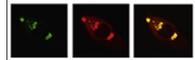


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## Research Report

# Stress-induced anhedonia correlates with lower hippocampal serotonin transporter protein expression

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## ABSTRACT

The serotonin transporter (5-HTT) regulates the extracellular concentration of serotonin, influencing neurotransmission. Evidence suggests that 5-HTT is altered during depression, but the precise changes in 5-HTT expression in the pathogenesis and treatment of depression are not clear. We investigated the protein expression of hippocampal 5-HTT in CD-1 mice exposed to unpredictable chronic mild stress for 10 continuous weeks. Following 6 weeks of the stress procedure, the mice were separated into anhedonic and non-anhedonic groups, which were then treated with fluoxetine (FLX, 10 mg/kg/day, i.p.) for 4 weeks. Behavioral state and therapeutic efficacy of the drug treatment were assessed using sucrose preference, physical state of the coat and body weight. Our results show that changes in hippocampal 5-HTT protein expression correlated with stress-induced behavioral states. Decreases in 5-HTT expression were associated with the stress-induced anhedonic state, whereas increases were associated with the stress-induced non-anhedonic state. Following FLX treatment, the changes in 5-HTT expression were reversed in a subpopulation of anhedonic mice, i.e., the treatment-responsive anhedonic mice. The treatment did not alter the changes in the treatment-resistant anhedonic mice or in the non-anhedonic mice. The data indicate that down-regulation of hippocampal 5-HTT protein expression is a signature change associated with anhedonia, a key endophenotype of clinical depression. Differential changes in 5-HTT expression may contribute to variations in the susceptibility to anhedonia.

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## 1. Introduction

Depression is a debilitating disease that is prevalent worldwide (Kessler et al., 2003; Berton and Nestler, 2006; Krishnan and Nestler, 2008). The disease is predicted to be the second leading

cause of disability in 2020 (Murry and Lopez, 1997). Despite considerable advances in our understanding of the disease's etiology, its underlying neural mechanisms remain largely unknown due to the complex nature of the disease. For example, differences in the vulnerability to depression and differences in

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the responses to antidepressants, two common observations associated with clinical depression, are still poorly understood.

Alterations in various brain neurotransmitter systems, such as serotonin (5-HT), have been widely implicated in the pathophysiology of depression. The serotonin transporter (5-HTT), an important member of 5-HT system, removes 5-HT from the extracellular space, thereby influencing neurotransmission. Clinical studies have suggested that 5-HTT is altered in patients with depression, but the available data remain inconsistent (Newberg et al., 2012; Savitz and Drevets, 2012). Preclinical studies show that two 5-HTT knock-out mouse lines, created using distinct genetic approaches, exhibit increased depressive-like behaviors in response to repeated stress (Zhao et al., 2006; Wellman et al., 2007), suggesting that 5-HTT is involved in the attenuation of depression. However, lifelong gene deletion may result in developmental and compensatory changes that can potentially confound interpretations of altered behavioral phenotypes observed in knockout mice (Gingrich and Hen, 2000). Animal models of depression, on the other hand, mimic various endophenotypes of clinical depression and respond to known antidepressants (Duman, 2010; Pollak et al., 2010; Yan et al., 2010). The unpredictable chronic mild stress (UCMS) model, the most widely used animal model of depression, also simulates differential susceptibilities to depression and differential responses to antidepressants (Strekalova et al., 2006; Wang et al., 2011), which can help us to identify molecular alterations underlying different elements of the disease.

The hippocampus, a brain region richly innervated by serotonergic neurons, is involved in neurogenesis and regulation of emotion (Sahay and Hen, 2007). Using UCMS as a model of depression, we measured 5-HTT protein expression in the hippocampus in CD-1 mice to determine (1) changes in 5-HTT expression associated with anhedonia, a core endophenotype of clinical depression, and (2) correlations in 5-HTT expression with the predisposition to anhedonia Fig. 1.

## 2. Results

### 2.1. Sucrose preference

UCMS has been shown to induce anhedonia in mice as measured by reduced sucrose preference (Pothion et al., 2004; Elizalde et al., 2008). We observed a decrease in sucrose preference starting from the 4th week of UCMS, and the preference scores stabilized within the next two weeks.

