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Behavioral and physiological consequences of enrichment loss in rats

Brittany L. Smith¹, Carey E Lyons², Fernanda Guilhaume Correa², Stephen C. Benoit¹, Brent Myers¹, Matia B. Solomon¹, and James P. Herman¹

¹University of Cincinnati, Department of Psychiatry & Behavioral Neuroscience

²University of Cincinnati, Summer Undergraduate Research Fellowship Program

Abstract

Significant loss produces the highest degree of stress and compromised well-being in humans. Current rodent models of stress involve the application of physically or psychologically aversive stimuli, but do not address the concept of loss. We developed a rodent model for significant loss, involving removal of long-term access to a rewarding enriched environment. Our results indicate that removal from environmental enrichment produces a profound behavioral and physiological phenotype with depression-like qualities, including helplessness behavior, hypothalamo-pituitary-adrenocortical axis dysregulation and overeating. Importantly, this enrichment removal phenotype was prevented by antidepressant treatment. Furthermore, the effects of enrichment removal do not occur following relief from chronic stress and are not duplicated by loss of exercise or social contact.

Keywords

environmental enrichment; loss; stress; weight gain; imipramine; depression

1. INTRODUCTION

Chronic stress often precipitates depression in humans and is commonly used to model depression-like characteristics in rats and mice. With at least half of clinical patients failing to find commensurate relief with antidepressant treatment (Fava 2003), it is necessary not only to further our understanding through use of current models, but also to expand how we model stress and depression-like phenotypes. Current rodent stress paradigms almost universally impose aversive physical and/or psychological challenges (e.g. repeated social defeat, chronic variable/unpredictable stress, repeated restraint, repeated footshock) (Krishnan & Nestler 2011). While these stimuli translate to unpleasant or traumatic experiences occurring in humans, they do not account for the impact of loss.

*Address correspondence to: James P Herman, PhD, Donald C. Harrison Professor, Department of Psychiatry and Behavioral Neuroscience, Director, UC Neurobiology Research Center, Director, Stress Neurobiology Laboratory, University of Cincinnati, 2170 East Galbraith Road, Cincinnati, OH 45237-0506, Phone: 513-558-4813, Fax: 513-558-9104, james.herman@uc.edu.

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According to the Holmes-Rahe Stress Inventory, the five life events that generate the highest incidence of stress and illness in people are related to loss (e.g., death of a spouse, divorce, marital separation, incarceration, and death of a close family member) (Christie-Seely 1983, Rahe 1968). The central theme of these stressful life events is major loss, whether it be loss of a loved one or loss of freedom. Loss has a profound negative impact on mental health in humans, who are sensitive to many different types of losses. Psychosocial loss, such as spousal loss, is strongly associated with the development of depression (Sikorski et al 2014, Wang et al 2015b). Personal physical loss, such as injury, increases symptoms of stress and depression (Wiseman et al 2015). Finally, devastating financial loss provokes the emergence of major depression and suicidality (Ganzini et al 1990, Wang et al 2015a). In order to expand the current rodent models of stress and improve our understanding of mental health, we sought to model significant loss in rats with respect to standard tests of depressive symptoms.

Since human aspects of financial, physical, and psychosocial loss may be impossible to model in rodents, we focused on the rewarding properties of previously enjoyed stimuli. To model loss, we identified a stimulus that encompasses a range of pleasurable and positive activities, such that removal would recapitulate a variety of human losses. Therefore, we exposed adult male rats to four weeks of environmental enrichment and subsequently placed them into isolated standard housing. With environmental enrichment (EE), rats encounter novelty, social interaction, and physical activity. All of these types of stimuli are independently rewarding to rats, suggesting that combining the three via EE is highly rewarding (Bevins & Bardo 1999, Belke 2000, Yates et al 2013, Greenwood et al 2011, Louilot et al 1986, Rebec et al 1997). Environmental enrichment induces a positive affective bias in rats and is even capable of displacing the rewarding effects of cocaine, further demonstrating the rewarding properties of EE (Solinas et al 2008, Puhl et al 2012, Stuart et al 2013). Consequently, removal of environmental enrichment is interpretable as a major loss of a long-standing array of positive stimuli. We hypothesized that this enrichment removal would precipitate a depression-like phenotype.

