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Regulation of miR-9 by Ethanol and the Effects of miR-9 Inhibition

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Abstract

Alcohol addiction is a complex disease that alters molecular pathways within the brain and leads to the development of alcohol tolerance. One of alcohol's primary targets in the brain is BK potassium channels. BK channels are alternatively spliced and the splice variant expression is regulated by miR-9. Exposure to alcohol upregulates miR-9, an miRNA that modifies the cellular composition of BK channel isoforms and contributes to alcohol tolerance. The molecular mechanisms by which miR-9 is upregulated upon alcohol exposure are currently unknown. We hypothesize that miR-9 upregulation occurs at the transcriptional and/or post-transcriptional level and that inhibition of miR-9 will diminish the acute and long-term effects of alcoholism. We will investigate whether alcohol upregulates miR-9 through interactions with the miR-9 promoter and attempt to identify alcohol sensitive regions of the miR-9 promoter. Another mechanism by which alcohol may upregulate miR-9 is by directing post-transcriptional miRNA maturation through interactions with the enzymes Drosha or Dicer. Lastly, we will inhibit miR-9 expression through RNAi to characterize its role in ethanol sensitivity. Identification of the mechanism underlying miR-9 upregulation after exposure to alcohol will not only provide further insight into alcohol addiction, but may also contribute to the development of potential therapeutic treatments for alcoholism.

Background

Drug addictions are pervasive diseases within modern society that result in substance dependence and are characterized by habitual drug use despite serious negative consequences. The most commonly abused drugs include heroin, nicotine, cocaine, and alcohol. Drug abuse is a societal burden costing the United States half a trillion dollars annually and affecting 7.5% of the US population over the age of 12 (1). Addictions are particularly complex diseases due to the involvement of multiple factors including genetics, neurobiology, and interactions with the environment (2). Excessive drug abuse is known to induce molecular changes within the brain that promote continued drug use and increase an individual's susceptibility to relapse after drug use has ceased (2, 3). Habitual drug use also leads to tolerance as a result of decreased sensitivity to the drug (2). One of the primary challenges in understanding addiction is identifying the molecular changes that underlie the development of dependence and tolerance. Identifying the molecular mechanisms and cellular adaptations involved in addiction is essential to understanding these neuronal disorders and in developing possible treatments.

In alcohol addiction, BK potassium channels are a principal target and may play a central role in alcohol tolerance (4-7). BK channels are large-conductance, calcium

and voltage-activated potassium channels (8, 9). These channels are widely expressed in the brain, and they influence the initiation and propagation of action potentials (10, 11). BK channels are alternatively spliced, and the production of different splice variants contributes to the functional diversity of BK channels in the brain (8). Alternative splicing of BK channels may also contribute to neuronal plasticity, which is essential to the development of addiction (8).

The specific effects of alcohol on BK channels are still not well understood, although recent studies have provided important insight into the role of BK channels in behavioral and neurobiological responses to alcohol. Deleting the gene encoding neuronal BK channels in *C. elegans* decreases alcohol sensitivity (4). Also, BK channels develop tolerance to alcohol in two mammalian brain regions significant to alcohol abuse and addiction: the supraoptic nucleus (SON) and the striatum (7). Exposure to alcohol rapidly alters the composition of BK channel splice variants within SON and striatal neurons (8), and different BK channel isoforms have different sensitivities to alcohol (8). These changes in BK channel mRNA expression are the result of post-transcriptional regulation by the microRNA (miRNA) miR-9, which is upregulated by alcohol (8). Upregulation of miR-9 may thus contribute to both the acute effects of alcohol and the long-term development of tolerance (8). In addition to BK channels, miR-9 is predicted to have multiple other targets that are involved in the nervous system's response to alcohol (8). These findings indicate that miRNA, and more specifically miR-9, may have a significant role in the development of alcohol addiction and tolerance.

miRNA are small (approximately 19-25 nucleotides long), noncoding RNAs that silence gene expression post-transcriptionally (12 - 15). Emerging evidence shows that miRNAs play a role in numerous biological processes, including development, proliferation, and apoptosis, and they have been implicated in the pathogenesis of various diseases including cancer (12 - 15). Most miRNA are expressed under the control of their own promoters and regulatory sequences, but some are clustered together on DNA and may be co-regulated (12 - 15). The biogenesis of miRNA is a multi-step process in which primary miRNA (pri-miRNA) are cleaved within the nucleus into precursor miRNA (pre-miRNA) by an enzyme complex including Drosha, an RNase III family nuclease (16 - 19). This is followed by processing of pre-miRNAs in the cytoplasm by Dicer, another RNase III, which cleaves the double stranded pre-miRNA to form mature miRNA (15, 21). The mature miRNA can regulate gene expression of complementary mRNAs by binding to the miRNA recognition element (MRE) in the 3'-untranslated region (3'UTR) of target mRNAs (8, 16).

