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A Simple Behavioral Paradigm to Measure Impulsive Behavior in an Animal Model of Attention Deficit Hyperactivity Disorder (ADHD) of the Spontaneously Hypertensive Rats

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Abstract

Impulsiveness is an important component of many psychiatric disorders including Attention-deficit/hyperactivity disorder (ADHD). Although the neurobiological basis of ADHD is unresolved, behavioral tests in animal models have become indispensable tools for improving our understanding of this disorder. In the punishment/extinction paradigm, impulsivity is shown by subjects that persevere with responding despite punishment or unrewarded responses. Exploiting this principle, we developed a new behavioral test that would evaluate impulsivity in the most validated animal model of ADHD of the Spontaneously Hypertensive rat (SHR) as compared with the normotensive "control" strain, the Wistar Kyoto rat (WKY). In this paradigm we call the Electro-Foot Shock aversive water Drinking test (EFSDT), water-deprived rats should pass over an electrified quadrant of the EFSDT apparatus to drink water. We reasoned that impulsive animals show increased frequency to drink water even with the presentation of an aversive consequence (electro-shock). Through this assay, we showed that the SHR was more impulsive than the WKY as it demonstrated more "drinking attempts" and drinking frequency. Methylphenidate, the most widely used ADHD medication, significantly reduced drinking frequency of both SHR and WKY in the EFSDT. Thus, the present assay may be considered as another behavioral tool to measure impulsivity in animal disease models, especially in the context of ADHD.

Key Words: Impulsivity, ADHD, SHR, WKY, Methylphenidate

INTRODUCTION

Impulsivity is defined as a predisposition toward rapid, unplanned reactions to internal or external stimuli without regard to the negative consequences of these reactions to themselves or others (Moeller *et al.*, 2001). It is an action without foresight and is one of the main constituents of a number of psychiatric disorders such as mania, substance abuse and attention deficit hyperactivity disorder (ADHD) (Winstanley *et al.*, 2006). In ADHD, increases in the different levels of impulsivity are suggested to determine its different subtypes (e.g. hyperactive-impulsive, predominantly inattentive and combined type) (Sonuga-Barke, 2002; Nigg, 2003). ADHD is the most common neuropsychiatric disorder of childhood and it has a worldwide prevalence rate of 3-18%, depending on age, gender and the definition and specific assessment methods used (Jensen, 2006).

Measuring impulsivity in the laboratory is a daunting task as it is a diverse behavior, covering a variety of phenomena that may have independent biological mechanisms (Evenden, 1999). Nevertheless, test methods to measure impulsiveness have been developed and they are of the following categories: punishment/extinction, reward-directed or rapid-decision paradigms. In punishment/extinction paradigm, impulsivity is shown by the subjects when they persevere with responding despite punishment or the unrewarded responses. Reward-directed paradigms demonstrate impulsiveness when subjects prefer a smaller-but sooner reward over a larger-later reward. In rapid-decision paradigms, impulsivity is assessed when subjects make premature or disinhibited responses (Dougherty *et al.*, 2005). On the other hand, Winstanely *et al.* (2006) summarized these different behavioral paradigms into

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two groups: those that measure impulsive choice, and impulsive action. Impulsive choice is elucidated by the making of impulsive decisions, that is, impulsive subjects opt for smaller and immediate rewards more often than delayed but larger rewards while impulsive action refers to the inability to withhold from making a response. Delay discounting paradigms represent successfully the experiments that measure impulsive choice. Examples of behavioral paradigms that measure impulsive action are the stop-signal reaction time (SSRT) and the go/no-go tasks. In addition, the five-choice serial reaction time task (5-CSRT) also measures motoric impulsivity while concurrently gauging sustained or divided attention (Winstanley *et al.*, 2006).