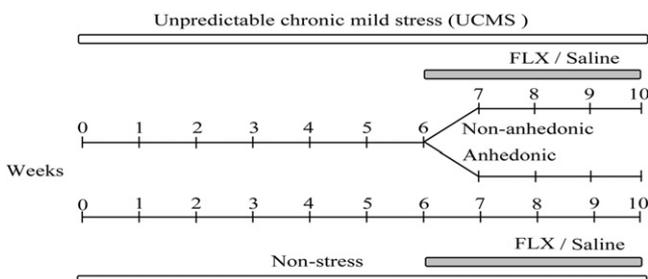


Fig. 1 – Time line illustration of the UCMS procedure, antidepressant treatment and experimental design.

At the end of the 6-week period, 58% of the mice (21 out of 36) became anhedonic, whereas 42% (15 out of 36) were non-anhedonic (see Section 5.2) (Fig. 2A). The sucrose preference measured in the non-anhedonic mice was significantly different from the anhedonic mice ( $F=8.14, p<0.01$ ) and similar to that of the non-stressed controls ( $n=14; F=2.87, p>0.05$ ). Post hoc analysis showed significant differences in sucrose preference between the anhedonic and the non-anhedonic mice at the 4th week ( $F=6.37, p<0.05$ ) and 5th week ( $F=7.16, p<0.01$ ) time points as well.

We next analyzed the three groups for their responses to drug treatment (Fig. 2B). Among the anhedonic mice treated with fluoxetine (FLX), 42% (5 out of 12) were FLX responsive (FLX(+)) (see Section 5.2). Random block ANOVA on sucrose preference revealed a significant three-way interaction

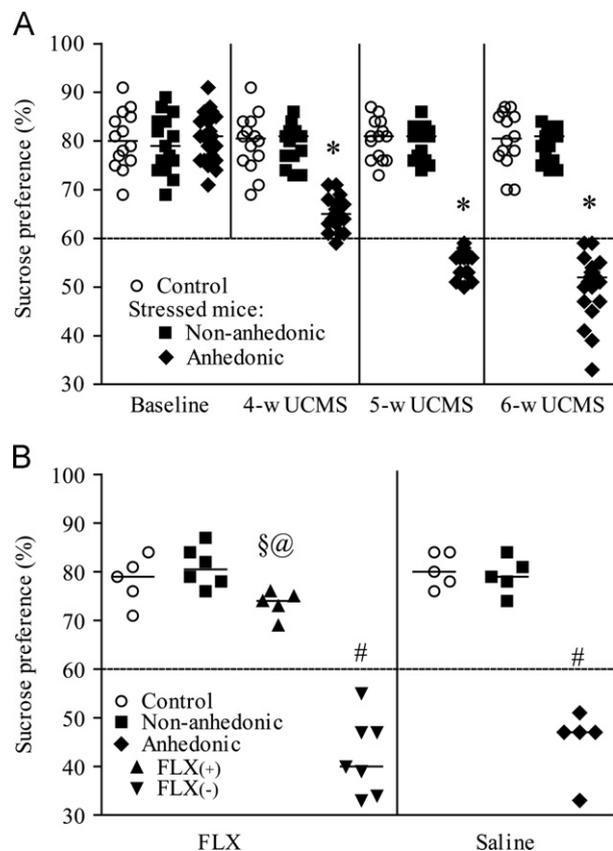


Fig. 2 – Impact of UCMS and FLX treatment on sucrose preference. (A) UCMS induced a consistent drop over 6 weeks in sucrose preference (<60% of baseline) in a subgroup of stressed mice, which were defined as anhedonic ( $n=21$  for the anhedonic;  $n=15$  for the non-anhedonic;  $n=14$  for the non-stressed controls). \* $P<0.05$  vs. non-stressed controls by one-way ANOVA. (B) Subsequent treatment with FLX (7–10 weeks) significantly increased the sucrose preference ( $\geq 60\%$  of baseline) in a subgroup of anhedonic mice, which were defined as FLX(+) ( $n=5-7$ /group). # $P<0.05$  vs. saline-treated anhedonic mice, by simple effects post hoc analysis after a significant interaction in the three-way ANOVA. § $P<0.05$  vs. FLX(-) mice by one-way ANOVA. Bars indicate medians of the groups.

