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Project Title: Investigating the Developmental and Brain Region-Specific Expression of Selected Deregulated Genes by Continuous Ethanol Exposure in Brain-Derived Neural Stem Cells

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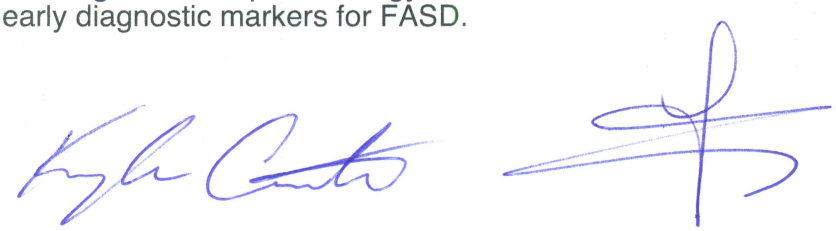
SUMMARY: (no more than 250 words single spaced)

Fetal Alcohol Spectrum Disorders (FASD) refer to a range of phenotype defects due to Fetal Alcohol Exposure (FAE), for which there is currently no known cure. Many secondary issues surround FASD, including substance abuse, mental health problems, dependent living, cognitive deficits and productivity-loss. Alcohol exposure *in-utero* is currently the leading cause of non-hereditary intellectual disability in the United States, with a prevalence of 0.2-1.5%, and an estimated cost of over $4 billion dollars per year for Fetal Alcohol Syndrome (FAS) alone, the most severe form of FASD. For designing future interventions, we must understand the mechanisms by which FASD develops, which can be accomplished by studying the genetic changes that occur as a consequence of FAE.

To study the effect of ethanol on gene expression, we performed RNA sequencing on murine neural stem cells (NSCs) that had undergone treatment with ethanol to identify genes that were differentially expressed. Selected genes were then validated using Real-Time PCR. For this study we chose four genes, two up-regulated and two down-regulated genes as a result of continuous ethanol treatment. Our goal was to analyze the developmental and brain region-specific expression of each gene in order to identify the developmental ages and specific regions of the brain where the candidate genes might play a role in brain development, and where deregulation of those genes might be involved in FASD pathogenesis. Our results may shed some light into the pathobiology or FASD and will have future applications for identifying early diagnostic markers for FASD.



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Title: Investigating the Developmental and Brain Region-Specific Expression of Selected Deregulated Gene by Continuous Ethanol Exposure in Brain-Derived Neural Stem Cells

Introduction & Background

Fetal Alcohol Spectrum Disorders (FASD) is a general term used to describe the range, or spectrum, of phenotypic effects that result from fetal exposure to ethanol during embryonic development. Following prenatal exposure to alcohol, the fetus will often develop a characteristic phenotype that falls along a continuum of severity. At the most severe end of the spectrum lies Fetal Alcohol Syndrome (FAS), which is characterized by facial dysmorphisms, growth deficits, and neurocognitive problems [1]. The facial dysmorphisms include short palpebral fissures, a smooth philtrum, and a thin upper vermillion [1]. This spectrum also includes partial Fetal Alcohol Syndrome (pFAS), Alcohol-Related Birth Defects (ARBD) and Alcohol-Related Neurodevelopmental Disorder (ARND).

It has long been known that ethanol can have deleterious effects on the body when used inappropriately, and that the fetus is particularly sensitive to these effects of ethanol. Exposure to ethanol *in utero* can cause defects in many organs including the central nervous system (CNS), with much variability in the effects due to many factors affecting susceptibility and resistance to this exposure, as well as the dosage and timing of the exposure to ethanol [2].

The clinical diagnosis of FASD requires documentation all three facial dysmorphologies (small palpebral fissures, thin vermillion, and smooth philtrum), a growth deficit, an abnormality in the CNS (structural or functional), and confirmed maternal alcohol exposure during pregnancy [3]. Currently, diagnostic teams use a 4-digit Diagnostic Code worldwide to diagnose FASD across the full spectrum. This four digit code assigns a number to each of four criteria; growth percentile for height and weight, the number of the characteristic facial features present, the level of dysfunction of the CNS, and the level of confirmed alcohol exposure *in utero*. These four criteria produce a 4-digit code, which is then cross-referenced to a table that summarizes all of the codes that fall under each of the disorders in the spectrum of FASD, ultimately providing a diagnosis based on the 4-digit code [4].

Exposure to alcohol *in utero* is currently the most common cause of non-hereditary intellectual disability in the United States, with Fetal Alcohol Syndrome having an estimated prevalence of 0.2-1.5 cases per 1000 births and other disorders in the FASD spectrum having a prevalence as high as 9-10 per 1000 live births. The prevalence of Fetal Alcohol Spectrum Disorders is even higher (15 per 1000 children) in the foster care system, and it is greatly overrepresented in the justice system, with more than 200 per 1000 individuals in the juvenile justice system having a disorder in the spectrum [3]. In the United States, the estimated cost related to FAS (not FASD) was $4 billion in 1998.

Another issue surrounding FASD children is secondary disabilities, which will commonly arise when an individual with FASD is not properly diagnosed and the necessary interventions are not put in place. These secondary disabilities include mental health problems, substance abuse, educational problems, criminal involvement, dependent living, and cognitive deficits [3].

The prevention of FASD can be summarized in a very simple manner; it is completely preventable if the mother abstains from alcohol during her pregnancy, and does not consume alcohol while planning to become pregnant so as to not enter ethanol withdrawal during the pregnancy. However, this is not a simple problem, as there are many social, demographic, and health factors that relate to the reason why women drink during pregnancy. It is for this reason that in 1993, the American Academy of Paediatrics put forth 8 recommendations for the prevention and elimination of prenatal alcohol exposure. These recommendations include the

following: 1) abstain from alcohol during or while planning a pregnancy; 2) educate women prior to and during child-birth years on the consequences of alcohol consumption in pregnancy; 3) inclusion in the curriculum for elementary, post-secondary, and adult learning centers; 4) increasing FASD awareness among healthcare professionals; 5) increasing awareness of PAE as a cause of birth defects; 6) referring infants and children suspected of having FAS/FAE to developmental paediatricians; 7) instituting a federal legislation requiring a health/safety message in all print broadcasting the advertisement of alcohol; and 8) creating a state legislation to make information available at marriage-licensing bureaus and other appropriate public places including those that sell alcohol [5].

Despite having been identified a long time ago, evidence-based interventions for those living with FASD have been lagging behind significantly [6]. There exists the potential for interventions in many fields surrounding FASD. These include interventions in classrooms, teaching support and resources, parenting interventions with consultation and skill development, adaptive skills training for patients with FASD including safety and social skills, and pharmacological interventions. Another important potential area for intervention is case-specific management of the many possible secondary disabilities that may develop. Prominent ones include substance abuse, comorbid disorders, sexuality and medical issues, and issues with the justice system [6].

Studies that involved brain neuroimaging in human FASD patients have shown that these patients have reduced volumes in the areas of the cortex, cerebellum, and basal ganglia [7]. FASD patients have also shown altered functioning in tasks that involve inhibitory control, working spatial memory, and working verbal memory [8]. It has also been shown in rodents that the hippocampus is a structure that is exceptionally susceptible to damage cause by ethanol, which leads to decreased cell number in the hippocampus, reduced density of the dendritic spine, decreased neurogenesis in the hippocampus, and altered electrophysiological properties of the neurons [9]. Also, in rodents with ethanol-induced damage, they showed behavioural deficits in tasks that required the proper functioning of the hippocampus, which suggests that the changes noted above have a functional impact [9].

