

Chronic Antidepressant Treatment in Normal Mice Induces Anxiety and Impairs Stress-coping Ability

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Antidepressants are clinically used for patients with major depression. Antidepressant treatments in certain groups of patients are effective for relieving depression as well as anxiety disorder. However, it is not clearly known whether the use of current antidepressants in healthy persons is beneficial for upcoming depression- and anxiety-inducing life events. To address this question, normal mice were intraperitoneally administered with imipramine or fluoxetine for more than 2 weeks, and behaviors related to anxiety and depression were evaluated. Mice treated with imipramine or fluoxetine for more than 14 days exhibited significantly decreased immobility time in the forced swim test and tail suspension test, but these mice exhibited enhanced anxiety in several behavioral tests. Furthermore, chronic antidepressant treatments followed by sub-threshold level of stress in normal mice profoundly aggravated antidepressant-induced anxiety-like behaviors without further affecting depression-related behaviors. Chronic antidepressant treatments followed by sub-threshold level of stress produced swollen vesicles and ulcerations on the lips as well as a watery and inflammatory nose. Mice given chronic antidepressant treatments displayed intestinal abnormalities evidenced by a highly enlarged and inflamed small intestine full of defecation materials. These results suggest that chronic antidepressant treatment in normal mice provokes anxiety-like behaviors and impairs their stress-coping ability.

Key words: Antidepressant, side effects, anxiety, stress-coping response

INTRODUCTION

Depression is one of the most prevalent psychiatric disorders and presents in patients as depressed mood, loss of interest, feelings of low self-worth, and diminished ability to experience pleasure [1, 2]. Patients with depression are normally treated with antidepressants, which include classical monoamine modulators, such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs),

selective serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs) [3, 4]. However, current antidepressant medications are only effective in ~60% of patients [5]. Moreover, current antidepressants produce severe side effects that include dizziness, nausea, lethargy, headache, anxiety, agitation, and constipation [6-10].

Acute treatment with antidepressants in rodent models produces anxiogenic effects in the elevated plus maze test [11-14], light/dark box test [14], and social interaction test [15-18]. Conversely, other studies have reported that administration of acute antidepressants to normal animals produces opposing anxiolytic effects [19, 20] or no effect [20, 21]. Specifically, several studies have reported that chronic treatment of normal animals with antidepressants induces anxiogenic behaviors in the elevated plus maze, light/

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dark box test, novelty suppressed feeding test, open field test, and social interaction test [22-25], whereas a number of other studies have reported that chronic antidepressant treatments produce anxiolytic effects [11, 12, 26, 27] or no effect at all [17, 19, 21, 25, 28, 29]. Others have shown that treatment of normal animals with antidepressants does not change immobility time in the forced swim test [19, 26, 29, 30], and may even increase immobility time in the forced swim test [31].

The conflicting results of antidepressant treatment effects in normal animals may be due in part to inconsistent experimental designs and skills in handling animals, antidepressant treatment procedures, and behavioral testing skills among different laboratories. Anxiety-like behaviors in animal models are easily induced by repeated stress [32, 33], repetition of the behavioral tests [34-36], improper animal handling [37-39], and stressful housing conditions [40, 41]. It is also unclear whether different antidepressants produce differential effects on depression- and anxiety-related behaviors [20]. Thus, the results of many previous studies are too complex to draw firm conclusions regarding anxiety. Nonetheless, in considering the effect of stress on anxiety, it is possible that antidepressant treatment during undefined sub-threshold stress, which alone does not induce depression-like or anxiety-like behavioral changes, affected animal's anxiety states in previous studies. Therefore, chronic antidepressant treatment effects in normal animals need to be investigated in a more controlled manner in the context of depression, anxiety, and stress.

In the present study, we demonstrated that chronic treatments with imipramine or fluoxetine in mice induces anxiety-like behaviors, and that such treatments impaired animals' ability to cope with stress.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6J mice were purchased from Daehan BioLink, Inc (Eumsung, Chungbuk, Korea). Mice were housed in pairs into clear plastic cages in an environment in which temperature (23°C) and humidity (50~60%) were controlled on a 12-hr light/dark cycle (light on at 7 A.M.) with *ad libitum* access to food and water. Animals were allowed to adapt to the new environment for 1 week before the start of experiments. All animals were handled in accordance with the animal care guideline of Ewha Womans University School of Medicine (IACUC 2013-01-007).

Antidepressant drug administration

Imipramine (Sigma-Aldrich, St. Louis, MO, USA) and fluoxetine

(Sigma-Aldrich) were dissolved in 0.9% saline and injected intraperitoneally (i.p) daily at a dose of 20 mg/kg body weight in a total volume of 120 µl. Vehicle-treated animals received daily intraperitoneal injections of 120 µl saline.

Restraint stress

Restraint was administered as described previously [32, 42]. Mice were restrained for either 2 h or 6 h daily for 3 days as indicated. To administer restraint stress, animals were individually placed head-first into well-ventilated 50-ml conical tubes (SPL Lifesciences, Pocheon, Korea), plugged with a ~3-cm-long middle tube and finally the cap of the 50-ml tube. Mice could not freely move to the front or the reverse direction inside the tube. Restraint was delivered to animals beginning at 10 A.M. Control mice remained in their original cages with no disturbance. After 2 or 6 h-restraint treatment, the mice were returned to their home cage in which they were housed in pairs. Antidepressant or saline treated mice were administered daily as indicated until the behavioral tests were finished.

Assessments of antidepressants- and stress-induced physiological symptoms

Antidepressant- and stress-induced changes in pathological phenotypes were quantified as described previously [43]. Briefly, six days after the last stress session (day 17), the extent of antidepressant- and stress-induced pathological symptoms were assessed using rating scales ranging from 0 to 3. The scale for hair was as follows: 0, no observable change; +1 (weak), unkempt hairs on the head; +2 (moderate), unkempt and caked hairs on the head and neck; and +3 (severe), unkempt and caked hairs throughout the whole body. The scale for lips was as follows: 0, no observable change; +1 (weak), a sign of swelling on the lips; +2 (moderate), swelling, ulceration, and signs of scabs; and +3 (severe), inflammation and ulceration with noticeable scabs. Finally, the scale for nose was: 0, no observable change; +1 (weak), runny nose; +2 (moderate), runny nose with swellings and ulceration; and +3 (severe), runny nose with inflammation and ulceration along with respiratory difficulties.