[group × treatment × week:  $F(8,108)=6.97$ ,  $p<0.01$ ;  $n=5-7$ /group]. In a subpopulation of the anhedonic mice, i.e. the FLX(+) mice, the FLX treatment reversed the decrease in sucrose preference observed prior to the treatment [treatment × week:  $F(4,108)=14.17$ ,  $p<0.01$ ; group × week:  $F(8,108)=17.04$ ,  $p<0.01$ ]. No changes were observed in the non-anhedonic mice [ $F(4,36)=1.32$ ,  $p>0.05$ ] or in the non-stressed controls [ $F(4,32)=1.07$ ,  $p>0.05$ ].

2.2. Physical state of the coat

A decrease in self-grooming behavior assessed by the physical state of the coat has been linked to a depression-like state in mice (Stemmelin et al., 2010; Yan et al., 2010). The coat of six areas of the body (head, neck, back, belly, forepaws, hindpaws) was scored as previously described (Stemmelin et al., 2010): 0 for normal physical state; 0.5 for medium degradation; 1 for high degradation. A global measurement of the coat state was obtained by summing the scores from the six different areas. We observed that the anhedonic mice ( $n=21$ ) showed deteriorations in the physical state of the coat

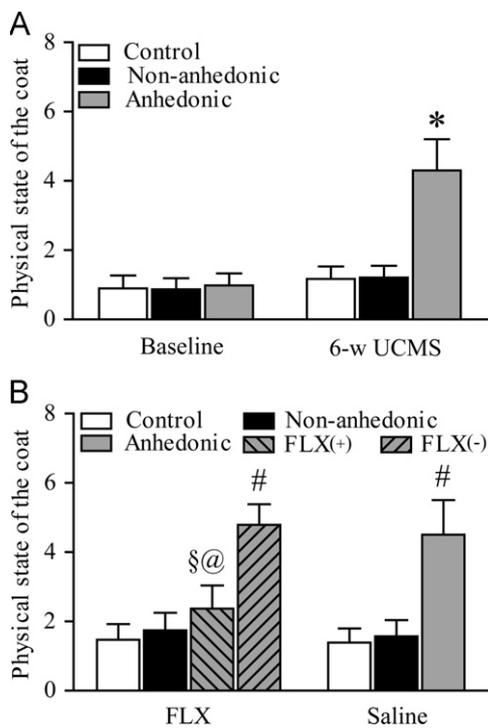


Fig. 3 – Impact of UCMS and FLX treatment on the physical state of the coat. (A) Six weeks of UCMS induced deteriorations in the physical state of the coat in anhedonic mice ( $n=21$  for the anhedonic;  $n=15$  for the non-anhedonic;  $n=14$  for the non-stressed controls). \* $P<0.05$  vs. non-stressed controls by one-way ANOVA. (B) Subsequent treatment with FLX (7–10 weeks) resulted in a significant improvement in the physical state of the coat in the FLX(+) mice ( $n=5-7$ /group). \* $P<0.05$  vs. non-stressed controls; <sup>§</sup> $P<0.05$  vs. saline-treated anhedonic mice, by simple effects post hoc analysis after a significant interaction in the three-way ANOVA. <sup>@</sup> $P<0.05$  vs. FLX(-) mice by one-way ANOVA.

compared to the non-anhedonic mice ( $n=15$ ;  $F=9.14$ ,  $p<0.01$ ) and to the non-stressed controls ( $n=14$ ;  $F=7.71$ ,  $p<0.01$ ) at the end of the 6 weeks of UCMS (Fig. 3).

A three-way interaction on the coat state was detected after the 4 weeks of FLX treatment [group × treatment × week:  $F(8,108)=5.55$ ,  $p<0.05$ ;  $n=5-7$ /group], indicating differential responses to the treatment across groups. The FLX treatment resulted in a significant improvement in the physical state of the coat in the FLX(+) anhedonic mice [treatment × week:  $F(4,108)=4.35$ ,  $p<0.05$ ; group × week:  $F(8,108)=4.91$ ,  $p<0.05$ ]. A significant difference was also detected between the FLX(+) and the FLX(-) anhedonic mice at the end of the treatment [ $F=6.75$ ,  $p<0.05$ ].