2. METHODS AND MATERIALS

2.1 Subjects

All experiments utilized adult male Sprague Dawley rats, weighing 250–275 g (approximately 9 weeks old) upon arrival from Harlan (Indianapolis IN USA). Rats were acclimated to the vivarium for 72 h – 1 week prior to experimental manipulations. All rats were housed with corn cob bedding and ad libitum water and chow (3.41 kcal/g, 0.51 kcal/g from fat; Harlan Teklad, Madison WI USA) unless noted. The vivarium was temperature and humidity controlled, with a 12-hour light cycle. All procedures were conducted in compliance with the National Institutes of Health Guidelines for the Care and Use of Animals, and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2 Housing Manipulations

Standard single housing consisted of one rat per opaque polycarbonate shoebox cage (20 cm height $\times$ 22 cm width $\times$ 43 cm length). Standard pair housing was conducted in the same fashion, except with two rats per cage. The rats placed in environmental enrichment were housed 10 per enrichment chamber. The enrichment chambers were 1 m height $\times$ 1 m width $\times$ 1 m length, with wire mesh walls and removable metal floor pans (Johnson et al 2013). A wire mesh loft measuring approximately 0.5m $\times$ 1m was suspended 0.5m from the floor pan, with metal ladder attached for climbing access. The assortment of toys included: various sized and textured huts and balls, different shaped tubes, small cars and shovels, Nylabones, plastic rings, dishes, cones, cups, crinkle paper, and nestlets. Cages were cleaned and supplied with a different set of toys each week.

Because the enrichment removal model is a new model developed by our group, housing manipulations were modified between the first experiment and the subsequent experiments to improve data collection for behavioral and hormonal endpoints. For Experiment 1 (see Figure 1 for list of experiments), all animals were housed in the same colony room for the duration of the study. Rats were divided into 3 groups, standard single housing (CON, $n = 12$), continuous environmental enrichment (EE, $n = 10$), or enrichment removed (ER, $n = 10$). EE and ER rats were housed in the enrichment chambers constantly, 24 h per day, with cages changed and toys rotated every 4–5 days. The ER rats were removed from enrichment after 4 weeks and placed into standard single housing. All rats were only handled on cage change days and behavioral testing days. This first experiment established support for the concept, and subsequent experiments took additional measures to obtain individual measurements from enriched rats. For a timeline of all experiments, see Figure 1.

2.3 Active Cycle Enrichment

Starting with Experiment 2 and continuing for the remainder of the experiments, we exposed rats to enrichment only during the dark phase, the active phase of their circadian cycle. This active cycle enrichment was developed to obtain undisturbed individual measurements from enriched rats, which cannot be readily accomplished in the group-housed situation. For active cycle enrichment, enriched rats were singly housed with cage enrichment (crinkle paper) during the light phase (09:00 – 20:00), in the same room as control standard-housed rats. Approaching lights off (20:00 – 21:00), enriched rats were briefly transported to a nearby colony room and transferred to the enrichment chambers during the dark phase (21:00 – 09:00). This cycle was repeated every day/night and took advantage of natural activity patterns in rats, so that the rats had access to enrichment during a time when they were inherently most active and were singly housed during a time when they naturally rested (Greenwood et al 2011). Furthermore, rats anticipate being transferred to EE in a similar fashion as how they anticipate natural rewards, suggesting that this manipulation is rewarding and not stressful (van der Harsta et al 2003). Continuously enriched rats (EE, $n = 10$) received active cycle enrichment every night for the duration of the study. After 4 weeks of active cycle enrichment, enrichment removal rats (ER, $n = 10$) were still carted to the enrichment room for the dark phase, but remained unhandled in standard single housing without cage enrichment. Standard pair-housed control rats (PCON, $n = 12$) were paired housed for 12 h during the dark phase, and singly housed for 12 h during lights on, to mimic

active cycle social interaction. The PCON group controlled for any potentially stressful effects from repeated intermittent isolation. Standard singly housed control rats (SCON, n = 11) were then handled every 12 h, at the same time the other rats were handled. This way, every single rat was habituated to handling and to being singly housed during the day. All behavioral and hormonal data were collected in a window 1 h – 6 h after lights turned on. To maintain consistency, the active cycle design was used for all housing conditions for the remainder of the experiments. See Figure 1 for a description of all experiments.

2.4 Antidepressant Drug Treatment

For experiment 3, rats received daily injections (for a total of 27 days) of 10 mg/kg imipramine hydrochloride (BioXtra 99%, Sigma Aldrich) dissolved in sterile saline (IMIP n = 21; n = 11 CON IMIP; n = 10 ER IMIP) or saline vehicle (SAL n = 21; n = 11 CON SAL; n = 10 ER SAL). Imipramine was used because of its known efficacy in reducing immobility in the FST after 5 days of treatment in our research group (Wulsin et al 2010, Solomon et al 2014). Rats were weighed daily and administered 0.1 ml/100g injection volume. Injections began the morning after the rats were first removed from enrichment, and were administered 0–2h after lights on. Out of the 27 total injections, 16 injections were administered intraperitoneally. Due to observed discomfort from repeated injections, the route was switched to subcutaneous for 10 days (both drug and vehicle groups). Injection route was not changed on any of the testing days.

