summarized in Table 4. In behavior sensitization rats, the majority of LC units showed a significant increase in ED 10 baseline activity compared to ED1 baseline activity, while in behavioral tolerance rats, the majority of LC units showed a significant decrease in ED10 baseline activity compared to D1 baseline activity respectively. Significant differences between behavioral sensitization and behavioral tolerance subgroups were observed in all the three doses of MPD treated groups (Chi-square test, $p<0.0001$).

3.2.6. Comparing the Effect of Rechallenge MPD on ED 10 to the Initial MPD Exposure on ED 1 in all LC Units (Table 2 C)

The effect of rechallenge administration of MPD on ED10 was compared to the effect of initial administration of MPD on ED1. 94% (88/94), 98% (104/106) and 94% (102/109) of the recorded LC units responded significantly ($p <0.05$) by changing their firing rate to 0.6, 2.5 and 10 mg/kg MPD respectively. The majority of LC units exhibited a significant decrease in their firing rate on ED 10 post MPD exposure compared to acute MPD on ED1 (Table 2C). The

further increase of LC neuronal activity on ED 10 compared with the increase of neuronal activity on ED 1 after MPD administration (Fig 5, upper panel) can indicate neurophysiological sensitization, while the no change of LC neuronal activity on ED 10 post MPD exposure compared to the activity on ED 1 post MPD exposure can indicate neurophysiological tolerance (Fig 5, lower panel). The percentage of increased, decreased and unresponsive LC units in the 0.6 mg/kg MPD treated group was significantly different from 2.5 and 10 mg/kg MPD groups (Chi-square test, $p < 0.001$. Table 2C, MPD ED 10 vs. MPD ED 1).

3.2.7. Comparing the Effect of Rechallenge MPD on ED 10 to the Initial MPD Exposure on ED 1 in LC Units Recorded from Behavioral Sensitization and Tolerance Rats (Table 5)

The effect of rechallenge MPD on ED10 compared to the initial MPD exposure on ED1 of LC units recorded from behavioral sensitization or tolerance rats were summarized in Table 5. 95 % (34/36), 97 % (35/36) and,89% (40/45) of the LC units recorded from behavioral sensitization rats, 93 % (54/58), 99 % (69/70) and 97% (62/64) of the LC units recorded from behavioral tolerance rats responded significantly (Chi-square test, $p<0.05$) by changing their firing rates on ED10 following rechallenge MPD compared to their firing rates following acute MPD exposure on ED1 in the 0.6, 2.5 and 10mg/kg MPD group respectively. The majority of the LC units recorded from behavioral sensitization rats exhibited further increase in their firing rate on ED 10 compared to the effect elicited by the initial MPD given on ED 1, while the majority of the LC units recorded from behavioral tolerance animals response to MPD rechallenge on ED 10 by decrease their neuronal activity compared to the initial effect of MPD on ED 1 (Table 5). The above difference was significant (Chi-square test, $p < 0.0001$).

4. DISCUSSION

MPD has been used for decades as a leading treatment for ADHD [1-3], but the neuronal mechanisms underlying its action are still unclear. The DA system has been focused in explaining the effect of MPD, however the potential contribution of NE has been overlooked and there are only few reports implicating NE in the pathophysiology of ADHD [4, 6]. It was reported that MPD can increase extracellular NE concentration by binding to NE transporter [7, 47]. The elevation of NE profoundly affects the performance and the attention of the subject, especially the maintenance of arousal which is known to be deficient in ADHD [48, 49].Therefore, the electrophysiological properties of LC neurons(the source of NE in the brain) and animal behavior following acute and chronic dose response MPD were concomitantly investigated in this study.