Animal models help to simplify and promote the understanding of disorders. Much of the understanding in ADHD due to animal models. A number of animal models have been developed for ADHD and the most validated are the Spontaneously Hypertensive rats (SHR). The SHR, derived from the main progenitor Wistar-Kyoto rats (WKY) and originally developed as animal models of hypertension, also display the salient features of ADHD (e.g. hyperactivity, inattention and impulsiveness) (Sagvolden, 2000). The SHR were readily shown to be impulsive in various delay discounting paradigms (Bizot *et al.*, 2007; Fox *et al.*, 2008). SHR are more active than WKY (Hard *et al.*, 1985; Hendley *et al.*, 1985; Wultz *et al.*, 1990; Mook and Neuringer, 1994; Berger *et al.*, 1998; Sagvolden *et al.*, 1998), and tend to prefer immediate smaller rewards rather than delayed larger rewards (Mill *et al.*, 2005). However, there is a difficulty in demonstrating impulsive actions in SHR using the 5-CSRT thus the SHR have been criticized to not fully represent the symptoms of ADHD (van den Bergh *et al.*, 2006). It also remains to be known if SHR show impulsiveness in two other tasks, the go/no-go and the SSRT. It could be that the complexity. If this indeed is true, there is a need to develop relatively easier behavioral models that could measure impulsiveness with ease, without sacrificing good results.

In the present study, we present a simple but effective behavioral paradigm that measures impulsivity in an animal model of ADHD of the SHR (as compared with WKY). We call this the Electro-Foot Shock water Drinking aversive test (EFSDT) and this operates according to the concepts exploited in punishment/extinction paradigms. We report the results of a pilot experiment and conducted pharmacological validation to ensure reliability of the present assay to measure impulsivity, at least in an animal model of ADHD.

MATERIALS AND METHODS

Subjects

We used 4-week old male SHR and WKY rats supplied by Charles River, Japan via Orient Co. (Korea). Rats were housed in cob containing plastic cages placed in a temperature-controlled room (21 ± 1°C) under a reverse light/dark cycle (lights on at 07:00 until 19:00). They were allowed free access to water and standard laboratory food except during the experiments. Test sessions were performed during the light cycle, 3 days per week, one session per day. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23 revised 1985) and the Animal Care and Use Guidelines of Konkuk University, Korea.

Methods for baseline factors

Locomotor activity in open-field and the EFSDT box: Open-field test experiment was conducted in apparatus made of Plexiglas (42×42×50 cm). Rats were placed in the center of the apparatus and allowed to move freely. Behavioral data in the open-field test was recorded for 20 minute and analyzed using Noldus EthoVision software. Those data were analyzed as indicator of hyperactive properties. We also measured the basal locomotor activity levels of rats during the first two days of the training phase. Rats were placed in the EFSDT box and the distance travelled for 10minute were recorded via the Ethvision system.

Water consumption: We evaluated the total water consumption levels during the first two days of the test. For 3 consecutive days of the phase the basal water intake was observed in the two groups after water deprivation. Each day, subjects were weighed and given water for 1 hour through 100 ml calibrated water bottle. At the end of an hour, the consumption of water was measured nearest milliliter. Food was available during each of testing phase. Water intake was calculated at ml consumption.

Pain sensitivity: Separate groups of rats were tested for electroshock sensitivity. This test was performed by examining the ability of an automated Freeze Monitor system to reliably record immobility behavior displayed by rats subjected to a variety of experimental manipulations. A footshock (2 mA, 1 second) was delivered through the grid floor of the chamber for 10 minute (1 second duration/20 second inter-shock-interval). Behavioral responses in an automated Freeze Monitor system are measured manually.

Elevated plus maze test: We conducted elevated plus maze test as an assay of anxiety-related behavior. Rats are placed in the intersection of the four arms of the elevated plus maze and their behavior is typically recorded for 8 minute. The time spent on the open and closed arms were measured and the percentage of time spent (duration) in the each arms [100 ×each arms/(open+enclosed)] was calculated.

