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Validation of Ninein as an Ethanol-related Quantitative Trait Gene: Reassessment, Design, and Functional Validation of Reference Genes for qPCR Analysis of Brain Tissue in Mice

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Validation of Ninein as an Ethanol-related Quantitative Trait Gene: Reassessment, Design, and Functional Validation of Reference Genes for qPCR Analysis of Brain Tissue in Mice

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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I am honored to have had help from several colleague during the course of the work presented in this thesis. Contributions not cited in the text are detailed below. All other work was performed entirely by me in the laboratory of Dr. Michael Miles.

Chapter 2

The nucleus accumbens data set used in the initial overlap analysis was generated by Dr. Michael Miles using existing microarray data from the laboratory (Kerns et al., 2005). Alejandro Andrade ran the qPCR to generate standard curves for *Canx* and *Sort1* primers.

Chapters 2 and 3

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Abstract

The increasing use of quantitative real-time polymerase chain reaction (qPCR) as a method for quantifying gene expression has led to an increased demand for standardization of data analysis methods to ensure accurate reporting and robust, reproducible results. The exponential nature of qPCR amplification results in the potential magnification of what are usually very small sources of error. Relative gene expression calculations circumvent this issue by normalizing target gene expression data to within-sample expression of a previously validated, stably expressed reference gene or genes. Multiple studies discussed herein have found that qPCR data are more reliable and reproducible when multiple reference genes are used, and that they are validated prior to use in experiments with new conditions. In this thesis, existing reference genes are evaluated to ensure they meet these criteria in experimental paradigms used frequently in our laboratory. Existing work on ethanol's anxiolytic-like effects in our laboratory utilized microarrays to identify *Ninein* as a cis-regulated, quantitative trait gene for these effects in nucleus accumbens (NAc) of BXD recombinant inbred mice and their progenitors, C57BL6/J (B6) and DBA/2J (D2) mice. Contrasting behavioral responses to ethanol in these mouse strains make them a frequent subject of study for determining genetic components underlying those behaviors. In the first data chapter, the case is made for eliminating one reference gene typically used for qPCR data normalization in qPCR experiments assessing strain differences in NAc gene expression in the laboratory, *Ppp2r2a*. The reference genes subsequently validated for use in qPCR analysis in ethanol-naïve NAc and amygdala of saline and ethanol-treated B6 and D2 mice are then used in an in-depth characterization of *Ninein* expression in B6 and D2 NAc and amygdala. Furthermore, evidence is provided for the first *in vivo* observation of murine *Ninein* transcript variant 6 in adult neural tissue. The data presented make the case for a more thorough re-evaluation of reference

genes for future qPCR experiments in the laboratory, as well as a potential mechanism for *Ninein*'s involvement in variation of anxiolytic-like responses to ethanol in B6 and D2 mice.

Chapter 1 Introduction and Background

Neurobiology of Alcohol Use Disorders

It has been recently estimated that 1 in every 19 people aged 12 or older in the United States suffer from an alcohol use disorder (AUD) (SAMHSA, 2019). In the Diagnostic and Statistical Manual of Mental Disorders (APA, 2013), AUDs are characterized by persistent alcohol use despite negative personal, social, psychological and/or physiological consequences, as well as craving, tolerance, and withdrawal. Neurobiologically, increased activity in the mesolimbic dopamine system - including the nucleus accumbens (NAc), ventral tegmental area (VTA), and prefrontal cortex (PFC) - mediates the acute rewarding effects of ethanol and other drugs of abuse. Neuroplasticity in these regions over a period of consistent alcohol use is thought to be the underlying factor in the transition from acute alcohol abuse to the chronic, compulsive drinking behavior seen in AUD (see Volkow & Koob, 2010 for review). Thus, existing work in the laboratory has focused on the mesocorticolimbic reward circuit due to the extensive evidence for its role in drug reward and dependence.

The extended amygdala also plays a key role in behavioral responses to both acute and chronic ethanol exposure. The amygdala has extensive interconnectivity with the mesolimbic system, including afferent projections from the PFC and hypothalamus, as well as efferent projections to the PFC, NAc, hypothalamus, and BNST. Changes in activity or plasticity in the amygdala have been associated with the rewarding effects of drugs and the preoccupation or craving associated with the late stages of addiction (Volkow & Koob, 2010), and acute doses of ethanol have been shown to alter c-Fos expression in the central amygdala and bed nucleus of the stria terminalis (BNST) (Davis et al., 2009; Sharko et al., 2016). In addition, both amygdala and BNST have been implicated in phasic and sustained fear in both rodents and humans (Davis et al., 2009). Together, these data suggest that activity in the amygdala could play a crucial role in the anxiolytic-like effect of ethanol.

Genetics of Alcohol-Related behavior

Twin studies show that approximately 48%-58% of the liability for AUD risk is associated with genetic factors (Prescott & Kendler, 1999; Tawa et al., 2016). Given the complex nature of AUD-related symptoms and behaviors, it is not surprising that multiple genetic loci have been associated with this risk (see Tawa et al., 2016 for review). Rodent models have become one of the prevailing methods for studying the genetic components underlying complex ethanol-related behavioral phenotypes due to divergent behavioral responses to acute and chronic ethanol in existing inbred strains with known genotypes. C57BL/6J (B6) and DBA2/J (D2) mice are two such examples of these contrasting behaviors. In multiple chronic drinking paradigms, B6 mice drink more and have a greater preference for ethanol than D2 mice (Crabbe et al., 1999; McClearn & Rodgers, 1959; Moore et al., 2010). B6 mice are also less sensitive to the locomotor activating (Phillips et al., 1994) and sedative-hypnotic (Linsenbardt et al., 2009) effects of ethanol, whereas D2 mice are less sensitive to ethanol's anxiolytic-like effects (Putman et al., 2016). Because of their extensive behavioral characterization, B6 and D2 mice have been used as progenitor strains for the BXD recombinant inbred (BXD RI) line of mice, comprised of more than 100 inbred strains with known B6 or D2 alleles at almost 7500 polymorphisms across all chromosomes (Taylor, 1978; Williams et al., 2001). Correlating variation in genotype with variation in behavior among these strains has allowed for quantitative trait locus (QTL) mapping of several ethanol-related phenotypes including withdrawal (Buck & Finn, 2001; Crabbe et al., 1999), hypnotic sensitivity measured with loss of righting reflex (Radcliffe et al., 2000), locomotor activation and motor incoordination (Demarest et al., 1999; DuBose et al., 2013; Phillips et al., 2010), metabolism (Grisel et al., 2002), anxiolysis measured in the elevated zero maze (Cook et al., 2015), and anxiolysis measured in the light-dark box (Putman et al., 2016).

Associations between anxiety and alcohol use have long been reported in scientific literature, and patients with AUD often cite the anxiolytic properties of ethanol as the reason for

persistent use and abuse (Newlin & Thomson, 1990). As such, it is not surprising that pre-existing anxiety disorders are associated with an increased risk for the onset of comorbid alcohol dependence (Crum et al., 2013; Swendsen et al., 2010). With mounting evidence for heritable components of both AUD and anxiety disorders (Hodgson et al., 2016; Prescott & Kendler, 1999), our laboratory sought to identify behavioral QTL (bQTL) and expression QTL (eQTL) underlying the anxiolytic effect of ethanol.

Ethanol-induced anxiolysis QTL 1 (*Etanq1*) was identified in nucleus accumbens using the BXD recombinant inbred panel of mice in the light-dark transition model of anxiety (Putman et al., 2016). Light-dark transition assays rely on the innate tendency of rodents to avoid bright lights or open fields (Buccafusco, 2009). In brief, a mouse is placed in a chamber with an open, brightly lit compartment and an enclosed dark compartment. Increases in time spent or distance traveled in the light compartment after a given experimental treatment are interpreted as anxiolytic-like behaviors. A mixed-model behavioral QTL analysis, incorporating the effects of genotype and treatment on percent distance traveled (%DTL) and percent time spent (%TSL) in the light after treatment with 1.8 g/kg ethanol or saline, was used to identify *Etanq1* on chromosome 12. Because of a potential interaction with a second QTL on Chr 1, the QTL was further refined using six additional BXD strains with either B6 or D2 alleles at its peak marker, and balanced for B6 and D2 alleles at a chromosome 1 locus with an epistatic interaction with *Etanq1*. Differences in %DTL after ethanol treatment were still significant despite variation at the Chr1 locus. Fine-mapping of the initial 18 Mb support interval, using 3 additional BXD strains with recombination events within *Etanq1*, narrowed the interval to about 3 Mb that includes 41 protein coding genes and genes with non-coding RNAs. Of 10 missense SNPs identified in this interval, the only SNP predicted to alter protein function in a deleterious manner (rs29159683) was within *Ninein* (*Nin*). To determine whether BXD genotype affected mRNA expression, microarrays were performed with tissue from prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral midbrain (VMB). Genes within *Etanq1* whose expression varied

with genotype, i.e. had cis-eQTLs, included *Sos2* in VMB, and *Nin*, *Atp5s*, *Trim9*, and *Sos2* in NAc. When correlating candidate gene expression with ethanol-induced anxiolysis, only *Nin*, *Sos2*, and *Trim9* expression in the NAc were significantly correlated with %DTL after ethanol injection. Relative expression of *Nin, Trim9, and Sos2* in NAc of B6 and D2 mice were measured using qPCR, and only *Nin* was differentially expressed between strains. Allele specific qPCR in B6D2F1 mice revealed higher expression of mRNA containing the D2 allele, confirming its cis-regulation, and western blots show higher expression of two provisional NIN protein isoforms in D2 NAc as compared to B6 (Putman et al., 2016).

Validating Quantitative Trait Genes from microarray data using quantitative real-time polymerase chain reaction (qPCR)

qPCR is frequently used to validate microarray results because of its high sensitivity, specificity, and wider dynamic range. In the context of quantitative trait gene (QTG) validation, qPCR serves as a less expensive and more rapid parallel method for evaluating expression differences of a smaller number of candidate genes between progenitor strains with more biological replicates for higher statistical power.

There are two widely used fluorescence methods available for these purposes, namely SYBR Green and TaqMan technologies. SYBR green dyes are non-specific and intercalate all double stranded nucleic acids. Thus, qPCR experiments utilizing this method rely on the assumption that the only double stranded products in the reaction are those produced by primer binding to a single specific target. In contrast, probes in TaqMan reactions contain a reporter fluorophore and corresponding quencher in close proximity. During the extension phase of the PCR reaction Taq polymerase cleaves the probe, separating the fluorophore from the quencher and allowing it to fluoresce. For this reason, TaqMan reactions are capable of amplifying targets with very low copy numbers, while the sensitivity of SYBR Green reactions is reliant upon the specificity and binding efficiency of target primers. The utilization of different

fluorophores on TaqMan probes for different targets can also allow for quantification of multiple targets in a single reaction, which can be useful when starting RNA quantities are very low. Reactions with SYBR Green dyes, on the other hand, are limited to amplification of a single target per reaction. The complex nature of TaqMan probes, however, are often cost prohibitive and frequently limited to pre-designed probes. Primers for SYBR Green PCR are typically much cheaper, allowing for quick, user-based design of primers for a variety of targets. As a result, SYBR Green is often the method of choice when using qPCR to quantify expression of multiple target QTGs.

The primary focus of behavioral QTL and QTG validation is usually quantifying relative differences in expression of target genes between experimental groups. In other words: Is the direction of variation in expression of the target gene directly or inversely correlated with the direction of variation in magnitudes of the behavior of interest? As opposed to: What specific copy number of a target mRNA corresponds to a specific magnitude of behavior? In cases where absolute quantification is either unnecessary or not possible due to the lack of standards with known copy number, relative quantity (ΔCt method) or normalized expression ($2^{-\Delta\Delta Ct}$ method) is used. Relative quantity is calculated as the fold change relative to either an "untreated" control sample or to a standard curve with serial dilutions of known cDNA mass. Relative quantity calculations rely on the assumption that the starting amount of cDNA template in all reactions is identical, which is often not the case due to small variations in quantity introduced during total RNA quantification prior to reverse transcription, and pipetting error during the setup of PCR reactions. To compensate for this error, the $2^{-\Delta\Delta Ct}$ method employs a previously validated internal control gene(s), or "reference gene", to normalize sample Ct values of target genes. The $2^{-\Delta\Delta Ct}$ method uses target gene Ct values normalized to reference gene Ct values to compare target gene expression in a treatment sample to an untreated control, where Ct is the cycle threshold of fluorescence in a given sample, ΔCt is the difference between target

gene Ct and reference gene Ct for the same sample, and ΔΔCt is the difference between the ΔCts of the treated sample and a control (Livak & Schmittgen, 2001).

The wide variety of RNA isolation methods, qPCR fluorophores, thermocycler protocols, and data analysis methods paired with the variation in reporting of validation methods and data often results in low reproducibility of qPCR experiments within and between labs. As a result, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE Guidelines) were developed (Bustin et al., 2009). The MIQE guidelines are recommendations for the submission of data collected at multiple phases of qPCR experiments prior to and after the actual target amplification, including experiment design, sample preparation, nucleic acid extraction methods, reverse transcription methods, target gene information, primer and reference gene validation, thermocycler protocol, reagents used, and data analysis methods.

One of the MIQE guidelines most frequently missing from publications is the validation of reference genes used for normalization (Bustin et al., 2013). The selection of appropriate reference genes is vital given their function in data normalization. In order for a reference gene to be valid, it must be stably expressed across all experimental groups and/or tissues being investigated, and its amplification efficiency should be comparable to that of the target gene (Livak & Schmittgen, 2001). Frequently, qPCR data are presented normalized to a single reference gene presumed to be stably expressed in all tissues, such as *Actb* or *Gapdh* (Czechowski et al., 2005). Use of a single, non-validated reference gene can lead to the appearance of expression differences 3- to 6-fold higher than are actually present (Hellemans & Vandesompele, 2014). Vandesompele and colleagues (2002) developed a method and software for calculating expression stability of candidate reference genes called GeNorm. GeNorm utilizes the pairwise variability of relative quantities of candidate reference genes between samples to designate the most stably expressed genes, as well as the minimum

number of reference genes necessary to accurately quantify target gene expression across all experimental groups.

The primary goal of the work in this thesis is to find suitable reference genes for comparison of gene expression between in the NAc and amygdala of B6 and D2 mice treated with acute doses of ethanol. Re-assessment and further characterization of *Ninein* expression, the suspected QTG underlying the QTL for ethanol-induced anxiolytic-like response to ethanol in mice (*Etanq1)*, will be presented as a functional validation of the use of proper reference genes for qPCR experiments.

Chapter 2 Reassessment and Design of Reference Genes for Comparison of Gene Expression in B6 and D2 Mice Exposed to Acute Ethanol

Introduction

Quantitative real-time polymerase chain reaction (qPCR) is a widely used method for measuring gene expression in a broad variety of cell types and tissues. Although absolute quantification of target mRNA copy number is possible, it requires the availability of calibrated external standards with known copy number or concentration. These standards can be obtained from plasmids containing the gene of interest (GOI), synthetic nucleotides with the sequence of the amplicon desired, or cell lines with known expression levels of the GOI (see Arya et al., 2005 for review). This can be problematic when these resources are either unavailable or costly to produce, and is often unnecessary in experiments where the research question is based on relative expression of a GOI between treatment or time groups. In these instances, measurement of relative quantities (ΔCt) or normalized expression($2^{-\Delta\Delta Ct}$) is used in lieu of absolute quantification (Livak & Schmittgen, 2001).

