

# Viral Delivery of Ethanol Response Related Genes in Mouse Brain: A Preliminary Study

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## ABSTRACT

Previous research on the genetic component of alcohol dependence has implicated certain genes to be important in the body's response to ethanol. This study proposes a novel way of validating these genes-of-interest's role in the ethanol response. The larger project that this study is a part of involves genes being ligated into adeno-associated viral vectors, packaged in virus and injected in mouse brain allowing behavioral studies to be run on animals that have a specific gene up or down regulated in areas of the brain. The portion of the proposed study discussed in this paper involves the construction and validation of the viral vectors, specifically one containing a mutant form of the GADD34 protein. This construct is predicted to increase the rate of protein translation in neurons and in this way affect drinking behavior.

## INTRODUCTION

According to the Center for Disease Control and Prevention 61% of adults reported drinking alcohol in 2006. In addition to the high rate of alcohol consumption, in 1993 the NCADD estimated the number of alcoholics in the United States to be approximately 12.1 million. The ways in which alcohol affects the body are important to study in order to create new treatments for alcoholism. In addition, the study of addictive drugs informs the ways in which chemicals and chemical balances in the brain can affect behavior.

This study is focused on a small portion of a larger project that is aiming to study how change in the expression of different genes implicated in ethanol response in the brain affect mouse drinking behavior. The goal of the project is to use adeno-associated virus to infect specific areas of mouse brain with viral vectors containing genes of interest. Then use behavioral studies to investigate the ways in which the change in a gene's expression level affects drinking and anxiety behaviors. Adeno-associated virus was chosen as the delivery mechanism because it infects neurons and does not induce a

significant immune response in the host (Kaplitt, Michael Ch. 14).

The genes that are being constructed into viral vectors have been implicated by both an Ethanol meta-analysis currently being constructed by Dr. Michael Miles and an ethanol deprivation study done by Dr. Rahul Khisti. The goal of the meta-analysis is to integrate data from the variety of studies done in different species on the genetic component of alcohol dependence in order to discover common networks and produce target gene lists (Miles 2008 *unpublished*). In the meta-analysis, Dr. Michael Miles used a scoring algorithm to integrate multiple ethanol related data-sets from “microarray, linkage, and single gene studies from human, mice, *Drosophila* and *C. elegans*” and generate a list of genes involved in response to alcohol across species. About 15 of the top candidates from this list are being inserted into viral vectors in the Miles lab. Additional genes of interest are also being derived from a study done on ethanol deprivation in mice.

In rodents, the ethanol deprivation effect is used to model ethanol craving. The effect describes the phenomenon of animals previously self-administering ethanol temporarily increasing their ethanol intake following a period of deprivation. Khisti et al. 2006 describes this effect in C57BL/6 mice. Following the deprivation period Dr. Rahul Khisti performed a yet unpublished microarray analysis on the gene expression levels in the nucleus accumbens, ventral tegmental area, medial pre-frontal cortex of mouse brain. The levels of gene expression were compared between mice sacrificed at the end of the deprivation period and control mice sacrificed after the period of increased drinking (Khisti et al. 2008).

This microarray analysis generated a list of genes differentially expressed in the brain between experimental and control groups. A class of genes that appeared a number of times in the analysis was translation initiation factors. Eukaryotic initiation factors are responsible for the initiation of mRNA translation. Dynamic protein translation is thought to be an important component of brain plasticity underlying drug use. Because regulation of protein synthesis components occurs during the ethanol deprivation effect according to the microarray results, altering the rate of protein translation in the brain could have an affect on drinking behavior in mice.

In order to alter the rate of protein translation a mutant form of a protein known as

GADD34 was selected because its ability to increase protein translation had already been demonstrated in Gomez et al. 2008. Growth-arrest and DNA-damage-inducible protein-34 (GADD34) works to regulate the phosphorylation state of eIF2 $\alpha$  (eukaryotic initiation factor 2 $\alpha$ ). EIF2 $\alpha$  is a subunit of the eIF2 complex, which mediates the first step of translation initiation: the attachment of the initiator methionine-tRNA to the 40S ribosomal subunit (Lang et al. 1999). GADD34 direct protein phosphatase 1 to dephosphorylate eukaryotic initiation factor 2 $\alpha$ , which leads to increased protein translation (**Figure 1**).