Cognitive ability: We measured number of drinking attempts in both strains during each training phase for cognitive ability. Rats were placed in the EFSDT box and the drinking attempts for 10minute were recorded.

Apparatus

The Electro-foot shock aversive water drinking test (EFSDT) box: Experiments were conducted in an EFSDT box measuring 60×60×30 cm. The box is made of wood, painted black, and divided into three compartments (start area, water area and a free area, Fig. 1). With the exception of the start area, the floor of the EFSDT box was made of grid electrified wire (Fig. 1). In the water area, a water bottle with a stainless steel nozzle was fitted from outside of the box so that the nozzle extended 4 cm into the box at a height of 6 cm above the floor. The Noldus Ethovision system (Noldus information technology, Wageningen, The Netherlands) was used to track movement of rats and frequency in each of the compartments of the EFSDT box.

Procedures

The EFSDT consisted of two phases; the training phase which lasted for two days and a testing phase which lasted for a day (Fig. 2). The procedures of each of the phases are described below.

Training phase: Prior to training, rats were individually housed and deprived of water for at least 18 hours. This is a moderate, but sufficient deprivation for motivating the animal to drink water. During the training phase, rats were removed from their home cage and placed individually in the start area of the EFSDT box. During this phase, whenever a rat licked water from the bottle for at least 5 second, an experimenter removed it from the water area and placed it back to the start area. Training lasted for 10 minute, for two consecutive days. No electroshock was presented with every drink made. After training, rats were granted free access to mineral water in their home cages for at least 4 hours.

Test phase: Subjects were deprived of water overnight (see above) before testing. In addition, they were orally administered with 8% NaCl solution (1 mg/kg) 30 minutes before tests for further motivation to drink water. The methods of the test phase are similar to those of the training phase. This time, however, an experimenter presented an electroshock punishment (2 mA, 0.5 second) whenever a rat has licked from the water bottle for at least 5 second. The number of drinking resulted in electroshocks (i.e. 5 second licking of the water bottle) was noted as the number of impulsive drinking and such data was used to demonstrate impulsivity in rats (i.e. persevering with drinking despite punishment). In addition, frequency of the animals in the water (shock) area the EFSDT box was also recorded via automatic systems (Noldus Ethovision b.v. Noldus information technology, Wageningen, The Netherlands). Such data, i.e. impulsive drinking attempt despite punishment, served as another measure of impulsiveness.

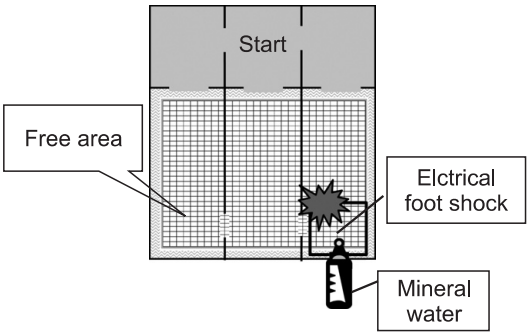

Fig. 1. Schematic diagram of electro-foot shock aversive water drinking test (EFSDT) apparatus. The box is made of wood and divided into three compartments (start area, water area and a free area. In water area, electroshock may be given to deter the thirst animal to drink water. Details of the apparatus and experimental protocol is described in materials and methods.

Pharmacological challenge: effect of methylphenidate to alleviate impulsivity in rats: The effect of methylphenidate, the most widely prescribed medication for ADHD, to reduce impulsivity in rats, was investigated. Methylphenidate was dissolved in physiologic saline and given intraperitoneally to rats at 2 and 5 mg/kg, 30 minute before testing (time corresponding to oral administration of 8% NaCl solution). The doses used are considered therapeutically relevant (Bizot *et al.*, 2007). As described in the test phase, the number of impulsive drinking (the number of more than 5 second water drinking, which resulted in electroshock) and the number of impulsive drinking attempt (the frequency of rats entering the water area) were recorded for analysis.

