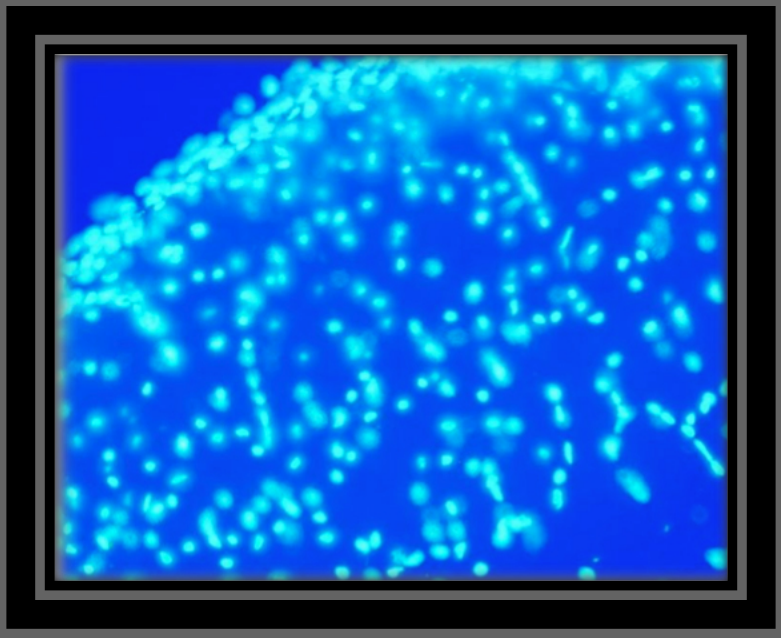
# U.R.N.

## UNDERGRADUATE RESEARCH NEWSLETTER



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# *Foreword*

This introduction is dedicated to the contributors. They are to be congratulated on their hard work—which is most of what good research is. Past introductions have highlighted how undergraduate research impacted their careers in science. That can't be done here, since I did not do research as an undergraduate—a second interest was poetry and instead I contributed to and helped edit the University of Missouri at Kansas City student literary magazine. I also worked part time for a chemical company, but that was not research, it was mostly cleaning glassware. While undergraduate research done by me did not impact my career, since being at the University of Miami I have seen how it has impacted the careers of students that have worked in my lab. This summer, the son of one of those students toured the University of Miami while deciding which University he wanted to attend. The parent had worked some twenty five years earlier as an undergraduate researcher in my lab. He went on to get an MD, and then later, since he wanted to do research more than general practice, he went on to get a PhD and is now an accomplished medical researcher. He wrote me an email saying that it was his experience as an undergraduate researcher at UM that pushed him in that direction.

Research does push you. Not always in the direction you want, but usually in the direction you should go. The contributors to this third annual edition of URN have experienced that. They have also, no doubt, experienced the frustration and hard work that go along with it. That is something research has in common with poetry, and all other pursuits of value—the hard work and frustration prior to the payoff. I like the saying of the poet Robert Frost in that regard—"no surprise for the writer, no surprise for the reader". It is difficult to write a poem or a research article without that surprise. Fortunately for the reader, the writers of this issue of URN have all experienced that surprise at the end of their hard work, and convey it in this volume.

This third issue of URN contains articles on a range of topics, from the effects of pre-natal nicotine exposure; to the role of retinoic acid in the nervous system. Other topics discuss aggregation of human insulin, analysis of synaptic vesicles in hippocampal neurons, and emotion processing skills in younger siblings of children with and without autism. Just looking at the titles in the table of contents there are already hints of surprises and insights lurking.

Those surprises and insights have the same source as those of Keats in his famous poem "On first looking into Chapman's Homer". There is a common feeling to creativity and discovery which crosses all disciplines. The last section of that poem illustrates how discovery motivates and pushes at us all. It serves to close this introduction to the third annual edition of URN:

*"Yet did I never breathe its pure serene*
*Till I heard Chapman speak out loud and bold:*
*Then felt I like some watcher of the skies*
*When a new planet swims into his ken;*
*Or like stout Cortez when with eagle eyes*
*He star'd at the Pacific—and all his men*
*Look'd at each other with a wild surmise—*
*Silent, upon a peak in Darien."*

Carl D. Hoff
Professor, Chemistry

# Pre-Natal Nicotine Exposure Influences the Post-Natal Expression of Estrogen Receptor-β in the Brain of Female Rats

Mohga Behairy (Class of 2012)

*Major*: Neuroscience
*Principal Investigator/Supervisor*: Dr. Ami Raval
*Department*: Neuroscience
*Senior Thesis*: Yes

*Nicotine has been previously found to have adverse effects on estrogen levels in adult women. In this study, we investigated how pre-natal nicotine exposure affects estrogen receptor levels in offspring. We exposed a group of pregnant rats to nicotine during gestation and examined the estrogen receptor (ER) protein levels in the offspring after birth. We found that pre-natal nicotine exposure reduces the post-natal neurological development of ER protein levels in female offspring.*

