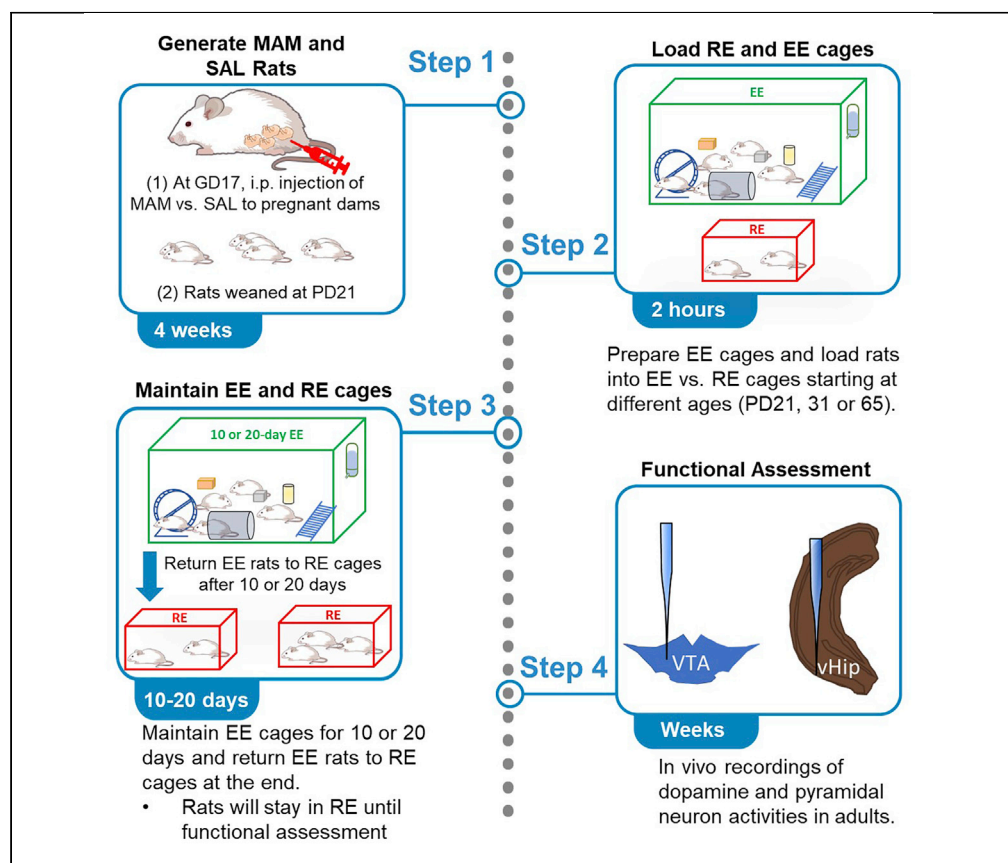


## Protocol

# Use of prepubertal environment enrichment to prevent dopamine dysregulation in a neurodevelopmental rat model of schizophrenia risk



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### Highlights

Protocol to deliver EE in postnatal windows of a rat model of schizophrenia risk

Detailed description of supplies and procedures to construct EE boxes

Details of animal loading/offloading, cage maintenance, and design of control groups

Expected electrophysiological outcomes and troubleshooting of common pitfalls

Here, we present a protocol for delivering environmental enrichment (EE) in discrete postnatal windows to prevent long-term dopamine neuron dysfunctions in a neurodevelopmental rat model of schizophrenia risk. We describe generation of the schizophrenia model through prenatal treatment of rats with methylazoxymethanol acetate (the MAM model) and the saline-treated controls. We then detail the 10-day or 20-day EE paradigms applied on male rats at different ages. This protocol also includes preparation of control groups in regular environment (RE) cages for comparison.

Zhu & Grace, STAR Protocols  
 3, 101215  
 March 18, 2022 © 2022 The Author(s).  
<https://doi.org/10.1016/j.xpro.2022.101215>



## Protocol

## Use of prepubertal environment enrichment to prevent dopamine dysregulation in a neurodevelopmental rat model of schizophrenia risk

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## SUMMARY

Here, we present a protocol for delivering environmental enrichment (EE) in discrete postnatal windows to prevent long-term dopamine neuron dysfunctions in a neurodevelopmental rat model of schizophrenia risk. We describe generation of the schizophrenia model through prenatal treatment of rats with methylazoxymethanol acetate (the MAM model) and the saline-treated controls. We then detail the 10-day or 20-day EE paradigms applied on male rats at different ages. This protocol also includes preparation of control groups in regular environment (RE) cages for comparison.

For complete details on the use and execution of this protocol, please refer to Zhu and Grace (2021).

