Journal of Visualized Experiments

The Unpredictable Chronic Mild Stress Protocol for Inducing Anhedonia in Mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58184R2
Full Title:	The Unpredictable Chronic Mild Stress Protocol for Inducing Anhedonia in Mice
Keywords:	Unpredictable Chronic Mild Stress; Anhedonia; Sucrose Preference; Animal Model; Depression; Antidepressants
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Mr. Benjamin Werth
Senior Science Editior
JoVE

March 21, 2018

Dear Mr. Benjamin Werth,

Enclosed please find our manuscript entitled *'Assessment of Hedonic Tone following the Unpredictable Chronic Mild Stress Mice Model for Depression'*.

Sincerely yours,
Ravid Doron, PhD

TITLE:

The Unpredictable Chronic Mild Stress Protocol for Inducing Anhedonia in Mice

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KEYWORDS:

Unpredictable Chronic Mild Stress; Anhedonia; Sucrose Preference; Animal Model; Depression; Antidepressants

SUMMARY:

Here we present the unpredictable chronic mild stress protocol in mice. This protocol induces a long-term depressive-like phenotype and enables to assess the efficacy of putative antidepressants in reversing the behavioral and neuromolecular depressive-like deficits.

ABSTRACT:

Depression is a highly prevalent and debilitating condition, only partially addressed by current pharmacotherapies. The lack of response to treatment by many patients prompts the need to develop new therapeutic alternatives and to better understand the etiology of the disorder. Pre-clinical models with translational merits are rudimentary for this task. Here we present a protocol for the unpredictable chronic mild stress (UCMS) method in mice. In this protocol, adolescent mice are chronically exposed to interchanging unpredictable mild stressors. Resembling the pathogenesis of depression in humans, stress exposure during the sensitive period of mice adolescence instigates a depressive-like phenotype evident in adulthood. UCMS can be used for screenings of antidepressants on the variety of depressive-like behaviors and neuromolecular indices. Among the more prominent tests to assess depressive-like behavior in rodents is the sucrose preference test (SPT), which reflects anhedonia (core symptom of depression). The SPT will also be presented in this protocol. The ability of UCMS to induce anhedonia, instigate long-term behavioral deficits and enable reversal of these deficits via chronic (but not acute) treatment with antidepressants strengthens the protocol's validity compared to other animal protocols for inducing depressive-like behaviors.

INTRODUCTION:

Major depressive disorder (MDD) is a debilitating condition, that has been indicated as the 11th cause of global burden from disease[1], with a lifetime prevalence of 11-16%[2, 3]. MDD has been associated with severe impairments on patients' social and occupational functioning, diminished quality of life, numerous mental and physical disorders and increased risk for mortality[4–7]. There are several efficacious pharmacotherapies and psychological interventions for MDD; however, more than the third of the patients do not achieve remission with the existing therapeutic options[8–11]. Therefore, better mapping of the pathophysiology of MDD and development of novel drugs are still of utmost importance. In order to address these tasks scientifically validated animal models needs to be utilized.

Unpredictable chronic mild stress (UCMS) is a renowned rodent paradigm used to induce depressive- and anxiety-like behaviors[12–15]. The main objective of UCMS is to generate behavioral deficits (such as anhedonia and behavioral despair[12, 15]) in mice and rats, and promote screenings for potential therapeutic pharmacological agents. The procedure was first introduced by Katz[16] and subsequently developed by Willner[17, 18], yielding vast behavioral and neurobiological outcomes reminiscing depressive symptomatology[12]. It was initially designed for rats and later accommodated to mice[13, 19]. In the procedure, adolescent animals are chronically exposed to different unpredictable mild stressors. Subsequently, pharmacological agents are administered. Behavioral and biological indices are obtained upon treatment termination. One of the more prominent tests conducted following UCMS is the sucrose preference test (SPT). The SPT is based on rodents' innate preference for the sweetened solution rather than water and is widely acknowledged as an essential translational model for assessing anhedonia[12, 18, 20, 21] (which is a core symptom in human depression[22, 23]).

While entering the fourth decade since its introduction, UCMS has been applied on mice and rats in myriad studies. The majority of these studies employed UCMS as a method to induce depressive-like behaviors[12, 13, 21, 24]. Studies have also employed the model to generate anxiogenic effects[25–29]. Sucrose and saccharin preferences are the main tests used to assess anhedonia following UCMS[12, 18, 30–33]. Other notable outcome measures that are highly incorporated in UCMS literature are: the tail suspension test (TST)[28, 34, 35], the forced swim test (FST)[28, 34, 36, 37] (both measuring stress coping / behavioral despair), the open field test (OFT; measuring exploratory behavior, anxiety-like behavior and locomotor activity)[25, 28, 38], the elevated plus maze (EPM; measuring anxiety-like behavior)[25, 39, 40] and additional tests measuring depressive-like behaviors, anxiety-like behaviors, cognitive functioning and social behavior[12]. Chronic administration of tricyclic antidepressants (TCAs; imipramine[35, 41–43], desipramine[18, 44, 45]), tetracyclic antidepressants (TeCAs; maprotiline[46, 47], mianserin[48]), selective serotonin reuptake inhibitors (SSRIs; fluoxetine[46, 47, 49], escitalopram[30, 50], paroxetine[51, 52]), melatonin[43, 49], agomelatine[53], the fatty acid amide hydrolase (FAAH) inhibitor URB597[54] and several natural compounds[30, 37, 50, 55–58] have been demonstrated to reverse the UCMS-induced depressive- and anxiety-like symptoms. Overall, these therapeutic effects have not been obtained via acute treatments[12] (*e.g.*, paroxetine[51, 52], imipramine[53, 54, 59, 60], fluoxetine[53], agomelatine[53], URB597[54], brofaromine[60]).

Stress exposure during childhood and adolescence is a major risk factor for the anterior formation of MDD (among several other psychiatric disorders) in adulthood[61–63]. The hypothalamic-

pituitary-adrenal (HPA) axis is a major neuroendocrine system regulating the bio-behavioral response to stress[64]. Long-term stress during the sensitive neurodevelopmental periods of childhood and adolescence impairs the equilibrium of the HPA axis. It might provoke a state of enhanced sympathetic activation, unbalanced reactivity and hypercortisolemia lasting through the resting state; thus, rendering individuals vulnerable to the depression or anxiety-related psychopathologies[65–68]. UCMS adequately translates this pathogenesis: stress application during mice' adolescence induces a long-term depressive-like susceptibility. Moreover, the behavioral deficits induced by UCMS, are underlain by significant alterations in HPA axis functioning (*e.g.*, by causing a reduction in hippocampal brain-derived neurotrophic factor [BDNF; a protein highly involved in the equilibrium of the HPA axis[69, 70]][30], or by impairing the regulation of corticosterone secretion to the blood[71, 72]), in similarity to the pathophysiology in humans[12, 50, 73].

UCMS has several bolstering features as a model for depression: *e.g.* (i) the elicitation of anhedonia (which is regarded an endophenotype of MDD[23, 74]); (ii) UCMS enables to assess wide variety of depressive-like behaviors such as behavioral despair, reduced social behavior, deterioration in fur state and more[34]; and (iii) chronic (2-4 weeks), but not acute, administration of antidepressants following stress exposure could produce a protracted therapeutic effect parallel to the effect obtained in human patients by the same agents[30, 75–77].

These features strengthen the validity of UCMS compared to other animal models of depression. The FST[78] and the TST[79] are two models that are used either to induce or to assess depressive-like behavior. As models for inducing depressive-like behaviors they have clear shortfalls compared to UCMS; they do not prompt long-term behavioral changes and might merely reflect an adjustment to acute stress rather than yield a durable depressive-like manifestation[76].

An alternative animal model of depression is the social defeat model. Unlike the FST and the TST this model (like UCMS) require the application of chronic stress (*id est* [*i.e.*], the recurrent subjection of the animal to aversive social encounters with dominant counterparts)[76, 77, 80–82]. The main advantage of the social defeat model is that it employs social stimuli as stressors, thus reflecting the role of psychosocial stress in the pathogenesis of human depression. Similar to UCMS, the social defeat model elicits long-term depressive-like behaviors and neuroendocrine alterations. Yet again parallel to UCMS, the social defeat-induced deficits could be reversed via chronic, but not acute, administration of antidepressants. Overall, there is large support for the utilization of both UCMS and social defeat as pre-clinical apparatuses for investigating the pathophysiology of depression[76, 77, 81, 82]. However, a major shortfall of the social defeat model is that it could only be applied on male rodents, as females do not exhibit sufficient aggressive behavior toward each other[83]. Contrastingly, UCMS has been shown to produce several depressive-like effects on both male and female mice[34].