The main findings of this study are: (1) The same dose of chronic MPD administration can elicit behavioral sensitization or tolerance. 70%, 55% and 64% of the rats expressed behavioral tolerance and others expressed behavioral sensitization after chronic 0.6, 2.5 and 10 mg/kg MPD respectively. (2) 43%, 87% and 83% of LC units responded significantly to acute in 0.6, 2.5 and 10 mg/kg MPD group respectively. The acute effect of MPD on LC units recorded from animals

expressing behavioral sensitization compared to the LC units population recorded from animal expressing behavioral tolerance was not significantly different in all MPD groups. (3) 94%, 100% and 95% of LC units changed their baseline activity on ED10 compared to that on ED1 in 0.6, 2.5 and 10 mg/kg MPD group respectively. In the behaviorally sensitized animals most of the LC units exhibited a significant increase in their ED10 baseline neuronal activity compared to ED 1 baseline. Conversely there were more LC units which showed a significant decrease in their baseline neuronal activity on ED10 compared to their baseline neuronal activity on ED1 recorded in behaviorally tolerant rats. (4) 94%, 98% and 94% of LC units responded significantly by changing their firing rate to 0.6, 2.5 and 10 mg/kg MPD rechallenge on ED 10 compared to the initial effect of MPD exposure respectively. (5) The response pattern (how many units response and how many responded by increase or decrease) of LC unit population recorded from animals expressing behavioral sensitization was significantly different from those LC units population recorded in animals expressing behavioral tolerance. In behavioral sensitization animals, the majority of the LC units responded to MPD rechallenge on ED 10 by further increase their response compared to the initial effect on ED 1. This heighten response can consider as neurophysiological sensitization. While in behavioral tolerance animals, the majority of the LC units exhibited a decrease in their response to MPD rechallenge on ED 10 compared to the initial effect on ED 1. This attenuation response can consider as neurophysiological tolerance. The significantly different neuronal population between behavioral sensitization and behavioral tolerance rats confirm our hypothesis.

4.1. The Effect of MPD on the Locomotor Activity of Rats

In this experiment, acute and chronic administration of 0.6mg/kg MPD had no significant effect on the locomotor activity of the rats. While acute and rechallenge administration of 2.5 and 10mg/kg MPD significantly increased the locomotor activity on ED1 and ED10 respectively. This finding is consistent with previous studies where administration of MPD (2.5mg/kg or above) resulted in an increase in locomotor activity [17-23, 25, 26, 50]. In order to find out whether the same MPD dose can elicit behavioral sensitization or tolerance, each animal's behavior was evaluated individually; i.e. the locomotor activity after MPD administration on ED10 was compared to the effect of MPD obtained on ED1. As we hypothesized, the same dose of MPD elicited behavioral sensitization in some rats and behavioral tolerance in others.

4.2. Effect of MPD on the LC Neuronal Activity

Previous studies have revealed that LC neurons are tonically active during active waking and moving in animals [13-16, 28]. LC neuronal activity contributes to the maintenance of muscle tone in waking, and that reduction in LC discharge plays a role in the loss of muscle tone in cataplexy and rapid-eye-movement sleep [51]. These data indicate that LC neurons are electrophysiological quiet during low vigilance states such as sleep or in the lack of sensory input, while during active walking or exposed to a strong stimulus, the LC neurons markedly increase

their firing rate. Consistent with the above studies, the present experiment found that most of the recorded LC units were unresponsive to acute and chronic 0.6mg/kg MPD administration, while following acute and chronic 2.5 and 10 mg/kg MPD administration, most of the recorded LC units responded to the drug by exhibiting a significant increase in their firing rate, since 2.5 and 10 mg/kg administration of MPD resulted in an increase in locomotor activity but not 0.6 mg/kg MPD administration. This increased activity of LC neurons to MPD in freely moving rats can be explained by two possible mechanisms. First, the sensory feedback from behaviors elicited by MPD may be attributed to the activation of LC neurons, since LC neurons are phasically activated by a variety of sensory stimuli [13, 52]. Second, the LC receives projections from many different brain regions that release a wide spectrum of neurotransmitters, such as opiates, glutamate, GABA, serotonin, epinephrine [53]. The interactions of these neurotransmitters with LC neurons may be the other possible explanation for the increased firing of LC neurons elicited by MPD in freely moving rats.