One of the most important frontiers in FASD research is that of genetics and epigenetics. The development of FASD requires both fetal exposure to alcohol and susceptibility to the teratogenic effects of ethanol, a factor that is likely influenced by the genetics of the fetus. The genetic make-up of the parents as well as the offspring have both been linked to the susceptibility or resistance to the teratogenic effects of alcohol exposure to the fetus. Examples of this include the *ADH1B*3* genotype, a polymorphism of the Alcohol Dehydrogenase (ADH) enzyme that offers a protective effect to the fetus against prenatal ethanol exposure when present in the mother's genetic makeup [10, 11]. The same type of genetic protection can also be seen in the *ADH2*3* mutation, another ADH enzyme variant that offers fetal protection against ethanol exposure when present in the maternal genetic profile, as well as in the genotype of the fetus [12].

A commonly overlooked cause of ethanol-induced changes in offspring is paternal consumption of alcohol. Several studies have looked into the effect that paternal drinking has on the offspring, and they have found that it has been associated with decreased cognitive capacity [13], an increase in ethanol intake preference for their male offspring [14], as well as behavioural variations characterized by increased aggressiveness and reduced fear [15]. Other studies have shown changes in methylation of imprinted genes in the offspring such as *H19, Peg3,* and *Rasgrf1* [16, 17]. These findings suggest that epigenetic changes that occur in parents as a result of ethanol consumption can be passed on to their offspring, resulting in some of the characteristics of FASD surfacing despite there being no alcohol consumption by the mother during pregnancy.

An important topic to understand with FASD is the gene expression changes that occur as a result of ethanol exposure. The interaction between ethanol and the genome of an organism has varying effects on many biological pathways. One such example is the Sonic Hedgehog (SHH) signalling pathway. It has been previously shown that ethanol causes a decrease in *Shh* expression in chicken, mice, and zebrafish [18]. Mutations or dysfunction in the SHH protein can lead to many deficits during development which include neural crest cell death, misspecification of the dorsal-ventral axis of the neural tube, and a loss of the midline craniofacial structures, all of which are features that can be seen in those with FAS [18]. To further support the involvement of *Shh* in the morphological changes that we see in the FASD phenotype, supplementation of SHH protein has been shown to alleviate the deleterious effects that ethanol has on the fetus [19]. Another interesting finding is that the co-receptor for SHH, CDON, has been shown to lead to holoprosencephaly through interaction with ethanol [19].

Currently, we are interested in gene expression profile changes that occur in murine brains that are exposed to ethanol, ethanol withdrawal, and binge ethanol exposure treatment. In this study, we aim to identify target genes that are potentially associated with the teratogenic effects of ethanol, which will be validated through Real-Time PCR. This process involves analyzing RNA sequencing data from murine neural stem cells (NSC) that had undergone various types of ethanol treatments and comparing this to data from the control group (no ethanol treatment). Once potential genes were identified from the RNAseq studies, we investigated the gene ontology of each gene and selected four deregulated genes (two upregulated and two downregulated) as a result of ethanol treatment. Further studies for these genes involved the use of Real-Time PCR analysis and quantification as well as gel electrophoresis to obtain a gene expression profile for these genes at various developmental stages, as well as in various brain regions at early adulthood.

Hypothesis - Based on previous studies having linked genetics and epigenetics to the generation of FASD [20], we hypothesize that 1) *there are genes, which when exposed to ethanol, become differentially expressed*, 2) *that the identification of these genes is possible using RNA sequencing studies, 3) the differential expression of those genes can be validated,* and 4) *We can analyze the relative expression of those genes in both their developmental pattern and their brain region-specific expression pattern.*

Three specific aims are proposed for this study to address our hypothesis: 1) to identify, through the use of RNA sequencing data, genes that are differentially expressed as a result of continuous ethanol exposure in mouse neural stem cells (NSCs).; 2) to validate selected differentially expressed genes using qRT-PCR.; 3) to analyze the developmental and brain-region specific expression patterns of selected genes using qRT-PCR and gel electrophoresis. Due to time constraints, my role was mainly limited to the first and third aims.

Rationale - The reasoning behind each of our specific aims is as follows. For our first aim, RNA transcripts will reflect the expression levels of these genes, therefore if we compare RNA sequencing data of NSCs that have been treated with ethanol to control NSCs, we can determine the fold change in the gene's expression as a consequence of ethanol treatment. For our second aim, qRT-PCR will be used to validate these genes in order to ensure that the changes in expression we observed are reproducible. Finally, the reason for our third aim is that if we can construct a developmental and brain region-specific expression pattern for each selected gene, we might find specific developmental points or brain regions where the gene is highly expressed and where deregulation of the gene might lead to developmental consequences.

Based on the results of the RNAseq studies and gene ontology, the genes we chose for analysis were *Scn3a, Sptbn2, Nfil3,* and *As3mt* (**Table. 1**). *Scn3a* encodes an α-subunit for a

voltage-gated sodium channel, a structure that is essential for the generation and propagation of action potentials in excitable tissues, and mutations in this gene have been linked to a number of neurological diseases and disorders [23]. This gene was chosen because of its involvement in neuronal functioning and neurological diseases. *Sptbn2* encodes β-III spectrin, a protein present in the brain that is well known to be essential to the functioning of the cerebellum, mutations of which have previously been found to cause Spinocerebellar Ataxia Type 5 (SCA5) [29]. This gene was chosen due to the deficits in the cerebellum commonly seen in FASD [25]. *Nfil3* encodes nuclear factor interleukin 3-regulated, which plays an essential role in the circadian clock system as well as the development and survival of immune cells. It has also been suggested to play a role in repressing the programmed cell death of developing neurons [32]. The neuroprotective role of *Nfil3* in developing neurons was the reason that this gene was chosen. The final gene, *As3mt,* encodes the enzyme arsenic^{3+} methyltransferase, which is the primary metabolizer of inorganic arsenic in humans and produces methylated metabolites through a process called arsenic biomethylation [34]. This process produces highly reactive and toxic compounds compared to arsenic [35]. The possible damage that could be caused by dysregulation of this gene is the reason that it was chosen.

Materials and Methods

RNAseq Studies - In order to identify potential genes for validation, primary brain-derived neural stem cells that had underwent a treatment of continuous ethanol (CE), ethanol withdrawal (EW), binge exposure to ethanol (BE), or no ethanol (NE) were sent for RNA sequencing to GénomeQuébec. NSC isolation, culture, ethanol treatments, sample collections and RNA-seq studies were completed by our lab prior the start of my training in our lab. The expression profiles of these NSC that we received were compared in order to determine the fold changes of various genes between the different conditions. The results from the comparative analysis were used to identify potential gene targets for further validation. This involved many different attempts to analyze the data using different selection criteria. In the end, genes were chosen based on three criteria based on multiple meetings between our lab, Dr. James Davie and Dr. Wayne Zu (U. Manitoba); a very low EdgeR adjusted p value, a small standard deviation among replicates, and a fold change of greater than +/-1.50.

RNA Extraction - RNA samples for three sets of developmental brain and specific brain regions of adult mice were collected using C57/BL6 mouse [21] and were provided to me. We divided these RNA samples into two groups; the developmental samples and the brain region samples. The developmental samples were whole brain extracts taken from six different developmental time points. These included embryonic day fourteen (E14), embryonic day eighteen (E18), post-natal day one (P1), post-natal day seven (P7), post-natal day twenty-one (P21), and post-natal day twenty-eight (P28). The brain region samples were extracted from different areas of the brain from 6 weeks mice. The sample regions included the Cerebellum, Thalamus, Hippocampus, Cortex, Striatum, Olfactory Bulb, and an additional Whole Brain sample. Developmental samples as well as brain regions samples were from in-house mice, while the whole brain sample was from Jacksons™.

