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Explore the impact of the use of two different odors in training of reversing intergenerational stress in mice

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Abstract. Current research has indicated that stress from relatives affects not only relatives but also their offspring, and that therefore, effective intergenerational genetics to prevent stress would be beneficial to public health, while odor-based fear adjustment provides a way to address the problem. So our research is aimed at examining the intergenerational genetic trauma and stress. We adjusted the odor fear of F0 mice before they were conceived, and we trained F0 mouse with two different odors (cumin and cinnamon). We expose mice in a particular group of experiments to a specific odor (cumin or cinnamon) and train them to associate the odor with current stimulation (conditioning). At the same time, we will also help mice to eliminate their fear of extinction in a way that shows only the smell after the conditioning training of other specific groups of mice. We then performed behavioral and neurosynthesis tests on F0 offspring of F1 mice under different conditions, with specific smell and no specific odor (two of which were used to train F0 mice), and tested the egg cells of offsprings of females. We hope to use the findings to further explain the previously undetected methods of intergenerational stress genetic reversal, further explaining how environmental information, as well as multiple environmental data, are transgenetic at the behavioral, neuro-anatomical and epigenetic levels. This will further reveal the potential for extinction training to be applied to family training to reverse the impact of family stress sources on offspring.

Keywords: Behavioral sensitivity, Neuroanatomical test, Extinction training, Olfaction, Olfactory sensory neurons, Ovum

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1. Introduction

The mouse's olfactory system is an ideal model of analyzing the transmission of genealogy pressure to offspring, which can well reveal the specific effects of this intergenerational transmission, and researchers can find appropriate ways to block this transmission by studying its epitome. Current research [1], primarily based on the initial use of mice to construct an intergenerational stress transmission system, explores the origins, genetic and even the influence of odor on conditional fear in mice, including behavioral and neuroanatomical, but currently lacks the following: will the conditional fear of mice and its transmission between generations be affected by the use of both odors and even more complex experimental conditions in extinction training?

Olfactory treatment is a fear conditioning treatment in which mice would be exposed to one odor presentation and conduct body electric shocks [2]. After training, mice would show the behavior of fear to the special odor. In the research, it was used to study the nervous system of the mice effected by the parental experiences. Extinction training is a training that reversed the effects of the structure and function of the nervous systems of the mice effected by its parents. Mice receiving olfactory training would receive electric-shock-free training and be exposed to one or two odors to extinguish its fear. In the research, it was used to study the effects to the offspring of the mice [1]. Sensitivity is the reaction of the mice that received olfactory training or extinction training after being exposed to the special odor. After training, mice would be exposed to the special odor again to test its behaviors. In the research, it was used to study the result of the training and analysis the effects brought by parental experiences. Using the above method, we can preliminarily construct the odor-related conditioning fear model of mice.

After the creation of a scary-stimulated-smell-related mouse model, we also need a more specific, macro-specific method of testing, and the results of which are modeled, the mice are indeed influenced by our experiments, and we need to further reveal through these results the quantitative extent to which the mouse is affected by the stimulus with the smell hint, and in this way to detect whether the F1 subtype shows the same stress type as the F0 gene. The above-mentioned can be determined by the behavioral analysis of experimental mice, the behavioral phenotype of the mutant mice can not only explain the genetic mechanisms of their behavior, but also macroscopically indicate the extent to which they are affected. We'll use three common behavioral analysis methods to detect [3]: improved shock reflex experiments, which show smell before frightening stimuli; and high-rise cross labyrinth experiments; and ultimately three chambers in the laboratory box—the first with an Eppendorf tube that has a particular smell, the second with an empty Eppendorf tube, and the third with a vacuum chamber where mice are free to roam [1].

Furthermore, to visualize the intergenerational effects on the F1 generation, we have opted to employ the population of olfactory sensory neurons (OSNs) stained with antibodies to discern any distinctions in the neuroanatomy between the F0 and F1 generations. The development of the olfactory system hinges on the growth of OSNs, which are widely regarded as the fundamental components of this sensory system. Scientific literature has documented that alterations in the odorant environment can indeed impact the population of OSNs expressing the mouse olfactory receptor106-1(MOR106-1),also known as OR genes S1 and mouse olfactory receptor23 (MOR23). Each OSN exclusively expresses a specific odorant receptor, thereby dictating the properties of odorant response of the OSN. Consequently, the most expansive odorant responsiveness in a mature OSN is associated with the expression of the OR gene, thereby guiding the focus of our research [4].