4.3. Correlations between the LC Neuronal Activity and Animal Behaviors Induced by MPD Administration

The neural basis of behavioral sensitization or tolerance remains poorly understood. Changes of mesolimbic DA transmission, as well as in transcription factors, such as delta FosB and CREB (cAMP response element binding protein) has been implicated in behavioral sensitization or tolerance [31, 54]. However, there is no electrophysiological study concerning the relation between the neuronal activity and behavioral sensitization or tolerance thus far. The LC plays a major role in attention and behavioral flexibility [55, 56]. The activity of LC neurons varies not only with arousal state (wake-sleep cycle) but also with other behavioral types [12]. In addition, LC neuronal activity is involved in behavioral depression [57] and behavioral sensitization to cocaine [58]. These studies indicated the associated activation of LC neurons with animal behaviors. In order to elucidate the role of LC neuronal activities in the animal behaviors induced by chronic MPD administration, LC neuronal activities recorded in animals which exhibited behavioral sensitization were compared to the LC neuronal activities recorded in animals which exhibited behavioral tolerance. It was observed that the percentage of increase, decrease and unresponsive LC units following acute MPD were not significantly different between behavioral sensitization and tolerance rats for all three MPD dosages groups. These findings suggested that the acute effect of MPD on LC neuronal activities was not correlated with behavioral changes of the animals. However, the chronic effect of MPD administration showed significant differences in the response to MPD rechallenge between the LC units recorded from animals expressing behavioral sensitization to those LC units recorded from animals expressing behavioral tolerance.

It was reported that chronic exposure to psychostimulants causes long-lasting molecular up and down regulating of transcription factor that result in cellular changes in the central nervous system, such as protein synthesis, gene alteration and dendritic spine density modulation [54, 59,

60], which may affect the brain neuronal activity and play a pivotal role in the process of establishing behavioral dependence/addiction [31, 61]. In this experiment, after six days MPD administration and 3 washout days, the majority of LC units significantly changed their baseline activity. Behavioral sensitization animals express mainly increase in their ED10 baseline activity compared to ED1 baseline activity, and behavioral tolerance animals exhibiting mainly decrease in their baseline activity on ED10 compared to their baseline activity observed on ED1. It is possible to postulate that in those animals expressing increase activity in their ED10 baseline after the six daily MPD exposures is the result that elicits upregulation of transcription factors, protein synthesis and increase the neuropil. While the offsite occurs in animals expressing attenuation in their ED 10 baseline compared to ED 1 baseline. In addition, the LC units showed a significant difference in response to rechallenge MPD administration on ED10 compared with the initial MPD administration on ED1 between behavioral sensitization and tolerance subgroups. The majority of the LC units recorded from behavioral sensitization animals exhibited electrophysiological sensitization. Conversely, the majority of the LC units recorded from behavioral tolerance animals exhibited electrophysiological tolerance. These data suggested that the enhanced LC neuronal activity may contribute to the expression of behavioral sensitization, while the attenuated LC neuronal activity may contribute to the expression of behavioral tolerance after chronic MPD administration. Additional possible postulation that the increase in the response to MPD on ED 10 compared to ED 1 is the result of increase the neuropil, while the decrease in activity in response to MPD on ED10 compared to ED 1 is a result of decrease in the neuropil. This possibility needs further studies.

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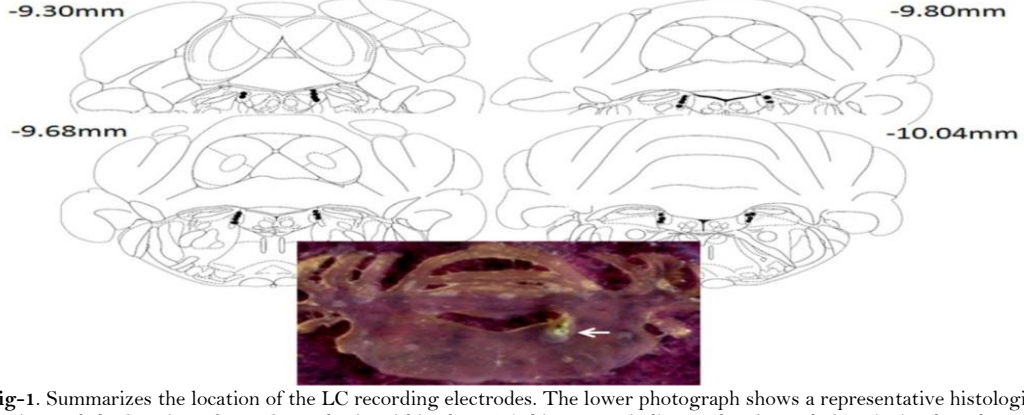

Fig-1. Summarizes the location of the LC recording electrodes. The lower photograph shows a representative histological section and the location of one electrode tip within the LC (white arrow indicates the electrode location). The others are reconstructs histologically sketched and summarize the electrode tip placements. The rat atlas plates represent the LC in serial coronal sections [35]. The number next to each section represents the posterior distance (mm) from bregma. Black

dots are designated to represent electrode placement of those that were found in the LC. Forty-eight pairs electrodes were securely placed in the LC and the recordings from these electrodes were evaluated.