The use of the ΔCt method for reporting gene expression is limited by the assumption that the amount starting quantity of template in each reaction well is equal. Instrumental error in nucleic acid quantification and pipetting error often introduce small variations in starting quantity that render this assumption invalid. As a result, the $2^{-\Delta\Delta Ct}$ method has been widely adopted as an alternative. In this method, GOI Cts in all experimental samples are normalized to reference gene Cts in the same samples prior to group comparisons in order to eliminate the error produced by small variation in starting quantities. Reference genes in these experiments require prior validation of stable expression across all experimental groups, and their primers must have amplification efficiencies similar to GOI primers (Livak & Schmittgen, 2001).

Because the expression level of a particular gene can vary across strains, sexes, tissues, cell types, and treatment groups, it is important to verify that the reference gene and

primers used meet both of these criteria. When the expression level of a reference gene is very low, primer efficiency can be greatly impacted. A reference gene proven to be stably expressed across, for instance, PFC in B6 and D2 mice with and without ethanol exposure may not be stably expressed in NAc under the same conditions. Similarly, a reference gene that is stably expressed between strains in basal conditions may not be stably expressed in the presence of drug treatment, or throughout development. Unfortunately, much of the existing qPCR literature utilizing reference genes for data normalization do not report validating these conditions prior to performing their experiments (Bustin et al., 2013). It is also common practice to use a single reference gene, when in reality qPCR data normalization is typically more accurate with the use of at least three (Derveaux et al., 2010; Vandesompele et al., 2002).

In an attempt to keep with these standards, this chapter describes the use of publicly available bioinformatic tools and published genome-wide datasets to identify candidates for use as reference genes in qPCR experiments comparing gene expression in B6 and D2 amygdala and NAc with and without an acute dose of ethanol. This is followed by experimental validation of these reference genes and their primers using relative quantification and GeNorm software.

Methods and Materials

In-silico evaluation

Databases, Bioinformatic Tools, and Software

Robust Multi-array Average (RMA) values and S-scores from nucleus accumbens (NAc) were obtained from GeneNetwork databases GN154, GN155, GN156, containing Affymetrix GeneChip Mouse Genome 430 2.0 arrays from B6, D2, and BXD male mice dissected four hours after an IP injection with saline or 1.8g/kg ethanol (Kerns et al., 2005). RMA values from basolateral amygdala were obtained from GeneNetwork database GN323, containing pooled Affymetrix GeneChip™ Mouse Gene 1.0 ST arrays from untreated male and female mice

(Mozhui et al., 2010). Microarray data from amygdala in ethanol treated mice were not available.

NCBI Primer BLAST tool was used to generate potential primer sequences, using the RefSeq accession number for one or all transcripts for target genes. NCBI Nucleotide BLAST, UCSC in-silico PCR and UCSC BLAT tools were used to scan for overlap of mRNA and primer sequences in non-specific locations. IDT UNAfold was used to evaluate the secondary structures of primers. Websites for all databases and in silico tools used are listed in Table 2.1

Candidate gene ID

The genes evaluated for potential use as reference genes were pooled from an overlap between those used in the mouse GeNorm kit (n = 10) available from Primerdesign Ltd. (Camberley, UK), genes currently used as reference genes in the laboratory (n = 8), and a BXD gene set from microarrays in NAc filtered for ethanol S-scores between -2 and 2 and with mas4 scores above 100 (n = 4985).

Candidate genes from the resulting list were eliminated if:

- B6 and D2 mice had different RMA values for that gene in NAc and/or amygdala, suggesting differential expression levels between the strains.
- Either strain had a significant S-score (|S-score|>2) when comparing saline to ethanol-treated mice, suggesting ethanol regulates expression of the gene,
- B6 and D2 mice had significantly different (|S-score|>2) S-scores for the target gene
- More than 30% of the target gene sequence shared greater than 80% sequence identity with other genes, other gene transcripts, or non-coding chromosomal regions
- The target gene had pseudogenes

Bioinformatics Tool	***URL***
GeneWeaver	https://geneweaver.org/
GeneNetwork	http://www.genenetwork.org/webqtl/main.py
NCBI PrimerBLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
UNAfold	https://www.idtdna.com/UNAFold?
NCBI Nucleotide BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
UCSC BLAT	http://genome.ucsc.edu/cgi-bin/hgBlat
UCSC in-silico PCR	http://genome.ucsc.edu/cgi-bin/hgPcr?db=hg38
NCBI RefSeq	https://www.ncbi.nlm.nih.gov/refseq/
GeNorm	https://genorm.cmgg.be/
RefSeq	https://www.ncbi.nlm.nih.gov/refseq/

Table 2.1 Websites used for bioinformatic analyses of candidate reference genes

Parameter	***Target Value***
Product size	Minimum: 70 bp
	Maximum: 400 bp
Primer Melt Temperature	Minimum: 57
	Optimum: 60
	Maximum: 65
	Maximum Difference: 2
Exon junction overlap	At least one primer must span an exon exon junction
Organism ID	Mus musculus (taxid: 10900)
Primer Length	Minimum: 19 bp
	Optimum: 20 bp
	Maximum: 25 bp
Primer GC Content	Minimum: 20%
	Maximum: 65%
3' GC clamp	1
Max Self Complementarity	Any: 5.00
	3': 3.00
Max Pair Complementarity	Any: 5.00
	3': 3.00
SNP Handling	Primer binding site may not contain a known SNP

Table 2.2 Primer design criteria

Primer Design

Existing primer pairs in the laboratory were redesigned if NCBI nucleotide BLAST, UCSC in-silico PCR, or UCSC BLAT searches revealed one or both primers:

- Were located in the 3'-untranslated region of a transcript
- Did not overlap an exon junction
- Had non-specific binding sites
- Did not bind all known mRNA transcript variants of the given gene

Otherwise, new primer pairs were designed with NCBI's PrimerBLAST using the default search parameters except for the criteria listed in Table 2.2. Primer annealing temperature, PCR product length, primer length, GC content, and self-complementarity were chosen in order to maximize efficiency in PCR reactions using SYBR Green Master Mix. Target mRNA specificity was optimized by eliminating primers that overlapped strain-polymorphisms (dbSNP) and requiring at least one primer to overlap an exon junction. Potential primer secondary structures and their melting points were evaluated using IDT-UNAfold, and primer sequences were discarded if the melt temperature of those secondary structures was not significantly less than the predicted annealing temperature of the primer.

In vivo validation

Animals

Eight-week old male C57BL6/J and DBA2/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed four per cage on ventilated racks with Teklad Sani-Chip bedding (currently Envigo, Cumberland, VA) and cotton nesting material. Animals were subject to a 12-hour light-dark cycle and had ad-libitum access to Teklad LM-485 7012 standard rodent chow and tap water. Two weeks after their arrival, mice were administered 0.9% saline, 1.8 g/kg or 4 g/kg ethanol via intraperitoneal injection. Four hours following the

injection, mice were sacrificed via cervical dislocation and decapitation in order to obtain brain tissue for dissection. All procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee in accordance with National Institute of Health guidelines.

Tissue Collection

Immediately after sacrifice, whole brains were removed and dissected as described by Kerns et al. (2005). In brief, whole brain tissue was chilled for 1 minute in 1X phosphate buffer on ice, then dissected to isolate amygdala, nucleus accumbens, prefrontal cortex, caudate putamen, septum, hypothalamus, cerebellum, entorhinal cortex, and ventral midbrain. Tissue sections were placed in individual tubes, flash frozen in liquid nitrogen, and stored in a -80°C freezer until RNA extraction.

Nucleic acid extraction and evaluation

Total RNA was isolated from amygdala and nucleus accumbens tissue as described previously (Kerns et al., 2005) using homogenization in STAT-60 (Tel-test, Inc., Friendswood, TX, USA) followed by RNA purification with a Qiagen RNeasy Mini Kit (Qiagen, Redwood City, CA, USA). RNA concentration in each sample was quantified based on UV-Vis absorbance at 260nm using a ThermoFisher Nanodrop 2000 Spectrometer. Sample quality was assessed using RNA Quality Indicator values acquired from analysis with a Bio-Rad Experion™ Automated Electrophoresis System with Experion RNA StdSens analysis kits using the included protocol. Per the Bio-Rad Experion™ software protocol, samples with RNA quality indicator (RQI) values lower than 7.0 were not included in qPCR experiments.

Quantitative Real Time PCR (qPCR)

cDNA was synthesized from 1μg total RNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), and qPCR was performed using iQ SYBR Green Supermix Kit and CFX Connect Real Time System (Bio-Rad, Hercules, CA, USA). All qPCR amplifications were carried out in sealed Bio-Rad hard-shell, semi-skirted, 96-well PCR plates with clear shells and white wells. All reaction mixtures were 20 μL; detailed parameters for each PCR experiment protocol can be found in the results section and Appendix 2. In brief, for each primer pair, temperature gradients were carried out with one technical replicate of one sample per strain across a temperature range of 54°C to 64°C. Optimum annealing temperature was determined based on gel electrophoresis experiments described below. Standard curves were obtained using 3 technical replicates of one sample per region per strain in 1:5 serial dilutions with nuclease free water resulting in cDNA quantities of 0.04ng, 0.2ng, 1ng, and 5ng. The annealing temperature in the thermocycler protocol for standard curves was set to the optimum temperature for each primer pair as determined in temperature gradient qPCR experiments. In order to obtain preliminary data, temperature gradients and standard curves were carried out using one biological replicate per region per strain. In future primer validation studies, it is vital that **at least three** biological replicates per region per strain are used for robust results and proper quantification of limit of detection (LOD) and limit of quantification (LOQ).

Gel Electrophoresis

Optimum annealing temperature for each primer pair was determined using agarose gel electrophoresis. Following qPCR, five microliters of reaction mixture from each sample at each temperature were run with 4% agarose and 1X GelRed® (Biotium, Fremont, CA) in 1X TBE at 90 volts. Bands were visualized using a Kodak Image Station and Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY). Optimum annealing temperature for a primer pair

was selected if the run temperature of a given reaction mixture produced a single band at the predicted molecular weight of the product, appeared to have the highest quantity of product relative to other run temperatures, and had no visible bands indicating the presence of primer dimers at low molecular weights. Primer pairs that did not meet all of these criteria were discarded.

Data Analysis

For standard curves, C_q values were calculated using the Bio-Rad CFX Manager 3.1 Software Single Threshold determination mode. C_q data and standard curves were analyzed and plotted using Microsoft Excel 2016 for Mac. Efficiency was calculated using the following equation:

$$\% \text{ Efficiency (E)} = -1+10^{\left(-1/Slope\ of\ standard\ curve\right)} * 100$$

The final candidate genes and total number of reference genes to be used were selected using the GeNorm macro V3.5 for Microsoft Excel previously downloaded from the GeNorm website. Although this version is no longer available from its originators, the updated version is included as part of Biogazelle's qbase+ software available at http://www.qbaseplus.com/.

Results

In silico Analysis

In silico analyses reveal five candidate reference genes.

Overlap analyses of the three gene sets revealed twelve genes in common between at least two of the sets (Figure 2.1). In addition to these twelve genes, *Ppp2r2a* was included as a candidate gene in further bioinformatic analyses due to its use as the sole reference gene for previously published qPCR experiments from our laboratory. This resulted in a total of thirteen genes used in the following bioinformatic analyses of candidate reference genes.

Reference genes (n=13) were evaluated for potential use in experiments comparing nucleus accumbens in B6 and D2 male mice exposed to saline or 1.8 g/kg ethanol, and amygdala from ethanol-naïve animals. As a preliminary screen, available nucleus accumbens microarray data from previously published experiments (Kerns et al., 2005) were examined for evidence of differential expression of candidate genes (n=13) between strains and ethanol dose group. The results of this analysis are summarized in Table 2.3 and Table 2.4. One *Actb* probeset located in the last two exons of the transcript indicated differential regulation of *Actb* by ethanol between B6 and D2 mice in nucleus accumbens ($|Sscore_{DBA/2J} - Sscore_{C57BL/6J}| > 2$). Available RMA values from microarrays in ethanol-naïve B6 and D2 mice indicate no difference in basal expression of any of the thirteen genes in amygdala(Mozhui et al., 2010).

Candidate genes (n=13) and their associated transcripts (n=27) were assayed for sequence similarity with unintended targets using the BLASTn algorithm. Of the original 13 candidate genes, five were eliminated due to overlap of their gene sequences with non-specific transcripts or chromosomal areas, while an additional three were eliminated because one or more of their transcript variants overlapped non-specific targets. This resulted in a list of five candidate genes remaining: *B2m*, *Canx*, *Ndufv1*, *Sdha*, and *Sort1*. None of these had pseudogenes. The workflow used and its results are shown in Figure 2.2.

Primer design

The laboratory had previously designed primers for four out of five of the final candidate genes – *B2m, Canx, Ndufv1, and Sort1*. In order to avoid binding of primers to parent gene loci on potential genomic DNA contamination of samples, primer pairs were discarded if neither primer overlapped an exon junction or one of the primers was located in the 3'-untranslated region of a target transcript. Existing primers for *B2m* were the only primers that did not require redesign based on these criteria. New primers for *Canx, Ndufv1, Sort1, and Sdha* were designed using NCBI PrimerBLAST, and subsequently evaluated using UCSC in-silico PCR, UCSC BLAT and/or NCBI BLAST, and IDT-UNA Fold. The alignment of all primers to their target mRNAs are shown in Figure 2.3 and Figure 2.4; primer sequences are listed in Table 2.5.