DNase1 Treatment - My original set of RT-PCR without reverse transcriptase showed the existence of genomic DNA in the total RNA extracts. Therefore, to remove the genomic contamination of RNA samples, I performed DNase I treatment to remove the DNA. The DNase I treatment consisted of taking 1ug of RNA sample and diluting it to 10.5µL. To this volume, we added 1.5uL of a 10x Buffer along with 1µL of a DNase1 enzyme solution. This was mixed and then incubated in a water bath at 37°C for 30 minutes. Following incubation, 2µL of DNase inactivation reagent was added to the mixture, which was then allowed to sit for 5 minutes at room temperature while being mixed occasionally. The mixture was then centrifuged at 10000g for 1.5

minutes, and the supernatant was transferred carefully into another tube. This yielded roughly 12 to 14µL of DNase I-treated RNA for each sample. DNase I treatment was performed on both the Developmental samples and the Brain Region samples. Each of these reagents for the reaction was provided in a DNase I treatment kit from Invitrogen®. All samples were quantified following the treatment using spectrometry.

RNA Quantification - RNA quantification was performed on all samples using a Nanodrop spectrophotometer both before and after DNase I treatment to calculate required volumes and determine the quality of the RNA sample. Ultrapure™ water was used as the blank/reference sample.

cDNA Synthesis - For cDNA synthesis, we used 500ng RNA, the volume for which was calculated based on RNA quantification, and incubate it for 5 minutes at 65°C mixed with random primers, dNTPs and Ultrapure™ water. Afterwards, to each sample we added a mixture of first strand buffer, DTT, RNase out, and Reverse Transcriptase enzyme and allowed it to stand for 5 minutes at room temperature. To promote the activity of the enzyme the mixture was transferred to a 50°C heat block where it incubated for 60 minutes prior to being transferred to a 70°C block for another 15 minutes. When the reaction was complete the samples were transferred to the -20°C freezer for long-term storage. This reaction yielded 20µL of cDNA solution per sample. Reactions without reverse transcriptase were used as No-RT controls.

Real-Time PCR - Real-Time PCR was used for the further validation of the four selected genes. This involved mixing either the cDNA samples or the No-RT samples with forward and reverse primers for a given gene, Ultrapure™ water, and SYBR™ green master mix [22] and then loading each sample into two separate wells (in duplicate) of a 96-well PCR plate. The RT-PCR method for each gene was previously optimized, and for every gene each sample was run both for Real-Time PCR quantification relative to *Gapdh*. PCR run methods are available on request.

The primer sequences used for the Real-Time PCR are as follows. For *Gapdh,* we used 5'-ATGTCGTGGAGTCTACTGG-3' for the forward primer, and 5'-GTGGTGCAGGATGCATTGC-3' for the reverse primer. The primers for *Scn3a* were 5'-TCCGAGCCTTATCCCGCTTTGA-3' as the forward primer and 5'-GAAGATGAGGCACACCAGTAGC-3' for the reverse. For *Sptbn2,* we used 5'-GTGGCAGAAACACCAGGCATTC-3' for the forward primer and 5'-CTCCAGCTTCTCTGACACTACG-3' for the reverse primer. *Nfil3* primers were 5'-CAGGACTACCAGACATCCAAGG-3' for the forward primer and 5'-AGGACACCTCTGACACATCGGA -3' for the reverse one. Finally, *As3mt* used the sequence 5'-TCCACGTTTGGTCACTGCCGAT-3' for the forward primer and 5'-GAAGAGGCGAAATGTGGCAGAC-3' for the reverse primer. Our expected fragment size for each product was 181bp for *Gapdh,* 101bp for *Scn3a,* 133bp for *Sptbn2,* 137bp for *Nfil3,* and 100bp for *As3mt.*

For Real-Time PCR quantification of each gene relative to *Gapdh*, every sample was run for 35 cycles with each of the four selected genes along with *Gapdh*. The relative expression of each gene is calculated using the CT value, referring to the number of cycles required for the fluorescence signal of the sample to cross the threshold.

Gel Electrophoresis - In order to make sure that we were getting a product of the correct size as well as to determine if there was anything additional being formed, the PCR products were subjected to gel electrophoresis. Aside from the products of *Scn3a*, which used 2.0% agarose (W/V), all gels were made using 1.5% agarose (W/V) by boiling 3.0g of agarose in 200mL of 1xTAE buffer and pouring it into a gel mould once it had sufficiently cooled. Eight-µl of PCR product mixed with 1.5µL of 6x loading dye was loaded into separate wells, one for each sample. Each gel also had a 100bp DNA ladder loaded into one of the wells. The gels were run at 100V

in 1xTAE buffer until sufficiently far, usually between 1 and 1.5 hours. The gels were then stained using Ethidium Bromide and imaged using an ultraviolet imager.

Results

RNAseq Studies - Our first aim in this study was to identify genes that were differentially expressed in mouse NSCs as a result of ethanol exposure using RNA sequencing methods. From our comparative analysis of the sequencing data, several notes were made about the gene expression changes: 1) the major changes in gene expression are caused by stem cell differentiation rather than exposure to or withdrawal from ethanol; 2) there are some genes that were affected by the ethanol treatment at Day 2 (D2) or Day 8 (D8) with a fold change greater than +/-1.50; 3) there were more genes to be differentially expressed at D8 following ethanol treatment that at D2 following ethanol treatment; 4) the gene expression profile at D8 following ethanol withdrawal after D2 was quite similar to the D8 control NSCs (no ethanol), but has a few differentially expressed genes compared to the continuous ethanol treatment group at D8.

Next, we studied the gene ontology of each of the top ranked genes and chose ones that were related to neural development, linked to neurodevelopmental/ neuropsychiatric disorders, or related to some other kind of disorder that could be linked with FASD. This gave us a list of 29 genes in total, with a roughly equal number of upregulated and downregulated genes. We selected the comparison of D8 continuous ethanol treatment (D8 CE) with the D8 control group to pick 5 upregulated genes and 5 downregulated genes for validation. These 10 genes were then validated in another comparison of D8 CE vs D8 control using qRT-PCR in a previous study from our lab (Romina Levy, Honour project thesis, 2015) [22]. We choose four genes, two upregulated and two downregulated in the D8 CE vs D8 Control comparison, for qRT-PCR analysis in the developmental and brain region samples. Three of these genes were chosen from the validated genes, which were *Sptbn2, As3mt,* and *Nfil3.* A fourth gene, *Scn3a,* was chosen for analysis due its significant upregulation in the CE treatment group from the RNAseq studies, its functional role in neurons and its link to certain phenotypes observed in FASD patients [23, 24, 25].

RNA quantification - Quantification was performed using a Nanodrop spectrophotometer, and the concentration of RNA in each sample following DNase I treatment is shown in **Table 2.** The ratio of 260nm/280nm was recorded and used to determine the quality of the RNA (**Table 2**). If the ratio of 260nm/280nm was less than 1.65, the samples were excluded from our studies, due to poor quality RNA. This led to the exclusion of multiple samples from our studies. From the Developmental set of samples, this included E18 sample for set 1 and E14 sample for set 3. Therefore, these two developmental stages had two sets of samples. From the brain regions RNA samples, this included the cerebellum sample for set 1 and hippocampus sample for set 2. RNA samples from the olfactory bulb and whole brain in set 3 were also excluded from the calculations as my No-RT reactions resulted in PCR products even following DNase I treatment.

