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piRNAs in the Olfactory Bulb Related to Fear Conditioning can Migrate from the Brain to the Germline

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Abstract: Small RNAs have been shown to be crucial in the mechanisms of transgenerational memory. Precisely, piRNAs have previously been thought to only exist in the germline and are related to transgenerational memory. To determine if the offspring inherits memory due to piRNA transmission, we conducted odor fear-conditioning tests and identified a piRNA that increased in abundance. That piRNA is thought to be involved in memory formation of the fear-conditioning test. We then used a virus vector to manipulate a single nucleotide of that piRNA sequence to see if it can migrate from the olfactory bulb to the germline. The data should theoretically indicate whether the mutant piRNA has migrated from the olfactory bulb to the germline of the mice.

Keywords: piRNAs; Odor fear-conditioning test; Fear-conditioning test; Germline

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1. Introduction

Recent papers suggest in C. elegans that, when exposed to pathogens, parents can transmit avoidance memories to their offspring for up to four generations [1]. This ability to inherit learned information from parents can be evolutionarily beneficial, increasing the offspring's chance of survival. The paper also demonstrated that exposure to the bacteria *Pseudomonas* induces changes in piRNA abundance [1]. Since other articles considered the possibility of other small RNAs – miRNAs – influencing environment enrichment of the next generation, we considered the possibility that piRNA was an essential part of the mechanism of transgenerational memory [2].

Piwi-interacting RNAs (piRNAs) are non-coding RNAs with 24–32 nucleotides that regulate gene expression and the formation of memories [3]. Dias and Ressler suggested in their paper that odor fear conditioning conducted in the F0 generation of mice can cause the F1 generation to larger dorsal and medial odor-responding glomeruli [4]. However, it is still unclear how this mechanism of transgenerational memory works. Thus, we considered that piRNA could play a significant role in this instance of transgenerational memory by transporting from the olfactory bulb to the germline.

In our experiment, we will mutate the piRNA involved in the learning and memory of odor fear-conditioning and check whether it is detected in the germline. If the mutant piRNA is detected and the offspring still inherit the memory, we can conclude that this instance of transgenerational memory works by mechanisms of piRNA.

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 receive the piRNA migrated from the brain region and thus inherit the memory of the odor used in the odor fear conditioning.

2. Experimental methods

2.1 Odor fear-conditioning in mice

For our experiment, we used adult male transgenic mice that had a 12-hour light/dark cycle, free access to food and water, and contained the fluorescence gene on the sensory neurons relating to acetophenone glomeruli (M71-GFP). M71 is the odor receptor for acetophenone, and thus the M71-GFP mice would have fluorescent neurons once the sensory neurons have detected the acetophenone odor. Through this method, we can observe the change in neurons before and after the fear-conditioning.

Then, we performed fear-conditioning on the mice by pairing an electric shock with two odors to see the mice's ability to discriminate. We would have three groups, each consisting of 6 mice, to perform the fear-conditioning: one pairing the acetophenone with the electric shock, one just with the acetophenone and no electric shock, and one pairing propanol (an odor which we chose at random) with an electric shock. The first group (acetophenone + electric shock) would be the experimental group in which we would test the change in piRNAs and neurons. The second group (acetophenone + no electric shock) is a control group to test whether there are changes in neurons after sensing acetophenone without fear-conditioning. The third group (propanol + electric shock) indicates whether other unrelated odors will trigger changes in the M71-GFP neurons.

Odorants used in the experiments consisted of 10% acetophenone or 10% propanol (both from Sigma-Aldrich) in propylene glycol. Regarding the fear-conditioning, mice then received two training sessions per week for three weeks to ensure solid and stable odor-shock association. Each odor shock training session consisted of 5 trials of 10s odor conditioned stimulus terminating with a 0.25 s, 0.4 mA foot shock, presented with an average 120s intertrial interval (ITI) (range 90 –150s) [5].

We also performed a discrimination test to check whether the mice would freeze or hide in the corners when only acetophenone was administered after the fear-conditioning.

2.2. Sequencing and identification of piRNA in the acetophenone-responding glomeruli

As explained in the introduction, specific odors have neural pathways corresponding to specific glomeruli. Odor fear-conditioning has been shown to have an increase in the neurons (see **Figure 1**) connected to the glomeruli in transgenic male mice [4]. Thus, we choose to take out the acetophenone-responding glomeruli in the first (acetophenone + FC) and second (acetophenone only) group of mice to determine the change in piRNAs after fear-conditioning. The piRNAs discovered in the second group will be used as a baseline for the expected increase in piRNAs in the first group.

After isolating the glomerulus where it has the neuron's axons, we took out the small RNA associated with the neuronal circuits and sequenced them. The piRNA clusters discovered in the acetophenone-responding glomeruli are sequenced using RNA sequencing, which uses reverse transcriptase to form cDNA from the piRNAs. Then, the high-throughput sequencing machine should give us the base sequence and amount of the piRNAs. However, we still needed to confirm whether the small RNAs found in the glomeruli were indeed piRNAs. We can initially use the sequencing to determine the length of the small RNAs found, and if they were similar to piRNA length, which is found to be predominantly 28 nucleotides, we could distinguish the small RNA to be piRNA [6]. After the sequencing, we expect to see a significant increase in one of the piRNAs, piRNA-X.