Pre-natal nicotine exposure has adverse effects on neurological development. Over half of women who smoke in the United States continue smoking during pregnancy (Cener for Disease Control and Prevention, 2011). Previous researchers have found that nicotine crosses the placental barrier and interferes with pre- and post-natal neurological development in various ways (i.e., episodic hypoxia, low brain weight, loss of brain cells, sudden infant death syndrome, etc; Ahlborg & Bodin, 1991; Cnattingius, 2004; Coleman, Britton, & Thornton, 2004; Farkas, MacKinnon, Ariano, Sitar, & Hasan, 2007). Researchers have also found that nicotine reduces endogenous circulating estrogen levels in adult women, leading not only to the early onset of menopause, but also to susceptibility towards various cardiovascular and cerebrovascular diseases that are normally prevented by circulating estrogen in premenopausal women (Baillargeon, McClish, Essah, & Nestler, 2005; Cassidenti, Vijod, Vijod, Stanczyk, & Lobo, 1990; Cramer, Harlow, Xu, Fraer, & Barbieri, 1995; Rusa et al., 1999). Nicotine reduces circulating estrogen levels in adult women by inhibiting estrogen biosynthesis in the brain (Barbieri, Gochberg, & Ryan, 1986) and by interfering with estrogen mediated intracellular signaling in the hippocampus via reduction of estrogen receptors (Raval, Bhatt, & Saul, 2009; Raval, Bramlett, & Perez-Pinzon, 2006; Raval et al., 2011; Raval, Bhatt, & Saul, et al., 2009). In this study, we investigated how estrogen mediated intracellular signaling develops in the brain after pre-natal nicotine exposure by examining the post-natal protein levels of estrogen receptors in rats. We hypothesized that ER-α and ER-β protein levels would be reduced in male and female rats that were exposed to nicotine during gestation.

Using osmotic pumps, we exposed one group of pregnant rats to nicotine, and as a control, another group of pregnant rats to saline. After birth, we collected tissues from the offspring on various post-natal days (PND). We used hippocampal, cortical, and striatal tissues to perform Western blotting using the following antibodies: Rabbit polyclonal anti-ER-α (1:1000), rabbit polyclonal anti-ER-β (1:1000), and mouse monoclonal anti-β-actin (1:10000). We quantitatively analyzed immunoblots using AlphaEase FC Software (version 6.0) to detect optical density values.

Contrary to our hypothesis, ER-α protein levels increased in the hippocampal tissues of male and female rats exposed to nicotine as opposed to saline, and ER-β protein levels increased in male rats exposed to nicotine as opposed to saline in both hippocampal and striatal tissues. However, as hypothesized, ER-α protein levels decreased in the striatal tissues of male and female rats exposed to nicotine as opposed to saline. Finally, as hypothesized, ER-β protein levels decreased in the hippocampal and striatal tissues of female rats that were exposed to nicotine as opposed to those exposed to saline during gestation.

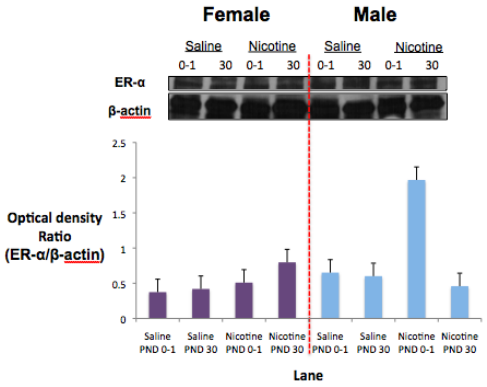


**Figure 1:** ***Hippocampal ER-α Immunoblots***
This figure shows the hippocampal immunoblots for ER- α and β-actin. The quantification of the bands is a ratio of the optical densities of ER-α/β-actin. There are eight lanes going from left to right on the immunoblots and represented on the graphs with lanes 1-4 being female and lanes 5-8 being male: lane 1 = PND 0-1 saline, lane 2 = PND 30 saline, lane 3 = PND 0-1 nicotine, lane 4 = PND 30 nicotine, lane 5 = PND 0-1 saline, lane 6 = PND 30 saline, lane 7 = PND 0-1 nicotine, and lane 8 = PND 30 nicotine.

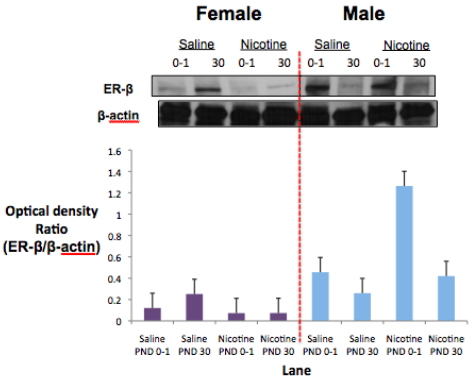


**Figure 2:** ***Hippocampal ER-β Immunoblots***
This figure shows the hippocampal immunoblots for ER- β and β-actin. The quantification of the bands is a ratio of the optical densities of ER- β/β actin.