Real-Time PCR - The CT value, which is the number of cycles required for the fluorescence signal of the sample to reach the threshold, was used to compare the expression of each gene relative to *Gapdh*, a well-known housekeeping gene. The difference in the CT values (ΔCT) was determined using the formula $\Delta CT = CT_{Gene} - CT_{Gapdh}$, and the relative expression of the gene of interest was calculated using the formula $Expression = 2^{-\Delta CT}$. The relative expression for each gene was then plotted as a function of either the developmental age or different brain regions using GraphPad Prism 6. It is important to note that the CT values for *Gapdh* varied for the analysis of each gene, as each one used a different fluorescence signal threshold for the analysis. The resulting graphs are shown in the figures section (**Figs. 2-5)**. Significant mean differences for

each set of samples were calculated using one-way analysis of variance (ANOVA) as well as *Tukey post hoc* multiple comparison analysis (**Tables. 3-4)**.

Developmental Expression Patterns - Part of our third aim in this study was to create a developmental expression pattern relative to Gapdh for each of the four selected genes. The resulting graphs, as mentioned in the previous section, can be seen in the figures section (**Figs. 2-5**). When we look at the expression of *As3mt* relative to *Gapdh* in the developmental samples (**Fig. 2)**, we see an almost saw-toothed pattern as we move from E14 to P28, with a large peak at E18. This indicates that *As3mt* is highly expressed, relative to *Gapdh*, in the early stages of embryonic development and could be important for proper growth and development of the brain at an early age. The pattern for *Nfil3* has a large peak at E18, however in this developmental pattern we see a steady decrease in expression as we move from E18 to P28 (**Fig. 3)**. The significantly higher expression that we see in the early ages for *Nfil3* could mean that this gene is important during early stages of brain development. For *Scn3a,* we also see a peak in expression at E18 with decreased expression towards the P28 developmental age (**Fig. 4)**. The increased expression during early embryonic development could suggest importance for proper brain development at a young age. The developmental expression pattern of *Sptbn2* relative to *Gapdh* (**Fig. 5**) shows an almost steady increase in expression from E14 all the way to P28, with only a small decrease in relative expression occurring at the age of P1.

Brain Region-Specific Expression Patterns - The other part of our third aim in this study was to create a brain region-specific expression pattern for each of the genes. The resulting graphs are plotted with the developmental samples in the figures section (**Fig. 2-5**). The plot of *As3mt* expression in the brain regions (**Fig. 2**) at 6 weeks of age shows a very low expression throughout, even in the whole brain. The highest level of expression is detected in the thalamus and olfactory bulb, with minimal expression in cortex and cerebellum. The pattern for *Nfil3* shows low levels of expression throughout the brain (**Fig. 3**). This was to be expected due to the low expression of *Nfil3* that is seen at 6 weeks. When comparing the expression levels between different brain areas, we see a high level of *Nfil3 expression* in the olfactory bulb and cerebellum, with low expression in striatum and thalamus. *Scn3a* expression relative to *Gapdh* (**Fig. 4**) in various brain regions shows almost no increase at all in the cerebellar region, with elevated expression in all other tested brain regions except for the cortex. Peak expression for this gene is seen in the striatum area of the brain. Looking at the pattern of expression for *Sptbn2* in the brain regions (**Fig. 5**) we see that it is elevated in the hippocampus, cortex, and striatum, with the highest expression detected in hippocampus. Low levels of expression relative are seen in cerebellum, thalamus, and olfactory bulb, with the lowest being in the olfactory bulb.

Gel Electrophoresis - The images for each of the gel electrophoresis experiments are shown in the figures section (**Figs. 1-5**). No contamination or any additional products were evident in any of the gels, and all PCR products were the expected size according to a DNA ladder that was run alongside them. For *As3mt* (**Fig. 2**), we can see that the 24-cycle gel in both the developmental set and the brain region set of samples did not have very much product visible. The same is seen with *Nfil3* (**Fig. 3**), with primarily only primer dimers being visible for the brain regions set of samples at 24 cycles and nothing visible in the developmental set of samples at 23 cycles. At 23 cycles, the PCR products for *Scn3a* (**Fig. 4**) in the brain regions set of samples also show only a minimal amount of product. For the 35 cycles RT-PCR product gels, we see pure products of the correct size throughout with no peculiarities except for the cortex sample in set 1 of the *As3mt* experiment (**Fig. 2**). The product for this sample is almost absent compared to the rest of the brain region samples, and perhaps it has something to do with the very low expression relative to *Gapdh* that we see in this region of the brain.

Discussion

The purpose of this study was to identify gene candidates with a possible involvement in the development of the FASD phenotype, validate them using qRT-PCR, and to analyze the developmental and brain region specific expression of selected genes in mice brain. This was accomplished first by analyzing RNA sequencing data from murine differentiated NSC that were exposed to ethanol or not (control) to identify potential candidate genes. Gene ontology was studied for each of these candidate genes, and we chose two upregulated and two downregulated genes based on their fold change, the significance of their fold change, and gene ontology. Three of our chosen genes had been previously validated in our lab (Romina levy, Honours project thesis, 2015) [22], with an additional gene, *Scn3a,* chosen due to its significance and magnitude of its differential expression by ethanol and known implications in various neurological disorders (see below). Our sets of developmental and brain region samples were then analyzed for each these genes using qRT-PCR and visualization using gel electrophoresis.

Our first gene, *Scn3a,* encodes an α-subunit for a voltage-gated sodium channel, a structure that is essential for the generation and propagation of action potentials in excitable tissues such as neurons or heart muscle [23]. Mutations in genes encoding these channels have been linked to the development of neurological diseases and other disorders, some of which include epilepsy, ataxia, and heart muscle disorders [23]. Interestingly, seizures and epilepsy have been found to have remarkably high prevalence in FASD patients [24]. Also of important note, cerebellar dysfunction and ataxia are commonly seen in alcoholics, and many of the symptoms seen in FASD patients are associated with deficits in the cerebellum [25]. This gene was found to be significantly upregulated as a result of continuous ethanol treatment in our RNAseq studies (**Table. 1**). Due to the link between the dysfunction of this gene and epilepsy as well as ataxia, it is reasonable to think that deregulation of this gene seen with continuous exposure would contribute to these features of the FASD phenotype.

Scn3a was also found to have the highest expression in the early stages of development and then steadily decreasing, indicating that this gene might be more important for development at early development, when the organism is susceptible to the teratogenic effects of ethanol (**Fig. 4)**. At 6 weeks of age, the highest expression of this gene was seen in the striatum, olfactory bulb, and hippocampus areas of the brain (**Fig. 4**). The hippocampus is important for memory due to its ability for long-term potentiation (LTP), an ability that has been shown to be affected by prenatal ethanol exposure [26]. The striatum coordinates with many other areas of the brain, and is involved in functions such as movement coordination, behavioural responses, and short-term memory [27], all of which can be disordered in FASD. The olfactory bulb is crucial for the sense of smell, and has been shown in mice to become smaller and cause impairments in odor discrimination as a result of ethanol exposure during the fetal period [28]. Due to the expression patterns seen in the brain regions, deregulation of *Scn3a* might play a role in the deficits of the striatum, hippocampus, and olfactory bulb that can result from fetal alcohol exposure.

