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Maternal Partial-knockout experiment on piRNA in mice

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Abstract. The role of PIWI-interacting RNAs (piRNAs) in maintaining genomic integrity and facilitating neurogenesis has been well documented, especially when it comes to the germline. The study explores the impact of piRNA knockout, specifically the PIWIL2 gene on cognitive abilities, neurogenesis, and postnatal hippocampal development in mice. We will selectively remove PIWIL2 RNA at the embryonic stage through the use of CRISPR technology to assess its effects on short-term memory and neural function across multiple generations. Behavioral assessments will be conducted using two Y-mazes: one with an electric shock to test for neurogenesis and the other one without to evaluate the retention of memory. Additionally, Magnetic Resonance Imaging (MRI) and RNA sequencing were employed to observe structural changes in the hippocampus and gene expression alterations. Preliminary results suggest that the absence of PIWIL2 correlates with cognitive decline, affecting neurogenesis and causing significant development changes in the hippocampus, thereby highlighting the critical role of piRNAs in neural health and its potential risks related to their dysregulation.

Keywords: piRNA (PiWi-interacting RNAs), CRISPR (Clustered regularly interspaced short palindromic repeats), Gene knockout, Hippocampus, Postnatal development.

1. Introduction

1.1. Previous Studies

Previous studies have shown that total knockout of PIWI (P-element induced wimpy testis) and its RNA in mice leads to infertility, leading to the conclusion that PIWI is crucial for germline development and gametogenesis of many metazoan (or animal) species. Research into the PRG-1 domain of the PIWI protein in C. elegans demonstrates that PIWI is crucial to the inheritance of avoidance behavior (from naive parental exposure to a certain bacteria) in offspring until F5, which demonstrates PIWI's importance as a potential media for epigenetic inheritance of stress, potentially crossing to other factors such as memory formation and neurogenesis abilities. However, recent research has discovered the cancerous damage of degenerative PIWI in humans, which could be due to the transposon activity permitted around the body. Therefore, this proposal will evaluate PIWI's role in conducting regular activities through a knockout experiment of PiWi-interacting RNAs in mice, in three facets: short-term

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memory, neurogenesis, and postnatal development of the hippocampus. In other words, this study aims to discover whether normal brain activity can still be sustained with the removal of PiWi-interacting RNAs, provided that degenerative PiWi proteins may induce cancer, removing PiWi proteins could lessen the risk for cancers, especially for people with old age or deteriorating functions. Most studies have set their focus on C. elegans or looked at both PiWi proteins and their respective RNAs., C. elegans has a domain separate from the series of human PiWi domains and is fairly well-discovered, and the knockout research of PiWi that discovered the consequence of infertility may be due to the knockout of multiple domains. Thus, the investigation of this study focuses on the knockout of only one of many domains between humans and mice (PIWIL2 gene) by silencing its respective RNA (mRNA).

1.2. PIWI Protein and its RNAs

PIWI proteins are primarily found in the germline and are associated with a unique class of small RNAs called PIWI-interacting RNAs (piRNAs). In mammals, zebrafish, and Drosophila, tens of thousands of piRNA species, typically 24-32 nucleotides in length, have been identified. Most piRNAs seem to originate from a limited number of long single-stranded RNA precursors, often encoded by repetitive intergenic sequences in the genome. They are also vital for germline development and gametogenesis in many metazoan species, playing key roles in germline determination, germline stem cell maintenance, meiosis, spermiogenesis, and transposon silencing. These functions likely involve piRNAs and are mediated through novel mechanisms of epigenetic and post-transcriptional regulation.

1.3. Outline

Research Question: How would the removal of piRNA (PIWIL2) affect the hippocampus and cognitive abilities of the mice?

Hypothesis: piRNA removal in the hippocampus of mice would cause cognitive decline in 3 facets: short-memory, neurogenesis, and postnatal development of the hippocampus.

This proposal will test the reduced PiWi protein's (knockout of PIWIL2) impact on the mouse's short-term memory, neurogenesis, and postnatal development of the hippocampus. The experiment will conduct the knockout of PIWIL2 using CRISPR technology, and test changes (if any) in the mouse in two aspects. This proposal will test its behavioral change on two mazes, the first Y-maze carries an electric shock to test the mouse's neurogenesis ability, and how it remembers the maze over 5 consecutive trials. Second is a same-size Y-maze without an electric shock, to see the lifespan of the mouse's short-term memory. Additionally, this proposal will evaluate the postnatal development in the mouse brain through Magnetic Resonance Imaging (MRI) and tracking the gene expression in the mouse brain after targeting possible affected areas.