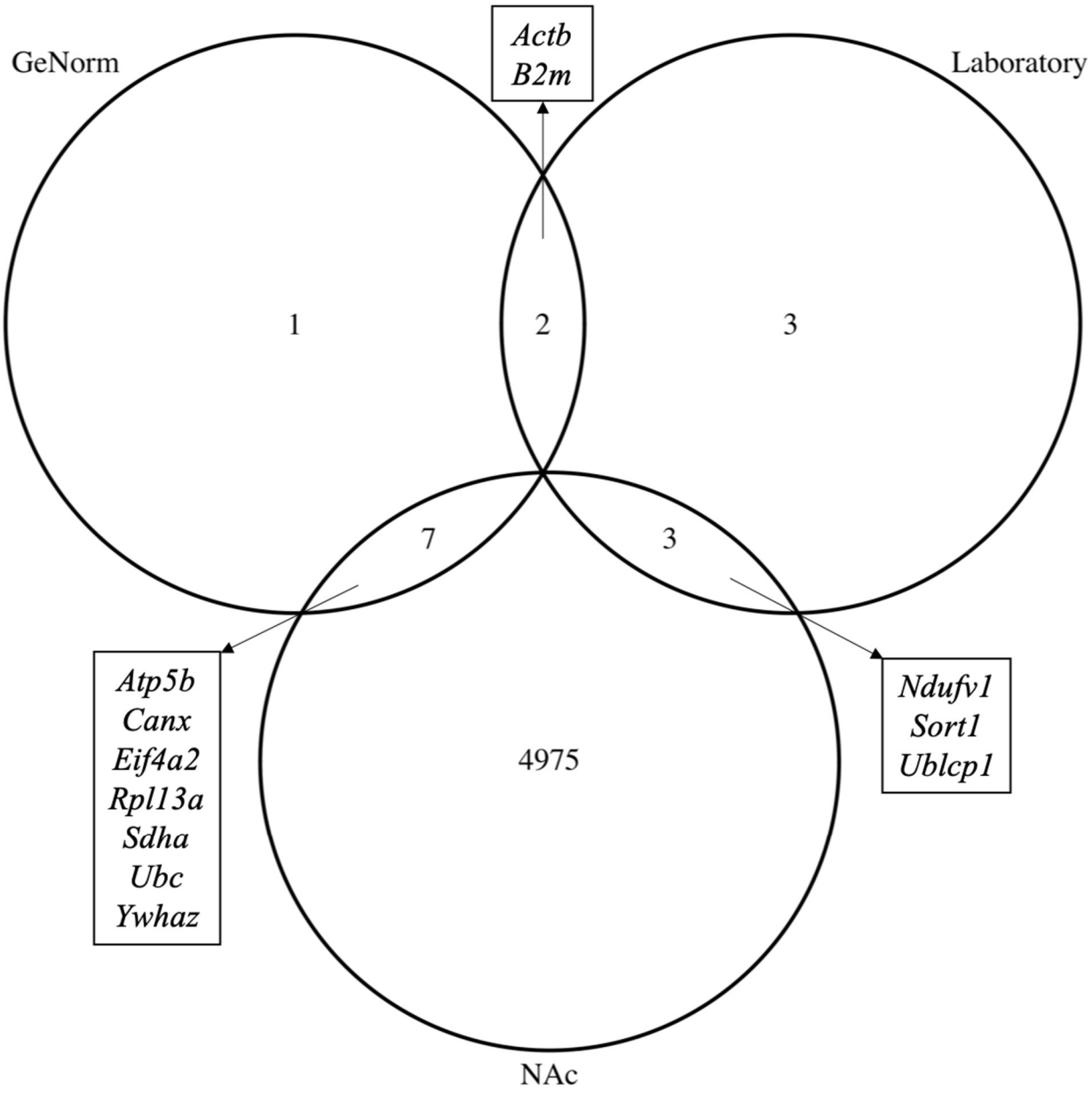


Figure 2.1 Overlap of Candidate Reference Gene Sets
Sets of genes from our laboratory (n=8), GeNorm kits (n=10), and an analysis of nucleus accumbens (NAc) microarray data from our laboratory filtered for genes with high expression levels (RMA values greater than 6.64) and no differential expression with acute ethanol exposure (within-strain saline vs. 1.8g/kg ethanol |Sscores|<2) (n=4985).

Gene Symbol	ProbeSetID / RecordID	ProbeTarget	Saline RMA		Ethanol RMA		Ethanol vs Saline Sscore	
			C57BL/6 J	*DBA/2 J*	*C57BL/6 J*	*DBA/2 J*	*C57BL/6J*	*DBA/2J*
Actb	1419734_at	mid 3' UTR	10.277	10.299	10.255	10.153	-0.053	-0.714
	1436722_a_at	exon 3, 4, 5 and proximal 3' UTR	14.491	14.306	14.556	14.302	0.814	-0.729
	AFFX-b-ActinMur/M12481_3_at	last two exons (Affymetrix control, 3'-most probe set used to calculate 3':5' ratio)	14.526	14.422	14.589	14.346	1.120	-1.160
	AFFX-b-ActinMur/M12481_M_at	exon 3 (Affymetrix control, middle probe set used to compute 3':5' ratio)	13.911	13.659	13.956	13.665	0.955	-0.661
	AFFX-b-ActinMur/M12481_5_at	exons 2 and 3 (Affymetrix control, 5'-most probe set used to calculate 3':5' ratio)	14.036	13.780	14.081	13.805	1.018	-0.377
Atp5b	1416829_at	exons 8 and 9	14.144	14.109	14.109	14.114	0.140	-0.372
B2m	1427511_at	first intron	5.773	5.779	5.817	5.737	0.461	0.150
	1452428_a_at	two exons	11.047	10.896	11.057	10.919	0.046	0.404
	1449289_a_at	last two exons and 3' UTR	11.052	10.898	11.035	10.979	-0.168	1.056
Canx	1445045_at	NA	4.992	5.032	5.006	5.049	-0.089	-0.274
	1428935_at	NA	8.775	8.737	8.784	8.672	-0.228	-0.684
	1415692_s_at	mid 3' UTR	12.380	12.382	12.300	12.430	-0.348	0.265
	1422845_at	last exon and proximal 3' UTR	10.972	10.874	10.809	10.995	-1.073	0.719
Eif4a2	1450934_at	last exon and 3' UTR	13.856	13.880	13.821	13.883	0.293	-0.465
Ndufv1	1456015_x_at	last exon and 3' UTR	11.987	12.076	12.011	12.064	0.464	-0.181
	1415966_a_at	last exon	12.274	12.370	12.296	12.397	0.307	0.047
	1415967_at	exons 7, 8, and 9 (of 10)	12.181	12.270	12.186	12.256	0.365	-0.187
Ppp2r2a	1429715_at	NA	8.088	8.078	8.092	8.078	-0.273	-0.024
	1437730_at	NA	12.097	11.940	12.065	11.900	0.079	-0.647
	1453260_a_at	last exon and last intron	10.998	10.773	10.944	10.825	-0.026	0.495
Rpl13a	1433928_a_at	NA	10.789	10.942	10.809	10.977	0.108	0.280
	1455001_x_at	NA	11.659	11.730	11.736	11.759	0.682	-0.293
	1455485_x_at	last three exons	13.888	13.842	13.947	13.848	0.794	-0.196
	1435873_a_at	last two exons	13.841	13.832	13.915	13.840	0.892	-0.055
	1417608_a_at	exons 3 and 4	13.302	13.236	13.324	13.238	0.905	0.300

Table 2.3 Nucleus Accumbens Microarray Data for Candidate Reference Genes

The *Actb* probe highlighted in red shows differential regulation by ethanol in B6 and D2 mice.

Gene Symbol	ProbeSetID / RecordID	ProbeTarget	Saline RMA		Ethanol RMA		Ethanol vs Saline Sscore	
			C57BL/6J	*DBA/2J*	*C57BL/6J*	*DBA/2J*	*C57BL/6J*	*DBA/2J*
Sdha	1426689_s_at	mid-distal 3' UTR	12.012	12.066	12.015	12.033	0.172	-0.301
	1426688_at	last two exons and proximal 3' UTR	12.496	12.481	12.497	12.503	0.277	-0.062
	1445317_at	intron (not expressed)	7.600	7.481	7.655	7.400	0.278	-0.045
	1433293_at	intron 1 (expressed)	8.686	8.671	8.807	8.656	0.934	0.318
Sort1	1423363_at	proximal 3' UTR	9.480	9.190	9.339	9.452	-0.800	1.069
	1450955_s_at	proximal 3' UTR	9.450	9.198	9.307	9.449	-0.579	0.992
	1423362_at	distal 3' UTR	11.856	11.817	11.794	11.811	-0.465	-0.252
Ubc	1432827_x_at	only coding exon	13.946	14.030	13.949	14.069	0.330	-0.124
	1420494_x_at	only coding exon and 3' UTR	13.433	13.457	13.485	13.513	0.676	0.134
	1425966_x_at	only coding exon and 3' UTR	12.196	12.205	12.206	12.233	0.113	0.109
	1437666_x_at	only coding exon and 3' UTR	13.486	13.507	13.503	13.531	0.763	-0.128
	1425965_at	NA	7.721	7.600	7.649	7.577	-0.186	-0.541
	1438137_at	NA	6.971	7.050	7.039	7.065	0.914	-0.444
	1458507_at	NA	7.332	7.509	7.443	7.479	0.113	-0.043
Ublcp1	Data Not Available							
Ywhaz	1416103_at	far 3' UTR	10.592	10.596	10.604	10.566	-0.110	0.125
	1436971_x_at	distal 3' UTR	13.694	13.703	13.681	13.702	0.352	-0.336
	1448218_s_at	mid distal 3' UTR	13.189	13.191	13.096	13.157	-0.179	-0.557
	1439005_x_at	multiple probe targets (antisense to human Ywhaz sequence)	10.293	10.092	10.262	10.040	0.076	-0.126
	1436981_a_at	antisense probe set in mid 3' UTR of Ywhaz (LTR element homology)	10.981	10.905	11.059	10.856	0.499	-0.008
	1416102_at	mid-proximal 3' UTR	12.626	12.479	12.520	12.557	-0.341	0.012
	1448219_a_at	mid 3' UTR	13.411	13.237	13.328	13.297	-0.235	-0.073
	1443893_at	intron 2	5.354	5.325	5.432	5.399	-0.161	-0.068

Table 2.4 Nucleus Accumbens Microarray Data for Candidate Reference Genes (continued)

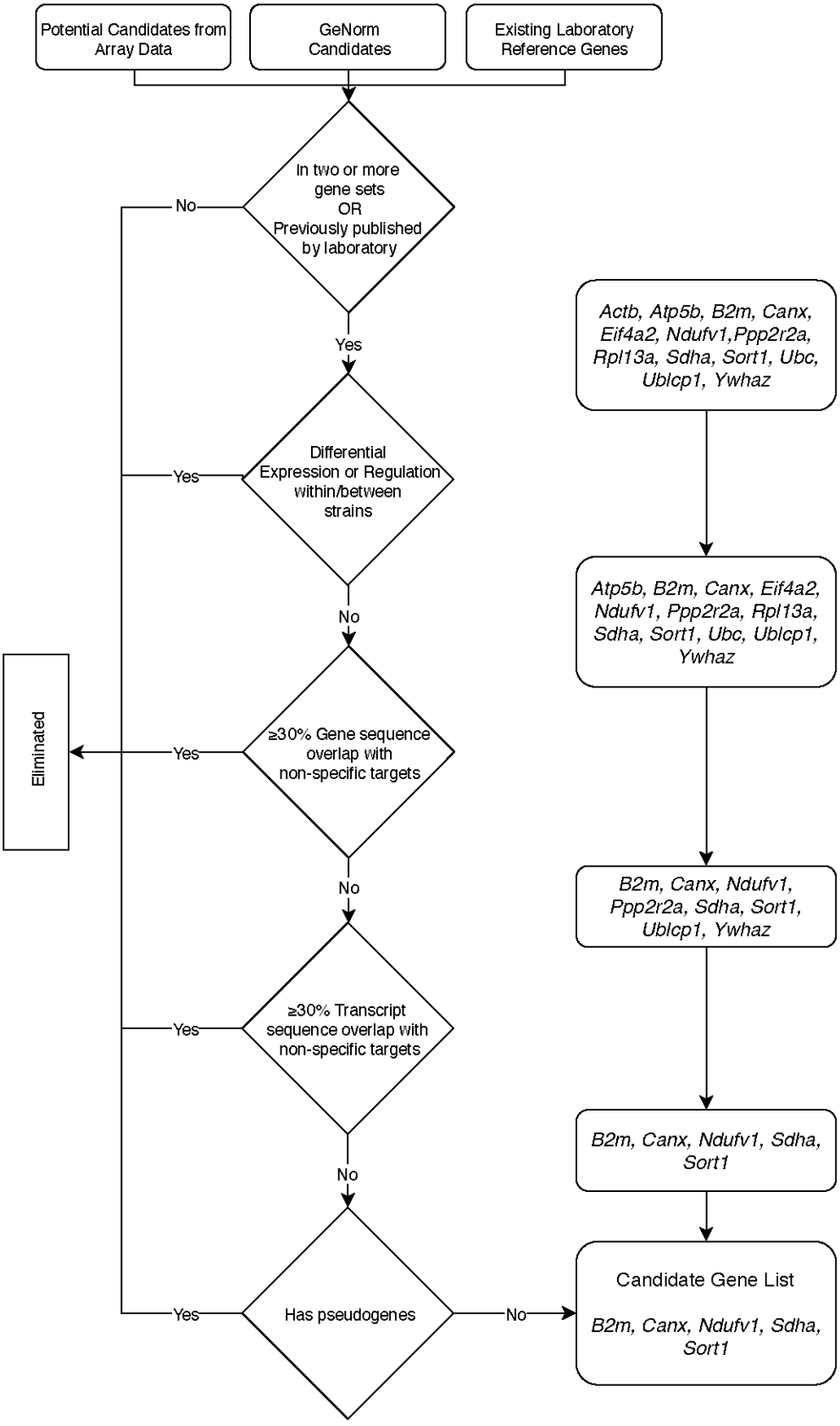


Figure 2.2 Workflow for Candidate Gene Selection

In vivo validation

RNA Sample Quality

Canx, Sort1, and *Ndufv1* primers were analyzed first due to their immediate availability. *Ppp2r2a* and *Ublcp1* were also evaluated because of their broader applicability to ongoing experiments in the laboratory. RNA sample quality for B6 and D2 amygdala and nucleus accumbens was assessed prior to any qPCR experiments. Figure 2.5 shows the virtual gel electrophoresis images and resulting RNA quality indicator (RQI) numbers from Experion runs of the four samples used to produce temperature gradients and standard curves.

Primer evaluation

Annealing temperatures of primer pairs were evaluated using a 3-step qPCR protocol with a plate temperature gradient spanning 10°C and centered around the predicted melt temperature of the primers as the annealing step. In order to reduce the number of agarose gels run, qPCR products from sample reactions at the annealing temperature with the lowest Cq and no-template control reactions at the lowest annealing temperature were evaluated. Primer pairs *Ublcp1* F/R2, and *Ublcp1* F/R3, were discarded because more than one distinct band was visible in the sample qPCR products (Figure 2.6). Standard curves were produced with samples from one B6 and one D2 mouse using the annealing temperature with the lowest Cq from remaining primer pairs for all genes except *Canx* and *Sort1*. Efficiency and R^2 were determined for each strain and brain region separately (Figure 2.7). *Ndufv1* F/R2 primers were excluded from further analyses in amygdala because both efficiencies were not between 80-100%. The remaining primers (*Canx* F/R3, *Sort1* F/R2, *Ppp2r2a* F/R, and *Ublcp1* F/R4) were used in subsequent experiments evaluating candidate reference genes in amygdala. *Canx* F/R3, *Sort1* F/R2, *Ndufv1* F/R2, and *Ublcp1* F/R4 were evaluated in NAc.

Figure 2.3 New and Existing Primer Alignments

Primers are displayed as two arrows flanking the intended amplicon. The direction of the arrow indicates the direction of the primer. Primers or amplicons highlighted in red indicate that they did not meet primer design criteria.

