Multigenerational Effect of piRNA on Neuronal Structure Change of Mice Induced by Odor-fear Conditioning

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ABSTRACT

This paper focuses on the biological relationship between piRNA and transgenerational memory in mice, and in it describes an experiment that can determine the existence of such relationship or not. piRNA is a type of non-coding RNA that is recently found and has potential importance in the field of epigenetics, especially transgenerational memory. The presence of such memory is usually checked with odor-fear testing for generations of mice. In animal body, it is discovered that piRNA needs the accompaniment of PIWI protein to be produced or to carry out its normal function. Therefore, in the experiment, we use loxP and CRE protein to eliminate the PIWI protein, which is essential for piRNA production, from all cells. The mice with no piRNA interbreed, and this paper looks at the mice's response in the second generation to odor-fear test as a dependent variable. If the response changes when piRNA is eliminated, that means piRNA is related to transgenerational memory, at least with the genes that are important to odor-fear testing. If such hypothesis is proved, future research can look at the detailed mechanism of how piRNA works to alter epigenetic information.

Keywords: *piRNA, transgenerational memory, epigenetics*

1.INTRODUCTION

Transgenerational memory is a relatively new area to study, there are only several related experiments carried out on C.elegans, mice and aplysia [1-3]. The researchers commonly propose the hypothesis that non-coding RNAs are responsible for the formation or passing of transgenerational memory [4]. Non-coding RNAs include tRNA, rRNA, miRNA, lncRNA, siRNA, dsRNA, piRNA and several kinds of nuclear RNA that can be found in the body but do not code the genetic information [5]. We are particularly interested in the function of piRNA and its significance in transgeneration inheritance. piRNA, which in full is PIWI-interacting RNA, forms a complex with PIWI protein and usually perform its function in germline cells and somatic cells [5].

Our motivation to study piRNA comes from an idea concluded from several research papers on epigenetics. The first paper is Dias and Ressler's essay on transgenerational neuronal change in mice induced by odor-fear experiment [2]. This paper describes an interesting phenomenon that worth studying. The researchers find out, when they condition wild-type mice to be afraid of odor, the direct offspring of mice also appears fear for the odor. They exam the neuronal structure of the F1 progeny mice, and observe an increase in the glomerular area, which contributes to the increase of sensitivity. They also observe a hypermethylation in the genome, which removes the methylation marks and makes the gene easier to be transcribed, but they did not use non-coding RNAs to explain this phenomenon. This makes us curious about the mechanism and regulation of transgenerational memory. Moreover, in Rajasethupathy and Kandel's paper [3], the researchers find that piRNA can methylate the genome. In their experiment, they prove that piRNA can methylate the promotor of CREB2 gene in aplysia cell. CREB2 expression reduces because of this methylation. This paper gives us the inspiration to suspect piRNA has some connection with methylation, specifically, it may be the direct cause, or a step that can serve as an indicator of hypermethylation in mice's CREB2 gene. Finally, in Moore's paper studying RNA [1], we observe several similarities that strengthened our suspicion. In Moore's paper, the researchers let parent C.elegans eat pathogenic bacteria, and find out that the offsprings of these C.elegans will avoid the specific kind

of pathogenic bacteria. We think this is related to the odor-fear conditioned mice because both animals show the following similarities: 1. Both phenotypes are transgenerational. 2. Both are odor-specific or pathogen-specific. 3. The heterozygote offsprings (conditioned father x unconditioned mother, or unconditioned father x conditioned mother) also show the phenotype.

Therefore, after looking at this research, we become interested in studying piRNA. We aim to test the significance of piRNA in mice, to see if mice is similar to C.elegans in Moore's paper, that forms a transgenerational memory connected with fear of a specific organism of object [1]. In this way, we designed an experiment, using odor-fear test described in Dias and Ressler's essay to exam whether is piRNA required for the transgenerational memory to form, pass on to homozygous offspring and heterozygous offspring, and function similarly in them as in their conditioned parents [2].

2.EXPERIMENT

We designed an experiment on mice to figure whether piRNA is required for transgenerational memory in mice. The mice will be conditioned in an odor-fear experiment, which teaches them a specific odor will be related with electric foot shock. Ideally, such electric shock will induce fear in an unconditioned mouse. After mice have learnt that electric shock will occur with a specific odor, they will show fear by spending less time in room with that specific odor.

