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Comparative Behavioral Correlation of High and Low-Performing Mice in the Forced Swim Test

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Abstract

Behavioral analysis in mice provided important contributions in helping understand and treat numerous neurobehavioral and neuropsychiatric disorders. The behavioral performance of animals and humans is widely different among individuals but the neurobehavioral mechanism of the innate difference is seldom investigated. Many neurologic conditions share comorbid symptoms that may have common pathophysiology and therapeutic strategy. The forced swim test (FST) has been commonly used to evaluate the "antidepressant" properties of drugs yet the individual difference analysis of this test was left scantly investigated along with the possible connection among other behavioral domains. This study conducted an FST-screening in outbred CD-1 male mice and segregated them into three groups: high performers (HP) or the active swimmers, middle performers (MP), and low performers (LP) or floaters. After which, a series of behavioral experiments were performed to measure their behavioral responses in the open field, elevated plus maze, Y maze, three-chamber social assay, novel object recognition, delay discounting task, and cliff avoidance reaction. The behavioral tests battery revealed that the three groups displayed seemingly correlated differences in locomotor activity and novel object recognition but not in other behaviors. This study suggests that the HP group in FST has higher locomotor activity and novelty-seeking tendencies compared to the other groups. These results may have important implications in creating behavior database in animal models that could be used for predicting interconnections of various behavioral domains, which eventually helps to understand the neurobiological mechanism controlling the behaviors in individual subjects.

Key Words: Forced swim test, Immobility, Locomotor activity, Object exploration, Novelty-seeking

INTRODUCTION

Behavioral neuroscience, also known as biological psychology (Rosenzweig *et al*., 2002), biopsychology, or psychobiology, is the approach of applying biological principles to the study of physiological, genetic, and developmental mechanisms of behavior in humans and other animals or simply, the study of brain mechanisms underlying the behavior. Knowing how and why things go wrong in the brain in various neurological and psychiatric disorders is as vital as understanding how the normal brain works to support cognition, emotion and sensorimotor function. Behavioral neuroscience had a strong history of contributing to the understanding of medical conditions and disorders, including those that fall under the umbrella of clinical psychology and biological psychopathology (also known as abnormal psychology). Although animal models usually do not meet the full face, construct and predictive validity of all mental illnesses, the field has solicited important therapeutic data on a variety of conditions such as Parkinson's disease, Huntington's disease, schizophrenia, autism, anxiety, drug abuse, alcoholism, and clinical depression.

There is no absolute and unified structure in assessing animal behaviors but some scientists categorized the behaviors into broad domains with corresponding behavioral test methods that can be performed (Karl *et al*., 2003; Crawley, 2007). The scope of the behavioral test is however not limited to one domain as some tests can draw various behavior parameters. For instance, the open field test measures the locomotor ac-

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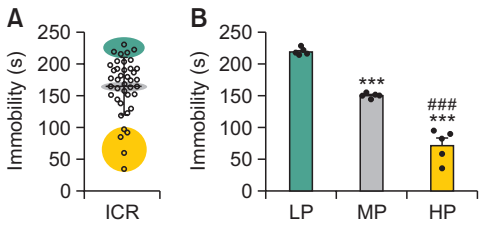

Fig. 1. FST screening and segregating into LP, MP and HP groups. (A) Scatter dot plot graph of immobility duration of ICR mice in FST and identification of 3 groups (n=40). (B) Comparison of immobility between LP, MP and HP (n=5). All data are presented as the mean ± SEM. ***p<0.001 vs. LP and ###p<0.001 vs. MP.

tivity of rodents based on the distance moved and movement duration, but the parameter of time spent in the center area may solicit information on the state of anxiety of the study subject (Prut and Belzung, 2003). Similarly, cognitive or decision-making tasks represent endpoints that may be aberrant in anxiety-related conditions wherein cognitive impairment is a core feature of depressive disorders frequently comorbid with anxiety disorders (Lezak *et al*., 2017), not to mention the neurodegenerative disorders such as Alzheimer's disease. Thus, one behavior aspect could be intertwined with another aspect and studying these connections may offer a tool of uncovering the brain regions or networks involved in various neurological conditions.

