The Role of Long Non-Coding RNA in Major Depressive Disorder: A Mouse Model, with Special Emphasis on the Prefrontal Cortex

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Abstract
Objectives: After many years of research, the understanding of the pathophysiology of major depressive disorder (MDD) is still incomplete. Due to the fact that only a subset of patients respond to the available drugs for depression, there is a need for a better understanding of the molecular mechanisms of depression to find new drug targets. The overexpression of certain genes and further research regarding the implications of different forms of RNA, specifically long non-coding RNA, may be the key to combatting depression in the future.

Methods: Molecular cloning was performed to make viruses in order to overexpress four lncRNAs in the prefrontal cortex of mice. The mice underwent surgeries to insert the lncRNAs into their brains and multiple behavioral tests were conducted. Following sacrificing of the mice, brain punches were taken, neuronal nuclei were isolated through fluorescence-activated cell sorting (FACS), and the RNA was purified. Promoter prediction was then done to determine transcription factors that regulate the lncRNAs and fluorescence in situ hybridization (FISH) was conducted.

Results: Large subsets of lncRNA were differentially expressed in depressed brains compared to controls. In addition, three lncRNAs had a significant effect on depression-like behaviors of female mice. However, there were no significant results indicating a connection between these three lncRNAs and depression in male mice. As shown by these results, differential regulation of these lncRNAs is sex specific, whether in female and male mice or humans. Through promoter prediction, two transcription factors, Npas2 and Egr1, were identified as potential regulators of these lncRNAs.

Discussion: Specific lncRNAs are potential key regulators in depression and further research could potentially uncover more. Additional bioinformatics will be necessary as well as RNAseq of the brain punches to reveal the amount of RNA present in each sample. Npas2 and Egr1 will need to be tested in cell lines to confirm whether or not they regulate the lncRNAs. Future research would involve overexpressing these lncRNAs in mice in different brain regions of the reward pathway and testing their behavior to find similar correlations between lncRNAs, sex, and depression-like behaviors.
1.0 Introduction

Major depressive disorder (MDD) can be defined as a mental state resulting in dysphoria, a negative emotional state characterized by severe despondency and discouragement. Anhedonia and depressed mood are considered core symptoms, but those affected often also experience abnormalities in sleep, appetite, cognitive ability, and more. It is a common mental state that currently affects about 350 million people worldwide and is largely more prevalent in women than in men (LaPlant et al., 2009). The susceptibility of depression in women and in men is due to a combination of both genetic and environmental factors (LaPlant et al., 2009; NIH, 2015; Marieb et al., 2013). After many years of research, the understanding of the pathophysiology of depression is still only partial. Due to the fact that only a subset of patients respond to the available drugs for depression, there is a need for a better understanding of the molecular mechanisms of depression to find new drug targets.

The reward pathways of the brain are responsible for mediating and reinforcing behaviors that induce a feeling of pleasure (NIH, 2015). The three main brain regions involved are the Ventral Tegmental Area, the Nucleus Accumbens, and the Prefrontal Cortex, which are considered part of the dopaminergic mesolimbic system (Fig. 1) (Ikemoto et al., 2013).

Dysfunction of the brain reward pathways is involved in depression and in drug addiction disorders (NIH, 2015).

In order to determine which genes are involved in depression and how altering their expression can affect the reward pathways, multiple studies have been done using mice models (Krishnan et al., 2007; Feng et al., 2015; Ikemoto et al., 2014). Many studies associated with depression involve isolating mice for a period of weeks and testing their social interaction with other mice, as well as putting them in other stressful situations. Researchers in the Nestler lab

![Fig. 1 Brain regions involved in the reward pathway (Felhaber, 2012)]
identified a series of protein coding genes that are both necessary and sufficient to induce depression and anxiety-like behaviors in mice stress models. Furthermore, individual differences in the response to stress were found as “susceptible” mice showed a significant decrease in body weight and sucrose preference, and displayed more depressive-like behaviors in response to stress unlike mice that were stress “resilient” (Krishnan et al., 2007). However, both groups of mice showed anxiety-like behavior, as seen when they were placed in a maze and didn’t spend much time in the open, perhaps because they felt increased vulnerability (Krishnan et al., 2007).

