Decreased parvalbumin mRNA expression in cerebellar Purkinje cells in autism

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Boston University
DECREASED PARVALBUMIN mRNA EXPRESSION IN CEREBELLAR PURKINJE CELLS IN AUTISM

by

SUJITHRA REPRAKASH

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DEDICATION

I would like to dedicate this work to my family and my friends, Jessica Hill and Julia Lai.
ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Jean-Jacques Soghomonian for his support and patience during my thesis work. He challenged me to strive for my best and to always think critically. Secondly, I would like to thank Dr. Jennifer Luebke for being a fantastic mentor and a role model. Furthermore, I would like to thank my PA mentor, Thai Vu, for training me for PA interviews. I also would like to thank all of the Graduate Medical Science department staff especially Natasha, Millie, and Sherill who gave me assistance and always answered my questions. Finally, I would like to thank my fellow lab members, Kunzhong Zang, Cynthia Wang, Afraa Alsamkari, and Chantal Chammas.
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PURKINJE CELLS IN AUTISM
SUJITHRA REPRAKASH

ABSTRACT

Earlier human and animal studies have indicated abnormal striatal GABAergic interneurons relating to autism spectrum disorder’s (ASD) core features such as stereotypic repetitive behaviors, impaired language and motor skills, and social interactions. Purkinje cells (PCs) in the cerebellum are of great interest in ASD; earlier research has reported a loss of PCs, irregularities within deep cerebellar nuclei, a lower level of GAD67 (glutamic acid decarboxylase) mRNA expressed on PCs, and reduced parvalbumin (PV)-positive interneurons in cortex and hippocampus. In this study, in-situ hybridization was used to quantify the levels of PV mRNA in PCs in post-mortem human autism and control cerebellum sections. Two-tailed t-test analysis of the data showed a significant decrease (p<0.05) in PV mRNA level on PCs in autism compared to control sections. In addition, when comparing two groups (seizure and no seizure) in autism sections, no statistical significance was observed. Post-mortem interval (PMI) and age was compared between the PV mRNA levels in autism and control. Only weak negative correlation was found among age and PV mRNA levels in both groups. This report of decreased PV mRNA level in autism cases further supported previous research findings related to PCs and also confirmed interference with the
inhibitory function of PCs to deep cerebellar nuclei and the cortex resulting in behavioral and motor impairments in ASD.
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INTRODUCTION

What is Autism?

Autism spectrum disorder (ASD) is a neurodevelopment disorder characterized by symptoms such as deficiency in social communication and the ability to develop and maintain relationships. ASD severity is determined from the intensity of stereotypic and repetitive behaviors and from the extent of social communication deficits. Symptoms of ASD are present from early childhood, and ASD is very often comorbid with intellectual disability (DSM-V, APA, 2013). Children with ASD also exhibit impaired development of motor skills and praxis (Qiu, Adler, Crocetti, Miller, & Mostofsky, 2010). Reports indicate that 1 in 88 children are diagnosed with ASD in the US, and prevalence rate is higher in males than females (Halladay et al., 2015; Thurm & Swedo, 2012). Epilepsy is also found to be a comorbid disorder frequently associated with ASD. In a study of 118 individuals diagnosed with infantile autism (IA) and 336 controls, epilepsy was significantly higher among the IA group (Mouridsen, Rich, & Isager, 2011). In a general population, epilepsy has a 2-3% incidence rate while normally it is 30% among ASD cases (Tuchman & Rapin, 2002). In a separate study, the prevalence rate of epilepsy was 19.44% in children with ASD (Kohane et al., 2012). However, when looking at the prevalence rate of ASD in children with epilepsy, one study found 32% of epileptic children fit the ASD criteria (Clarke et al., 2005). Nevertheless, a different study done by Matsuo and colleagues reported only 15.2% of their epilepsy cases had ASD, which is much lower than
the previous study mentioned (Matsuo, Maeda, Sasaki, Ishii, & Hamasaki, 2010). ASD is a rather heterogeneous disorder that is a result of genetic, neurobiological, and environmental interactions during primary brain development (Kong et al., 2014). While the causes of autism are widely being explored, no specific causation for ASD has been identified.

Even though both genetic and environmental factors are implicated in ASD, 90% of ASD cases are categorized as idiopathic and only 10% of the cases have been linked with genetic mutations (Zafeiriou, Ververi, Dafoulis, Kalyva, & Vargiami, 2013). Some of the major genetic variations linked to ASD are Fragile X syndrome (FXS), tuberous sclerosis complex (TSC), Rett syndrome, and neurofibromatosis type 1 (NF1) (Takano, 2015). Another important genetic marker of ASD is Shank 2 and 3 deletion syndromes. Genetic variations within Shank 3, genes involved in TSC, neuroligins 3 and 4 (NLGN3; NLGN4) are implicated in idiopathic ASD (Kelleher et al., 2012). Other whole-genome microarray research reported that deletions and duplications known as copy number variation (CNV) were seen in 5%-10% of ASD cases (Betancur, 2011). Kohane and his colleagues reported that FXS and TSC occur in 0.5% and 0.8% in ASD cases (Kohane et al., 2012). This is consistent with previous studies showing that the genetic mutations underlying ASD are not common within the population. When looking at the comorbidity of ASD in individuals with FXS and TSC, the rate was 30% and 50-61% correspondingly (Kong et al., 2014). One curious finding is the prevalence rate of epilepsy in these genetic syndromes.
According to Tuchman and Rapin, the seizure risk can be up to 94% in Rett syndrome. They also stated that in some of the genetic syndromes involving inheritance, autism and epilepsy do occur side by side (Tuchman & Rapin, 2002). When looking at ASD cases, the occurrence of TSC is uncommon; however, it is more common when epilepsy and ASD are both present in these cases (Spence & Schneider, 2009).