## BEFORE YOU BEGIN

## Generating the MAM model and the saline-exposed control rats

⌚ Timing: 4 weeks

Sprague-Dawley rats prenatally treated with either a mitotoxin, methylazoxymethanol acetate (MAM; 20 mg/kg, i.p.), or saline (SAL) are generated based on our previous published article and protocol (Lodge, 2013; Moore et al., 2006). Furthermore, below are additional considerations.

1. Dams should be ordered to arrive the facility at gestational day (G) 14 or 15 to allow time for the rats to acclimate to the environment before the MAM injection at G17.
2. After birth on postnatal day (P) 0, the offspring stay with the dams until the weaning day (P21).
3. For group design, in each experimental group we recommend using multiple (>3) litters and representative (n = 1 or 2) pups from each litter to avoid data overrepresentation (i.e., litter effect).
4. To ensure effective counterbalancing for litters, each experimental cohort should include rats from at least 3 MAM-treated and 3 SAL-treated dams.

## Preparing relevant equipment and supplies

⌚ Timing: 1–2 days



5. We frequently referred to two studies in the design of EE boxes (Ma et al., 2016; Fischer et al., 2007). In particular, Ma et al. (2016) was conducted at the University of Pittsburgh and therefore is a primary reference.
6. The EE boxes and objects should be purchased from institution's approved vendors. At the University of Pittsburgh, Department of Neuroscience, we used two major sources to order supplies:
  - a. [www.thatpetplace.com](http://www.thatpetplace.com)
  - b. [www.grainger.com](http://www.grainger.com)

### Important considerations

The protocol below describes critical steps to use prepubertal environmental enrichment (EE) in a neurodevelopmental model of schizophrenia (the MAM model) to prevent dopamine (DA) neuron-related electrophysiological abnormalities and alteration of ventral hippocampal (vHipp) activity. We have not directly tested this protocol to prevent other phenotypes of the MAM model, such as sensorimotor gating, social, and/or memory deficits, although others have suggested potential preventative efficacy (Bator et al., 2018). Beyond schizophrenia and the MAM model, others have used diverse EE paradigms in a variety of neurodevelopmental disorder (NDD) models, and their translational potential is reviewed elsewhere (Ball et al., 2019). Altogether, current evidence suggests that early EE is a promising approach to prevent symptom-related phenotypes in models of NDDs. Nevertheless, comprehensive characterization and optimization of EE parameters (e.g., exposure age, duration, recency, etc.) are recommended before adopting this protocol to other models of NDDs.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Experimental models: Organisms/strains</b>		
Rat: Hsd:Sprague Dawley® SD®	ENVIGO	<a href="https://www.envigo.com/model/hsd-sprague-dawley-sd">https://www.envigo.com/model/hsd-sprague-dawley-sd</a>
<b>Chemicals, peptides, and recombinant proteins</b>		
Methylazoxymethanol acetate	MRIGlobal Chemical Carcinogen Repository	Cat# 213
Sterile saline	Covetrus	Cat# C1880725
<b>Other</b>		
Storage Tote	STERILITE	Mfr. Model # 19453V04
SAINT-GOBAIN Fiberglass Door and Window Screen (cage cover)	Saint-Gobain	Mfr. Model # FCS10113-M
Cable Ties	Powerfirst	Mfr. Model # 36J154
Large running wheel (for age >P40)	Kaytee	MPN: 100533403
Small running wheel (for age <P40)	Kaytee	MPN: 100079362
Grainger plastic tube materials (running tunnel)	Grainger	Mfr. Model #: <ul style="list-style-type: none"> <li>• 1WHT9</li> <li>• 1WJA7</li> <li>• 1WHZ4</li> </ul>
Ladder	Penn Plax	MPN: BA243
Feeder from standard rodent cage	Allentown	N/A (compatible with the 40-cage rack system: RS10147U40MVSPCD3-R)
Kimwipes (nestlets)	KIMTECH	Cat#34155
Lab rodent chow (Labdiet 5001)	Labdiet	Cat#: 0001319
Play ball pack	Marshall Pet Products	MPN: 572017
Lattice Balls with Bells	Penn Plax	MPN: BA-511
Color Play Dangly	Oxbow	MPN: 2951

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Atomic Ball	Ware	MPN: 089348
Rat chew	Kaytee	MPN: 100504115
Wood puzzle	Kaytee	MPN: 100501560
Example ceramic objects: 11oz mug, single color	Various sources	N/A
Example ceramic objects: figurine (approximately 1 × 1.375 × 1.125 inches; single color)	Unknown	N/A