2.3. Body weight

Weight changes have been observed in mouse models of depression as well (Ducottet et al., 2003; Strekalova et al., 2006). We plotted body weight measured across different groups in Fig. 4. Following 6 weeks of the UCMS, body weights

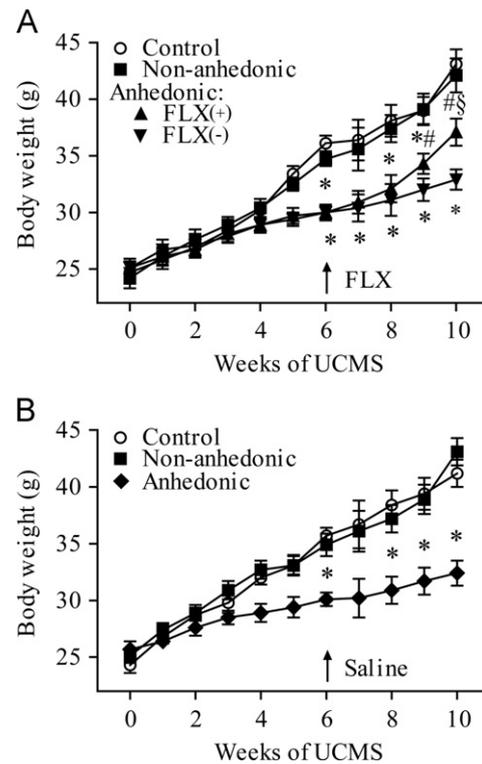


Fig. 4 – Impact of UCMS and FLX treatment on body weight. Mice were exposed to UCMS procedure for 10 weeks, followed by FLX (A) or saline (B) treatment over the last 4 weeks. Six weeks of UCMS induced a decrease in body weight in the anhedonic mice, and subsequent FLX treatment prevented the decrease in body weight in the FLX (+) mice ( $n=21$  for the anhedonic,  $n=15$  for the non-anhedonic,  $n=14$  for the non-stressed controls during the first 6 weeks;  $n=5-7$ /group during drug treatment). \* $P<0.05$  vs. week-matched non-stressed controls, <sup>§</sup> $P<0.05$  vs. week-matched saline-treated anhedonic mice, by simple effects post hoc analysis after a significant interaction in the two-way ANOVA. <sup>§</sup> $P<0.05$  vs. FLX(-) by one-way ANOVA.

of the anhedonic mice ( $n=21$ ) were lower than those of the non-anhedonic mice ( $n=15$ ;  $F=6.23$ ,  $p<0.01$ ) and the non-stressed controls ( $n=14$ ;  $F=7.43$ ,  $p<0.01$ ).

During the drug treatment, there was no three-way interaction detected, only a significant two-way interaction [treatment  $\times$  week: ( $F(4,108)=20.74$ ,  $p<0.01$ );  $n=5-7$ /group]. Post hoc analysis indicated that the FLX treatment caused a significant increase in the body weight only in the FLX(+) anhedonic mice.

#### 2.4. Hippocampal 5-HTT protein expression

Prior to the UCMS procedure, the hippocampal 5-HTT protein levels (calculated as a ratio of 5-HTT to  $\beta$ -actin levels) were similar ( $n=4$ /group;  $F=0.97$ ,  $p>0.05$ ) (Fig. 5A). Six weeks of UCMS induced a decrease in the hippocampal 5-HTT protein levels in the anhedonic mice ( $0.12 \pm 0.02$ ;  $F=8.27$ ,  $p<0.01$ ) and an increase in the non-anhedonic mice ( $0.36 \pm 0.04$ ;  $F=10.15$ ,  $p<0.01$ ), compared to the non-stressed controls ( $0.22 \pm 0.01$ ) ( $n=4$ /group) (Fig. 5B).

