Transgenerational Memory and piRNAs

Zijing Liang

The Hun School of Princeton, Princeton, New Jersey 08540, the United States, cicizijingliang@gmail.com

ABSTRACT

Previous studies suggest that memory inheritance is observed on some mice progenies with parents who have experienced fearful memory. Further investigation also shows that there are increments of piwi-RNA, which shows to have effects in enhancing the progenies' ability to avoid harmful bacteria, in both the nervous system and the sperm cells of these parents. In this work, the relationship between the presence of harm-signaling odors and the increments piRNA's presence in olfactory bulb and sperm cells is investigated. Meanwhile, this work also examines the effect of piRNA's increment in parent generation on progenies' enhancing cognitive ability in distinguishing the harm-signaling odors. The results of this study could help establish the correlation between the change in piRNA abundance in response to fearful stimuli and the inheritance of this memory.

Keywords: *piwi-RNA, transgenerational memory, olfactory bulb, fear conditioning, M71*

1. INTRODUCTION

When an odor is sensed by an organism like a mouse, this specific odor is first detected by its unique sensory neuron located in the olfactory epithelium (OE); it is then passed on to the glomeruli in the olfactory bulb (OB). If this odor is paired with emotions like fear, the odorant stimulus is then transferred to other brain regions like the hippocampus and amygdala to initiate memory production and learning [1]. This type of learning can be considered fear conditioning (FC). Since fear-conditioned memory is acquired and does not involve any change of one's DNA sequence, it is generally thought that the memory would not pass on to offsprings or change behaviors as in their parents. However, a recent study reveals that this assumption might not be valid. Similar to odor, pathogenic bacteria *Pseudomonas aeruginosa* (PA14) is presented to *C.elegans* in the study. Due to the harm caused by PA14, *C.elegans* soon learns to avoid the pathogenic bacteria. And, most surprisingly, this avoidance behavior is also found in its progeny up to four generations, providing progeny protection for better survival. Further investigation also finds that this inheritance of memory is likely to relate to a type of small RNA called piRNA, which shows increased abundance when *C.elegans* is exposed to PA14 and produce memory-inherited offspring [2].

In another study focusing on memory inheritance, the abundance of another type of small RNA called miRNA shows a similar increment in both nervous system and sperm cells of the mice that produce offsprings with enhanced cognitive abilities through a learning task called environment enrichment [3]. While this study is related to miRNA, our study focuses on piRNA, due to the difference in the experiment setting of fear conditioning and avoidance behavior. Meanwhile, as shown by the studies demonstrating the function of piRNA, piRNA participates in memory production by binding with a protein called CREB2, which controls the memory-related synaptic plasticity [4]. Therefore, if there is an increase of piRNA abundance in both the FC-related neuronal circuit in the nervous system, which is related to the production of memory, and sperm cells, which are connected to offspring's inheritance from parent generation, it is reasonable to infer that piRNA acts an essential role in the transgenerational memory.

However, it is unlikely that piRNA discovered in sperm cells is produced in this same site, so a question has been raised: where does the piRNA in the sperm cells come from? Based on the background information, if piRNA in sperm cells carries the memory to the progeny, there is a significant chance that this increased piRNA comes from the brain where memory is produced initially. Additionally, since the olfactory bulb is the first site in the brain where odors are processed, research should be conducted first on this part of the FC circuit, which we choose as the method to produce memory for examination. Thus, a research question is established by our team: Can the piRNA associated with fear-conditioning neuronal circuit in the brain migrate into their sperm cells? Building on the question, we

hypothesize that if the specific piRNA associated with the fear-conditioning neuronal circuit changes in abundance in the olfactory bulb, the sperm cells would be able to get these piRNA migrated from the brain region and thus inherit the memory of the odor used in the odor fear conditioning.

2. EXPERIMENTAL APPROACH

2.1 Odor Fear Conditioning in Mice

Adult male transgenic mice of a strain named M71-GFP are used in this experiment. M71 is the odor receptor of an odor called acetophenone. The corresponding neurons in the OB of these mice will become fluorescent once they detect acetophenone. By using this type of mice, we can recognize the neurons related to acetophenone, which we use for odor fear conditioning, by comparing the neurons before and after FC. These mice are all placed in a 12-hour light/dark cycle with free access to food and water before the experiment [5].

Fear conditioning is conducted through pairing electric shocks with odors. Three groups, each having 6 mice, are set for this experiment. For mice in the first group, the experiment group, acetophenone (10% acetophenone in propylene glycol) paired with 0.25s, 0.4 mA electric shock is present. Besides the experiment group, the second and third groups are set as control groups. For the second group, acetophenone, which alone doesn't cause harm to the mice, is presented; for the third group, propanol, an unrelated odor that would not trigger a change in M71 neurons, paired with electric shock is presented. Discrimination tests, during which only acetophonone will be present, are also conducted on the mice of all three groups to observe whether the mice establish fear-related behaviors like avoidance or freezing.

