Exosome Related Neural Communication and Memory Inheritance

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ABSTRACT
Transgenerational memory is a newly discovered phenomenon and observed in several different species. Evidence suggests olfactory bulbs and ncRNAs are typically involved. However, it is unknown that the carrier that brings the memory to the gametes. Exosomes, an overlooked substance, is now reconsidered as the most promising carrier of the information. By focusing on parts of the brain related to transgenerational memory, inhibiting exosome release, determining the cargo, and determining the effects of exosomes, it is likely to reveal the true value of exosomes. However, in brain communication between hippocampus and olfactory bulb through exosome is not determined, and the pathway is not clear. This paper provides certain methods based on existing results for future research.

Keywords: *Exosome, transgenerational memory, ncRNA, cellular communication, olfactory bulb*

1. INTRODUCTION

1.1 Background

In 2014, Lamarck found epigenetic inheritance of ancestral odor fear conditioning. When a mouse associates an odor (acetophenone) to danger (foot shock), two generations of offspring (F1 and F2) are more sensitive to the odor, as they were able to detect acetophenone at a lower concentration, an enhanced detection sensitivity specific to the F0-conditioned odor [1]. Behaviorally, when OPS (odor potentiated startle) is computed, there is enhanced OPS to acetophenone compared with the control group, suggesting an increase in sensitivity, but not necessarily more frightened. Anatomically, an observation is made that shows in F1, there is an increase in number of acetophenone-responsive OSNs, and that glomerular area is bigger than control group. Epigenetically, for Olfr 151, one CpG di-nucleotide at the 3' end is significantly less methylated, and as the CpG mechanism goes, less methylation means more transcription [1].

Similarly, in 2019, scientists found out C. elegans can "transfer" memories to their offspring: four generations (F1, F2, F3, F4) have the same odor avoidance as the first generation(P0), the fifth generation(F5) goes back to normal (no avoidance). However, since the odor they avoid is a pathogen, and the underlying mechanism is different from simply smelling an odor. It involves "heat shock response", by increasing the heat shock factor 1 (HSF-1)–dependent expression of genes encoding molecular chaperones, they learn to avoid the odor [2-4]. However, HSP is released when cells are protecting themselves from macromolecules. In conclusion, it is inappropriate to examine C. elegans in this research paper, but it also proves to us the wide magnitude of memory inheritance, and clearly demonstrates the "dying off effect".

1.2 Evolution

Evolutionally speaking, the phenomenon is not hard to explain. It brings the species two advantages.

For one, the memory inheritance allows off springs to better fit the environment: they could be more aware of odors that forbode danger. C. elegans offspring could escape the pathogen, and mouse offspring might be more sensitive to odors of foxes and fear the odor.

However，this memory is only beneficial when the environment stays the same. In most places, environment do change, which makes the mechanism more harmful. Thus, in addition to the born-to-fear effect, the mechanism also allows adaptation just in case the environments change, and the odor does not necessarily forbode danger anymore. In this case, the odor aversion is a poor strategy. In the fifth generation of C. elegans, they return to normal, the memory dies off, which allows C. elegans to try the odor associated food again to presumably broaden the food choice.

Combined, the "born-to-fear effect" and the "dying off effect" allows the specie to protect themselves and broaden the food choice in search for nutrition at the same time.

1.3 Background information and reasoning

Though it seems very logical and reasonable when observed in an evolutionary viewpoint, as this phenomenon is receiving increasing attention, scientists are making effort to answer the question: what is going on in the biological bodies during the whole process? In this research, the focus is on the brain, due to its importance of the whole phenomenon to occur.

1.4 Olfactory bulb

In the olfactory bulb, the molecular bases of odor discrimination at the level of olfactory receptors appear to correlate well with the receptive field in the olfactory bulb where the input signal is further processed to create the specific odor maps [5]. The olfactory system forms a unique spatial organization such that the axons of olfactory neurons expressing the same receptor converge onto fixed glomeruli. OSNs project their axons into specific glomeruli in the olfactory bulb (OB) where they form excitatory synapses onto a complex circuit of interneurons and mitral/tufted (M/T) cells [6-9]. This convergence forms the basis of the glomerular odor map whereby odor information is represented by distinct spatial-temporal patterns of M/T cell apical dendrite glomerular activity [5]. As far as individual neurons are concerned, a model is proposed saying each receptor code contains a zip code and has elements that act positively to promote expression in a subset of ORN classes, and also has elements that restrict expression to a single ORN class. As mentioned before, FC induces epigenetic change and neurogenesis in the olfactory bulb.