Analysis of hippocampal 5-HTT protein expression following FLX treatment revealed a significant two-way interaction [group  $\times$  treatment:  $F(2,27)=8.85$ ,  $p<0.05$ ;  $n=5-7$ /group] (Fig. 5C). The FLX treatment normalized the 5-HTT protein levels in the FLX(+) anhedonic mice, but had no effect on the non-anhedonic mice or on the non-stressed controls [treatment:  $F(1,27)=7.35$ ,  $p<0.05$ ; group:  $F(2,27)=14.18$ ,  $p<0.01$ ]. The FLX(-) mice showed a similar level of 5-HTT protein expression compared to the level detected prior to the treatment ( $F=0.51$ ,  $p>0.05$ ), but it was lower compared to their FLX(+) counterparts ( $F=17.63$ ,  $p<0.01$ ).

### 3. Discussion

Our study reports a correlation between hippocampal 5-HTT protein expression and UCMS-induced behavioral states. Specifically, decreases in hippocampal 5-HTT expression were associated with the anhedonic state, whereas increases were associated with the non-anhedonic state. Changes in 5-HTT in the anhedonic mice were completely reversed by FLX treatment in nearly half the population, similar to the percentage of patients reported to be responsive to antidepressants (Trivedi et al., 2006).

A large body of evidence from clinical studies has suggested that the 5-HTT is altered in patients with depression. This is primarily based on human studies of 5-HTT binding using positron emission tomography (PET). These studies showed both increases and decreases in 5-HTT binding, as well as negative findings (Newberg et al., 2012; Savitz and Drevets, 2012). These results may be due in part to methodological differences, disease heterogeneity, prior drug exposure, gender and age of the participants (Malison et al., 1998; Newberg et al., 2005; Reimold et al., 2008).

Preclinical studies have also reported changes in 5-HTT associated with endophenotypes of depression. Decreases in both 5-HTT density and mRNA were observed in various brain regions in rats with olfactory bulbectomy, a lesion model of depression, and with chronic food-restriction, another model of depression induced by food restriction