Two possible results might be seen after the experiment. First, if the mice in the experiment group display fear-related behaviors while mice in the other two groups don't, the odor fear conditioning can be considered successful so the following experiment can be processed. Second, if the situation above doesn't occur, the experiment might undergo errors, so a new round should be conducted until the first result appears.

2.2 Sequencing and Identification of piRNA

After the fear conditioning experiment is successfully conducted, it can locate the specific glomeruli connecting with the neurons in the circuit. Since piRNA generally increases to form piRNA clusters, the glomeruli need to be isolated from the neurons in order to obtain the increased piRNA clusters. After taking out the piRNA found on the FC related neurons, RNA sequencing should be applied. With RNA sequencing, a certain type of piRNA, which is referred to as piRNA-X in this study, would show the most significant increase in abundance. Afterwards, RNA sequencing is also to ensure the piRNA-X we identify is indeed a type of piRNA but no other types of small RNA since this technology can show the size of the isolated piRNA, which we can use to compare with other known piRNA strands.

A possible result of this experiment is that piRNA-X shows an increase compared to the baseline mice (mice that do not undergo FC) after exposure to acetophenone fear conditioning. If this result takes place, it is reasonable to infer that the increase of piRNA in the olfactory bulb is related to the formation of fear conditioning memory. There is a big chance that this result would occur based on the conclusion of the previous study conducted on another organism called *Aplysia*, where memory formation is caused by the increase in piRNA abundance [4]. However, there are still potential limitations to this experiment because multiple piRNAs could all show an increase in abundance. Still, we can solve this by doing the following experiment on the strand that offers the most significant increase or conduct multiple trials on all the strands. Overall, if a rise in piRNA is observed during this step, the relationship between piRNA and memory formation in mice can be supported, so this step is considered as successful.

2.3 Determination of piRNA

Following the previous step, a further examination of the relationship between piRNA and memory production is also needed. We can achieve this goal by conditionally removing piRNA-X in the olfactory bulb to see if it is required in memory formation. CRE recombinase is used here to conditionally knock out the gene that encodes for piwi proteins working with the piRNA in OB. After this step, similar fear conditioning tests and discrimination tests are then conducted.

According to our expectation, if, after the inhibition of piRNA-X, the mice are not able to form a memory of the odor FC test, the piRNA-X identified can be considered as the correct piRNA relating to memory formation. If the mice still form new memory without piRNA-X, this step should be repeated with the improved procedure, or other strands of piRNA should be tested similarly.

2.4 Creation of Mutant piRNA

After confirming that piRNA is the correct one that participated in the memory production, we now need to add a "tag" onto the piRNA so we can trace whether the piRNA found later in sperm cells indeed originated from the OB. The CRE loxP system is utilized in this study to create a minor change, a change that would not disturb the original function, in piRNA-X. This change is accomplished by introducing a virus vector to a group of

mice embryos. The virus would add a CRE loxP system and the mutated piRNA-X gene into the existing DNA sequence. Using the CRE enzyme in the OB region of the mice, the gene that will code for piRNA-X containing one mutated nucleotide will be activated and produce many piRNA-X with tags. This procedure of changing only one nucleotide of piRNA-X would not alter its original function but only add a tag on it, so if later this mutated piRNA is found in sperms, it must come from OB.

Nevertheless, this method still has many limitations. Most importantly, since piRNA has a relatively small size with generally 26-28 nucleotides, alteration of even one nucleotide may still cause a significant change to the entire function of piRNA-X and the normal function of the mice. In order to solve this problem, two additional steps are also made. Firstly, after the virus vector and loxP system introduces a mutated sequence into the mice's DNA, the wild type piRNA-X gene is kept ensuring the normal living of the mice. Secondly, in order to examine whether the mutated piRNA-X is still capable of the same function, immunoprecipitation (IP), which can check if piRNA can still bind with piwi proteins as before by using antisense and a technique called RT-PCR, is used. If all the steps aforementioned are passed, it can be considered that a tagged piRNA is created successfully [6].

2.5 RT-PCR Test

Since the previous steps have finished the investigation of piRNA-X in the olfactory bulb, an experiment focusing on the sperm cells is then needed to finally confirm the migration of piRNA-X from the brain all the way to the germline. An RT-PCR test can be used for this step to examine whether the mutated piRNA-X indeed appears in the sperms. An RT-PCR test uses reverse transcriptase to transfer piRNA, which cannot be directly tested in a PCR machine, into cDNA, and the test will then make multiple copies of the cDNA that indicates the presence of piRNA. However, since the amount of piRNA is minimal, there is a small chance of giving false results so multiple trials are needed to enhance the accuracy of this experiment.