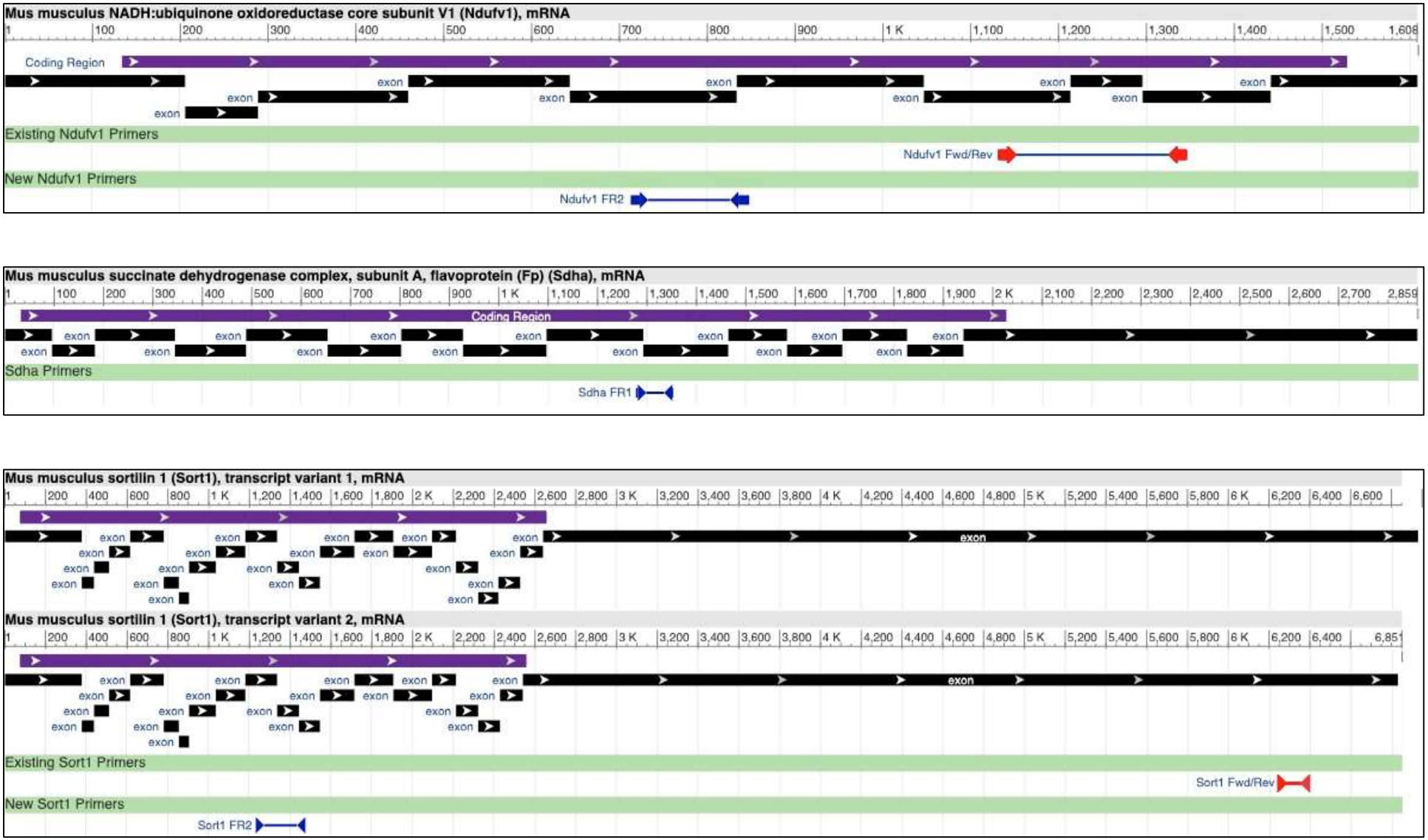
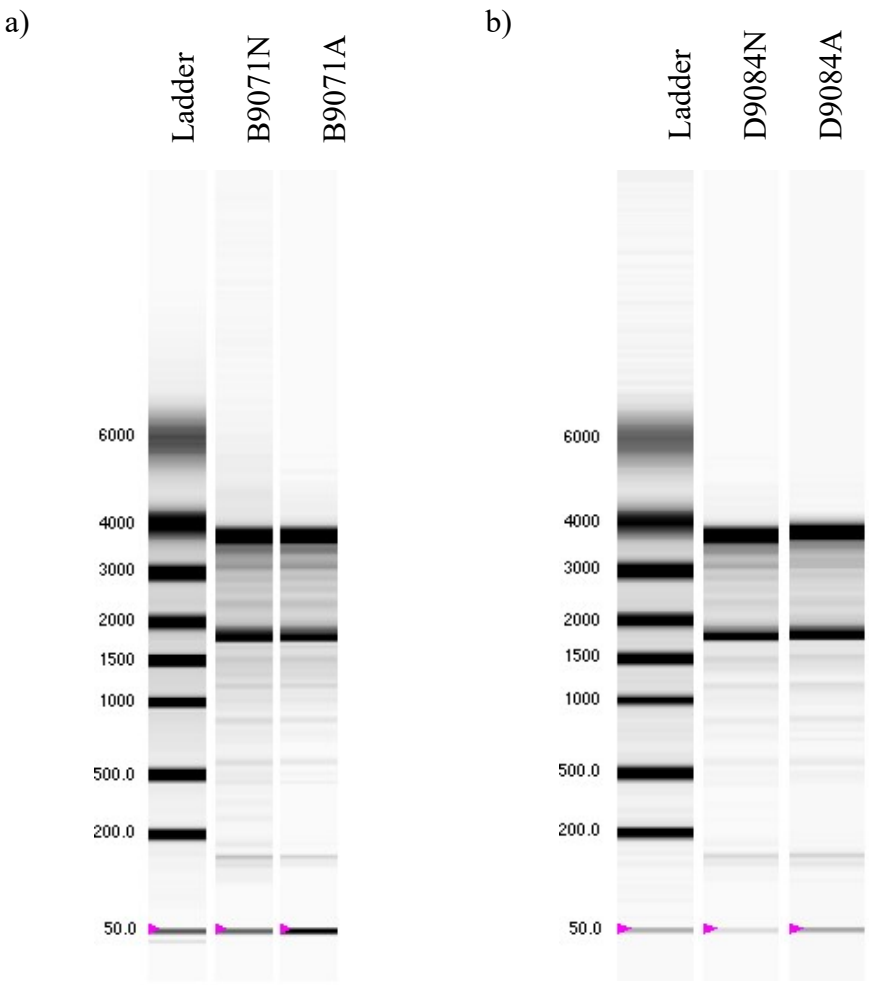


Figure 2.4 New and Existing Primer Alignments (continued)

Primers are displayed as two arrows flanking the intended amplicon. The direction of the arrow indicates the direction of the primer. Primers or amplicons highlighted in red indicated that they did not meet primer design criteria.

Existing Primers			
Target Gene	**Primer Pair Name**	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
B2m	B2m F/R1	GGAGAATGGGAAGCCGAACA	TCTCGATCCCAGTAGACGGT
Canx	Canx Fwd/Rev	TGGCAGTCAAGATGAGGAAG	GAAGGGGAGGGAGAGGAAAT
Ndufv1	Ndufv1 Fwd/Rev	GACCGTGCTAATGGACTTCG	GGCATCTCCCTTCACAAATC
Sort1	Sort1 Fwd/Rev	TTTATCAGTATGCCCCGAAT	CCATCAAACACAGGGACTCA
Ublcp1	Ublcp1 F2/R2	GCTAAAATGAAAGAGCTGGGCG	ACACCAAGAGGCTTCACGTC
New Primers			
Target Gene	**Primer Pair Name**	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
Canx	Canx F2/R2	CAGATGACTGGGATGAAGACGC	TTCCTTGGTTTCCAGATTCCCTG
	Canx F3/R3	GCTTTGCCAGTGTTCCTTG	ATTTCATCCTCCTCTGCTTTAGG
Ndufv1	Ndufv1 F2/R2	ATGTGTTTGTGGTGCGTGGG	GGCATCCAAACACTCCCACATC
Sdha	Sdha F1/R1	AACTACAAGGGACAGGTGCTG	CCTCCCCACAGGCATACAG
Sort1	Sort1 F2/R2	CTCTATACCACCACAGGCGG	GAAGGCTGCACTCGTTCTTG
Ublcp1	Ublcp1 F3/R3	AATGAAAGAGCTGGGCGTGA	CCCAAATGACACCAAGAGGC
	Ublcp1 F4/R4	TCCTGGTGCTGGATGTTGAC	TCACGCCCAGCTCTTTCATT

Table 2.5 New and Existing Reference Gene Primer Sequences



Strain	Region	Sample Name	RNA Area	RNA Concentration (ng/µL)	Ratio [28S:18S]	RQI	RQI Classification
D2	NAc	9084N	20.48	152.02	1.33	9.4	■
D2	Amygdala	9084A	50.96	59.91	1.57	8.9	■
B6	NAc	9071N	148.34	74.15	1.17	8.8	■
B6	Amygdala	9071A	65.49	32.74	1.14	9.3	■

Figure 2.5 RNA Quality Asessment of Samples Used for Standard Curves

a) Virtual gel image from NAc and amygdala of a B6 mouse
b) Virtual gel image from NAc and amygdala of a D2 mouse

Canx and Sort1 are optimum reference genes to use in experiments evaluating gene expression in ethanol dose-response experiments with B6 and D2 mice

Variability in expression of candidate reference genes was examined in amygdala from B6 and D2 mice i.p. injected with 0 g/kg, 1.8 g/kg, or 4 g/kg ethanol (n = 7-8 per group). Mean Cq values for each gene in amygdala are shown in Figure 2.8. No significant differences were found between experimental groups for any of the genes in amygdala (two-way ANOVA, α = 0.05). GeNorm analysis of these results determined the optimum number of reference genes to be 2-3 ($V_{2/3}$ = 0.121, $V_{3/4}$ = 0.189) and *Canx and Sort1* to have the lowest amount of variability in expression across experimental groups among the genes tested (M = 0.345, Figure 2.10). In NAc from saline treated B6 and D2 mice, no significant differences were found between Mean Cq for any of the genes studied (Figure 2.9); the most stable genes were *Canx* and *Ublcp1* (Figure 2.11).

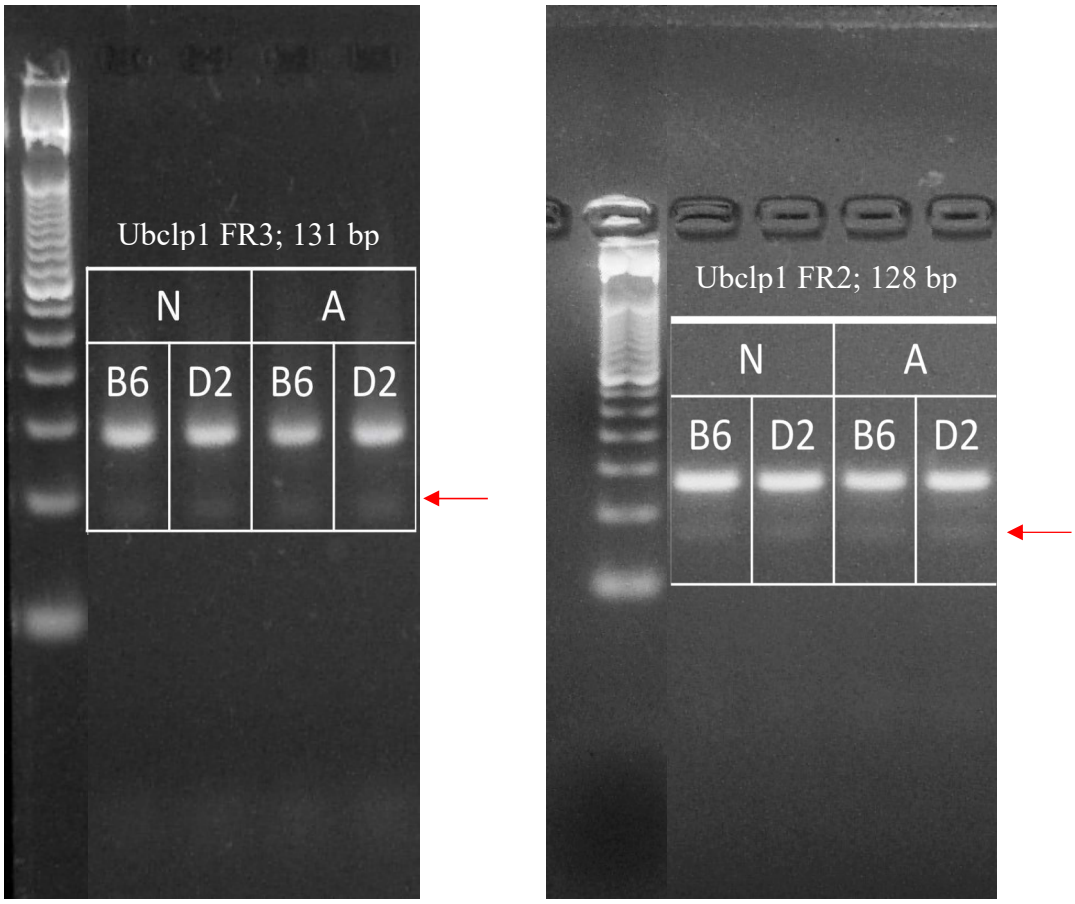


Figure 2.6 Gel electrophoresis of *Ublcp1* primer products

Red arrows show location of potential unintended product. N = nucleus accumbens, A = amygdala. *Note: The ladder and samples shown in each image were run on the same gel. The whole gel images have been cropped to remove wells with samples from unrelated experiments*

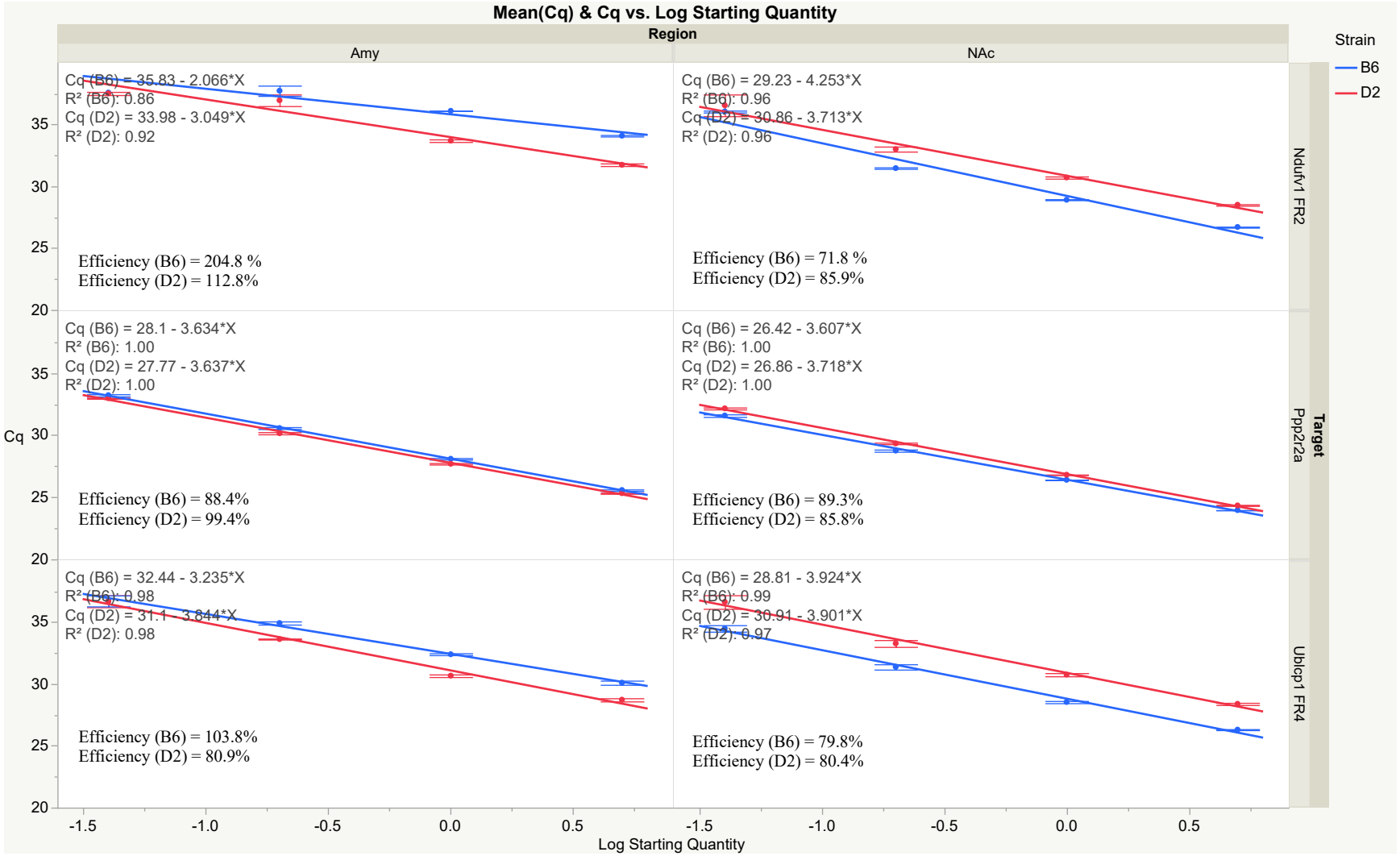


Figure 2.7 Standard Curves for Candidate Reference Genes in NAc and Amygdala

Mean Cq ± SEM of three technical replicates of one sample per strain in each brain region.