In addition to neuroanatomical test, we will also test eggs in mice. Methylation is an epigenetic modification that can influence gene expression without changing the underlying DNA sequence. We will examine and compare the methylation status of genes encoding cumin and cinnamon odorant receptors in the egg of F0 female mice, including F0-Trained, F0-Exposed, F0-Extinguished-1, and F0-Extinguished-2 [1].

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2. Method

In the present study, we design an olfactory treatment and extinction training experiment to model F0 genealogy mice. In the modeling process, we will use two different odors specifically applied to mice in extinctions training. Then, we'll use three different behavioral testing experiments to detect the impact of fear level and generational transmission of parental stress in F1 mice. At the same time, in order to make the role and impact of this intergenerational genetics more understandable, we have also designed experiments to detect specific OSNs in mice at the level of neurosynthesis to indicate the specific expression of relevant OR genes. Finally, at the cellular level, we design an experiment to detect F0 progenitor female egg cells, aimed at studying the degree of methylation of the genes responsible for encoding the receptors related to the odor and odor involved in the experiment.

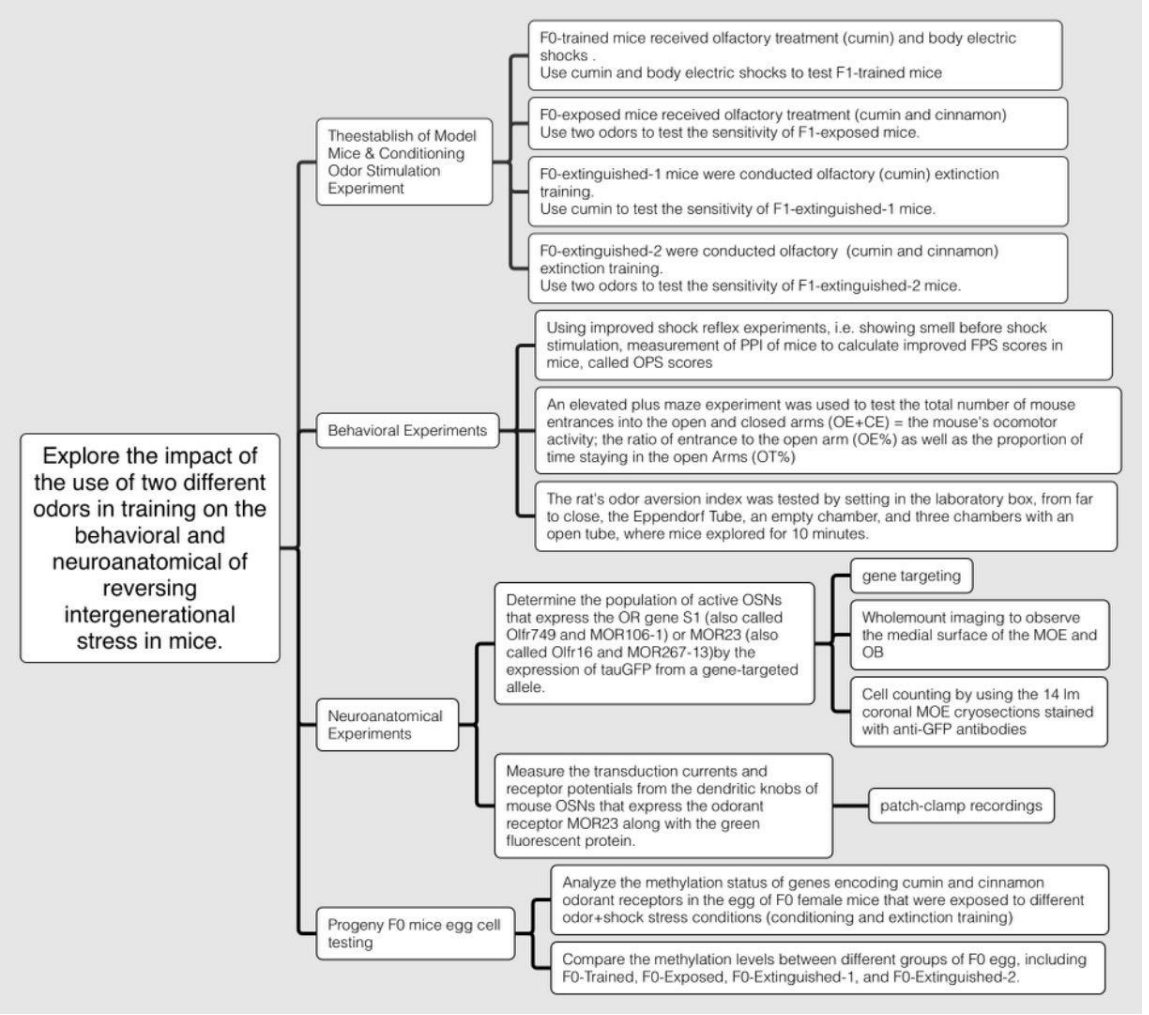

Figure 1. Technology Roadmap

2.1. The establish of Model Mice & *Conditioning Odor Stimulation Experiment*

2.1.1. Mice. The experiments are conducted by using 2-month-old female mice. F0 mice are C57B1/6J and M71-LacZ, which are kept in 129/Sv ×C57BL/6J background. F1 mice are horned by F0 mice that are mated with the same sort of F0 female mice. F1 mice are weaned after three weeks. Mice are housed in the cages that are up to scratch in which they are subject to a twelve hours light and dark cycle with limitless food and water. The maximum number of mice in one cage is five. The experiments are performed in the duration of the light cycle.

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Figure 2. The establish of model mice

2.1.2. Behaviour. All behaviour is performed in a double-blind principle. Using computer algorithms software that can monitor automatically to acquire the data.