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2. Literature Review

2.1. PIWI-Interacting RNA: Its Biogenesis and Functions

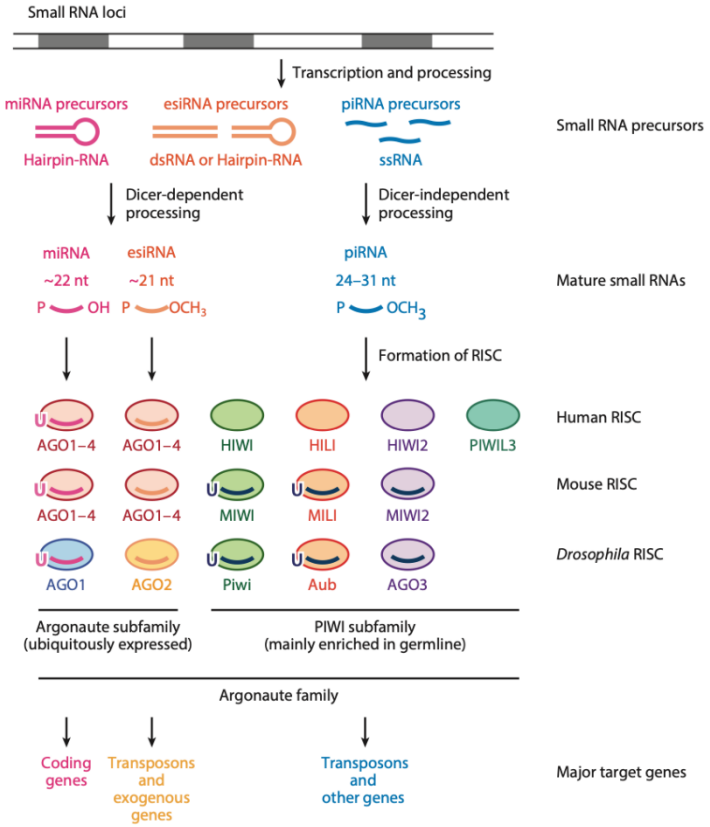


Figure 1. features of miRNAs, siRNAs, and piRNAs but mentions no known association between piRNAs and human PIWI proteins (1)

PIWI-interacting RNAs (piRNAs) are a specialized class of small RNAs, which are 24-31 nucleotides in length. piRNAs play a crucial role in maintaining the genome integrity inside the germline. The RNAs associate with PIWI proteins, to form piRNA-induced silencing complexes (piRISCs), which repress transposons via both transcriptional and post-transcriptional mechanisms. “DNA transposons move from one genomic location to another by a cut-and-paste mechanism. They are powerful forces of genetic change and have played a significant role in the evolution of many genomes” (5). However, this comes with the downside of hindering genomic stability, leading to malignantly mutated genes. Beyond transposon silencing, piRNAs also have regular cellular gene function and contribute to many cellular processes including gametogenesis and reproduction. In particular, piRNAs have shown the ability to pass on “self” and “non-self” memories across multiple generations, indicating their role in transgenerational inheritance. The research suggests a broader contribution of piRNAs to other cellular processes over time, the main factor the research proposal aims to address. (1)

2.2. Knockout Gene-Based Evidence for PIWI-Interacting RNA Pathway in Mammals

This specific study by Yinuo Li, Yue Zhang, and Mingxi Liu, pointed out piRNA’s essential role across mammal species. If the piRNA had been completely knocked out in male mice, it would cause infertility due to piRNA pathway factors leading to meiotic arrest during spermatogenesis. However, it is uncertain if most piRNA factors present in spermatids play a role in suppressing LINE1 after meiosis. Researchers

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used this finding to infer that removing piRNA in mammals would cause reproductive issues. At the same time, when the researchers applied the same experiment on hamsters, they discovered that controversially hamsters' reproductive abilities would only be affected if the piRNA removal took place in female egg cells. The piRNA pathway consists of small RNAs (24-31 nucleotides long) that interact with PIWI proteins to silence transposable elements, primarily in the germline. This pathway is crucial for maintaining genomic stability during germ cell development. MILI (PIWIL2) is key in piRNA processing and transposon repression; knockout leads to spermatogenesis blockage. (2)

2.3. PiWi-interacting RNAs in Human Cancer

This article explores the role of piRNAs in human cancer. piRNAs are known for their role in germline transposon control, yet in this research, it has been discovered that piRNAs have also been implicated in various aspects of cancer biology, involving cell proliferation processes, apoptosis, invasion, metastasis, and maintaining cancer stemness. The article discusses how dysregulation can contribute to cancer, and highlights that aberrant expression of piRNAs and PIWI proteins have been observed in multiple cancer types, suggesting its potential as diagnostic and prognostic biomarkers, and therapeutic targets. The research proposal aims to discover the potential of a PIWI knockout to eradicate risks involving its dysregulation that leads to cancer. (3)