The forced swim test (FST) is one of the most common behavioral tests performed in rodents to evaluate the antidepressant potential of drug candidates. The mouse version of the forced swim test is a relatively short and low-cost behavioral test that requires no training of the mice and can be performed with minimal equipment. The mice that display high immobility throughout the trial period are interpreted as showing despair-like or depression-like behavior (Can *et al*., 2012). In screening for new therapeutics of depression, wild-type or normal mice show wide variation in performance and groups, and that high immobility was regarded as positive controls having depressive-like behavior. Thus, a rising number of researchers are contesting the validity of FST as a meaningful tool for depression-related studies. Recently, some researchers presented new insights that FST could be a test that evaluates the stress coping strategy rather than a depression-like behavior (Commons *et al*., 2017).

More generally, behavioral variability within a species is typically neglected in behavior research. The high variations in experimental results from individual subjects could be a big obstacle to various researchers but it may also remind the outstanding point that behavioral individuality needs to be accepted as part of research variables. Intriguingly, the identification of variability in the immobility profile of control mice in the FST could be one good starting point for the study of behavior domain interconnections. Considering the scarcity of study on the possible connection of FST responses to other behavioral domains, this paper targets to find out whether the divergent responders in FST could be classified and would also be accompanied by phenotype-dependent differences in other behavioral domains.

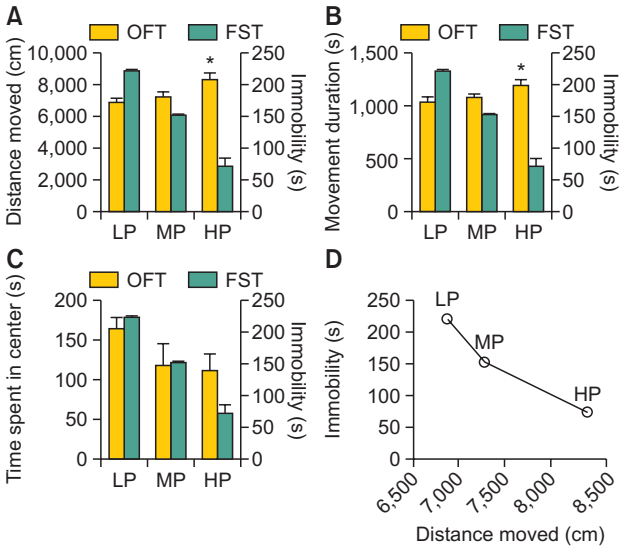

Fig. 2. Assessment of locomotor activity in OFT. (A-C) OFT parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. (A) Distance moved, (B) movement duration and (C) time spent in the center were measured. (D) A line graph depicting the means of immobility in FST and distance moved in OFT was also shown. All data are presented as the mean ± SEM. *p<0.05 vs. LP in the OFT. Significant differences in the FST immobility time can be referred to Fig. 1B.

MATERIALS AND METHODS

Animals

Male CD-1 mice (7 weeks old) were delivered from OrientBio (Seongnam, Korea). They were housed in a Plexiglas cage in groups of 10 and kept in a temperature- (22 ± 2°C) and humidity-controlled (55 ± 5%) animal room on an artificial 12-12 h light/dark cycle with lights on at 7 am. Habituation for a week in the new environment was done prior to initiation of experiments. Food and water were freely available except during the experiment and a special arrangement of behavioral tests. All experiments were performed between 09:00 to 16:00 and a minimum of 30 min acclimatization to the testing room was observed prior to every behavioral test. The whole behavioral study lasted for 6 weeks. All procedures and animal treatment were carried out in accordance with the Principles of Laboratory Animal Care (National Research Council, 2010) and were approved by the Animal Care and Use Committee of Konkuk University (KUIACUC), Korea (KU18054). All efforts were made to reduce the number of animals.