## STEP-BY-STEP METHOD DETAILS

### Constructing the EE boxes

⌚ Timing: 1–2 h

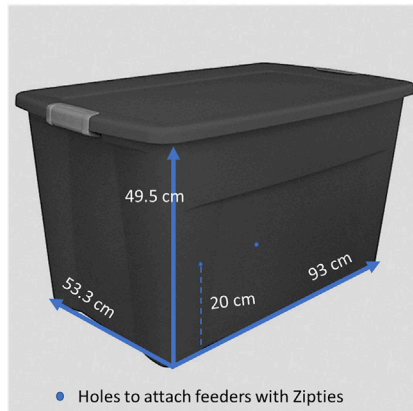
An EE cage should be designed and assembled to incorporate core enriching elements: cognitive stimulation, complex social interaction, and increased physical activity (van Praag et al., 2000).

1. Before building the EE box, all the equipment, supplies, and procedures described below have to be approved by the institutional animal care and use committee (IACUC).
2. Equipment and supplies need to be first thoroughly cleaned by the central animal facility, and then disinfected by the experimenters with 70% ethanol just before EE box is assembled and loaded.
3. Assemble the EE boxes.
  - a. We use 30-gallon Sterilite storage totes, see [Figure 1](#) for dimensions.
  - b. Drill four holes on the long walls of the EE box according to the locations in [Figure 1](#).
  - c. Use four cable ties to attach the four corners of the feeder to the walls.
4. Evenly spread 1-inch thick of standard bedding materials onto the EE box floor.
5. Fill the feeder with standard rodent chows and a water bottle to provide ad libitum access to food and water.
6. Attach the ladder to the feeder.
  - a. Spread 10–15 pellets of rodent chow randomly on the bedding materials.
  - b. These steps are designed to encourage foraging behaviors, which has been linked to the success of enrichment (Hutchinson et al., 2005).
7. Add 10 pieces of Kimwipes to serve as nesting materials.

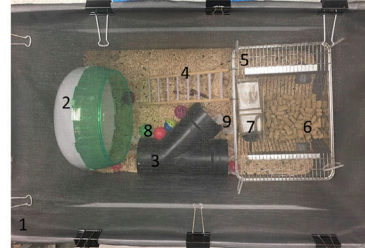
**Optional:** This can be replaced by other long-fiber nesting materials provided by the animal facility.

8. Place a running wheel and up to 3 pieces of tunnels to EE cages, see [Figure 1](#) for an example.
  - a. See [key resources table](#) for running wheel size information.
  - b. The orientation of the tunnels and the running wheels are changed together with the cage cleaning in step 20 and 21.
9. Randomly spread 6–10 objects onto the bedding materials. At a given moment, the objects in the EE box should be of at least two textures [ceramic (see [key resources table](#) for examples and general dimension), plastic, or wooden], colors, and shapes (ball, square/rectangular, triangle, etc.).
  - a. Change the types of objects three times a week during weekdays (Monday, Wednesday, and Friday). This step is typically brief and does not require the complete removal of animals during the item changing.
  - b. Within each week, avoid using the same objects for more than twice.
  - c. When removed, objects should be rinsed with water and disinfected with 70% ethanol, and then stored in airtight Ziplock bags to avoid contamination.

Dimensions of an EE cage



Top view of an EE cage



**Figure 1. Dimensions and a top view of an example EE box**

(left) EE box converted from a 30-gallon storage box; blue points represent where the feeder is attached on the inside. (Right) a top view of a finished EE box. Numbers indicate different supplies in an EE cage, including (1) cage cover made from window screen; (2) Kaytee running wheel; (3) tunnels made from Grainger vent pipes; (4) Penn Plax ladder to connect the feeder; (5) feeder from a typical automated rat housing cage (Allentown), attached to walls with cable ties; (6) rodent chow; (7) water bottle; (8) toys with varied color, shape, and textures; (9) Kimwipes as nesting materials.

### Loading animals into the EE boxes

⌚ Timing: 2 h

Duration and age of exposure are critical parameters of EE protocols, as they can determine the success of enrichment, depending on the endpoints being tested (Leger et al., 2015). In our lab, 10- or 20-day continuous EE paradigms have been given to male MAM rats and SAL controls. Here we describe important experimental steps for housing rats at different ages in EE cages. This protocol used animals aged between P21-40 and P65-84.

10. For every age group, house 5 age-matched rats per EE cage. (max age in EE box: P84).
11. For the prepubertal P21-30 or P21-40 EE groups, on P21 directly load the weanlings to the EE cage.
  - a. SAL and MAM rats are mixed and loaded into the same EE boxes, and therefore clear marking is essential. Check everyday to ensure visible tail marks.
  - b. Mark the tails with differently colored bands using a permanent marker to differentiate individual animals.