Predictable chronic mild stress (PCMS) is another rodent model that enforces a regimen of daily recurring exposure to restraint stress[28, 84–87]. Several studies have shown that PCMS increased anxiety-like behaviors[28, 87]; albeit, there are contradictory reports vis-à-vis PCMS ability to induce long-term depressive-like behaviors. Unlike UCMS, PCMS has produced less satisfactory results referring to its ability to induce an anhedonic-like state[28, 84, 86]. This is consistent with the human

phenomenology, in which unpredictable stressors are more harmful than predictable ones[88].

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee of the Academic College Tel-Aviv-Yaffo.

1. Animals

1.1. Use pre-adolescent (*i.e.*, 3 weeks old) Institute of Cancer Research (ICR) outbred male mice.

1.2. Randomize mice to two equally sized stress group (UCMS vs. naïve). Use 15 mice per treatment group (*e.g.*: if there are 3 pharmacological treatment groups use 90 mice overall; 2 [UCMS vs. naïve] × 3 [treatments] × 15 [mice] = 90)

1.3. House mice according to the stress group; namely, house naïve mice with naïve mice only, and house UCMS group mice with mice from the UCMS group only.

1.4. House animals in standard home cages (30 × 15 × 14 cm; 5 mice per cage; each cage containing mice from all treatment groups [*i.e.*, pharmacological treatment groups]; maintain mice in the same cage throughout the experiment, except when indicated otherwise).

1.5. Fill home cages with fresh sawdust (replaced twice a week) and add a piece of cotton wool for enrichment.

1.6. House animals in the home cage for an acclimation period of one week. Allow *ad libitum* access to rodent chow and water (except during UCMS stressor applications).

1.7. Keep a consistent 12 h light / dark cycle (except when indicated otherwise). During UCMS procedures maintain naïve mice in their home cage.

2. UCMS

2.1. Designate a separate room in the lab, for the sole use of the UCMS protocol.

2.2. Design a 4-week stressor regimen in which each of the seven stressors (*i.e.*, wet cage, dampened sawdust, tilted cage, empty cage, social stress, mice restraint and disruption of light / dark cycle) is utilized once a week, on a different day each week (for a possible design see **Supplemental Table 1)**.

2.3. Following 1 week of acclimation (see **1.6.**) initiate stressors application (ensure that mice are approximately 4 weeks old).

2.4. Each day, before the stressor application, transfer the cages of the UCMS group from the housing room to the UCMS room.

2.5. During stressor applications, block access to the rodent chow and water for the UCMS group (except during reversal of light / dark cycle).

Note: This could be obtained by the replacement of the original cage lid (which contains food and water), to an empty cage lid.

2.6. Apply the following stressors according to the regimen designed earlier (see **2.2.**):

2.6.1. Wet cage

2.6.1.1. Place mice together with their home cage counterparts in an empty cage (*i.e.*, cage without sawdust).

2.6.1.2. Fill the empty cage with water kept at 24 ± 1 °C to a depth of 1 cm (pour with caution to avoid direct water spillage on mice). Keep mice in the wet cage for 4 h.

2.6.1.3. Transfer each mouse to a separate individual transient drying cage with a heat lamp above it, a heating pad under it and paper towel bedding. Place a thermometer in the transient cage to verify the temperature does not exceed 37 °C.

2.6.1.4. Keep each mouse in the transient cage until it is dry and looks invigorated (approximately 10-15 min). Return mice to home cage with same counterparts.

2.6.2. Dampened sawdust

2.6.2.1. Pour water kept at 24 ± 1 °C to the home cage until the sawdust is moderately dampened (pour with caution to avoid direct water spillage on mice).

Note: It is not necessary to use fresh sawdust before pouring the water.

2.6.2.2. After 4 h, dry mice in transient cages as described in **2.6.1.3.** Place mice with home cage counterparts in a sterile cage with fresh sawdust.

2.6.3. Tilted cage

2.6.3.1. Tilt cages at 45° against the wall for 4 h.

Note: During this period, stressor mice remain in their home cage with their counterparts.

2.6.4. Empty cage

2.6.4.1. Transfer mice, along with their specific home cage counterparts, from the home cage to an empty cage for 4 h.

2.6.5. Social stress

2.6.5.1. Transfer mice, along with their specific home cage counterparts, from the home cage to a cage which was housed by a different group of mice for a period of at least 3 d prior to stressor application. Keep mice in the unfamiliar cage for 4 h.

Note: To avoid uncertainty place a sticker on each cage to indicate mice origin cage.

2.6.6. Mice Restraint

2.6.6.1. Place each mouse separately in a clean mouse restrainer for 4 h. Return mice to home cage with same counterparts.

2.6.7. Disruption of light / dark cycle

2.6.7.1. Transfer mice, in their home cage with their specific counterparts, to the UCMS room. Keep the light on, for 24 consecutive h.

Note: Only during this stressor mice will be allowed *ad libitum* access to rodent chow and water.

2.7. Following stressor application, return cages of the UCMS group from the UCMS room to the housing room.

2.8. During the 4 weeks of stress exposure, keep the naïve group in their home cages located in the housing room.

Note: Naïve mice are not transferred to the UCMS rooms because exposure to other mice undergoing stress procedure could induce a stressogenic effect, even without direct exposure to the stressor[89, 90].

2.9. Monitoring of Animals during UCMS

2.9.1. During stressor applications (except during disruption of light / dark cycle) monitor mice every 30 min by an experienced experimenter. If an atypical distress (*e.g.*, trembling, lethargy, lack of movement) is observed (special caution should be placed on potential hypothermia during 'wet cage' and 'dampened sawdust') relieve the mouse from the stressor immediately.

2.9.2. Inspect each mouse daily for wounds or other physical or behavioral abnormalities. If such are inspected consult with the laboratory's veterinarian to decide whether the mouse should be excluded from the experiment.

2.9.3. Weigh each mouse every 3 d. Robust reduction in body weight (*i.e.*, >10% reduction from baseline weight or >15% reduction from last measured weight) must be reported to the

laboratory's veterinarian and the mouse must be excluded from the experiment.

3. Screening for Antidepressants

3.1. On the day following cessation of the UCMS protocol, start administration of putative therapeutic pharmacological agents (*i.e.*, escitalopram [15 mg/kg; i.p.; 3 weeks; on administration per day] [30, 50], or NHT [30 mg/kg; i.p.; 3 weeks; one administration per day] [30, 50]).

3.2. Include a control treatment group and administer saline (i.p.; 3 weeks; one injection per day) to this group.

3.3. Calculate drug dose according to mice' weight, as obtained in the preceding weighing.

3.4. Administer drugs chronically (usually one drug administration per day via intraperitoneal injection [i.p.] for 3 weeks).

Note: There is no need to anesthetize the mice before the i.p. injection.

3.5. Continue to weigh mice every 3 d; the last weighing should be 3 d before the last drug administration.

4. Assessment of Hedonic Tone in the SPT

4.1. Following the treatment phase, remove each mouse from the home cage and place it individually in a cage filled with fresh sawdust and a piece of cotton wool for enrichment.

4.2. Prepare two bottles, one with distilled water and another with 2% sucrose solution (other substance could be used: *e.g.*, saccharin[91], ethanol[30]).

Note: Each bottle should contain the same volume of fluid. Neither prior acclimation nor habituation phases are needed before the introduction of fluids.

4.3. Weigh the two bottles and set them at the cage lid to allow mice *ad libitum* access to both solutions for a period of (one of the following): 24 / 48 / 72 / 144 h.

4.4. Place the two bottles at both ends of the cage lid. Place rodent chow between the two bottles to allow *ad libitum* access to food.

4.5. Replace bottles every 24 h, using sterile bottles with fresh fluid.

Note: As mice are housed individually there is no need to change the sawdust even after 144 h[92].

4.6. Switch nozzles' positions every 12 h (when test duration is 24 h) or once a day (when test duration exceeds 24 h) to counterbalance the possibility that the results were confounded by

position preference.

4.7. Weigh bottles each day to estimate consumption from each bottle.

4.8. Calculate sucrose preference as theratio of sucrose intake from total fluid intake (*i.e.*, sucrose / sucrose + water).

REPRESENTATIVE RESULTS:

In order to corroborate the efficacy of the UCMS procedure for inducing depressive-like deficits, a manipulation check was conducted. Male ICR outbred mice were randomly assigned to either UCMS or naïve conditions (4 weeks, as described in **2.2.**). Subsequently, the SPT (6 d, as described in **4.**) was administered to assess whether mice after undergoing UCMS demonstrated hedonic deficits. Shortly after, mice were sacrificed and the hippocampus was dissected out entirely for BDNF (a protein highly implicated in the pathophysiology of depression[70, 93]) assessment via enzyme-linked immunosorbent assay (ELISA). See **Figure 1** for the study design.