FXS patients have mutations in the FMR1 gene where there is a decreased production of Fragile X Mental Retardation Protein (FMRP). This protein binds to RNA that is involved in brain synaptic plasticity, long term potentiation, and learning and memory (Uzunova, Hollander, & Shepherd, 2014). The abnormalities found in the brain due to the lack of FMRP are seen in various regions of the brain in both excitatory and inhibitory pathways such as the amygdala, the hippocampus, the striatum, and the cerebral cortex (Takano, 2015). Other small cellular irregularities were also found in autopsy studies in the cerebellum (Tuchman & Rapin, 2002). In FXS cases linked to ASD, many brain areas are affected, but the main region of interest is the cerebellar vermis lobules VI-VII. Patients with FXS who are not diagnosed with ASD do not share these abnormalities found in the cerebellar regions, but patients with ASD do share this aspect (Fatemi et al., 2012). In particular, altered parieto-temporal lobe, cerebellar cortex, striatum, frontal cortex, and hypothalamus were strongly associated in mouse models of ASD (Ellegood et al., 2015). Phenotypically, FXS patients exhibits not only hyperactivity, attention deficits, aggression, but also
intellectual disability in males who have the full mutation (Zafeiriou et al., 2013). Furthermore, ASD was reported in FXS cases that had pre-mutation along with slight cognitive defects and normal IQ (Moss & Howlin, 2009). Pre-mutations are carriers that do not exceed certain amount of CGG repetitions in FMR1 gene and do not express FXS completely (Greco et al., 2002).

The tuberous sclerosis complex (TSC) is another genetic syndrome that has been associated with ASD. Not only is it linked with ASD, but also it is implicated with cerebellar abnormalities (Fatemi et al., 2012). TSC is an autosomal dominant disorder which affects many systems in the body and occurs by alterations with the TSC1 or TSC2 genes; these genes are responsible for the transcription of the proteins hamartin or tuberin (Takano, 2015). Other characteristics also include tumors, neurological deficits, epilepsy and cognitive dysfunctions. Mutations in the TSC genes lead to abnormalities within the rapamycin complex 1 activation (mTORC1), which is responsible for cell growth and protein translation (Uzunova et al., 2014). GABA interneurons dysfunction was also suspected in individuals with TSC, epilepsy, and ASD when a GABA-potentiating agent, vigabatrin, was introduced and those individuals had infantile spasms. Other studies found that the deletion of TSC1 gene increased mTORC1 signaling in GABAergic interneurons in the cortex and hippocampus, and Purkinje cells degenerated in TSC mouse model with Purkinje cell-TSC2 deletion (Takano, 2015). This showed further evidence that TSC1/TSC2 genes and GABAergic interneurons may play a role in ASD and epilepsy. When looking at
the neurofibromatosis type 1 (NF1) in ASD patients, the prevalence rate is approximately 1.2% which is smaller than other genetic syndromes and ASD. However, the prevalence rate of ASD in patients with NF1 is 4%. Phenotypic features associated with NF1 include behavioral deficits, learning impairment, and attention-deficit hyperactive disorder (Zafeiriou et al., 2013). Learning disability is reported in about 50% of individuals with NF1. NF1 is an autosomal dominant disorder that occurs due to mutations within the NF1 gene; this gene encodes for neurofibromin, mainly expressed in Schwann cells, oligodendrocytes, and neurons. The neurofibromin activates the Ras-GTPase pathway and help with the regulation of mTORC1 (Takano, 2015). When mutations occur with the NF1 gene, mTORC1 is not very well regulated similar to TSC syndrome.

In Rett syndrome patients, which have similar deficits as ASD, gene mutations that are responsible for methyl-CpG binding protein 2 (MeCP2) causes impairment with language and motor skills. These individuals develop ataxia, cognitive deficits, seizures, and stereotypic hand movements (Ito-Ishida, Ure, Chen, Swann, & Zoghbi, 2015). One mice study explored the loss of MeCP2 from GABAergic interneurons in the cortex and striatum; they found that the mice with the deletion had impaired motor coordination, stereotypic repetitive behaviors and other features relating to Rett syndrome. They also had a decrease in GABA release associated with a loss of MeCP2 in GABAergic interneurons which may lead to hyperexcitability (Chao et al., 2010). Additionally,
loss of MeCP2 gave rise to smaller soma within calretinin-, parvalbumin-, and somatostatin-positive cortical interneurons. Compared to neurons that are not GABAergic, GABAergic interneurons have more MeCP2 protein levels (Takano, 2015). One interesting aspect would be the subtypes of interneurons and the loss of MeCP2 with Rett’s phenotypic features. Mice that lacked the production of MeCP2 protein in PV-positive and somatostatin-positive interneurons showed the most phenotypic features of Rett syndrome (Ito-Ishida et al., 2015). Moreover, changes in the GABA\textsubscript{A} receptor have been seen in mouse models of Rett syndrome phenotypes and few other genetic syndromes. There was a decreased subunit β\textsubscript{3} expression in GABA\textsubscript{A} receptors in individuals with Rett syndrome and were also seen in ASD cases (Eagleson et al., 2010). These studies further emphasize the significance of MeCP2 protein and GABAergic signaling found in the brain. Thus, investigating these genes closely by using specific agonists or antagonists would reveal more about GABAergic interneurons and the mechanisms involved in ASD. Correspondingly, there is a strong comorbidity between genetic mutations, epilepsy, and ASD, so looking at the interactions between the mutations and signaling more closely would reveal additional information about syndromic ASD and idiopathic ASD even though only a small percentage of ASD is associated with genetic syndromes. Ultimately, investigating the pathophysiological components of each genetic mutation would be valuable to understand all aspects of ASD and to conceive newer treatments for ASD.
**Basal Ganglia and Autism: GABA**

Invasive habits and rituals that dominate daily life can be seen in neuropsychiatric disorders such as ASD, and especially in Rett syndrome. Over the years, studies have shown that the basal ganglia circuitry is mainly involved in the acquisition of habits and repetitive behaviors. Impaired activity in these circuits may be a factor in neurological disorders (Graybiel, 2008). One of the core features of ASD is repetitive and stereotypic behaviors. Some neural pathways related to the deficits seen in ASD are considered to be the dorsolateral prefrontal cortex projection to the caudate head and medial inferior frontal gyrus to ventromedial caudate and to dorsal globus pallidus (GP). Lesions to these particular areas has been linked to social impairments. Additionally, cognitive impairments in ASD has been connected to neural pathways involving the thalamus (Schuetze, Park, Cho, MacMaster, & Chakravarty, 2016). Using MRI, shape irregularities were reported in the basal ganglia in children with ASD. Decreased motor skills were associated with the abnormalities in the right posterior putamen along with decreased praxis in the bilateral anterior and posterior putamen. Another observation made in the same study was the association between bilateral medial caudate head deformation and higher social impairment (Qiu et al., 2010). Dyspraxia was highly correlated with social, communication, and behavioral impairments in high functioning children with ASD (Jeste, 2011). Although the majority of MRI studies have reported that the striatum, pallidum, and thalamus in cases with ASD were larger than in the
control subjects, some researchers reported the opposite or no significance between the two groups (Schuetze et al., 2016).