Forced swim test (FST)

All mice were identified by tail markings before initiating the FST. Subject mice were allowed to swim in transparent cylindrical Plexiglas tanks (30 cm in height×20 cm in diameter) filled with 15 cm-deep water. After carefully putting each mouse in the tank, the timer and video recorder were simultaneously started. Each trial lasted 6 min and the last 4 min were used for analysis. At the end of each test, animals were removed from the water, dried with a paper towel and transferred back to their home cages. The immobility duration was scored for each mouse similar to the technique used by the previous report (Can *et al*., 2012).

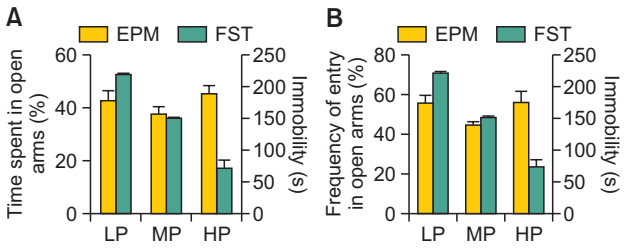

Fig. 3. Assessment of anxiety-related behaviors in the EPM. EPM parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. The percentage of (A) time spent in open arms and (B) frequency of entry in open arms were calculated. All data are presented as the mean ± SEM. No significant difference was observed between the three groups in the EPM test parameters. Significant differences in the FST immobility time can be referred to Fig. 1B.

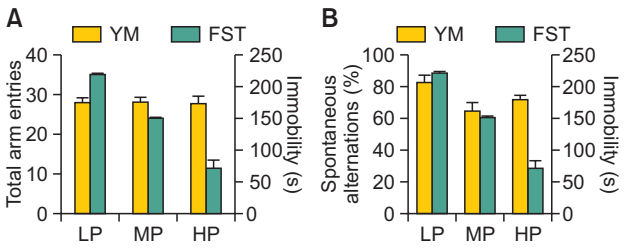

Fig. 4. Assessment of spontaneous alternation behaviors in the Y maze. Y maze parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. The (A) total arm entries and (B) spontaneous alternations were calculated. All data are presented as the mean ± SEM. No significant difference was observed in the Y maze test parameters. Significant differences in the FST immobility time can be referred to Fig. 1B.

Classification into high-performing, middle-performing, or low-performing groups

After the manual analysis of the video recording, the mice were classified into three groups: high performing (HP) or active swimmers characterized by an immobility profile lower than the standard deviation of the mean, middle performing (MP) with immobility duration nearest to the mean, and low performing (LP) group which has an immobility duration more than the upper standard deviation (Fig. 1). The identified groups were then housed together per group in a new cage and were allowed to habituate for a few days before starting the next behavioral tests. Most importantly, each group is evenly represented throughout the whole experiments performed.

Open field test (OFT)

Open field test was performed to evaluate both exploratory and locomotor activity and provide an initial screen for anxiety-related behavior in rodents. At the start of OFT, each study subject was gently placed in the center area of five large square chambers (42 cm×42 cm) and allowed to explore the arena for 25 min. An automated tracking system that used EthoVision software automatically analyzed the movements of the subject mice including the distance moved and movement duration as well as the time spent in the center area.

Elevated plus maze test (EPM)

EPM assesses the anxiety responses of rodents in an elevated open platform (Pellow *et al*., 1985). The maze consists of four arms (two open without walls and two enclosed by 15 cm high walls) and measures 50 cm long and 10 cm wide with an elevation of 1 m from the floor. A subject mouse was placed at the junction of the open and closed arms, facing the open arm opposite to where the experimenter is standing. Automatic tracking of movements was done through EthoVision software for 8 min. The parameters measured in this test are the percentage of entry and the percentage of time spent in the open arms (Walf and Frye, 2007).

Y maze (YM)

Y maze is commonly used to assess the spatial working memory in rodents using the spontaneous alternation task (Wolf *et al*., 2016). The apparatus is made of three-enclosed arms with identical size (5×35×10 cm). Each subject is placed in one arm facing the central section and permitted to explore the maze for 8 min. An entry is counted when all four paws of the mouse enter an arm and an alternation is counted when the subject consecutively enters three different arms. The percentage of spontaneous alternation was calculated as total alternations/[total entries–2]×100 (Sarter *et al*., 1988).