2.5 Chronic Stress Procedure

For Experiment 4, animals received chronic variable stress (CVS, n = 10) or repeated restraint stress (RR, n = 10) for 4 weeks during the light phase, which are standard methods used in our laboratory (Flak et al 2012, Kopp et al 2013, Smith et al 2016). CVS consisted of 2 stressors per day, assigned in an unpredictable fashion, and separated by a minimum of 2 h. Stressors included: 30 min hypoxia (8% oxygen, 92% nitrogen), 1 h cold room exposure without bedding, 1 h orbital shaker, 1–2 h exposure to static radio, 5 min open field in a guinea pig cage, and overnight wet bedding. RR consisted of 30 min restraint stress, repeated once per day at 1 h into the light phase, whereby rats were placed in clear Plexiglas® restraint tubes. Animals recovered from CVS or RR for 2 weeks prior to testing.

2.6 Running and Social Housing

For Experiment 5, rats were placed into social housing conditions (SOC, n = 16) or given running wheel access (RUN, n = 13). SOC or RUN were given during the dark phase only, at the same time as environmental enrichment. For SOC, rats were placed 4 per cage in a clear guinea pig cage (20 cm height × 38 cm width × 47 cm length). The same 4 rats were housed together for the duration of SOC. For RUN, rats were placed 1 per cage in a clear guinea pig cage with a running wheel (Lafayette Instrument Co., Lafayette IN USA). Running activity was recorded via computerized Animal Wheel Monitoring System (Lafayette Instrument Co., Lafayette IN USA), see Figure S4a for running distances. During the light phase, rats were singly housed in standard cages.

2.7 Forced Swim Test

For the forced swim test (FST), rats were placed in Plexiglas® cylindrical tanks (45 cm H × 20 cm diameter) filled 31±3 cm with water (24±2°C). We employed a single 6-min exposure to the FST, with all animals per study tested on the same day. This version of the FST detects the effects of antidepressants and verifies animal models of depression-like behavior (Wulsin et al 2010, Overstreet 2002, Overstreet et al 2004, Cryan et al 2005a, Cryan et al 2005b). Videos were scored manually with the assistance of Hindsight, by an individual blind to the experimental groups. Quantified behaviors included: swimming, diving, climbing, and immobility.

2.8 Sucrose Preference Test

Animals were habituated to dual water bottle access for 3 days prior to sucrose presentation. Following habituation, rats were given one bottle with 1% sucrose (Sigma Aldrich) solution and the second bottle with tap water. The position of the sucrose bottle was alternated to avoid side preferences (Hong et al 2012). Bottles were weighed within 1 h after lights on and again within 1 h before lights off. Data were analyzed both as total sucrose consumed (g) and percent sucrose consumption/total fluid consumption.

2.9 Reward-Based Feeding Behavior and High-Fat Diet

For Experiment 5, reward-based feeding behavior was assessed by a paradigm developed by (Choi et al 2010 and Davis et al 2012). Briefly, rats were food deprived for 23 h and then given access to chow for 2 h to allow satiation. After 2 h of chow access, rats were given a second hopper with high-fat diet (HFD; 4.41 kcal/g, 1.71 kcal/g from fat; Research Diets; New Brunswick NJ USA). Both hoppers were weighed 24 h later and then weekly for the remainder of the study.

2.10 Open Field Test

The open field test (OFT) began with the animal being placed in the corner of a square open field apparatus (1 m² arena, 30 cm walls) (McKlveen et al 2013). The OFT was conducted under dim light (26 lux) and lasted 5 min in duration. Velocity, distance travelled, center vs. periphery distance, and center vs. periphery time were scored automatically with Clever TopScan software (CleverSys, Reston, VA). The OFT was utilized as a measure of locomotor activity for Experiments 1 and 3. For Experiment 1, a novel object (empty plastic pipette tip box) was placed in the center of the open field to also assess novel object interaction.

2.11 Social Interaction Test

In Experiment 1, rats were exposed to novel conspecific interactors in a standard shoebox rat cage and allowed to interact for 10 min (Myers et al 2016). Using Hindsight, videos were scored for four behaviors: nonsocial, dominant, submissive, social. Four behaviors were identified: 1.) Nonsocial: experimental animal faces away from interactor and does not initiate contact 2.) Dominant: experimental animal places two paws on top of the interactor 3.) Submissive: experimental animal allows interactor to place two paws on him 4.) Social:

all other behaviors initiated by the experimental animal that engages voluntarily with the interactor.

2.12 NMR

Noninvasive nuclear magnetic resonance (NMR) spectroscopy (EchoMRI; Echo Medical Systems, Houston TX) was used to obtain whole body composition (fat and lean mass values provided by the software) as previously validated and described by Kovner et al 2010 and Packard et al 2014.

2.13 Novel Stress Challenge and Blood Collection

The novel stress challenges were initiated 2.5 – 3 h into the light phase. Rats were placed in clear Plexiglas® restraint tubes for 30 min. Blood samples were taken via tail clip at 0, 15, 30, 60, and 120 min after stressor initiation and kept on ice in tubes filled with 10µL of 100 mM EDTA. Blood was centrifuged at 6,000 rpm for 15 min at 4°C. Plasma was collected and stored at –20°C until use for radioimmunoassay (RIA).