In order to reverse some of these physical abnormalities, researchers have been experimenting with the over and under expression of different neural substrates. The overexpression of certain genes and further research regarding the implications of different forms of RNA, specifically long non-coding RNA, may be the key to combatting depression in the future.

Recent scientific research regarding non-coding RNAs has shown that they can play an important role in many biological processes (Fig. 2) (Quinn et al., 2016; NIH, 2015; Fenoglio et al., 2013). Different classes of non-coding RNA have been created based on their characteristics and functions. For example, long non-coding RNAs (lncRNAs) are classified by their length: greater than or equal to 200 nucleotides (NIH, 2015). There are currently known to be at least two times more lncRNA genes in the human genome than protein coding genes. These include lincRNAs, antisense, sense intronic, and sense overlapping lncRNAs. Additionally, most of these lncRNAs are found only in primates, and 40% of them are brain specific.

LncRNAs are transcribed in the genome and their dysregulation has been linked to many disorders (Fenoglio et al., 2013). LncRNAs have been found to play multiple roles in different epigenetic mechanisms throughout the body. They can act as scaffolds for large complexes or decoys, sequester molecules in the cells or regulators, and act as activators or repressors. Molecularly, lncRNAs can interact with DNA, RNA or proteins.
Different classes of lncRNAs have also been shown to regulate and modify genes that result in neurodevelopmental disorders such as schizophrenia, Rett syndrome, and Fragile X syndrome, but their implications in depression must be researched further (Barry, 2014). For example, researchers have found associations between Ube3a-as and Angelman Syndrome, BC200 BACE1-AS and Alzheimer’s, and DISC2 Gomafu and other psychiatric disorders, implicating that lncRNAs have varying biological functions brain pathology (Fenoglio et al., 2013). Although it is known that lncRNAs can regulate genes and the chromatin state, the role of lncRNAs in depression is unknown. Such studies can make a breakthrough and even be able to reverse the effects of depression directly from the source (Chu et al., 2011).

2.0 Research Question and Hypotheses

This research is focused on determining whether or not certain types of lncRNAs can influence a mouse’s susceptibility or resilience to depression and its response to treatment. Through bioinformatically analyzing lncRNA expression patterns in the depressed brains of human and mouse models, it was possible to identify key lncRNAs and their target genes. Following up with correlation analysis within the RNAseq data between the expression levels of lncRNAs and protein coding genes and other further bioinformatics made it possible to determine four lncRNAs that may act as biomarkers for depression. It is expected that lncRNAs RP11-298D21.1, RP4-630C24.3, RP11-148L24.1, and linc00473 will increase mice’s susceptibility or resilience to depression-like behaviors, showing to be key regulators of MDD.
3.0 Materials and Methods

3.1 Molecular cloning to make viruses for lncRNAs overexpression

In order to introduce these four promising human lncRNAs into the mouse brain, viruses had to be used. Each lncRNA was cloned into a bacterial plasmid (Fig. 3) that was further used to generate synthetic Herpes Simplex Virus (HSV). These plasmids, and later on viruses, were validated both in cell lines and in mice to confirm their ability to overexpress the depression-related lncRNAs.

![Plasmid DNA replication](image)

*Fig. 3 Plasmid DNA replication (Lodish et al., 2000)*

The overexpression vectors were then sub cloned to replace the Green Fluorescent Protein (GFP) reporter with RPL10-GFP using restriction enzymes. RPL-10 is ribosomal RNA that is expressed in cell nuclei and will enable improving the FACS to sort only the infected neurons.

3.2 Surgeries and Mouse Behavioral Tasks

Mice were assessed at baseline conditions, then the 51 female mice were split into groups based on which virus they would receive, and a control group received only GFP. The mice were anesthetized by my mentor with a small dose of ketamine. Then, she placed two small needles on the prefrontal cortex of the brain and small amounts of the viruses were injected at a time.