Three-chamber sociability and social preference test

The three-chamber social test is a method to evaluate the social ability of a subject mouse by exploring a stranger mouse in a wire cage over the empty cage, and preference to a novel stranger mouse over an already familiar one (Moy *et al*., 2004; Gonzales *et al*., 2016). The three chambers (23×40×22 cm per chamber) made of Plexiglas were separated by walls with two 10-cm^2 openings in each side of the center area. Cylindrical wire cages of the stranger mice were placed in the middle of each side chambers. The stranger mice were allowed to habituate in the three chambers for 5 min before starting the sociability test. Then, a stranger mouse is placed in one of the wire cages and the other left empty to commence the sociability test that lasted for 10 min, followed by placing a new stranger mouse in the previously empty cage to measure the preference for social novelty for another 10 min. The duration in each compartment and the approach area were automatically measured by EthoVision software. The duration of sniffing to the wire cages was counted by an observer blind to the group conditions. Lastly, the sociability and social preference indices were calculated as previously described (Kim *et al*., 2011).

Novel object recognition test (NORT)

The NORT test gauges the exploration of the subject mouse to a novel object over a familiar one without the presence of rewards to extract their natural propensity for novelty (Baxter, 2010). The test was conducted in an open field arena (42×42 cm) with two kinds of objects, generally consistent in height and volume, but different in shape and appearance. We followed the protocol from (Ennaceur, 2010) with modifications according to the laboratory condition. During the habituation period, the animals were allowed to explore an empty arena for 10 min. Then, two identical objects were diagonally placed at an equal distance from the nearest corner of the arena to familiarize the subject mouse to these objects for another 10 min. One familiar object was then replaced with a novel ob-

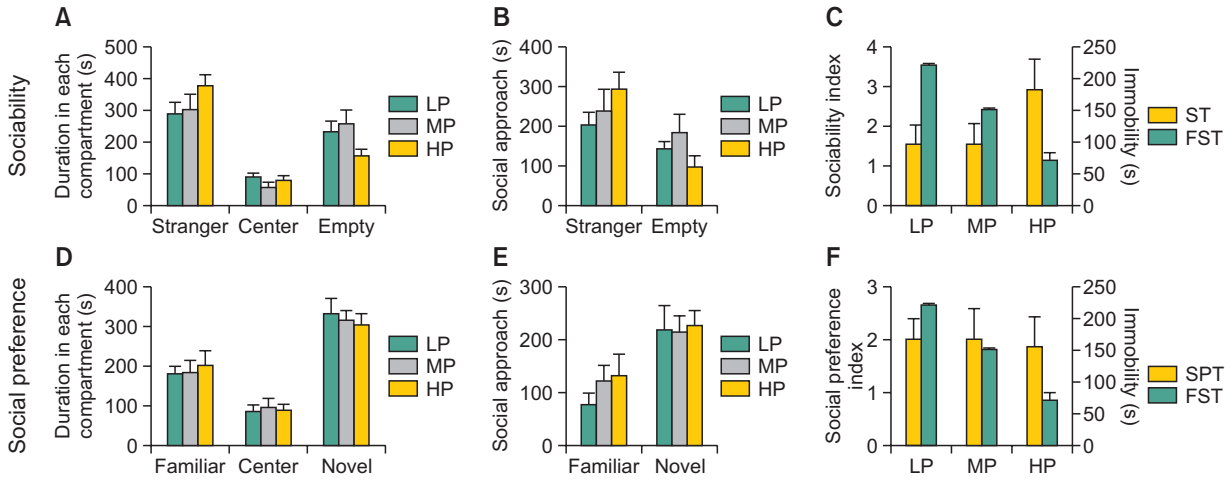

Fig. 5. Assessment of sociability and social preference in the three-chamber social approach task. The sociability (A-C) and social preference (D-F) parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. The (A, D) duration in each compartment, (B, E) social approach and (C) sociability- or (F) social preference indices were measured. All data are presented as the mean ± SEM. No significant difference was observed in the social test parameters. Significant differences in the FST immobility time can be referred to Fig. 1B.

ject and the subject mouse was again allowed to explore the objects for 5 min to test the short-term recognition memory (Tanaka and Curran, 2001). The time spent exploring each object was counted and the recognition between familiar and novel object was calculated as previously described (Vogel-Ciernia and Wood, 2014).

