PiRNAs Involved in the Memory Formation of Fear-conditioning Tests Migrating from the Brain to the Germline

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Abstract: Small piRNAs regulate and manipulate gene expressions and are important for forming memories. These piRNAs are thought to be germline-specific and can help offspring inherit memories from their parents. In this paper, we conducted odor fear-conditioning tests to identify a piRNA that increased in abundance and is involved in the memory formation of the fear-conditioning test to determine how the offspring can inherit memory. A mutant piRNA is created using a virus vector and introduced into the mice brains to see if it can migrate from the brain to the germline. If the the mutant piRNA is found in the sperm cells, then we know that the piRNAs can migrate from the brain to the sperm cells and thus inherit the memory of the odor used in the odor fear conditioning test.

1 INTRODUCTION

In a recent study, Moore et al. reported that C.elegans worms, upon exposure to PA14 (Moore, 2019), can transmit avoidance memory to their offspring for several generations. This transgenerational memory can provide their offspring with advantageous mechanisms to increase their chances of survival. Besides, the study has helped us think of a possible way how memories can be transferred from parent animals to their offspring i.e., via piRNAs.

piRNAs are a class of 26-28 nucleotide small non-coding RNAs and are associated with piwi proteins to regulate gene expression and form memories (Rajasethupathy, 2012). In Kandel's study, it was identified that piRNAs increased in amount in the neurons of the Aplysia adult brain and thus amplified its sensitivity to serotonin by inhibiting the transcription of CREB2. Furthermore, the offspring of the Aplysia were tested to see if the neurons can also display long-term potentiation (LTP) from serotonin stimulus, and was found that the offspring will also display LTP. However, it is still unclear how this transgenerational memory is achieved.

In this paper, we are going to create a mutant piRNA to the piRNA that is involved in the memory formation of odor-fear conditioning in the olfactory bulb and see if it can be detected in the germline. If it is detected and the progeny has the memory, then we can suggest that transgenerational memory is achieved via piRNAs.

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

2 MATERIALS AND METHODS

2.1 Odor-Fear Conditioning Tests

All mice used in the experiments are M71-GFP mice strains. These mice are transgenic and have fluorescent neurons when M71 receptors sense acetophenone in the olfactory bulb. In this way, we can observe changes and take out these specific neurons more easily. The adult mice were kept in standard cages with a 12-hour light/dark cycle with free access to food and water.

The experiment utilized 2 different odorants to see if the mice can discriminate between them: the first odorant consisted of 10% acetophenone and the control odorant consisted of 10% propanol in propylene glycol.

Before the fear-conditioning tests, the mice were given 3 weeks to habituate to the startle chambers to ensure a strong odor-shock association. They

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received 2 training sessions per week and each training session consisted of 5 trials of 10 s odor conditioned stimulus followed by a 0.24 s, 0.4 mA electric footshock. The trials were separated by a 120 s time gap (Jones, 2008).

When the solenoid switch is closed, clean air can flow freely with no difference in airflow. Backflow of the air is prevented by several one-way valves (yellows arrows). To remove the odor an exhaust hose (green arrow) is utilized. The electric shock is generated by a machine-driven animal shocker and delivered through electric bars installed in the cage floor. During the behavioral fear-conditioning testing, the startle is recorded by a 105dB white noise outburst, and the activity and startle amplitude are measured by a piezoelectronic device underneath the floor of the cage. (Jones, 2008)

The first experiment consisted of 6 mice with an acetophenone stimulus + electric footshock. The second experiment consisted of 6 control mice and was given 10 s of acetophenone stimulus only. This control experiment was carried out to see if any changes in the neurons and glomeruli sizes were found without given footshocks. The third experiment also consisted of 6 control mice and was given 10 s of propanol odor + electric footshock to see if whether other odors can also be detected by the M71 receptors.

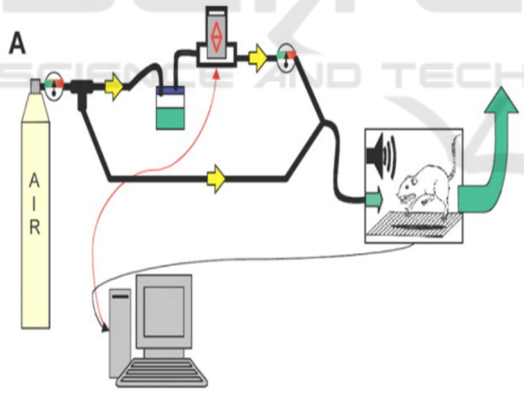

Figure 1: The flow of the air through the odorant jar and into the startle chamber is controlled using a SR-Lab Response Software which uses a solenoid switch (red arrows) to control the flow of compressed air.