Following surgeries, the mice underwent a series of behavioral tests. The females underwent a forced swim test, sucrose preference test, novelty suppressed feeding, elevated plus maze, and a splash test. During the forced swim test, the mice were each put in a beaker of water in a cubicle and the time it took for them to stop swimming was measured. The purpose of this test was to establish a possible connection between the virus the mouse was infected with and its motivation. Sucrose preference tests and splash tests are tests of a mouse’s ability to feel pleasure. In the sucrose preference test, mice were isolated in a cage with a choice of water and
a sucrose solution. Every day the weight of the bottles was measured to determine if the mice were experiencing the reward of consuming the sucrose water or if that reward was being suppressed by the virus. During the splash test, a few drops of sucrose solution were dropped on the mouse’s fur and the time it spent continuing to groom itself was measured. The purpose of both the novelty suppressed feeding test and the elevated plus maze were to determine whether the mice were willing to spend more time in the open or if they felt more comfortable spending their time in a corner or sheltered in some way. This vulnerability is associated with depression. Next, the results were analyzed statistically to see if certain groups of the mice had demonstrated increased depressive-like behaviors.

3.3 Brain punches, FACS and RNA purification

Following the behavioral tests, the mice were sacrificed by my mentor and their brains taken out for punching. Once the brain was outside of the mouse, it was put on ice and sliced thinly. Next, it was looked at under a microscope to see how accurately the GFP and virus were placed in the prefrontal cortex. The areas that lit up green were punched out of the brain and put into small tubes on ice. Next, neuronal nuclei were isolated from the brain punches using fluorescence-activated cell sorting (FACS) to isolate only the infected neurons from the brain punch. Subsequently, RNA was purified using a direct-Zymo micro kit. Each tube was centrifuged and 1 volume of 70% ethanol solution was added. The solutions were mixed by pipetting and then transferred to a spin column. This was then washed and centrifuged once again to remove the ethanol and excess solution. A buffer was then added to the columns and centrifuged to isolate the RNA, and finally, the RNA was eluted to a collection tube.

3.4 Promotor Prediction

In order to determine the transcription factors that regulate the depression related lncRNAs, we used a software called Homer, manufactured by the Salk Institute, to identify motifs upstream to the lncRNAs. These lists of transcription factors were overlaid with the list of transcription factors that are known to be regulated by depression and are predicted to be upstream regulators of the MDD data set. Through this overlay, we identified protein coding genes Egr1 and Npas2 as regulators of the four lncRNAs.

3.5 Fluorescence in Situ Hybridization (FISH)
This was followed up with fluorescence in situ hybridization (FISH) and a stellaris single molecule kit was used according to the manufacturer instructions to determine the sub cellular localization of lncRNA RP4-630C24.3.

4.0 Results

The expression pattern of lncRNA in the six depression related brain regions was tested both in male and female post mortem tissue and compared to healthy controls using RNAseq. Large subsets of lncRNA were differentially expressed in depressed brains compared to controls, exhibiting a robust brain site and sex expression pattern (Fig. 4).

![Fig. 4 Differential regulation of lncRNAs in both male and female brain regions. Yellow=upregulated, Blue=downregulated, Gray=no regulation](image)

In addition, the degree of overlap in the list of lncRNAs differentially regulated by depression in each specific brain between male in female is strictly low (Fig. 5).
A handful of lncRNAs were differentially regulated in all brain sites in depressed male or females compared to controls (Fig. 6). From these, lncRNAs RP4-630C24.3 and RP11-148L24.1 were chosen for further studies. Additional bioinformatic analysis pointed out RP11-298D21.1 and linc00473 as potential key regulators of depression.

To determine the molecular targets of these lncRNAs, they were overexpressed in human neuro progenitor cells (NPC) followed by RNAseq. Next, the effects of the overexpression of the
depression related lncRNAs on the transcriptome was compared to the transcriptional profile of depressed females using Rank Rank Hypergeometric Overlap (RRHO) (Fig. 7).

David gene ontology software was then used to classify the genes that were overlapping between RP11-298D21.1 and the human MDD dataset into functional biological clusters (Fig. 8).
The three out of the four depression-related lncRNAs are upregulated in all brain regions in female depressed subjects compared to controls and not in male subjects, except for linc00473, which is down regulated in depressed females compared to controls. All the four lncRNAs are primate specific and not expressed in mouse brains. We hypothesized that expressing the upregulated depression-related lncRNAs in mouse PFC will mimic the human phenotype, thus increasing depression-like behaviors in females but not male mice.