2.4. PIWIL2 (Mili) Sustains neurogenesis and prevents cellular senescence in the postnatal hippocampus

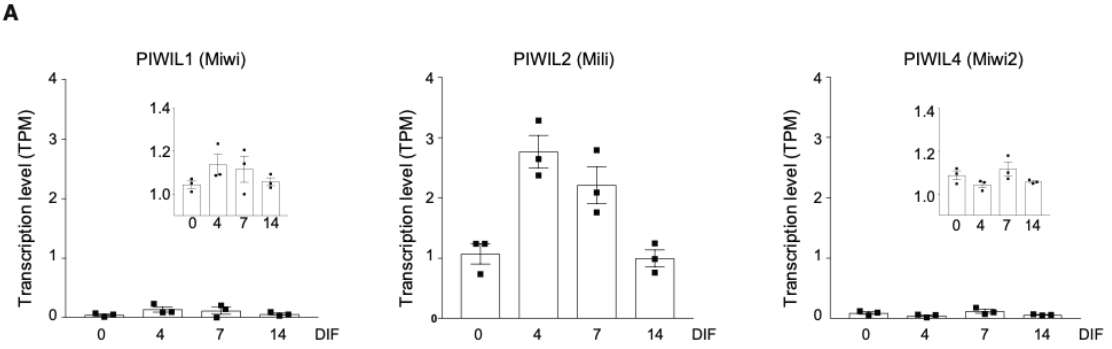


Figure 2. Levels of Piwil1 (Miwi), Piwil2 (Mili), and Piwil4 (Miwi2) transcripts in RNA seq. (4)

The graphs in Figure 2 illustrate the transcription levels of PIWI proteins: PIWIL1 (Miwi), PIWIL2 (Mili), and PIWIL4 (Miwi2) during the differentiation of neural progenitor cells over 14 days. PIWIL2 demonstrates a significant increase in transcription levels, peaking at day 4, suggesting its part in the early stages of neurogenesis. Contrastingly, PIWIL1 (Miwi) and PIWIL4 (Miwi2) maintain low and relatively stable transcription levels during the differentiation period, which indicates a limited role in the neurogenesis process or a function in later stages of development or role in other cell types. The patterns highlight the role of PIWIL2 in early neural differentiation, while Miwi and Miwi2 appear to be less involved in this stage. (4)

2.5. Piwi/PRG-1 Argonaute and TGF-b Mediated Transgenerational Learned Pathogenic Avoidance

This study written by Rebecca S. Moore, Rachel Kaletsky, and Coleen T. Murphy mainly introduced the idea of how the nematode C. elegans can pass on learned behaviors to their offspring, which may provide an evolutionary advantage. Specifically, after C. elegans learn to avoid the harmful bacteria Pseudomonas aeruginosa (PA14), this avoidance behavior is inherited by their descendants for up to four generations. The expression of the DAF-7 protein in specific sensory neurons is necessary for this inherited behavior, and the Piwi Argonaute protein PRG-1, along with its associated molecules, is crucial for this transgenerational inheritance. (5)

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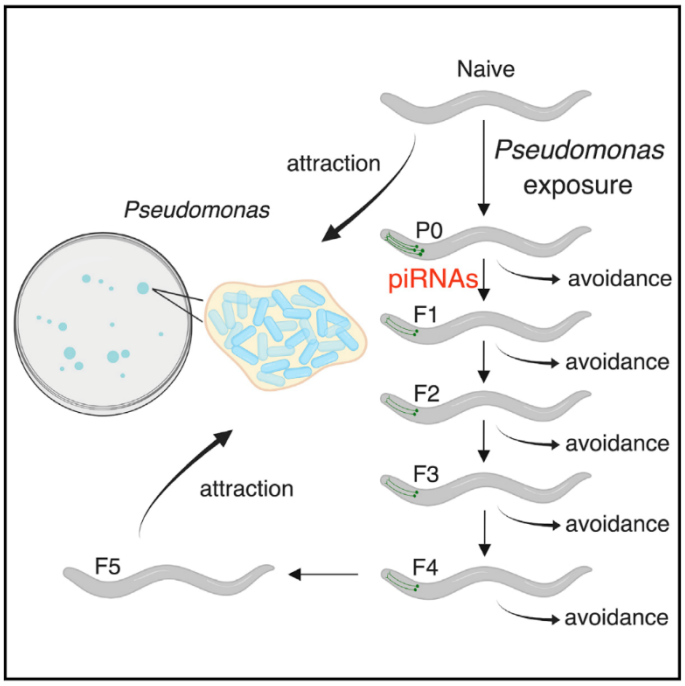


Figure 3. Worms can transmit avoidance behavior to their young for four generations, providing progeny with a mechanism to survive dangerous conditions. (6)