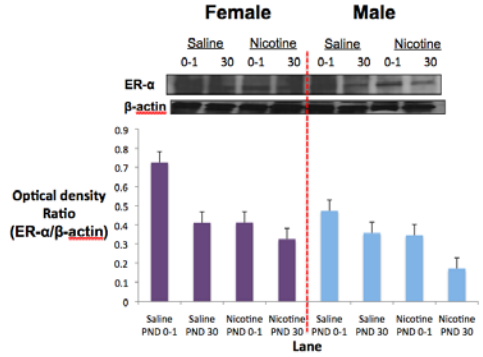


**Figure 3:** ***Striatal ER-α Immunoblots***
This figure shows the striatal immunoblots for ER- α and β-actin. The quantification of the bands is a ratio of the optical densities of ER- α/β-actin.

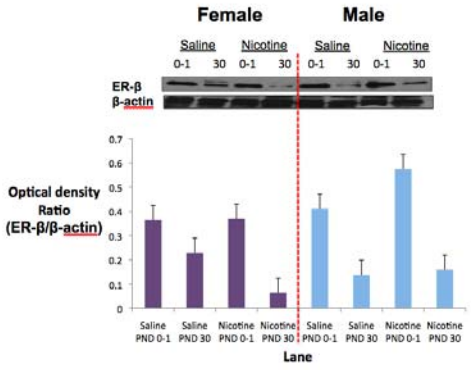


**Figure 4:** ***Striatal ER-β Immunoblots***
This figure shows the striatal immunoblots for ER-β and β -actin. The quantification of the bands is a ratio of the optical densities of ER-β/β-actin.

Our main findings showed decreased ER-β protein levels in the striata and hippocampi and reduced ER-α protein levels in the striata of female rats that were pre-natally exposed to nicotine, and it is important to note the many detrimental effects of reduced ER protein levels in females.

Evidence suggests that apart from the nucleus, estrogen receptors are located on the plasma membrane and are essential in mediating rapid non-genomic actions of estrogen (Kalita,

Szymczak, & Kaczmarek, 2005; Toran-Allerand, 2004; Vasudevanm & Pfaff, 2008). Interestingly, a necessary step in estrogen receptor(s) localization to membrane is palmitoylation of the receptor (Acconcia et al., 2005; Marino & Ascenzi, 2008; Pedram et al., 2007). We observed reduced ER-β protein levels in the hippocampi and striata as well as reduced ER-α protein levels in the striata of female rats exposed to nicotine pre-natally, and although further investigation is necessary, this suggests the possibility of defects in palmitoylation of estrogen receptors.

Estrogen receptors are also known to regulate estrogen-mediated mitochondrial structure and function (Bettini & Maggi, 1992; Klinge, 2008; Mirebeau-Prunier et al., 2010). Researchers have demonstrated that the reduction of ER-β protein levels in the mitochondria of rats exposed to nicotine causes mitochondrial dysfunction (Raval et al., 2012). It is important to note that mitochondrial dysfunction has also been associated with ischemic neuronal death (Kristian, 2004; Raval et al., 2012).

The hippocampus plays important roles in long-term memory and represents the most vulnerable region of the brain under disease conditions such as ischemia. Long-term potentiation (LTP), a cellular correlate of learning and memory, is characterized as a long lasting (hours to days) synaptic strengthening that results from brief (1-2 sec) tetanic (~100 Hz) stimulation (Bliss & Collingridge, 1993). LTP is readily observed in many brain regions (Kirkwood, Dudek, Gold, Aizenman, & Bear, 1993), but is particularly prominent in the hippocampus (Bliss & Collingridge, 1993). In the hippocampus of female experimental animals, estrogen enhances the magnitude and persistence of LTP as a result of its intracellular signaling cascade (Adams et al., 2004; Sarkar, Smith, Logan, & Simpkins, 2010; Smith & McMahon, 1992). The intracellular cascade activated by estradiol includes phosphorylation of CREB via calcium-calmodulin kinase II (CaMKII) activation (Boulware, Kordasiewicz, & Mermelstein, 2007; Jover-Mengual et al., 2007; Raval, Bhatt, & Saul, et al., 2009; Ward, 1999). The previous studies from our lab which have demonstrated that chronic nicotine exposure reduced pCREB (which requires the activation of estrogen receptors) in the hippocampus of adult female rats, are findings which allow us to infer that the observed loss of ERs due to prenatal nicotine exposure could also influence memory and cognition in the female offspring of smoking mothers (Jover-Mengual et al., 2007; Raval et al., 2006; Raval et al., 2009; Raval, Saul, et al., 2009; Raval et al., 2011; Wu et al., 2005; Yang et al., 2010).

Although we can use previous research to scientifically deduce the adverse effects of reduced estrogen levels on post-natal development, the data we have tell us only that post-natal estrogen receptors are altered in females due to pre-natal nicotine exposure. This limitation in our experiment was solely due to time constraint and future study is required.