Data analysis

Statistical analysis was performed using GraphPad Prism Version 5. Un-paired Student's *t*-test was used to compare responses of SHR and WKY. When appropriate, one-way ANOVA, followed by Dunnet's test was used for other comparisons. Results were considered statistically significant when p values were $p<0.05$.

RESULTS

Baseline factors

In the present assay, we first determined several basal factors, which might affect the interpretation of the experimental results if different between SHR and WKY. General activity was measured in the open field. Consistent with the hyperactive phenotype of SHR, the locomotor activity (Fig. 3A) of SHR in the open field is significantly higher than that of WKY. We also measured the basal locomotor activity levels of rats during the first two days of the training phase. In Fig. 3B, it is noticeable that locomotive activity during the two days of the training phase did not vary between strains. Both SHR and WKY demonstrated decreased activity levels during the second day of training, however, this response was not different in the two strains. In our observation, it took an average of first drinking latency of 13.05 ± 1.67 second for SHR and 25.20 ± 6.70 second for WKY to locate the water area and eventually to drink water from the water bottle nozzle in the training phase. This result means SHR are more impulsive than WKY. There is also possibility that differences in thirst levels could influence motivation to drink water. We evaluated the possible participation of this confound thus, the total water consumption levels during the first two days of the test, as well as during the day which corresponds to the EFSDT were measured. In Fig. 3C, the level of water consumption did not vary in different strains, reflecting the lack of difference in

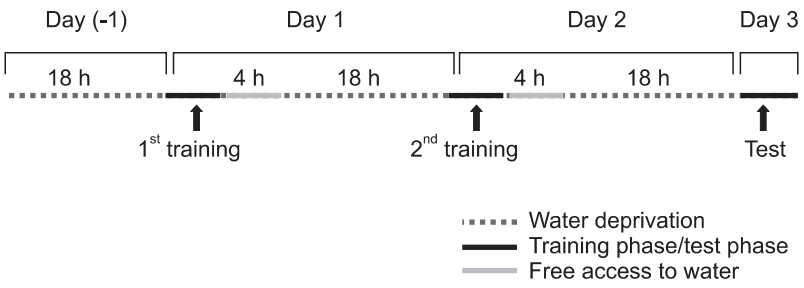

Fig. 2. Experimental schedule. The day before training rats were deprived of water for 18h and the rats were trained for two consecutive days to find the water area to drink water. On day 3, the thirst animal was punished by light electroshock in water area to deter the drinking attempt. For details, see methods.

thirst levels between SHR and WKY. Meanwhile, differences in sensitivity to pain or electro-shock may also affect performance in the EFSDT (i.e. those rats which are more sensitive to pain or punishment would rather not attempt to drink than those which are not). To address this, separate groups of rats were tested for electroshock sensitivity. As shown in Fig. 3D, no difference between freezing times was observed between SHR and WKY, indicating that pain sensitivity to electroshock are similar in the two strains. There is also possibility that differences in anxiety levels could influence try to go the water area to drink water. To evaluate this possibility, we conducted elevated plus maze test. As shown in Fig. 3E, no difference between the percentage of time spent (duration) in each arms was observed between SHR and WKY, indicating that anxiety