3. Methodology

3.1. CRISPR and PIWIL2 Gene

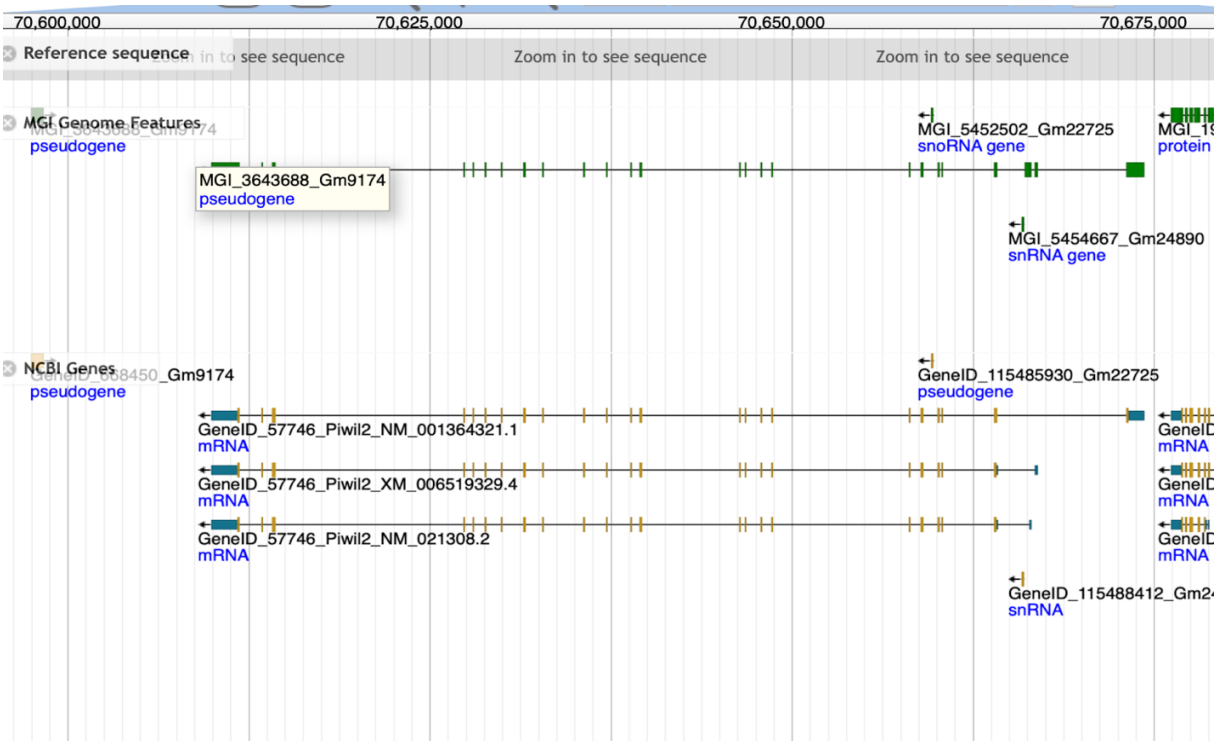


Figure 4. PIWIL2 gene domain shows which section of RNA CRISPR would be edited. (1)

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CRISPR(Clustered regularly interspaced short palindromic repeats) is a technology that research scientists use to selectively modify the DNA of living organisms, mainly applied to prokaryotic organisms like bacteria. For CRISPR to work, there would be a crRNA which is an RNA sequence that's getting CRISPRed. A tracrRNA would then target recognition and act like a primer to help activate the Cas 9 protein. PAM(protospacer adjacent motif) is used to locate where the Cas 9 protein needs to cut and prevent cleavage that is not needed, Cas 9 is an enzyme that acts like a molecular scissor. After the Cas 9 protein successfully made cuts on the DNA strand, the DNA strand would have DSB (DNA double-strand breaks). There are two methods to repair the DNA strands after cuts, one is NHEJ(Non-Homologous End Joining), which would generate insertions or deletions to repair the DSB; the other one is HDR (Homology Directed Repair) by inserting, labeling, or knocking out genes to alter the gene expression.

This proposal would apply CRISPR by using CRISPR to remove PIWIL2 RNA at the embryonic stage of the F1 offspring, optimally it would have a cognitive affection since Piwil2 (Mili) maintains neurogenesis and prevents cellular senescence in the postnatal hippocampus.

3.2. Magnetic Resonance Imaging (MRI)

MRI uses magnets and radio waves to produce images on a computer. MRI does not use ionizing radiation. Images produced by an MRI scan can show organs, bones, muscles, and blood vessels. MRI could be used to see the abnormalities of postnatal development of the mice brains and to observe what had been directly impacted by the removal of PIWIL2 in the hippocampus of the mice. Compared to CT (computed tomography), MRI is better at observing soft tissues. As previous studies have proved that the removal of piwi might cause fundamental changes in mice, this proposal would use MRI to see if there are structural changes after the knockout of piwil2 located in the hippocampus.