Independent samples *t*-test revealed a significant difference between the groups in their sucrose preference ($t_{(23)} = 2.32$, $p < 0.05$). The UCMS group demonstrated diminished sucrose preference compared to the naïve group (see **Figure 2A**). This suggests that the UCMS protocol was effective in inducing anhedonia. Independent samples *t*-test on hippocampal BDNF levels revealed a significant difference between the groups ($t_{(23)} = 2.43$, $p < 0.05$). The UCMS group demonstrated diminished hippocampal BDNF levels compared to the naïve group (see **Figure 2B**). This suggests that the UCMS protocol led to the diminution in hippocampal BDNF levels, as evident in human depression[94].

In another study from our lab, we examined the potential antidepressant-like effects of two drugs following UCMS protocol.[30] Following the UCMS procedure (as described in **2.**) male ICR outbred mice received chronic (3 weeks) treatment with the SSRI escitalopram (15 mg/kg; i.p.), NHT (30 mg/kg; i.p.; for more information regarding NHT see[25, 30, 50, 75]) or saline. Following treatment phase, the SPT was conducted and hippocampal BDNF levels were assessed. See **Figure 3** for the study design.

Two-way analysis of variance (ANOVA) on sucrose preference revealed significant treatment ($F_{(2,92)} = 4.01$, $p < 0.05$) and UCMS × treatment interaction ($F_{(2,92)} = 4.92$, $p < 0.01$) effects (see **Figure 4A**). Sidak post-hoc analysis revealed that the UCMS-saline group demonstrated a significant decrease in sucrose preference compared to the naïve-saline group ($p < 0.001$); no decreases were observed in the UCMS-escitalopram and in the UCMS-NHT groups compared to the naïve groups (not significant [*N.S.*]). Additionally, the UCMS-saline group demonstrated decreased sucrose preference compared to both the UCMS-escitalopram ($p < 0.05$) and the UCMS-NHT ($p < 0.001$) groups. These suggest that both escitalopram and NHT normalized the UCMS-induced anhedonia.

Two-way ANOVA on hippocampal BDNF levels revealed significant UCMS ($F_{(1,22)} = 8.92$, $p < 0.01$), treatment ($F_{(2,22)} = 18.36$, $p < 0.001$) and UCMS × treatment interaction ($F_{(2,22)} = 5.19$, $p < 0.05$)

effects (see **Figure 4B**). Sidak post-hoc analysis revealed that the UCMS-saline group demonstrated a significant decrease in hippocampal BDNF levels compared to the naïve-saline group ($p < 0.001$); no similar decreases were observed in the UCMS-escitalopram and in the UCMS-NHT groups compared to the naïve groups (*N.S.*). Additionally, the UCMS-saline group demonstrated decreased hippocampal BDNF levels compared to both the UCMS-escitalopram and the UCMS-NHT groups ($p < 0.001$ in both contrasts). These suggest that both escitalopram and NHT normalized the UCMS-induced reduction in BDNF levels in the hippocampus.

FIGURE AND TABLE LEGENDS:

Figure 1: A diagram depicting a possible experimental design. Following 1 week of acclimation, mice were randomly assigned to either UCMS or naïve conditions (persisting 4 weeks). Subsequently, sucrose preference was examined and mice were prepared for BDNF assessment. SPT = sucrose preference test; CD = cervical dislocation.

Figure 2: The effects of UCMS on sucrose preference and hippocampal BDNF levels. A. Mice subjected to 4 weeks of UCMS demonstrated a significant reduction in sucrose preference compared to naïve mice. **B.** Mice subjected to 4 weeks of UCMS demonstrated a significant reduction in hippocampal BDNF levels compared to naïve mice. $n = 12–13$ mice per group. Results are expressed as mean ± SEM. $*p < 0.05$

Figure 3: A diagram depicting a possible experimental design. Following 1 week of acclimation, mice were randomly assigned to either UCMS or naïve conditions (persisting 4 weeks). Subsequently, mice received chronic treatment with saline, escitalopram (15 mg/kg; i.p.) or NHT (30 mg/kg; i.p.), lasting 3 weeks (one administration per day). Following treatment, sucrose preference was examined and mice were prepared for BDNF assessment. SPT = sucrose preference test; CD = cervical dislocation.

Figure 4: The effects of chronic treatment with escitalopram and NHT on UCMS-induced reductions in sucrose preference and hippocampal BDNF levels. A. Both escitalopram and NHT prevented the UCMS-induced reduction in sucrose preference; $n = 15–17$ mice per group. **B.** Both escitalopram and NHT prevented the UCMS-induced reduction in hippocampal BDNF levels; $n = 4–6$ mice per group. Results are expressed as mean ± SEM. $*p < 0.05$ $***p < 0.001$. This figure has been modified from a previously published study from our lab and is reprinted under PLoS ONE open access license ("CC-BY") which allows reprint[30].

Supplemental Table 1: Schedule of unpredictable chronic mild stress (UCMS).

DISCUSSION:

Insofar as MDD is a widespread highly debilitating disorder, only partially addressed by current therapeutic options, the scientific quest for better treatments is still a pressing issue. Along with innovations in psychological techniques, additional pharmacotherapies are required for the large portion of patients who do not respond to the existing drugs. Meticulous animal models for depression are the key element in this task. Such models facilitate screenings for innovative

antidepressants and expand the understanding of the etiology of the disorder. UCMS is one of the more prominent rodent models of depression. Its' stature is exhibited by vast publications and notable insights[12, 18, 82, 95–97].

Anhedonia is one of the core symptoms of MDD[22, 23, 74]. A more severe anhedonic tone has been associated with poorer prognosis for MDD patients[74, 98]. A major strength of UCMS as a model of depression is its ability to generate anhedonia[31] as exemplified in the SPT. Sucrose is an innate reinforcer for various rodent species[51, 52, 59, 99]; this explains the overall support of SPT as a realistic model of hedonic tone in rodents[14, 31, 100]. Due to the focal role of anhedonia in any animal model of depression, it has been suggested that when considering implementing UCMS into a lab, the first step should be a verification of the procedure ability to induce an anhedonic state[12]. This will facilitate a better standardization across labs and could be the foundation for future studies shedding more light on the disorder.

Another feature that supports the validity of UCMS as a model of depression is that the behavioral and molecular alterations induced by UCMS are reversed by chronic, but not acute, treatment with agents that have been previously verified as effective antidepressants[12]. The protracted therapeutic effect is similar to the effects of antidepressants in humans, which usually start manifesting only after 2-3 weeks of treatment[101, 102]. In this regard, UCMS possess a superior face validity compared to the FST[78] and the TST[103], in which the effects are obtained also following acute treatment. Unlike the TST and the FST, this shortfall is not evident in the social defeat model of depression, which (along with UCMS) stand out as an excellent animal model of depression. However, compared to the FST and the TST, UCMS and other chronic stress models are much more lengthy and expensive.

Notable mice strains have been employed in UCMS studies. Among the more frequent strains are the C57BL/6 and the BALB/cJ[21, 34]. We have utilized male ICR outbred mice as numerous studies have demonstrated the efficiency of UCMS in this strain. Moreover, the utilization of ICR outbred mice bolsters the ecological validity of the protocol, due to the high between animals genetic variability of this strain (compared to transgenic mice strains)[30, 75, 104–106].

For suggested doses of other drugs not included in this protocol, but were used in other UCMS protocols see: flouxetine[46, 47, 49, 104, 105], paroxetine[51], imipramine[35, 41–43], desipramine[18, 44, 45], maprotiline[46, 47], mianserin[48], melatonin[43, 49], URB597[54] and other natural compounds[37, 55–58].

There are several additional outcome measures frequently applied in UCMS protocols, among them: (i) FST: a measure for behavioral despair (see reference[78] for protocol delineation); (ii) TST: another measure for behavioral despair (see reference[103] for protocol delineation); (iii) splash test and evaluation of coat state: two indicators of grooming behavior and putative measures of apathy (see reference[107] for protocol delineation); (iv) sociability/preference for social novelty: measures for social behavior[108] (see reference[109] for protocol delineation); and (v) sexual behavior: another measure for hedonic tone (see reference[75] for protocol delineation). Furthermore, UCMS is used to assess neuromolecular, endocrine and other biological measures pertinent to depression[50, 72, 110–116] (specifically, see references[117, 118] for BDNF assessment via

ELISA protocol delineation).