Sptbn2 is the gene that encodes β-III spectrin, a protein present in the brain that is known to be essential to the functioning of the cerebellum. Mutations in this gene have previously been found to cause Spinocerebellar Ataxia Type 5 (SCA5) [29]. Also, a study that involved disruption of the *Sptbn2* gene in mice demonstrated that the knockout mice had cerebellar ataxia, progressive Purkinje cell loss in the cerebellum, and a reduction in the levels of the glutamate transporter specific to the cerebellar Purkinje cells [30]. As we just discussed, deficits in the cerebellum are commonly associated with FASD patients [25]. *Sptbn2* was found to be significantly upregulated in the continuous ethanol treatment mice in our RNAseq studies (**Table. 1**). Due to the prominent link between *Sptbn2* and cerebellar ataxia as well as cerebellar dysfunction and degeneration, *Sptbn2* dysregulation could be a contributing factor to the cerebellar symptoms seen in FASD patients.

Sptbn2 was also found to have an increasing expression from E14 to P28 in the developmental samples (**Fig. 5**). This indicates that if this gene is required for proper brain development, it is likely more important in the later stages of development. At 6 weeks, peak expression for this gene was in the hippocampus, cortex, and striatum (**Fig. 5**). As discussed above, the striatum is an important structure for many of the brain's functions, and the olfactory bulb has been shown in mice to be affected by prenatal exposure to ethanol. It has also been shown that children with FASD have reductions in both cortical and subcortical grey-matter, negatively affecting the higher-order functioning of the brain [31]. The peak expression at 6 weeks in the cortex, striatum, and hippocampus could indicate that dysregulation of this gene is involved in the FASD pathology seen in these regions of the brain.

Nfil3 encodes the basic leucine transcription factor in mammals, called nuclear factor interleukin 3-regulated, which plays an essential role in the circadian clock system as well as the development and survival of immune cells. It has also been suggested that NFIL3 plays a role in repressing the programmed cell death of developing neurons [32]. To support this, it was shown to promote the survival of chicken embryo motor neurons even when they had been deprived of neurotrophic factors or when their death receptors had been activated [33]. Also in support of this is the fact that NFIL3 overexpression in the neural tubes of chicken embryos caused a reduction in the number of motor neurons that were dying at later stages [33]. In the developing cerebellum, ethanol activates dsRNA-activated protein kinase (PKR), which is involved in ethanol-induced neuroinflammation and neurotoxicity [25], a process for which *Nfil3* could be a protective factor due to its ability to repress programmed cell death in developing neurons. *Nfil3* was found to have significant downregulation in the continuous ethanol group of our RNAseq studies (**Table. 1**). Due to the possible neuro-protective effect of this gene, the downregulation that is seen with continuous ethanol treatment might leave the developing brain more susceptible to neuronal cell death, and could contribute to FASD pathology.

Nfil3 was also found to have a peak expression at E18 in the developmental samples, followed by decreasing expression until P28 (**Fig. 3**). This might indicate that expression of this gene is critical in the early stages of development of the brain. Expression of this gene at 6 weeks is relatively low throughout the entire brain, with a small peak seen in the olfactory bulb as well as the cerebellum (**Fig. 3**). As mentioned in our discussion for *Scn3a,* prenatal ethanol exposure was shown in mice to cause dysfunction of the olfactory bulb [28], and deficits in cerebellar functioning are commonly seen in FASD [25]. Even though the expression of *Nfil3* is low in these areas at 6 weeks, the expression pattern could indicate some involvement of this gene in the proper development of the cerebellum and olfactory bulb.

As3mt encodes the enzyme arsenic^{3+} methyltransferase, which is the primary metabolizer of inorganic arsenic in humans and produces methylated metabolites through a process called arsenic biomethylation [34]. In the CNS, this biomethylation process of inorganic arsenic yields many products that are highly toxic and reactive compared to the original compound [35]. This production of methylated metabolites could play a role in DNA methylation and epigenetic changes when cells are exposed to arsenic. Elevated exposures to inorganic arsenic have also been linked to cognitive deficits, which include attention, language abilities, and ADHD-type symptoms [36, 37]. Features of these deficits can also be seen in FASD patients. This gene was found to be significantly downregulated in the ethanol treatment group of our RNAseq studies (**Table. 1**). This is an interesting finding seeing as how a decrease in *As3mt* expression would mean a reduced rate of production of biomethylated products were there to be arsenic introduced into the system. Though, perhaps at low levels of arsenic exposure, the body is capable of safely disposing of the biomethylated metabolites, and a build-up of inorganic arsenic might be more harmful.

Peak developmental expression of *As3mt* relative to *Gapdh* is seen at E18, with lower relative expression at all other ages (**Fig. 2**). This could indicate that the *As3mt* gene is also important for brain development in the early ages, and downregulation as a result of ethanol might play a role in some of the phenotypic changes we see in FASD. In the brain region-specific expression pattern for this gene, relative expression in each region is very low with the highest expression being seen in the olfactory bulb (**Fig. 2**). As mentioned just before, the olfactory bulb is seen to have deficits in mice exposed to ethanol prenatally, and the dysregulation of the *As3mt* gene might result in the reduced size and abnormal functioning seen in those mice.

Our hope for future studies is that by building a better understanding of the genetic changes that occur as a result of ethanol exposure, we can understand the mechanisms by which the phenotypic changes of FASD occur. This, in turn, gives potential in the future for developing some means to reverse or greatly prevent these changes whenever it is known that a fetus was exposed to alcohol. Also, through the study of genetic changes occurring in FASD we might discover biomarkers that can be used to diagnose FASD in earlier stages, when interventions would have the greatest impact on future outcomes. In the meantime, we need to focus on building on understanding the basic changes at the gene expression level that are caused by ethanol. Future directions in our lab will include the validation of the genes from this study in humans, as well as looking into loss and gain-of-function studies for these genes. We will also aim to conduct further studies for other genes that are differentially expressed as a result of ethanol treatment.

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Abbreviations – FASD: Fetal Alcohol Spectrum Disorders, FAS: Fetal Alcohol Syndrome, pFAS: partial Fetal Alcohol Syndrome, ARBD: alcohol-related birth defects, ARND: alcohol-related neurodevelopmental disorder, PAE: Prenatal Alcohol Exposure, FAE: Fetal Alcohol Exposure, ADH: alcohol dehydrogenase, SHH: Sonic Hedgehog protein, RNA: Ribonucleic Acid, PCR: Polymerase Chain Reaction, NSC: Neural Stem Cell, qRT-PCR: Real-Time Polymerase Chain Reaction, CE: Continuous Ethanol, BE: Binge Ethanol, EW: Ethanol Withdrawal, NE: No Ethanol, E14: Embryonic Day 14, E18: Embryonic Day 18, P1: Postnatal Day 1, P7: Postnatal Day 7, P21: Postnatal Day 21, P28: Postnatal Day 28, dNTP: Deoxynucleotide Triphosphate, LTP: Long-term Potentiation, PKR: dsRNA-activated protein kinase, CNS: Central Nervous System, Fig.: Figure.

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Figures

Table 1: *Mean Fold Change of Selected Genes with Differential Expression Resulting from Continuous Ethanol Treatment Determined by RNA Sequencing*

Differential Expression	Gene Symbol	RNA sequencing mean FC (n=6)*	EdgeR adjusted *p*-value
Up- regulated	*Scn3a*	2.15	1.40E-06
	Sptbn2	1.93	0.00042
Down-regulated	*As3mt*	-1.72	9.20E-08
	Nfil3	-1.70	2.10E-14

Note. Significance criteria*:* -1.50 < FC< 1.50 and $p < 0.05$
*Calculated based on three biological replicates each with two technical replicates, giving a total of six replicates per gene.