1.5 Genetics

With or without FC, genetic regulation plays a crucial role. Researchers found out several activities regulated gene expression. The "activity" is depolarization, or the calcium influx. Activity-dependent, complementary expression of axon guidance factors Kirrel 2 and Kirrel3 contributes to the specificity of OSN axon coalescence by mediating homophilic adhesion. The map of glomeruli, the locations, are also dependent on OR signaling and activity-dependent gene expression [10]. OSNs also depend on activity-dependent gene expression for survival.

1.6 Second messengers and possible pathways

Several routes can take place in activity-dependent gene expression. For one, calcium can follow the pathway of CREB, cAMP response element binding protein. It can silence and translate genes, and it is activity dependent. However, CREB-dependent memories of pathogenic experience are not transmitted across generations. Thus, considering inherited memory, another possible, hypothesized pathway would be through ncRNA.

This work does not rule out other possible pathways, as gene knock-out studies suggested that the cAMP cascade comprised of three components (i.e., stimulatory G protein alpha subunits G alpha olf, adenylyl cyclase type III, and cyclic nucleotide-gated channels) was dominant in transmitting the odorant signal in the olfactory neurons [11]. However, some odorants activate a cAMP cascade (as "cAMP"-elevating odorants), while another signaling pathway has been shown to exist, leading to an increase in IP_3 (by "IP_3"-elevating odorants) (Breer and Boekhoff, 1992) [5]. Thus, the possible pathways are still widely unknown.

1.7 Role of ncRNA

Presumably, as G proteins trigger ncRNA, ncRNA regulate the gene expression. ncRNA has a high correlation with gene regulation. Take piRNA as an example: piRNA, smRNA, and PIWI-clade Argonaute protein mediate gene silencing, the process is called the Ping-Pong Cycle. The mechanism goes that in cells, as piRNA is amplified, it represses active transposons. It cuts a fragment of RNA out from its sequence, which might be important in exosomes.

No matter how clear the mechanism of RNA is, it is noteworthy that the change is limited to individual cells. The route by which the change is transferred from the brain to the germline is rather troubling. Equally troubling is the part of neurogenesis. Here we turn our attention to exosome.

1.8 Exosomes

Exosomes are extracellular vesicles. They have a diameter of 40-100 nm. They are released upon fusion of endosomes to the membrane [14]. Many cell types can secrete exosomes, including cells from the central nervous system (CNS): microglia, oligodendrocytes, astrocytes and neurons [12,13]. They were previously regarded as "garbage bins", cargos of which are proteins, RNA fragments, lipids unwanted and thrown away by cells. However, this mode of thinking has been changed. It is discovered that exosomes allow direct protein transfer, lipid transfer, and RNA transfer. Thus, they might be able to play a role in intercellular communication and transfer.

Exosomes released upon synaptic activation do not bind to glial cells but selectively to other neurons. This suggests that they can help neurons communicate [12,13]. Fusion of endosomes to the plasma membrane

allows insertion of post-synaptic receptors and thereby reinforcing synaptic efficacy [12].

In addition to the synaptic change, there are genetic modifications too. miRNA binds to complementary sequences on target mRNA and control post-transcriptional gene expression. Thus, miRNA-loaded exosomes are used to modify the expression of specific genes. The canonical role of miRNA is regulation of gene expression that might be performed at the post-transcriptional level by repression of translation or induction of mRNA degradation [3].