There are several critical steps within the UCMS protocol: (i) it is vital that mice from all treatment groups will be housed together and not in separate cages. For example, if there are 3 treatment groups (*e.g.*, escitalopram, NHT, and saline) there will be 2 mice from 2 of the groups and 1 mouse from the remaining group in each cage. The hybrid group housing will thwart the possibility that the results were underlain by the housing conditions and not the treatment *per se*. (ii) naïve mice must be housed separately from stressed mouse, since housing with stressed mice is stressful[90, 119, 120] and, therefore, could impede or attenuate the stress manipulation. (iii) previous UCMS protocols have instructed single housing rather than group housing[32, 121]; we have suggested the latter as single housing might cause further susceptibility to stress in mice and rats[122–125]. (iv) the UCMS schedule must be designed diligently to ensure unpredictability (*i.e.*, random exposure to each stressor once a week); however, schedules could be modified throughout the experiment as long as the unpredictability is kept.

ACKNOWLEDGMENTS:

This research was supported by the Israel Ministry of Science, Technology & Space (grant no. 313552), by the National Institute for Psychobiology in Israel (NIPI-208-16-17b) and by the Open University Foundation.

DISCLOSURES:

The authors have nothing to disclose.

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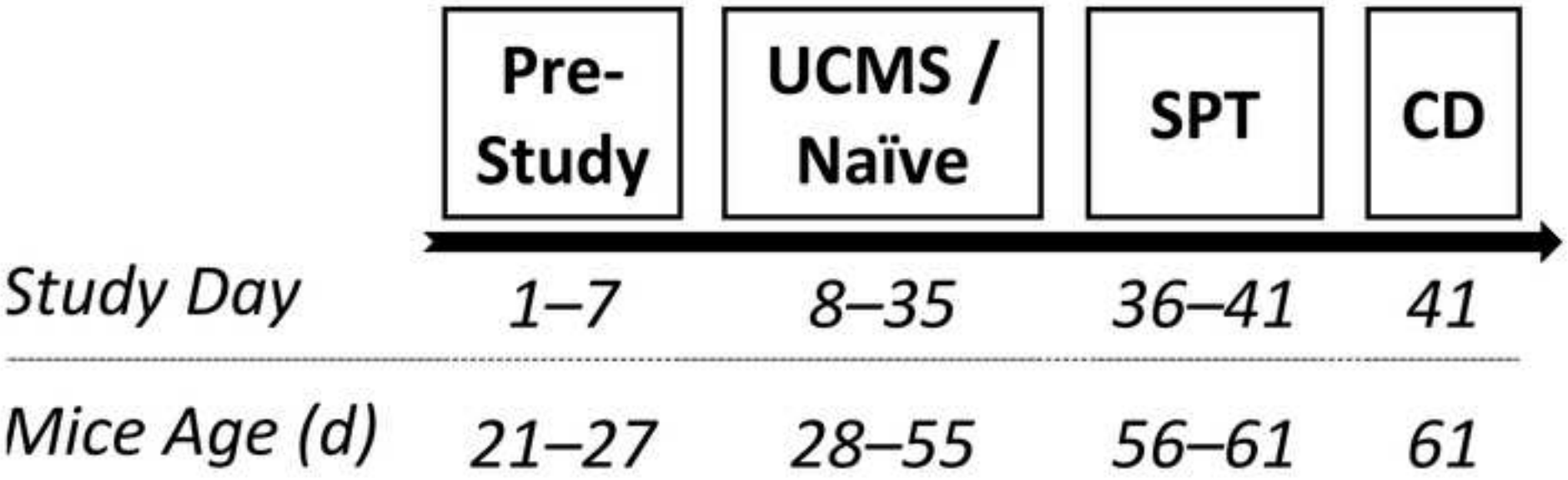

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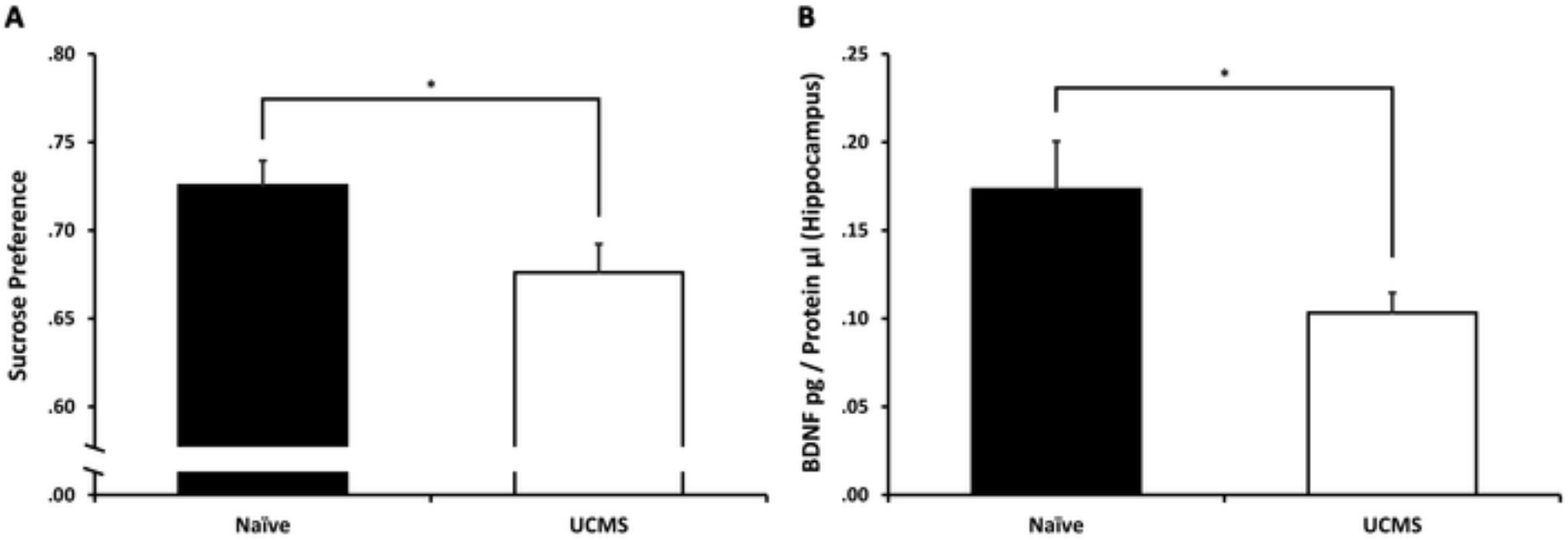

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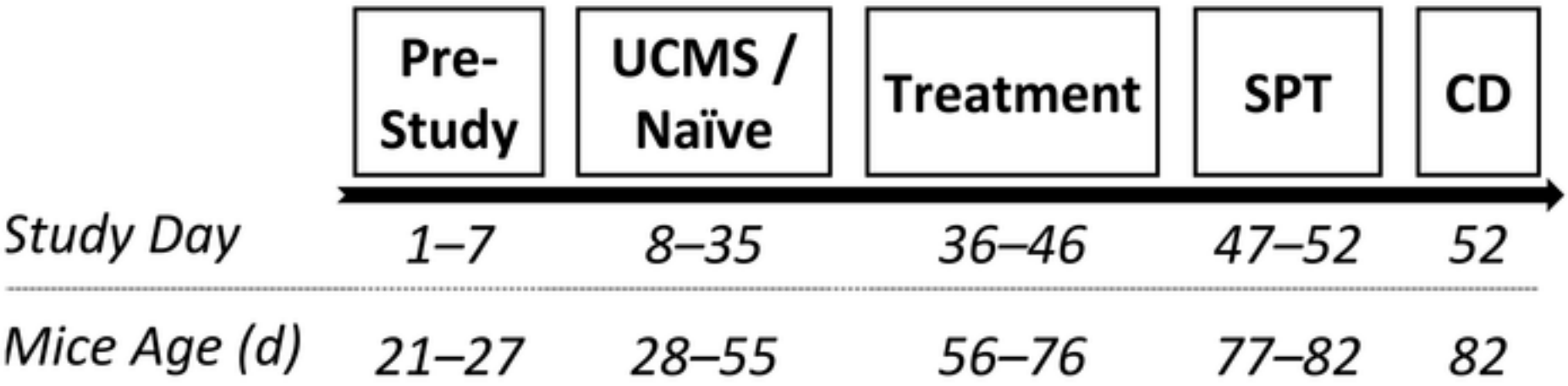