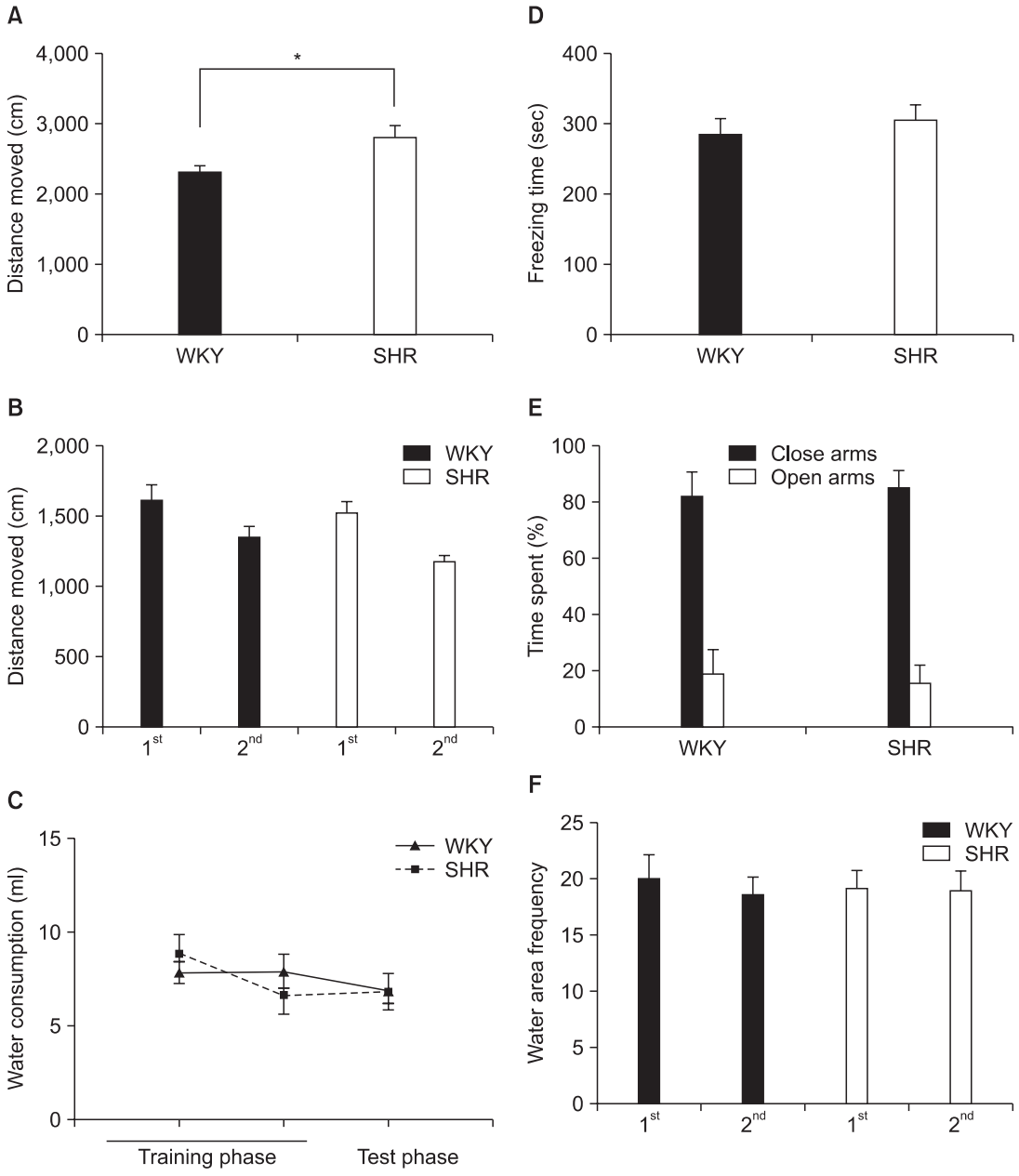

Fig. 3. Baseline data of SHR and WKY. (A) Hyperactive phenotype of SHR in open-field locomotive measurement. The distance moved in an open field was measured for 20 min. All data are expressed as mean ± S.E.M. SHR showed hyperactive phenotype compared with WKY. *Statistically significant difference compared with WKY (n=10, $p<0.05$). (B) Locomotive activity in EFSDT apparatus during two training session. (C) The amount of water consumption during training as well as test session. No difference was observed between SHR and WKY (n=10). (D) Sensitivity against electroshock. Freezing time after electroshock was measured as described. No difference was observed in electroshock sensitivity between the two strains (n=10). (E) Measurement of anxiety level. Anxiety level was measured using elevated plus maze as described. The time spent either in closed arm or open arm sector was not different in SHR and WKY (n=10). (F) Normal recognition of water area during the training session. To test whether SHR and WKY recognize water area in training session, we measured the number of frequency entering water area during the 1st and 2nd training session. No differences were observed (n=10).