**⚠ CRITICAL:** Since MAM or SAL injection are maternal manipulations, to avoid litter effects we recommend using 1 or 2 representative weanlings of each litter in each EE boxes. This way each EE cage can hold rats from at least 3 litters, minimizing potential litter batch effects.

12. For P31-40 or P65-84 enrichment, on P21 directly load the weanlings into regular environment (RE) cages at a group size of 2 or 3 in a counterbalanced manner for litters.
  - a. House these rats until P31 or P65, and then load them into the EE boxes in a group of 5.

**Note:** For the duration of our EE protocols (maximum of 20 days), we did not observe biting even in the adult P65-84 group, consistent with the findings that Sprague Dawley rats are relatively “peaceable” (Henry et al., 1993).

13. Place the EE cages on a rack in the same colony room where RE cages are housed for either 10 or 20 days based on individual experimental needs.

△ **CRITICAL:** this step controls for light schedule, temperature/humidity, noise exposure, etc.

### Creating appropriate control groups

⌚ **Timing:** 5 min, per cage

Environmental enrichment is multifaceted, and its design is based on each experimental need. Thus, experimenters should be cautious in selecting control groups to avoid confounds (Ball et al., 2019). In our study, the primary goal was to determine whether there are preventative benefits of EE on dopamine- and hippocampus-related pathophysiology in the MAM model (Zhu and Grace, 2021). Since MAM rats display prominent dysfunctions even in paired- or triple-housed social conditions (Modinos et al., 2015), we did not include a social-deprivation control group. Moreover, we did not create control groups specifically for each enrichment element, because our emphasis was to determine whether relatively complete enrichment experiences (with all enriching elements) are sufficient to prevent later pathophysiology in the MAM model.

14. Wean all the rats for RE groups on P21.
  - a. RE groups are weaned and housed in 2–3 rats per cage.
  - b. Same control for litter effect (see step 11 and 12) also applies, i.e., when possible, each RE cage should contain one representative pup from each litter. No more than two pups from a single litter are included in a single RE cage.
15. Place the RE cages onto the automated rack system.

**Note:** Except for the weekly cage change conducted by institution's animal care staff, rats in RE groups are generally undisturbed throughout the study. However, to control for handling stress, brief handling (e.g., picking animals from home cages, body weight recording, examine signs of injury, etc.) are given to RE rats on the same schedule of EE cage maintenance (see step 17).

**Optional:** depriving animals from and/or varying certain EE elements can be used to create subgroups in the control animals. This would allow studies to determine the necessary/critical factors (e.g., cognitive, social and/or physical exercise) in the designed experiments. For example, dissociable effects of social vs. physical EE have been tested in rats, and the physical exercise of EE is deemed uniquely influential to brain plasticity-related changes (Brenes et al., 2016).

### Cleaning, maintaining, and animal observing during EE paradigms

⌚ **Timing:** 5–15 min

Throughout the EE periods, perform daily, twice-a-week, and weekly maintenance to the EE cages according to the Steps outlined below. In our study, RE cages are maintained mainly by the institutional animal care staff, which entails a weekly cage change to refill food and completely replace cage, beddings, and nestlets.

16. Quietly observe the EE animals daily for signs of injury or distress.
  - a. This can be done without removing EE cage mesh cover, but if necessary, gently transfer the animals to a separate cage for further inspection.
  - b. Inspect levels of food and water for refill.
17. The bedding materials of EE cages are changed/cleaned twice a week.
  - a. A major change is scheduled every 7 days following the initial loading of the animals.

- b. A minor change occurs three days after the initial animal loading and after every major change.
- 18. For both the minor and the major cage changes, temporarily transfer all EE cage-mates into a static housing cage.
  - a. Provide the cage with ad libitum water and food.
  - b. Provide the cage with 3–5 pieces of Kimwipes as nesting materials.
  - c. Place the temporary transfer cages in the same colony room. Perform cage changes in a separate room to avoid potential noise exposure.
- 19. For the minor change [less than 5 min], simply remove all the feces and dirty bedding, and spread new bedding when necessary.
- 20. For the weekly major change [10–15 min], clean around half of the used beddings and replace with fresh ones. Replace all other needed supplies detailed in step 6–8 with new ones. Clean the walls and the running wheel with 70% ethanol and examine whether the running wheel is functional.
  - a. Major cage change is typically conducted on the same days for RE cage changes to control for handling effects.
- 21. Replace the rats back to the EE cages.