The basal ganglia consist of subcortical nuclei that play a role in motor, associative, cognitive, mnemonic, and limbic functions. There are several structures in the basal ganglia such as the striatum, the globus pallidus (GP) in mice also known as globus pallidus externus in primates (GPe), the entopenduncular nucleus (EP) in mice or globus pallidus internus (GPI) in primates, the subthalamic nucleus (STN), nucleus accumbens, and the substantia nigra (SN) which has two parts: pars compacta (SNc) and pars reticulata (SNr) (Bolam et al., 2000). In primates and cats, the striatum is made up of two parts: caudate and putamen and in between them is the internal capsule. The striatum receives input projections from the cortex mostly while the globus pallidus or GPe and the SNr project outside the basal ganglia (Albin, Young, & Penney, 1989). Even though, the striatum receives substantial input from the cortex, some cortical projections also relay information to the STN. The information received by the striatum is assimilated from other areas such as amygdala, hippocampus, intralaminar thalamic nuclei, and dorsal raphe (Bolam et al., 2000). All of these structures are involved in the direct and indirect pathways that regulate the thalamocortical circuitry.

The direct and indirect pathways are briefly summarized in figure 1 in the context of striatum, SNr, SNc, STN, ventral anterior and ventral lateral nuclei of thalamus (VA/VL), and cortex. The striatum mostly contains spiny neurons while
the pallidum consists of aspiny neurons (Yin & Knowlton, 2006). It has been suggested that interneurons play a crucial role in controlling hyperexcitability; dysfunction with these interneurons has been linked to epilepsy, higher risk of seizures, TSC, and Rett syndrome (Takano, 2015). Although both projection neurons and different types of interneurons exist in the striatum and are GABAergic, about 90-95% of the neurons are medium size densely spiny neurons. To communicate, these neurons use gamma-aminobutyric acid (GABA) as their main neurotransmitter. Additionally, the cortical input received by the spiny neurons are regulated by local striatal interneurons and by SNc inputs (Bolam et al., 2000).

Studies have indicated that aberrant GABAergic pathways are linked to ASD. The researchers observed decreased levels of GAD67 (Glutamic acid decarboxylase) mRNA in Purkinje cells (PCs) of ASD cases; GAD67 is one of the isoforms that is prominent in PCs, and this enzyme produces the neurotransmitter GABA from glutamate which is used for excitatory signaling (Yip, Soghomonian, & Blatt, 2007). Furthermore, Zhang and his associates illustrated that mice deficient of GAD67 in striatal neurons expressing Gpr88 protein demonstrated social impairments such as repetitive grooming and learning impairments (Zhang, Hill, Labak, Blatt, & Soghomonian, 2014). This conclusion is consistent with the notion of abnormal GABAergic interneurons in the pathology of ASD.
Interneurons

A small percentage that make up the striatal neurons are interneurons. These GABAergic interneurons have different subpopulations, but the main population expresses parvalbumin (PV), a calcium binding protein, and have a high rate of firing (Bolam et al., 2000). Calcium ions (Ca$^{2+}$) are crucial for signal transmission in the brain through intracellular and transmembrane transport as well as neurotransmitter release, regulation of gene expression, synaptic plasticity, and axonal elongation. Some of these functions are regulated by calcium binding proteins during neuronal differentiation and development (Ulfig, 2002). There are over 250 types of calcium binding proteins in the body, and PV is one of the most well-known in the central nervous system. Along with calretinin (CR) and calbindin (CB), PV is involved in calcium homeostasis in subtypes of neurons and in buffering of Ca$^{2+}$. GABAergic interneurons in the cerebral cortex such as chandelier and basket cells also expresses PV (Eyles, McGrath, & Reynolds, 2002). Thus, these calcium binding proteins are used as biomarkers to investigate the neuroanatomy of the brain. In addition, PV is expressed during later in brain development and has been mostly linked with mature neurons while the other two are expressed during early brain stages (Ulfig, 2002).

One study looked at the morphological properties of GABAergic interneurons in the somatosensory cortex of FMR1 KO and wild type mice. They reported a substantial decreased amount of PV-positive interneurons and larger soma size of PV neurons in KO mice (Selby, Zhang, & Sun, 2007). This further
illustrates the important connection between GABAergic inhibitory system and ASD. As mentioned earlier, mutated SHANK genes seem to play an important role in ASD since ASD features were often associated with mutated SHANK1, SHANK2, and SHANK3. PV-positive interneurons were reduced by 20-25% in the previous human with ASD and mouse models of ASD studies even though the gene that encodes for PV (PVALB) was not altered (Filice, Vörckel, Sungur, Wöhr, & Schwaller, 2016). Several research studies involving genetic mutations and calcium conductance in humans have been associated with the pathophysiology of ASD. Plasma membrane calcium ATPases (PMCA)s transport calcium from the cytoplasm to the extracellular space during signaling. The ATP2B2 gene that encodes for PMCA is normally found in the dendritic spines of PCs and in PV-positive GABAergic interneurons. Variants for this gene indicated a lower expression of PMCA in post-mortem males with ASD (Fatemi et al., 2012). In a mice study that investigated a gene encoding for the homeobox-containing transcription factor, Engrailed-2 (En2), and the GABAergic interneurons, the researchers detected decreased interneurons expressing PV, somatostatin, and neuropeptide Y in the En2 knockout mice cortex and hippocampus. The En2 knockout mice also exhibited lower PCs in the cerebellum and amygdala abnormalities in previous animal studies (Sgadò et al., 2013). An additional mice study which looked at Ca²⁺ dependent activator protein for secretion 2 (CADPS2) and brain-derived neurotropic factor (BDNF) supported GABAergic interneuron dysfunction in ASD. Mutations of the gene encoding for
CADPS2 resulted in impaired BDNF release. Since BDNF is crucial for
differentiation of PV-positive cortical interneurons, they reported that PV-positive
interneurons in the neocortex and hippocampus were significantly lower than
wild-type mice and the affected mice had behavioral phenotypes similar to ASD
features (Sadakata, 2007). In this same study, they reported another important
implication relating to ASD neuropathology. Purkinje cells expressing CB were
reduced in the CADPS2 knockout mice. By introducing exogenous BDNF, the
viability of PCs increased significantly which also reinforced the importance of
BDNF for PC functionality in the cerebellum and calcium signaling (Sadakata,
2007). Wöhr and other colleagues found that PV knockout mice exhibited
phenotypic features of ASD such as impairment with social interactions and
communication and repetitive behavior. Even heterogeneous mice group
presented social and communication deficits which indicated ASD features can
result from lower expression of PV (Wöhr et al., 2015). An earlier
immunohistochemistry study showed strong PV antiserum labeling on all PCs in
rats. They also found PV antiserum labeling on stellate and baskets cells which
are found in the molecular layer of cerebellum which was opposite of granule
cells in the granular cell layer (Celio & Heizmann, 1981). There are obvious links
between PV-positive interneurons and ASD pathology, but how exactly PV plays
a role in the pathophysiology is yet to be determined.
Cerebellum Pathophysiology