levels are similar in those two strains. Finally, it is possible that differences in cognitive ability between rat strains could affect responses in EFSDT. However, as shown in Fig. 3F, no difference was observed in the ability of the rats to locate the water area of the box suggesting the normal cognitive function, at least in the impulsivity test setting.

Impulsivity in SHR as measured by the EFSDT

As stated in the Methods, two measures were used to demonstrate impulsivity in the subjects: (1) the number of impulsive attempt (the frequency entering in water/shock area of the EFSDT box). (2) the number of impulsive drinking (5 second water drinking resulted in electroshock punishment). As shown in Fig. 4A, SHR entered the water/shock area more frequently than their WKY counterparts [t (18)=2.733, p<0.01]. In Fig. 4B, SHR also received more electroshocks [t(18)=9.107, p<0.001] compared with WKY, indicating higher impulsive drinking frequency in this strain compared with WKY rats.

Effects of methylphenidate

Fig. 4 also shows the effects of methylphenidate treatment in SHR and WKY performing the EFSDT. As shown in Fig. 4A, methylphenidate treatment (2 and 5 mg/kg) did not affect staying frequency of rats in the water (shock) area, indicating lack of effect of the drug in reducing impulsive drinking attempts in both strains. Meanwhile, in Fig. 4B, methylphenidate treatment (2 and 5 mg/kg) significantly reduced impulsive drinking in SHR [F (2, 17)=22.90, p<0.001] and also in WKY [F (2, 17) =4.82, p<0.05].