2.14 Radioimmunoassay

Plasma adrenocorticotrophic hormone (ACTH) was measured by radioimmunoassay (RIA) with a specific antiserum (diluted 1:120,000; graciously provided by Dr. William Engeland - University of Minnesota, Minneapolis MN) with ¹²⁵I ACTH as a tracer label (Amersham Biosciences, Piscataway NJ). Plasma corticosterone concentrations were measured with ¹²⁵I RIA kit (MP Biomedicals Inc, Orangeburg NY USA). Samples were run in duplicate for both assays, when possible.

2.15 Statistics

Group effects were analyzed by one-way ANOVA. Interaction effects of group × time were analyzed by two-way repeated measures ANOVA. Interaction effects between group × drug treatment × time were analyzed by three-way repeated measures ANOVA. Sigma Stat (Systat Software, San Jose CA USA) was used for one-way and two-way ANOVAs and Statistica (StatSoft/Dell) was used for three-way ANOVAs. Post hoc testing was performed only if the omnibus F values were significant (or significant interaction effects for two-way and three-way ANOVAs), utilizing Fisher's Least Significant Difference (LSD). Our sample sizes for EE and ER were limited to 10 rats (maximum allowed in one enrichment chamber). With this small sample size, Fisher's LSD is most appropriate for limiting familywise error rate while also maintaining power (Howell 2013). Additionally, we limited our post hoc analysis to comparisons of interest and avoided making extraneous comparisons. For example, we analyzed group differences within a single time point only, instead of across multiple time points. Outliers were determined *a priori* by values that fall outside 1.96± the mean times the standard deviation, above the upper quartile + 1.5 times the interquartile range, or below the lower quartile – 1.5 times the interquartile range. Data are graphed as mean ± standard error of the mean.

3. RESULTS

3.1 Identifying an enrichment-removal phenotype

To introduce enrichment removal as a model for loss, it was first necessary to demonstrate that enrichment removal produces a behavioral phenotype that differs from both continuously enriched rats and singly housed rats (Timeline, Figure 1). Animals were tested for immobility in the forced swim test (FST) 9 days after enrichment removal. Four videos were lost due to equipment malfunction (Figure 2 legend displays sample size). Enrichment-removed (ER) rats had increased FST immobility [$F_{(2, 25)} = 4.062$; $p = 0.03$] (Figure 2a) in comparison to continuously enriched rats (EE) and singly housed rats (CON) ($p < 0.05$, Fisher's LSD post hoc). There were no differences in any other behaviors in the FST (see Table S1). There were also no differences in distance traveled in the open field test [$F_{(2, 29)} = 0.868$; $p = 0.431$] (Figure 2b), indicating that differences in the FST were not due to differences in general activity. For results from the social interaction test, see Figure S1. There were no differences in raw or relative adrenal, heart, or thymus weight in this experiment or any of the following experiments, demonstrating that our experimental manipulations did not have lasting effects on the integrity of stress-sensitive organ systems.

3.2 Active cycle enrichment for individualized assessment of group-housed enriched rats

In order to further characterize the enrichment removal phenotype, we developed a strategy for individualized assessment of enriched rats. We enriched rats only during the dark phase, the active phase of the animals' circadian cycle (active-cycle enrichment). Active-cycle enrichment enabled us to conduct all testing, such as sucrose preference, during the light phase, when rats were singly housed. Active cycle enrichment also allowed acquisition of undisturbed baseline ACTH and corticosterone levels as well as precisely-timed assessment of ACTH and corticosterone responses to a novel stressor. We verified the active cycle enrichment removal group differences in the FST [$F_{(3, 38)} = 3.498$; $p = 0.025$], with enrichment loss causing increased FST immobility by 15 days after enrichment removal ($p < 0.05$, Fisher's LSD post hoc) (Figure 3a). There were no differences in any other behaviors in the FST (see Table S1), but climbing was not distinguished from swimming behavior. Because this effect was not present as early as 5 days (Figure S2a), 9–15 days of environmental enrichment removal are required to elicit the FST phenotype. Next, we tested the impact of enrichment removal on HPA axis responsiveness to a novel stressor (restraint). There were significant interaction effects of group $\times$ time on ACTH [$F_{(12, 149)} = 2.758$; $p = 0.002$] and corticosterone concentrations [$F_{(12, 156)} = 3.953$; $p < 0.001$]. Active cycle EE rats had higher peak ACTH levels 15 and 30 min after restraint initiation ($p < 0.05$, Fisher's LSD post hoc) (Figure S2b), and higher corticosterone at 60 min ($p < 0.05$, Fisher's LSD post hoc) (Figure 3b). In contrast, the ER rats had significantly lower corticosterone levels at the 30 min time point ($p < 0.05$, Fisher's LSD post hoc) (Figure 3b), suggesting blunted peak stress responsiveness. We then assessed sucrose preference (SPT) 11 days after enrichment removal. ER rats had increased sucrose intake relative to EE rats and singly housed controls [$F_{(3, 36)} = 3.519$; $p = 0.025$] ($p < 0.05$, Fisher's LSD post hoc) (Figure 3c). Prior to enrichment removal, active cycle enriched rats gained less weight than singly housed rats and active cycle pair housed rats (group $\times$ time [$F_{(12, 156)} = 5.255$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 3d). However, enrichment removal caused significant weight