Delay discounting task (DDT)

DDT is a measure of impulsivity in animals with the principle of forgoing small and immediate rewards to gain greater rewards at a later time (Matta *et al*., 2012). Performed in standard operant chambers [Coulbourn Instruments, Allentown, PA, USA] that are placed in sound-proof boxes with fans for ventilation that also mask the external noise. Each chamber has a food pellet dispenser; two nose-poke apertures located on both sides of one wall, and a centrally positioned house light (2.5 W, 24 V) at the top of the chamber. A nose poked in the aperture would result in the delivery of a food pellet (45 mg) while the quantity will depend on the schedule. The Graphic State Notation Software controlled the experimental parameters and collected the data. The test subjects were deprived of food a day before the start of the experiment to increase their will to perform for food.

During the first 2 days of the training phase, the test subjects were introduced into the chamber with only the right aperture. Each nose poke on the aperture delivers a pellet. On days 3 and 4 of the training phase, the chamber is modified and will contain only the left aperture while on day 5, the subjects were introduced into a chamber containing both the left and the right apertures. It is best to start the test phase proper after 1 trial of having both the left and right apertures because the test subjects may develop a side preference the longer they are exposed to a chamber with two apertures. The left aperture would deliver one pellet instantly and the right aperture would deliver 5 pellets and illuminate the stimulus light. Over the course of the test phase, an increasing delay per day (0 s 10 s 30 s 50 s) is applied on the right lever's delivery of the pellets. The computer program will tally the times the test subjects poked their nose on the corresponding apertures (Mitchell, 2014).

Cliff avoidance reaction (CAR)

The cliff avoidance reaction is also used to assess the impulsivity of mice using a round plastic platform (diameter, 20 cm; thickness, 2 cm) elevated to a height of 50 cm (Yoshida *et al*., 1998). The test was initiated by gently placing an animal on a platform such that the forelimbs approached its edge. The latency to fall from the platform was recorded within the 10 min trial period. Other parameters measured are the duration of entry into the edge area defined as an outer ring that is within 5 cm distance from the edge of the round platform (Chen *et al*., 2015) and the frequency of head dips over the edge of the apparatus to evaluate the spontaneous anxiety in the cliff avoidance paradigm.

Statistical analysis

All collected data were expressed as the mean ± the standard error of the mean (SEM) and the GraphPad Prism software was used for statistical analysis. The normality in the baseline FST behavioral data was derived using the D'Agostino-Pearson test and a *p*-value less than 0.05 was considered to be statistically significant. One-way analysis of variance (ANOVA) was used to analyze the differences between HP, MP, and LP in every test conducted followed by Tukey as a post hoc analysis. Pearson's Correlation Coefficient compared the relationship between two quantitative, continuous variables like the immobility time in FST and distance moved in OFT and other parameters for the rest of the behavioral experiments.

RESULTS

Variable response in FST and grouping into HP, MP, and LP

The FST performed on 8-week old outbred CD-1 male mice produced a significantly wide range of response segregating