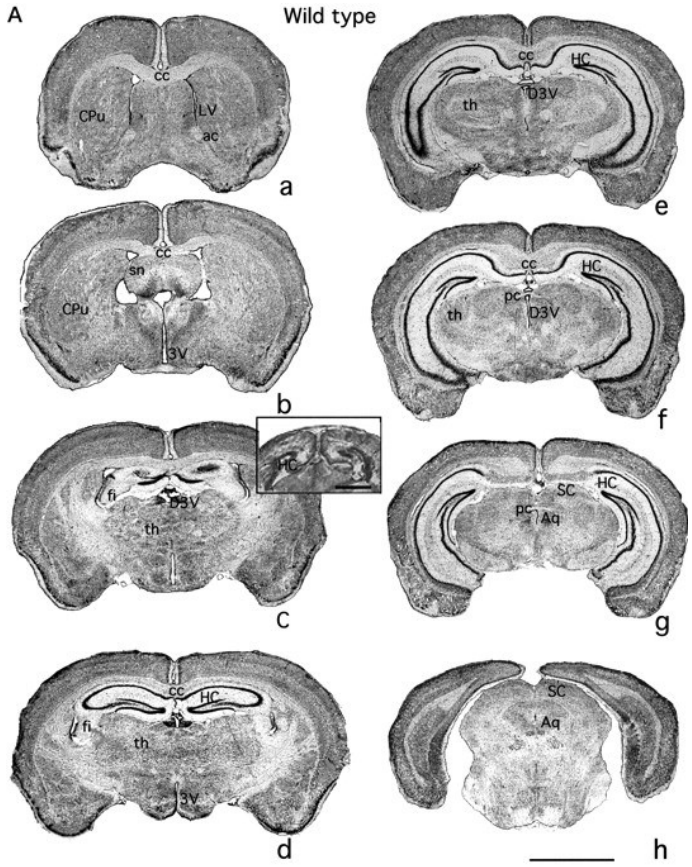


Figure 5. How MRI would show all types of apparent abnormalities of brain development of mice.

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3.3. RNA sequencing

It has been previously mentioned that the PiWi has the function of inhibiting transposons and thus if PiWi quantities are reduced, transposon activity could be permitted, leading to the generation of mutated genes. Given the knockout could induce targeted damage, an MRI would be able to detect the affected area in the brain. The brain would contain a bunch of normal neural cells and a bunch of mutated neural cells. To know what genetic mechanism is causing the difference, we will need to look at the differences in gene expression.

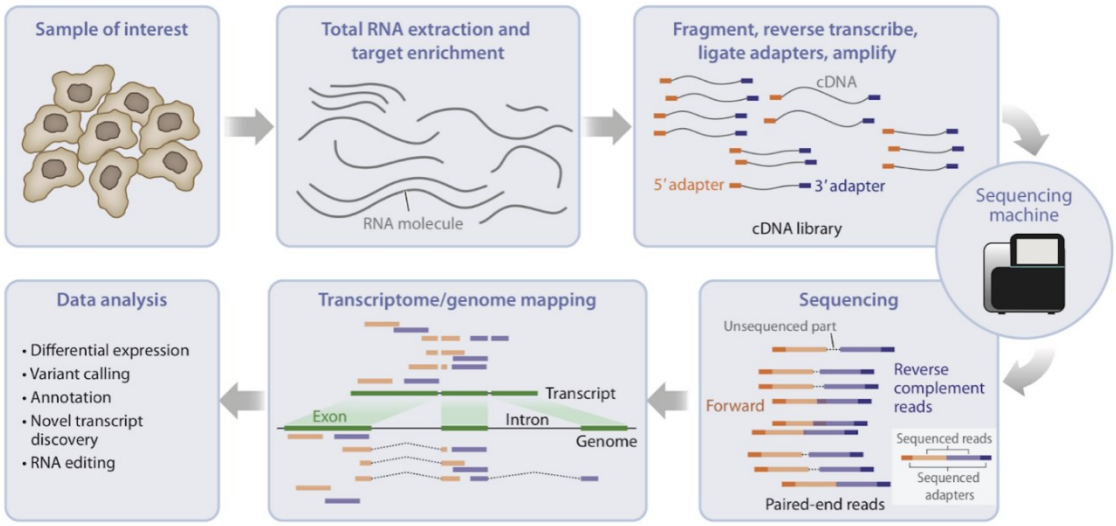


Figure 6. Description of how RNA sequencing works.

Process of RNA sequencing: begins by selecting the sample of interest, which contains cells where DNA will be extracted. Next, the total RNA is extracted from these cells, and specific RNA molecules such as mRNA are usually enriched to focus on the genes being expressed. RNA is then fragmented into smaller pieces, and each fragment is reverse-transcribed into its complementary DNA (cDNA). Adapters are ligated to the ends of the cDNA fragments and are amplified to create a library ready for sequencing. The cDNA library is loaded onto a sequencing machine, generating short reads from the cDNA, and mapped to the transcriptome or genome, to align the reads to known exon and intron regions, which allows researchers to reconstruct the transcripts. Finally, the data is analyzed to determine differential gene expression, identify genetic variants, annotate transcripts, discover new/novel transcripts, and even detect RNA editing events. (7)

Zooming a neural cell, is composed of chromosomes, genes, and their respective mRNA transcripts that indicate if a gene is active or inactive. High throughput sequencing (a specific term for RNA-seq technology) tells two things: 1) which genes are active, and 2) how much they are transcribed. We could use RNA sequencing to measure gene expression in normal cells, then measure gene expression in mutated cells to evaluate the difference, and spot what is different in the mutated cells, comparing gene by gene to track subtle and significant differences. (Starmer)

4. Experimental Design

4.1. Animal Groups/ Generations

The animal this proposal would use is both-gender adult mice. As Figure 1. shows there are two groups of mice. One would be the “Knockout” group, with piwil2 removal; the other group would be “Wild-type”, with normal mice with piwil2 RNA. The previous Parent (PP) generation is the grandparent generation of the F1(first generation offspring) mice, they would both be kept the same, and both have normal piwil2 in the hippocampus. Parent generation (P0) would be normal mice for two groups,

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however, in the "knockout" group F1's embryonic stage inside the P1 mother, piwil2 RNA would be removed. Due to this topic not being largely investigated, also because maternal changes would usually have a direct impact on the offspring, therefore this proposal would be based on maternal embryonic piwil2 removal impact on the F1.