In conclusion, our findings could provide a link between smoking during pregnancy and the susceptibility of female offspring developing the same complications that a cigarette smoking adult woman is prone to due to the dysfunction of estrogen mediated intracellular signaling caused by nicotine exposure. Nicotine dependence poses unique and severe risks for nicotine-attributed cerebrovascular diseases in children and future investigations targeted on the effects of nicotine on ER-β-mediated synaptic as well as mitochondrial function might reveal the consequences of nicotine specific to pre-natally exposed women.

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# Parent-Child Discourse, Emotion Processing Skills, and Social Deficits in Younger Siblings of Children With and Without Autism Spectrum Disorder

Charissa DiNobile (Class of 2012)

*Major:* Pschology
*Principal Investigator/Supervisor:*
Dr. Heather Henderson
*Department:* Psychology, Child Division
*Fellowship/Awards/Recognition:* Magna Cum Laude
*Senior Thesis:* Yes

*The first-degree relatives of individuals with autism spectrum disorder (ASD) are at increased risk for exhibiting subclinical deficiencies in social competencies, i.e., the broad autism phenotype (BAP) (Wainer, Ingersoll, & Hopewood, 2011). Accordingly, children at risk for the BAP may display limited emotion processing skills, especially during episodes of early social interaction such as parent-child discourse (Baker, Fenning, & Crnic, 2010; Fenning, Baker, & Juvonen, 2011). Past research on this topic is limited to studies involving the infant siblings of children with ASD, and in this study, I compared the emotion processing skills of the preschool-age younger siblings of children with ASD with those of typically developing children. Further, I examined the relation among emotion comprehension and social responsiveness in these children. Parent-child dyads recruited from an ongoing study at the University of Miami's Psychology Department participated in short emotion discourse interactions, which were subsequently coded and scored based upon the emotion processing abilities of the child. Additionally, each parent completed a measure of social responsiveness about his/her child. Data analyses revealed that the younger siblings of autistic children displayed significantly lower emotion processing skills than those of typically developing children, especially when these skills involved perspective taking (i.e., the ability to understand the thoughts, feelings, and motivations of others). Moreover, no significant correlation was found among overall emotion comprehension scores and social responsiveness scores of the younger siblings of children with ASD, whereas strong correlations were found among these variables in the younger siblings of typically developing children. Based on these findings, it can be concluded that early intervention in regards to emotion comprehension may be necessary for the younger siblings of children with autism. Moreover, such intervention could help support, promote more typical trajectories of socioemotional development in early childhood.*

The first-degree relatives of individuals with autism spectrum disorder (ASD) are at increased risk for displaying subclinical autistic tendencies (Wainer et al., 2011). They may exhibit deficits in areas considered to be the global domains of ASD, which include impairments in reciprocal social interaction and communication (American Psychological Association, 2000; Baron-Cohen, 2002). Furthermore, these individuals may display limited emotion processing skills—a deficit that might underlie their more general social limitations (Pons, Harris, & de Rosnay, 2004). Several researchers who explored this concept in BAP-risk children (e.g., Cassel et al., 2007) found significant differences in the emotional reactions of the infant siblings of children with ASD and those of typically developing children. However, these studies are limited by the extremely young age of participants and the consequent inability to measure siblings' comprehension of emotion concepts. The goals of my study are (a) to compare the preschool-age younger siblings of typically developing children (TD-sibs) and children with ASD (ASD-sibs) on emotion understanding during parent-child discourse, and (b) to examine the correlation between emotion understanding and parent-reported social deficits in these children.

The current study included participants recruited from an ongoing NICHD, Autism Speaks, and Marino Autism Research Institute funded longitudinal study of the younger siblings of children with autism at the University of Miami in Coral Gables, Florida. All child

participants were between 47 and 89 months of age at the time of testing and were separated into groups (ASD-sibs and TD-sibs) based on an older sibling's diagnosis of ASD. All parent participants filled out the Social Responsiveness Scale (SRS), which yields both composite and subscale scores pertaining to their child's social behavior (Constantino, 2005). Each dyad also independently completed an emotion discourse task, consisting of a 3-min conversation about a time when the child experienced an upsetting situation. Using the Emergent Social Cognition Observation System (ESCOS), the video-recorded discourse was coded and subsequently quantified based upon the quality of parent-child interactions regarding emotion and the ability of the child to comprehend emotional information. ESCOS evaluates discourse in terms of the child's comprehension and yields both a composite score and scores of its three core domains: Internal state understanding, perspective taking, and causal reasoning and problem solving (Fenning et al., 2011).