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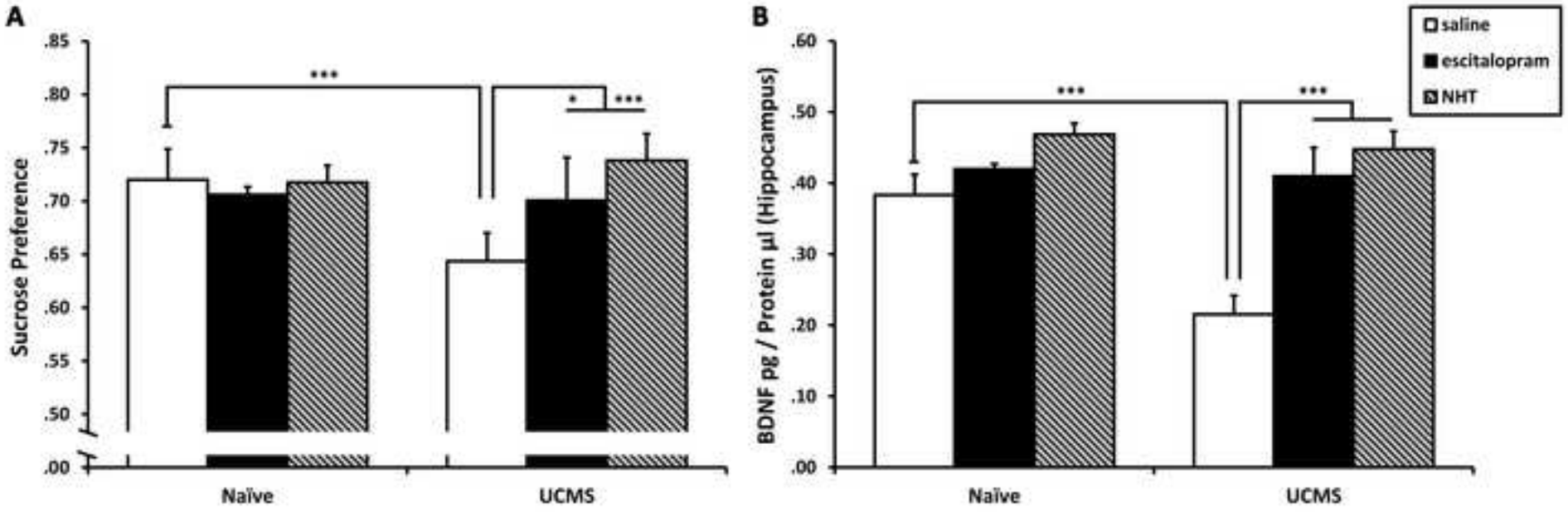

Materials Table

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Name of Material/ Equipment	Company	Catalog Number
Heating lamp	Ikea	AA-19025-3
Heating pillow	Sachs	EF-188B
Mice restrainer		
Portable electronic balance (*.** g)		
Standard rubber stopper, size 5	Ancare	#5.5R
Straight open drinking tube (2.5")	Ancare	OT-100
2% sucrose solution		
50ml conical centrifuge tube		
Pre-adolescent (approximately 20-days old) ICR outbred mice	Envigo	Hsd:ICR (CD-1)

Comments/Description

To avoid spillage during SPT
To avoid spillage during SPT (insert drinking tube into rubber stopper)

For the SPT

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We would like to thank the editor for the enlightening comments which helped us to improve this manuscript significantly.

Editorial Comments

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.
Answer: We have formatted the manuscript accordingly.

2. Please address all the specific comments marked in the manuscript.
Answer: Corrected.

3. Please change the title to reflect the protocol presented in the manuscript.
Answer: Title has been changed to reflect the protocol. The new title is: *"The Unpredictable Chronic Mild Stress Protocol for Inducing Anhedonia in Mice"*.

4. Please ensure that after formatting, the highlight is no more than 2.75 pages including heading and spacings as this is the upper limit for filming.
Answer: Highlights have been modified according to the limits.

5. The manuscript protocol talks about stress induction regime, however, no results are presented for this section. Please include a result to show how different stressors induce stress in mice. Also, please provide some marker studies to prove that indeed stress is induced in animals undergoing stressor treatment.
Answer: Results have been added to present both the efficacy of UCMS in inducing reduction in mice sucrose preference and in altering the density of a prominent biological marker of depression, namely hippocampal BDNF levels.

6. Screening for Antidepressant only present results for escitalopram and NHT. However there is a mention of other antidepressants as well in the protocol.Either move this to the discussion and specifically focus on the part you are doing or present the results for everything.
Answer: Doses of other antidepressants were moved to the discussion.

7. Sucrose consumption is not the standard test for depression. Please also show some marker stainings or western blots to corroborate the same.

Answer: Results of hippocampal BDNF levels (assessed via ELISA) were added to the results section to corroborate to anhedonic-like effect demonstrated in the sucrose preference test. However, anhedonia is a core factor in human depression, even conceptualized as an endophenotype of the disorder[1, 2]. Hence, the sucrose preference test is regarded an essential translational tool for modeling human depression in rodents[3–6].

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Table 1. Schedule of unpredictable chronic mild stress (UCMS).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Restrainer	Wet cage	Cage replacement	Tilted cage	Dampened sawdust	Empty cage	Disruption of l / d cycle
Week 2	Tilted cage	Cage replacement	Dampened sawdust	Empty cage	Wet cage	Restrainer	Disruption of l / d cycle
Week 3	Wet cage	Empty cage	Restrainer	Tilted cage	Cage replacement	Dampened sawdust	Disruption of l / d cycle
Week 4	Cage replacement	Restrainer	Dampened sawdust	Empty cage	Wet cage	Tilted cage	Disruption of l / d cycle

Note. l/ d = light / dark

TITLE:

The Unpredictable Chronic Mild Stress ~~Mice~~ Protocol for Inducing Anhedonia in Mice

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KEYWORDS:

Unpredictable Chronic Mild Stress; Anhedonia; Sucrose Preference; Animal Model; Depression; Antidepressants

SUMMARY:

Here we present the unpredictable chronic mild stress protocol in mice. This protocol induces a long-term depressive-like phenotype and enables to assess the efficacy of putative antidepressants in reversing the behavioral and neuromolecular depressive-like deficits.

ABSTRACT:

Depression is a highly prevalent and debilitating condition, only partially addressed by current pharmacotherapies. The lack of response to ~~the~~ treatment by many patients prompts the need to develop new therapeutic alternatives and to better understand the etiology of disorder. Pre-clinical models with translational merits are rudimentary for this task. Here we present a protocol for the unpredictable chronic mild stress (UCMS) method in mice. In this protocol, adolescent mice are chronically exposed to interchanging unpredictable mild stressors. ~~Reminiscing~~ Resembling the pathogenesis of depression in humans, stress exposure during the sensitive period of mice adolescence instigates a depressive-like phenotype evident in adulthood. UCMS can be used for screenings of antidepressants on variety of depressive-like behaviors and neuromolecular indices. Among the more prominent ~~outcome measures~~tests to assess depressive-like behavior in rodents is the sucrose preference test (SPT), ~~a realistic model~~which reflects ~~for~~ anhedonia (core symptom of depression). The SPT will also be presented in this protocol. The ability of UCMS to ~~elicit~~ induce anhedonia, instigate long-term behavioral deficits and enable ~~reverse~~ reversal of these deficits via chronic (but not acute) treatment with antidepressants strengthens the ~~model's~~ protocol's validity compared to other animal ~~models~~ protocols ~~of~~ for inducing ~~depression~~depressive-like behaviors.

INTRODUCTION:

Major depressive disorder (MDD) is a debilitating condition, that has been indicated as the 11th cause of global burden from disease[1], with a lifetime prevalence of 11-16%[2, 3]. MDD has been associated with severe impairments on patients' social and occupational functioning, diminished quality of life, numerous mental and physical disorders and increased risk for mortality[4–7]. There are several efficacious pharmacotherapies and psychological interventions for MDD; however, more than third of the patients do not achieve remission with the existing therapeutic options[8–11]. Therefore, better mapping of the pathophysiology of MDD and development of novel drugs are still ~~at~~ of utmost importance. In order to address these tasks scientifically validated animal models needs to be utilized.

Unpredictable chronic mild stress (UCMS) is a renowned rodent ~~model~~ paradigm used to induce depressive- and anxiety-like ~~of depression~~behaviors[12–15]. The main objective of UCMS is to ~~induce~~ generate ~~depressive like symptoms (~~behavioral deficits (such as anhedonia and behavioral despair[12, 15]) in mice and rats, and promote screenings for potential therapeutic pharmacological agents. The procedure was first introduced by Katz[16] and subsequently developed by Willner[17, 18], yielding vast behavioral and neurobiological outcomes reminiscing depressive symptomatology[12]. It was initially designed for rats and later accommodated to mice[13, 19]. In the procedure, adolescent animals are chronically exposed to different unpredictable mild stressors. Subsequently, pharmacological agents are administered. Behavioral and biological indices are obtained upon treatment termination. One of the more prominent tests conducted following UCMS is the sucrose preference test (SPT). The SPT is based on rodents' innate preference for sweetened solution rather than water, and is widely acknowledged as an essential translational model for assessing anhedonia[12, 18, 20, 21] (which is a core symptom in human depression[22, 23]).