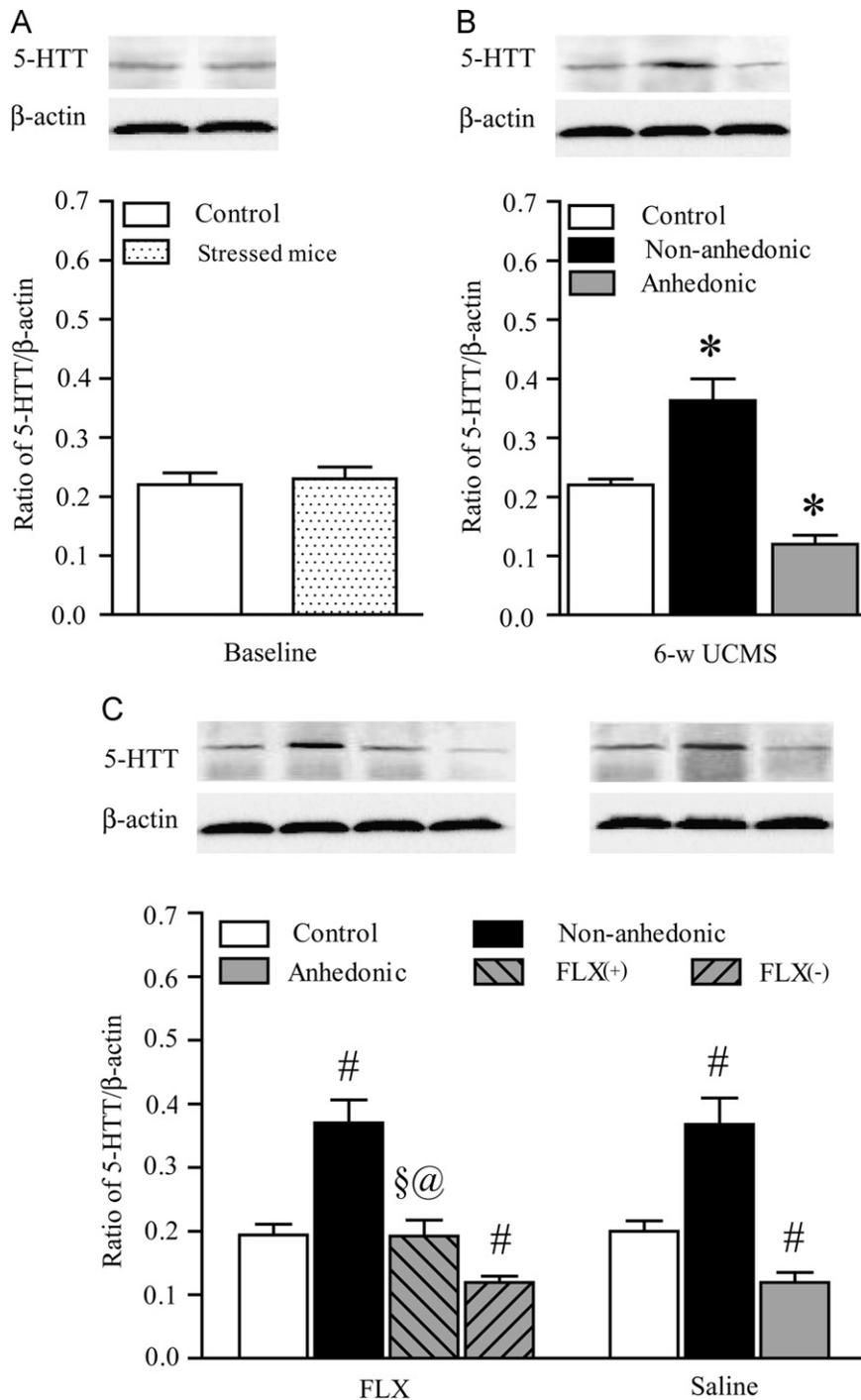
(Grecksch et al., 1997; Jahng et al., 2007). In contrast, an increase in 5-HTT density was reported in the hippocampus in Wistar rats treated with reserpine, a pharmacological model of depression (Iritani et al., 2006). Several factors may contribute to the apparent disagreement between Iritani et al. (2006) study and ours. First, a single injection of reserpine has been shown to alter 5-HTT mRNA in the midbrain for at least a week (Xiao et al., 1999), which may in turn affect 5-HTT protein expression. Hence, it is difficult to differentiate whether the changes in 5-HTT density were due to the reserpine's pharmacological effects or due to the depressive state. Second, differences in behavioral tests, basal 5-HT levels and responses to selective serotonin reuptake inhibitors (SSRIs) have been well documented in different rodent strains commonly used in studies of depression (Lucki et al., 2001; David et al., 2003; Ripoll et al., 2003; Scholl et al., 2010). Therefore, species differences (rat versus mouse) may be another source of experimental variation.

Our study reported a decrease in the 5-HTT protein expression in the hippocampus following exposure to the UCMS, a behavioral model of depression known to simulate the disease with a more natural induction. Moreover, the decrease was specifically reversed by FLX treatment at a dose commonly reported to be effective in producing antidepressant effects in mice (Ducottet et al., 2003; Isingrini et al., 2012). Taken together, the results indicate that the decrease in 5-HTT protein expression is a signature change associated with the anhedonic state, a core endophenotype of human depression.

We also found that the stress-induced non-anhedonic mice showed up-regulation of the hippocampal 5-HTT protein expression rather than the down-regulation seen in the stress-induced anhedonic mice. The distinct differences in 5-HTT protein expression between anhedonia-susceptible and anhedonia-resistant mice may offer insight into the neurobiological basis of the variations in susceptibility to clinical depression.

The differences in hippocampal 5-HTT protein expression between the stress-induced anhedonic and the non-anhedonic mice may be due to genetic differences. Recent studies show that a functional polymorphism in the human 5-HTT gene promoter (known as 5-HTT gene-linked polymorphic region, 5-HTTLPR) is linked to an increased risk of major depression (Caspi et al., 2003; Daniele et al., 2011). Although no 5-HTTLPT homolog has been found in rodents, it is possible that other functional polymorphisms of the 5-HTT gene, or other related genes involved in 5-HTT regulation, may be responsible for variations in 5-HTT protein expression in genetically distinct sub-populations of CD-1 mice used in the present study.