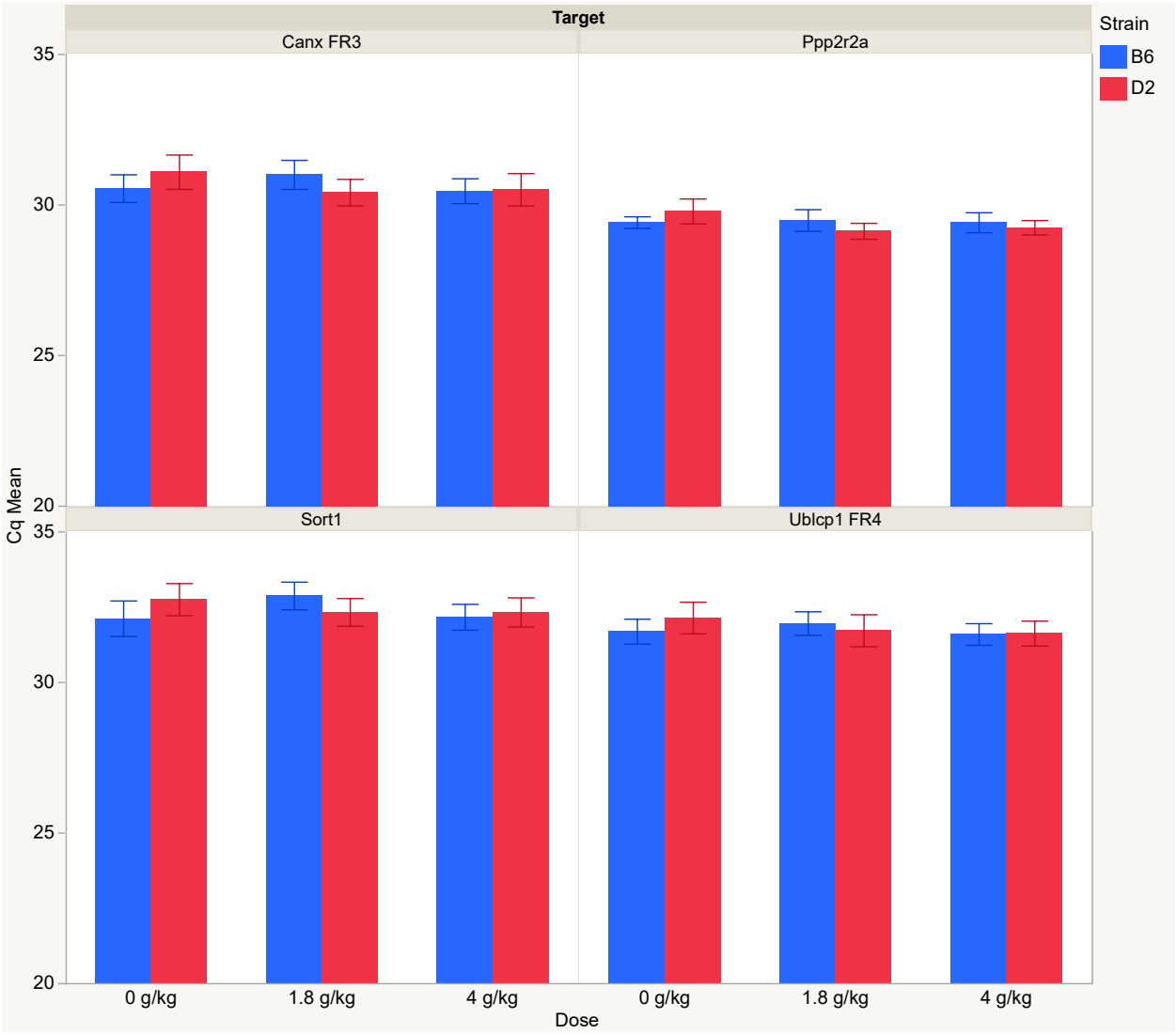


Figure 2.8 Mean Cq of Candidate Reference Genes Across Ethanol Doses and Strains In Amygdala

Data are presented as mean ± SEM (n=6-8 per group). No significant differences were identified with two-way ANOVA with Tukey's post-hoc tests (α = 0.05).

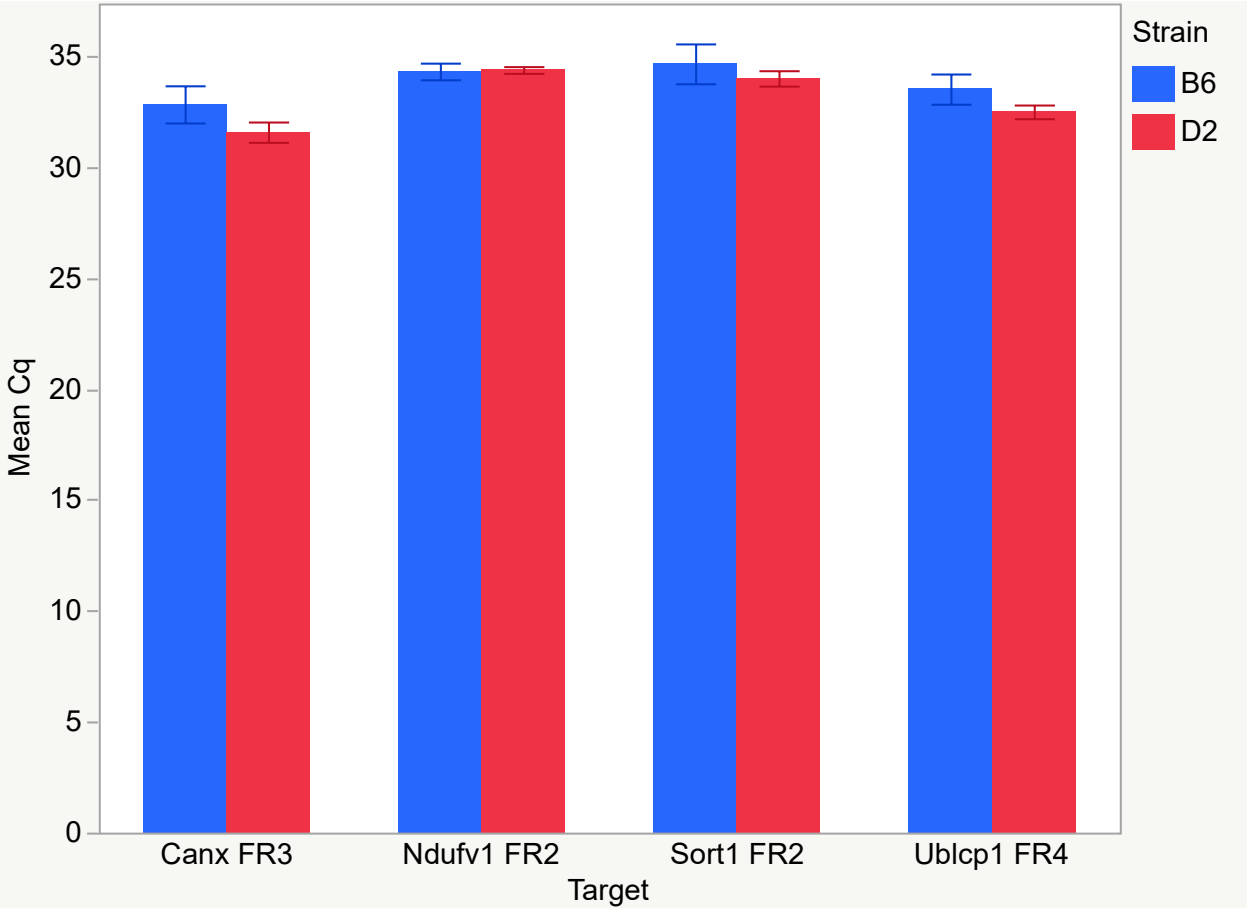


Figure 2.9 Mean Cq of Candidate Reference Genes in Saline-treated B6 and D2 Nucleus Accumbens

Data are presented as mean ± SEM (n=6-8 per group). No significant differences were identified with two-way ANOVA with Tukey's post-hoc tests (α = 0.05)

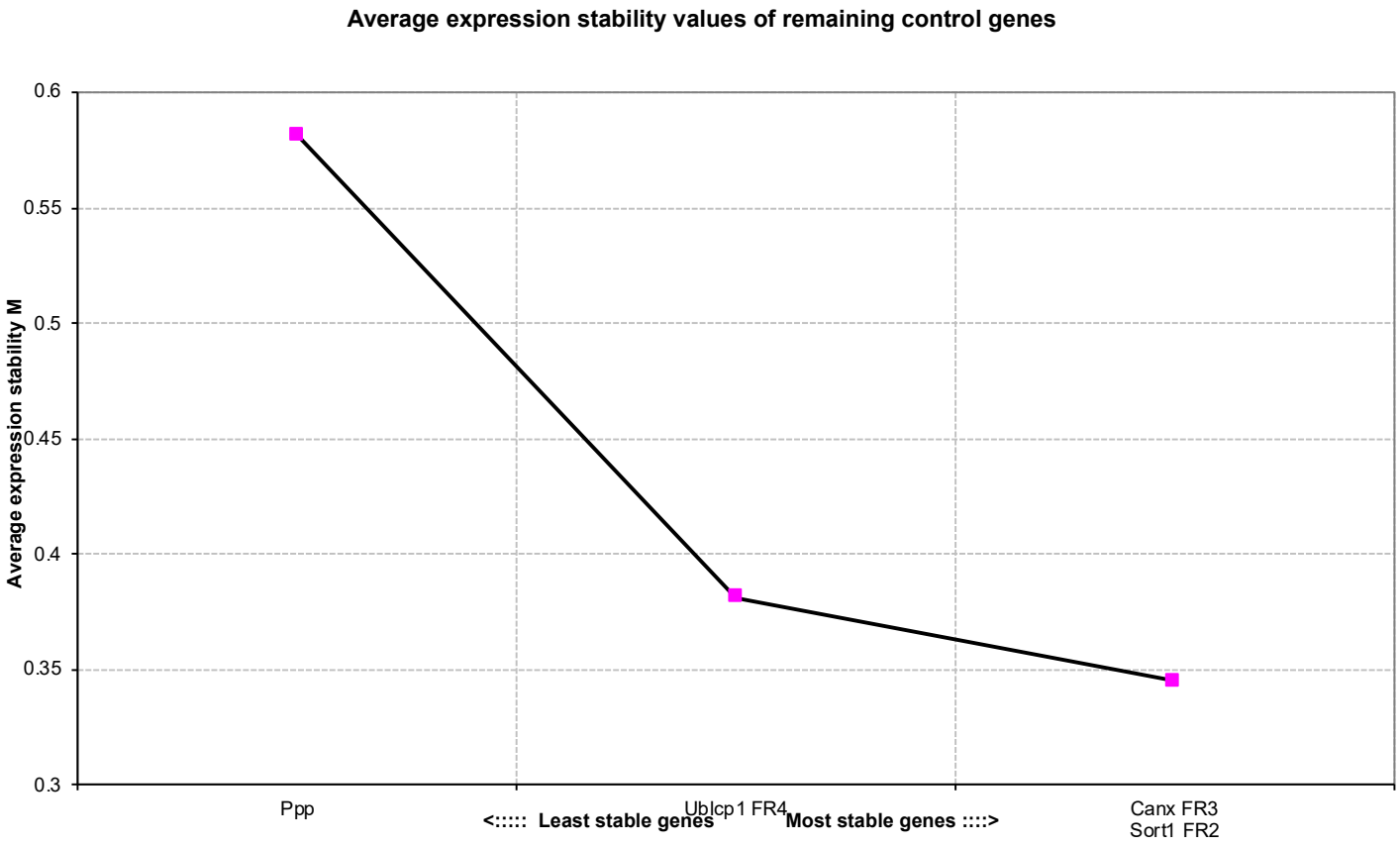


Figure 2.10 GeNorm Ranking of Most Stable Reference Genes in Amygdala

Average expression stability values of candidate reference genes in amygdala (n=46) from B6 and D2 mice exposed to saline, 1.8g/kg ethanol, or 1.4 g/kg ethanol.