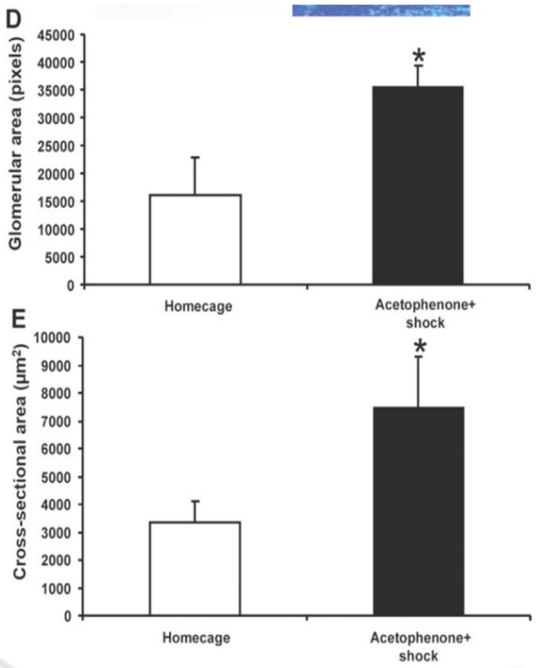

Figure 2: The glomerular surface area and cross-sectional area were larger in the acetophenone + shock trained group than in the homecage group ($p < 0/05$) (Jones, 2008).

2.2 Sequencing and Identification of piRNA

First, the glomeruli with acetophenone-sensitive neurons are isolated. These should be easy to identify as the neurons with the M71 receptors will fluoresce in the presence of acetophenone. After obtaining the RNA clusters a reverse transcriptase enzyme is used to form cDNAs of the RNAs. These cDNAs are then transferred into an Illumina high-throughput sequencing machine to be sequenced. Following having sequenced the cDNAs, we can identify the 26-28 nucleotide long piRNAs.

This experiment needs to be done before and after the fear-conditioning test. The second experimental group of the fear-conditioning test (the group of mice with exposure to acetophenone only) can be used to find the initial amount of piRNA in the brain and the first experimental group of the fear conditioning test can be used to find the piRNA that is involved in the formation of acetophenone memory. By using RNA sequencing, the machine will be able to show which piRNA has increased in an amount most, thus we can find which piRNA was associated with the test. The piRNA identified with the most significant increase in abundance is then called piRNA-X.

As shown in figure 3 the glomelum should have an increased M71 axon density and glomerular size

after the odor fear conditioning test in the dorsal and medial areas. Therefore, these acetophenone-sensitive neurons can be identified and isolated to find the piRNA that has increased in abundance.

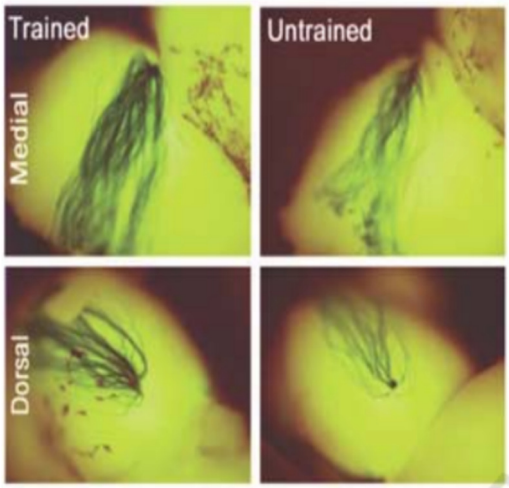

Figure 3: Increased M71 axon density and glomerular size with fear conditioning in the medial and dorsal pairs of M71 glomeruli (Jones, 2008).

2.3 Determination of the piRNA-X

To ensure that the found piRNA-X is indeed associated with the neuronal circuit and memory formation, the piRNA-X is conditionally removed in the olfactory bulb. The piwi protein that works together with the piRNA is knocked out by using CRE recombinase to remove the gene encoding for the piwi protein. Therefore, piRNA-X will no longer have an effect. Lastly, a fear conditioning test is conducted to see if the inhibition of the piRNA-X has any effect on the memory formation of acetophenone.

2.4 Mutant piRNA

To see if piRNAs can migrate from the brain to the germline a mutant piRNA-X is created using a virus vector to add a piRNA DNA to a group of mice embryos. The mutant piRNA-X differs from the initially found piRNA-X by a nucleotide achieved by using the CRE loxP system. Then the mutant piRNA strand can be activated using the CRE recombinase enzyme in the olfactory bulb region of the adult mice.

Following this, immunoprecipitation is used to see if the mutant piRNA-X can still perform the same task as the original piRNA-X. First, a crosslinker is used to link the piwi protein to the mutant piRNA-X and test if they can interact. Then specific antibodies are used to separate the piwi-piRNA complexes from the cells. We elute to separate the mutant piRNA-X from the piwi protein and check if the mutant piRNA-X is present with the RT-PCR test.