gain relative to all other groups (group [$F_{(3, 116)} = 6.630$; $p < 0.001$]; time [$F_{(3, 116)} = 50.043$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 3e), which was associated with a gain in fat mass (but not lean mass, $p > 0.05$, Figure S2c) relative to the EE group [$F_{(3, 39)} = 3.684$; $p = 0.02$] ($p < 0.05$, Fisher's LSD post hoc) (Figure 3f). Given the possibility that behavioral testing could differentially affect body weight following enrichment removal, we assessed body weight within a cohort of animals that did not undergo behavioral testing (Figure S5). Once again, animals gain less body weight while being enriched [$F_{(8, 120)} = 5.995$; $p < 0.001$] ($p < 0.05$, Fisher's LSD post hoc) (Figure S5a), and exhibit a marked increase in body weight after being removed from enrichment, replicating our prior observation [$F_{(6, 71)} = 3.144$; $p = 0.009$] ($p < 0.05$, Fisher's LSD post hoc) (Figure S5b).

3.3 Enrichment removal-induced FST immobility is prevented by chronic imipramine treatment

The presence of increased depression-like behavior in combination with weight gain, enhanced sucrose preference and blunted stress reactivity is reminiscent of so-called 'atypical' depressive symptoms in humans (Gold & Chrousos 2002). To determine whether the effects of enrichment removal in the FST may be considered a depression-like phenotype, we chronically treated rats with the antidepressant drug imipramine after cessation of enrichment. There was a significant interaction of group $\times$ treatment on duration of immobility in the FST [$F_{(1, 37)} = 7.991$; $p = 0.008$]. We found that 14-day imipramine treatment decreased immobility specifically in ER animals ($p < 0.05$, Fisher's LSD post hoc) (Figure 4a). Imipramine increased the latency to become immobile in both ER and control singly housed rats [$F_{(1, 37)} = 13.568$; $p < 0.001$] ($p < 0.05$, Fisher's LSD post hoc) (Figure S3a). Imipramine also increased climbing duration with a significant group $\times$ treatment interaction [$F_{(1, 37)} = 4.388$; $p = 0.043$]. This effect was specific to the ER IMIP group ($p < 0.05$, Fisher's LSD post hoc) (Figure 4b) and no differences were observed in diving or swimming behavior (see Table S1). Again, enrichment decreased body weight gain prior to enrichment removal (group $\times$ time [$F_{(3, 111)} = 3.379$; $p = 0.02$], group [$F_{(1, 37)} = 37.386$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 4c). ER animals treated with saline had a dramatic increase in body weight gain, which was successfully blocked by treatment with imipramine (group $\times$ time [$F_{(3, 111)} = 8.118$; $p < 0.001$], treatment $\times$ time [$F_{(3, 111)} = 12.32$; $p < 0.001$], group $\times$ treatment [$F_{(1, 37)} = 9.085$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 4d). There was a significant time $\times$ treatment interaction on ACTH [$F_{(4, 148)} = 7.8085$; $p < 0.001$] and significant group $\times$ treatment interaction on corticosterone concentrations [$F_{(1, 37)} = 5.383$; $p = 0.026$] after restraint challenge. ER rats had decreased peak ACTH and corticosterone responses to stress relative to singly housed rats ($p < 0.05$, Fisher's LSD post hoc) (Figure S3b), suggesting a hypoactive HPA response to a novel stress challenge. Imipramine masked the enrichment removal-induced decrease in peak corticosterone ($p < 0.05$, Fisher's LSD post hoc) (Figure S3c-d).

3.4 Loss of enrichment versus recovery from chronic stress

Because enrichment causes physiological changes reminiscent of chronic stress (i.e., elevated HPA responses, decreased body weight), we wanted to verify that our removal phenotype arises from loss of a rewarding stimulus and is not merely due to recovery from stress exposure. We therefore compared loss of enrichment (loss of a rewarding

manipulation) to recovery from either unpredictable (chronic variable stress) or habituating (repeated restraint) chronic stress regimens (Flak et al 2012, Kopp et al 2013, Smith et al 2016). Loss of enrichment increased immobility in the FST, whereas no changes were observed following recovery from repeated restraint stress or chronic variable stress [$F_{(3, 36)} = 3.823$; $p = 0.018$] ($p < 0.05$, Fisher's LSD post hoc) (Figure 5a), verifying that behavioral effects were due to the loss of a positive environment rather than stress. ER animals also had decreased swimming duration in the FST [$F_{(3, 36)} = 4.135$; $p = 0.013$], compared to both CON and CVS animals ($p < 0.05$, Fisher's LSD post hoc, Table S1). There were no differences in climbing behavior (data not shown). Repeated restraint stress and chronic variable stress caused decreased body weight gain compared to unstressed singly housed rats (group $\times$ time [$F_{(12, 144)} = 2.908$; $p = 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 5b). After cessation of chronic stress and enrichment, enrichment-removed rats again had significantly increased body weight gain compared to all other groups by 3 weeks after removal (group $\times$ time [$F_{(6, 71)} = 3.144$; $p = 0.009$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 5c). Rats recovering from repeated restraint stress or chronic variable stress did not gain any more weight than unstressed controls (Figure 5c), indicating that the weight gain seen after enrichment removal was not simply a recovery of lost body weight.