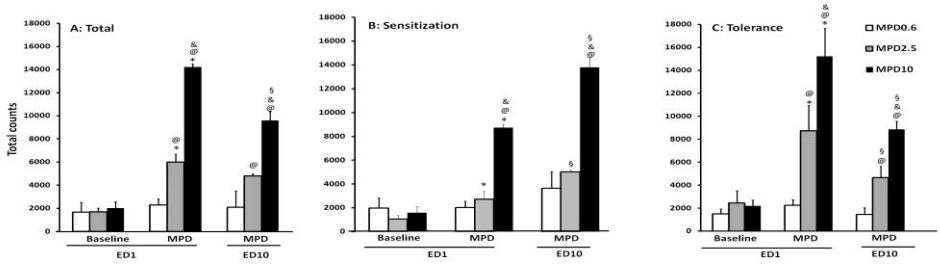

Fig-2. The figure summaries the locomotors activity of rats. A: total movement count of all rats to acute and chronic administration of 0.6, 2.5 and 10 mg/kg MPD. B: total movement count of the rats exhibited individually behavioral sensitization after chronic exposure of MPD. C: total movement count of the rats exhibited individually behavioral tolerance after MPD. *: p<0.05 vs. BL1; §: p<0.05 vs. ED1; @: p<0.05 vs. MPD 0.6; &: p<0.05 vs. MPD 2.5. ED1 and ED10- indicate the data recorded on experimental day 1 and day 10 respectively; MPD-methylphenidate; BL-baseline activity.

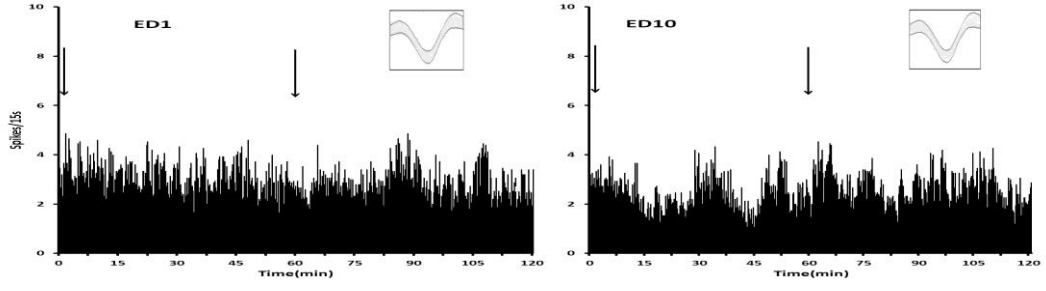

Fig-3. Representative sequential frequency firing rate of a LC unit recorded before and after saline injection on ED1 (A) and ED10 (B). The first 60 min shows the baseline activity after the first saline injection, followed by an additional 60 min recording after the second saline injection on ED1 and ED10. Arrow indicates the time of saline administration. Insert superimpose of 20 spikes sorted in same template on ED1 and ED10 respectively.