In their study “A decrease in cellular energy status stimulates PERK-dependent eIF2 $\alpha$  phosphorylation and regulates protein synthesis in pancreatic beta-cells” Gomez et al. demonstrated that the “inhibition of eIF2 $\alpha$  phosphorylation in glucose-deprived cells by the overexpression of dominant-negative PERK or an N-terminal truncation mutant of GADD34 leads to a 53% increase in the rate of total protein synthesis” (Gomez et al. 2008).

The N-terminal truncation of GADD34 is known as GADD $\Delta$ N, and constitutively directs protein phosphatase-1 to eIF2 $\alpha$ , leading to eIF2 $\alpha$  dephosphorylation and increase in protein translation (Gomez et al. 2008). Therefore we hypothesize that increasing protein synthesis by altering GADD34 expression in mouse brain will modify behavioral responses to ethanol.

## METHODS

### Recovery of the GADD $\Delta$ N Plasmid

Gomez et al. 2008 were kind enough to send the Miles lab the pAdTrack-GADD $\Delta$ N plasmid they had constructed. The plasmid was recovered from the filter paper by warming the paper in 30  $\mu$ L of TRIS. 50  $\mu$ L of Invitrogen Max-efficiency DHS $\alpha$  competent cells were transformed with 2  $\mu$ L of recovered pAdTrack-GADD $\Delta$ N plasmid. Cells were thawed on ice, and 2  $\mu$ L of plasmid was added the mixture which was then incubated on ice for 30 minutes. Next, the cells were heat-shocked for 45 seconds in a 42  $^{\circ}$ C water bath. After two minutes on ice, 450  $\mu$ L S.O.C was added and the cells were incubated at 37 $^{\circ}$ C for 1 hour at 225 rpm. After 1 hour, 30  $\mu$ L of cells were plated on a LB plate with kanamycin and incubated overnight at 37 $^{\circ}$ C.

The plasmid DNA was then isolated using a Quiagen QIAprep Spin Miniprep Kit by following the recommended procedure for a microcentrifuge.

## Construction of the pAAV-IRES-hrGFP-GADDΔN Plasmid

### *Digestion of viral vector backbone and pAdTrack-GADDΔN plasmid*

.75 μL of the adeno-associated viral vector pAAV-IRES-hrGFP was digested with 1 μL of restriction enzymes BamHI and XhoI in a 30 μL digestion using 3 μL 10X NEB buffer 3, 3 μL 10X BSA, and 21.25 μL nuclease free water. 10 μL of the recovered pAdTrack-GADDΔN plasmid was digested with 1 μL of BglII and XhoI in a 30 μL digest using 3 μL 10X NEB buffer 3, 3 μL 10X BSA and 12 μL of nuclease free water. The pAdTrack-GADDΔN plasmid digest was incubated at 37°C for 2 hours. The pAAV-IRES-hrGFP backbone digest was incubated at 37°C for 2.5 hours, at which point 1 μL of CIP (calf intestinal phosphatase) was added to the digest to prevent religation of the linearized plasmid. The backbone was then incubated for an additional 30 minutes at 37°C.

### *Gel electrophoresis of digest product*

The digest of the pAdTrack-GADDΔN plasmid was separated on a 1% agarose gel. The band containing the GADDΔN insert was then excised and purified using Quiagen's QIAquick Gel Extraction Kit and following the recommended protocol for a microcentrifuge. The digested pAAV-IRES-hrGFP vector was purified using Quiagen's QIAquick PCR Purification Kit and following the recommended protocol for a microcentrifuge.

### *Ligation*

The GADDΔN insert was ligated into the pAAV-IRES-hrGFP viral vector using a 20 μL ligation reaction containing 2 μL 10X NEB T4 ligase buffer, 1 μL NEB T4 DNA ligase, 15.25 μL nuclease free water, .75 μL digested pAAV-IRES-hrGFP vector, and 1 μL purified GADDΔN insert. The reaction was incubated at room temperature for 20 minutes.

### *Transformation*

50 μL of Invitrogen Max-efficiency DHSα competent cells were transformed with 2 μL of the ligation reaction using the previously discussed transformation protocol. 100 μL of cells were plated on an LB plate with ampicillin and incubated overnight at 37°C.

### *Recovery and analysis of pAAV-IRES-hrGFP-GADDΔN*

Transformed colonies were picked and grown up over night in 3 mL LB with ampicillin. Quiagen's QIAprep Spin Miniprep Kit was used to recover the pAAV-IRES-hrGFP-GADDΔN plasmid by following the suggested procedure for a microcentrifuge. Next, a diagnostic digest was used to determine whether the GADDΔN insert was in fact in the purified plasmid and whether it was in the correct orientation.