3.5 Dissecting the components of environmental enrichment

To extend our characterization of enrichment removal as a model for loss, we tested whether loss of individual components of environmental enrichment (social housing or exercise (running wheel)) would be sufficient to produce the phenotype elicited by enrichment removal. With running access during the active cycle, rats run increasingly greater distances over the 4 weeks (Figure S4a). Rats with running wheels or in environmental enrichment gained less weight than control rats, with running rats gaining even less weight than enrichment rats (time [$F_{(4, 192)} = 616.553$; $p < 0.001$], group [$F_{(3, 192)} = 17.255$; $p < 0.001$], group $\times$ time [$F_{(12, 192)} = 12.453$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 6a). Socially housed rats gained more weight than control rats ($p < 0.05$, Fisher's LSD post hoc) (Figure 6a). Enrichment-removed and running-removed rats gained more body weight than controls, starting 1 week after removal and maintained this effect for the rest of the experiment (time [$F_{(6, 288)} = 1480.115$; $p < 0.001$], group [$F_{(3, 288)} = 23.758$; $p < 0.001$], group $\times$ time [$F_{(18, 288)} = 16.32$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 6b). Chow intake was assessed for 2 days immediately following stimulus removal, to establish as close to a baseline food intake as possible. This baseline was used to calculate percent change in chow intake. ER rats ate significantly more food than all other groups in weeks 5 and 6 (1 and 2 weeks post-removal) (time [$F_{(2, 96)} = 34.386$; $p < 0.001$], group [$F_{(3, 96)} = 12.439$; $p < 0.001$], time $\times$ group [$F_{(6, 96)} = 12.231$; $p < 0.001$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 6c). (For raw food intake, see Figure S4b.) In the reward-based feeding paradigm, the enrichment, running, and social removed groups all ate significantly more high-fat diet than the control group (group [$F_{(3, 48)} = 4.843$; $p = 0.005$]); ($p < 0.05$, Fisher's LSD post hoc) (Figure 6d). Overall, loss of a positive stimulus drives reward-based feeding behavior. In particular, environmental enrichment removal alone causes general hyperphagia, including excessive consumption of both chow and palatable diet, suggesting that these effects require removal from the entire enrichment experience.

We should note that in this experiment, there was no effect of any enrichment treatment of immobility in the forced swim test (Figure S4c, Table S1). Indeed, immobility times in all groups were near maximal, suggesting a ceiling effect that would obscure any immobility enhancement effect of running, social or enrichment removal (Figure S4c). We believe this discrepancy across experiments may have a procedural basis, which is discussed in the figure legend for Figure S4c. We include these data in consideration of reproducibility. However, it is important to note that the increased immobility in the FST replicated across the prior 4 studies, and the body weight phenotype across all studies.

4. DISCUSSION

Our results are consistent with enrichment removal representing loss and negative affect in rats. Removing rats from environmental enrichment produces a marked and unique phenotype characterized by increased immobility in the forced swim test, blunted peak ACTH and corticosterone responses to a novel stressor, increased body weight gain, and increased consumption of chow, sucrose drink, and high-fat diet. Altogether, this phenotype resembles that seen in a sizable proportion of depressed patients, which includes symptoms of hyperphagia, weight gain, and hypoactivation of the HPA axis in the context of depressed mood. (Gold & Chrousos 2002). Moreover, the enrichment removal phenotype presents these key features of depression that are seldom observed in current animal models, such as hyperphagia and downregulation of the HPA axis (Krishnan & Nestler 2011). This enrichment removal protocol adds to current animal models of depression by expanding the range of possible phenotypes, while providing a model of loss with face validity.

With active cycle enrichment, we show that EE decreases body weight gain, increases the peak glucocorticoid response to acute stress, but does not change behavior in the FST and SPT. This may seem contradictory to previous work demonstrating antidepressant-like effects of enrichment, indicated by decreased immobility in the FST, increased sucrose preference, and potentially decreased basal corticosterone (Green et al 2010, Brenes et al 2006, Brenes et al 2008b, Stairs et al 2011, Belz et al 2003, Skwara et al 2012). Enrichment also prevents the HPA-stimulatory effects of prior stress by blocking the stress-induced increase in glucocorticoid responses to an acute challenge (Francis et al 2002). However, it is important to consider that all of these studies exposed rats to EE during the adolescent period. In adults, EE does not affect behavior in the SPT or immobility in the FST, and actually increases both basal and stress-induced HPA activation (Konkle et al 2010, Bakos et al 2009, Moncek et al 2004). With adult active cycle enrichment, there are no basal increases in corticosterone or ACTH but the effect of EE on stress-induced peaks are consistent with previous reports (Konkle et al 2010).