Although the scientific community has gained significant knowledge regarding miRNA biogenesis and biological function, very little is known about miRNA regulation. Currently, miRNA regulation is known to occur at two levels. miRNA can be regulated transcriptionally through regulatory sequences present in promoters or transcription factors (22). For example, miR-1 is regulated by the transcription factors MyoD, Mef2, and SRF (23). The expression of miRNA can also be regulated post-transcriptionally by affecting the activity of Drosha and Dicer in miRNA maturation (Lee Y., et al. 2002; Obernosterer G.,, et al. 2006). For instance, SMAD protein interaction with Drosha regulates miR-21 expression (24). Furthermore,

Dicer has been implicated in regulating the expression of miR-138 (25).

As stated previously, miR-9 is up-regulated by alcohol exposure (8); however, the mechanism by which this occurs is unknown. Additionally, miR-9 is known to play a significant role in the brain's response to alcohol and the development of tolerance (8), yet it is not known whether miR-9 is required for ethanol sensitivity. Given the importance of miR-9 in neuroadaptation to alcohol and its potential role in the development of alcohol addiction, it would be extremely valuable to identify the mechanism underlying miR-9 regulation by alcohol and to determine whether miR-9 is required for alcohol sensitivity.

Our interest lies in the role of miRNA in the development of alcohol addiction. We propose to test the hypotheses that alcohol is capable of upregulating the expression of miR-9 at the transcriptional and/or post-transcriptional level and that miR-9 is required for ethanol sensitivity.

Relevance

Broader Relevance: Drug abuse is an escalating economic, health, and social concern in the United States, and it frequently leads to drug addiction. Drug addictions cost the U.S. over half a trillion dollars annually and alcoholism accounts for $185 billion of the total expense (21). Alcohol addiction has potent health risks ranging from mental diseases to physical disorders, and in extreme cases may cause death (26, 27). Thus, there is a need to elucidate the molecular mechanisms associated with the development of alcohol addiction. The findings of this proposal will clarify the molecular basis underlying alcohol addiction, which may contribute to the development of potential therapeutic treatments.

Intellectual Merit: This proposal first seeks to identify the molecular mechanisms that lead to alcohol addiction. An understanding of these pathways may enhance further research regarding other drug addictions. We also propose to investigate the newly discovered miRNAs. Current evidence links the miRNA miR-9 to alcohol sensitivity and to the development of alcohol tolerance. miRNAs are ubiquitously expressed in mammals as regulatory molecules and are linked to disease pathogenesis (28, 29). Hence, discovering the mechanism by which miRNAs are regulated will provide more insight into the functional role of miRNAs in mammals. With these findings, we hope to foster knowledge within the scientific community.

Specific Aims

The long-term goal of this study is to clarify and find possible treatments for the molecular basis of alcohol addiction. This study will investigate how miR-9 is upregulated upon exposure to ethanol and the effects of miR-9 inhibition on ethanol sensitivity. The hypotheses are that miR-9 upregulation by ethanol depends on a specific molecular mechanism that affects transcription and/or miRNA maturation and that inhibition of miR-9 will diminish the acute effects of alcohol exposure while concomitantly reducing alcohol tolerance.

1.   To characterize the role of the miR-9 promoter in miR-9 upregulation due to ethanol exposure: miR-9 will be inserted with the miR-9 promoter into the pcDNA6.3V5-pL-DEST gateway vector, which will be transfected into a HEK293 cell line. We will then observe the effects of ethanol exposure on the transcription. We will do the same procedure with miR-131, another miRNA, and luciferase, a protein encoding gene. We will also determine if there are alcohol sensitive region(s) of the miR-9 promoter.

2.   To characterize miR-9 upregulation by examining ethanol's effects on post-transcriptional miRNA maturation: We will assess whether alcohol exposure upregulates pri-miR-9, pre-miR-9, and/or mature miR-9 expression in rat striatal and SON neurons. We will then use *in vitro* and *in vivo* assays to determine if alcohol upregulates miR-9 through interactions with either Drosha or Dicer.