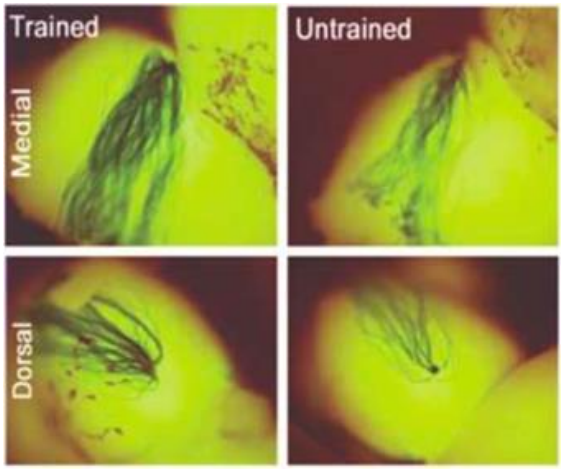

Figure 1. Increased M71 axon density and glomerular size before and after fear conditioning [5]

2.3. Determining piRNA

We conditionally removed the piRNA-X in the Olfactory Bulb to determine whether piRNA-X is required for learning and memory. First, we knock out the gene that encodes for the piwi protein that works with the piRNA by the CRE recombinase method. Therefore, piRNA-X has no effect and cannot work any longer. Fear-conditioning is then conducted to see if the inhibition of piRNA allows for memory formation after the odor fear-conditioning test.

2.4. Mutating the piRNA-X

We determined that piRNA-X showed the most significant increase after the fear-conditioning, so we selected that particular piRNA to perform the mutation on. To see if the piRNA-X can migrate from the brain to the germline, we change a single nucleotide of the sequence. To mutate the piRNA, we used a virus vector to add DNA with piRNA-X with one mutant nucleotide to a piRNA cluster through the CRE loxP system. Then, we introduce the virus to a group of mice embryos. Then, using the CRE enzyme in the olfactory bulb region in the adult mice, we can activate the mutant piRNA strand so that it is expressed in the olfactory bulb. Because there is a possibility that the piRNA does not move to the germline after it is mutated, we need to check the sperm cells for the presence of piRNA-X.

We used immunoprecipitation to check if the piwi protein and piRNA-X interact and to isolate the piRNA-X in preparation for an RT-PCR test. The RT-PCR uses a reverse transcriptase-polymerase chain reaction to confirm whether the mutant piRNA has migrated from the olfactory bulb to the germline, indicated by if the cDNA of the mutant piRNA is found in the sample.

2.5. Checking offspring of mutant mice

In order to check and see if the offspring still inherited the memory, we conducted an experiment where we presented the conditioned stimulus (acetophenone) to three different experimental groups: one consisting of offspring of parents who undergo fear conditioning and have mutated piRNA, one consisting of offspring of parents who undergoes fear conditioning but don't have modified piRNA, and a final group consisting of offspring of parents with normal piRNA but didn't experience fear conditioning. Since we assume the mutant piRNA has the same function as the non-modified piRNAs and doesn't interfere with their function, this step lets us confirm that hypothesis.

3. Possible results

3.1. Odor fear-conditioning in mice

From the fear-conditioning tests performed on the F0 generation, we can expect the following results to

happen. The first group (acetophenone + electric shock) should exhibit signs of learning and memory, so we should expect enhanced freezing behaviour when presented with the conditioned stimulus (acetophenone). However, there may be a situation where the mice in group 1 do not exhibit freezing, so the fear-conditioning needs to be repeated. The second group (acetophenone only) should not show signs of freezing when exposed to the conditioned stimulus (acetophenone). The third group should not have fluorescent M71-GFP neurons when presented with the propanol odor.

3.2. Sequencing and identification of piRNA in the acetophenone-responding glomeruli

After the RNA sequencing, the sequencing machine should give us the sequences and amount of each piRNA, and we would expect to see a significant increase in one of the piRNAs, piRNA-X.

3.3. Determining piRNA

After the inhibition of piRNA-X, we would expect that the mice will not form a memory of the odor fear-conditioning test, thus not freezing when presented with the conditioned stimulus (acetophenone). This means that we have found the correct piRNA involved in the fear-conditioning neuronal circuit.

3.4. Mutating the piRNA-X

The result of the PCR test depends on if the cDNA of the mutant piRNA is found in the sample. The machine gives a positive result if the cDNA of the mutant piRNA is located in the sample, and thus we can conclude that piRNA has migrated from the olfactory bulb to the germline. Conversely, the machine gives a negative result if the cDNA of the mutant piRNA is not found in the sample, and thus piRNA did not migrate from the olfactory bulb.

3.5. Checking offspring of mutant mice

We expect that the F1 generation of the first and second experimental groups would exhibit increased freezing behaviour when presented with acetophenone, while the F1 generation of the third group would not display any sign of freezing when exposed to acetophenone.

4. Discussion

A possible limitation when using RNA sequencing to separate the piRNA clusters and obtain piRNA sequences is that there could be several piRNAs that show an increase after the odor fear-conditioning. Then, we would have to choose which piRNA to eliminate. It is possible that if we would have to mutate multiple piRNAs if the inhibition of the first piRNA does not stop the learning and memory after fear-conditioning.

Another possible limitation is that changing the nucleotides may cause a change in the entire function of the piRNA. Since the piRNAs are only around 28 nucleotides long, changing one nucleotide for our experiment may be detrimental to the functioning of the piRNA itself.

Future studies could benefit by studying the method and pathway of the migration of piRNAs from the brain to the germline. A current hypothesis is that piRNAs travel through exosomes around the body to the germline. Furthermore, our study focuses on the olfactory bulb as the origin of transgenerational memory, but future studies can focus on other brain regions like the hippocampus or the amygdala.

5. Conclusion

Our hypothesis is accepted if the mutant piRNA is present in sperm, the piRNA associated with fear conditioning has migrated. However, if the mutant piRNA is not present in the sperm, then the piRNA related to fear conditioning has not migrated, our hypothesis is rejected.

Disclosure statement

The author declares no conflict of interest.

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