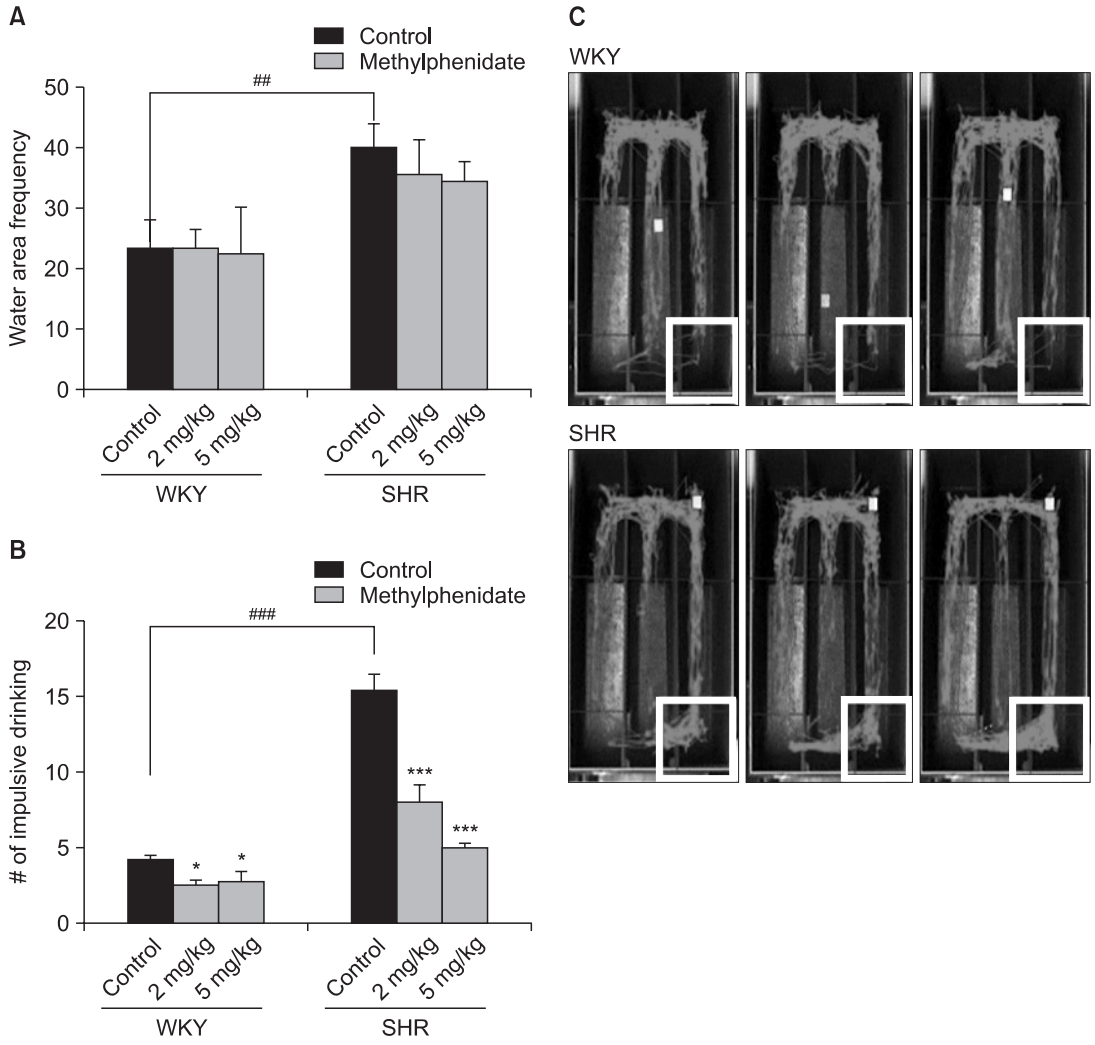

Fig. 4. Measurement of impulsivity by EFSDT and the effect of methylphenidate on impulsive behavior. WKY and SHR were trained and subjected to EFSDT as described. Before test, animals were intraperitoneally injected with methylphenidate (2 and 5 mg/kg). Control animals were injected with normal saline. Two parameters were determined as indices of impulsivity that is the frequency entering water/shock area (the number of impulsive attempt) (A), the number of impulsive drinking (B), which lasts for 5 s resulting in electroshock punishment and representative trace (C) of rats during electro-foot shock aversive water drinking test. Distance moved and movement duration of three rats from each control group in water/shock area was recorded by Ethovision 3.1 software. SHR control (bottom) made more drinking attempts in electro-shock area (inset box) than control strain, the WKY control (top) rats. All data are expressed as mean ± S.E.M. $^{\#}$represents significant difference as compared with WKY ($^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, n=10). *Represents significant difference compared with control in each strain (***p<0.001, n=10).

Representative trace of rats during EFSDT

In Fig. 4C, plotted tracks were depicted from three representative SHR and WKY rats which have performed the EFSDT. The square box corresponds to the water/shock area of the EFSDT box. Ethovision tracking shows more frequent entrances of SHR (bottom) in the water area of the box compared with WKY.

DISCUSSION

Behavioral test for impulsivity has been continuously devised and modified in behavioral neuropharmacological study of ADHD. We suggest a simple behavioral assay that could measure impulsivity in an ADHD animal model, i.e. the electro-foot shock water aversive drinking test (EFSDT). The idea is derived from the measurement of impulsivity through punishment/extinction paradigms. Moreover, the present test may measure impulsive action, one component of impulsive behavior. Optimal animal models should be similar to clinical cases in terms of etiology, biochemistry, symptomatology, and treatment (McKinney *et al*., 1969). Recently, criteria for assessing models for ADHD were proposed (Sagvolden, 2000). The best animal model for ADHD should ideally mimic ADHD in all respects: 1) impulsiveness; 2) sustained attention-deficit; and 3) like ADHD children, the model should not display hyperactivity in a novel environment but rather develop over time.