Behavioral assessments

Mice were brought to the testing room 30 min prior to the start of each behavioral test, and remained in the same room throughout the test. Behavioral assessments were performed using a computerized video-tracking system (SMART; Panlab S.I., Barcelona, Spain) for tracking mouse behavior or a webcam recording system, as previously described [32, 44]. Behavioral tests were performed 1 h after the last antidepressant or vehicle injection.

Forced swim test

The forced swim test (FST) was performed as described previously [32]. Briefly, mice were placed in a Plexiglas cylinder (height: 27 cm, diameter: 15 cm) containing water at 23°C and a depth of 15 cm such that mice could neither escape nor touch the bottom. Mice were subjected to a 15-min pre-swim. After a 15-min pre-test, the animals were dried quickly with a towel and returned to their home cages. One day later, mice were forced to swim again for 6 min. The animals were habituated for the first 1 min and behavior was noted over the next 5 min. Immobility was defined as the summation of the time during which animals remained floating with all limbs motionless. This scoring method was applied by following the procedure described previously [45].

Tail suspension test

The tail suspension test (TST) was carried out as described previously [46] with minor modifications. Briefly, mice were individually suspended 50 cm above a table surface by fastening the tip (1 cm long) of their tail with tape. Immobility was defined as the total time during which all limbs were motionless. Immobility time was measured for 6 min-periods.

Sociability test

The sociability test (ST) was carried out by following the procedure described previously [47] with a minor modification. The sociability test was administered in the open field of a cream-colored FOAMAX (Expanded PVC; LG Ltd., Korea) chamber (45×45×40 cm). The testing chamber was lit with indirect illumination (50 lux). Mice were placed in the open field and left to explore the test arena. The time spent in the interaction zones and corner zone was assessed.

Open field test

The open field test (OFT) was performed as described previously [32, 48]. The apparatus of the open field test was built using cream-colored FOAMAX (45×45×40 cm). For the test, the intensity of the illumination in the open field chamber was adjusted to 80 lux by indirect illumination. Each mouse was placed individually at the center of the open field, and locomotion was recorded for the indicated period. Horizontal locomotor activity was assessed according to the total distance moved by the animal. The inner area, which was 30 percentage of the open field, was defined as the center zone.

Elevated plus maze test

The elevated plus maze (EPM) test was performed as described previously [32, 48]. The elevated plus maze apparatus consisted

of four arms (30×7 cm each) made of black-colored FOAMEX and placed at right angles to each other. Two of the arms had 20-cm high walls (enclosed arms), whereas the other two of the arms had no walls (open arms). The whole apparatus was elevated 50 cm above the test room floor. Illumination at the center of the apparatus was adjusted to 40 lux. For testing, each mouse was initially placed at the center of the platform and left to explore the arms for 5 min. The number of entries into the open and enclosed arms and the time spent in the each arm were recorded. The entry into any arm was scored as an event if the animal placed all four paws in the corresponding arm.

Novelty suppression feeding test

The novelty-suppressed feeding (NSF) test was administered as described previously [49] with minor modifications. Twenty-four hours before starting the test, mice were singly caged and food was removed from cage, but water was available *ad libitum*. The testing apparatus consisted of a cream-colored FOAMAX chamber (45×45×40 cm). Illumination in the test chamber was adjusted to 50 lux by indirect illumination. For the test, a small piece of familiar food pellet was placed on a white circular filter paper (150 mm in diameter) in the center of the open field, and food deprived mouse was placed in a corner of the open field chamber. The latency for beginning a feeding episode was recorded (with maximal cutoff time of 5 min). Immediately after a mouse began to eat the food pellet, the mouse was removed from the open field and placed into her home cage. After the mice returned to their home cage, the amount of food consumed for 5 min was immediately measured.

Marble burying test

The marbles burying test (MBT) was administered as described previously [33, 50] with minor modifications. Empty cages were filled with smooth bedding (JRS 3-4; J. Rettenmaier & Söhne, Rosenberg, Germany) to a height of 5 cm from the cage floor. Next, 12 identically shaped grass marbles were placed on top of the new bedding using equal spacing throughout the cage. Mice were individually tested by allowing them to freely explore the cage with marbles over a period of 30 min. Finally, the total number of buried marbles was counted. Buried marbles were defined as those in which the visible area of the marbles was less than 25% of the total marble surface.

Rota-rod test

The rota-rod test was performed as described previously [48]. The rota-rod consisted of a rotating cylinder (diameter 4.5 cm) with a speed controller. Mice were placed on top of the cylinder,

which had a coarse surface to allow for a firm grip. The rotarod was accelerated to a setting of 10 rpm, and maintained at the indicated speed for 3 min, and then the mice were subjected to tests at successively higher speeds. A cutoff time of 5 min and an inter-trial interval of 60 min were used. The time spent on the rod without falling down was measured.

Statistical Analyses

Two-sample comparisons were performed using Student's *t*-test, and multiple comparisons were made using two-way ANOVA followed by Newman-Keuls *post hoc* test. GraphPad PRISM 6.0 software (GraphPad Software, Inc, CA, USA) was used for statistical analyses. All data are presented as the mean±SEM, and statistical significance was accepted at the 5% level unless otherwise indicated.

RESULTS

Chronic antidepressant treatments in normal mice induces anxiety-like behaviors

To understand whether chronic treatment with antidepressants affects depression- and anxiety-related behaviors, eight-week-old male C57BL/6J mice were intraperitoneally administered with imipramine (IMI, 20 mg/kg/day), a tricyclic antidepressant (TCA), for more than 14 consecutive days as described (Fig. 1A). Control animals received injections of saline (Veh) instead of the antidepressant for the same period of time. The behavioral states of mice on days 15~17 were examined. Chronic IMI-treated mice exhibited reduced immobility in the forced swim test (FST) [$t_{(14)}=2.332$, $p=0.0352$] (Fig. 1B). The chronic IMI-treated mice interacted with a social target in the sociability test similar to Veh-treated control mice, and there was no statistical difference between chronic IMI-treated mice and Veh-treated control mice [$t_{(24)}=1.267$, $p=0.2173$]. However, chronic IMI-treated mice spent more time in the corner zone than Veh-treated control mice [$t_{(24)}=2.529$, $p=0.0184$] (Fig. 1C and D). In the novelty suppressed feeding (NSF) test, the IMI-treated mice showed increased latency to eating a food pellet placed at the center of the open field [$t_{(12)}=2.959$, $p=0.0119$] (Fig. 1E), as well as reduced food consumption in a subsequent food consumption test [$t_{(14)}=4.525$, $p=0.0005$] (Fig. 1F).