This proposal would be observing 5 generations of offspring, to investigate how each generation's cognitive and memory abilities would be impacted. The removal of piwil2 might only affect a few generations as if piwil2 is essential it is possible that something would be evaluated and become a substitute for piwil2 to maintain piwil2's functions, this is why this proposal would choose a longer experiment period of 5 generations.

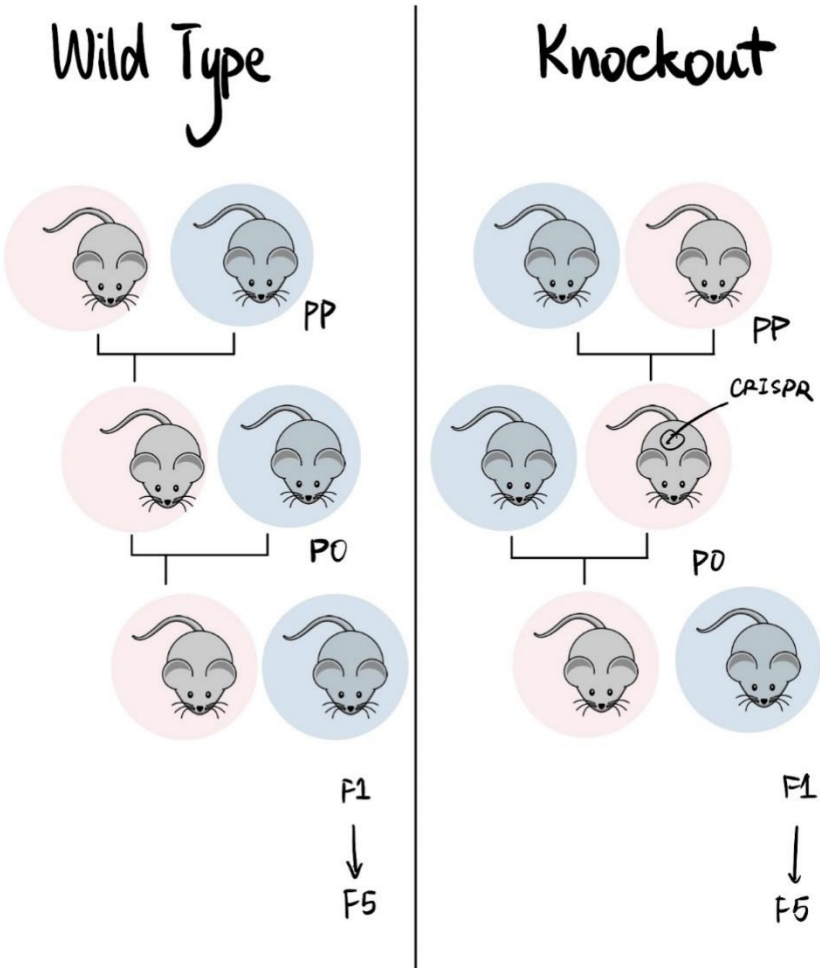


Figure 7. Experiment mice generations description diagram.

4.2. Controls

For the controls in our experiment, we will be regulating the following:

1. Positive Control Group (Knockout): The knockout mice group will have the PIWIL2 gene taken away from the hippocampus from the embryo of the P0 group. Provided the results resulting from previous studies, there are expected results from our experiments that test for behavioral performance tests in both mazes that test for early neurogenesis and short-term memory, with its unknown factor being its biological differences.
2. Negative Control Group (Wild type): this group of mice labeled "wild-type" has not undergone any treatment, and will be undergoing the same procedures as the knockout group, and we will be using

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it as a “normal” reference to compare with the knockout group which has undergone CRISPR treatment.

3. The CRISPR method: is consistently used to knock out the PIWIL2 gene in the knockout group, to ensure that effects observed are specifically due to the knockout of the PIWIL2 gene and not due to other factors.
4. Previous Parents (Parents of P0) will be chosen from the same strain (C57BL/6 for general studies) and the previous parent for both the knockout and wild-type groups will have similar healthy characteristics. Mice will be checked to specific pathogen-free (SPF) to avoid confounding variables from infections.
5. The number of mice: involved in both the wild type and knockout group will be the same starting from the Previous Parent and controlled throughout the entirety of the experiment in all generations. In other words, the offspring in each generation will be randomly selected with a fixed number of male and female offspring from its former ancestor.
6. Third Group with Naive Male Mouse: This third group will be used for both knockout groups and wild-type groups in the breeding process for the next generation. A female mouse from the respective groups will be paired with a naive male mouse from the third group to keep the breeding procedure consistent.
7. And all miscellaneous environmental factors for the environment: Y-Maze dimensions (2 meters for all arms of the Y-maze) and physical characteristics (specified in 4.3 & 4.4), temperature and humidity, lighting, diet, and water.