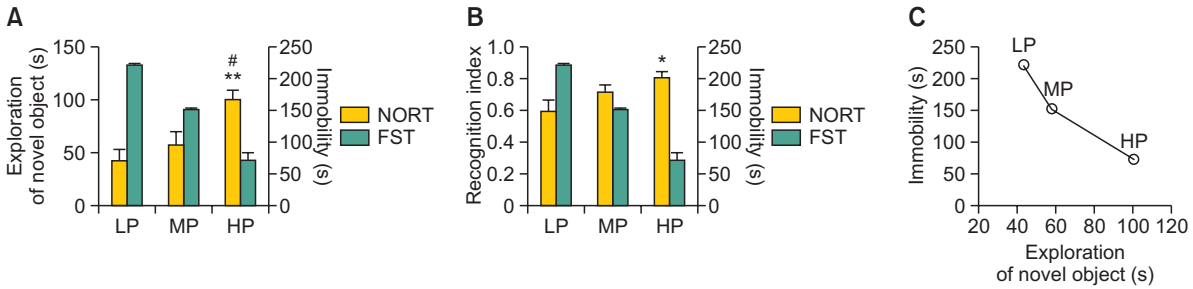

Fig. 6. Assessment of novel object recognition behaviors in the NORT. NORT parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. The (A) exploration of novel object and (B) recognition index were calculated. (C) A line graph depicting the means of immobility in FST and exploration of novel object in NORT was also shown. All data are presented as the mean ± SEM. *p<0.05 and **p<0.01 vs. LP in the NORT; $^{\#}p$<0.05 vs. MP in the NORT. Significant differences in the FST immobility time can be referred to Fig. 1B.

them according to the immobility time during the 6-min trial period (Fig. 1). The high-performing group is considered active swimmers with 50-100 s immobility time during the test. The low-performing group, on the other hand, spent floating for about 200-230 s. The middle-performer having a mean of 150-s immobility were also obtained to serve as a midline reference group throughout the whole series of behavioral study.

HP group displayed higher locomotor activity than the MP and LP groups in the OFT

To determine whether there are differences in locomotor activity among FST-screened mice, an open field test was performed (Fig. 2). There were significant differences among groups in one-way ANOVA of the two parameters in the OFT (Fig. 2A, 2B). Tukey's post hoc comparison revealed that the HP group showed higher distance moved (Fig. 2A) and movement duration (Fig. 2B) compared to LP but not with MP group. The time spent in the center area is not different between the groups (Fig. 2C). The immobility time in FST and distance moved in OFT showed a negative correlation pattern among three groups suggesting these two behaviors might have common regulatory components (Fig. 2D).

No difference in anxiety-like behavior in the EPM among FST-screened groups

To determine a possible relationship between the immobility profile in the FST and anxiety-like behavior, we performed the EPM test. However, the results showed no significant difference among the three groups both in the percentage of time spent in the open arms (Fig. 3A) and the frequency of entry in the open arms (Fig. 3B).

No observed spatial working memory differences in the Y maze among FST-screened groups

To investigate whether the variable responses in FST is linked to spatial working memory through the measure of arm entry alternations, Y maze test was performed (Fig. 4). All groups showed a similar number of arm entries (Fig. 4A) during the trial and no significant difference was also found in the one-way ANOVA between groups when the spontaneous alternations (Fig. 4B) were calculated.

No difference in sociability and social preference behaviors among FST-screened groups

To find out whether differences in FST performance can lead to differences in social behavior profiles, we performed three-chamber social assays (Fig. 5). In the sociability test, no difference was observed in all parameters that were analyzed including the duration in each compartment (Fig. 5A), approach duration (Fig. 5B) and sociability index (Fig. 5C), although the sociability index of HP group tends to be higher than MP and LP groups. In the same way, social preference for novelty was also identical in all groups from all parameters of the duration in compartment (Fig. 5D), approach duration (Fig. 5E) as well as the social preference index (Fig. 5F).

Higher novel object exploration and recognition index of HP group than in other groups

Looking at object exploration profile, we observed that the HP group in the FST displayed an increased exploration time to the novel object which was significantly different compared to MP (p<0.05) and LP (p<0.01) groups (Fig. 6A). In addition, the recognition index further revealed that the HP group recognized better than the LP group (p<0.05) but not significantly different from the MP group (Fig. 6B). The increase in recognition index in HP group suggest that the increased novel object exploration in the HP group is not simply mediated by the increased locomotive activity but instead mediated by the specific increase in the novelty-seeking behavior. The immobility time in FST and exploration time of novel objects also showed a negative correlation pattern among the three groups suggesting these two behaviors might be regulated by shared neurobiological substrates (Fig. 6C).