Table 2: *RNA quantification results of all samples following DNase1 treatment, measured using spectrophotometry.*

	Set 1		Set 2		Set 3	
Sample	[RNA] (ng/uL)	260nm/280nm	[RNA] (ng/uL)	260nm/280nm	[RNA] (ng/uL)	260nm/280nm
E14	125.4	1.90	344.1	1.70	293.3	1.56*
E18	325.5	1.62*	112.1	1.94	52.8	1.90
P1	74.5	1.90	91.9	1.90	103.2	1.89
P7	103.2	1.92	86.7	1.90	72.8	1.98
P21	103.7	1.93	80.0	1.90	74.1	1.83
P28	88.0	1.86	81.3	1.91	82.3	1.88
Cerebellum	247.1	1.50*	251.1	1.83	68.3	1.79
Thalamus	67.4	1.85	73.1	1.80	62.9	1.84
Hippocampus	79.7	1.67	190.1	1.64*	66.9	1.84
Cortex	63.9	1.87	173.6	1.68	71.9	1.86
Striatum	65.6	1.87	69.4	1.83	76.1	1.94
Olfactory Bulb	77.7	1.81	68.4	1.83	121.5	1.83
Whole Brain	81.5	1.86	67.9	1.85	127.1	2.02

Note. Inclusion Criteria*:* 260nm/280nm > 1.65
*Does not meet inclusion criteria.

Table 3: *Mean differences in expression of Scn3a, Sptbn2, Nfil3, and As3mt between developmental ages. Significant mean differences were determined using one-way ANOVA and Tukey post hoc multiple comparison analysis between the different ages of mice for each gene.*

	Scn3a			*Sptbn2*			*Nfil3*			*As3mt*		
Comparison	MD	*p*-value	SIG	MD	*p*-value	SIG	MD	*p*-value	SIG	MD	*p*-value	SIG
E14 vs E18	-0.01740	0.1406	ns	-0.04627	0.3872	ns	-0.02201	0.0336	*	-0.01357	0.1655	Ns
E14 vs P1	-0.01777	0.1284	ns	-0.05827	0.1869	ns	-0.00748	0.8183	ns	-0.00235	0.9970	Ns
E14 vs P7	-0.00724	0.8578	ns	-0.07083	0.0785	ns	0.00805	0.7721	ns	-0.00467	0.9387	Ns
E14 vs P21	0.00739	0.9632	ns	-0.09956	0.0058	**	0.01076	0.5234	ns	0.00117	0.9999	Ns
E14 vs P28	0.00344	0.9933	ns	-0.13730	0.0007	***	0.01015	0.5798	ns	-0.00241	0.9966	Ns
E18 vs P1	-0.00037	> 0.9999	ns	-0.01200	0.9941	ns	0.01452	0.2391	ns	0.01122	0.3189	Ns
E18 vs P7	0.01015	0.6191	ns	-0.02456	0.8839	ns	0.03005	0.0037	**	0.00890	0.5458	Ns
E18 vs P21	0.02247	0.0382	*	-0.06013	0.1652	ns	0.03276	0.0018	**	0.01474	0.1161	Ns
E18 vs P28	0.02083	0.0587	ns	-0.09100	0.0179	*	0.03216	0.0022	**	0.01116	0.3238	Ns
P1 vs P7	0.01052	0.5860	ns	-0.01256	0.9927	ns	0.01553	0.1876	ns	-0.00232	0.9971	Ns
P1 vs P21	0.02284	0.0347	*	-0.04813	0.3491	ns	0.01824	0.0935	ns	0.00352	0.9810	Ns
P1 vs P28	0.02120	0.0533	ns	-0.07900	0.0434	*	0.01763	0.1098	ns	-0.00006	> 0.9999	Ns
P7 vs P21	0.01232	0.4313	ns	-0.03557	0.6418	ns	0.00271	0.9973	ns	0.00584	0.8609	Ns
P7 vs P28	0.01068	0.5716	ns	-0.06644	0.1071	ns	0.00210	0.9992	ns	0.00226	0.9974	Ns
P21 vs P28	-0.00164	0.9998	ns	-0.03087	0.7561	ns	-0.00061	> 0.9999	ns	-0.00358	0.9796	Ns
Vs = versus, MD = mean difference, SIG = significance												

Note. Significant mean differences in expression are noted as $p<0.001$ (***), $p<0.01$ (**) or $p \leq 0.05$ (*). (ns) indicates that the mean difference is not significant. $p \leq 0.05$ was considered statistically significant.

Table 4: *Mean differences in expression of Scn3a, Sptbn2, Nfil3, and As3mt between different brain regions. Significant mean differences were determined using one-way ANOVA and Tukey post hoc multiple comparison analysis between the different ages of mice for each gene.*

	Scn3a			*Sptbn2*			*Nfil3*			*As3mt*		
Brain Region	MD	*p-value*	SIG	MD	*p-value*	SIG	MD	*p-value*	SIG	MD	*p-value*	SIG
CB vs Thal	-0.00413	0.5495	ns	0.66410	0.5345	ns	0.03148	0.5293	ns	0.01371	0.7553	ns
CB vs Hip	-0.00591	0.1988	ns	0.46260	0.8401	ns	0.03037	0.5665	ns	0.01508	0.6743	ns
CB vs Ctx	-0.00225	0.9437	ns	0.47490	0.8244	ns	0.03138	0.5328	ns	0.01704	0.5545	ns
CB vs Str	-0.01003	0.0101	*	0.52730	0.7506	ns	0.03193	0.5145	ns	0.01524	0.6643	ns
CB vs OB	-0.00702	0.1555	ns	0.66670	0.6428	ns	0.02661	0.7837	ns	0.01216	0.8935	ns
CB vs WB	-0.00365	0.7658	ns	0.50910	0.8491	ns	0.02964	0.6975	ns	0.01437	0.8023	ns
Thal vs Hip	-0.00178	0.9814	ns	-0.20150	0.9968	ns	-0.00111	> 0.9999	ns	0.00137	> 0.9999	ns
Thal vs Ctx	0.00188	0.9759	ns	-0.18930	0.9977	ns	-0.00010	> 0.9999	ns	0.00333	0.9998	ns
Thal vs Str	-0.00590	0.2001	ns	-0.13680	0.9996	ns	0.00045	> 0.9999	ns	0.00154	> 0.9999	ns
Thal vs OB	-0.00289	0.9000	ns	0.00262	> 0.9999	ns	-0.00487	> 0.9999	ns	-0.00155	> 0.9999	ns
Thal vs WB	0.00048	> 0.9999	ns	-0.15500	0.9996	ns	-0.00185	> 0.9999	ns	0.00066	> 0.9999	ns
Hip vs Ctx	0.00366	0.6696	ns	0.01224	> 0.9999	ns	0.00100	> 0.9999	ns	0.00196	> 0.9999	ns
Hip vs Str	-0.00412	0.5520	ns	0.06466	> 0.9999	ns	0.00155	> 0.9999	ns	0.00016	> 0.9999	ns
Hip vs OB	-0.00111	0.9992	ns	0.20410	0.9981	ns	-0.00376	> 0.9999	ns	-0.00293	> 0.9999	ns
Hip vs WB	0.00226	0.9654	ns	0.04649	> 0.9999	ns	-0.00074	> 0.9999	ns	-0.00072	> 0.9999	ns
Ctx vs Str	-0.00778	0.0533	ns	0.05241	> 0.9999	ns	0.00055	> 0.9999	ns	-0.00179	> 0.9999	ns
Ctx vs OB	-0.00476	0.5170	ns	0.19190	0.9987	ns	-0.00477	> 0.9999	ns	-0.00488	0.9989	ns
Ctx vs WB	-0.00139	0.9971	ns	0.03424	> 0.9999	ns	-0.00174	> 0.9999	ns	-0.00267	> 0.9999	ns
Str vs OB	0.00301	0.8815	ns	0.13950	0.9998	ns	-0.00532	> 0.9999	ns	-0.00309	> 0.9999	ns
Str vs WB	0.00638	0.2265	ns	-0.01817	> 0.9999	ns	-0.00229	> 0.9999	ns	-0.00088	> 0.9999	ns
OB vs WB	0.00337	0.8716	ns	-0.15760	0.9997	ns	0.00303	> 0.9999	ns	0.00221	> 0.9999	ns