As shown in Fig. 8 the female mouse behavioral tests, the three lncRNAs had a significant effect on depression-like behaviors. In the novelty suppressed feeding test, female mice expressing lncRNAs RP11-148L24.1, RP11-298D21.1, and RP4-630C24.3 in their PFCs had a longer latency to interact and longer latency to feed during the test, but not at the home cage, suggesting increased anxiety and depression-like behaviors compared to the controls. In addition, in the elevated plus maze test the RP11-298D21.1 group spent significantly less time in the open arms, indicating increased anxiety. Female mice expressing RP11-148L24.1 spent more time immobile in the forced swim test, indicating decreased motivation. Finally, lncRNAs RP11-298D21.1 and RP4-630C24.3 groups also demonstrated anhedonia, as shown by a decrease in sucrose consumption particularly on the third day of the sucrose preference test compared to the
other groups. As for the male mice, there were no significant results indicating a connection between these three lncRNAs and depression, shown through the behavioral tests (Fig. 9).

Fig. 8 Female mouse behavioral test results 8.1 novelty suppressed feeding test 8.2 novelty suppressed feeding post test 8.3 novelty suppressed feeding time to interact 8.4 elevated plus maze time spent in the open 8.5 forced swim test 8.6 Sucrose preference test
As a result of these behavioral tests, it has been uncovered that differential regulation of these lncRNAs is sex specific, whether in female and male mice or humans. Increased anxiety and depression-like behaviors were shown in female mice that expressed human depression-related lncRNAs in the prefrontal cortex. However, as shown by the graphs, the overexpression of depression-related human lncRNAs had little to no effect on male mice susceptibility to anxiety and depression-like behaviors.

To identify upstream regulators of the depression related lncRNAs, the predicted promoters of these lncRNA were analyzed using bioinformatic tools to identify putative transcription factors binding sites. The list of these predicted transcription factors was overlaid with data on differential expression in MDD to identify two transcription factors, Npas2 and
Egr1, as potential regulators of these lncRNAs. Further experimental work is need in order to elucidate the interaction between the depression-related lncRNAs and Npas2 and Egr1.

5.0 Discussion

As shown through the above behavioral tests, lncRNAs are regulated in depression. The three lncRNAs, RP4-630C24.3, RP11-148L24.1, and RP11-298D21.1, were shown to have a significant effect on female mice’s susceptibility to depression-like behaviors. However, these lncRNAs did not show a significant effect on male mice, as shown in the behavioral tests. This indicates that these lncRNAs are not only regulated in depression but in a sex-brain-site specific manner.

The data collected from the overexpression of these lncRNAs in the mice’s PFCs mimics the female dataset but not the males, showing that, like in humans, females are more easily susceptible to depression and anxiety (LaPlant et al., 2009; NIH, 2015; Marieb et al., 2013). Additionally, the overexpression data of lncRNA RP11-298D21.1 in the NPCs mimics the transcriptional profile of the human female MDD dataset. Overall, these results indicate that specific lncRNAs are potential key regulators in depression and that further research could potentially uncover more.

The next step, after bioinformatically analyzing all of the data, will be RNAseq of the brain punches that were overexpressed with the lncRNA to reveal the amount of RNA present in each sample. This transcriptional profile will then be compared to the human MDD dataset to find additional similarities.

The two transcription factors that were identified as potential regulators of these lncRNAs, Npas2 and Egr1, will be tested in cell lines to confirm whether or not they regulate the lncRNAs. They will be overexpressed and knocked down in cells, and the expression levels of the lncRNAs will be measured to see the effects of these transcription factors.

Further research would involve overexpressing these lncRNA in mice in different brain regions of the reward pathway and testing their behavior. A few of these brain regions might include, but are not limited to, the Nucleus Accumbens (NAc) and the Ventral Tegmental Area (VTA). Once again the mice should undergo a forced swim test, a novelty suppressed feeding test, an elevated plus maze test, a marble burying test, and a sucrose preference test because as shown before, not all of the tests show significant results.
The datasets of the tests in other brain regions should then be overlaid with the human MDD dataset as well to find similar correlations between sex and depression-like behaviors.
References