2.5 Testing for the Migration of piRNA-X

The RT-PCR test is used to check if the mutant piRNA-X is present in the sperm cells. First, a constant sequence of nucleotides is joined to the 3' end of the mutant piRNA-X. A primer complementary to the constant region binds to it and the reverse transcriptase can come along and form the DNA strand. A second primer specific to the mutant piRNA-X binds to the DNA strand and reverse transcriptase forms the cDNA of the mutant piRNA-X. The sample is then placed into the PCR machine.

2.6 Further Experiments + Controls

Lastly, the offspring of the experimental groups are tested to see if they were able to inherit the memory of the acetophenone fear-conditioning test. The first group of mice consists of the offspring of parents who have undergone the fear-conditioning test and have the mutated piRNA-X. The second experimental group consists of offspring of parents who have undergone the fear-conditioning test but do not have the mutant piRNA-X. The third experimental group consists of offspring of parents who have not undergone the FC test but have the mutant piRNA-X. These experiments are to prove that the mutant piRNA-X does not interfere with the regular function of the piRNA-X and the original piRNA-X is functional with the addition of the mutant piRNA-X too.

3 POSSIBLE RESULTS

3.1 Odor Fear-conditioning

From the first FC tests, some predictions of the results can be drawn. We expect that the first group of mice will show strong freezing behavior when exposed to acetophenone after the fear conditioning test. If the mice do not display a freezing behavior the same procedure will be repeated. The second group of mice should not show sensitivity to the odor and the third group of mice should not have fluorescent neurons (Jones et al., 2008).

Figure 4 shows how the results of the freezing behavior look like in the mice in response to odor

stimulus after training.

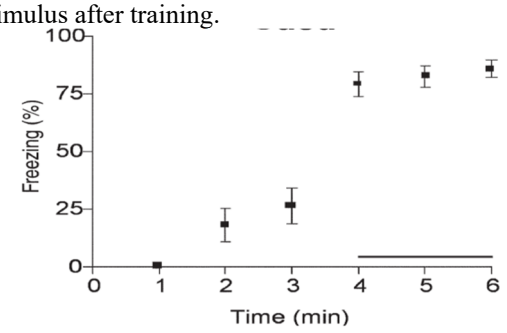

Figure 4: Freezing in response to CS presentation on Day 2 after training. (Sweatt, 2010).

3.2 Sequencing and Identification of piRNA

After DNA sequencing, the machine should give us the nucleotide base sequence of the piRNA that has increased in abundance the most, the piRNA-X.

3.3 Determination of piRNA

We expect that after the inhibition of piRNA the mice will not be able to form a memory of the odor FC test, they will not display freezing behavior. Therefore, we have found the correct piRNA that is involved in the FC neuronal circuit.

3.4 Mutant piRNA

The PCR machine can give 2 different results: positive or negative. If the PCR machine shows a positive result then we know that the cDNA is present in the sample and the mutant piRNA-X has migrated from the brain to the germline. If the PCR machine gives a negative result, then the cDNA is not present in the sample and the piRNA has not migrated from the olfactory bulb to the germline.

3.5 Offspring

We would expect that the offspring of the first and second experimental groups would display similar increased freezing behavior to acetophenone, whilst the offspring of the third experimental group would not display freezing behavior on exposure to acetophenone.

4 DISCUSSION

One possible limitation of using DNA sequencing to obtain the piRNA sequences is that several piRNAs can be identified that have increased in amount. In this case, we would have to choose which piRNA to mutate and eliminate. If after the inhibition of the 1st piRNA the mice can still exhibit enhanced sensitivity to acetophenone then a 2nd piRNA must be inhibited until the mice stops exhibiting freezing behavior and the piRNA then can be mutated.

Furthermore, one other limitation of creating the mutant piRNA is that changing the base sequence of the piRNA can result in the mutant piRNA having a different function to the original strand or can lead to damage to the animal since piRNAs are only 26-28 nucleotides long and a slight change to the sequence can lead to consequences.

5 CONCLUSION

In conclusion, if the mutant piRNA is present in the sperm cells, then the piRNA associated with fear odor conditioning has migrated from the brain to the sperm cells and memory of the odor used in th fear conditioning test can be inherited by offspring.

If the mutant piRNA is not present in the sperm cells, then the piRNA associated with odor fear conditioning has not migrated from the brain, and offspring will not inherit the memory of acetophenone.

This work could therefore confirm that piRNAs are related to transgenerational memory and migrate from the brain to the germline. In the future, the pathway and mechanism of the migration of piRNAs are nevertheless yet to be confirmed. We are currently thinking that perhaps piRNAs are transported in exosomes around the body or piwi-piRNA complexes can travel around the body to the germline. Moreover, future studies can also focus on structures such as the hippocampus and amygdala which are known to be involved in FC memory formations, and possibly the transgenerational memory.

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