We examined whether chronic treatment of fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI), produces similar behavioral effects as IMI. Normal C57BL/6J mice were treated with FLX (20 mg/kg/day) for more than 14 consecutive days as indicated above (Fig. 1A). Chronic FLX-treated mice showed reduced immobility in the tail suspension test (TST) [$t_{(13)}=7.898$,

$p<0.0001$] (Fig. 1G), and reduced locomotion in the open field test [$t_{(12)}=4.218$, $p=0.0012$] (Fig. 1H). Chronic FLX-treated mice had reduced entry numbers to the open arms [$t_{(13)}=6.593$, $p<0.0001$] and spent significantly less time in the open arms [$t_{(11)}=2.241$, $p=0.0466$] in the elevated plus maze (EPM) test (Fig. 1I and J). These results suggested that chronic treatment with IMI or FLX in normal C57BL/6J mice suppresses depression-related behaviors, but induces enhanced anxiety compared with control animals.

Chronic antidepressant treatments in normal mice deteriorated the stress coping ability in regulating anxiety-related behaviors

Next, we examined whether chronic antidepressant treatments in normal mice affect the stress coping abilities of mice in regulating anxiety-related behaviors. To address this, chronic IMI- or FLX-treated mice were exposed to restraint for 6 h daily for 3 days (6h×3d RST), according to the schedule depicted in Fig. 2A.

In the EPM test, chronic IMI treatment or 6h×3d RST treatment tended to decrease, but not significantly, entry numbers to the open arms and the amount of time spent in the open arms. Chronic IMI treatment followed by 6h×3d RST treatment significantly reduced entry numbers and the time spent in the open arms in the EPM test compared with chronic IMI treatment (Fig. 2B and C). Two-way ANOVA analysis detected the main effects of chronic IMI treatment [$F_{(1,26)}=17.62$, $p=0.0003$ for entries; $F_{(1,23)}=8.242$, $p=0.0086$ for time] and 6h×3d RST treatment [$F_{(1,26)}=14.01$, $p=0.0009$ for entries; $F_{(1,23)}=9.456$, $p=0.0054$ for time], whereas there was no effect of IMI×RST interaction [$F_{(1,26)}=3.297$, $p=0.0809$ for entries; $F_{(1,23)}=1.667$, $p=0.2094$ for time] (Fig. 2B and C). Chronic FLX treatment significantly decreased entries to the open arms and tended to reduce the amount of time spent in the open arms in the EPM test. The 6h×3d RST treatment effects were not significant in entries and the time as described above. Whereas chronic FLX treatment followed by 6h×3d RST treatment profoundly reduced open-arm entries and the time spent in the open arms compared with chronic FLX treatment (Fig. 2B and C). Two-way ANOVA analysis detected the main effect of chronic FLX treatment [$F_{(1,27)}=69.86$, $p<0.0001$ for entries; $F_{(1,24)}=14.72$, $p=0.0008$ for time], and 6h×3d RST treatment in open arm entries [$F_{(1,27)}=6.431$, $p=0.0173$], but not in the time spent in the open arms [$F_{(1,24)}=3.359$, $p=0.0793$]. There was no FLX×RST interaction [$F_{(1,27)}=0.3101$, $p=0.5822$ for entries; $F_{(1,24)}=0.1739$, $p=0.6804$ for time] (Fig. 2B and C).

Chronic IMI treatment in normal mice slightly reduced the total locomotion in the open field test (OFT), but did not change the amount of time spent in the center of the open field. The 6h×3d RST treatment in normal mice tended to increase the total locomotion and the amount of time spent in the center of the