3.   To characterize ethanol sensitivity through the inhibition of miR-9: We will inhibit miR-9 functionality in rat SON and striatal neurons through RNA interference to evaluate miR-9's role in ethanol sensitivity. We will use *in vivo* assays to examine any physiological changes in the cells that are caused by alcohol.

Research Design and Methods

1.   To characterize miR-9 upregulation in DNA transcription through interactions between ethanol and the miR-9 promoter:

Rationale: We suspect that alcohol behaves like steroid hormones and heat shock proteins, which regulate gene transcription through regulatory sequences (30, 31). Therefore, we believe that alcohol upregulates miR-9 by targeting the miR-9 promoter. We will examine the role of the miR-9 promoter in response to alcohol exposure using the pcDNA6.3V5-pL-DEST gateway vector, a vector without a promoter that can be expressed in mammalian cells. This vector will be transfected into HEK293 cells, which lack endogenous miR-9 (8). We will also couple the miR-9 promoter with miR-131 and luciferase in order to confirm that alcohol affects the promoter independent of the gene with which it is coupled.

Design and Method:

1. The Effects of Ethanol on the miR-9 Promoter and Gene Transcription: In order to determine the effects of ethanol on the miR-9 promoter, we will observe the transcription of miR-9 after alcohol exposure. In addition to using the miR-9 gene, we will use the miR-131 gene since it encodes a miRNA that is exclusively expressed in the brain (8). Secondly, we will use the protein encoding gene luciferase, a well-documented reporter gene, because its transcription is easily measured through bioluminescence (32). Since the miR-131 and luciferase genes are not known to be affected by alcohol, they should not be upregulated upon alcohol exposure unless they are coupled with the miR-9 promoter.

The miR-9 promoter will be inserted into the pcDNA6.3V5-pL-DEST gateway vector with the miR-9, miR-131, or luciferase gene (33). The vectors will then be transfected into HEK293 cells. We will also transfect HEK293 cells with an empty vector to serve as a control. The cells will be bathed in an ethanol-free medium or a medium containing 5mM, 20mM, or 50mM ethanol for 24 hours. Total miRNA transcription will be measured using reverse transcription followed by real-time PCR using methods previously described (34, 35). Transcription of the luciferase gene will be measured with a luminometer (36). Lastly, transcription will be analyzed using Northern blot.

Prediction: We expect that alcohol will upregulate gene transcription via the miR-9 promoter, irrespective of the gene with which the promoter is associated. Thus, we believe the results will show that gene transcription will increase as the concentration of ethanol increases.

2. Identification of the miR-9 Promoter Region Affected by Alcohol: The proximal promoter (hereafter referred to as the promoter) region is the location of transcription factor binding and transcriptional regulation (37). We will attempt to identify the specific region of the miR-9 promoter where ethanol binds and upregulates transcription. Little is known regarding the structure of the miR-9 promoter.   Therefore, to determine the region responsible for ethanol's effects, we will splice the promoter into three equal sections – identified as sections 1, 2, and 3. Each piece, as well as the core promoter, will be individually inserted into the pcDNA6.3V5-pL-DEST gateway vector with the miR-9, miR-131, or luciferase gene. We will then combine sections 1 and 2, 1 and 3, and 2 and 3 with the core promoter and insert these combinations into the vector with each of the aforementioned genes (38). These vectors will then be transfected into HEK293 cells. We will also transfect HEK293 cells with an empty vector to serve as a control. The cells will be bathed in an ethanol-free medium or mediums containing 5mM, 20mM, or 50mM ethanol for 24 hours (7, 8). We will measure the transcription levels of the three genes when combined with the different promoter recombinants using the analyses described in section one.

Prediction: We anticipate that one of the promoter regions (1, 2, 3, or a combination) will increase gene transcription when exposed to ethanol. This will demonstrate that alcohol interacts with one specific region of the promoter, thus upregulating miR-9 transcription. If ethanol does not upregulate miR-9 through the promoter, it must be upregulated through an alternative mechanism such as post-transcriptional regulation of miRNA maturation.