4.3. Demonstration Y-Maze 1

Hypothesis: Compare the “knockout” group and the “wild-type” group, the “knockout” group should take a longer time to pass through the Y-maze, will have a weaker memory of the electric shot, and have a higher possibility to go to the end with the electric shot. As piwil2 is removed in the hippocampus, it should show an obvious difference in cognitive abilities and memories by the time records and the track of mice passing through the maze.

The Y-maze one is different from Y-maze 2 with a plate carrying an electric shock, which is designed to test neurogenesis in the brain for the mouse to form spatial recognition of the maze’s area and where the electric plate and the exit is located shown in Figure.1. After receiving the electric shot, regular mice should have a shorter reaction time compared to the “knockout” group, in the next trial it should remember there was an electric shot and wouldn’t go to that end to prevent the harm. However, assuming piwil2 removal would have negative impacts on the mice’s cognitive abilities(reaction time) and weaker memories(wrong path), the results of the time difference in seconds between the two groups are shown in Table 1 below. Table 2 shows how this proposal would present the recorded time differences in seconds between each generation compared to the generation above. Except for these two tables, this proposal would also keep track of the specific route each mouse had chosen each time by drawing down the route on printed-out same-scale Y-maze.

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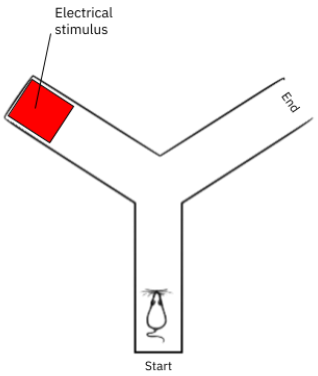


Figure 8. Y-maze 1 with electric shot plate (red).

4.4. Demonstration of Y-Maze 2

Hypothesis: With Control Group- assuming the piwil2 gene is intact in the hippocampus, the memory of the y-maze structure could last until day 5 and the generation with the maximum ability is unknown due to the inheritance of PIWI continuously across generations. Assuming that PIWI can inherit "non-memory" functions, a positive trend (decreased time) is expected.

With Knockout Group- provided that the piwil2 gene is taken away from the hippocampus, the long-term memory of the y-maze structure is unknown but would be worse than the control group because of the lack of ability to conduct neurogenesis in the brain. Assuming that PIWI is taken away from the generations, the disparity between the control group and the knockout group is exacerbated further down the generation line and a negative trend (increased time) is expected.

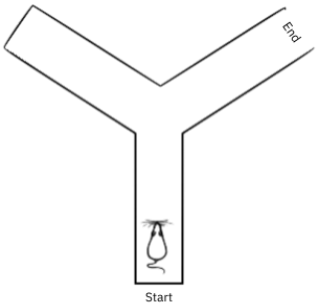


Figure 9. demonstration of Y-Maze 2

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At the start of the experiment the mouse was subjected to the Y-maze (Figure 8) with the electric board 5 times. Assuming that the repeated exposure to electric shocks would generate a trigger for short-term memory formation in the hippocampus, then the mice would be able to remember the Y-maze structure which is nearly identical to the last Y-maze except without the electrical board. This tests its memory from its spatial memory on the first day, and it should take minimal time for the mouse to leave the maze if it carries the memory from the electric shock. Table 3 displays the data table for the second Y-maze, with more columns to be added if the mouse's memory lasts for more than 5 days.

5. Expected results

The results this proposal is expecting could be observed from a few aspects:

1. Y-maze NO.1 with the electric shot plate:

The "knockout" group with piwil2 removal would take longer time in seconds to get out of the maze compared to the "wild-type" group. The route of the "knockout" group mice would be comparatively more complicated. As more generations, the cognitive and memory abilities might decrease or improve.

2. Y-maze NO.2: Assuming short-term memory is constructed from the neurogenesis of the mice on day one, the "knockout group" would take longer time in seconds to get out of the maze compared to the "wild-type" group. The route of the "knockout" group would involve more exploration of the maze if its association of the Y-maze with the electric shock isn't well defined. As more generations pass, the memorization capabilities might deteriorate for the knockout group until the PIWIL2 protein may regrow.
3. MRI: It is possible that removing piwil2 RNA would cause changes in postnatal development in the form of shape and visualization.
4. Gene expression: RNA sequencing can be used to infer what had been changed in the DNA after the removal of piwil2 RNA, which of the corresponding DNA as well as protein would be changed.
5. Transgenerational effect of piwil2 removal: If piwil2 can't be substituted, it might be possible that in later generations it would slowly grow back. If piwil2 can be substituted, in later generations other types of mutations in the DNA might happen for getting a substitution of piwil2 RNA.