The accuracy and reliability of this experiment has been improved by adopting Dias and Ressler's method [2]. Initially, scientists look for "startle" reaction in mice when treated with one specific odor [6]. However, such qualitative observation may easily ignore small startle reactions or mistreat other reactions as startle reactions due to human errors, such as anticipation or tiredness. On the other hand, by measuring the time mice spent in room with or without the specific odor and calculating an aversive fear index, we are able to collect quantitative data rather than qualitative ones, reducing human errors and supporting our conclusion to be more convincible.

To ensure the mice in the experiment are physically fit and can show expected response to odor-fear testing, all selected mice must first go through a series of pre-trials to be qualified. Moreover, in order to enhance the memory of odor-fear testing, repeated trials are required.

2.1 Selection of mice

We use adult mice (14 weeks old) in this experiment. The mice should live in cages with 12h light/dark cycle, with water and food available.

In order to make the results more reliable, the parent mice and offsprings must be separated. This is because the female mice will normally perform mother-caring behavior, but in this experiment, we need to avoid environmental perturbation to ensure fair testing [2].

2.2 Pre-trials

In the experiment, we are going to knock out the PIWI protein and the piRNAs in mice. However, PIWI-piRNA complex serves an important job in repressing the activity of transposons [5]. Therefore, the mice with no PIWI protein or piRNA may lost the fertility. Furthermore, eliminating the PIWI protein and piRNA in brain may cause the mice to be incapable of learning. Based on this possibility, the learning ability also needs to be tested before the actual experiment.

To select the mice that have proper fertility and learning ability, we have to go through a series of pre-trials (figure 1).

In first pre-trial, we use prg-1 mutant mice which cannot produce PIWI-piRNA complex in all cells. We will test their fertility and learning ability.

In the second pre-trial, we will use loxP technology to eliminate only the piRNA in the brain. One of the parent mice must carry two loxP sites on its chromosome, and the sites are on either side of the piRNA cluster [3]. Another parent must carry a CRE recombinase gene on the corresponding area, and a brain specific promotor near it. (Figure 2) The cross-over of two chromosomes will result in offspring mice with no piRNA cluster expressed.

The third pre-trial also uses loxP technology to knock out the piRNA cluster in the germline cells, inhibiting the expression of piRNA in germline. Again, we will test the fertility and learning ability of these mice after the third pre-trial.

Only the mice that have both fertility and learning ability are qualified for experiment. For convenience, we will call the qualified mice "F0 mice" in subsequent paragraphs.