*Optional:* cage changes should be combined with experiments such as blood draw, body weight recording, behavioral assays, etc. to avoid excessive handling.

### Return EE rats to RE cages

⌚ Timing: 30 min–1 h

- 22. In the afternoons of P30, P40 or P84, return EE rats to RE cages at a group size of 2–3 rats/cage

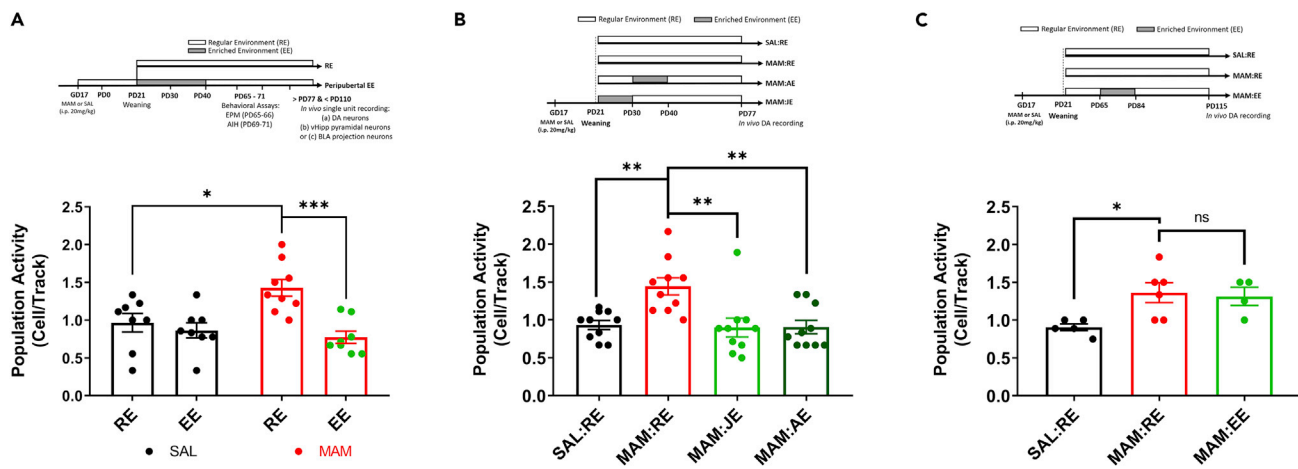
**Note:** When possible, counterbalance for litters.

**Note:** Rats returned both prepubertally (i.e., on P30 or P40) or as adults are expected to acclimate to the RE cages in a peaceful manner. Thus, we did not find signs of biting during the return of adult EE rats in our study, at least for the duration until the completion of experiments.

- 23. Thereafter, rats previously exposed to EE stay in RE cages until the end of all experiments.
  - a. Record the delay between EE completion and each testing date, as these data could be useful to study the recency effect of EE.
- 24. Dispose the used bedding according to institution’s regulations. Inform the animal facility staff to clean the EE cages.
  - a. In our study, EE cages were reused after cage cleaning and disinfection by ethanol (step 2).

### EXPECTED OUTCOMES

We did not directly examine several known behavioral benefits of EE in our study (Ball et al., 2019), as these effects (e.g., on sensorimotor gating, social approach, memory deficits, etc.) had been consistently reported by several other studies on models of schizophrenia risks (Akillioglu et al., 2012; McOmish et al., 2008; Burrows et al., 2015; Lee et al., 2012), including the MAM model (Bator et al., 2018). Instead, our study emphasized a novel potential of EE, and tested whether prepubertal EE can prevent physiological alterations in the MAM model proposed to underlie psychosis, namely the aberrant ventral tegmental area (VTA) DA neuron and elevated vHipp pyramidal neuron firings (Grace, 2016). Such preventative efficacy can be assessed by in vivo single unit recordings, of which the technical details can be found in our recent publications (Gomes et al., 2019a; Zhu et al., 2021).



**Figure 2. Example of prepubertal EE effects during discrete windows on DA neuron population activity**

(A) P21-40, prepubertal EE has been shown to prevent MAM-induced increased DA neuron population activity measured in adults (>P77).

(B) In a separate experiment, 10-day prepubertal EE, regardless of age of exposure, was shown to prevent adult DA-related pathophysiology. AE: adolescent enrichment; JE: juvenile enrichment.

(C) These preventative effects of prepubertal EE were not found in adult, P65-84 EE group. Statistical analyses were conducted using one- or two-way ANOVA with Tukey's post hoc analysis. Data are presented as mean  $\pm$  SEM. Significant results with  $p < 0.05$ ,  $< 0.01$ , or  $< 0.001$  are denoted by \*, \*\*, or \*\*\*, respectively. ns: not significant. Effects of MAM are highlighted in red, and effects of EE are highlighted by light or dark green.