Post-mortem FXS brains with pre-mutation or carriers of FXS showed cerebellar neurodegeneration, a decrease in Purkinje cells (PC), swelling of PC axons, and spongiosis of white matter in the cerebellum (Greco et al., 2002). PC loss in the cerebellum has been shown in various early studies of ASD. This loss is mostly seen in the lateral hemispheric region of the cerebellum cortex. In this region, 75% of ASD cases showed a significant loss of PCs (Hampson & Blatt, 2015). In a clustering analysis done in animal models for ASD, Ellegood et al. reported three circuits associated with ASD: cortex to basal ganglia, brain regions for social interaction and autonomic regulation, and cerebellum (Ellegood et al., 2015). Loss of PCs were mainly in the posterolateral neocerebellar cortex and nearby archicerebellar cortex of cerebellar hemisphere. Not only PCs were abnormal, but irregularities were also found within the deep cerebellar nuclei (Fatemi et al., 2012).

As shown in Figure 2, the PCs are the main output neurons in cerebellum. Both climbing fibers from inferior olivary nucleus (ION) and parallel fibers from granule cells (GCs) provide excitatory input to PCs (Yip et al., 2007). The cerebellum has three layers starting from inner to outer layer: granular cell layer (GCL), Purkinje cell layer (PCL), and molecular layer (ML). In the granular layer, the granule cells provide excitatory input to the Purkinje cell in the molecular layer via parallel fibers. The climbing fibers from the inferior olivary nucleus also gives excitatory input to the Purkinje cells in the molecular layer. Mossy fibers
from the pontine nuclei, spinal cord, and vestibular system carry excitatory input to the granule cells and the deep cerebellar nuclei. The climbing fibers also give excitatory input to the deep cerebellar nuclei. The deep cerebellar nuclei receive inhibitory input from PCs in the Purkinje cell layer. The information from the deep cerebellar nuclei will then travel to brainstem, the thalamus, premotor, and motor cortices (Hampson & Blatt, 2015).

In this study, PCs in human post-mortem cerebellum tissue samples from 11 cases with ASD and 11 controls were compared and analyzed. By utilizing the in-situ hybridization methodology, parvalbumin mRNA on PV-expressing PCs was labeled. Once labeled, the level of PV expression (no. of pixels/µm²) on the PCs was measured blindly and statistically analyzed for any significance between control and ASD groups. Other properties such as post-mortem interval (PMI) and age were also analyzed within each group. Analysis of seizures was only completed in the autism group since controls did not have any reported seizures. My thesis was completed to explore the pathology of PCs in human cerebellum of ASD. By looking at the expression of PV in the cerebellum, this study may encourage future research about the specific role of PV and Calcium signaling in interneurons and PCs which may lead to more specific treatments to improve the main symptoms of ASD.
In direct pathway, information relays from striatum (C+P) to GPi/SNr and then to thalamus (VA/VL) whereas information from striatum go to GPe and STN before going to GPi/SNr in indirect pathway. The negative and positive signs mean inhibitory and excitatory signals, respectively.
Figure 2. Cerebellum Structure
METHODS

Subjects and Tissue Preparation

For this study, 44 post-mortem cerebellum sections were obtained from 11 controls and 11 cases diagnosed with ASD. Two adjacent sections were processed from each case, so the total number of sections used per group was 22. The tissue samples were stored in a -80°C freezer before using them for the experiment. The tissue samples were collected along with age, post-mortem interval (PMI), and history of seizures presented in table 1.

Linearization of Parvalbumin DNA

First step was to linearize a transcription vector pPCR-Script AMP SK(+) that included the human parvalbumin complementary DNA (cDNA). The vector plus a restriction enzyme, SacI, was incubated at 37°C for 2 hours. In order to confirm the linearization was successful, gel electrophoresis was run with 1µL aliquots of uncut cDNA, cut cDNA before extraction, and cut cDNA after extraction on 0.8% Agarose gel at 75 Volts for 35 minutes (Lanoue, Blatt, & Soghomonian, 2013)

Synthesis of 35S Labeled RNA Probes

After the linearization step, the parvalbumin DNA (cDNA) was stored at -20°C until the experiment. During day 1 of in-situ hybridization, 35S radiolabeled complimentary RNA probe was transcribed from the linearized cDNA. For this in
vitro transcription step, 2.5µM $^{35}$S-uracil triphosphate (UTP; specific activity 1250 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) and 10 µM unlabeled UTP with adenosine triphosphate (ATP), cytosine triphosphate (CTP), and guanine triphosphate (GTP) were added to the linearized parvalbumin cDNA and then with the RNA polymerase T7 for 2-hour incubation at 37˚C. The cDNA template was broken down by adding DNAse I enzyme. The radioactive cRNA probe was extracted using phenol/chloroform combination and ethanol precipitation. Alkaline hydrolysis was completed for 38 minutes at 60˚C to shorten the length of the cRNA probe, so the accessibility of the probe during hybridization is better (Cox, DeLeon, Angerer, & Angerer, 1984; Yip, Soghomonian, & Blatt, 2007).