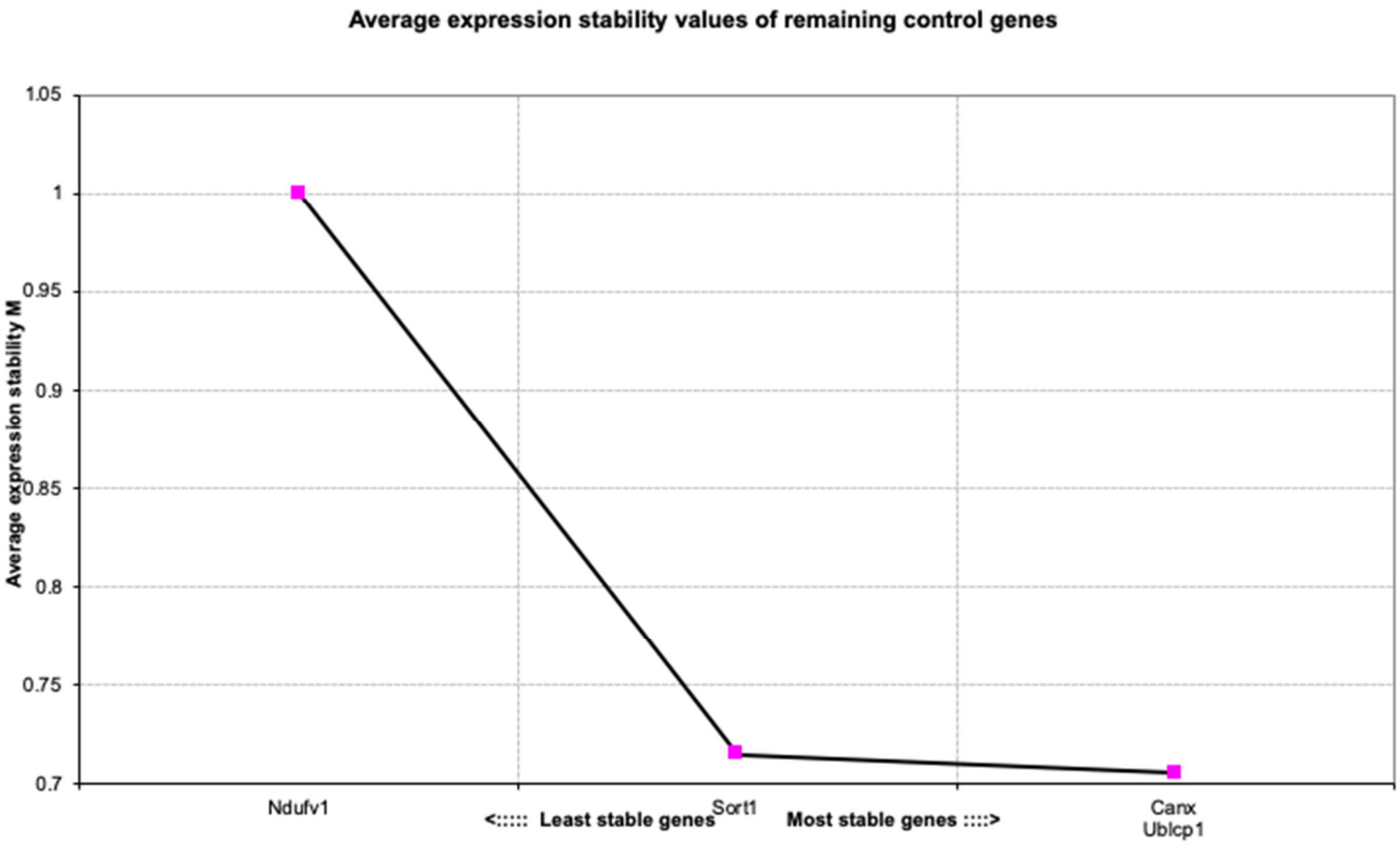


Figure 2.11 GeNorm Ranking of Most Stable Reference Genes in Nucleus Accumbens

Average expression stability values of candidate reference genes in nucleus accumbens (n=14) from saline treated B6 and D2 mice

Discussion

Prior to the evaluation and collection of the data presented in this chapter, the most commonly used reference genes to evaluate target gene expression in NAc and other brain regions of ethanol treated B6 and D2 male mice in our laboratory were *Ppp2r2a* and *Ublcp1*. These genes and their primers were chosen based on prior experiments validating their use as reference genes in ventral midbrain samples (Megan O'Brien, Miles laboratory; data not shown). In light of the developing standards for reference genes proposed by (Vandesompele et al., 2002), and in an effort to comply with MIQE guidelines (Bustin et al., 2009), new and existing laboratory reference genes were assessed for use in future experiments in both amygdala and NAc of B6 and D2 mice. Of the 13 genes initially considered, only *B2m*, *Canx*, *Ndufv1*, *Sdha*, and *Sort1* met all of the *in-silico* criteria used for selection of optimum candidates. Primers were then designed and validated in B6 and D2 NAc and amygdala using melt curves and standard curves to determine efficiency.

Based on GeNorm analyses, *Canx* and *Sort1* were the two genes with the least variability in amygdala of B6 and D2 mice from an acute ethanol dose-response experiment. At a minimum, these two reference genes should be used in further qPCR experiments comparing gene expression in amygdala from ethanol treated mice of these strains, followed in order by *Ublcp1* and *Ppp2r2a.* In NAc, candidate reference genes were only evaluated from saline-treated B6 and D2 mice. For experiments comparing basal gene expression in NAc of B6 and D2 mice, *Ublcp1* and *Sort1* are the most stably expressed between strains, followed in order by *Canx* and *Ndufv1*. Any comparison of B6 and D2 gene expression in NAc involving ethanol exposure will require further evaluation of candidate reference gene stability under those conditions.

It is important to note that *Ppp2r2a* was not present in the NAc microarray data from saline and ethanol treated BXD strains filtered for genes that were not differentially regulated by

ethanol (Kerns et al., 2005), particularly because of its use as the sole reference gene in previous experiments in NAc of B6 and D2 mice in the laboratory. In fact, qPCR data from ethanol-naïve B6 and D2 NAc suggests *Ppp2r2a* could be differentially expressed between the strains (Figure 2.12). Furthermore, ~50% of each of four *Ppp2r2a* transcript variants has greater than 90% sequence homology with a non-coding area of Chromosome 12, which makes it very difficult to design primers that will bind specifically to cDNA from *Ppp2r2a* transcripts in the presence of genomic DNA contamination. This would be less of an issue if total RNA samples were DNAse-treated prior to reverse transcription, but unless that is a laboratory-wide practice, target sequence similarity with genomic DNA needs to be considered when designing primers. Regardless, because of the apparent differential expression of *Ppp2r2a* in B6 and D2 NAc under basal conditions, it is the recommendation of the author that *Ppp2r2a* no longer be used as a reference gene in NAc for comparison of B6 and D2 gene expression. Similarly, existing qPCR experiments comparing gene expression in B6 and D2 NAc that use *Ppp2r2a* as a reference gene should be repeated with appropriately validated reference genes.

The difference in *Ndufv1* primer efficiency between NAc and amygdala in both strains, and the apparent differential expression between strains of *Ppp2r2a* in NAc, but not amygdala highlights the need for validation of reference genes as well as their primers for any experiments with new or unique conditions. *Ndufv1* may be stably expressed in amygdala, but the low total expression level of this gene makes that impossible to quantify in this region. Consequently, reference gene primers validated in one experimental condition (in this case, brain region) may not be appropriate for another. Conversely, the elimination of a candidate reference gene because of variability between groups in one experiment does not preclude its use in a different experiment where, for example, the same strains are used but a different brain region is being examined. Perhaps the most important conclusion gathered from this work is that accurate reporting of gene expression differences using relative quantification in qPCR requires

evaluation of **multiple** reference genes across all groups for the **specific experimental paradigm** being used.

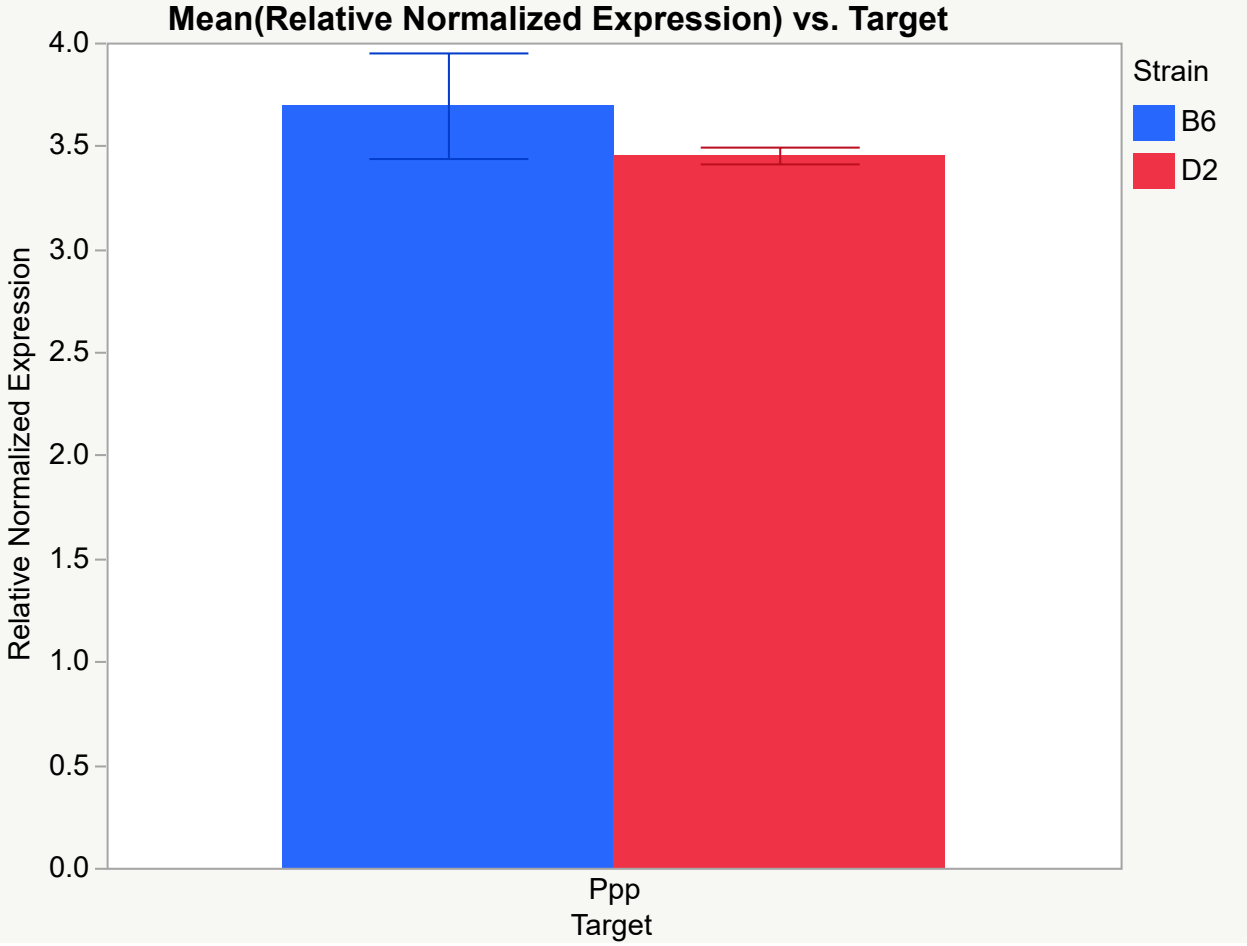


Figure 2.12 Ppp expression in NAc Normalized to Ublcp1

Data are presented as mean ± SEM (n=4 per strain). $P_{student's\text{-}t} = 0.45$

Chapter 3 Ninein Characterization as a Functional Validation of the Use of Proper Reference Genes

Introduction

Ninein was identified by our laboratory as a quantitative trait gene for ethanol induced anxiolytic-like behavior in BXD mice. Ninein is a microtubule associated protein (MAP) typically found at the minus end of microtubules in the centrosome, but has recently been identified in the cytoplasm of all parts of embryonic cortical neurons in mice, playing a role in axon outgrowth and branching (Srivatsa et al., 2015). There are 3 known NIN protein isoforms, and 6 known *Nin* transcript variants in mice (Table 3.1). The D2 allele at a potentially deleterious SNP (rs29159683) and another missense SNP (rs29192398) in these variants results in the creation or alteration of an exon splice enhancer sequence that promotes the splicing of exon 18 (RefSeq NR_104397.2). The absence of exon 18 has been shown to result in the dissociation of NIN peptides from the centrosome, and the presence of another exon (exon 29) results in diffusion of NIN peptides throughout the cytoplasm. Perhaps more striking, NIN peptides translated from these alternatively spliced variants are present almost exclusively in differentiated neurons (Zhang et al., 2016). In NCBI's current genome assembly, *Nin* transcript variants containing exon 29 are labeled non-coding because the presence of this exon creates a downstream frameshift resulting in a 'premature' stop codon, making these transcripts suspected targets for nonsense-mediated mRNA decay. While the stop codon present in transcript variants containing exon 29 is upstream of stop codons in other variants, the findings of Zhang et al. suggest that the resulting protein is not dysfunctional, but rather localizes differently than other isoforms. This may be problematic in mitotic cells where the primary function of NIN is in the centrosome, but non-deleterious in post-mitotic cells such as neurons where the stability of microtubules in or near dendritic spines plays an integral role in synaptic plasticity.

The provisional proteins NIN4 and NIN5 (see figure 7c in Putman et al. 2016), that were differentially expressed in NAc of B6 in D2 mice, have molecular weights corresponding to the predicted protein products of *Nin* transcript variants 5 and 6, containing exon 29. However, as of the time of this writing, there is no evidence for the presence of either of these transcripts in amygdala or nucleus accumbens of B6 or D2 mice *in vivo*.

In this chapter, basal expression of specific *Nin* transcript variants is examined in both nucleus accumbens and amygdala of B6 and D2 mice using appropriately selected reference genes from the previous chapter. The aim of these experiments is to validate the use of proper reference genes while providing a structural/functional basis for the role of *Ninein* in *Etanq1*-related behaviors.

Primer Set	Exon(s) Amplified*	Variant(s) Targeted	Protein Product	Functional Significance	Tissue Presence
NinEx5,6,7	5,6,7	All	Varies	Varies	Varies
NinEx16,17	16,17 ("large exon")	1-5	Varies	Localizes *Nin* to centrosome	Somatic, Nervous
NinTV1		1	NIN isoform 1	Microtubule anchoring at centrosome	Somatic, Nervous
NinTV2,3		2,3	NIN isoform 2	Microtubule anchoring at centrosome	Somatic, Nervous
NinTV4		4	NIN isoform 3	Microtubule anchoring at centrosome	Somatic, Nervous
NinTV5,6	29	5,6	Unknown	Peptides localize away from centrosome;	Neurons
NinTV6	Absence of "large exon"	6	Unknown	Peptides localizes away from centrosome AND throughout cytoplasm	Neurons

Table 3.1 Targeted Ninein transcript variants and protein isoforms

Methods and Materials

Experimental Subjects

C57BL/6J (n=8) and DBA/2J (n=8) male mice were obtained at 8 weeks of age from Jackson Laboratories (Bar Harbor, ME) and housed 4 per cage on ventilated racks with Teklad Sani-Chip bedding and cotton nesting material. All animals had ad libitum access to Teklad LM-485 7012 standard rodent chow and water under a 12-hour light-dark cycle. After two weeks, animals were sacrificed via cervical dislocation followed by decapitation. Immediately following, whole brains were removed and dissected as described in Kerns et. al, 2005. Tissue sections were placed in individual tubes, flash frozen in liquid nitrogen, and stored in a -80°C freezer until further use. All experimental procedures were approved by Virginia Commonwealth University Institutional Animal Care and Use Committees in accordance with NIH guidelines.

Nucleic acid extraction and evaluation

Total RNA was isolated from amygdala tissue as described previously (Kerns et al., 2005) using homogenization in STAT-60 (Tel-test, Inc., Friendswood, TX, USA) followed by RNA purification with a Qiagen RNeasy Mini Kit (Qiagen, Redwood City, CA, USA). RNA concentration in was quantified based on UV-Vis absorbance at 260nm using a ThermoFisher Nanodrop 2000 Spectrometer. Sample quality was determined using RNA quality indicator (RQI) values acquired from a Bio-Rad Experion™ Automated Electrophoresis System with Experion RNA StdSens analysis kit. Samples with RQI values lower than 7.0 were not included in qPCR experiments.

qPCR

cDNA was synthesized from 500 ng total RNA using Bio-Rad iScript cDNA Synthesis Kit. RT-qPCR was performed using Bio-Rad Universal SYBR Green Supermix in conjunction with

Bio-Rad CFX Connect Thermocycler. Bio-Rad CFX Manager Software was used for calculation of Cq values and relative normalized expression. Cqs were determined using the single threshold method, and relative normalized expression was determined using ΔΔCq method with two reference genes: *Ublcp1* and *Canx*. Target gene and transcript primers were designed and validated using the same procedure described in 0. Specific primer sequences can be found in Appendix 2.