Dysfunction within the 5-HT system does not fully explain depression, as indicated by our results and others. Other neurotransmitter systems or neural substrates may contribute to its pathophysiology. Dopamine (DA)'s involvement in depression has been highlighted recently. Phasic firing of ventral tegmental area (VTA) DA neurons is enhanced during depression (Chaudhury et al., 2013). The augmented firing of VTA DA neurons is observed only in depression susceptible mice, not in resilient individuals (Krishnan et al., 2007;



**Fig. 5 - Effect of UCMS and FLX treatment on the hippocampal 5-HTT protein expression.** 5-HTT protein expression was determined by Western Blot and presented as a ratio of 5-HTT to  $\beta$ -actin levels. (A) No differences were found between the stressed mice and their controls at the baseline. (B) Six weeks of UCMS induced a decrease in the hippocampal 5-HTT protein levels in the anhedonic group, and an increase in the non-anhedonic group ( $n=4/\text{group}$ ). \* $P < 0.05$  vs. non-stressed controls by one-way ANOVA. (C) Subsequent FLX treatment (7–10 weeks) increased the hippocampal 5-HTT protein levels in the FLX (+) anhedonic mice compared to saline controls ( $n=5-7/\text{group}$ ). # $P < 0.05$  vs. non-stressed controls; \$ $P < 0.05$  vs. saline-treated anhedonic mice by simple effects post hoc analysis after a significant interaction in the two-way ANOVA. @ $P < 0.05$  vs. FLX(-) mice by one-way ANOVA.

Chaudhury et al., 2013). Two recent studies strongly implicate the VTA-nucleus accumbens pathway in mediating the susceptibility to depression induced by repeated stress from social defeat and UCMS, respectively (Chaudhury et al.,

2013; Tye et al., 2013). Thus, elucidating how DA and 5-HT systems interact with one another during the development of depression-like states will increase our understanding of the pathogenesis of the disease.

## 4. Experimental Procedures

### 4.1. Animals

Fifty-eight male CD-1 mice (8–12 weeks of age) were used. Two weeks before experiments, each mouse was housed individually in a temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity ( $55 \pm 2\%$ ) controlled room with a 12 h light–dark cycle (lights on at 8 AM). Animals were given free access to food and water for the duration of the experiments unless otherwise specified.

All experimental procedures were carried out in accordance with the guidelines for human care of animals set forth by the National Institute of Health (NIH) in the United States and approved by the Institutional Animal Care and Use Committee at China Medical University.

### 4.2. Experimental design

UCMS was applied to 36 mice for 10 weeks. During the last 4 weeks of CMS, FLX-HCl prepared in saline was injected intraperitoneally at 10 mg/kg (Ducottet et al., 2003; Isingrini et al., 2012) once daily (between 9 and 10 AM) in a volume of 5 ml/kg. Fourteen mice, serving as a control group, were not exposed to stress (Fig. 1).

After 6 weeks of UCMS, mice were divided into anhedonic and non-anhedonic groups based on their responses in the sucrose preference test. The mice with sucrose preference  $<60\%$  were defined as anhedonic, whereas the mice with sucrose preference  $\geq 60\%$  were defined as non-anhedonic (Strekalova et al., 2006). Grooming behavior by assessing the physical state of the coat was also used to assist in verifying the anhedonic state (Stemmelin et al., 2010; Yan et al., 2010). Starting in the 7th week, mice in each group were further divided into 2 subgroups, one receiving FLX and the other one receiving saline for 4 weeks. The response to the treatment was then determined by their sucrose preference. The mice with sucrose preference that increased from  $<60\%$  to  $\geq 60\%$  were defined as FLX(+), whereas the mice with sustained  $<60\%$  sucrose preference were defined as FLX(–).

Hippocampal 5-HTT protein levels were measured at baseline, at the end of the first 6 weeks of UCMS, and after drug treatment, respectively.

### 4.3. UCMS procedure

A modified version of UCMS procedures described previously was employed (Pothion et al., 2004; Elizalde et al., 2008). The following stressors were presented: low intensity stroboscopic illumination (in dark 8 h), untuned radio noise (8 h), cage tilt  $45^\circ$  (8 h), wet bedding (200 ml of water per cage, 8 h), aversive odor (rat soiled bedding, 8 h), paired housing (with new partner of the same strain each time, 2 h), water and food deprivation (8 h), reversal of the light/dark cycle, removal of nesting material (8 h), forced swimming in icy water (3 min), tail suspension (5 min), and confinement in a tube (diameter = 4 cm, length = 5 cm; 2 h). Two or three of the stressors were randomly applied daily throughout the UCMS period.