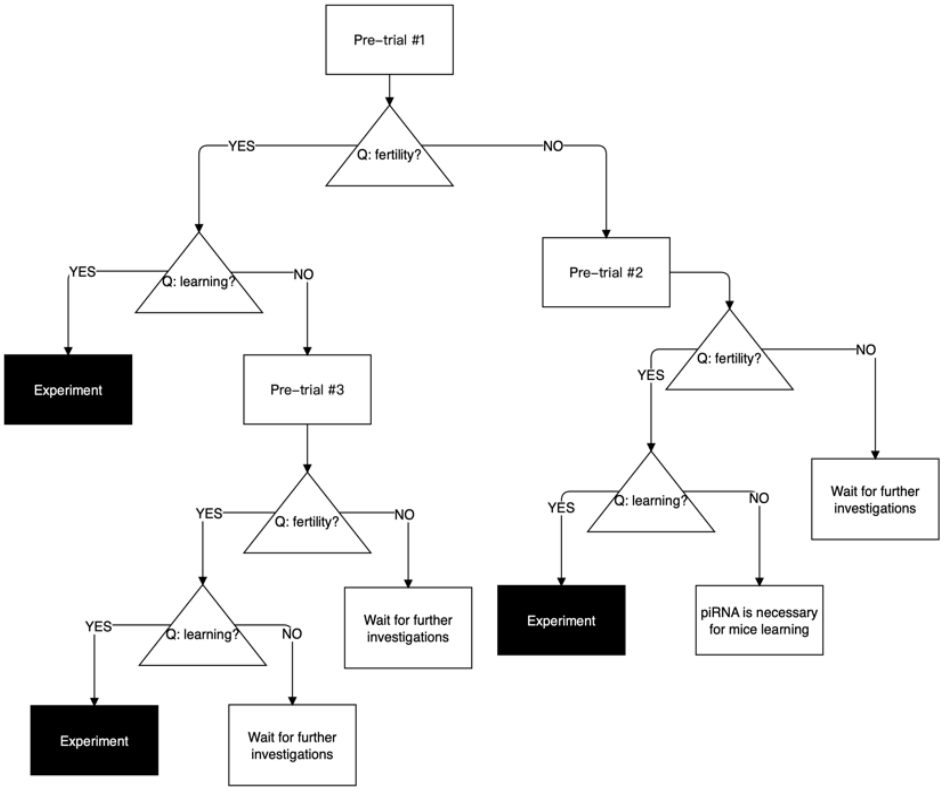

Figure 1. The flow diagram of pre-trials. The aim of pre-trials is to select the mice that has both fertility and learning ability, thus they can produce offspring and respond to odor-fear testing. "Wait for further investigations" means we are unable to explain the phenomenon with existing studies on piRNA. "Experiment" means the tested mice have passed the pre-trials; they can go through the actual experiment.

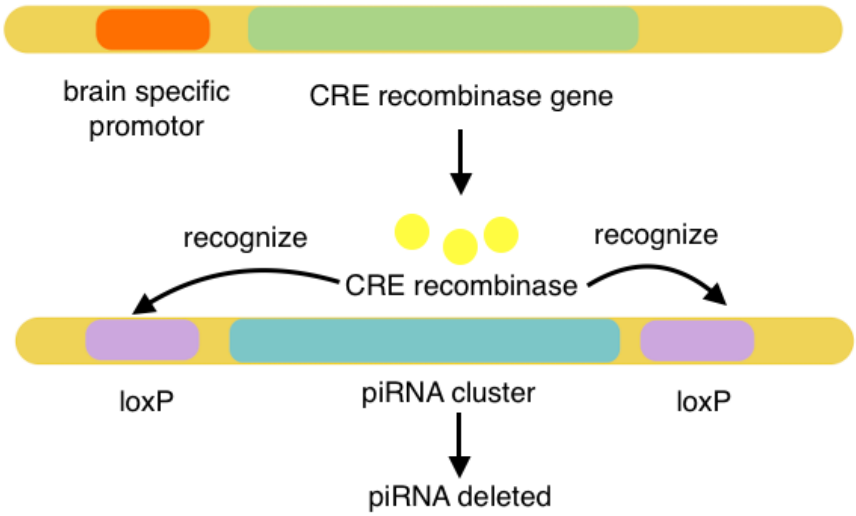

Figure 2. The images briefly review the nature of loxP technology used in piRNA deletion. Primarily, we perform genome screening and found two loxP sites that contain the piRNA cluster between them. Secondly, we add artificial strands of CRE recombinase gene (shown above) into the cell. The CRE recombinase genes will go through transcription and translation inside the cell, producing CRE recombinase. When we want to delete piRNA only in the brain, we will add a brain-specific promotor before the CRE recombinase gene; if we want to delete piRNA only in the germline, we will add a germ-line specific promotor. In this way, we can regulate the deletion of piRNA precisely. The CRE recombinase being produced recognizes the two loxP sites and delete the DNA strand between them. Therefore, the piRNA strand is deleted, and no piRNA can be produced.

2.3 Odor-fear conditioning for F0 mice

The mice need to have 2 sessions of odor-fear conditioning per week for 3 consecutive weeks, and with 5 trials per session. In each section, the odor acetophenone will be released into the mice's cage, and the mice will receive a mild foot shock [2].

2.4 Odor-fear test

The mice need to be placed in a three-room chamber, and they will be allowed 10 minutes to observe the environment. In the left chamber, there will be a tube containing acetophenone. The middle chamber will be empty. The right chamber will contain an empty tube.

The researchers need to observe mice activity and record the time they spent in room with odor and room without odor. An aversive fear index can reveal the sensitivity of mice, higher index shows less sensitivity.

The location of the mice will be defined by where its front paws are. If front paws are in different chambers, record according to the paw that is nearer to the mice's main body.

Aversive fear index [2]: time spent in odor room (s) - time spent in open room (s)

2.5 Control groups and experimental groups

The negative control group: offspring of wild-type naive mice, which means they are unconditioned, and their parental mice are also unconditioned. The negative control group will not show fear in the odor-fear test.