2. To characterize miR-9 upregulation by examining whether ethanol regulates post-transcriptional miRNA maturation.

Rationale: Several miRNAs are regulated at the post-transcriptional level through processes that modulate miRNA maturation and involve interactions with enzymes that participate in miRNA biogenesis, such as Drosha and Dicer (3, 16, 25). Therefore, we believe upregulation of miR-9 after alcohol exposure may be occurring post-transcriptionally through interactions with Drosha or Dicer. Rat striatal and SON neurons will be used to test expression of pri-miR-9, pre-miR-9, and mature miR-9 since miR-9 is known to be expressed in these cells (8). *In vitro* and *in vivo* assays will then be used to evaluate interactions of miR-9 with Dicer and Drosha since these are well-described methods for assessing enzyme activity in miRNA processing.

Design and Method:
1. Ethanol's Effect on miR-9 Biosynthesis: In order to investigate the effects of ethanol on miR-9 biosynthesis, we will compare the relative abundance of pri-miR-9, pre-miR-9, and mature miR-9 in untreated and ethanol-treated rat striatal and SON neurons. The cells will be cultured in either ethanol-free medium (control group) or medium containing 20mM ethanol for 24 hours. Total RNA will be extracted from cells by Trizol (Invitrogen) (24). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) will then be carried out using primers designed for rat pri-miR-9, pre-miR-9, and mature miR-9 (24). The products will be run on an agarose gel and analyzed using a densitometer (8). Data analysis will be performed using software by BioRad (24). In the ethanol-treated cells, the expression level of miR-9 will be measured prior to exposure to ethanol and after exposure for 15 min, 30 min, 6 hrs, and 24 hrs.   Expression level within control cells will be measured at the same time intervals.

Prediction: If ethanol upregulates miR-9 through interactions with Drosha, then it will accelerate the conversion of pri-miR-9 to pre-miR-9, which will result in the expression of more pre-miR-9 and mature miR-9 in cells exposed to ethanol.   If, on the other hand, ethanol upregulates miR-9 through interactions with Dicer, then it will accelerate the conversion of pre-miR-9 to mature miR-9, resulting in the expression of more mature miR-9 after exposure to ethanol.

2. Ethanol's Effect on Drosha Activity: Ethanol's effects on Drosha activity will first be evaluated using an *in vitro* pri-miRNA processing assay (24). Radiolabeled pri-miR-9 will be prepared by *in vitro* transcription and will then be incubated with nuclear extracts prepared from rat striatal and SON neurons that have been untreated (control) or treated with 20mM ethanol. The reaction mixtures will be subjected to denaturing gel electrophoresis and the amount of pri-miR-9 and pre-miR-9 will be quantified using a phosphoimager (24).   To demonstrate that ethanol is interacting with Drosha and is independent of the substrate, a second miRNA, miR-131, will be subjected to the same procedure; miR-131 is expected to behave like miR-9. We will perform a second *in vitro* assay to verify that ethanol is acting directly on the Drosha enzyme. The assay will be executed as previously described (39, 40) and will use immuno-purified Drosha that will be incubated with miRNA (either miR-9 or miR-131) in the presence or absence of 20mM ethanol. The products will then be subjected to qRT-PCR (as described in part 1), run on a gel, and measured using a densitometer.

Next, Drosha-miR-9 interactions will be evaluated *in vivo* using RNA immunoprecipitation performed as previously described (41, 42). Rat striatal and SON neurons will be bathed in either an ethanol-free medium (control) or a medium containing 20mM ethanol. The cells will be lysed and incubated with anti-Drosha antibodies. They will then be exposed to Protein A-agarose beads, and miR-9 will be separated by qRT-PCR and analyzed as described in part 1.

Prediction: If ethanol upregulates Drosha activity, then more pri-miRNA should be converted to pre-miRNA in the ethanol-treated *in vitro* assays than in the untreated assays. Additionally, the *in vivo* immunoprecipitation should show that there is more Drosha bound to pri-miRNA in ethanol-treated neurons than in untreated neurons. If the results indicate that ethanol does not upregulate Drosha activity, then this would suggest that upregulation is occurring through a different mechanism such as through interactions with Dicer or the miR-9 promoter.

3. Ethanol's Effect on Dicer Activity: Ethanol's effects on Dicer activity will be evaluated using the same procedures described in part 2. The only modifications will be that in the first *in vitro* assay, cytoplasmic extract will be incubated with the pri-miR-9 instead of nuclear extract since Dicer activity occurs in the cytoplasm rather than in the nucleus (16). Also, we will use immuno-purified Dicer in the second *in vitro* assay and anti-Dicer antibodies in the *in vivo* immunoprecipitation.