~~In the SPT, animals' innate preference to consume sucrose solution rather than water is examined (as a measure of hedonic tone)[12, 18, 21].~~

While entering the fourth decade since its introduction UCMS has been applied on mice and rats in myriad studies. The majority of these studies employed UCMS as a method to induce depressive-like behaviors[12, 13, 21, 24]. Studies have also employed the model to generate anxiogenic effects[25–29]. Sucrose and saccharin preferences are the main tests used to assess anhedonia following UCMS[12, 18, 30–33]. ~~.~~ Other notable outcome measures that are highly incorporated in UCMS literature are: the tail suspension test (TST)[28, 34, 35], the forced swim test (FST)[28, 34, 36, 37] (both measuring stress coping / behavioral despair), the open field test (OFT; measuring exploratory behavior, anxiety-like behavior and locomotor activity)[25, 28, 38], the elevated plus maze (EPM; measuring anxiety-like behavior)[25, 39, 40] and additional tests measuring depressive-like behaviors, anxiety-like behaviors, cognitive functioning and social behavior[12]. Chronic administration of tricyclic antidepressants (TCAs; imipramine[35, 41–43], desipramine[18, 44, 45]), tetracyclic antidepressants (TeCAs; maprotiline[46, 47], mianserin[48]), selective serotonin reuptake inhibitors (SSRIs; fluoxetine[46, 47, 49], escitalopram[30, 50], paroxetine[51, 52]), melatonin[43, 49], agomelatine[53], the fatty acid amide hydrolase (FAAH) inhibitor URB597[54] and several natural compounds[30, 37, 50, 55–58] have been demonstrated to reverse the UCMS-induced depressive- and anxiety-like symptoms. Overall, these therapeutic effects have not been

obtained via acute treatments[12] (*exempli gratia* [*e.g.*], paroxetine[51, 52], imipramine[53, 54, 59, 60], fluoxetine[53], agomelatine[53], URB597[54], brofaromine[60]).

Stress exposure during childhood and adolescence is a major risk factor for anterior formation of MDD (among several other psychiatric disorders) in adulthood[61–63]. The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system regulating the bio-behavioral response to stress[64]. Long-term stress during the sensitive neurodevelopmental periods of childhood and adolescence impairs the equilibrium of the HPA axis. It might provoke a state of enhanced sympathetic activation, unbalanced reactivity and hypercortisolemia lasting through resting state; thus, rendering individuals vulnerable for depression or anxiety-related psychopathologies[65–68]. UCMS adequately translates this pathogenesis: stress application during mice' adolescence induces a long-term depressive-like susceptibility. Moreover, the behavioral deficits induced by UCMS, are underlain by significant alterations in HPA axis functioning (*e.g.*, by causing a reduction in hippocampal brain-derived neurotrophic factor [BDNF; a protein highly involved in the equilibrium of the HPA axis[69, 70]][30], or by impairing the regulation of corticosterone secretion to the blood[71, 72]), ~~reminiscing~~ in similarity to the pathophysiology in humans[12, 50, 73].

UCMS has ~~additional~~ several bolstering features as a model for ~~MDD~~depression: ~~*exempli gratia* (~~*e.g.*~~)~~ (i) the elicitation of anhedonia (which is regarded an endophenotype of MDD[23, 74]); (ii) UCMS enables to assess wide variety of depressive-like behaviors such as behavioral despair, ~~anhedonia,~~ reduced social behavior, deterioration in fur state and more[34]; and (iii) chronic (2-4 weeks), but not acute, administration of antidepressants following stress exposure could produce a protracted therapeutic effect parallel to the effect obtained in human patients by the same agents[30, 75–77].

These features strengthen the validity of UCMS compared to other animal models of depression. The FST[78] and the TST[79] are two models that are used either to induce or to assess depressive-like behavior. As models for inducing depressive-like behaviors they have clear shortfalls compared to UCMS; they do not prompt long-term behavioral changes and might merely reflect adjustment to acute stress rather than yield a durable depressive-like manifestation[76].

An alternative animal model of depression is the social defeat model. Unlike the FST and the TST this model (like UCMS) require application of chronic stress (*id est* [*i.e.*], recurrent subjection of the animal to aversive social encounters with dominant counterparts)[76, 77, 80–82]. The main advantage of the social defeat model is that it employs social stimuli as stressors, thus reflecting the role of psychosocial stress in the pathogenesis of human depression. Similar to UCMS, the social defeat model elicits long-term depressive-like behaviors, and neuroendocrine alterations. Yet again parallel to UCMS, the social defeat-induced deficits could be reversed via chronic, but not acute, administration of antidepressants. Overall, there is large support for the utilization of both UCMS and social defeat as pre-clinical apparatuses for investigating the pathophysiology of depression[76, 77, 81, 82]. However, a major shortfall of the social defeat model is that it could only be applied on male rodents, as females

do not exhibit sufficient aggressive behavior toward each other[83]. Contrastingly, UCMS has been shown to produce several depressive-like effects on both male and female mice[34].

Predictable chronic mild stress (PCMS) is another rodent model that enforces a regimen of daily recurring exposure to restraint stress[28, 84–87]. Several studies have shown that PCMS increased anxiety-like behaviors[28, 87]; albeit, there are contradictory reports vis-à-vis PCMS ability to induce long-term depressive-like behaviors. Unlike UCMS, PCMS has produced less satisfactory results referring to its ability to induce an anhedonic-like state[28, 84, 86]. This is consistent with human phenomenology, in which unpredictable stressors are more harmful than predictable ones[88].

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee of the Academic College Tel-Aviv-Yaffo.

1. Animals

1.1. Use pre-adolescent (*i.e.*, 3 weeks old) Institue of Cancer Research (ICR) outbred male mice.

1.2. Randomize mice to two equally sized stress group (UCMS vs. naïve). Use 15 mice per treatment group (*e.g.*: if there are 3 pharmacological treatment groups use 90 mice overall; 2 [UCMS vs. naïve] × 3 [treatments] × 15 [mice] = 90)

1.3. House ~~naïve~~ mice ~~in separate cages from stressed mice.~~according to stress group; namely, naïve mice should be housed with naïve mice only, and mice in the UCMS group should be housed with mice from the UCMS group only.

1.4. House animals in standard ~~group~~home cages (30 × 15 × 14 cm; 5 mice per cage; each cage containing mice from all treatment groups [*i.e.*, pharmacological treatment groups]; maintain mice in the same cage throughout the experiment, except when indicated otherwise).

1.5. Fill home cages with fresh sawdust (replaced twice a week) and add a piece of cotton wool for enrichment.

1.6. House animals in home cage for an acclimation period of one week. Allow *ad libitum* access to rodent chow and water (except during UCMS stressor applications).

~~1.7. Allow *ad libitum* access to rodent chow and water (except during UCMS stressor application).~~

1.~~8~~7. Keep a consistent 12 h light / dark cycle (except when indicated otherwise). During UCMS procedures maintain naïve mice in their home cage.

~~1.9. During UCMS procedures, maintain naïve mice in their home cage.~~

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2. UCMS

2.1. Designate a separate room in the lab, for the sole use of the UCMS protocol.

2.2. Design a 4 week stressor regimen in which each of the seven stressors (*i.e.*, wet cage, dampened sawdust, tilted cage, empty cage, social stress, mice restraint and disruption of light / dark cycle) is utilized once a week, on a different day each week. For a possible design see **Table 1**.

2.3. Following 1 week of acclimation (see **1.6.**) initiate stressors application (mice should be approximately 4 weeks old).

2.4. Each day, before stressor application, transfer the cages of the UCMS group from the housing room to the UCMS room.

2.5. During stressor applications, block access to rodent chow and water for the UCMS group (except during reversal of light / dark cycle)~~;~~.

Note: ~~this~~ This could be obtained by replacement of the original cage lid (which contains food and water), to an empty cage lid.

2.6. ~~**Stressors.**~~ Apply the following stressors according to the regimen ~~you have~~ designed earlier (see **2.2.**):

2.6.1. Wet cage~~.~~

2.6.1.1. Place mice together with their home cage counterparts in an empty cage (*i.e.*, cage without sawdust).

2.6.1.2. Fill the empty cage with water kept at 24 ± 1 °C~~;~~ to a depth of 1 cm (pour with caution to avoid direct water spillage on mice). Keep mice in the wet cage for 4 h.