The positive control group: offspring of wild-type conditioned mice. The offspring themselves are also conditioned. The positive control group will show fear in the odor-fear test.

The experimental group: F1 mice, refering to the offspring of F0 mice. The offspring themselves are unconditioned, but their parental mice are conditioned.

3.EXPECTED RESULT

We expect three possible results for the experiment. They either be: hypersensitivity to the odor; hyposensitivity to the odor, or no observational difference in sensitivity to the odor compared with the negative control group; no observational difference in sensitivity compared with the positive control group. These results indicate different roles of piRNA in transgenerational memory that I will explain in subsequent paragraphs.

The first possible result is, the F1 mice shows an increased sensitivity to odor, which means their sensitivity will be higher than the positive control group. The sensitivity can be revealed by the aversive fear index, lower aversive index means higher sensitivity. If their sensitivity are higher than the positive control group, it reveals that the piRNA serves a repressive function in the forming or passing of the transgenerational memory. A possible hypothesis is that piRNA methylates the critical gene in this process, meaning less transcription and translation will occur on this gene. Therefore, when we remove the piRNA, this repression is removed, leading to hypersensitivity in the odor-fear testing.

Furthermore, the second possible result shows that transgenerational memory disappears, or the sensitivity decreases drastically. The mice behave the same compared with the negative control group. The second result indicates that piRNA is required in the formation or passing of the transgenerational memory, and it serves a positive role. It does not mean that piRNA do not methylate any genes, instead, it provides a cue that the pathway being involved is not straight-forward. A hypothesis is that piRNA may methylate the gene that represses transgenerational memory. However, it may work in different mechanism which requires further research.

In addition, the third possible result is, the F1 mice have the same sensitivity as the positive control group. The removal of piRNA does not result in any change of their behaviour. This result clearly means that piRNA is not required for the transgenerational memory pathway, and the hypermethylation is caused by other elements.

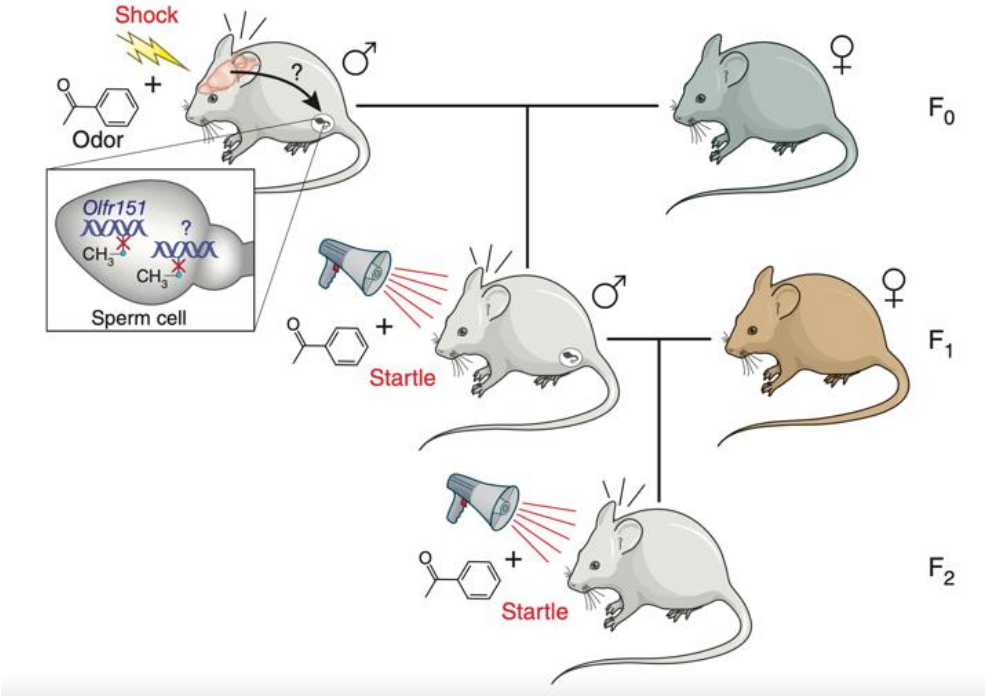

Figure 3. This image is from Katie Vicari [6], it shows a simple model of sound-fear conditioning. When F0 mice experiences a loud sound and an electric shock at the same time, the influence of this conditioning will affect its sperm and show up in the next generation F1. If treated with the odor, F1 mice will appear startled even if it hasn't been conditioned. The same will happen to its next generation F2 mice, supporting the transgenerational nature of this memory. Noticeably, although this image only illustrates the transgenerational memory passed when the offspring is heterozygous, which has a conditioned father and an unconditioned mother but not vice versa, Szyf's paper and experiment methods suggested that a series of trials on heterozygotes with unconditioned father and unconditioned mother had also be done.