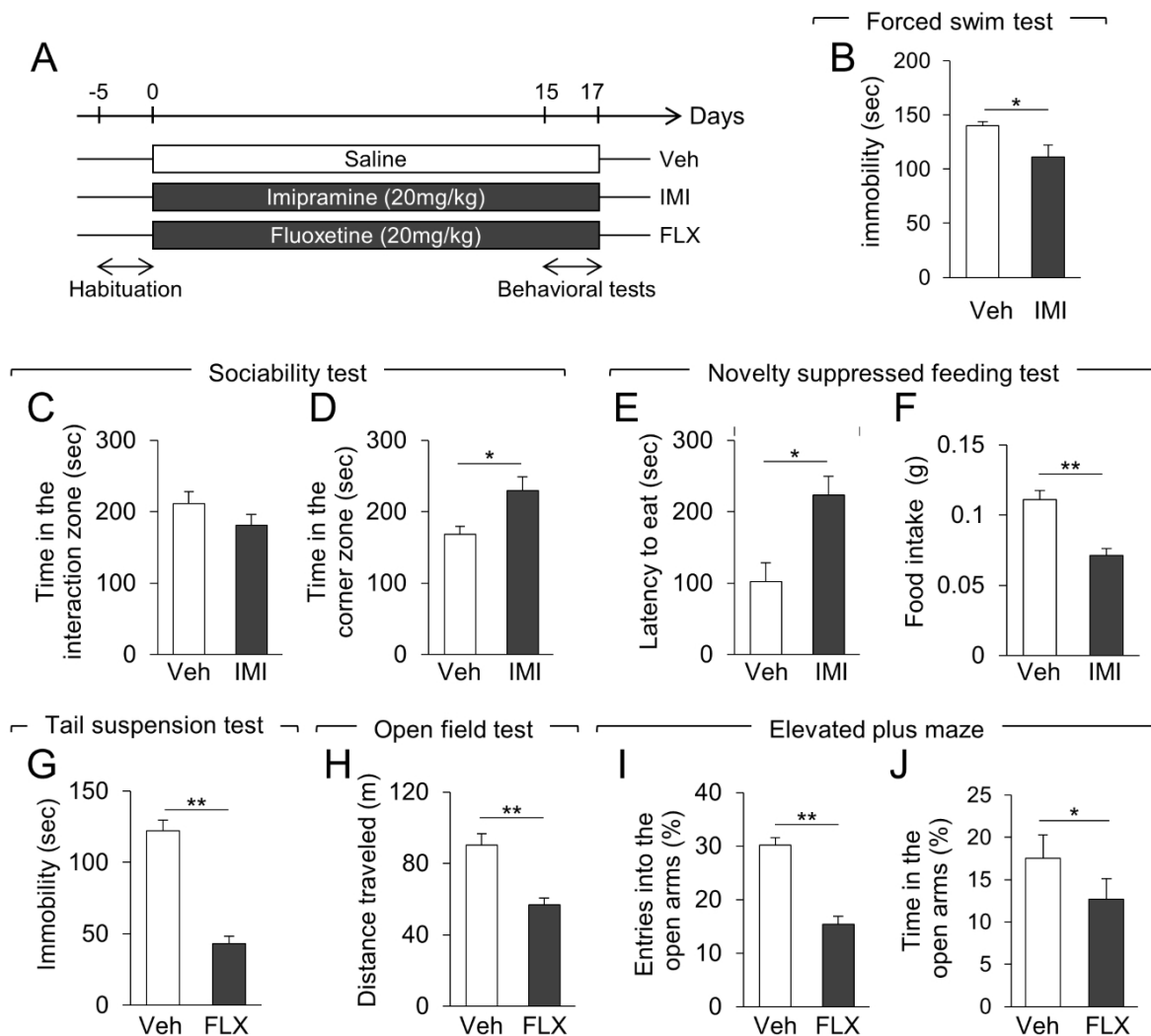


Fig. 1. Chronic antidepressant treatments produced anti-depressive-like effects, but induced anxiety-like behavior. (A) Experimental design for chronic administration of saline (Veh), imipramine (IMI) or fluoxetine (FLX) daily for 14 consecutive days and more, followed by behavioral tests. Behavioral tests were carried out in the following order: novelty suppressed feeding test (NSF), forced swim test (FST), and sociability test in the IMI treatment groups, and elevated plus maze (EPM), tail suspension test (TST), and open field test (OFT) in the FLX treatment groups. (B~F) Mice treated with chronic IMI showed decreased immobility in the FST (B). In the sociability test, IMI-treated mice spent a similar amount of time as that of control mice in the interaction zone (C), but significantly more time in the corner zone (D), a behavioral sign of anxiety. In the novelty suppressed feeding (NSF) test, IMI-treated mice showed increased latency to eating a food pellet in the center (E), as well as decreased food consumption examined for 5 min immediately after administering the NSF test (F). (G~J) Mice treated with chronic FLX showed decreased immobility in the TST (G). In the open field test, FLX-treated mice exhibited decreased locomotor activity (H). In the EPM test, FLX-treated mice had decreased entries to the open arms (I) and spent less time in the open arms (J). Data are presented as the mean±SEM (n=8 animals for FST; n=12~14 animals for sociability test; n=6~8 animals for NSF; n=7~8 animals for TST; n=7 animals for OFT; n=6~8 animals for EPM). * and ** denote differences between the two groups at $p<0.05$ and $p<0.01$. Student's *t*-test was used.

open field. Mice treated with chronic IMI followed by 6h×3d RST treatment did not significantly change the total locomotion and the amount of time spent in the center of the open field compared with chronic IMI treatment (Fig. 2D and E). Two-way ANOVA analysis detected the main effect of chronic IMI treatment in total locomotion [$F_{(1,27)}=6.152$, $p=0.0197$], but not in time spent in the center [$F_{(1,24)}=2.234$, $p=0.1481$]. There was no effect of 6h×3d RST

treatment [$F_{(1,27)}=1.567$, $p=0.2213$ for locomotion; $F_{(1,24)}=0.1301$, $p=0.7215$ for center], and no IMI×RST interaction [$F_{(1,27)}=0.3161$, $p=0.5786$ for locomotion; $F_{(1,24)}=0.1923$, $p=0.6649$ for center] (Fig. 2D and E). Chronic FLX treatment in normal mice reduced total locomotion in the open field test and the amount of time spent in the center of the open field (Fig. 2D and E). Chronic FLX treatment followed by 6h×3d RST treatment did not change the