Upon completion of coding, ESCOS and SRS scores were calculated for ASD-sibs and TD-sibs (see Table 1). To test my hypothesis that ASD-sibs would score significantly lower on measures of emotion comprehension than TD-sibs, independent t-tests (see Table 2) were conducted using ESCOS composite and domain scores. A significant difference was found among ESCOS composite scores of ASD-sibs and TD-sibs, t(42) = -2.36, p = .023, with ASD-sibs scoring significantly lower than TD-sibs as predicted. Specifically, ASD-sibs scored significantly lower on the domain of internal state understanding, t(42) = -2.14, p = .038, than TD-sibs, as well as on the domain of perspective taking, t(42) = -2.28, p = .028. However, the two groups did not differ significantly on causal reasoning and problem solving, t(42) = -1.68, p = .101, though ASD-sibs did generally score lower than TD-sibs on this domain.

My second hypothesis was that ESCOS scores would predict SRS scores in ASD-sibs; however, this hypothesis was not supported. Pearson correlations were performed using both ESC-OS and SRS composite and

**Table 1**

***Descriptive Statistics of Study Variables***

| Variable | Mean Score (SD) | |
|---|---|---|
| | ASD-sibs | TD-sibs |
| ESCOS Composite Score | 8.37 (3.09) | 10.59 (2.96) |
| Internal State Understanding | 2.89 (1.01) | 3.59 (1.12) |
| Perspective Taking | 2.26 (1.40) | 3.24 (1.35) |
| Causal Reasoning/Problem Solving | 3.22 (1.05) | 3.76 (1.03) |
| SRS Composite Score | 37.92 (21.08) | 27.13 (15.34) |
| Social Awareness | 6.79 (3.40) | 6.25 (4.30) |
| Social Cognition | 7.21 (4.73) | 3.94 (3.40) |
| Social Communication | 11.71 (8.16) | 8.88 (6.68) |
| Social Motivation | 7.04 (4.13) | 5.19 (4.37) |
| Autistic Mannerisms | 5.17 (5.40) | 2.88 (3.36) |

*Note:* $N$(ASD-sibs) = 27; $N$(TD-sibs) = 17

**Table 2**

***Mean ESCOS Score Comparison among ASD-sibs and TD-sibs***

| Variable | Condition | | *t* | *df* | Significance (2-tailed) |
|---|---|---|---|---|---|
| | ASD-sibs | TD-Sibs | | | |
| Internal State Understanding | 2.89 (1.01) | 3.59 (1.12) | -2.09* | 31.5 | .045* |
| Perspective Taking | 2.26 (1.40) | 3.24 (1.35) | -2.30* | 35.2 | .027* |
| Causal Reasoning/ Problem Solving | 3.22 (1.05) | 3.76 (1.03) | -1.69 | 34.6 | .101 |
| Composite | 8.37 (3.09) | 10.59 (2.96) | -2.38* | 35.3 | .023* |

*Note:* $N$ (ASD-sibs) = 27; $N$ (TD-sibs) = 17; * $p < .05$, ** $p < .01$

Domain scores of ASD-sibs (see Table 3 and Figure 1a) were nonsignificant with the exception of the relation among ESCOS perspective taking and SRS social communication, r(22) = -.438, p = .032. Identical Pearson correlations were conducted for TD-sibs as a means for comparison (see Table 4 and Figure 1b), and significant correlations were found among SRS composite and ESCOS composite scores, r(14) = -.558, p = .025, as well as among scores on several ESCOS and SRS subscales.

**Table 3**

***Pearson Correlation Coefficients among ESCOS Scores and SRS Scores in ASD-Sibs***

| | ES-C | ES-InS | ES-PeT | ES-CR | SRS-C | SRS-Aw | SRS-Cog | SRS-Com | SRS-Mo | SRS-Ma |
|---|---|---|---|---|---|---|---|---|---|---|
| ES-C | – | | | | | | | | | |
| ES-InS | .862** | – | | | | | | | | |
| ES-PeT | .908** | .644** | – | | | | | | | |
| ES-CR | .898** | .711** | .716** | – | | | | | | |
| SRS-C | -.335 | -.117 | -.402 | -.360 | – | | | | | |
| SRS-Aw | -.143 | -.132 | -.090 | -.181 | .687** | – | | | | |
| SRS-Cog | -.123 | .104 | -.222 | -.176 | .894** | .609** | – | | | |
| SRS-Com | -.349 | -.123 | -.438* | -.351 | .971** | .567** | .860** | – | | |
| SRS-Mo | -.376 | -.289 | -.376 | -.352 | .438* | .127 | .173 | .396 | – | |
| SRS-Ma | -.294 | -.056 | -.369 | -.340 | .886* | .565** | .802** | .868** | .115 | – |

*Note:* $N$ = 24; * $p < .05$, ** $p < .01$; ES-C = ESCOS Composite, ES-InS = ESCOS Internal State Understanding, ES-PeT = ESCOS Perspective Taking, ES-CR = ESCOS Causal Reasoning and Problem Solving, SRS-C = SRS Composite, SRS-Aw = SRS Social Awareness, SRS-Cog = SRS Social Cognition, SRS-Com = SRS Social Communication, SRS-Mo = SRS Social Motivation, SRS-Ma = SRS Autistic Mannerisms