PCR Product Isolation and Sequencing

Following qPCR, reaction mixture from one amygdala sample containing primers that would amplify *Nin* transcript variant 6 was run with 4% agarose and 1X GelRed® (Biotium, Fremont, CA, USA) in 1X TBE at 90 volts. Bands were visualized using a Kodak Image Station and Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY). The resulting band corresponding to the predicted molecular weight of an exon-18-lacking product was isolated using a QIAquick Gel Extraction Kit (Qiagen, Redwood City, CA, USA). Purified qPCR product and transcript variant 6 qPCR primers were sent to Eurofin Genomics DNA sequencing services (Louisville, KY, USA) for sequencing. The resulting sequence overlap was analyzed using NCBI Nucleotide BLAST.

Statistical Analyses

All statistical analyses were performed using JMP Pro 13 statistical software (SAS, Cary, NC, USA). Strain-mean relative normalized expression for each gene was compared using student's t-tests, and groups were considered significantly different if $p<0.05$.

Results

Functional validation of reference genes for measuring basal gene expression in B6 and D2 mice

qPCR was used to compare two reference genes in nucleus accumbens (NAc) of ethanol-naïve B6 and D2 male mice. Figure 3.1a shows the mean Cq values for *Actb* and *Ublcp1* in these samples. Although the strain-mean Cq difference does not reach statistical significance (p=0.3081, student's t-test), the variability of *Actb* expression between both mouse strains results in the appearance of otherwise non-existent strain differences in expression of a target transcript, *Ninein* transcript variant 1 (*Nin*TV1), when it is used as a reference gene. Figure 3.1b shows the same *Nin*TV1 qPCR data (Cq) normalized to *Actb*, both *Actb* and *Ublcp1,* and *Ublcp1* alone. The significant difference in expression between strains observed when *Actb* is used as a reference gene alone (p=0.0016), and paired with *Ublcp1* (p<0.0001), is no longer evident when *Ublcp1* is used as the sole reference gene (p=0.3002). In addition, when *Actb* is paired with *Ublcp1* the M-value reflecting pair-wise reference gene stability is higher than the recommended value for heterogenous samples ($M_{Actb/Ublcp1}$ = 1.2063). Further, a two-way ANOVA revealed a significant effect of reference gene(s) used (p=0.0003) and a significant interaction of reference gene(s) used with strain effect (p=0.0069) on relative normalized *Nin*TV1 expression. The significance of this interaction is eliminated when examining target genes that exhibit much larger differences in expression between strains, as is the case with *Stab2* (Figure 3.1c, $p_{reference\ gene(s)}$=0.6796, p_{strain}<0.0001, $p_{interaction}$=0.5539).

Detailed characterization of Ninein expression in B6 and D2 mice

Basal expression of all six *Nin* transcript variants, exons of interest, and total *Nin* expression was also examined via qPCR in nucleus accumbens and amygdala. Basal expression of these targets normalized to *Ublcp1* in nucleus accumbens is shown in Figure

3.2a. *Nin* transcript variant 4 is the only transcript with significant differences in expression between strains in NAc (p=0.02638). Additionally, total *Nin*, transcript variants 2 and 3 together, and transcript variants 5 and 6 together had near significant differences in expression in nucleus accumbens ($p_{TotalNinein}$=0.0649, $p_{Nin\text{TV2,3}}$=0.0890, $p_{Nin\text{TV5,6}}$=0.0886). In all cases, expression of these transcripts was higher in D2 NAc than B6 NAc. Of particular importance is the amplification of transcript variants 5 and 6, which have not been previously observed in this brain region *in vivo* in adult mice.

After seeing amplification of *Nin* transcript variants 5 and 6 in NAc, new primers were designed specifically to target transcript variant 6 in amygdala. The forward primer of this set overlapped the junction between the exon immediately upstream and immediately downstream of the "large exon" present in *Nin* transcripts 1-5. This exon is responsible for the localization of Ninein at the centrosome, and its absence results in re-localization of Ninein away from the centrosome in neurons *in vitro* (Yu et al., 2009; Zhang et al., 2016). As of this writing, we are aware of no published record of this transcript found *in vivo* in amygdala in adult mice. Expression of the large exon in *Ninein* and transcript variants 1, 4, 5, and 6 normalized to *Canx* and *Ublcp1* is shown in Figure 3.2b. Significant differences between strains were found for *Nin*TV1 (p=0.0028) and *Nin*TV5,6 (p=0.0024). *Nin*TV6 primers amplified a product in both strains, and while there was a small difference between strains it did not reach statistical significance (p=0.2827). The product of this reaction was isolated and sequenced; its sequence overlap with *Nin* transcript variants is shown in Figure 3.3. NCBI's BLAST algorithm identified a 99% overlap, including one mismatch and zero gaps, with 100% of the sequenced PCR product with the intended *Nin*TV6 target. The next five closest matches are with *Nin*TVs 1-5 in regions that share exons with *Nin*TV6 downstream of the junction covered by the forward primer.

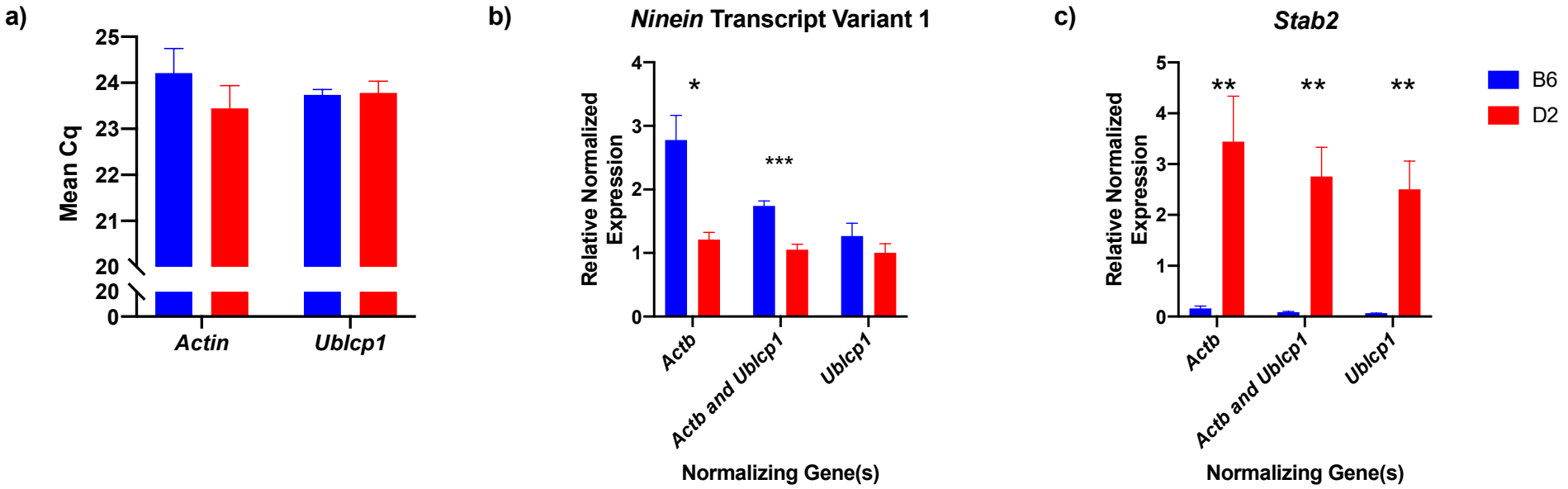


Figure 3.1 *Actb*1 and *Ublcp1* as Reference Genes for B6 and D2 Nucleus Accumbens

Data are presented as strain-mean ± SEM. **a)** Strain-mean Cq of *Actb* and *Ublcp1* in nucleus accumbens. No significant differences were observed using student's t-test (α=0.05). **b) and c)** Comparison of expression of *Nin*TV1 and *Stab2* normalized to *Actb* alone, *Actb* and *Ublcp1,* and *Ublcp1* alone. Significance determined using two-way ANOVA with Tukey's post-hoc tests (α=0.05). *p<0.05, **p<0.01, ****p<0.0001.

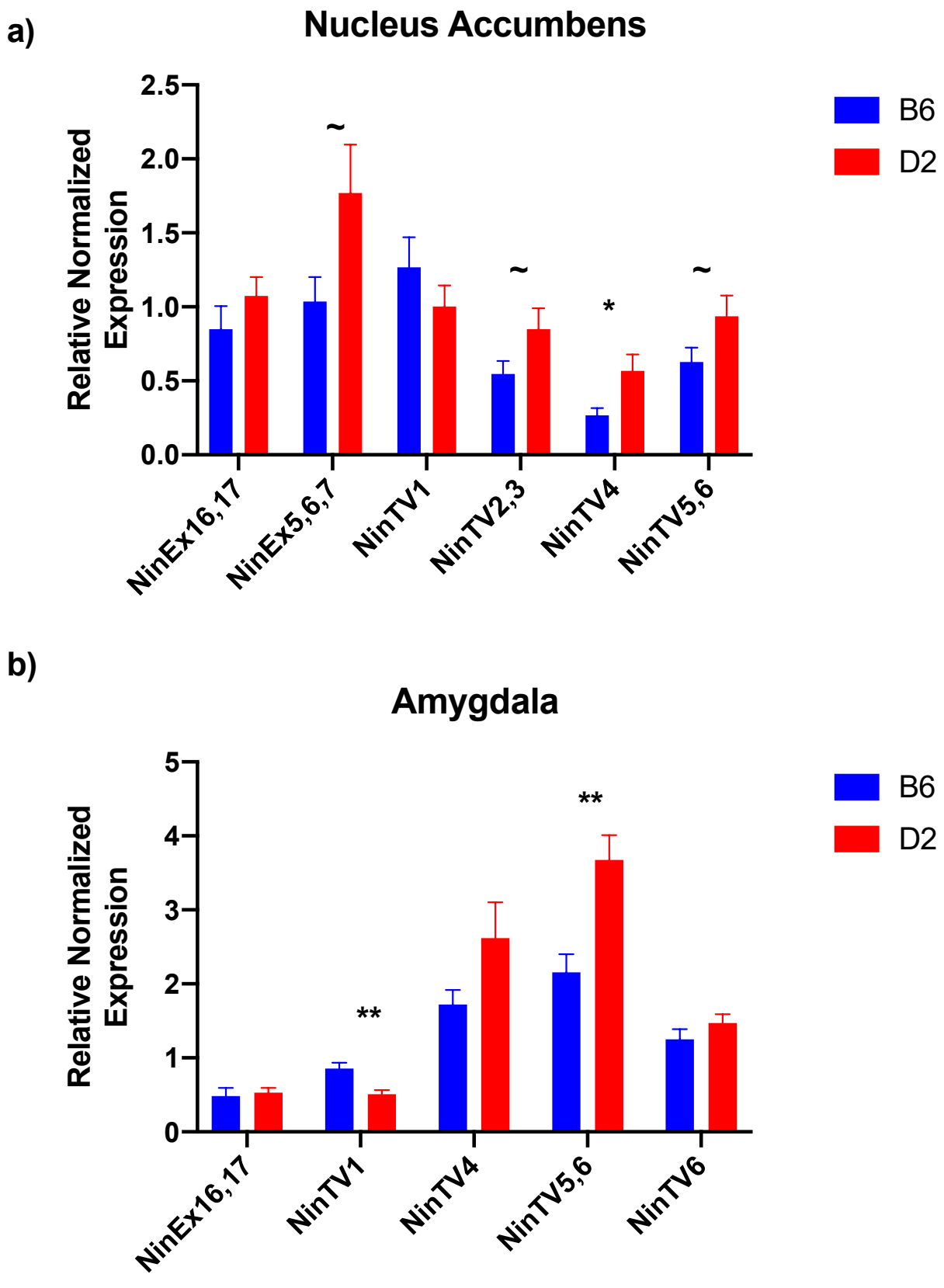


Figure 3.2 Characterization of *Ninein* Expression in Nucleus Accumbens and Amygdala

Strain means ± SEM of relative normalized expression of *Ninein* exons and transcript variants. Significant differences determined using student's t-tests with α=0.05. *p<0.05, **p<0.01, ~p<0.1

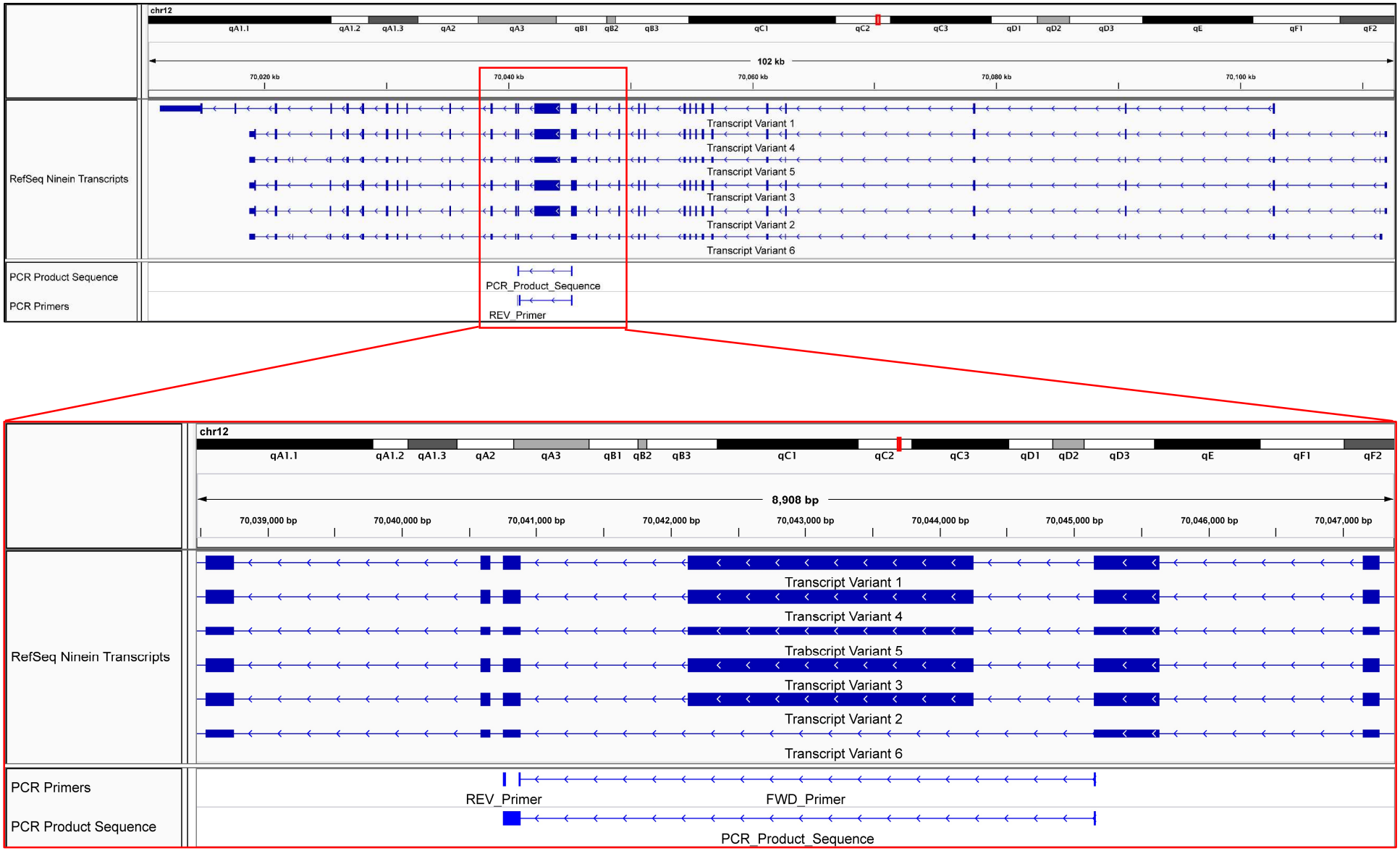


Figure 3.3 Sequenced PCR Product Overlap with *Nin* Transcript Variants

The PCR product isolated from PCR in B6 amygdala uniquely overlaps the sequence of *Nin*TV6, which excludes the "large exon" responsible for localization of NIN to the centrosome. Thick blue bars represent exons, while thin arrowed lines represent introns. It should be noted that due to limitations in the ability to incorporate multiple features in the file type necessary to display these images, that the single mismatch present at chr12:70055152 is not shown in this diagram.