1.9 Directions

Though exosomes sound like a promising way of intercellular communication, its cargo, effect, and route of transmission remains largely unknown. This work examines the difference in cargo, effect of exosomes to the target cells -- neurons and stem cells, and the route of transmission -- diffusion or through nervous fibers. It is hypothesized that exosomes are excreted during FC, cargo of which will change, and by diffusion, it goes to stem cells and other neurons, inducing neurogenesis and genetic change, respectively. To test the hypothesis, experiments will be conducted to test the difference of cargos of exosomes before and after FC (and possibly make a list of all the RNAs downregulated or upregulated), to examine the genetic expression of receiving neurons (and possibly make a list of all the RNAs downregulated or upregulated) and study the necessity of exosomes in neurogenesis and memory inheritance.

2. MATERIALS AND METHODS

In order to exclude the possibility of communication, only male rats are used in the experiment. Male mouse will undergo FC, pairing an odor (acetophenone) to an electric shock from the floor of the cage. Before everything is done, several pilot experiments were done.

2.1 Pilot experiment one: fMRI and parts of the brain involved

The brain is too big to be examined considering the complexity and uncertainty of the hypothesized theory. Thus, the focus is on olfactory bulb, due to its direct correlation with odor, its well-studied mechanism, and its well-defined change in response both anatomically and cellularly. Another focus is the hippocampus, due to its importance in memory formation.

However, one problem exists that in hippocampus, the structure is too large to make any RNA regulation significant. Thus, fMRI is required to detect the exact neurons (exact place of them, to be more accurate), extract them, and do further research.

2.2 Pilot experiment two: exosome inhibition

mTORC1, CD9, CD63, CD81; ESCRT, Alix, TSG101; Integrins; Hsp, actin and flotillins are genes or proteins discovered to be able to direct exosome inhibition. In this experiment, it is tested whether the up regulations and down regulations might influence other protein's expressions and need to test which of them can be used in the upcoming experiments—maybe some are not usable in the brain, or some don't have a satisfactory effect.

Our priority is to first make sure the method chosen won't affect anything but exosome secretion, and then look at the effectiveness.

2.3 Experiment one: exosome cargo

Two groups of 10 healthy mature mice will be cultured in a suitable environment and have enough food and water support for a 10-day period pre-expose in the content. One will undergo FC while the other would not. Acetophenone odor will be introduced with electric shock to another group of mice and wait until fear conditioning is fully formed in the mice. Then, parts of the olfactory bulb and hippocampus related to FC from the brain are transferred to incubation plates, where ionomycin induces exosome release. After purification, the cargo is revealed. A comparison will be made between FC group and control group. What has to be noticed is that a 10-day period is existent between FC and RNA regulation completion, thus this work will examine the cargos and expressions throughout the 10-day period after FC.

2.4 Experiment two: effects of exosome

Two (different) groups or rats will both experience FC. However, one group goes with exosome inhibited. Considering the possibility of communication of olfactory bulb and hippocampus, this work inhibited these two parts both separately and at the same time. Thus, three experiments are done separately. Then the behavioral, neuronal, anatomical, and transgenerational effects are tested: whether they elicit FC, how is the proteins different (both protein type, and protein percentage), whether fMRI shows any difference in size of the OSN responsible (which is also an indication of stem cell deriving), and whether the offspring has any change in behavior.

3. RESULTS AND CONCLUSION

3.1 effects of FC on exosome cargo and cell expression

Cell expression is expected to change the percentage of proteins, as shown in previous studies. However, the exosome cargo is not examined, and thus, unsure

whether it will change. However, because activity regulated genes are present and involved in the experiment, it is very likely that cargos will change, with new molecules, or upregulations and down regulations. Another aspect is the number of exosomes released: are they released more often or less often?

If cargoes change, then a comparison will be done to examine the list of change in exosomes and a list of regulated RNAs. For the overlap, the most direct interpretation is that the cargo goes into the cells and starts duplicating, transcribing, or even being amplified. For the non-overlap, it can also be interpreted that as a ncRNA, it regulated other RNAs. However, these interpretations need future examination, and the significance of this experiment it to point out the RNAs that have the value to be examined as far as inherited memory is concerned. As a matter of fact, our experiment might also shed light to the connection between RNA regulation and exosome cargo. As mentioned before, ping-pong cycle might have a link in producing the cargo in exosome. Thus, a mutual link is hypothesized: RNA regulation produces the cargo of exosome, and exosome regulated RNA expression.