**Table 4**

***Pearson Correlation Coefficients among ESCOS Scores and SRS Scores in TD-Sibs***

| | ES-C | ES-InS | ES-PeT | ES-CR | SRS-C | SRS-Aw | SRS-Cog | SRS-Com | SRS-Mo | SRS-Ma |
|---|---|---|---|---|---|---|---|---|---|---|
| ES-C | – | | | | | | | | | |
| ES-InS | .869** | – | | | | | | | | |
| ES-PeT | .794** | .440 | – | | | | | | | |
| ES-CR | .887** | .829** | .491* | – | | | | | | |
| SRS-C | -.558* | -.508** | -.355 | -.575* | – | | | | | |
| SRS-Aw | -.237 | -.127 | -.245 | -.210 | .784** | – | | | | |
| SRS-Cog | -.226 | -.170 | -.168 | -.238 | .634** | .773** | – | | | |
| SRS-Com | -.606* | -.610* | -.309 | -.665** | .845** | .498* | .279 | – | | |
| SRS-Mo | -.356 | -.272 | -.172 | -.497* | .613* | .210 | .266 | .469 | – | |
| SRS-Ma | -.348 | -.416 | -.297 | -.149 | .446 | .256 | -.007 | .340 | .029 | – |

*Note:* $N = 16$; * $p < .05$, ** $p < .01$; ES-C = ESCOS Composite, ES-InS = ESCOS Internal State Understanding, ES-PeT = ESCOS Perspective Taking, ES-CR = ESCOS Causal Reasoning and Problem Solving, SRS-C = SRS Composite, SRS-Aw = SRS Social Awareness, SRS-Cog = SRS Social Cognition, SRS-Com = SRS Social Communication, SRS-Mo = SRS Social Motivation, SRS-Ma = SRS Autistic Mannerisms

My findings suggest that BAP-risk individuals may display deficits in emotion processing skills in addition to their deficits in overall social competence. Further, the lack of a strong correlation among ESCOS and SRS scores in BAP-risk children suggests that they may possess a deficit that contributes to deficient integration of social and emotion concepts. Future research is needed to more closely examine these deficits, and empirical research as to the cause of these deficits would likely provide targets for both prevention and intervention. For the families of individuals who exhibit detrimental BAP characteristics, a more in-depth understanding of the subclinical, phenotypic presentation of autistic symptomology may provide a more comprehensive understanding of the BAP individual as well as an inclination to seek appropriate intervention.

**Figure 1a**

***Relationship among ESCOS Composite Scores and SRS Composite Scores in ASD-sibs. Correlations among composite scores of ESCOS and the SRS were nonsignificant, $r(22) = -.34$, $p = .110$.***

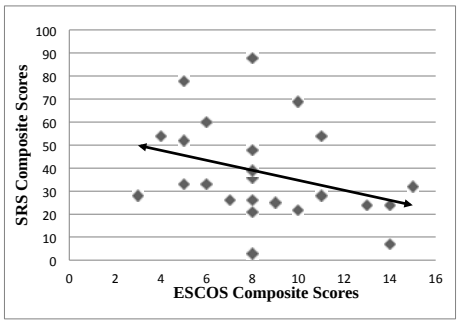
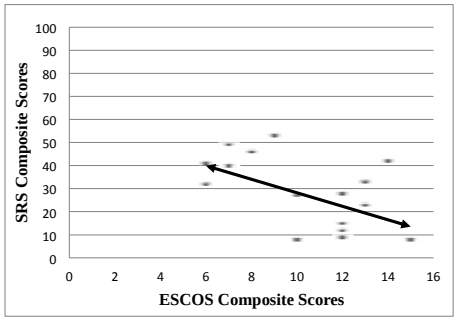



**Figure 1b**

***Relationship among ESCOS Composite Scores and SRS Composite Scores in TD-sibs. Correlations among composite scores of ESCOS and the SRS were significant, $r(14) = -.59$, $p = .025$.***

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# Retinoic Acid is Required in Partitioning the Hindbrain and Spinal Cord

Keun Lee (Class of 2014)

*Major:* Neuroscience
*Principal Investigator/Supervisor*: Dr. Isaac Skromne
*Department:* Biology
*Senior Thesis:* No

*The spinal cord is a unique vertebrate feature that originates together with the hindbrain from the most posterior region of the developing nervous system. The mechanisms that partition the nervous system into the hindbrain and spinal cord are unknown. It is important to understand the mechanisms that partition the hindbrain and spinal cord territories to gain insight into the causes of neural tube defects, the second most common birth malformation in humans. Previous studies have shown that a signaling molecule called Retinoic Acid (RA) is required for hindbrain development, but its contribution to spinal cord development has not been established. Here we show that RA elimination, using chemical and genetic approaches in the zebrafish embryo, affect hindbrain and spinal cord size. Our results suggest a fundamental function of RA in partitioning the hindbrain and spinal cord territories.*

Spinal cord and hindbrain arise from the same precursor cells, but the mechanisms that confer the cells spinal cord or hindbrain identities are unknown. Hindbrain identity is regulated by a potent signaling molecule, Retinoic Acid (RA, Begemann et al., 2004; Mayes, 2005). However, RA function in the spinal cord has not been investigated.