2.1.3. The olfactory treatment of F0 mice. First, F0 mice are exposed to two odors, cumin and cinnamon. They are trained on three successive days including five time of the presentation of these two odors for ten seconds each. This group is called F0-exposed mice. Then, another batch of F0 mice wpare exposed to cumin. They are trained on three successive days including five times of the presentation of cumin odor for ten seconds and received 0.25-second 0.4-mA body electric shocks. This group is called F0-trained mice. F0-extinguished-1 mice are treated like the F0-trained mice and F0-extinguished-2 mice are treated like F0-exposed mice. They are exposed to thirty times of the presentation of the odors that they have been conditioned without body electric shocks for ten second each. The experiment method for the two groups of mice is extinction training.

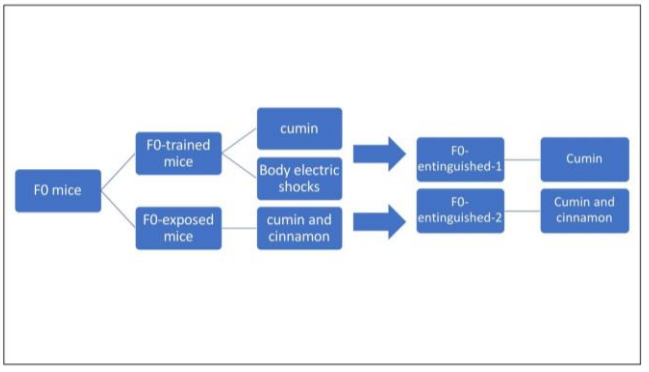

Figure 3. Specific the establish and olfactory treatment of model mice F0

2.1.4. The olfactory treatment of F1 mice. Test the F1 mice by using the identical methods of testing the F0 mice respectively to detect the sensitivity of the F1 mice to odors and the fear conditions.

2.2. Behavioral Experiments

In order to study the impact of the stress experienced by F0 rodents on F1 rodents in terms of the degree of fear of their respective smells and their frightening responses, we used three commonly used experiments to test the behavior of F1 mice. All behavioral tests were conducted using offspring of mice between 8-12 weeks of age, and in order to guarantee the double-blind experiment, the researchers responsible for testing during the experiment were blind to details such as the genetic subgroup of the test mice and their genetic epitome. Data collection for all behavioral tests is done by automated procedures without manual operation. All experimental procedures occurred between 08:00 and 20:00, under the light half cycle. Improved shock reflex experiments were first carried out, and 24 hours later

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a high-rise cross labyrinth test was conducted, and three days later, mouse exploration experiments on different chambers were conducted in the laboratory box. On the test day, the experimental object is delivered in advance to the dimly illuminated behavioural laboratory, ensuring that the behavior of the object is not interfered with by other factors for at least one hour.

2.2.1. Odor-potentiated startle. We use the odor-potentiated startle (OPS) assay mentioned earlier to measure the baseline behavioral sensitivity of F1 subgenre mice, which measures the acoustic startle response of mice when noise breaks out. Tests and training are carried out in startle chambers, where electric shock can be delivered to the floor of each room via a DC shock generator [5].