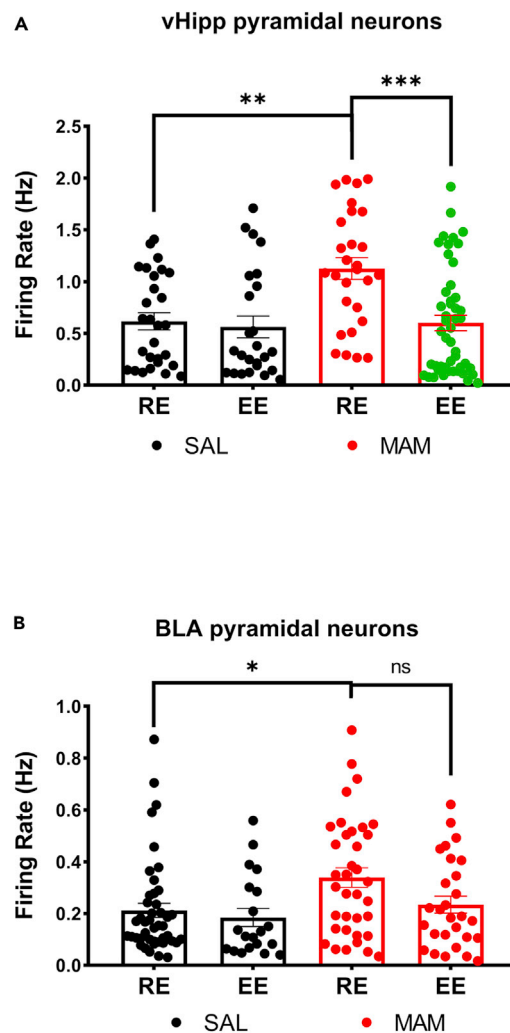
P21-40 prepubertal EE is expected to have no influence on unit firing properties of DA or BLA/vHipp pyramidal neurons of RE control animals (Figures 2 and 3). In contrast, EE during P21-30, P31-40, or P21-40 is expected to all prevent MAM-induced aberrant DA tonic activity (i.e., the number of spontaneously active neurons, or "population activity"; Figures 2A and 2B). These effects are not expected for the adult enrichment groups (Figure 2C), indicating that a prepubertal EE timing is critical to achieve prevention of pathophysiology regarding the electrophysiological activity of DA neurons. P21-40 EE is also expected to prevent the adult vHipp pyramidal neuron heightened firing rate in the MAM model (Figure 3A), and to have no influence on BLA-related pathophysiology (Figure 3B) nor adult anxiety-like behaviors in an elevated plus maze [data not shown, see (Zhu and Grace, 2021)].

We posit that a lack of BLA-related anxiolytic benefits does not contradict previous reports nor necessarily indicate inability of EE to rescue anxiety-like behaviors. Indeed, as indicated by (Leger et al., 2015), the anxiolytic effect of EE depends critically on EE duration and is task-specific (light dark box test vs. elevated plus maze). Alternatively, this null result might also suggest that EE may have other dissociable behavioral benefits independent of the amygdala-mediated anxiety, which constitutes a promising future direction. Thus, on top of the sensitive periods of EE's benefits on DA neuron physiological states, it is possible to optimize the present EE protocols to achieve multi-domain prevention. Future studies should explore this direction as NDDs tend to have complex symptomology spanning multiple behavioral domains (Sheffield et al., 2018).

## LIMITATIONS

The present protocol aims to determine the age of exposure effects, focusing on prepuberty as a sensitive period. Thus, the described protocol involves early cessation of EE before the testing, and therefore unavoidably introduces removal of EE. Novel evidence suggests such EE loss can lead to certain depression-related phenotypes (Smith et al., 2017). Therefore, for other experimental goals, such as to dissect optimal windows for EE to deliver maximum behavioral benefits, one must apply this protocol with necessary modifications. Noteworthy, the benefits of EE, especially the behavioral effects, are critically dependent on the duration, recency, and age of EE exposure. Thus, as pointed by previous evidence, it is not simply that the more chronic EE paradigm would





**Figure 3. Example of effects of P21-40 prepubertal on adult pyramidal neuron functions in the ventral hippocampus and the basolateral amygdala**

(A) 20-day prepubertal EE was found to prevent the MAM-induced increase in firing rate of putative pyramidal neurons in the vHipp.