Discussion and Future Directions

Proper selection of reference genes for a particular qPCR experiment is vital for obtaining accurate results. In the case of *Ninein* in B6 and D2 nucleus accumbens and amygdala, quantifying differences in expression of specific exons or transcripts requires the ability to detect very small differences in target quantities. When comparing *Actb* and *Ublcp1* as reference genes in nucleus accumbens it is clear that, despite the lack of statistical significance, higher variability in expression of a reference gene such as *Actb* greatly alters the appearance of strain differences in expression of *Ninein* transcript variant 1. This is true even when paired with *Ublcp1*, which has less variability in mean Cq across strains. On the other hand, small variability in reference gene expression across experimental conditions is less relevant when observing targets with very large differences in expression. For example, in the case of *Stab2,* the change in expression between B6 and D2 mice is apparent regardless of the reference gene(s) used for normalization. Identifying small differences in gene expression will be crucial in ethanol-related behavioral experiments where the differences in single gene expression are often less than 30%, and the response of the network of genes that are co-regulated with a GOI likely contributes more to a given behavioral phenotype than the GOI alone (Kerns et al., 2005; van der Vaart et al., 2017; Wolen et al., 2012).

Interestingly, the Bio-Rad CFX Manager software used for calculating normalized expression of targets relative to reference genes shows a higher coefficient of variance (CV) for *Ublcp1* compared to *Actb*, which suggests higher variability in *Ublcp1* expression across samples. This is notable because, when looking at the mean Cq data, the standard deviation of mean Cq of *Ublcp1* across all samples (0.536) is almost three-fold less than that of *Actb* (1.461). It is also clear in the comparisons shown in Figure 3.1b that the inclusion of *Actb* as a reference gene dramatically changes the conclusions that can be drawn from looking at normalized gene expression data. This discrepancy highlights the need for careful examination

of data and calculations produced by qPCR analysis software, rather than a blind reliance on its output. Perhaps more importantly, this further illustrates the need for validation of more than two potential reference genes when normalized expression is to be used as the primary metric for comparing target gene expression across experimental conditions. Because the analysis of *Ninein* in nucleus accumbens was done prior to the evaluation of multiple other reference genes, *Actb* and *Ublcp1* were run based on their previous use for similar experiments in the laboratory. qPCR data regarding detailed nucleus accumbens *Nin* characterization in this chapter are presented normalized only to *Ublcp1*. Future experiments aiming to reproduce or expand upon these results in nucleus accumbens from control and ethanol treated animals should be preceded by a more thorough evaluation of potential reference genes in all experimental groups, as described in the previous chapter.

That said, basal D2 expression of *Nin*TV4 in nucleus accumbens was significantly greater than B6 expression. This was accompanied by similar trends towards higher D2 expression of total *Nin*, *Nin* variants 2 and 3 together, and *Nin* variants 5 and 6 together. In amygdala, B6 expression of *Nin*TV1 was significantly greater than that of D2, while D2 expression of *Nin* variants 5 and 6 together was higher than B6. There was also a small trend towards greater expression of *Nin*TV4 in D2 amygdala versus B6. First, it is important to verify that the expression of these transcript variants does correspond to expression of the associated proteins which can be verified by western blot. While the general function of known Ninein isoforms 1 through 3 is minus-end microtubule anchoring to the centrosome (Bouckson-Castaing et al., 1996; Mogensen et al., 2000), there is little known about the differences in function or localization, if any, between these 3 isoforms. Assuming gene expression and protein expression are directly related, the expression differences of *Nin* variants 2 and 3 (Ninein isoform 2) and variant 4 (Ninein isoform 3) contrasted with the differences in *Nin*TV1 expression (Ninein isoform 1) in both NAc and amygdala provides a basis for the hypothesis that there is some functional difference between the isoforms. Because *Nin* transcript variants

1-4 were initially observed in fibroblasts (Bouckson-Castaing et al., 1996; Mogensen et al., 2000), then characterized in epithelial cells (Moss et al., 2007), and cortical neural progenitor cells and post-mitotic neurons (Zhang et al., 2016) during embryonic development, it is difficult to elucidate implications regarding strain differences in these transcripts in adults without further cell-specific experiments.

Prior to the experiments in this chapter, the only evidence of *Nin* transcript variants 5 (TV5) and 6 (TV6) was confined to embryonic cortical neurons *in vitro* (Zhang et al., 2016). While the difference in expression of NinTV5,6 relative to NinTV6 suggests, at a minimum, the presence of TV5, the data presented do not directly confirm this. Sequencing of the amplified product of *Nin*TV6, however, does directly confirm the presence of this transcript despite it being labeled as a target for nonsense mediated decay (NMD) in the most recent version of the mouse genome (GRCm38.p6, 2017). Although it is possible that the *Nin*TV5,6 and *Nin*TV6 products we see in these qPCR experiments are just present prior to being removed via NMD, Putman et al. (2016) suggests otherwise. A western blot in nucleus accumbens using an antibody that binds to Ninein isoforms 1-3, and what would be the predicted protein products of *Nin*TV 5 and 6 shows more than the three bands that would be expected if known Ninein isoforms were the only proteins translated from existing transcripts. In this blot, there are two visible bands between 117KDa and 170KDa that could correspond to the predicted molecular weight of the protein product of *Nin*TV6 (~147KDa) labeled NIN4 and NIN5. Further, D2 mice have significantly higher levels of both of these provisional proteins, which parallels the higher levels of *Nin*TV5,6 observed in NAc. Evaluation of strain differences in *Nin*TV6 expression in NAc via qPCR and sequencing of the proteins at bands NIN4 and NIN5 are needed to further validate this theory.

Because we know that peptides resembling *Nin* variants 5 and 6 are unique to differentiated neurons (Zhang et al., 2016), higher expression of these variants in D2 mice could imply either a greater quantity of neurons or a greater potential for plasticity in existing neurons.

In amygdala, the significant difference in expression of *Nin*TV5,6 versus the lack of significance in expression of *Nin*TV6 alone suggests that a majority of the difference observed results from differences in expression of *Nin*TV5. Since *Nin*TV6 is a splice variant of *Nin*TV5, it is impossible to quantify *Nin*TV5 directly using qPCR with a single fluorophore; multiplex qPCR would be better suited for this task. Splicing of the "large exon" of *Nin*TV5 to form *Nin*TV6 results in dissociation of Ninein from the centrosome and diffuse localization throughout the cytoplasm. This also highlights the importance of the ratio of *Nin*TV5 to *Nin*TV6. If there is a higher quantity of *Nin*TV5 relative to *Nin*TV6, and this ratio is larger in D2 mice than in B6 mice, this suggests a greater potential for either quick axonal outgrowth and branching in nascent adult neurons, or a greater potential for synaptic plasticity in D2 mice due to increased stabilization of microtubules in newly formed dendritic spines or axon terminals. In these scenarios, *Nin*TV5 would act as a readily available precursor for *Nin*TV6 that is spliced and translated upon cellular responses to a stimulus that result in the formation of new synapses or neurons guided by microtubule outgrowth and stabilization. It would be interesting to look at expression of known *Ninein* transcription and splice factors such as *Sip1* and *Qki5*, respectively (Hayakawa-Yano & Yano, 2019; Srivatsa et al., 2015) in order to shed more light on whether *Nin*TV6 in adult NAc and amygdala plays a role in adult neurogenesis or in synaptic plasticity in postmitotic neurons.

Validating *Ninein* as a quantitative trait gene for ethanol-induced anxiolytic-like behavior will require characterization of *Nin* transcript variant expression in nucleus accumbens before and at different time points after restraint stress, ethanol exposure, and both. It will also require validation of the NIN4 and NIN5 sequence. In addition, incorporating female mice into these experiments is pertinent given the sex and sex*strain effects on anxiety-like behavior in certain BXD strains with and without an acute dose of ethanol (Putman, 2008). One study in rats identified different light-dark transition-related behavioral QTL not only between sexes, but also across estrous cycle stages (Izídio et al., 2011), which raises the possibility that *Etanq1* may not be reproducible in female mice of the same BXD strains. This suggests that female anxiolytic-

like response to ethanol may be mediated by a different and/or more complicated mechanism than occurs in males.

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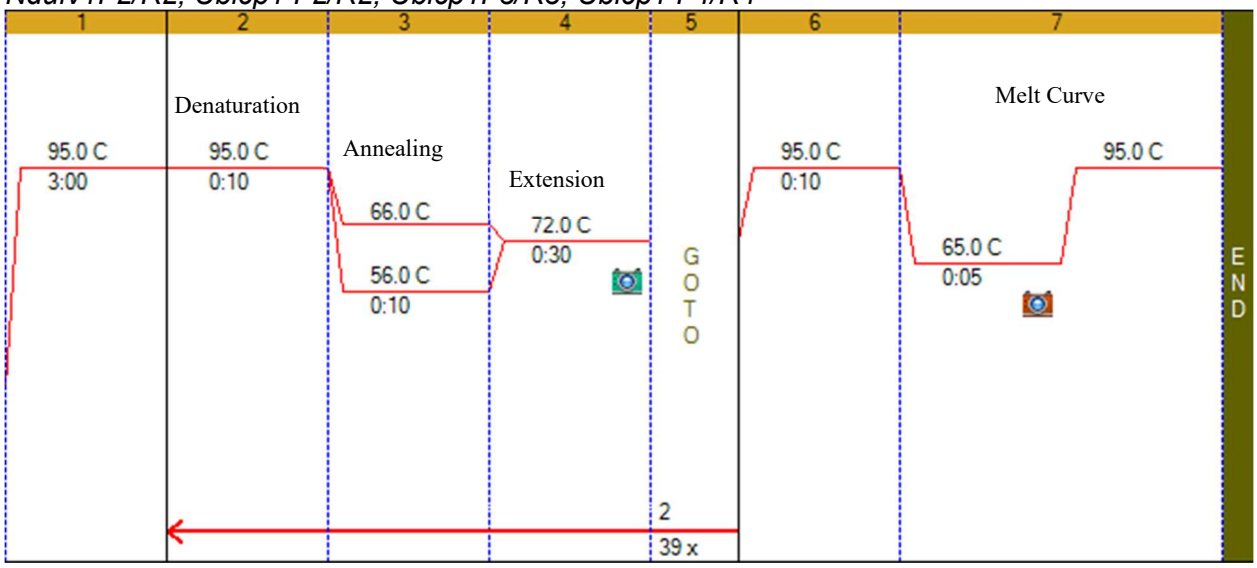
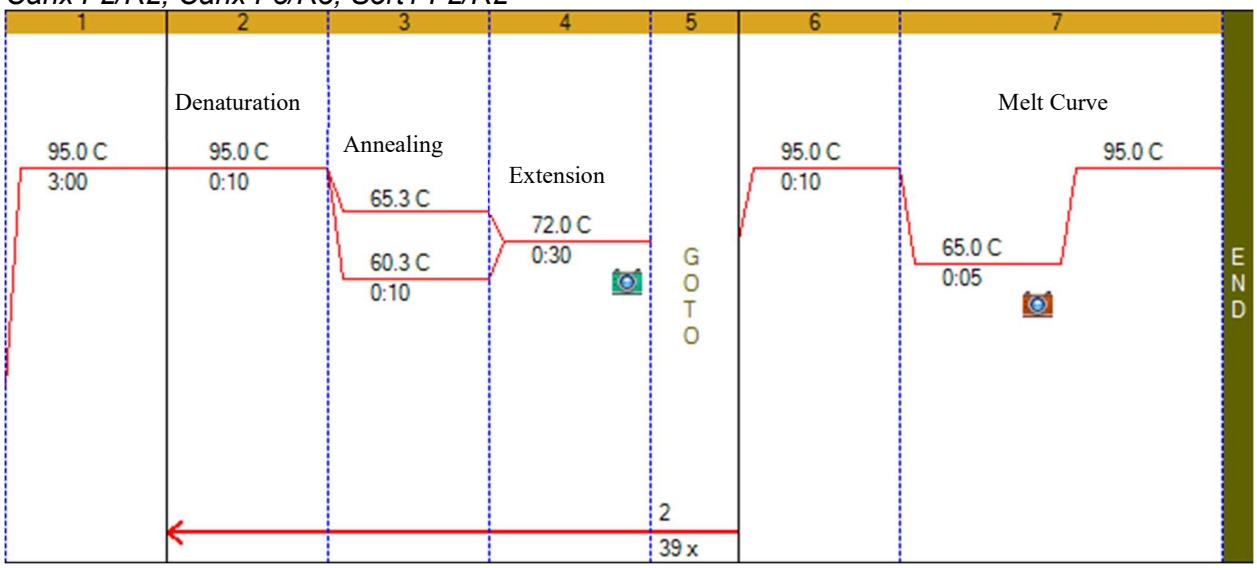
Appendix 1 qPCR Temperature Gradient Protocols

Ndufv1F2/R2, Ublcp1 F2/R2, Ublcp1F3/R3, Ublcp1 F4/R4



Canx F2/R2, Canx F3/R3, Sort1 F2/R2



Appendix 2 *Ninein* Primer Sequences

Primer Set	Forward (5' -> 3')	Reverse (5' -> 3')
NinEx5,6,7	TGGTTTCCATCTGCGAACAG	CATCGAAAGACTGCATAGAGAGA
NinEX16,17	AGTTAGGCAAAAAGACGCCC	AGCTTTTCAGATTCATATCTCAGGA
NinTV1	CTGCCCGAGTTTCAAGAGTC	ATTCAGGTCATCTGGGTTCC
NinTV2,3	ACAGGTGAGACTGGACGAGAA	AGCAAAGCCTGTGGTGTGTT
NinTV4	GAAGAAACAGATGCAGCCCCT	TTCAGAGGTGCCCAATCCTTCT
NinTV6	TCCAGGAGGGAAGATATGAATCTG	GGGAGTTCTTTTGGCTGAGTTC