To investigate RA role in the spinal cord development, RA synthesis was blocked in developing zebrafish embryos using DEAB, a well-known pharmacological inhibitor. While extensively used to block RA synthesis, two important caveats associated with the use of pharmacological inhibitors, they have no effect on previously synthesized RA and treatments are not tissue-specific; all cells of the developing embryo are affected. To overcome these limitations, we developed an alternative method to eliminate RA activity by increasing the production of Cyp26, a RA-degrading enzyme. Enzymes can be expressed in all cells of an embryo or in specific tissues. Here we provide evidence that increasing Cyp26 expression in all cells of an embryo is an effective method to eliminate RA, and sets the stage for tissue specific RA elimination. Furthermore, using this method we uncovered a novel role of RA in establishing the spinal cord.

To examine the effectiveness of eliminating RA by increasing RA degradation, we also treated embryos with DEAB, Cyp26, or both, to see if RA inhibition effect were additive. Embryos were treated with either 0.1% DMSO (controls) or 0.1 mM DEAB in 0.1% DMSO at 5.3 hours post fertilization, as previously described (Begemann et al. 2004). Cyp26 (variant c) mRNA was prepared by in vitro translation from plasmid following standard procedures (mMESSAGE mMACHINE kit by Ambion). Cyp26 was overexpressed in zebrafish embryos by injecting 2.5 ng of mRNA at the 1-cell stage of development. To detect gene expression, in situ hybridization was performed to visualize the effect of eliminating RA signaling on gene activity.

Embryos overexpressing Cyp26 or exposed to DEAB developed similar hindbrain defects that are known to be associated with the loss of RA activity (Fig.1). Cyp26 overexpression resulted in the disappearance of a posterior hindbrain marker, vhnf1, and the expansion of an anterior hindbrain marker, hoxb1a (Fig.1 E and F). This was similar to the effect of blocking RA synthesis with DEAB (Fig.1 C and D, Maves and Kimmel 2005). This result suggests that Cyp26 overexpression effectively blocks RA activity in the hindbrain.

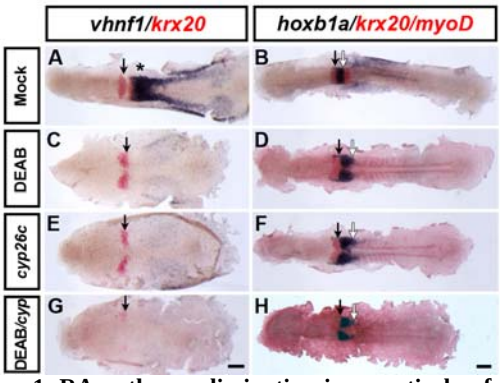


**Figure 1. RA pathway elimination irrespectively of the method used, cause similar posterior hindbrain defects.** Expression of posterior hindbrain gene vhnf1 is lost (A, C, E, G, asterisks) and expression of anterior hindbrain gene hoxb1a is posteriorly expanded (B, D, F, H, white arrows) in RA-deficient embryos (C-H) compared to wild type siblings (A-B). Expression of the gene krx20 (red) was used to align embryos (black arrows). (A-B) Embryos treated exposed to vehicle alone (0.1% DMSO, negative controls). (C-D) Embryos exposed to the RA-synthesis inhibitor DEAB (0.1 mM in 0.1% DMSO, positive control). (E-F) Embryos injected to over-express Cyp26c1. (G-H) Embryos injected to over-express Cyp26c1 and treated with DEAB. Anterior is to the left. Scale bar 0.1 mm.

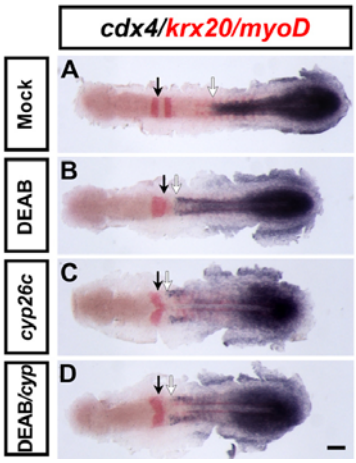


**Figure 2: RA-pathway inhibition shifts spinal cord territory anteriorly.** Expression domain of the spinal cord marker cdx4 (purple) is shifted anteriorly in RA-deficient embryos (B-D) compared to wild type siblings (A). Expression of the gene krx20 (red) was used to align embryos (black arrows). (A) Wild type, (B) DEAB-treated, (C) Cyp26c1 over-expressing and (D) Cyp26c1 over-expressing, DEAB treated embryos. Anterior is to the left. Scale bar 0.1 mm.