The subjects are adjusted for 5-10 minutes in the shock room on 3 separate days. On the day of the test, the mice will be first exposed to 15 Startle-alone experiments (100 milliseconds of 100 decibels of noise) and then to 10 Odor + Startle experiment, in which 10 Startle-alone experiments are randomly mixed. The Odor + Startle trial includes 10 seconds of odor presentation and 100 milliseconds of 100 decibels of noise eruption. For each mouse, the Odor-Potentiated Startle (OPS) score can be calculated by subtracting the first Odor + Startle response from the last Startle-alone response in the experiment, which is subtracted by the alarm response in the final Startle-alone response and multiplied by 100 to obtain the OPS percentage score (%OPS) [6].

2.2.2. Elevated plus maze experiment. The elevated-plus maze is performed with a 12 x 2 inch arms in the dark light (50 lux), which consists of a high-rise platform with two closed arms with walls and two open arms without walls connected by an open center. The mouse is placed onto the center between the cross maze arms, and are allowed to explore freely for ten minutes. Record the total number of mice entering open and closed arms (OE+CE), representing the mouse's ocomotor activity. Then calculate the ratio of mice entering the open arm (OE%) and the time they stay in the open Arm (OT%). The time and distance spent on the open and closed arms is measured using Noldus Information Technology as a measure of the level of anxiety of mice [7].

2.2.3. Odor sensitivity. Mice were placed in a three-room box and allowed to explore for 10 minutes between all three rooms. The specified concentration of the odor (either cumin or cinnamon) used for training F0 progenitor mice is placed in one of the rooms, the second room is empty, and the third one has an empty Eppendorf tube. Record association time of mice in an empty room or an odor room. The aversion index of disgust is calculated by deducting the time spent in the smell room from the time used in the open room. Independent mice were used in the cumin and cinnamon experiments (F1-cumin, n = 15; F1-cinnamon, n = 15).

2.3. Neuroanatomical Experiments

To investigate the repercussions of intergenerational stress experienced by the F0 generation (parental) on the olfactory sensory neurons (OSNs) of the F1 generation (offspring), we employed the cryosectioning method to label the subcellular location of proteins, specifically the green fluorescent protein (GFP) fused with OSNs expressing olfactory receptor genes, identified by morphological criteria. To visualize the GFP's location within the OSNs, we employed wholemount imaging, providing ultrastructural insights into OSNs in a MOR23-13-IRES-tauGFP gene-targeted mouse strain and the MOR106-1-IRES-tauGFP gene-targeted mouse strain mentioned previously.

2.3.1. *Gene-targeting.* The inducible inactivation of a target gene in mice was accomplished through a gene-targeting approach, which can be reversed by controlling the expression of Cre recombinase. To elucidate further, Cre can excise a DNA polymerase gene segment flanked by loxP recombinase recognition sites [8]. Notably, all these procedures are conducted during the embryonic stage. In this study, MOR23 and MOR106-1 neurons express GFP under the control of the MOR23 and MOR106-1 odorant receptor promoters, respectively. Consequently, coding sequence of the mouse has the coding sequences of MOR106-1 and MOR23.These sequences were employed to screen a mouse genomic

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library, leading to the generation of two 10.6 kb targeting vectors. Additionally, PacI sites were engineered at the S1 and MOR23 stop codons by introducing 3 nucleotides. Furthermore, an internal ribosome entry site (IRES) will be encoded by a cassette (IRES-tauGFP-ACNF).A self-excising neo-selectable marker in parallel with a tauGFP fusion protein were inserted into these PacI sites of each OR gene [9].

2.3.2. Odor Cryosectioning. The cryosectioning process entails six key steps, commencing with tissue embedding preparation and culminating in GFP visualization. Chemical fixation involving formaldehyde (FA) and/or glutaraldehyde (GA) is essential to preserve the ultrastructure of olfactory sensory neurons in fixed tissue. Following fixation, the tissue is rapidly frozen within embedding materials and sectioned under freezing conditions, facilitated by a cryostat, with the assistance of an internal microtome containing a large refrigerated chamber. Subsequently, the tissue is mounted between a slide and a coverslip, primed for observation via the immunofluorescence method [10]. The cryosectioning process entails six key steps, commencing with tissue embedding preparation and culminating in GFP visualization.