Prediction: If ethanol upregulates Dicer activity, then more pre-miRNA should be converted to mature miRNA in the ethanol-treated *in vitro* assays than in the untreated assays. The *in vivo* immunoprecipitation should also show more Dicer bound to pre-miRNA in ethanol-treated neurons than in untreated neurons. If the results indicate that ethanol does not upregulate Dicer activity, then upregulation must be occurring through an alternative mechanism such as through interactions with Drosha or the miR-9 promoter.

3. To characterize the effects of miR-inhibition.

Rationale: miR-9 alters the composition of BK channel isoforms in neurons and is linked to alcohol sensitivity and the development of tolerance (8). Therefore, the next logical step would be to observe the effects of miR-9 inhibition *in vivo*. To examine the functional role of miR-9, we will inhibit this short non-coding RNA through RNA interference (RNAi). We will use two methods of RNAi: the first uses 2'-OMethyl (OMe) modified short interfering RNAs (siRNA) (42, 43) and the second utilizes locked nucleic acid (LNA). siRNA and LNA are commonly used inhibitors of miRNA (42, 43), but LNA proves more effective in miRNA inhibition than siRNA because it can bypass the plasma membrane and is more stable *in vivo* (44). We will use rat SON and striatal neurons, which naturally express miR-9, to study the effects of miR-9 silencing on ethanol sensitivity by employing siRNA and LNA (7).

Design and Method:

1. Antisense siRNA (Antigomir) activity: We propose to study the effects of miR-9 inhibition using synthetic antisense siRNA (45). To prepare our antisense OMe siRNA, we must first obtain the reverse complement sequence of miR-9, which is available from http://microrna.sanger.ac.uk/sequences/ (45). Once an oligonucleotide template has been made, commercially available 2'O-Me-Nucleotides can be purchased from Dharmacon (43, 45-46). The oligonucleotides will be purified by high-performance liquid chromatography (HPLC) and then used to synthesize siRNA by standard solid phase oligonucleotide synthesis protocols (47). The production of the siRNA will be verified using PCR (48, 49). Transfection of the siRNA into the SON and striatal neurons will require the siRNA to be placed into a plasmid and then into 5 μL of Lipofetamine 2000 (Invitrogen) for 4hrs. These cells will then be removed and exposed to 20 mM ethanol to observe the effects of inhibited miR-9. Control cells will be placed in an ethanol-free medium. Furthermore, we will use anti-miR-131 siRNA as our siRNA control molecule. This siRNA should not inhibit miR-9 function since it does not have a binding sequence complementary to miR-9. To observe the effects of miR-9 inhibition, both loss-of-function and control cells will be analyzed 15 min, 30 min, 6 hrs, and 24 hrs after ethanol exposure. To measure the physiological changes of the cell, we will perform three analyses. The first will be whole-cell patch clamp method to measure the differences in potassium current changes between control and ethanol exposed cells (4, 8). Secondly, the use of siRNA causes the degradation of the target miRNA (48, 49); thus, we will measure miR-9 levels in cells using Northern blot. Lastly, miR-9 changes the BK channel isoform composition (8). We will then employ Western blots to evaluate the BK channel isoforms.

Prediction: Inhibition of miR-9 with siRNA will reduce alcohol tolerance in these cells and prevent changes in BK channel isoform composition. However, it is possible that the cells will maintain alcohol tolerance after miR-9 inhibition because alcohol may have other targets in the cell that regulate ethanol sensitivity. Furthermore, miR-9 inhibition may prove fatal to the cell as it has multiple targets within neurons (8).

2. Antisense LNA activity: We will also study LNA inhibition of miR-9. LNA has more therapeutic potential than siRNA because siRNA require plasmids and vectors to bypass the cell membrane. These transport vehicles may be toxic to cells and therefore could not be used therapeutically. Anti-miR-9 LNA oligionucleotides can be purchased from Biosynthesis (50). Transfection of the LNA into SON and striatal neurons will be achieved with a 40% confluent Lipofection (GIBCO/BRL) medium for 15 minutes (50). The same experimental conditions, controls, and analyses will be used as in part 1. However, our control will be anti-miR-131 LNA, which should not bind to miR-9 as it does not have a complementary sequence.

Prediction: Inhibition of miR-9 with antisense LNA will reduce alcohol tolerance in these cells and prevent changes in BK channel isoform composition. However, alcohol tolerance may remain because ethanol may have multiple targets that influence ethanol sensitivity. Alternatively, it is possible that miR-9 inhibition may prove toxic, as miR-9 has multiple targets within neurons.

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