15μL of the purified plasmid was digested in a 30 μL reaction with 1 μL XbaI and 1 μL XhoI, in addition to 7 μL nuclease free water, 3 μL 10X NEB buffer 2, and 3 μL 10X BSA. The digest reaction was separated on a 1 % agarose gel and analyzed for presence of insert and insert size.

## Validation of the pAAV-IRES-hrGFP-GADDΔN Plasmid

### *Sequence analysis of pAAV-IRES-hrGFP-GADDΔN*

In order to ensure that the insert shown to be present in the electrophoresis of the digested product was in fact the GADDΔN insert, a sample of the purified plasmid was sent to the core DNA sequencing facility for sequencing.

### *Analysis of the pAAV-IRES-hrGFP-GADDΔN in cell culture*

To test whether the pAAV-IRES-hrGFP-GADDΔN plasmid would express the mutant GADDΔN protein in cell culture, AAV-293 cells were transfected with the plasmid. Cells were cultured in a 6 well plate and 2 wells were transfected with an empty pAAV-IRES-hrGFP vector, 2 with the pAAV-IRES-hrGFP-GADDΔN vector and 2 were left as mock transfections. Plasmids were transfected using Arrest-In™ Transfection Reagent and following the suggested protocol.

After 24 hours the cells were viewed under a fluorescence microscope to check for the expression of the reporter gene GFP in the pAAV-IRES-hrGFP and pAAV-IRES-hrGFP-GADDΔN transfected cells. 48 hours after transfection the cells were harvested and lysed using RIPA lysis buffer. After sonication of the lysed samples, a BCA protein concentration assay from Pierce was used to determine the concentration of the samples.

### *Western Blotting*

To ensure that the GADDΔN protein was being produced in the AAV-293 cells, a western blot of the protein samples from transfected cells was probed with an antibody against GADD34. Samples for the polyacrylamide gel were prepared by adding 20 μg of protein sample to 6 μL 5X SAB with 5% beta-mercaptoethanol and the appropriate amount of water. The samples were heated at 99° C for 2 minutes. Samples were loaded into a pre-cast BIO-RAD 10% TRIS-HCl polyacrylamide gel, and run at 150 V.

The samples were then transferred to a PVDF membrane at 100V for 1 hour. The membrane was blocked in 5% dry milk in TBST for 1 hour at room temperature with shaking. Next, it was incubated with the primary antibody against GADD34 from Santa Cruz Biotechnology, Inc. at a 1:500 dilution in TBST with 5% dry milk overnight at 4 °C with shaking. The membrane was then washed 3 times with 1X TBST for 15 minutes each before incubating it in the Secondary antibody (an ECL Anti-rabbit IgG with Horseradish Peroxidase from GE Healthcare) at a 1:100,000 dilution in TBST with 5% dry milk for 2 hours at room temperature with shaking. After being washed 3 times with 1X TBST for 10 minutes each, the blot was treated with ECL by following the recommended protocol. The blot was then developed using an Xomat.

## RESULTS

The goal of this portion of the project was to construct a viral vector with the GADDΔN insert and verify its presence and functionality. The digest of the pAAV-IRES-hrGFP-GADDΔN purified plasmid revealed an insert of approximately the correct size (**Figure 2**). Plasmid was sent to the core facility for DNA sequencing, which yielded approximately 900 bases of confident sequence. Using NCBI's BLAST we checked to

see whether the sequence matched up with the GADD34 murine gene. The results showed that the sequence matched with gene Myd116 which is GADD34 in mouse. The digest and sequencing results verified that the insert in the viral vector was the correct size, correct orientation and the correct gene.

In order to see if the mutant protein was produced in vivo, AAV-293 cells were transfected with the pAAV-IRES-hrGFP-GADDΔN plasmid and a western blot was used to detect expression of the mutant protein. After antibody staining and developing the membrane, two dark bands were clearly stained in the lanes that contained the protein samples from the cells that had been transfected with the pAAV-IRES-hrGFP-GADDΔN plasmid. No bands were seen in other lanes that contained cells transfected with the empty pAAV-IRES-hrGFP plasmid and no plasmid (**Figure 3**).