CB = Cerebellum, Thal = Thalamus, Hip = Hippocampus, Ctx = Cortex, Str = Striatum, OB = Olfactory Bulb, WB = Whole Brain.
Vs = versus, MD = mean difference, SIG = significance

Note. Significant mean differences in expression are noted as $p<0.001$ (***), $p<0.01$ (**), or $p \leq 0.05$ (*). (ns) indicates that the mean difference is not significant. $p \leq 0.05$ was considered statistically significant.

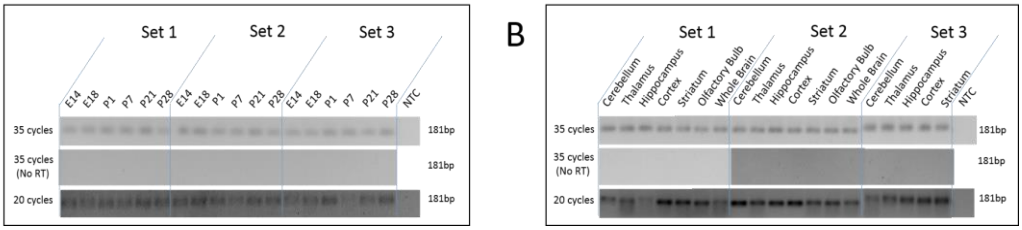

Figure 1. Gel Electrophoresis results for *Gapdh* PCR products. A) qRT-PCR products from the developmental set of samples for *Gapdh*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 20-cycle qRT-PCR was performed on the samples, which shows an increase in *Gapdh* product levels as we increase in age, however some of the samples are not consistent between the sets. **B)** qRT-PCR products from the brain region set of samples for *Gapdh*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 20 cycle qRT-PCR was performed on the samples, which shows the highest amounts of product in the hippocampus, cortex, cerebellum, and striatum, though this is not consistent between the three sets.

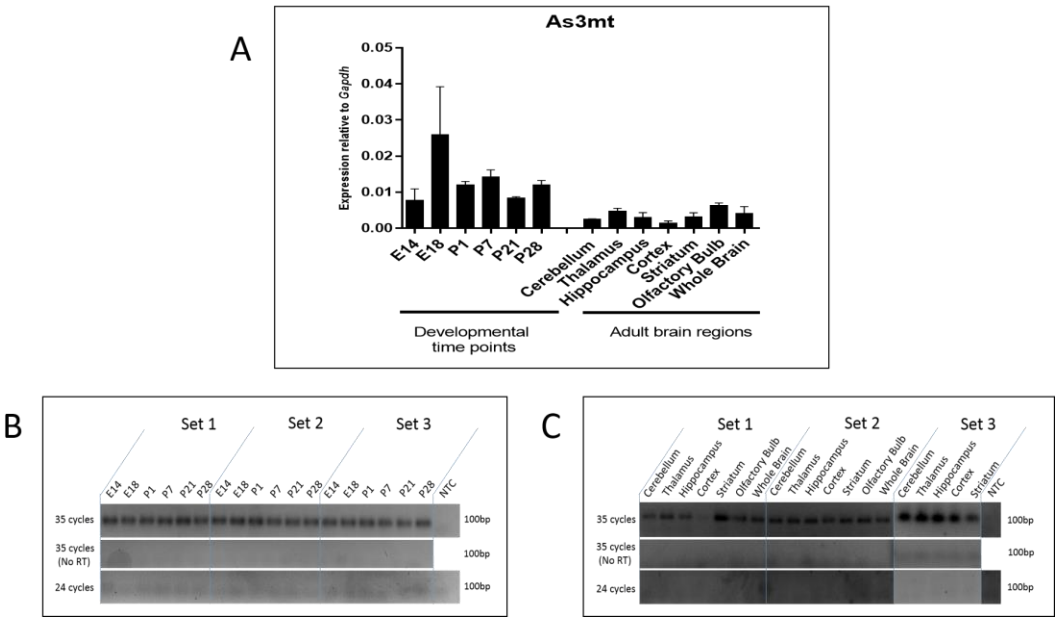

Figure 2. qRT-PCR and Gel Electrophoresis results for *As3mt*. A) Quantitative RT-PCR was performed using cDNA that was reverse-transcribed from RNA from developmental mice brains as well as mouse brain regions. Threshold cycle values (CT) were normalized to *Gapdh*. One-way ANOVA and *Tukey* were used to determine significant differences between developmental ages or brain regions. SEM is shown as one-way error bars. Significant mean differences are indicated as $p<0.001$(***), $p<0.01$(**), or $p \leq 0.05$(*). **B)** Gel electrophoresis of qRT-PCR products from the developmental set of samples for *As3mt*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 24 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen. **C)** Gel electrophoresis of qRT-PCR products from the brain region set of samples for *As3mt*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 24 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen.