2.6.1.3. Transfer each mouse to a separate individual transient drying cage with a heat lamp above it, a heat pad under it and paper towel bedding. Place a thermometer in the transient cage to verify the temperature does not exceed 37 °C.

2.6.1.4. Keep each mouse in the transient cage until it is dry and looks invigorated (approximately 10-15 min). Return mice to home cage with same counterparts.

2.6.2. Dampened sawdust~~.~~

2.6.2.1. Pour water kept at 24 ± 1 °C to the home cage until the sawdust is moderately dampened (pour with caution to avoid direct water spillage on mice).

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Note: It is not necessary to use fresh sawdust before pouring the water.

2.6.2.2. After 4 h dry mice in transient cages as described in **2.6.1.3.**. Place mice with home cage counterparts in a sterile cage with fresh sawdust.

2.6.3. Tilted cage

2.6.3.1. Tilt cages at 45° against the wall for 4 h.

Note: During this stressor mice remain in their home cage with their counterparts.

2.6.4. Empty cage

2.6.4.1. Transfer mice, along with their specific home cage counterparts, from the home cage to an empty cage for 4 h.

2.6.5. Social stress

2.6.5.1. Transfer mice, along with their specific home cage counterparts, from the home cage to a cage which was housed by a different group of mice for a period of at least 3 d prior to stressor application. Keep mice at the unfamiliar cage for 4 h.

Note: To avoid uncertainty place a sticker on each cage to indicate mice origin cage.

2.6.6. Mice Restraint

2.6.6.1. Place each mouse separately in a clean mouse restrainer for 4 h. Return mice to home cage with same counterparts.

2.6.7. Disruption of light / dark cycle

2.6.7.1. Transfer mice, in their home cage with their specific counterparts, to the UCMS room. Keep the light on, for 24 consecutive h.

Note: Only during this stressor mice will be allowed *ad libitum* access to rodent chow and water.

2.7. Following stressor application, return cages of the UCMS group from the UCMS room to the housing room.

2.8. During the 4 weeks of stress exposure, keep the naïve group in their home cages located in the housing room.

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Note: Naïve mice are not transferred to the UCMS rooms because exposure to other mice undergoing stress procedure could induce a stressogenic effect, even without direct exposure to the stressor[89, 90].

2.9. Monitoring of Animals during UCMS

2.9.1. During stressor applications (except during disruption of light / dark cycle) mice must be monitored every 30 min by an experienced experimenter. If an atypical distress (*e.g.*, trembling, lethargy, lack of movement) is observed (special caution should be placed on potential hypothermia during 'wet cage' and 'dampened sawdust') the mouse must be relieved from the stressor immediately.

2.9.2. Inspect each mouse daily for wounds or other physical or behavioral abnormalities. If such are inspected consult with the laboratory's veterinarian to decide whether the mouse should be excluded from the experiment.

2.9.3. Weigh each mouse every 3 d. Robust reduction in body weight (*i.e.*, >10% reduction from baseline weight or >15% reduction from last measured weight) must be reported to the laboratory's veterinarian and the mouse must be excluded from the experiment.

3. Screening for Antidepressants

3.1. On the day following cessation of the UCMS protocol, start administration of ~~relevant~~ putative therapeutic pharmacological agents (~~*e.g.*~~*i.e.*, escitalopram [15 mg/kg; i.p.; 3 weeks; on administration per day][30, 50], or NHT [30 mg/kg; i.p.; 3 weeks; one administration per day][30, 50]).

~~TCAs, TeCA, SSRIs or other putative pharmacological agents with a hypothesized antidepressant or anxiolytic effect).~~

Note: ~~Here are suggested drug doses of the specific drugs used in this protocol: escitalopram (15 mg/kg; i.p.; 3 weeks)[27, 50], novel herbal treatment (NHT; 30 mg/kg; i.p.; 3 weeks)[27, 50]. not included in this protocol~~

3.2. Include a control treatment group and administer saline (i.p.; 3 weeks; one injection per day) to this group.

3.~~2~~3. Calculate drug dose according to mice' weight, as obtained in the preceding weighing.

3.~~3~~4. Administer drugs chronically (usually one drug administration per day via intraperitoneal injection [i.p.] for 3 weeks) ~~to assess antidepressant- and anxiolytic-like effects~~.

Note: There is no need to anesthetize the mice before the i.p. injection.

3.~~4~~5. Continue to weigh mice every 3 d; last weighing should be 3 d before the last drug administration.

~~3.5. Here are suggested drug doses of the specific drugs used in this protocol: escitalopram (15~~

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~~mg/kg; i.p.; 3 weeks)[27, 50], novel herbal treatment (NHT; 30 mg/kg; i.p.; 3 weeks)[27, 50]. For doses of other drugs see: flouxetine[46, 47, 49, 87, 88], paroxetine[51], imipramine[35, 41–43], desipramine[18, 44, 45], maprotiline[46, 47], mianserin[48], melatonin[43, 49], URB597[54] and other natural compounds[37, 55–58].~~

4. Assessment of Hedonic Tone in the SPT

4.1. Following the treatment phase remove each mouse from the home cage and place it individually in a cage filled with fresh sawdust and a piece of cotton wool for enrichment.

4.2. Prepare two bottles, one with distilled water and another with 2% sucrose solution (other substance could be used: *e.g.*, saccharin[91], ethanol[30]).

Note: Each bottle should contain the same volume of fluid. Neither prior acclimation nor habituation phases are needed before introduction of fluids.

4.~~4~~3. Weigh the two bottles and set them at the cage lid to allow mice *ad libitum* access to both solutions for a period of (one of the following): 24 / 48 / 72 / 144 h.

4.~~5~~4. Place the two bottles at both ends of the cage lid. Place rodent chow between the two bottles to allow *ad libitum* access to food.

4.~~6~~5. Replace bottles every 24 h, using sterile bottles with fresh fluid.

~~4.7.~~Note: As mice are housed individually there is no need to change the sawdust even after 144 h[92].

4.~~8~~6. Switch nozzles' positions every 12 h (when test duration is 24 h) or once a day (when test duration exceeds 24 h) to counterbalance the possibility that the results were confounded by position preference.

4.~~9~~7. Weigh bottles each day to estimate consumption from each bottle.

4.~~10~~8. Calculate sucrose preference as ratio of sucrose intake from total fluid intake (*i.e.*, sucrose / sucrose + water).

REPRESENTATIVE RESULTS:

In order to corroborate the efficacy of the UCMS procedure for inducing depressive-like deficits a manipulation check was conducted. Male ICR outbred mice were randomly assigned to either UCMS or naïve conditions (4 weeks, as described in **2.2.**). Subsequently the SPT (6 d, as described in **4.**) was administered to assess whether mice undergone UCMS demonstrated hedonic deficits. Shortly after mice were sacrificed and the hippocampus was dissected out entirely for BDNF (a protein highly implicated in the pathophysiology of depression[70, 93]) assessment via enzyme-linked immunosorbent assay (ELISA). See **Fig. 1** for study design.

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Independent samples *t*-test revealed a significant difference between the groups in their sucrose preference ($t_{(23)}$ = 2.32, p < 0.05). The UCMS group demonstrated diminished sucrose preference compared to the naïve group (see **Fig. 2A**). This suggests that the UCMS protocol was effective in inducing anhedonia. Independent samples *t*-test on hippocampal BDNF levels revealed a significant difference between the groups ($t_{(23)}$ = 2.43, p < 0.05). The UCMS group demonstrated diminished hippocampal BDNF levels compared to the naïve group (see **Fig. 2B**). This suggests that the UCMS protocol led to diminution in hippocampal BDNF levels, as evident in human depression[94].

~~The following~~In another study from our lab we examined the potential antidepressant-like effects of two drugs following UCMS protocol.~~ results are derived from previously published study from our lab[30]. Male ICR outbred mice were assigned to either the UCMS group or the naïve group~~ Following the UCMS procedure (as described in **2.**) male ICR outbred mice received chronic (3 weeks) treatment with the SSRI escitalopram (15 mg/kg; i.p.), NHT (30 mg/kg; i.p.; for more information regarding NHT see[25, 30, 50, 75]) or saline. Following treatment phase the SPT was conducted and hippocampal BDNF levels were assessed. See **Fig. 3** for study design.
~~. Following stress procedure mice received chronic treatment with the SSRI escitalopram (15 mg/kg; i.p.), NHT (30 mg/kg; i.p.; for more information regarding NHT see[22, 27, 50, 71]) or saline for 3 weeks. Subsequently the SPT was conducted (see **Fig. 1** for study design).~~

Two-way analysis of variance (ANOVA) on sucrose preference revealed significant treatment ($F_{(2,92)}$ = 4.01, p < 0.05) and UCMS × treatment interaction ($F_{(2,92)}$ = 4.92, p < 0.01) effects (see **Fig. ~~2~~4A**). Sidak post-hoc analysis revealed that the UCMS-saline group demonstrated a significant decrease in sucrose preference compared to the naïve-saline group (p < 0.001); no decreases were observed in the UCMS-escitalopram and in the UCMS-NHT groups compared to the naïve groups (not significant [*N.S.*]). Additionally, the UCMS-saline group demonstrated decreased sucrose preference compared to both the UCMS-escitalopram (p < 0.05) and the UCMS-NHT (p < 0.001) groups. These suggest that both escitalopram and NHT normalized the UCMS-induced anhedonia.