### 4.4. Sucrose preference test

Anhedonia was evaluated by weekly monitoring of sucrose preference throughout the 10 weeks duration of the experiment. Mice were given two standard drinking bottles containing 2.5% sucrose and tap water, respectively. The night before the sucrose preference test, mice were food and water deprived. The two bottles were presented next morning between 8:00 and 9:00 AM, with the positions of the two bottles (right/left) interchanged randomly from trial to trial to prevent possible effects of side-preference on drinking behavior. Intake of each solution was determined by the difference in bottle weight measured between pre- and post-drinking session. Sucrose preference (%) was calculated as: [sucrose solution intake (ml)/total fluid intake (ml)]  $\times 100$ .

### 4.5. Physical state of the coat

Grooming behavior was assessed by monitoring the physical state of the coat at week 0, 6, 7, 8, 9, 10.

### 4.6. Body weight

Mice were weighed weekly. The measurement was not recorded after the food or water deprivation—one of the stressors used in the UCMS procedure.

### 4.7. Protein expression of hippocampal 5-HTT

Mice were decapitated and brains were removed. The hippocampi were homogenized in ice-cold lysis buffer (0.1% SDS, 1% Triton-100, 10 mM HEPES, 5 mM NaF, 0.25 M sucrose, pH 7.4) to make a cell lysate. Protein concentration was measured using the Bradford method (Bradford, 1976), with bovine albumin as the standard. Samples containing 100  $\mu\text{g}$  of protein were loaded onto 12% polyacrylamide gel, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) overnight. The membrane was blocked with 5% milk in Tris-buffered saline with Tween-20 (TBS-T) for 2 h at room temperature. A goat anti-5-HTT polyclonal antibody (1:300, Santa Cruz) was incubated with the membrane for 4 h at room temperature. The membrane was washed once for 30 min and twice for 20 min with TBS-T, and incubated with a horseradish peroxidase-conjugated anti-goat IgG secondary antibody (1:500, Santa Cruz) for 2 h at room temperature. After the same washing steps, the specific immunoreactive staining was visualized by enhanced chemiluminescence (ECL) detection reagents (Sigma, USA), and captured by Tanon-4200 gel imaging system (Tanon Science, China).  $\beta$ -actin, a housekeeping protein, was detected with polyclonal rabbit antibody (1:1000, Santa Cruz) and secondary anti-rabbit antibody (1:1000, Santa Cruz). 5-HTT was revealed as a band of 70 kDa,  $\beta$ -actin was revealed as a band of 42 kDa. Band density was analyzed with GIS image analysis software (Tanon Science, China). To eliminate possible variations in the efficiency of protein extractions and sample loading,  $\beta$ -actin was used as an internal control. Hence, the expression level of 5-HTT was normalized to its corresponding  $\beta$ -actin level in each sample.

#### 4.8. Statistical analysis

All measurements were analyzed across groups at baseline and following the first 6 weeks of UCMS using one-way ANOVA.

During the drug treatment, the data collected from the FLX(+) mice were used for the anhedonic group. Sucrose preference, physical state of the coat and body weight were analyzed using random block ANOVA with two between-subject variables (group and treatment) and one within-subject variable (week). Simple effect tests with Bonferroni correction factors were performed to distinguish the influence of one variable on another when a significant interaction was detected. Protein expression of 5-HTT was analyzed using two-way ANOVA (group and treatment). Differences between the FLX(+) and the FLX(−) mice were analyzed using one-way ANOVA. The level of significance was  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.

## 5. Conclusion

Our results suggest that anhedonia correlates with a lower hippocampal 5-HTT protein expression. Moreover, differential changes in the protein expression may contribute to variations in the susceptibility to develop anhedonia. Ongoing studies are examining functional activities of hippocampal 5-HTT to gain a more complete understanding of the involvement of hippocampal 5-HTT in the pathophysiology of depression.

## Financial disclosures

All authors have no conflicts of interest to declare.

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