**In-situ hybridization**

For day 2, the protocol was performed as described in a previous study Soghomonian et al., 1994. The tissue samples stored in -80˚C were defrosted under the hood and then fixed in 3% paraformaldehyde. Then, the sections were sequentially washed in 2 x SSC, 0.1M PBS, 0.1M Triethanolamine, and acetic anhydride, and 1.0 M tris-glycine. In order to dehydrate the sections, they were then immersed in 70%, 80%, and 95% ethanol. Adequately sized coverslips were cut from parafilm with scissors cleaned with 100% ethanol. 7ng of probe mixed in with hybridization solution (40% formamide, 10% dextran sulfate, 4x SSC, 10mM dithiothreitol, 1.0% sheared salmon sperm DNA, 1.0% yeast tRNA, 1x Denhardt's
solution) were applied on each section. During hybridization, the tissue sections were incubated for 4 hours at 52°C.

After 4 hours, the cover slips were removed by immersing them in 2x SSC and the sections washed in a new 2x SSC solution. Post hybridization incubations included 50% formamide in 2x SSC for 25 minutes in the 52°C water bath, 2x SSC washes, RNAse A for 30 minutes in the 37°C water bath, 50% formamide in 2x SSC wash again for 5 minutes. Finally, the sections were left overnight in 2x SSC with 0.05% Triton X. On day 3, sections were consecutively washed in 2x SSC with 0.05% Triton X, 300mM ammonium acetate, 70% and 80% ethanol/300mM ammonium acetate, 95% ethanol, xylene, and 100% ethanol. Once the sections were dry, they were placed in a metal cassette. In a dark room, Kodax Biomax-MR X-ray films were set on top of the sections to capture the exposure of the radioactivity of the tissue. The metal cassettes were covered in aluminum foil in the dark to ensure no light exposure. The films were exposed for 16 days and developed.

**Emulsion radioautography**

Emulsion procedure was completed in a dark room. Slides were dipped in Kodak TB3 nuclear emulsion melted in a 37°C water bath. Then sections were left to dry for 3 hours. They were then placed in slide boxes that were light tight and had desiccant beads. The boxes were covered in aluminum foil to keep light out completely. After 21 days of exposure, the emulsion covered slides were
developed in D19 solution on ice at 13°C-15°C. Slides were developed by immersing them in D19 for 3.5 minutes and fixed for 4 minutes. After Hematoxylin and eosin (H&E) staining, coverslips were placed on the slides using Eukitt (Sigma-Aldrich Chemicals, St. Louis, MO).

Quantitation

Emulsion radioautographs were analyzed under bright field microscopy with a 60x objective. The images of labeled neurons were captured using a Sony CCD video camera and Macintosh computer and analyzed in Image J software (NIH image). There were two slides per subject and 50 Purkinje cells per section were quantified. An identical circular outline was used for each Purkinje cell for measuring the area covered by silver grains. The area covered by silver grains was expressed as a number of pixels per cell. The quantitation was carried out by an experimenter blind to the experimental groups. In addition, sections were analyzed in random in order to avoid biases. Furthermore, on the first slide analyzed, the number of grains was counted manually and the corresponding number of pixels measured. This was carried out in order to demonstrate a linear relationship between the two measures was found. This validated our computerized analysis by showing that the area covered by silver grains was correlated with the number of silver grains. The collected numbers of pixels per cell for each slide were then averaged per subject and then compared between autism and control subjects. After isolation of the data into two groups, a two-
tailed t-test was performed in Prism 7th Ed, 2016. During the comparison, few assumptions were made about the silver grains as follows: (1) the silver grains expressed intracellular parvalbumin mRNA content and the number of silver grains was proportional to the amount of radioactivity in each cell, (2) parvalbumin mRNA was only in the Purkinje cell soma, and (3) hybridization did occur in the Purkinje cell soma (Lanoue et al., 2013; Yip et al., 2007).
Table 1. Case Information with Age, PMI, History of Seizures

<table>
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<tr>
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<th>Group</th>
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<td>4104</td>
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<td>5</td>
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<td>Gun shot - chest</td>
<td>M</td>
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<tr>
<td>4271</td>
<td>Control</td>
<td>19</td>
<td>21</td>
<td>--</td>
<td>Epiglottitis/unknown</td>
<td>M</td>
</tr>
<tr>
<td>5251</td>
<td>Control</td>
<td>19</td>
<td>18.6</td>
<td>N</td>
<td>Pneumonia</td>
<td>M</td>
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<td>5718</td>
<td>Control</td>
<td>22</td>
<td>21.5</td>
<td>N</td>
<td>Unknown</td>
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<tr>
<td>4364</td>
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<td>27</td>
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<td>N</td>
<td>Accident</td>
<td>M</td>
</tr>
<tr>
<td>4272</td>
<td>Control</td>
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<td>17</td>
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<td>M</td>
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<tr>
<td>4188</td>
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<td>13</td>
<td>N</td>
<td>Unknown</td>
<td>M</td>
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<td>4362</td>
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<td>25</td>
<td>--</td>
<td>N</td>
<td>Accident</td>
<td>M</td>
</tr>
<tr>
<td>4275</td>
<td>Control</td>
<td>20</td>
<td>16</td>
<td>N</td>
<td>Accident</td>
<td>M</td>
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<tr>
<td>4267</td>
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<td>N</td>
<td>Accident</td>
<td>M</td>
</tr>
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<td>39</td>
<td>14</td>
<td>N</td>
<td>Cardiac Tamponade</td>
<td>M</td>
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<tr>
<td>4899</td>
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<td>14</td>
<td>9</td>
<td>Y</td>
<td>Drowning</td>
<td>M</td>
</tr>
<tr>
<td>5176</td>
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<td>23</td>
<td>18</td>
<td>N</td>
<td>Subdural Hemorrhage</td>
<td>M</td>
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<tr>
<td>1401</td>
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<td>20</td>
<td>N</td>
<td>Pneumonia</td>
<td>F</td>
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<td>N</td>
<td>Burns/unknown</td>
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</tr>
<tr>
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<td>29.98</td>
<td>--</td>
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<td>M</td>
</tr>
<tr>
<td>5278</td>
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<td>13</td>
<td>Y</td>
<td>Drowning</td>
<td>F</td>
</tr>
<tr>
<td>6677</td>
<td>Autism</td>
<td>30</td>
<td>16</td>
<td>N</td>
<td>Congestive Heart Failure</td>
<td>M</td>
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<tr>
<td>3511</td>
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<td>16</td>
<td>Y</td>
<td>Train Accident</td>
<td>M</td>
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<tr>
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<td>31</td>
<td>28.4</td>
<td>Y</td>
<td>Pancreatitis/cancer</td>
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<tr>
<td>6337</td>
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<td>22</td>
<td>25</td>
<td>Y</td>
<td>Aspiration/Choked</td>
<td>M</td>
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</tbody>
</table>
RESULTS