3.2 effect of exosome on gene expression

Previously, the experiments examined the variation of gene expression before and after FC. This experiment would examine the gene expression with or without exosome. One thing to be mentioned it the group that tests the gene expression after FC and the group with exosome is the same group, based on the fact that the situations are exactly same.

These experiments not only reveal the effect of exosome on gene expression, but also highlights the possibility of in brain communication through exosome. Suppose an RNA is found only in exosome of hippocampus not olfactory bulb. An OSN expressing the RNA is a strong indication that there is communication involved, and this exact hypothesis is tested in experiment two.

3.3 effect of exosome on neurogenesis

As FC stimulates stem cells to derive into OSNs responsible for the exact odor, the information cannot be passed to stem cells via electric signals and neurotransmitters. Considering the gene regulation involved in derivation, the focus is on exosomes.

Two groups will be used: with exosome or without exosome. When placed in fMRI, acetophenone will be present to trigger OSNs. If these two groups show a difference, then exosomes are responsible for stem cell derivation. This further proves exosomes are gene regulators, and that they perform as intercellular communicators. However, another possibility is that there is no difference. One explanation is the plasticity—substitutes of exosomes are used. In this case, this work might discover a unique and unexpected communicator. Another explanation is that stem cells is regulated by Wnt pathway, which doesn't include exosomes. However, Wnt is often paired with early brain plasticity, so its involvement in adult rats is an area worth studying.

3.4 Inherited memory

In these two groups, this paper compares the epigenetic differences in the sperm. In addition, fMRI and behavioral tests will be presented to their offspring. This process experiments three generations after P0, as is consistent with the existing literature.

The measurements of sensitivity in the offspring, an indication of the memory inheritance, is Fear-potentiated startle (FPS), a behavioral test to assay for fear learning. It is a readout of sensitivity, not fear [8].

4. CONCLUSION

Although the previous literatures are very inspirational and professional, they, combined, is not painting a whole picture. One piece of puzzle missing is the cargo of exosomes and the effects of them, few papers address this problem. The significance of this is revealing the route of the RNA transfer as well as its effect. When fully understood, the origin, pathway, and results of RNA fragments in the brain can be known, and this is a bridge linking environmental stimulation and genetic expression as well as a bridge linking different parts of the brain (reasoning: in 4.3).

In this paper, the main fucus is intercellular communication from multiple perspectives. The research concludes the effects of FC on exosome cargo and cellular gene expression, as well as the effects of exosomes on neurons and stem cells. The effects will presumably explain the increase in OSN, increase in sensitivity of OSN, as well as the inheritance. In addition, this highlights the effect of in-brain communication.

We hope this would shed light to a fully understood in-brain communication, not just through nervous fibers, but also exosomes. However, the work is not able to determine the way exosomes transfer: whether diffusion or transduction. Both are possible, as diffusion is through blood, while transduction is through fiber. Professor Samuel Kunes designed an experiment that can be used when trying to find the exact route of exosomes. The main idea is to put a tracer in the exosome. He describes the technique to modify a piRNA, creating a fake piRNA using CRE-Loxp. Thus, any cell that has this fake piRNA must be in the route. In this case, it is crucial to determine what piRNA to modify. And the fact is, our study exactly sequences the cargo, and finds the correct piRNA to modify. This experiment is presented for future research teams to refine and use.

Limitations of the experiments are existing. This research is focused on olfactory bulb and hippocampus, while amygdala is also involved, due to the nature of fear. Other structures might be important too. Thus, future research should look at the entire brain, trace the exosomes: both their origins and routes, and design a map of exosome-based brain communication in FC. This might be a huge project like how people are now drawing the neuron-based map of the brain, but this is a direction lying here.

Another future direction is the effect of exosomes throughout the whole body. This is equally valuable as this will present us a picture of how brain controls the whole body, not only through hormone and nervous fibers, but also exosome. Right now, exosomes have an increasing importance in treating diseases, and we would like it to be extended and refined.

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