(B) No effect of EE was observed in the BLA pyramidal neuron firing rate of the MAM rats. Two-way ANOVA on EE and MAM exposure. Data are presented as mean  $\pm$  SEM. \*, \*\*, and \*\*\* denote significant differences at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively. Effects of MAM are highlighted in red, and effects of EE are highlighted by light green.

be more effective (Leger et al., 2015), nor that earlier EE exposure age would always be more beneficial (Harburger et al., 2007).

Prior research on MAM-induced DA-related pathophysiology has been heavily conducted in males, with female endpoints examined much less frequently (Perez et al., 2014). Thus, to parallel the vast previous research basis, the present protocol focuses on male rats, and whether the same window of opportunity for EE applies to females is an open question that needs to be addressed by a dedicated future study to reveal potential sex differences.

At a translational level, animal EE studies have provided critical mechanistic insights that have supported clinical trials [see review in (Ball et al., 2019)] and investigations of key components of enriching living conditions in humans [for example, see (Kühn et al., 2017)]. In contrast, the

reverse-translation of successful human EE-related approaches to manipulatable rodent models can be challenging. This is in part due to the complexity of certain human EE elements, which are sometimes not feasible to be directly reverse-translated. For example, in terms of alleviating schizophrenia-related symptoms, although many cognitive therapy methods are based on EE concepts and determined to be relatively beneficial (Wykes et al., 2011), whether the complexity of these approaches can be readily modeled by providing cognitive stimulation in rats using varying toys and objects is worth future investigations.

### TROUBLESHOOTING

As mentioned above, the success of EE depends on multiple factors, each of which are possibly to be confounded if the studies were not well controlled.

#### Problem 1

Expected effects of MAM on dopamine neuronal firing not seen in the adult MAM:RE animals (see [generating the MAM model and the saline-exposed control rats](#) and step 13).

This can be either expressed as SAL controls showing MAM-like DA dysfunction (i.e., increased population activity), or MAM rats not displaying previously documented DA-related pathophysiology [part of the “MAM phenotype”, (Lodge, 2013)].

#### Potential solution

The former scenario is likely induced by uncontrolled prepubertal environmental stress in SAL or both groups, which can by itself induce MAM-like DA neuron dysfunction (Gomes et al., 2019a). This can be potentially avoided by reducing noise/light exposure (step 13) and/or social stress (such as to avoid single housing). The latter scenario can be driven by multiple factors, one of which could be insufficient exposure to the MAM compound prenatally. In fact, although being a rare case, some MAM-exposed litters do not develop the well-known DA dysfunction, potentially attributable to injection failure (See [generating the MAM model and the saline-exposed control rats](#)). This can be typically avoided by using multiple litters in design (we recommend at least three litters per group), and within each litter use 1 or 2 representative pups per experiment.

#### Problem 2

Overly extensive MAM-induced phenotypes compared previous literature (see [generating the MAM model and the saline-exposed control rats](#)).

The complete MAM phenotypes (Lodge, 2013) include histological, behavioral, and neurophysiological abnormalities consistent with human schizophrenia symptomology (Modinos et al., 2015). Although this protocol focuses on the electrophysiological alterations, region-specific histological deficits such as cortical thinning in the vHipp or the prefrontal cortex (PFC) might serve as an easy validation of the effectiveness of MAM injection. The specificity and extent of histopathology in the limbic vHipp is critical to the manifestation of DA-related phenotypes. For example, in neonatal vHipp lesion model, a broader, non-specific vHipp pathology involving the entorhinal cortex and the dorsal hippocampus failed to produce the expected amphetamine-induced hyperlocomotion (behavioral index of overall DA dysfunction) as in the specifically vHipp lesioned rats (Swerdlow et al., 2001). Thus, another explanation for the failure to observe MAM-induced pathophysiology or EE-related rescue might be overly severe and/or non-specific MAM-induced pathology. Severe MAM-induced pathology has been observed in MAM offspring with earlier injection date [e.g., MAM G15 vs G17 models (Moore et al., 2006)] and/or with increased MAM doses (Cattabeni and Luca, 1997) (see [generating the MAM model and the saline-exposed control rats](#)). Moreover, unexpected MAM-phenotype has been observed in rats that presumably undergo prepubertal shipment stress [e.g., shipping 5-week-old offspring (Howe et al., 2015)]. The MAM offspring has increased stress vulnerability during prepuberty (Zimmerman et al., 2013). Thus, we postulate that uncontrolled environmental stress from shipping could act upon the MAM-induced prepubertal stress

vulnerability, possibly leading to severe adult pathology that are too extreme to recapitulate important schizophrenia features, such as subtle limbic histopathology and dopamine dysfunction.