No difference in measures of impulsivity among the FST-screened groups

The percentage of choice for the large reward in the delay discounting task was similar between the three groups (Fig. 7). In the cliff avoidance reaction, most of the mice didn't jump out of the edge during the 10-min trial showing no inclinations of impulsivity in these animals. In measuring the time spent and head dipping around the edge area of the platform, there were tendencies of higher values in the HP compared to both MP and LP groups but not significantly different from each other (p>0.05).

DISCUSSION

The state of the mind, emotion and physical actions are

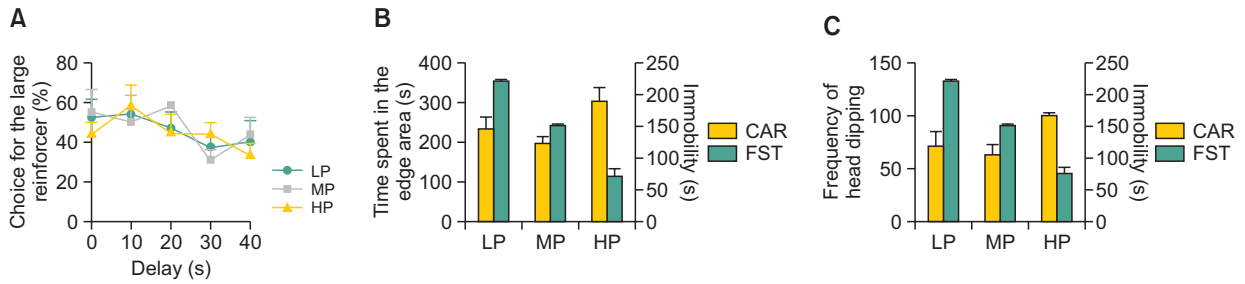

Fig. 7. Assessment of impulsivity in DDT and CAR. CAR parameters were depicted in the left Y-axis in comparison to immobility profile in the right side of Y-axis. The (A) percentage of choice for the large reinforcer, (B) time spent in the edge area and (C) frequency of head dipping were calculated. All data are presented as the mean ± SEM. No significance was observed in the DDT and CAR parameters. Significant differences in the FST immobility time can be referred to Fig. 1B.

interconnected and are often complex if not simple. A number of parameters used in behavioral tests such as FST assess the animal response in terms of their actions, which could be affected by various parameters such as neurophysiological and emotional factors of individual subjects. At present, the vast majority of literature interprets the results of FST as being associated with depression or the so-called "depression-like behavior". For this reason, Commons challenged the idea of assuming a connection between animal behavior and human psychopathology that discourages critical thoughts. Rather, the FST is an interesting and unique test that contributes to the understanding of neural networks regulating the behavioral response towards the acute stressful situation, which may be defective in many neurological disorders such as depression, ASD, and other disorders (Commons *et al*., 2017). This study shows (1) that CD-1 outbred mice have diverse response in FST as we classified into three groups such as HP, MP, and LP; (2) that the HP group have increased locomotor activity in the OFT and higher novelty-seeking behavior in the NORT compared to MP and LP; and that (3) these identified innate behaviors are correlated to the grouped response in FST.

A fundamental inter-strain difference of response in the FST has already been established (Petit-Demouliere *et al*., 2005). Thus, the wide variation of response in outbred mice, CD-1 (ICR), receiving no treatment, in FST should be taken into account. This disparity could be a confounding factor in predicting the clinical efficacy of antidepressant drugs (Bogdanova *et al*., 2013). For example, treatment with the norepinephrine reuptake inhibitor desipramine resulted in a significant decrease in immobility in the rat FST only in low responders, while the selective serotonin reuptake inhibitor fluoxetine caused a significant reduction in immobility in both low and high- responders (Jama *et al*., 2008). In another study, the tricyclic antidepressant clomipramine reduced the floating and increased the swimming response only in high responding male rats; while in females, a reduced floating and increased climbing were shown only for low responders (Pitychoutis *et al*., 2011). This finding may require further investigation as mice and rats do not always share the same characteristics, nonetheless, this may contribute to the elimination of unidentified factors affecting the reliability and validity in the preclinical pharmacological study.