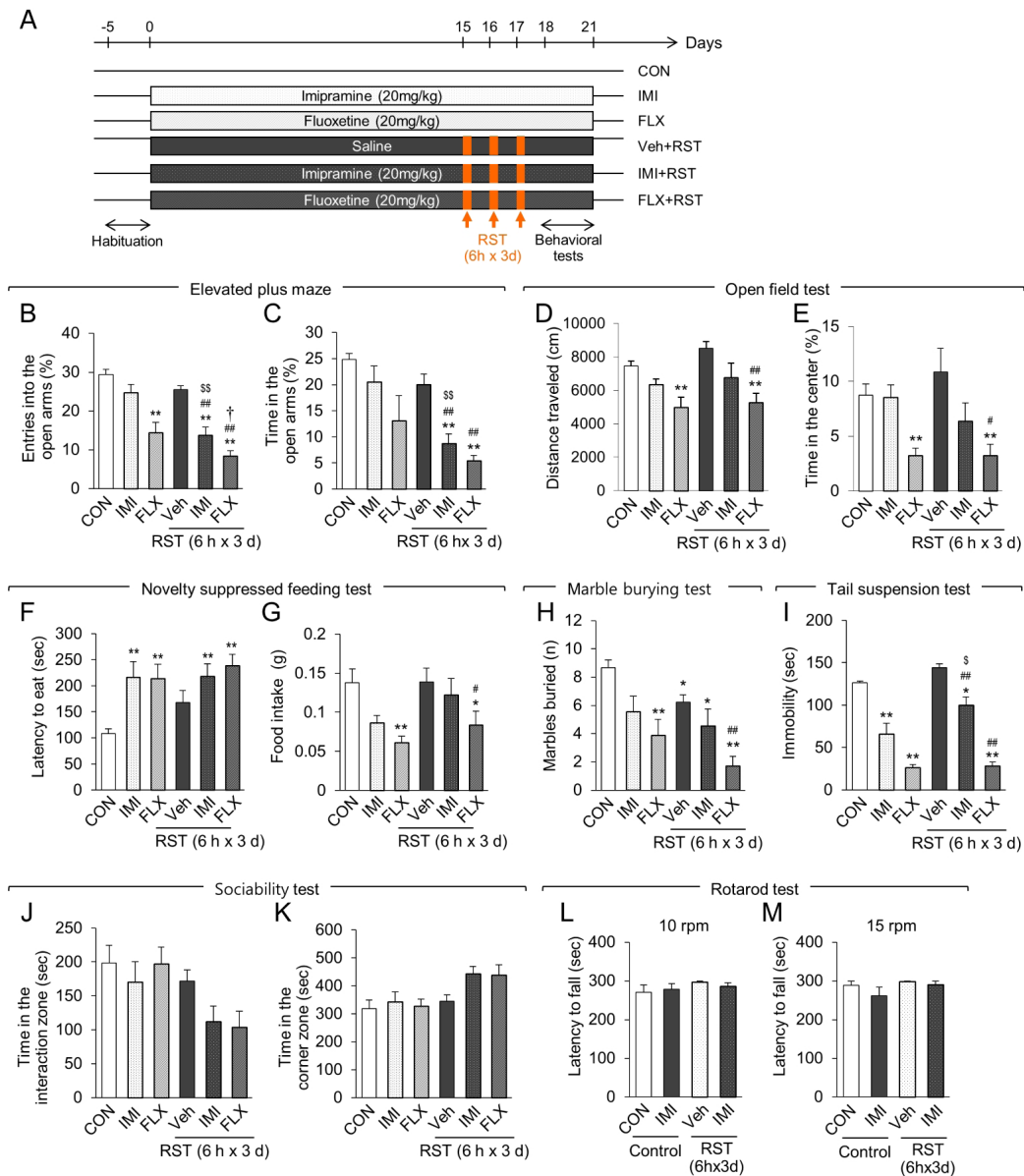


Fig. 2. Chronic antidepressant treatments impaired the animals stress-coping ability in regulating anxiety-related behavior, but not in depression-related behavior. (A) Experimental design of chronic administration of saline (Veh), imipramine (IMI) or fluoxetine (FLX), following treatment with restraint for 6 h daily for 3 consecutive days (RST, 6h x 3d), and subsequent behavioral tests. Behavioral tests were carried out for more than three independent sets of experimental groups. CON, untreated naive mice. Behavioral tests were carried out in the order of the EPM test, sociability test, MBT, NSF test, OFT, TST, and rotarod test. (B, C) The percentage of entry numbers to the open arms (B) and the percentage of the time spent in the open arms (C) in the EPM test, among untreated mice (CON), mice treated with chronic IMI or FLX, and mice treated with chronic saline (Veh), IMI or FLX followed by 6h x 3d RST. (D, E) The traveled distance of animals in the open field test (D), and the time spent in the center zone of the open field (E), among untreated mice (CON), mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 6h x 3d RST. (F, G) Latency to eating a food pellet in the center of the open field in the novelty suppressed feeding (NSF) test (F), and the amount of food consumption assessed immediately after the NSF test (G), among untreated mice (CON), mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 6h x 3d RST. (H) The number of marbles buried in the marble burying test (MBT), among untreated mice (CON), mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 6h x 3d RST. (I) Immobility time in the tail suspension test, among untreated mice (CON), mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 6h x 3d RST. (J, K) Interaction time with a social target in the interaction zone (J) and the corner zone (K) in the sociability test, among untreated mice (CON), mice treated with chronic IMI or FLX or mice treated with chronic Veh, IMI or FLX followed by 6h x 3d RST. (L, M) Performance on the rota-rod rotating at 10 rpm (L) and 15 rpm (M), exhibited by untreated mice (CON), mice treated with chronic IMI, and mice treated with chronic Veh or IMI followed by 6h x 3d RST. Data are presented as the mean ± SEM (n=4~9 animals for EPM; n=6~9 animals for OFT; n=8~10 animals for NSF; n=9~10 animals for MBT; n=8 animals for TST; n=7~8 animals for sociability test; n=7~8 for rotarod test). * and ** denote differences from CON group; † and †† denoted differences from the Veh+RST group; ‡ and ‡‡ denoted differences from the IMI group, at p<0.05 and p<0.01, respectively. ‡ denoted difference from the FLX group, at p<0.05. Two-way ANOVA and Newman-Keuls *post hoc* test was used in statistical analysis.

reduced locomotion and the amount of time spent in the center of the open field compared with chronic FLX treatment (Fig. 2D and E). Two-way ANOVA analysis detected the main effect of chronic FLX treatment [$F_{(1,29)}=33.26$, $p<0.0001$ for locomotion; $F_{(1,27)}=22.16$, $p<0.0001$ for center], whereas there was no effect of 6h×3d RST treatment [$F_{(1,29)}=1.793$, $p=0.1910$ for locomotion; $F_{(1,27)}=0.001775$, $p=0.9667$ for center] and no FLX×RST interaction [$F_{(1,29)}=0.5954$, $p=0.4466$ for locomotion; $F_{(1,27)}=0.00299$, $p=0.9568$ for center] (Fig. 2D and E).

Chronic IMI or FLX treatment in normal mice significantly increased the latency for eating a food pellet placed in the center of the open field in the novelty suppressed feeding (NSF) test (Fig. 2F). In normal mice, the 6h×3d RST treatment tended to increase the latency in the NSF test. Chronic IMI or FLX treatment followed by 6h×3d RST treatment increased the latency, but the level was comparable to that by chronic IMI or FLX treatment (Fig. 2F). Two-way ANOVA analysis detected the main effects of chronic IMI or FLX treatment [$F_{(1,31)}=12.76$, $p=0.0012$ for IMI; $F_{(1,33)}=17.84$, $p=0.0002$ for FLX], but no or weak effect of 6h×3d RST treatment [$F_{(1,31)}=1.926$, $p=0.1751$ for IMI; $F_{(1,33)}=4.134$, $p=0.0501$ for FLX], and no antidepressant×RST interaction [$F_{(1,31)}=1.730$, $p=0.1981$ for IMI×RST; $F_{(1,33)}=0.6891$, $p=0.4124$ for FLX×RST] (Fig. 2F). In the subsequent assessment of food consumption, mice treated with chronic IMI showed slightly reduced food consumption, whereas mice treated with chronic IMI followed by 6h×3d RST treatment did not. Mice treated with chronic FLX exhibited reduced food consumption, and mice treated with chronic FLX followed by 6h×3d RST treatment also showed reduced food consumption, although the amount of food consumed was slightly enhanced after 6h×3d RST treatment (Fig. 2G). Two-way ANOVA analysis detected the main effect of chronic FLX treatment [$F_{(1,33)}=17.03$, $p=0.0002$], but not IMI treatment [$F_{(1,32)}=4.014$, $p=0.0537$]. There was no effect of 6h×3d RST treatment [$F_{(1,32)}=1.175$, $p=0.2865$ for IMI; $F_{(1,33)}=0.5669$, $p=0.4568$ for FLX], and no interaction between chronic IMI or FLX treatment and 6h×3d RST treatment [$F_{(1,32)}=1.037$, $p=0.3162$ for IMI×RST; $F_{(1,33)}=0.4668$, $p=0.4992$ for FLX×RST] (Fig. 2G).