Two-way ANOVA on hippocampal BDNF levels revealed significant UCMS ($F_{(1,22)}$ = 8.92, p < 0.01), treatment ($F_{(2,22)}$ = 18.36, p < 0.001) and UCMS × treatment interaction ($F_{(2,22)}$ = 5.19, p < 0.05) effects (see **Fig. 4B**). Sidak post-hoc analysis revealed that the UCMS-saline group demonstrated a significant decrease in hippocampal BDNF levels compared to the naïve-saline group (p < 0.001); no similar decreases were observed in the UCMS-escitalopram and in the UCMS-NHT groups compared to the naïve groups (*N.S.*). Additionally, the UCMS-saline group demonstrated decreased hippocampal BDNF levels compared to both the UCMS-escitalopram and the UCMS-NHT groups (p < 0.001 in both contrasts). These suggest that both escitalopram and NHT normalized the UCMS-induced reduction in BDNF levels in the hippocampus.

FIGURE AND TABLE LEGENDS:

Figure 1: A diagram depicting a possible experimental design. Following 1 week of acclimation,

mice were randomly assigned to either UCMS or naïve conditions (persisting 4 weeks). Subsequently, sucrose preference was examined and mice were prepared for BDNF assessment. SPT = sucrose preference test; CD = cervical dislocation.

Figure 2: The effects of UCMS on sucrose preference and hippocampal BDNF levels. A. Mice subjected to 4 weeks of UCMS demonstrated a significant reduction in sucrose preference compared to naïve mice. **B.** Mice subjected to 4 weeks of UCMS demonstrated a significant reduction in hippocampal BDNF levels compared to naïve mice. *n* = 12–13 mice per group. Results are expressed as mean ± SEM. **p* < 0.05

Figure ~~1~~3: A diagram depicting a possible experimental design. Following 1 week of acclimation, mice were randomly assigned to either UCMS or naïve conditions (persisting 4 weeks). Subsequently, mice received chronic treatment with saline, escitalopram (15 mg/kg; i.p.) or NHT (30 mg/kg; i.p.), lasting 3 weeks (one administration per day). Following treatment, sucrose preference was examined and mice were prepared for BDNF assessment. SPT = sucrose preference test; CD = cervical dislocation.

Figure 4: The effects of chronic treatment with escitalopram and NHT on UCMS-induced reductions in sucrose preference and hippocampal BDNF levels. A. Both escitalopram and NHT prevented the UCMS-induced reduction in sucrose preference; *n* = 15–17 mice per group. **B.** Both escitalopram and NHT prevented the UCMS-induced reduction in hippocampal BDNF levels; *n* = 4–6 mice per group. Results are expressed as mean ± SEM. **p* < 0.05 ****p* <0.001. This figure has been modified from a previously published study from out lab[30].

~~**Figure 2: The effects of chronic treatment with escitalopram and NHT on UCMS-induced reduction in sucrose preference.** Both escitalopram and NHT prevented the UCMS-induced reduction in sucrose preference; *n* = 15–17 mice per group. Results are expressed as mean ± standard error of the mean (SEM). **p* < 0.05 ****p* <0.001. This figure has been modified from a previously published study from our lab[27].~~

DISCUSSION:

Insofar as MDD is a widespread highly debilitating disorder, only partially addressed by current therapeutic options, the scientific quest for better treatments is still a pressing issue. Along with innovations in psychological techniques, additional pharmacotherapies are required for the large portion of patients who do not respond to the existing drugs. Meticulous animal models for depression are key element in this task. Such models facilitate screenings for innovative antidepressants, and expand the understanding of the etiology of the disorder. UCMS is one of the more prominent rodent models of depression. Its' stature is exhibited by vast publications and notable insights[12, 18, 82, 95–97].

Anhedonia is one of the core symptoms of MDD[22, 23, 74]. A more severe anhedonic tone has been associated with poorer prognosis for MDD patients[74, 98]. A major strength of UCMS as a model of depression is its ability to generate anhedonia[31], as exemplified in the SPT. Sucrose is an innate reinforcer for various rodent species[51, 52, 59, 99]; this explains the overall support of SPT as a realistic model of hedonic tone in rodents[14, 31, 100]. Due to the focal role of anhedonia in any

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animal model of depression, it has been suggested that when considering implementing UCMS into a lab, the first step should be a verification of the procedure ability to induce an anhedonic state[12]. This will facilitate a better standardization across labs and could be the foundation for future studies shedding more light on the disorder.

Another feature that supports the validity of UCMS as a model of depression is that the behavioral and molecular alterations induced by UCMS are reversed by chronic, but not acute, treatment with agents that have been previously verified as effective antidepressants[12]. The protracted therapeutic effect is similar to the effects of antidepressants in humans, which usually start manifesting only after 2-3 weeks of treatment[101, 102]. In this feature, UCMS possess a superior face validity compared to the FST[78] and the TST[103], in which the effects are obtained also following acute treatment. Unlike the TST and the FST, this shortfall is not evident in the social defeat model of depression, which (along with UCMS) stand out as an excellent animal model ~~for~~ of depression. However, compared to the FST and the TST, UCMS and other chronic stress models are much more lengthy and expensive.

Notable mice strains have been employed in UCMS studies. Among the more frequent strains are the C57BL/6 and the BALB/cJ[21, 34]. We have utilized male ICR outbred mice as numerous studies have demonstrated the efficiency of UCMS in this strain. Morever, the utilization of ICR outbred mice bolsters the ecological validity of the protocol, due to the high between animals genetic variability of this strain (compared to transgenic mice strains)[30, 75, 104–106].

For suggested doses of other drugs not included in this protocol, but were used in other UCMS protocols see: flouxetine[46, 47, 49, 104, 105], paroxetine[51], imipramine[35, 41–43], desipramine[18, 44, 45], maprotiline[46, 47], mianserin[48], melatonin[43, 49], URB597[54] and other natural compounds[37, 55–58].

There are several additional outcome measures frequently applied in UCMS protocols, among them: (i) FST: a measure for behavioral despair (see reference[78] for protocol delineation); (ii) TST: another measure for behavioral despair (see reference[103] for protocol delineation); (iii) splash test and evaluation of coat state: two indicators of grooming behavior and putative measures of apathy (see reference[107] for protocol delineation); (iv) sociability/preference for social novelty: measures for social behavior[108] (see reference[109] for protocol delineation); and (v) sexual behavior: another measure for hedonic tone (see reference[75] for protocol delineation). Furthermore, UCMS is used to assess neuromolecular, endocrine and other biological measures pertinent to depression[50, 72, 110–116] (specifically, see references[117, 118] for BDNF assessment via ELISA protocol delineation).

There are several critical steps within the UCMS protocol: (i) it is vital that mice from all treatment groups will be housed together and not in separate cages. For example, if there are 3 treatment groups (*e.g.*: escitalopram, NHT and saline) there will be 2 mice from 2 of the groups and 1 mouse from the remaining group in each cage. The hybrid group housing will thwart the possibility that the results were underlain by the housing conditions and not the treatment *per se*. (ii) naïve mice must be housed separately from stressed mouse, since housing with stressed mice is stressful[90, 119, 120] and therefore could impede or attenuate the stress manipulation. (iii)

previous UCMS protocols have instructed single housing rather than group housing[32, 121]; we have suggested the latter as single housing might ~~lead~~ cause ~~to a~~ further susceptibility to stress in mice and rats[122–125]. (iv) the UCMS schedule must be designed diligently to ensure unpredictability (*i.e.*, random exposure to each stressor once a week); however, schedules could be modified throughout the experiment as long as the unpredictability is kept.

ACKNOWLEDGMENTS:

This research was supported by the Israel Ministry of Science, Technology & Space (grant no. 313552), by the National Institute for Psychobiology in Israel (NIPI-208-16-17b) and by the Open University Foundation.

DISCLOSURES:

The authors have nothing to disclose.

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