## DISCUSSION

The long term goal of this study is to answer the question of whether increasing protein synthesis by altering GADD34 expression in mouse brain will modify behavioral responses to ethanol. The work completed so far only deals with the beginning portion of the study in which the viral vectors are constructed and verified. After this initial step, the viral vectors will need to be packaged in adeno-associated virus and injected into mouse brain before behavioral studies can be done. The major question answered by this beginning research is whether the GADDΔN insert was ligated into the pAAV-IRES-hrGFP viral vector in the correct orientation and whether that construct will produce the mutant GADDΔN protein in cell culture.

The results of the diagnostic digest of the plasmid reveals two bands, one of which is the correct size for the viral vector backbone and one of which is approximately the size we would expect the mutant gene to be. These results are a preliminary indication that some piece of DNA of approximately the correct size has been ligated into the viral vector. The sequencing results reveal a sequenced portion of the plasmid and a read of approximately 900 nucleotides. When this sequence is put into BLAST we see that the strands are oriented in the same direction, indicating that the insert is in the proper orientation, and that the insert has the same sequence as the Myd116 gene, which is the mouse version of the GADD34 gene. These results indicate with confidence that

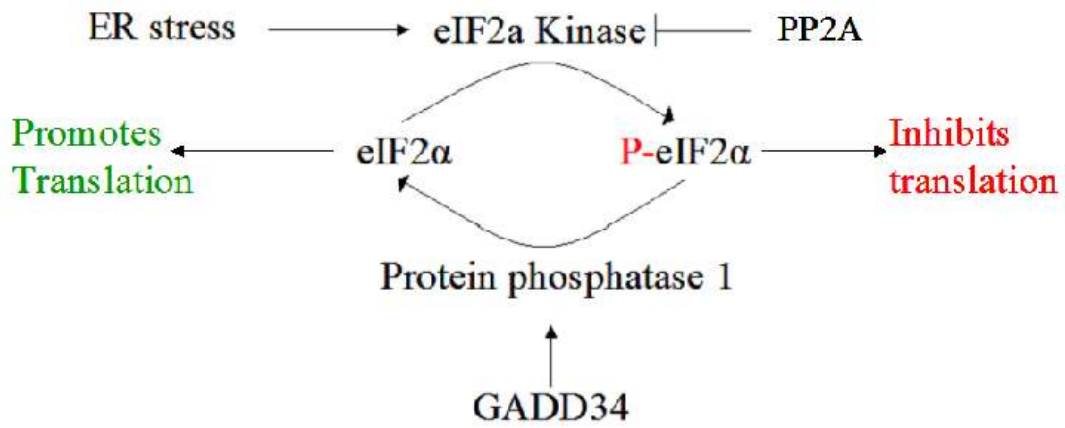
the DNA insert in the viral vector is in fact the GADD $\Delta$ N insert and it is in the correct orientation.

In order to see whether the mutant GADD $\Delta$ N protein was being produced in cell culture, and whether the GFP reporter gene in the viral vector was functioning, AAV-293 cells were transfected with the pAAV-IRES-hrGFP-GADD $\Delta$ N plasmid. Looking under the microscope 24 hours after transfection revealed that greater than 30% of the cells were transfected and producing GFP, indicating that the transfection was successful. The results of the western blot probed against the GADD34 protein revealed two dark bands in the lanes with the samples that had been transfected with the pAAV-IRES-hrGFP-GADD $\Delta$ N plasmid. These results indicate that the GADD $\Delta$ N protein was being over expressed in the cells transfected with the viral vector containing the GADD $\Delta$ N gene. GADD34 is an endogenous protein in the AAV-293 cells, so we were initially expecting a light band in all of the lanes from the larger endogenous GADD34 protein. Possible reasons this band was absent are that GADD34 is produced in response to cell stress, particularly as part of the unfolded protein response. Therefore if the cells were fairly healthy when they were lysed it is possible that the level of endogenous GADD34 was very low and therefore not detectable. It is also possible that using a stronger detection agent such as ECL plus may have revealed very light bands.