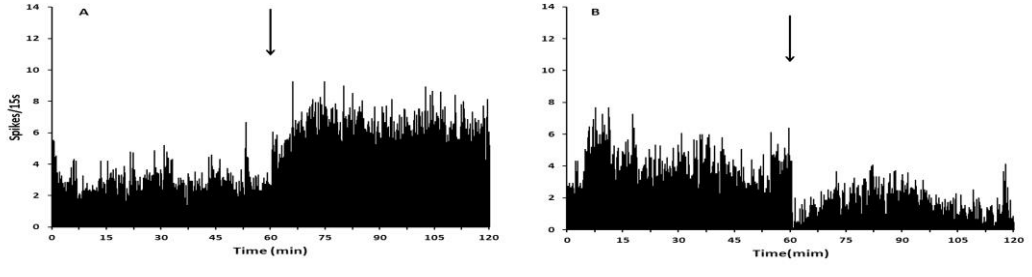

Fig-4. Show two (A and B) representative sequential frequency firing rate of two LC units on ED1. The first 60 min shows the baseline activity after saline injection followed by an additional 60 min after initial 10 mg/kg MPD administration. Arrow indicates the time of 10 mg/kg MPD administration. Unit in A exhibited an increase in firing rate after acute 10 mg/kg MPD administration. Unit in B exhibited a decrease in firing rate after acute 10 mg/kg MPD

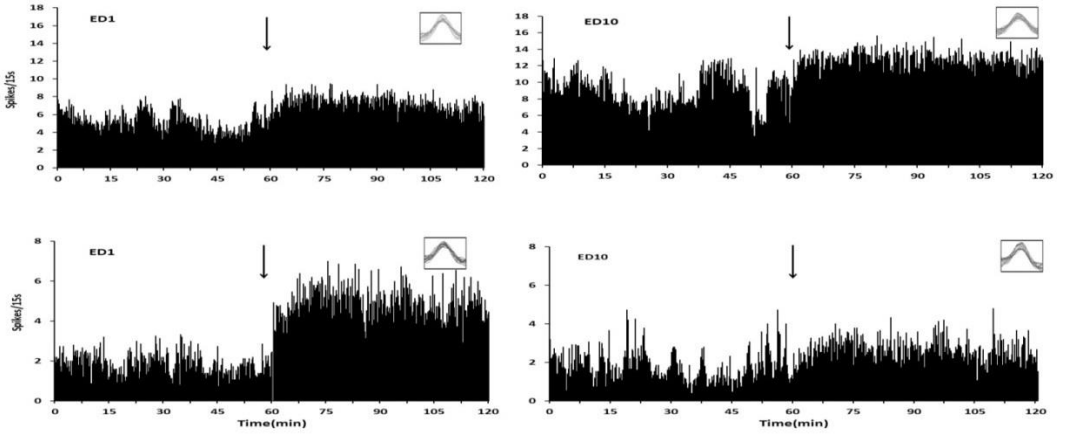

Fig-5. A representative sequential frequency firing rate of two LC units recorded on ED1 and again on ED10. The first 60 min shows the baseline activity after saline injection, followed by an additional 60 min after 10 mg/kg MPD administration. Arrow indicates the time of 10 mg/kg MPD administration. The upper panel shows a LC unit that exhibited a significant increase in its activity on ED1, the baseline activities on ED10 after 6 daily MPD exposure and three washout days compared to ED1 baseline exhibit increase, and rechallenge of MPD on ED10 exhibited further increase in the firing rate of this LC unit compared to MPD initial response on ED1. This phenomena can interpreted as that this LC unit expresses neurophysiological sensitization. The lower panel shows a LC unit that exhibited significant increases in its activities after initial MPD on ED1. The baseline activity on ED10 was attenuated compared to ED1 baseline. The activity following MPD on ED10 exhibited attenuation compared to the activity following MPD on ED1. This phenomena can interpreted as neurophysiological tolerance. Insert superimpose of 20 spikes sorted in same template on ED1 and ED10 respectively.

Table-1. Experimental protocol (injection and recording)

Groups	ED1 *	ED2-6	ED7-9	ED10*
Saline control	Saline/Saline	Saline	W.O	Saline/Saline
0.6mg/kg MPD	Saline/0.6 MPD	0.6 MPD	W.O	Saline/0.6 MPD
2.5mg/kg MPD	Saline/2.5 MPD	2.5 MPD	W.O	Saline/2.5 MPD
10mg/kg MPD	Saline/10 MPD	10 MPD	W.O	Saline/10 MPD

The table described the experiment protocol after 3-5 day post electrode implantation. ED: experiment day.*indicate: behavioral and electrophysiological recording days. W.O.: washout days, on these three days no injection were given. All injections were given in the morning at 0.8 ml volume in the animal's home cage.