Two possible results will yield from this experiment. Ideally, cDNA of mutated piRNA-X can be discovered in sperm cells using the RT-PCR test. If this result appears, the overall hypothesis of this study will fail to be rejected: piRNA found increasing in the sperm cells originates in the olfactory bulb. If cDNA of mutated piRNA-X is not found in germline cells, the hypothesis is then rejected, by which piRNA did not migrate from the olfactory bulb.

2.6 Odor Fear Conditioning in Offspring

Besides using techniques like IP to ensure if the function of piRNA-X is not disturbed by the mutation, a check of the mice's offspring generation is also needed to see if the inheritance of memory still occurs. A similar procedure mentioned in the first part of the experiment is applied to this step. Three groups are planned for this experiment: the first group is the experimental group consisted of offspring with parents who have modified piRNA-X and undergo fear conditioning; second group, the first control group, consists of fear-conditioned, unmodified parents' offspring; the third group, the second control group, are offsprings of parents with normal piRNA-X and don't experience FC.

If this experiment is conducted as expected the mice of the F1 generation from the experimental group will exhibit fear-related behaviors in response to the presence of acetophenone while electric shock is not presented to them, and this experiment can be proven to be successful. On the other hand, if F1 mice do not display this behavior, the experiment needs to be repeated with adjustion. Since we intend to make mutant piRNA-X that function the same as wild-type piRNA-X while keeping many normal piRNA-X to ensure the normal living of the mice, this step can verify that the mutant piRNA functions correctly, and the maintained normal ones are also normally functioning.

3. CONCLUSION

Overall, the experimental approach can be divided into three main parts: identification of piRNA-X that is related to the formation of fear conditioning memory in OB region, minor mutation of piRNA-X serves to add a tag for tracing, and confirmation of the presence of mutated piRNA to determine whether migration takes place. If all three parts consisted of the steps mentioned in the previous section yield expected results, the hypothesis that piRNA, potentially carrying the memory information for transgenerational inheritance, increased in germline cells is migrated from the olfactory bulb in mice is supported. This result will be significant for determining piRNA's role in transgenerational memory since it appears in both the sites of memory production and inheritance.

Additionally, if the piRNA indeed migrates from the olfactory bulb to the sperms, many further questions are raised: How did piRNA pass on to the offsprings and affect their memories? Do other brain regions like the hippocampus and amygdala within the FC circuit also contribute to the migration? What is the pathway of this migration? What carries the piRNA from the brain to the gametes? Some possible answers to the question about the carrier and pathway include exosomes and piwi protein, which will not be easily diluted on the way from the brain to the germline as the tiny piRNA we are targeting in the study is [7].

REFERENCES

[1] Jones, S. V., Choi, Dennis C., Davis, Michael., Ressler, Kerry J. (2008) Learning-Dependent Structural Plasticity In The Adult Olfactory Pathway. Journal Of Neuroscience, vol 28, no. 49: 13106-13111.

[2] Moore, Rebecca S., Kaletsky, Rachel., Murphy, Coleen T. (2019) Piwi/PRG-1 Argonaute And TGF-B Mediate Transgenerational Learned Pathogenic Avoidance. Cell, vol 177, no. 7: 1827-1841.

[3] Benito, Eva., Kerimoglu, Cemil., Ramachandran, Binu., ..., Edbauer, Dieter., Dean, Camin., Fischer, André. (2018) RNA-Dependent Intergenerational Inheritance Of Enhanced Synaptic Plasticity After Environmental Enrichment. Cell Reports 23: 546-554.

[4] Rajasethupathy, Priyamvada., Antonov, Igor., Sheridan, Robert., Frey, Sebastian., Sander, Chris., Tuschl, Thomas., Kandel, Eric R. (2012) A Role For Neuronal Pirnas In The Epigenetic Control Of Memory-Related Synaptic Plasticity. Cell, vol 149, no. 3: 693-707.

[5] Dias, Brian G, Ressler, Kerry J. (2013) Parental Olfactory Experience Influences Behavior And Neural Structure In Subsequent Generations. Nature Neuroscience, vol 17, no. 1: 89-96.

[6] Poria, D., Ray, P. (2017) RNA-protein UV-crosslinking Assay. BIO-PROTOCOL, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5450928/[7] Mosher, Rebecca A. (2021) Small Rnas On The Move In Male Germ Cells. Science, vol 373, no.6550: 26-27.