Chronic IMI or FLX treatment in normal mice tended to suppress the number of marbles buried in the marble burying test (MBT). The 6h×3d RST treatment in normal mice decreased the number of marbles buried. Chronic FLX treatment followed by 6h×3d RST treatment suppressed the number of marbles buried to the level that was lower than that by chronic FLX treatment (Fig. 2H). Two-way ANOVA analysis detected the main effects of chronic IMI or FLX treatment [$F_{(1,32)}=6.273$, $p=0.0175$ for IMI; $F_{(1,33)}=33.17$, $p<0.0001$ for FLX], and the main effect of 6h×3d RST treatment in FLX group, but not in IMI group [$F_{(1,32)}=3.260$,

$p=0.0804$ for IMI; $F_{(1,33)}=8.234$, $p=0.0071$ for FLX]. There were no antidepressant×RST interaction [$F_{(1,32)}=0.5734$, $p=0.4545$ for IMI×RST; $F_{(1,33)}=0.02505$, $p=0.8752$ for FLX×RST] (Fig. 2H).

Next, we examined whether chronic antidepressant treatment followed by short-term acute stress affects depression-related behaviors in normal mice (Fig. 2I, J and K). Chronic IMI treatment in normal mice significantly decreased immobility time in the tail suspension test (TST) (Fig. 2I). The 6h×3d RST treatment in normal mice did not significantly change immobility time in the TST. Chronic IMI treatment followed by 6h×3d RST treatment also decreased immobility time in the TST compared with chronic IMI treatment (Fig. 2I). Chronic FLX treatment in normal mice significantly reduced immobility time in the TST. Chronic FLX treatment followed by 6h×3d RST treatment suppressed immobility time in the TST compared with chronic FLX treatment (Fig. 2I). Two-way ANOVA analysis detected the main effects of chronic IMI or FLX treatment [$F_{(1,28)}=34.42$, $p<0.0001$ for IMI; $F_{(1,28)}=683.2$, $p<0.0001$ for FLX] and 6h×3d RST treatment [$F_{(1,28)}=8.421$, $p=0.0071$ for IMI; $F_{(1,28)}=5.791$, $p=0.0230$ for FLX]. There were no antidepressant×RST interaction [$F_{(1,28)}=0.8216$, $p=0.3724$ for IMI×RST; $F_{(1,28)}=3.579$, $p=0.0689$ for FLX×RST] (Fig. 2I).

Chronic IMI or FLX treatment in normal mice did not change the interaction time with a social target and the time spent in the corner zone in the sociability test. Likewise, the 6h×3d RST treatment did not change the interaction time with a social target and the time in the corner zone in the sociability test (Fig. 2J and K). Chronic IMI or FLX treatment followed by 6h×3d RST treatment tended to suppress the interaction time with a social target and tended to increase the time spent in the corner zone in the sociability test compared with chronic IMI or FLX treatment (Fig. 2J and K). Two-way ANOVA analysis showed no effect of chronic IMI treatment [$F_{(1,25)}=2.510$, $p=0.1257$ for interaction zone; $F_{(1,25)}=3.354$, $p=0.0790$ for corner zone] and chronic FLX treatment [$F_{(1,25)}=1.753$, $p=0.1975$ for interaction zone; $F_{(1,25)}=2.478$, $p=0.1280$ for corner zone] (Fig. 2J and K). There was the main effect of 6h×3d RST treatment in FLX group [$F_{(1,25)}=5.207$, $p=0.0313$ for interaction zone; $F_{(1,25)}=4.348$, $p=0.0474$ for corner zone], but not in IMI group [$F_{(1,25)}=2.344$, $p=0.1383$ for interaction zone; $F_{(1,25)}=3.572$, $p=0.0704$ for corner zone] (Fig. 2J and K). There were no antidepressant×RST interaction in IMI group [$F_{(1,25)}=0.3338$, $p=0.5686$ for interaction zone; $F_{(1,25)}=1.201$, $p=0.2835$ for corner zone] and in FLX group [$F_{(1,25)}=1.630$, $p=0.2135$ for interaction zone; $F_{(1,25)}=1.629$, $p=0.2135$ for corner zone] (Fig. 2J and K).

Chronic IMI treatment in normal mice did not change the riding performance on the rota-rod test. Likewise, the 6h×3d RST treatment did not change the performance on the rota-rod

test. Chronic IMI treatment followed by 6h×3d RST treatment in normal mice also did not change the performance in the rota-rod test (Fig. 2L and M), indicating that chronic IMI treatment did not have reduced general motor activity. Two-way ANOVA analysis revealed no main effect of chronic IMI treatment [$F_{(1,27)}=0.01137$, $p=0.9159$ for 10 rpm; $F_{(1,28)}=1.669$, $p=0.2070$ for 15 rpm] and 6h×3d RST treatment [$F_{(1,27)}=1.811$, $p=0.1896$ for 10 rpm; $F_{(1,28)}=2.026$, $p=0.1657$ for 15 rpm], and no IMI×RST interaction [$F_{(1,27)}=0.5724$, $p=0.4559$ for 10 rpm; $F_{(1,28)}=0.5578$, $p=0.4614$ for 15 rpm] (Fig. 2L and M).

Chronic antidepressant treatments impaired stress-coping ability, resulting in diverse physiological symptoms

We next examined whether chronic antidepressant treatments in normal mice affected stress coping ability (Fig. 3). C57BL/6J mice that were exposed to restraint for 2 h daily for 3 days (2h×3d RST) actively cared for their coat states, thus maintaining well-kept hairs, in home cage returned after stress treatment procedure (Fig. 3A and B). On the contrary, chronic IMI or FLX treated mice neglected this behavior, such that their coat remained dirty and unkempt. Mice treated with chronic IMI followed by 2h×3d RST treatment showed more adverse coat state than chronic IMI group, thus indicative of more unhealthy overall appearance after 2h×3d RST treatment, whereas mice treated with chronic FLX followed by 2h×3d RST treatment looked unhealthy, but exhibited no more adverse change in coat state compared with chronic FLX group (Fig. 3B). Two-way ANOVA analysis detected the main effects of chronic IMI or FLX treatment [$F_{(1,18)}=30.77$, $p<0.0001$ for IMI; $F_{(1,17)}=23.22$, $p=0.0002$ for FLX] (Fig. 3B). There was the main effect of 2h×3d RST treatment in IMI, but not in FLX, group [$F_{(1,18)}=14.14$, $p=0.0014$ for IMI; $F_{(1,17)}=0.513$, $p=0.4836$ for FLX]. There was a significant interaction between IMI and RST, but not between FLX and RST [$F_{(1,18)}=10.01$, $p=0.0054$ for IMI×RST; $F_{(1,17)}=0.06783$, $p=0.7976$ for FLX×RST] (Fig. 3B).