1) Embed tissue for preparation:

   a) The mouse olfactory epithelium is extracted and has been fixed by using fixative buffer which contained 4 % paraformaldehyde solution in 1× phosphate buffered saline (PBS), pH 7.4.

   b) Then the material will be rinse with 0.5 M EDTA overnight [4].

   c) The tissue should be placed and cryoprotected in 30% sucrose solution for 12h at 4 ℃.

   d) Remove the tissue and rinse and washed by excess sucrose with ddH2O.

   e) Submerge the tissue completely from the sucrose solution to an embedding mold contained O.C.T compound, followed by placing the entire mold into the dry ice for freezing the block and should be undo the cryosection immediately.

2) Prepare cryostat and section:

   a) Place the sample from the dry ice into the cryostat chamber, in parallel with sterilizing organize materials included slides, cryostat chuck and paint brush at around -20 ℃ for 15min.

   b) A cold razor blade is used for removing the molding from the sample.

   c) A small amount of room temperature O.C.T. at the cold cryostat chuck is required for freezing the sample to secure the sample on the cryostat chunk.

3) Cryostat sectioning:

   a) Place the cryostat chuck onto the sterilized microtome and angle the specimen for better observation

   b) Cut the sections in 12micrometer thick for each slide.

   c) Retrieving the section under the security of the block of the crank.

   d) incubation with the following primary antibodies: MOR23, GFP and olfactory marker protein (OMP) in 2% normal donkey serum overnight at 4 ℃.

   e) wash the sections in 0.1% Triton in phosphate-buffered saline.

   f) incubation the sections with primary antibody: fluorescein-conjugated donkey anti-chicken and Cy5-conjugated donkey anti-goat [11].

4) Thin sections are collected on a slide:

   a) Retrieve the sections onto the surface of a cold slide and the area of the place where the sections at by a finger.

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b) Keep storage of the slide in-80 ℃ and air-dried the slide at room temperature overnight.

c) Mount the on the slide by Vectashield containing method and cover the cover slide [11].

5) Thick Free-Floating cells are collected and mounted:

a) Prepare multi-welled plates filled with the 25 % glycerin/30 % ethylene glycol solution.

b) Covered and stored the sections at -20 ℃ after removal the O.C.T. compound and cryoprotectant from the tissues.

c) Prepare a 100mm Petri dish and filled with PBS. Place the slide on the Petri shish and followed by sections

d) Draw off PBS slowly using a 5 or 10 mL pipette into the Petri dish contained the slide which has sections on it. Excess PBS will be added using pipettes with increasingly smaller sizes (P1000, then P100).

e) Dry excess PBS from around sections with a dry paintbrush.

f) Apply DAKO mounting medium and coverslip the slide.

2.3.3. Whole-amount imaging and cell counting. The alteration in the quantity of the olfactory sensory neurons in both F0 and F1 generation is required for investigation, to pursue this aim, whole-amount imaging is used for capturing the pictures of the tissue to improve the accuracy of the cell counting. GFP-containing neurons were excited using a BP460-490 filter with light from a mercury HBO 100W Olympus lamp. Whole mounts from dissected tissue were prepared sagittally for the observation of the medial surface of the mouse olfactory epithelium (MOE) and olfactory bulb (OB). GFP fluorescence staining the OSNs was visualized using a GFP filter, while DAPI staining was also observed using an Olympus BX51WI microscope coupled with an Olympus DP72 camera [11]. Detailed images encompassing the mouse olfactory epithelium were captured to facilitate the calculation of the ratio of neurons contained GFP to the total olfactory sensory epithelium surface. The cell bodies of OSN expressed S1 and MOR23 were enumerated from coronal MOE cryosections stained with anti-GFP antibodies, using a confocal microscope [4].

2.4. Progeny F0 mice egg cell testing

DNA methylation refers to a type of chemical modification of DNA that adds a methyl group to the cytosine residue of CpG dinucleotide. To gain further insights into how gene expression is regulated and the way it is affected by environmental elements like stress, DNA methylation in the egg of F0 female mice of each group is tested using Methylated DNA Immunoprecipitation. First, fragmented DNA is incubated with an antibody specific to 5-methylcytosine (5mC). The antibody-DNA complexes are captured and then the methylated DNA fragments are eluted from the antibody. Finally the enriched methylated DNA is analyzed and compared utilizing DNA sequencing.

3. Discussion

Smell exposure and deprivation experiments are important environmental experiences affecting gene expression related to odor in mice and their physiology and behavior. Experiments on F0 mice involve the presentation of odors that lasted for three days, and previous studies have not been able to confirm whether the use of odor A in mice and the extinction training of mice with odor B has a corresponding effect on the behavioral and neuroanatomical of the offspring of F1 mice.