A) Decreased parvalbumin mRNA levels found in autism cerebellum post-mortem tissue

Parvalbumin mRNA labeled with the $^{35}$S Parvalbumin probe was represented by clusters of silver grains as shown in figure 3. More silver grains were visually observed in PC found in control sections (Figure 3B). Once identified on the Purkinje cells in both groups, the silver grain densities were measured as number of pixels with Image J software. The same circular outline was used for every Purkinje cell in both autism and control sections. 100 Purkinje cells were quantified for each case and mean averages were calculated (Table 2). There were 11 controls and 10 autism sections. One outlier was identified and removed from the autism group using Prism 7th Ed, 2016. The mean mRNA levels for autism and control were $114.7 \pm 14.48$ and $228.2 \pm 46.75$, respectively. Two-tailed T-test Analysis of these densities showed a significant decrease in parvalbumin mRNA levels in autism compared to the control group (Figure 4).

B) Analysis of parvalbumin mRNA levels in Autism cases with reported history of seizure and no seizure

There were 5 cases that reported no seizure and 5 cases that reported seizures. One outlier was removed within the seizure group before analysis. The mean ± SEM values for PV mRNA levels in no seizure and seizure groups were
127.5 ± 27.62 and 100.1 ± 12.68 (Table 3). Two tailed t-test analysis of the PV mRNA mean values exhibited no significance (Figure 5).

C) Correlation results between age, PMI, and average parvalbumin mRNA levels in autism and control

To look further into age and PMI, a correlation analysis was performed between age, PMI, and average PV mRNA levels in both groups. As shown in figure 6, no correlation was observed between PMI and mRNA levels in neither groups. However, a negative relationship was observed between age and PV mRNA levels within autism and control sections (r = -0.3743 and r = -0.2335, respectively), but this observation was not statistically significant.
Figure 3. Photographs of Purkinje cell (arrows) in cerebellum under 60X after *in situ* hybridization with radiolabeled S\(^{35}\) parvalbumin RNA probes, developed with emulsion, and stained with H&E. Figure 3A represents the silver densities found in a PC of an autism section. Figure 3B represents the PC found in a control section. Identical circular outline surrounding only the cell was used to measure the densities in both sections. Any outside silver grains were considered artifact and were not included in the measurements.
Table 2. The mean values of $^{35}$S labeled PV mRNA levels from 100 Purkinje cells (PCs) for each case subject. Mean ± SEM is included for autism and control.

<table>
<thead>
<tr>
<th>Autism Case Number (n=10)</th>
<th>Mean mRNA Levels</th>
<th>Control Case Number (n=11)</th>
<th>Mean mRNA Levels</th>
</tr>
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<tbody>
<tr>
<td>6401</td>
<td>98.23</td>
<td>4268</td>
<td>247.46</td>
</tr>
<tr>
<td>4899</td>
<td>133.32</td>
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<td>234.84</td>
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<td>5176</td>
<td>138.15</td>
<td>4271</td>
<td>594.4</td>
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<td>1401</td>
<td>197.94</td>
<td>5251</td>
<td>128.07</td>
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<td>164.64</td>
<td>5718</td>
<td>80.01</td>
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<td>147.77</td>
</tr>
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<td>102.22</td>
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<td>192.83</td>
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<tr>
<td>6337</td>
<td>72.43</td>
<td>4275</td>
<td>171.18</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>114.7 ± 14.48 (*p&lt;0.05)</td>
<td></td>
<td>228.2 ± 46.75</td>
</tr>
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</table>
**Figure 4.** Average parvalbumin mRNA levels in Purkinje cells labeled with $^{35}$S RNA probe and represented as number of pixels in control and autism groups. The average found for control was $228.2 \pm 46.75$, ($n=11$) and for autism was $114.7 \pm 14.48$, ($n=10$). Two tailed t-test analysis showed a significance between two group (*$p<0.05$).
Table 3. Mean values of PV mRNA levels in autism cases with reported seizures and no seizures.

<table>
<thead>
<tr>
<th>Case Number</th>
<th>No Seizure (n=5)</th>
<th>Case Number</th>
<th>Seizure (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6401</td>
<td>98.23</td>
<td>4899</td>
<td>133.32</td>
</tr>
<tr>
<td>5176</td>
<td>138.15</td>
<td>5278</td>
<td>92.61</td>
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<tr>
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<td>164.64</td>
<td>6337</td>
<td>72.43</td>
</tr>
<tr>
<td>6677</td>
<td>38.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean ± SEM</strong></td>
<td><strong>127.5 ± 27.62</strong></td>
<td></td>
<td><strong>100.1 ± 12.68</strong></td>
</tr>
</tbody>
</table>
Figure 5. The levels of PV mRNA measured on the Purkinje cells in autism is represented by number of pixels. The autism group was further divided in two groups; patients with a history of seizure (n=5) and no seizure (n=4). Two tailed t-test analysis of the means showed no significance.
Figure 6. PV mRNA levels were compared in both autism and control groups according to age and post-mortem interval values. Figure 6A & 6C exhibits the correlation between age, PMI, and mRNA levels in autism (n=10). Figure 6B & 6C represents correlation between age (n=11), PMI (n=9) and mRNA levels in control. Weak negative correlation was found between age and PV mRNA levels in autism and control, while no correlation was found between PMI and mRNA levels in neither groups.
DISCUSSION