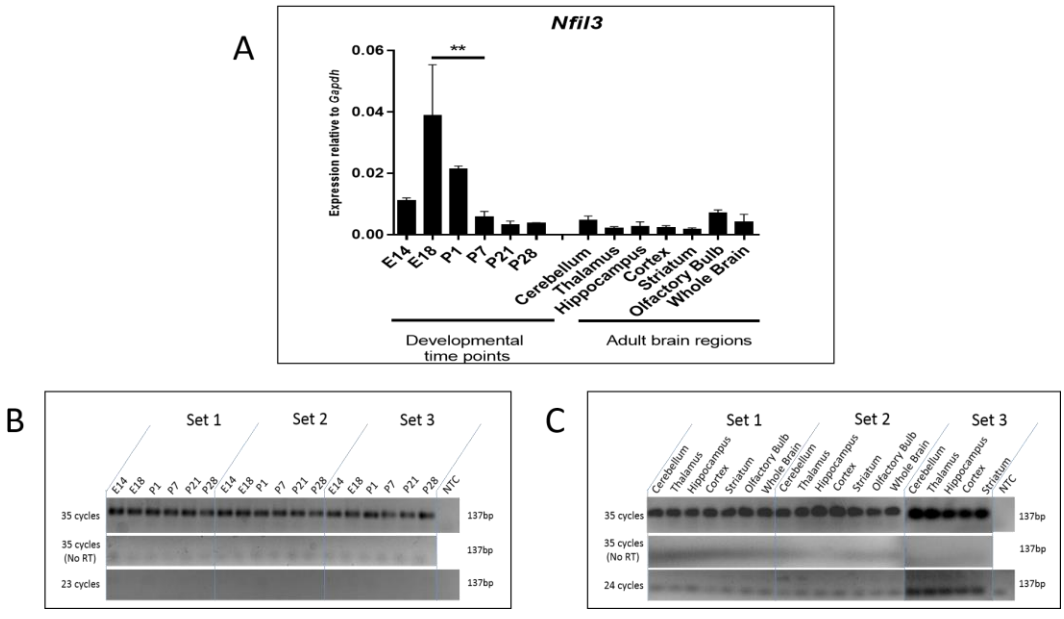

Figure 3. qRT-PCR and Gel Electrophoresis results for *Nfil3*. A) Quantitative RT-PCR was performed using cDNA that was reverse-transcribed from the RNA from developmental mice brains as well as mouse brain regions. Threshold cycle values (CT) were normalized to *Gapdh*. One-way ANOVA and *Tukey* were used to determine significant differences between developmental ages or brain regions. SEM is shown as one-way error bars. Significant mean differences are indicated as $p<0.001$(***), $p<0.01$(**), or $p \leq 0.05$(*). **B)** Gel electrophoresis of qRT-PCR products from the developmental set of samples for *Nfil3*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 23 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are visible. **C)** Gel electrophoresis of qRT-PCR products from the brain region set of samples for *Nfil3*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 24 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen and only primer dimers are visible for most samples.

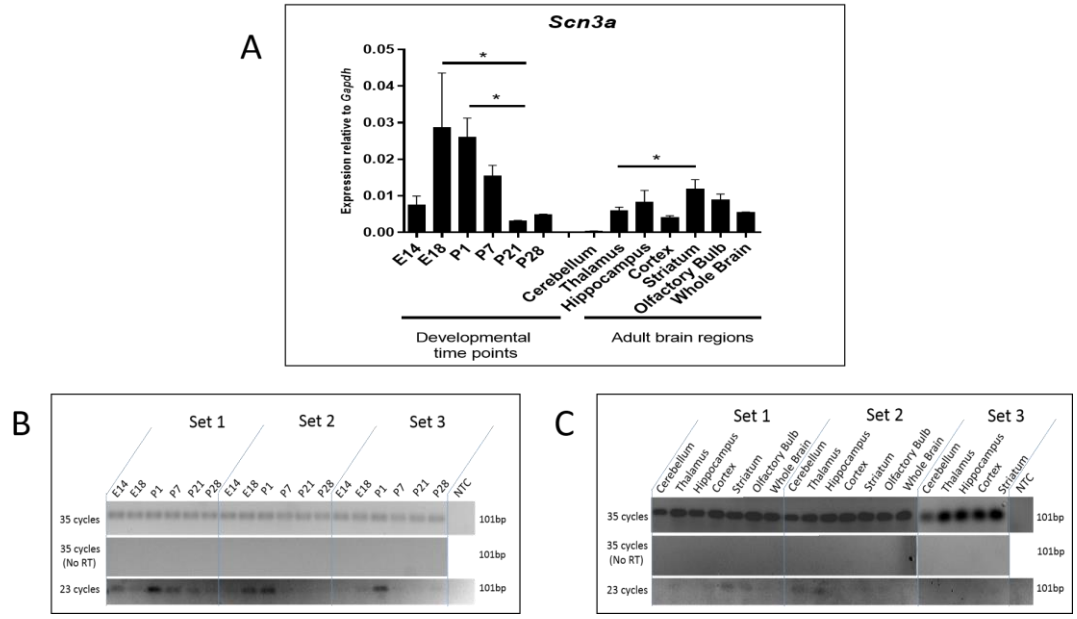

Figure 4. qRT-PCR and Gel Electrophoresis results for *Scn3a*. A) Quantitative RT-PCR was performed using cDNA that was reverse-transcribed from RNA from developmental mice brains as well as mouse brain regions. Threshold cycle values (CT) were normalized to *Gapdh*. One-way ANOVA and *Tukey* were used to determine significant differences between developmental ages or brain regions. SEM is shown as one-way error bars. Significant mean differences are indicated as $p<0.001$(***), $p<0.01$(**), or $p \leq 0.05$(*). **B)** Gel electrophoresis of qRT-PCR products from the developmental set of samples for *Scn3a*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 23 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen in many samples. One notable finding is that the most product is seen in P1 for all three sets of samples. **C)** Gel electrophoresis of qRT-PCR products from the brain region set of samples for *Scn3a*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 23 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen.

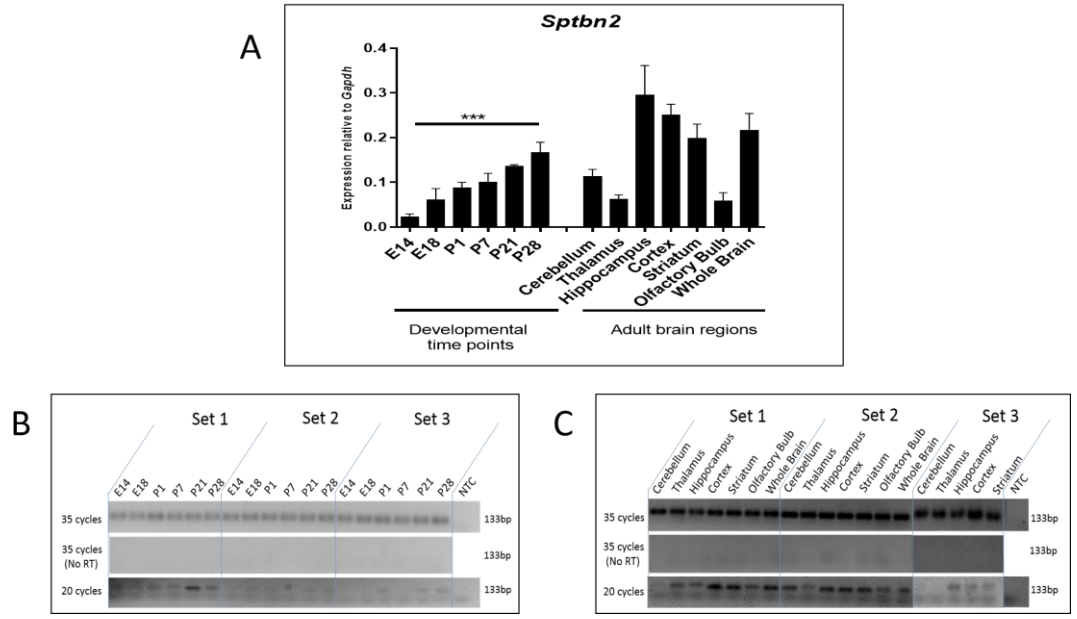

Figure 5. qRT-PCR and Gel Electrophoresis results for *Sptbn2*. A) Quantitative RT-PCR was performed using cDNA that was reverse-transcribed from RNA from developmental mice brains as well as mouse brain regions. Threshold cycle values (CT) were normalized to *Gapdh*. One-way ANOVA and *Tukey* were used to determine significant differences between developmental ages or brain regions. SEM is shown as one-way error bars. Significant mean differences are indicated as $p<0.001$(***), $p<0.01$(**), or $p \leq 0.05$(*). **B)** Gel electrophoresis of qRT-PCR products from the developmental set of samples for *Sptbn2*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 20 cycle qRT-PCR was performed on the samples, however only minimal amounts of product are seen. **C)** Gel electrophoresis of qRT-PCR products from the brain region set of samples for *Sptbn2*. The 35 cycle qRT-PCR also had No-RT samples run along with it to ensure there was no genomic contamination. 20 cycle qRT-PCR was performed on the samples, which shows high levels of product in the hippocampus, cortex, and striatum in all three sets.