In experiments with F0 mice, they are exposed to the odors and body electric shocks simultaneously. It is expected that the experimental results will reveal that F1 mice showed their sensitivity of fear to the odors as a result. For the F0 mice receiving extinction training, the experiments are expected to show that their fears of the odors are allayed.

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In behavioral testing, we aimed primarily at observing the sensitivity and detestability of F1 mice to odors, as well as the frightening reaction of F1, when stimulated by a specific odor (two of the odors used for training F0 mice). We reasonably predict that offspring of F1 mice who were given odor-electric shock pair stimulation during training would show a greater frightening reaction in behavioral testing than in the absence of odor. At the same time, extinction training of F0 mice that had previously undergone odor fear adjustment reversed the behavioral and neuro-anatomical transmission of the fear effect in their offspring of F1 mice.

And with regard to neuroanatomical testing, the methodologies delineated in the preceding sections are ideal for exploring the neuroanatomical disparities between two generations of mice subjected to gene-targeting via cryosection immunolabeling. By comparing the quantities of MOR23 and MOR106-1 OSNs, we postulate that intergenerational stress experienced by the parental generation can impact their offspring. This is of substantial relevance in the realm of pathology, as it may shed light on the inheritance of mental disorders from one generation to the next. However, it is worth noting that our study focused on the olfactory sensory neurons in mice rather than neurons in the mouse brain. Consequently, this paves the way for our next research phase, wherein we will redirect our attention towards the neuroanatomy of mouse brain neurons to investigate whether intergenerational stress can be transmitted from the parental generation to the succeeding one.

DNA methylation of genes that are encoding odorant receptors in F0 female mice egg was analyzed to help attain more insights into the influences of epigenetic marks on the reversibility of transgenerational impacts of stress. It is expected that the DNA methylation level of the trained would be higher than the exposed but lower than the extinguished.

In summary, the core of our research programme is to explore the difference between the use of two different types of odors in two categories of environmental events with conditioning and extinction training to train F0 mice, based on previous studies, to find the two odour stimuli being applied simultaneously to the parent generation as an intervention and to intervene using only one odour stimulation. The findings are expected to be applied to better utilizing genetic interventions to reverse intergenerational stress.

4. Conclusion

For F0 mice, two groups of these mice are trained with two different odors respectively and two groups of mice receive extinction training respectively. The steps are identical to train F1 mice.

In behavioral testing, in order to detect whether the behavioral sensitivity of the F0 mice and its offspring of F1 mice changes and possible causes, as well as to test whether the use of two different odors in training actually reverses the intergenerational generation pressure transmitted by F0 to the F1, we trained F0 mice with two different smells, and also applied these two odors to the behavior testing of F1. The study aims to quantify the stress transmission of two generations of mice and to demonstrate more clearly and intuitively the stress levels inherited by the offspring (the behavioral characteristics of the F1 mice and its degree of aversion to the corresponding odors).

Our investigation also employed multi-faceted approaches that included gene-targeting techniques, cryosectioning, immunofluorescence, and whole-mount imaging to explore the intergenerational impact of stress on olfactory sensory neurons (OSNs)expressing the MOR106-1 and MOR23 odorant receptor genes. The research highlights the utility of gene-targeting methods for manipulating gene expression in mice by inducible inactivation and Cre recombinase, providing valuable insights into the genetic regulation of OSNs. Moreover, the tissue preparation and sectioning done in the cryosectioning process, conjugated with immunofluorescence staining allowed visualization of the distribution of GFP within OSNs expressing MOR106-1 and MOR23 from the molecular level. Additionally, the study employed whole-mount imaging to capture comprehensive images of the mouse olfactory epithelium and olfactory bulb, facilitating the accurate quantification of OSNs expressing GFP.

To learn more about the epigenetic changes that may be involved in the intergenerational impacts of stress and effects of extinction training, we tested and compared the level of DNA methylation of genes

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which encode cumin and cinnamon odorant receptors in F0 female mice egg of each group, since previous studies only examined in F0 sperm.

Overall, our research is aimed at applying more accurate, intuitive experimental design and data processing methods, on the basis of previous research, to further explore potential genetic factors that can influence intergenerational stress, and from the point of view of behavioral and neuroanatomical to study in depth how these influencers work.

Acknowledgments

Fangjie Zhu, Jiani Gu, Lang Lin, and Weitong Zhang contributed equally to this work and should be considered co-first authors.

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