The active cycle enrichment protocol replicates the effects of EE and ER, demonstrating the efficacy of enrichment access during the active cycle. This protocol was initiated to allow undisturbed individual measures for EE and ER rats. Removal of rats from the EE for stress testing likely induces an HPA axis response on its own, and may create a disturbance in the colony environment. In addition, capture for behavioral/stress testing can take variable amounts of time, which may affect performance and accuracy/reproducibility of sampling time-courses. In the EE group, our active cycle enrichment removes this confound and

demonstrates increased peak glucocorticoid responses to stress, followed by efficient recovery. This suggests that continuous EE produces an enhanced HPA axis response. This may be suggestive of EE driving the HPA axis as a 'eustressor' (given its rewarding nature), perhaps as an adaptive response keyed to increased energetic need engendered by the high activity environment (Crofton et al 2015, Konkle et al 2010, Peña et al 2009).

Enrichment loss induces depression-like behavior in the FST but also increases sucrose consumption and decreases the peak glucocorticoid response to acute stress. Typically, decreased sucrose consumption and increased HPA reactivity are associated with animal stress models that induce depression-like symptoms (Krishnan & Nestler 2011). However, HPA hypoactivity in humans is associated with alternative forms of depression, such as atypical, seasonal, and postpartum depression (Tsigos & Chrousos 2002). Blunted HPA reactivity is also a characteristic of other stress related disorders, such as chronic fatigue syndrome (Tsigos & Chrousos 2002). Furthermore, increased sucrose preference can actually be associated with increased immobility in the FST, or increased depression-like behavior (Brenes et al 2006, Brenes et al 2008a). These data suggests that ER may recapitulate a depression endophenotype consistent negative mood in the context of hypophagia and HPA hypofunction (e.g., atypical depression)(Gold & Chrousos 2002). We acknowledge that our designs prohibited analysis of the HPA axis across all studies, limiting the breadth of our conclusions on the HPA hypofunction in ER animals. Future studies are needed to address this in a more thorough manner. Despite this limitation, the use of the loss model will be important in understanding mechanisms of negative mood symptoms that are underrepresented by the current selection of animal models.

One of the most consistently striking effects of enrichment removal is the increased rate of body weight gain and food intake that persists for weeks after enrichment loss. Animals removed from environmental enrichment are hyperphagic, evidenced by increased intake of chow, sucrose drink, and high-fat diet. This general hyperphagia and weight gain are unique to enrichment removal because they are not recapitulated by removal of other rewarding or aversive stimuli (loss of exercise, loss of social contact, recovery from chronic stress).. Additionally, antidepressant treatment attenuates the increase in body weight seen with enrichment removal. This aspect of the enrichment removal model may provide novel insights into the development of obesity and help explain the high comorbidity of obesity and atypical depression (Chou & Yu 2013).

Our design involves removal of all groups to individual housing in the resting (light) phase of the daily activity cycle). There is an ongoing debate regarding the extent to which isolation comprises a stressor, and hence one may be concerned that individual housing may comprise an additional stress component that contributes to the EE phenotype. However, we do not believe this to be the case, as our data indicate that neither daily removal from paired housing nor daily removal from social housing produces our body weight/anhedonia phenotype. Moreover, the fact that the ER food intake effect is not seen following removal of other stimuli, either positive (running wheels) or negative (restraint or CVS) suggests that it is not associated with simple removal of stimuli (i.e., 'boredom').

The development of the enrichment removal phenotype is unique in that it represents an internal and psychogenic phenomenon that does not require the administration of an exogenous stimulus. Rather, it is dependent on the absence of a previously known stimulus. This notion is exemplified by our data demonstrating that in adult males, isolated animals that had enrichment removal fare worse (in terms of depression-like characteristics) than isolated or pair housed animals that had never experienced enrichment. Demonstrating that animals are significantly affected by loss enhances the scope of animal research and has important implications for future studies that remove rewarding stimuli. It is of the utmost importance to expand the enrichment removal model to include female rats, especially given that disorders triggered by loss are often more prevalent in the female population. Future studies will include need to include females in the experimental design.