Table-2. The effect of saline and MPD on all the LC neuronal activity

	Groupes	N	Increase	Decrease	Unresponsive
A. Initial (Acute) effect	saline	48	0	0	48(100%) $
	0.6 mg/kg	94	24(26%)	21(22%)	49(52%) *
	2.5 mg/kg	106	73(69%)	73(69%)	14(13%) #
	10 mg/kg	109	60(55%)	31(28%)	18(17%)

B. ED10 BL vs. ED1 BL	saline	48	0	0	48(100%) $
	0.6 mg/kg	94	31(33%)	57(61%)	6(6%) @
	2.5 mg/kg	106	46(43%)	60(57%)	0
	10 mg/kg	109	42(39%)	61(56%)	6(5%)
C. ED10 vs. ED1	saline	48	0	0	48(100%) $
	0.6 mg/kg	94	28(30%)	60(64%)	6(6%) @
	2.5 mg/kg	106	40(38%)	64(60%)	2(2%)
	10mg/kg	109	39(36%)	63(58%)	7(6%)

MPD-methylphenidate; ED-experimental day; BL- baseline activity; vs. – compare; $: p<0.001 compare with 0.6, 2.5 and 10mg/kg MPD group; * p<0.001 compare with 2.5 mg/kg and 10 mg/kg MPD group; #p <0.05 compare with 10 mg/kg MPD group; @ p <0.001 compare with 2.5 mg/kg and 10 mg/kg MPD group; §p <0.001 compare with 10 mg/kg MPD group.

Table-3. The acute effect of MPD on LC units on ED1 in behavioral sensitization and tolerance animals

			Effect of MPD compared to baseline activity on EDI		
Groups	Behavior	N	Increase	Decrease	No change
0.6mg/kg	Sensitization	36	11(31%)	7(19%)	18(50%)*
	Tolerance	58	13(23%)	14(24%)	31(53%)*
2.5mg/kg	Sensitization	36	26(72%)	7(19%)	3(9%) #
	Tolerance	70	48(69%)	12(17%)	10(14%) $
10mg/kg	Sensitization	45	24(53%)	12(27%)	9(20%)
	Tolerance	64	36(56%)	19(30%)	9(14%)

* p <0.0001 compared with 2.5 and 10 mg/kg MPD group; #p<0.001 between 2.5 and 10 mg/kg MPD group; $ p<0.05 between 2.5 and 10 mg/kg MPD group;

Table-4. The effect of MPD on the baseline activity of LC units on ED10 compare to the baseline activity in behavioral sensitization and tolerance animals

			Baseline on ED10 compare to baseline on ED1		
Groups	Behavior	N	Increase	Decrease	No change
0.6mg/kg	Sensitization	36	17(47%)	17(47%)	2(6%)* #
	Tolerance	58	13(22%)	40(69%)	5(9%)#
2.5mg/kg	Sensitization	36	24(67%)	12(33%)	0*
	Tolerance	70	22(31%)	48(69%)	0
10mg/kg	Sensitization	45	26(58%)	17(38%)	2(4%)*
	Tolerance	64	16(25%)	44(69%)	4(6%)

* p<0.0001 between behavioral sensitization and tolerance subgroups;#p<0.0001 between 0.6 and 2.5 mg/kg MPD group.

Table-5. The effect of MPD to LC units on ED10 compared to that on ED1 in behavioral sensitization and tolerance animals

			Baseline on ED10 compare to baseline on ED1		
Groups	Behavior	N	Increase	Decrease	No change
0.6mg/kg	Sensitization	36	15(42%)	19(53%)	2(5%)*#
	Tolerance	58	13(22%)	41(71%)	4(7%)@
2.5mg/kg	Sensitization	36	19(53%)	16(44%)	1(3%) *$
	Tolerance	70	22(32%)	47(67%)	1(1%) $
10mg/kg	Sensitization	45	26(58%)	14(31%)	5(11%)*
	Tolerance	64	13(20%)	49(77%)	2(3%)

* p<0.0001 between behavioral sensitization and tolerance subgroups; #p<0.0001 between 0.6 and 10 mg/kg MPD group;@p<0.01between 0.6 and 10mg/kg MPD group; $p<0.01 between 2.5 and 10mg/kg MPD

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