4.DISCUSSION

If we perform this experiment, we will be able to answer the following questions:

First, is piRNA in the brain required for mice to learn the odor-fear conditioning and correctly respond to it (corrected defined by comparing with positive control group)?

Second, is piRNA required for transgenerational memory to be formed, passed, and functioned in the offspring of F0 mice, which had been conditioned to fear a specific odor?

In research, we also noticed some questions that we had not previously encountered, or seen any papers encountered before. These are the new question derived from our essay:

First, if "piRNA does not affect F0 learning but affects F1 memory" is true in mice, does this principle apply to all kinds of animals? Does it differ according to extent of development in animals? What about human?

Second, how does piRNA pass the transgenerational memory? Is it contained in genome, sperm, or egg cytoplasm?

Third, what exact function does piRNA serve in the development of transgenerational memory? In other papers, there are clues that piRNA can methylate or dethylate certain genes to regulate their activity. However, contrasting evidence are presented in different papers, and this is a question worth looking into.

Fourth, what the entire pathway of transgenerational being processed, formed, passed and functioned, involving the role of piRNA?

Certainly, more research on the direct connection between piRNA and transgenerational memory can help answer the questions that our experiment is unable to provide evidence for. These are some questions remain unanswered, and would be the directions of future research:

First, how does piRNA in the brain pass its information to the germline cells? What biological activities are involved in this process?

Second, what substance is passed from parent to offspring that led to a development of transgenerational memory?

Third, and also an important one related with neuroscience and psychology, is transgenerational

memory a “cognitive memory”, a knowledge, or a tendency of behavior?

It was very interesting, for me, to study the inheritance of phenomenon that were previously thought cannot be inherited, which, when put in word, are “nurture” characteristics. Conditioned fear for a particular odor is definitively a nurture feature that was not included in mice's genome normally. The existence of such unusual inheritance gives rise to more future considerations: How are these traits different from Lamarck's giraffe example, and Weismann's experiment on cutting mice tails? These are the questions that require revisitation.

In case of can the same principle apply to human, interestingly, I have read an article several years ago, that suggests the aftermath of famine, a nurture factor as well, can impact more than one generation. The paper shows, if parents had lived long years through famine, their children would tend to obtain more energy from a certain mass of food compared with normal children, meaning those will eat less. Such an unusual discovery is potentially parallel to the examples discussed above, as they both involve an inheritance of nurture factor. However, whether they have the same inheritance pathway, or whether they are results of piRNA activities, remain unanswered.

5.CONCLUSION

We designed an experiment to determine whether piRNA is required in the transgenerational memory pathway in mice through the application of odor-fear conditioning, and whether piRNA's function in such pathway is counted towards positive or negative. This experiment involves several generations of mice, three pre-trials to select qualified mice, repeated odor-fear conditioning trials and a final odor-fear test to obtain the results.

Although we take the idea of odor-fear testing on mice from Szyf’s paper [6], we had adopted the experiment method in Dias and Ressler's paper [3], which enables us to collect both qualitative and quantitative data, improving both the accuracy and the reliability. The data will be processed by calculating an aversive fear index. In addition, by performing the pre-trials, we can also have side discoveries on the connection between piRNA and infertility, which may support further investigation in this area.

Though we are unable to perform the experiment on ourselves due to lack of equipment and shortage of time, we had already outlined the method, explained the pre-trials of mice selection, and predicted three possible results of the experiment with a detailed explanation on each. We sincerely anticipate some labs to perform this experiment and investigate on the direct relationship between piRNA and transgenerational memory, even on how piRNA functions. The performance of this experiment can truly provide a new insight into the role of piRNA, and even a new discovery in the field of epigenetics.

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