6. Conclusion

This study provides compelling evidence of the crucial role that PIWI-interacting RNAs (piRNAs), particularly those associated with the PIWIL2 gene, play in maintaining cognitive functions, neurogenesis, and hippocampal development in mice. Through the selective knockout of PIWIL2 RNA using CRISPR technology, we observed significant impairments in short-term memory, a reduction in neurogenesis, and structural changes in the hippocampus, as confirmed by behavioral tests, MRI imaging, and RNA sequencing. These findings suggest that piRNAs are not only essential for germline development but also have broader implications for brain function and overall neural health. The observed decline in cognitive abilities across generations highlights the potential long-term consequences of piRNA dysregulation, raising concerns about its role in age-related cognitive decline and its association with cancerous growths. Future research should explore the mechanisms by which piRNAs influence neural pathways and assess the potential therapeutic benefits of targeting piRNA pathways in neurodegenerative diseases and cancer.

Acknowledgement

Rixi Lam, Jinxuan Ma contributed equally to this work and should be considered co-first authors.

References

[1] Iwasaki, Yuka W et al. "PIWI-Interacting RNA: Its Biogenesis and Functions." Annual Review of Biochemistry vol. 84 (2015): 405-33. doi:10.1146/annurev-biochem-060614-034258

DOI: 10.54254/2753-8818/72/2024.18893

[2] Li, Yinuo, et al. "Knockout Gene-Based Evidence for PIWI-Interacting RNA Pathway in Mammals." Frontiers in cell and developmental biology vol. 9 681188. 14 Jul. 2021, doi:10.3389/fcell.2021.681188
[3] Lin, Yuan, et al. "PIWI-interacting RNAs in human cancer." Seminars in cancer biology vol. 75 (2021): 15-28. doi:10.1016/j.semcancer.2020.08.012
[4] Van de Berge, Koen, and Lieven Clement. "Introduction to RNA-Seq Sequencing: Preprocessing." Statomics.github.io, 21 Nov. 2023, statomics.github.io/SGA/sequencing_intro.html.
[5] Gasperini, Caterina, et al. "Piwil2 (Mili) sustains neurogenesis and prevents cellular senescence in the postnatal hippocampus." EMBO reports vol. 24, 2 (2023): e53801. doi:10.15252/embr.202153801
[6] Moore, Rebecca S, et al. "Piwi/PRG-1 Argonaute and TGF-β Mediate Transgenerational Learned Pathogenic Avoidance." Cell vol. 177, 7 (2019): 1827-1841.e12. doi:10.1016/j.cell.2019.05.024
[7] www.ncbi.nlm.nih.gov/gene/57746#:~:text=Piwil2%20(Mili)%20sustains%20neurogenesis%20and
[8] John Hopkins Medicine. "Magnetic Resonance Imaging (MRI)." Www.hopkinsmedicine.org, 6 Dec. 2021, www.hopkinsmedicine.org/health/treatment-tests-and-therapies/magnetic-resonance-imaging-mri#:~:text=MRI%20is%20a%20type%20of.
[9] "CRISPR." Genome.gov, www.genome.gov/genetics-glossary/CRISPR#:~:text=CRISPR%20.
[10] Thomson, Travis, and Haifan Lin. "The biogenesis and function of PIWI proteins and piRNAs: progress and prospect." Annual Review of Cell and Developmental Biology vol. 25 (2009): 355-76. doi:10.1146/annurev.cellbio.24.110707.175327
[11] Muñoz-López, Martín, and José L. García-Pérez. "DNA Transposons: Nature and Applications in Genomics." Current Genomics, vol. 11, no. 2, 1 Apr. 2010, pp. 115–128, https://doi.org/10.2174/138920210790886871.
[12] Starmer, Josh. "StatQuest: A Gentle Introduction to RNA-Seq." YouTube, 31 Aug. 2017, www.youtube.com/watch?v=tlf6wYJrwKY. Accessed 20 May 2020.

Appendix

Table 1. Table used to record the time taken each time to get out of the maze. No real data, only for visualize how data would be presented after lab.

Generation 00	Gender	Time to get out of the maze with an electric trigger						
		1st Time	2nd Time	3rd Time	4th Time	5th Time	Average	Standard Deviation
F1	Male							
	Female							
F2	Male							
	Female							
F3	Male							
	Female							
F4	Male							
	Female							
F5	Male							
	Female							

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Table 2. Calculate the difference between each generation compared to the last generation The difference smaller changes over time. No real data, only for visualize how data would be presented after lab.

Generation	Gender	Difference in time between trials of mouse's maze time (s)			
		Difference (1-2)	Difference (2-3)	Difference (3-4)	Difference (4-5)
F1	Male				
	Female				
F2	Male				
	Female				
F3	Male				
	Female				
F4	Male				
	Female				
F5	Male				
	Female				

Table 3. Data chart for Y-maze 2. No real data, only for visualize how data would be presented after lab.

Generation	Gender	Time to escape maze (s)														
		Day +1			Day +2			Day +3			Day +4			Day +5		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
F1	Male															
	Female															
F2	Male															
	Female															
F3	Male															
	Female															
F4	Male															
	Female															
F5	Male															
	Female															