Chronic IMI or FLX treatment in normal mice produced normal appearance of the lips and nose, although weak inflammatory signs on the lips and nose appeared in 1 animal among 5 animals. Chronic 2h×3d RST treatment produced no detectable sign on the lips and nose (Fig. 3C and D). Whereas chronic IMI or FLX treatment followed by 2h×3d RST induced the appearance of swollen vesicles and ulcerations on the lips (Fig. 3C) as well as watery and inflammatory noses in 5 out of 8 animals for IMI and 3 out of 7 animals for FLX, although the mean values of quantified symptoms were not statistically significant (Fig. 3D). Two-way ANOVA analysis showed no main effect of chronic IMI treatment [$F_{(1,18)}=0.9677$, $p=0.3383$ for lips; $F_{(1,18)}=3.086$, $p=0.096$ for nose] and chronic FLX treatment [$F_{(1,17)}=2.878$, $p=0.1081$ for lips;

$F_{(1,17)}=3.085$, $p=0.097$ for nose]. There was no main effect of 2h×3d RST treatment in IMI group [$F_{(1,18)}=0.9677$, $p=0.3383$ for lips; $F_{(1,18)}=3.086$, $p=0.096$ for nose] and in FLX group [$F_{(1,17)}=0.9105$, $p=0.3534$ for lips; $F_{(1,17)}=0.2455$, $p=0.6266$ for nose]. There was no interaction between antidepressant and RST on lip symptom [$F_{(1,18)}=0.9677$, $p=0.3383$ for IMI×RST; $F_{(1,17)}=0.9105$, $p=0.3534$ for FLX×RST] and nose symptom [$F_{(1,18)}=3.086$, $p=0.096$ for IMI×RST; $F_{(1,17)}=0.2455$, $p=0.6266$ for FLX×RST] (Fig. 3C and D).

Chronic IMI or FLX treatment in normal mice decreased the number of defecations in the novel open field environment. The 6h×3d RST treatment in normal mice also decreased the number of defecations in the open field. Chronic IMI or FLX treatment followed by 6h×3d RST treatment in normal mice reduced the number of defecations in the open field compared with CON group (Fig. 3E). Two-way ANOVA analysis showed the main effect of chronic FLX treatment, but not chronic IMI treatment [$F_{(1,32)}=3.940$, $p=0.0558$ for IMI; $F_{(1,33)}=15.16$, $p=0.0005$ for FLX]. There was the main effect of 6h×3d RST treatment [$F_{(1,32)}=5.146$, $p=0.0302$ for IMI; $F_{(1,33)}=6.767$, $p=0.0138$ for FLX], and the significant antidepressant×RST interaction [$F_{(1,32)}=5.146$, $p=0.0302$ for IMI×RST; $F_{(1,33)}=6.600$, $p=0.0149$ for FLX×RST] (Fig. 3E).

Mice treated with chronic IMI or FLX followed by 6h×3d RST treatment usually had a large abdomen. Surgical opening of the abdomen revealed a highly enlarged and inflamed small intestine full of defecation materials (Fig. 3F).

DISCUSSION

The results of the present study showed that chronic treatment with imipramine or fluoxetine in normal mice produce enhanced anxiety in several behavioral tests. Moreover, chronic antidepressant treatment increased sensitivity of animals to subsequent sub-threshold levels of stress. These results raise several important questions concerning chronic treatment of imipramine or fluoxetine both in practice and research applications.

Chronic antidepressant treatments in the normal mice induced anxiety-like behaviors

Major depression and anxiety disorders have high levels of comorbidity [51, 52]. Among patients with major depression, 59.2% have anxiety disorders, while 45.5% of patients with anxiety disorders have major depression [51]. Mice treated with chronic stress show both depression- and anxiety-like behaviors in various animal models [25, 32, 33, 53], and these anxiogenic phenotypes can be rescued by treatment with antidepressants [12, 27]. Thus, depression is tightly associated with anxiety in certain patients with depression or in stress-induced depression animal models.