Analysis of the mean values found on Purkinje cells (PCs) showed an overall decreased PV mRNA level in the autism cerebellum sections. Additionally, Yip and other colleagues established *in situ* hybridization data indicating a lower level of GAD67 mRNA on Purkinje cells in autistic cerebellum (Yip et al., 2007). Further examination by Yip et al. indicated that GAD67 mRNA levels in interneurons in the cerebellum molecular layer (Stellate and Basket cells – Figure 2) were higher in autism than control cases. These interneurons provide inhibitory input to PCs which then send inhibitory signaling to deep cerebellar nuclei (Yip, Soghomonian, & Blatt, 2008). Higher expression of GAD67 in these cells may contribute to the decreased GAD67 mRNA levels found in PCs, which ultimately interrupts cerebellar output function of PCs and deep cerebellar nuclei transmission. Although the number of labeled PCs in the cerebellum was not investigated in this study, it may be interesting to determine if a loss of PCs occurs as has been described in earlier ASD studies (Hampson & Blatt, 2015). There was no correlation found between PMI and mRNA levels in neither autism nor control (Figure 6C & 6D). According to Yip et al., post-mortem tissue has very stable RNA (Yip et al., 2008). This supports the notion that the post-mortem period does not affect mRNA quality considerably. On the contrary, a trend for negative correlation was found for both groups when age was compared with the level of PV mRNA (Figure 6A & 6B). Since this trend was not significant, it can be concluded that age may not greatly affect mRNA levels in either groups.
Since epilepsy and seizures have been strongly associated with dysfunction of GABAergic interneurons (Takano, 2015), additional analysis of autism cases with reported seizures and the levels of PV mRNA was performed in order to explore the link between seizures and physiological changes within PCs. The analysis revealed no significance between the two means found within the autism group (Table 3; Figure 5). Although no significant difference was established between the PV mRNA levels on PCs, a closer look at table 1 revealed that about half of the autism cases (n=5) had a history of seizures while almost 80% of control cases did not. The no seizure group had a slightly higher mean value but this was not significant. However, it may be worth considering a bigger autistic population sample looking specifically at the connection between seizures and expression of parvalbumin in interneurons and PCs.

Chattopadhyaya and Cristo also suggested that seizure threshold may be lowered by changes in the GABA function since GABAergic abnormalities were found with high comorbidity rate of autism and epilepsy (Chattopadhyaya & Di Cristo, 2012).

Cerebellum abnormalities have been reported in many animal and human studies. Substantially, it is easier to investigate ASD phenotypes in KO animal studies than in human post-mortem tissues due to accessibility and a limited supply of human brain tissue. This limitation contributes to the depth of further exploration using real ASD cases, so we have to rely on transgenic animal models that are closest to ASD phenotypes. Han et al. observed social
impairment and other core ASD features in a mice model, $Scn1A^{+/-}$. They found GABAergic impairment in the forebrain where this gene encodes for voltage-gated sodium channel (Na\textsubscript{v}1.1). By adding low doses of clonazepam, social behavior and fear memory impairment was restored. They also stated that these transgenic mice have abnormal action potentials in the interneurons located in the hippocampus and in Purkinje cells as well as decreased Na\textsuperscript{+} currents (Han et al., 2012). One potential methodological limitation in this current study is that the thresholding of silver grains during the quantitative analysis may be subjective because the thresholding is done manually and can be influenced by bias. However, in a pilot quantification, we confirmed that the number of pixels measured after thresholding was correlated with the actual number of silver grains.

In summary, our study provides further evidence for cerebellum abnormalities, specifically with the PCs, in autism. Furthermore, GABAergic interneurons function is very complex due to the existence of subtypes with varying morphology and physiological components (Chattopadhyaya & Di Cristo, 2012). In another study involving human cerebellum tissue, GAD65 mRNA was labeled on two dentate cell subtypes: large and small. Only the large dentate cells in autistic sections had reduced GAD65 labeling. This further proves the heterogeneity of ASD and the selectivity of altered inhibitory and excitatory pathways in the cerebellum in ASD (Yip, Soghomonian, & Blatt, 2009).
Our experiment revealed a statistically significant reduction in the PV mRNA levels in Purkinje cells of autistic post-mortem sections compared to control cases. In addition, no significant difference was reported between seizure and no seizure groups within the autism sections. Finally, no correlation was presented between post-mortem interval (PMI) and PV mRNA levels while a weak negative correlation was observed in terms of age and PV mRNA levels in both groups. Our study opens new avenues for future research to better understand the inhibitory pathways in the cerebellum as well as to gain a deeper understanding of the pathophysiology of PCs in autism. One possible future study could look at the loss of PCs and different calcium binding protein or transporters found specifically on PCs and try to see if ASD symptoms can be alleviated using agents targeting those pathways. Combining earlier studies along with other findings can clarify the pathophysiological implications of parvalbumin expression in ASD and GABAergic circuitry in the entire brain. Ultimately these research studies will get a few steps closer to finding other innovative therapies for individuals suffering from ASD.
REFERENCES


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Sadakata, T. et al. (2007). Autistic-like phenotypes in Cadps2-knockout mice and aberrant CADPS2 splicing in autistic patients, 117(4). http://doi.org/10.1172/JCI29031DS1


doi:10.1007/978-3-642-59425-0


CURRICULUM VITAE

SUJITHRA REPRAKASH (Date of Birth: 1989)
Phone: 217-4-4933  Email: suji.reprakash@gmail.com

EDUCATION

Boston University School of Medicine, MA (2014 – present)
- M.S in Anatomy and Neurobiology (Expected completion, May, 2016)

University of Illinois at Chicago, IL (2007 – 2010)
- B.S. in Biological Sciences – GPA: 3.72/4.00
- Magna cum laude with College Honors

HONORS & AWARDS

Carle Foundation Hospital Recognition – Sterile Processing and Distribution Department (SPD) (2014)
- Received monetary award and recognition for my initiative to help the SPD department improve training practices and to lead shifts when supervisor was not available.

Liberal Arts and Sciences Undergraduate Research Initiative Award (2010)
- Received a $2500 to help further my independent research with Dr. A. Don Murphy on the neural pathways of a gastropod, Helisoma Trivolvis, and an inorganic dye called Ruthenium Red. Dr. A. Don Murphy also received $1500 towards his laboratory.

Chancellor’s Student Service & Leadership Award (2009)
- Awarded for my Volunteering commitment and service to the University of IL at Chicago and to the community.