Albeit with the inconsistent results (Alsop, 2007), SHR fulfills many of these validation criteria and they exhibit all the behavioral characteristics of ADHD in most cases. Because some of the current behavioral models of impulsivity developed for use with rodents based on human neuropsychological tests such as the five-choice serial reaction time task, the stop-signal reaction time task and delay-discounting paradigms (Winstanley *et al*., 2006) does not provide simple and easy way to analyze impulsive phenotype, we conducted EFSDT in the most validated animal models of ADHD, the SHR. In EFSDT, impulsivity is measured by the persistence of the subjects to obtain a biological reward (water) despite the presentation of an aversive consequence (electroshock). Impulsivity is also shown by the number of impulsive drinking attempts (demonstrated by frequency in the water/shock area of the EFSDT apparatus) despite risk of electroshock. Using this protocol, we showed that the SHR showed significantly higher levels of impulsivity as compared with the normotensive strain, the WKY. Methylphenidate, the most widely used medication for ADHD, reduced impulsive drinking frequency (or impulsivity) in both strains, providing pharmacological validation on the reliability of the present protocol. Thus, we have not only shown the efficacy of the present paradigm to measure impulsivity but also demonstrated it effectively in the animal model of ADHD. The ability of methylphenidate to reduce impulsiveness in this rat strain has also been shown in this rat strain using other animal models of impulsivity (Bizot, 2007). In this study, the effect of methylphenidate on impulsive drinking was not restricted to the SHR as it also reduced impulsive drinking in WKY (Fig. 4B) suggesting methylphenidate is effective in reducing basal level of impulsive action even though further study is needed to validate the conclusion.

Another interesting finding in this study is that although SHR have higher impulsive drinking attempt (i.e. the frequency in water/shock area) compared with WKY, methylphenidate did not affect the impulsive drinking attempt (Fig. 4A). Whether the lack of methylphenidate's effect on impulsive drinking attempt may reflect the drug's inability to control the initial stage of impulsive action although it has profound effects on sustained impulsive action (Fig. 4B) would be an interesting topic to follow up in the future.

The various behavioral measures of impulsivity have been grouped into (1) those that measure impulsive choice or decision-making and (2) those that measure impulsive action or motoric impulsivity. Impulsive choice is manifested when for example, subjects prefer a smaller immediate reward to a larger-but delayed reward. This principle is exploited in the delay discounting paradigms and tests of this kind have successfully demonstrated impulsivity in both human and non-human subjects. On the other hand, impulsive action is shown by the inability of the subjects to withhold from making a response that could result to the delivery of a primary reinforcer (e.g. food or water) or other highly desirable rewards (Winstanley *et al*., 2006). While it is easy to gauge impulsive action in humans, it is challenging to do so in animals probably due to the complexity of the present behavioral assays (e.g. SSRT and go/no-go tasks).

Impulsivity has a multi-faceted nature (Winstanley *et al*., 2006), and the various behavioral expressions of impulsivity can broadly be divided into two categories (Evenden, 1999, Winstanley *et al*., 2006), namely behaviors resulting from deficits in the ability to withhold responding and thereby reflecting poor inhibitory control (impulsive action), and behaviors that do not result from inhibitory control deficits but result from insensitivity to delay of gratification or delay aversion and consequently lead to impulsive decision-making exemplified by increased preference for immediate reward over more beneficial but delayed reward (impulsive choice).

In conclusion, the EFSDT may also be considered as behavioral assessment tool that could measure impulsivity in animals. Some advantages of the present assay include the simplicity and measure more animal than ever in short time. Lastly, methylphenidate has been shown to alleviate impulsivity in the animals using this behavioral assay which further shows the applicability of the present protocol to preclinical studies in ADHD.

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