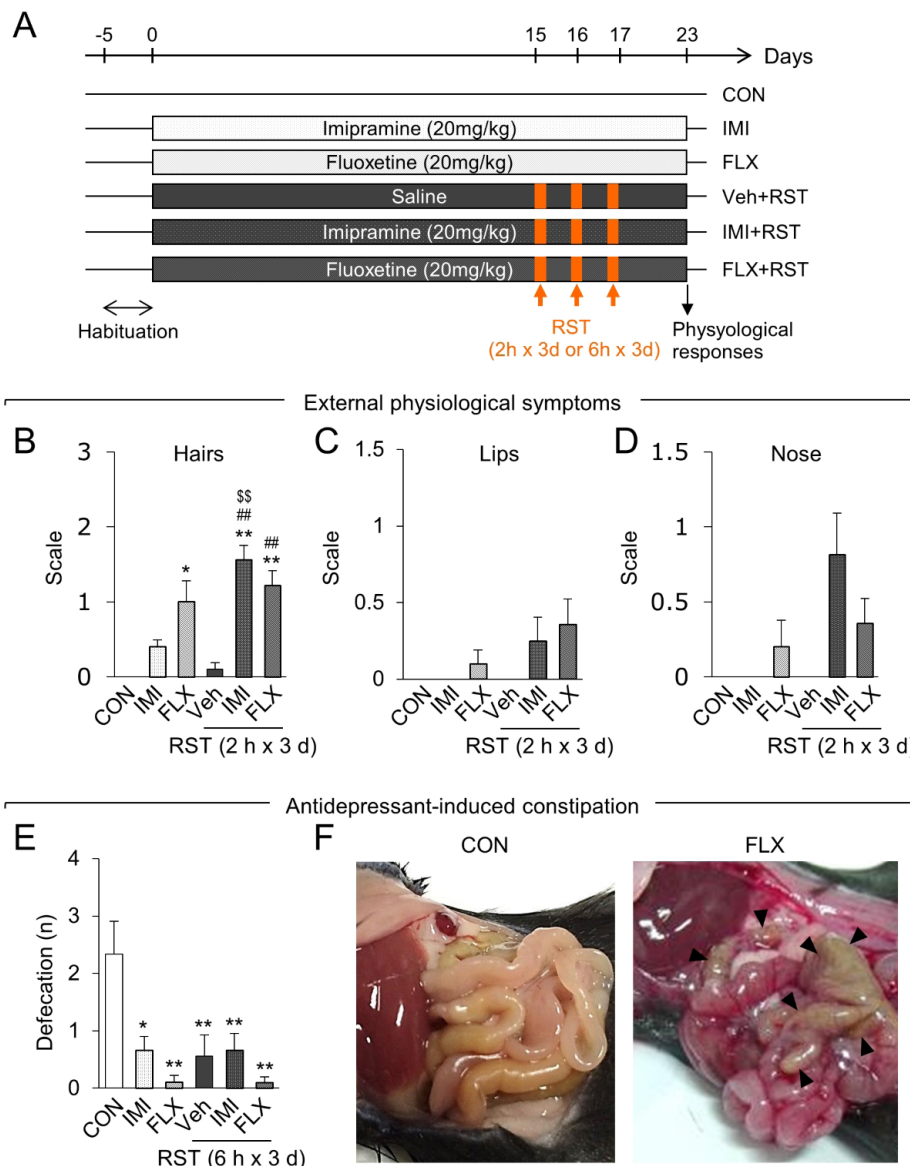


Fig. 3. Chronic antidepressant treatments deteriorate the external physiological stress-coping response of mice. (A) Experimental design of chronic administration with saline (Veh), imipramine (IMI), or fluoxetine (FLX) following treatment with restraint for 2 or 6 h daily for 3 consecutive days (RST, 2h x 3d; RST, 6h x 3d), and subsequent assessment of physiological responses. CON, untreated mice. (B~D) Extent of physiological symptoms presenting on the coat (B), lips (C), and nose (D), among mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 2h x 3d RST. CON, untreated mice. External physiological symptoms were evaluated using a rating scale of 0~3 as described in the Materials and Methods. (E, F) Number of defecation boli in the novel open field environment, among mice treated with chronic IMI or FLX, and mice treated with chronic Veh, IMI or FLX followed by 2h x 3d RST. CON, untreated mice. (F) Enlarged small intestine of mice treated with chronic FLX followed by 6h x 3d RST. White arrow heads indicate parts of the small intestine clogged with defecation materials (E). Data are presented as the mean \pm SEM (n=4~8 animals for external physiological symptoms; n=9~10 animals for antidepressant-induced constipation). * and ** denote differences from the CON group, at p<0.05 and p<0.01, respectively. ## denotes differences from the Veh+RST group; \$\$ denotes differences from the IMI group, at p<0.01. Two-way ANOVA and Newman-Keuls *post hoc* test was used.

On the contrary, the results of the present study suggest that certain depression- and anxiety-related behaviors within the same animals may express in opposite manners after chronic treatment with imipramine or fluoxetine.

The results of the present study indicate that chronic treatment

with imipramine or fluoxetine in normal animals induces anxiety without affecting depressive-related behaviors (Fig. 1 and 2). Although the present study did not explore the detailed mechanism underlying antidepressant-induced enhanced anxiety, we can speculate upon following possibilities. Chronic imipramine

or fluoxetine treatment may induce differential transcriptional changes in different brain regions, resulting in the suppression of depression-related behaviors while enhancing anxiety-related behaviors. Consistent with this possibility, chronic restraint stress produces differential gene expression profiles in the hippocampus and amygdala [32, 54]. It is also possible that a single or a set of its related genes is induced by chronic antidepressant treatment commonly in different brain regions, which in turn facilitates suppression of depression-related behaviors while increasing anxiety-related behaviors. BDNF is one candidate gene which may produce such functions: (i) BDNF is induced by treatment with imipramine or fluoxetine in various regions of the brain, including the hippocampus [55, 56] and *in vitro* culture condition [57]. (ii) In the social defeat stress model, BDNF expression in the hippocampus is downregulated, while viral vector-mediated expression of BDNF effectively rescues stress-induced depression-related behaviors [58]. (iii) In a social defeat stress model or in a repeated restraint model, BDNF expression in the hippocampus is downregulated, whereas its expression in the nucleus accumbens [21, 59] and amygdala [60-62] is up-regulated. On the other hand, blocking the effects of BDNF in the nucleus accumbens [21, 59, 63] and amygdala [64] in stress-induced depression models rescues anxiety- and depression-like behaviors. (iv) Transgenic overexpression of BDNF in the hippocampus and amygdala induces both antidepressant and anxiogenic effects [65]. Furthermore, mice with the heterozygous state of BDNF in the whole brain do not have depressive-like behavior [66-69]. However, the precise role of BDNF in anxiety and depression needs to be studied further and remains to be controversial as summarized recently [70].

Chronic antidepressant treatments in normal mice weakens the stress coping abilities in maintaining anxiety-related behaviors and in regulating physiological symptoms

The results of the present study argue that chronic antidepressant treatment in normal mice deteriorate the animals ability to withstand upcoming stress. Indeed, mice treated with antidepressants for 14 and more days showed weak or undetectable levels of physiological symptoms including changes in maintaining a clean coat state and normal immune responses as characterized by the appearance of the lips and nose (Fig. 3). On the other hand, animals treated with chronic antidepressants followed by a sub-threshold level of stress exhibited dirty and unkempt appearance of coat states (Fig. 3). These antidepressant-primed and stress-induced failures in maintaining proper coat state and immune responses that presented on the lips and nose, although the signs of lips and nose were subtle, were similar to those exhibited by AC5 KO mice exposed to short-term

stress [43]. Specifically, AC5 KO mice exposed to restraint for 2 h daily for 5 days exhibit failure in keeping a proper coat state and have significant lips and nose signs. In addition, AC5 KO mice exposed to short-term stress have increased intestinal abnormalities as evidenced by a highly inflated and inflamed small intestine full of digested food (unpublished observations). In considering that AC5 KO mice displayed exaggerated stress responses [43], it will be interesting to investigate whether the hypothalamus-pituitary-adrenal gland (HPA) system is overactivated in mice undergoing chronic treatment with antidepressants.

In conclusion, we show that chronic treatment with imipramine or fluoxetine in normal mice results in a strong induction of anxiety, despite the fact that long-term treatment with these antidepressants confers anti-depressant-like effects. Chronic antidepressant treatments in normal mice also deteriorate the animal's stress coping ability, resulting is heightened sensitive to subsequent moderate stress. These results raise concerns regarding that long-term use of current anti-depressants and suggest that special considerations be made with respect to their use in practice and research applications.

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