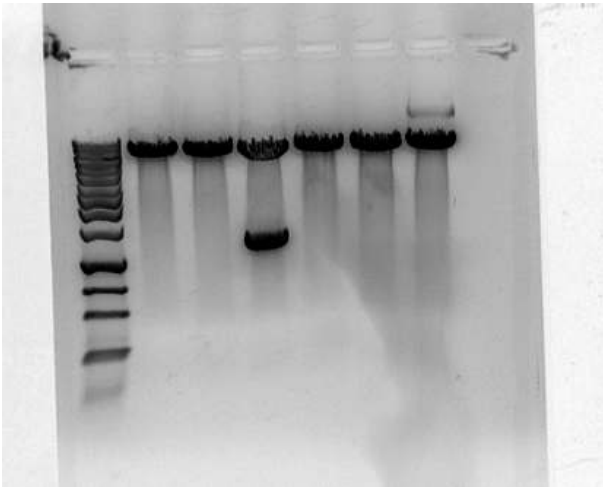
## CONCLUSION

The experiments done in this study have verified that the GADD $\Delta$ N mutant gene has been successfully inserted into the pAAV-IRES-hrGFP viral vector and that the mutant protein is expressed in cell culture. The next step in this project is to package the newly constructed plasmid in adeno-associated virus in cell culture. Once viral packaging is occurring at a rate that produces a high enough titer of virus, the virus can be harvested, purified and injected into mouse brain.

We would expect that drinking behavior and possibly anxiety behavior would be affected by the increase in protein translation that the mutant GADD34 protein would result in. If a change in behavior is seen, it would help support the evidence that changes in eukaryotic translation initiation factors are an important part of the brain's response to ethanol.

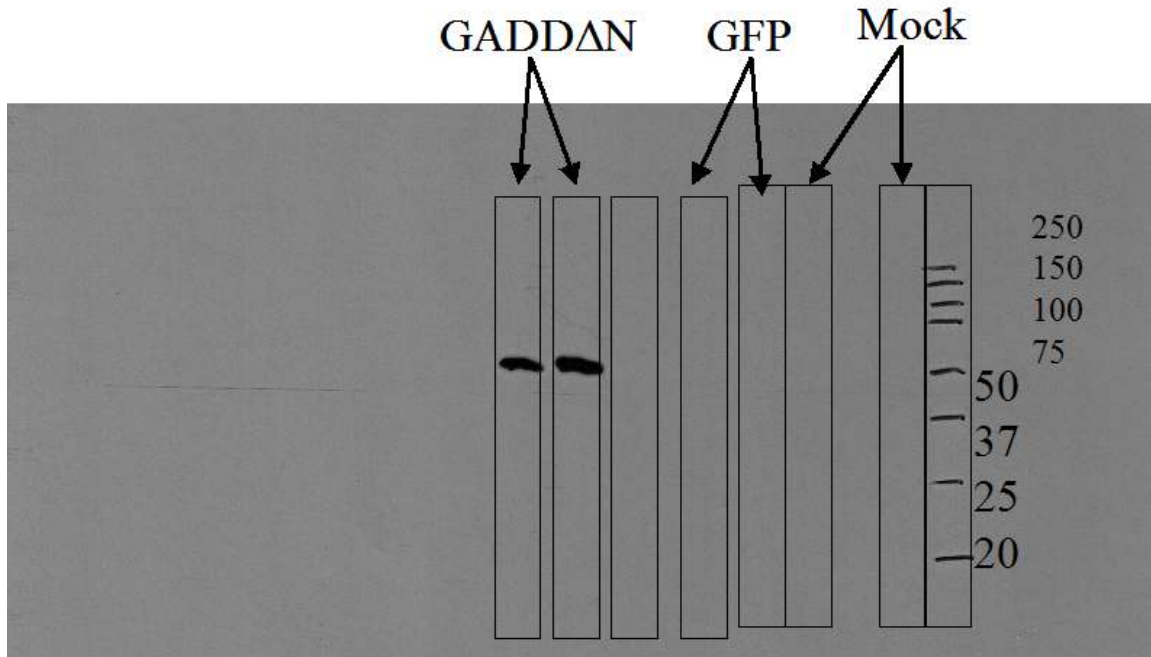


**Figure 1:** GADD34 direct protein phosphatase 1 to dephosphorylate eukaryotic initiation factor 2 $\alpha$ , which leads to increased protein translation.



**Figure 2:** Digest of the pAAV-IRES-hrGFP-GADD $\Delta$ N purified plasmid revealed an insert of approximately the expected size.





**Figure 3:** A western blot was used to detect expression of the mutant protein. Two dark bands were clearly stained in the lanes that contained the protein samples from cells transfected with the pAAV-IRES-hrGFP-GADDΔN plasmid. No bands were seen in other lanes that contained cells transfected with the empty pAAV-IRES-hrGFP plasmid and no plasmid.

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