#### **Potential solution**

At the end of the studies, we recommend confirmatory experiments to a subset of SAL:RE and MAM:RE rats to examine the extent of MAM-induced cortical thinning. In our laboratory the cortical thinning in the MAM animals is relatively region-selective to the PFC and the vHipp at approximately 5–10% (Moore et al., 2006). Therefore, more severe cortical thinning might indicate improper control of MAM dose or timing, as well as shipping stress. In the injection phase, the experimenters should ensure the dosage to be exactly 20 mg/kg and the injection to be conducted on G17. Furthermore, we do not recommend directly ordering MAM offspring from vendors (see [generating the MAM model and the saline-exposed control rats](#)), as this often involves shipping animals in the most stress vulnerable prepuberty (P21–30).

#### **Problem 3**

MAM phenotypes observed but no benefits of EE on DA neuron electrophysiology (step 13).

If MAM effects are consistently observed in MAM:RE rats but no effect is observed in MAM:EE rats, this would directly point to failures of EE paradigms per se. Such failure of EE could be induced by uncontrolled stress especially in EE cages, which presumably counteracts the electrophysiological prevention described above. This is because the MAM-induced pathophysiology is causally linked to prepubertal stress-response (Gomes et al., 2019b; Du and Grace, 2013; Zimmerman et al., 2013), and as such increased anxiety of MAM:EE rats during these critical windows between P21–50 could potentially increase later life DA output, counteracting the benefits of EE exposure presumably via a distinct mechanism.

#### **Potential solution**

We place the EE and RE cages nearby and in the same colony room to control for husbandry conditions not directly related to the designed cage differences, such as noise or light exposure. If needed, use a luminescence and a sound meter to measure the light and noise levels in the EE boxes and compare that with the RE cages. We also recommend not putting EE cages directly under a light source. Given the large area of the EE cages, excessive light exposure can be anxiogenic especially in the center of the floor, in a way mimicking an open field-testing chamber. In our studies, MAM-EE rats had similar EPM anxiety response compared to MAM-RE rats [not shown, see (Zhu and Grace, 2021)]. However, if there is worse EPM anxiety-related behaviors from EE-exposed animals, detailed troubleshooting on above-mentioned factors should be conducted.

#### **Problem 4**

Non-reproducible effects among EE cages (step 16–18).

We recommend running two or more EE cages at a time, as this would allow the examination of inter-cage differences. Such differences could reflect inconsistent EE conditions and/or schedule of maintenance.

#### **Potential solution**

Purchase each type of EE objects in multiple sets (see [key resources table](#)) to ensure that the study has a sufficiently large roster of objects to choose from. Ensure that all the supplies in each EE cages are identical. For each session of EE cage maintenance, ensure that same cleaning procedure is applied across cages (step 16–18).

#### **Problem 5**

EE rats do not access running wheel (step 8).

In our cohorts we frequently observed the EE rats exercising on the running wheel during our daily inspection, which generally started after P25. We observed wheel running occurred more often during the dark phase. If rats were found not using the running wheels, this could lead to failure of EE.

### Potential solution

Although a previous study indicated strong mouse preference for large running wheels with plastic cover, less is known for rats ([Banjanin and Mrosovsky, 2000](#)). Nevertheless, since exercise is a core enriching element, researchers should conduct necessary pilot experiments to select the most suitable running wheel configuration based on animal's preference. Common wheel options are in upright or angled "disc" shape that might be of different colors and materials. In our experience Sprague Dawley rats prefer the plastic upright running as indicated in step 8 and [key resources table](#).

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Xiyu Zhu] ([xiz82@pitt.edu](mailto:xiz82@pitt.edu)).

#### Materials availability

All materials described in the present study are commercially available.

#### Data and code availability

The results in expected outcomes are available in a recently published article ([Zhu and Grace, 2021](#)).

### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant No. MH57440 (to A.A.G.). We thank our technicians Niki MacMurdo and Christy Smolak for the purchasing the equipment and supplies. We also thank undergraduate students Kaetlyn Conner, Madhura Leninakan, and Junhao Xu for their assistance in data analysis.

### AUTHOR CONTRIBUTIONS

A.A.G. obtained the funding. X.Z. and A.A.G. codesigned the experiments. X.Z. performed the experiments, analyzed the data, and drafted the initial manuscript. X.Z. and A.A.G. interpreted the data, and A.A.G. edited the final manuscript.

### DECLARATION OF INTERESTS

A.A.G. has received consulting fees from Alkermes, Lundbeck, Takeda, Roche, Lyra, Concert, and SynAgile, and research funding from Lundbeck. X.Z. reports no biomedical financial interests or potential conflicts of interest.

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