The results in the OFT revealed that the HP mice in FST (that is the active swimmers) also traveled more distance in the open field arena compared to LP mice. This result confirms previous findings in a reciprocal fashion where the grouping was done with the response in OFT before doing the FST (Jama *et al*., 2008). The areas in the brain that control locomotor activities vary widely and that the locomotor activity in the open field task likely has several mediators depending on the emotionality (limbic areas), exploratory activities (higher neocortical regions) and function of senses such as olfaction (Stafstrom, 2006). Based on previous reports regarding HP and LP rats, the increased levels of intra- and extra-cellular dopamine (DA) in the nucleus accumbens (NAc) complemented by lower levels of DA in prefrontal cortex under basal condition is directly proportional to the amount of locomotor activity in response to novelty (Hooks *et al*., 1992; Verheij *et al*., 2008). This may be understood as: the more active an individual copes in a stressful situation, the more active one involves in physical activity (Stults-Kolehmainen and Sinha, 2014; Wermelinger Ávila *et al*., 2018). Psychological stress and physical activity are noted to have a dynamic, bi-directional relationship. It is a widely held view that physical activity has anti-depressive and anxiolytic effects, and reduces the sensitivity to stress. This concept has also been strengthened by other studies showing that physical performance can provide a protective and resilient effect. However, it is uncertain at the moment whether physical activity per se or the neurobiological/neuropsychological underpinnings of the increased activity (i.e., increased OFT activity in the present investigation) and/or high resilience to a stressful situation (i.e., high performance in FST) determines the behavioral appearance of individuals. In this sense, the behavioral correlation model of the present investigation may provide further insights into the mechanistic study targeting delineation of the neural correlates determining the behavioral outcomes.

Another interesting finding is the increased novelty-seeking behavior of HP mice as evidenced by more time spent in the novel object and higher recognition index especially compared to the LP group. This result introduces the connection of active swimming in the FST with novelty-seeking behaviors and that these behaviors can affect each other in various situations and treatment conditions. Our previous study suggested that high novelty-seeking responses in NORT as well as in OFT in rats would also respond more positively to certain drug treatment (dela Peña *et al*., 2015). In addition, low novelty-seeking can induce anhedonia in rats amidst chronic mild stress (Stedenfeld *et al*., 2011). A novelty-seeking personality trait can be described as the response of an organism to novel stimuli or situations with regards to the aptness to explore, and prefer or react positively to the novelty (Cloninger, 1986). Thus, our

current results indicate some patterns of behaviors in mice exposed to a series of behavioral tests, in which the grouping was based on their three levels of responses to FST. It remains to be determined whether this type of behavioral connections is maintained among different mice strains or a particular strain would show different behavioral inter-correlation for certain conditions such as depression-like, novelty-seeking, anxiety, social behavior, and many others. Knowing the behavioral inter-correlation of FST performance and novelty-seeking behavior, it would be an interesting experimental idea to adapt behavioral-conditioning such as increasing the motivation to seek novelty (that is doing new things) or increasing physical activity as plausible ways to reduce despair or depressive-like behaviors in experimental animals as in humans.

Although it is unclear what is the underlying mechanism governing the individual difference in behavioral performance in this study as well as the neural substrates determining the interconnected regulation in some domains of the behavioral paradigms, it is obvious that we can take advantage of the observed individual inter-correlation of the behavior to excavate genes and proteins involved in the regulation of the behavior. Future study will also tell us whether the inter-correlative behavior will be maintained through the neurological, pharmacological, physical and psychological perturbation to the individual subjects, a theme closely related to the personalized brain functional landscape.

CONFLICT OF INTEREST

The authors do not have any conflict of interest regarding this study.

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