Distinction for Commitment in NSCS for Leadership, Service, & Scholarship (2009)
- Was awarded $200 for my commitment and volunteering services in the National Society of Collegiate Scholars organization.

Recognition for valuable participation in Emerging Leaders Program (2008)

The National Society of Collegiate Scholars Honors (2008)

Science Olympiad Medals (2006 & 2007)
- Was awarded 2nd place medal for Ecology in the Regional competition in 2006.
- Won 1st place in Genetics and Biology for both Regional and State Competition in 2007.

**Mu Alpha Theta Honors – National High School and Two-Year College Mathematics Honor Society (2006)**
- Joined with honors due to my academic success. Helped students with trigonometry, algebra, and geometry.

**PROFESSIONAL EXPERIENCE**

**Carle Foundation Hospital, Urbana IL (Aug 2012 – Apr 2014)**

*Sterile Processing and Distribution (SPD) Technician*

- Trained new employees in each area of the department. Worked with OR nurses to ensure new SPD staff received adequate training. Created training videos and instructions on how to restock Omni cells in OR, clean endoscopes, and sterilize plasma and steam instruments. Promoted to lead position for evening shift and night shift in the absence of supervisor. Selected supplies, processed trays, cleaned and sterilized instruments for all surgeries in OR. Answered phone and filled requests for other floors in the hospital.

**Consolidated Communications Call Center, Mattoon-Champaign IL (Mar 2011 – Aug 2012)**

*Customer Call Center Operator*

- Provided 24/7 live answering service and after-hours emergencies services to local businesses in Central IL. Helped with tech support for Volo Broadband and CMI Broadband. Placed substitute teachers for Springfield, Urbana, and Oswego school districts.

**Bath & Body Works, Champaign IL (Jun 2010 – Mar 2011)**

*Floor Sales Associate*

- Performed cashier duties and assisted customers finding items matching their preferences. Replenished under-stock cabinets and helped set up decorations for new upcoming themes.

**America Reads Challenge (ARC), Chicago IL (Aug – Dec 2010)**

*Tutor*

- Tutored 2nd grade students in math, reading, and grammar at the Galileo Scholastic Academy for Math and Science. Graded tests and homework. Worked one-on-one with students for homework and test preparations.

**RESEARCH**
Boston University – School of Medicine, Boston, MA (May 2015 – Present)
Graduate Research Student
- Thesis work with post-mortem human cerebellum tissue and radioisotope markers to identify structures within Purkinje cells in cerebellum.

University of Illinois – Dept. of Biological Sciences Snail Lab, Chicago, IL (Apr 2009 – Dec 2010)
Undergraduate Research Assistant
- Completed a Capstone research project on the neural effects of ruthenium red dye on the salivary gland cells in Dr. A. Don Murphy’s Lab. Finalized a dissection guideline for other students for BIO 489 and assisted them with recordings of brain activity. Learned to properly dissect snail salivary glands and record brain activity via electrodes. Performed lab duties such as feeding snails, cleaning tanks and autoclaving gravel and equipment.

VOLUNTEER
Anatomy and Neurobiology Graduate Student Committee (Sep 2014 – Present)
- Master Student Representative for academic year 2015-2016.
- Organizing a social event after orientation in September 2015 for incoming students.

Graduate and Professional Leadership Council - Boston University School of Medicine, Boston, MA
Student Representative for Vision and Dental Insurance for Graduate Students (April 2015 – Present)
- Research and collect information on better vision and dental insurance for graduate students.

Carle Foundation Hospital, Champaign, IL
Carle Auditory Oral School (Feb 2011 – April 2014)
- Helped hearing impaired students reach “target” words for language skills development. Assisted teachers during class, lunch, nap, and recess with any duties and supervision.

Pediatrics Unit (May – Aug 2012)
- Cleaned play rooms and stocked nurses' stations and carts. Transported patients after discharge. Supervised patients in the game room. Fed pediatric patients who were in social care. Help make arts and crafts projects for children.

Emergency (May 2011 – Aug 2011)
- Attended to patients who waited in the hallway for rooms and gave them blankets. Helped nurses with lifting patients onto beds and wheelchairs. Stocked supplies in each room and in the cabinets in the hallway. Cleaned
the equipment and beds in between patients. Stocked the oxygen room with full tanks.

Clinical Observational Unit (Jun – Aug 2010)
- Assisted nurses with any clerical tasks and front desk duties such as answering phones, transferring calls to nurses’ charge phones, answering patient call lights, and restocking forms. Sanitized beds and IV equipment in between patient change.

PACU (Jun – Aug 2010)
- Took patients’ temperatures after they came out of surgeries and placed warm blankets on patients. Cleaned equipment in-between patients and stocked any shelves with supplies and warm blankets. Helped transport patients to other units. Provided bedside company to patients who were waiting for rooms in other units.

Champaign County Christian Health Care (CCCHC), Champaign IL
Triage (Jun – Aug 2012)
- Recorded patients’ blood pressure, heart rate, breathing rate, height, weight, and patient history.

- Managed patient flow from triage, physicians, social work, and discharge. Checked in patients and provided assistance with patient forms.

University of IL at Chicago - Music Therapy, Chicago IL (Feb 2009 – June 2009)
- Played clarinet for the patients and the staff at the Johnson R. Bowman Health Center at Rush University Medical Center.

Rush University Medical Center, Chicago IL (Apr 2009 – Dec 2010)
- Served as a wheelchair patient transporter. Administered help services including delivering packages, refilling water, and carrying out any tasks for nurses.

Jordan James Boys & Girls Family Center, Chicago IL (Nov 2007 – Apr 2008)
-Supervised Elementary and Middle school children during extracurricular activities and classroom. Tutored students with homework.

SHADOWING
Boston Medical Center (May 2015 – Present)
- Observed Thai Q. Vu, Chief PA-C in the Neurosurgery department and his interactions with other PAs and physicians.

Education to Careers/Professional Programs (Oct 2006 – Feb 2007)
• Shadowed Dr. Bradley Morgan, a pathologist, at Carle Foundation Hospital. Observed many autopsies done by Dr. Morgan. Autopsies included an amputated leg, breast tissue, gallbladders, and livers. Also shadowed various sections of the pathology lab such as the hematology, microbiology, histology, and cytology.