5. CONCLUSION

Rodents are significantly affected by removal from environmental enrichment and display features of depression, especially those observed in atypical depression. Our results demonstrate that removal of long-standing enrichment is an appropriate model for studying the neurobiological effects of loss, which likely will enable a more mechanistic investigation into the development of psychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. List of experimental timelines

Experimental timeline for the 5 experiments and a 6th cohort without behavioral testing, run concurrently with experiment 4. EE = environmental enrichment; FST = forced swim test; SPT = sucrose preference test; RR= repeated restraint; CVS = chronic variable stress; RUN = running wheel access; SOC = social housing; HFD = high fat diet

Figure 2. Enrichment removal produces depression-like behavior

(a) ER animals have significantly increased immobility in the FST * $p < 0.05$ vs CON, # $p < 0.05$ vs EE (b) No differences in locomotion in the open field (CON $n = 11$; ER $n = 9$; EE $n = 8$)

Figure 3. Enrichment removal produces depression-like behavior, blunted peak corticosterone following acute novel stress, increased sucrose intake, increased weight gain and fat mass
(a) ER animals have increased immobility in the FST $*p < 0.05$ vs SCON, $^{\wedge}p < 0.05$ vs PCON, $^{\#}p < 0.05$ vs EE **(b)** ER animals have decreased corticosterone at 15 and 30 min after restraint onset, while EE animals have increased corticosterone at 60 min after restraint onset $*p < 0.05$ vs SCON, $^{\wedge}p < 0.05$ vs PCON, $^{\#}p < 0.05$ vs EE, $^{\&}p < 0.05$ vs ER **(c)** ER animals consume more sucrose on the first day of the sucrose preference test $*p < 0.05$ vs SCON, $^{\#}p < 0.05$ vs EE **(d)** During enrichment, EE and ER animals gain less weight $*p < 0.05$ vs SCON, $^{\wedge}p < 0.05$ vs PCON within the same week **(e)** With enrichment removal, ER animals gain weight at a faster rate $*p < 0.05$ vs SCON, $^{\wedge}p < 0.05$ vs PCON, $^{\#}p < 0.05$ vs EE within the same week **(f)** ER animals regain fat mass after 2 weeks of removal, while EE maintain a decreased fat mass $*p < 0.05$ vs SCON, $^{\wedge}p < 0.05$ vs PCON, $^{\&}p < 0.05$ vs ER (SCON $n = 11$; PCON $n = 12$; EE $n = 10$; ER $n = 9$)

Figure 4. Antidepressant treatment imipramine prevents depression-like behavior and weight gain induced by enrichment removal

(a) ER animals treated with imipramine (ER IMIP) had decreased immobility in the FST $^{\wedge}p < 0.05$ vs CON IMIP, $\#p < 0.05$ vs ER SAL, $\sim p = 0.073$ vs CON SAL (b) ER IMIP animals had increased climbing duration in the FST $^*p < 0.05$ vs CON IMIP (c) ER animals gain less weight than CON during enrichment and before starting IMIP treatment $^*p < 0.05$ vs respective CON within the same week ** denotes ES SAL and ES IMIP (d) After enrichment removal, ER SAL animals have an increased weight gain, while ER IMIP animals do not differ from CON SAL animals $^*p < 0.05$ vs CON SAL within the same week (CON SAL $n = 11$; ER SAL $n = 9$; CON IMIP $n = 11$; ER IMIP $n = 10$)

Figure 5. Enrichment removal phenotype does not generalize to recovery from chronic stress
 (a) ER animals have increased immobility in the FST, but not animals recovering from chronic stress $*p < 0.05$ vs CON, $\#p < 0.05$ vs CVS (b) CVS and RR animals gain less weight during chronic stress regimens $*p < 0.05$ vs CON, $^{\wedge}p < 0.05$ vs ES within the same week (c) After enrichment or stress removal, ER animals gain a higher percentage of body weight, while animals recovering from CVS or RR do not differ from CON animals $*p < 0.05$ vs CON, $^{\wedge}p < 0.05$ vs RR, $\#p < 0.05$ vs CVS within the same week (CON $n = 10$; ER $n = 10$; CVS $n = 10$; RR $n = 10$)

Figure 6. Enrichment removal, but not removal of other rewarding stimuli, increases chow intake

(a) During active cycle stimulus exposure, RUN and ER rats gain less weight than CON and SOC rats gain more weight than CON * $p < 0.05$ vs CON, ^ $p < 0.05$ vs SOC, # $p < 0.05$ vs ER within the same week (b) Upon removal of rewarding stimuli, RUN and ER rats gain more weight than CON with chow diet (weeks 5–7) and RUN, ER, and SOC rats gain more weight than CON with high fat diet (weeks 7–10) * $p < 0.05$ vs CON, ^ $p < 0.05$ vs SOC within the same week; *^denotes both RUN and ER groups (c) ER rats eat progressively more chow in the two weeks after enrichment removal, while RUN rats begin to eat less chow * $p < 0.05$ vs CON, ^ $p < 0.05$ vs SOC, & $p < 0.05$ vs RUN; see Supplemental Figure 4b for raw food intake (d) 2.5 weeks after stimulus removal, ER, RUN, and SOC rats eat more

high fat diet than CON rats in a reward based feeding test * $p < 0.05$ vs CON (CON n = 13; ER n = 10; RUN n = 13; SOC n = 16)

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