To assess the function of RA in spinal cord development, we next removed RA activity in embryos and analyzed the expression of the spinal cord marker, cdx4 (Skromne et al., 2007). The loss of RA activity caused an anterior expansion of cdx4 (Fig. 2). This result suggests that RA limits the size of the spinal cord by inhibiting cdx4 expression.

Using loss of function strategies, we identified RA as a strong candidate to partition the nervous system's hindbrain and spinal cord. In addition, we showed that the Cyp26 overexpression is an effective method to eliminate RA activity. In future studies, Cyp26 overexpression will be used to reduce RA activity exclusively in neural tissue or in adjacent tissues using tissue-specific promoters. This novel approach will allow us to analyze to what extent RA functions directly in the nervous system or indirectly in other tissues during the establishment of the hindbrain and spinal cord.

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# Human Insulin Self-Aggregation Study as Langmuir monolayer

Wei Liu (Class of 2013)

*Majors*: Chemistry
*Principal Investigator/Supervisor*: Dr. Roger Leblanc
*Department*: Chemistry
*Senior Thesis*: No

*Insulin is a hormone with the crucial function of regulating glucose metabolism in human body. In this project, the self-aggregation process of insulin is studied by surface chemistry approach. Human insulin was spread at the air-water interface and formed Langmuir monolayer. The aggregation property was then investigated using surface isotherms and spectroscopic methods in different conditions. The experimental data suggested that the aggregation process of insulin was likewise seen to carry specific orientations at air-water interface in different conditions.*

Insulin is an important protein which controls the blood glucose level in human body. There are several forms of insulin including monomer, dimer and hexamer. Hexamer is the most stable form of insulin which is used in various diabetes drugs. However, the only active form in glucose metabolism process is the monomer. Therefore, it is very important to investigate the aggregation of insulin. The Langmuir monolayer is an ideal in vitro model to study the target compound in biological membranes. Thus, surface chemistry methods involving Langmuir monolayer and spectroscopy were selected to conduct this research.

Consider the pH effect to protein structure, different samples of insulin in acidic and basic conditions were spread at air-water to form Langmuir monolayer. Zn2+ concentration was controlled in the subphase because zinc ions are the core of hexamer formation. The insulin samples as Langmuir monolayer were studies by surface pressure-area isotherm, surface potential-area isotherm, UV-Vis spectroscopy, fluorescence spectroscopy, Infrared reflection-absorption spectroscopy (IRRAS) and circular dichroism (CD) spectroscopy.

Figure 1A shows that: (i) the lift-off area per molecule increases with the increase in the concentration of Zn (II) ions; (ii) the slope of each isotherm in the region of 400 to 700 Å2 molecule-1 is approximately the same, but the limiting molecular area, which indicates the closest packing of the human insulin molecule, increases with the concentration of Zn (II) ions, i.e. from 660 (pure water) to 700, 780 and 850 Å2 molecule-1. From previous studies we can deduce that our data shows formation of aggregates at the air-water interface. Figure 1C showed a behavior opposite to the one seen on Fig. 1A: with the increase of the Zn (II) concentration, the lift-off (from 800 to 500 Å2 molecule-1) and limiting molecular area (from 630 to 450 Å2 molecule-1) are reduced. The presence of Zn (II) ions have a great influence on the different conformations since both the basic (BI) and acidic human insulin (AI) aqueous spreading solvent on pure water surface give a similar surface pressure-area isotherm.

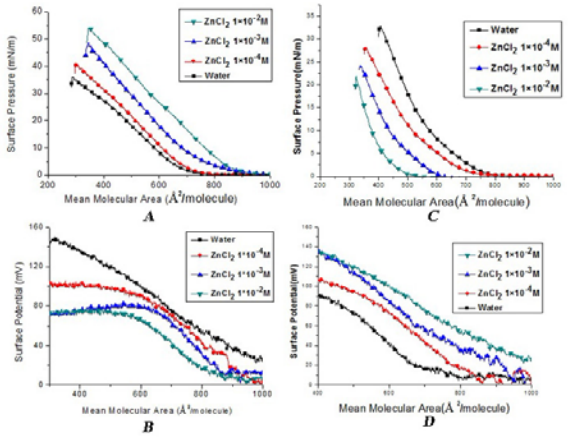


**Figure 1:** Surface pressure- and surface potential-area isotherms of human insulin Langmuir monolayer with different subphase conditions at pH 2 ( A,B ) and pH 9 ( C,D) respectively.

Figures 2C and 2D present the UV-vis spectra of the insulin Langmuir monolayer at two surface pressures, 5 and 25 mN/m, respectively. From these figures, in presence of Zn (II) ions a shift is observed from 224 to 234 nm, while the peak at 276 nm remains the same at both surface pressures. From our results we have suggested a mechanism for the observed

shift in the 224-234 nm range of the absorption spectra. In this mechanism, the Zn (II) ions would induce an aggregated state of the human insulin in 2-D, regardless of the pH and of the surface pressure imposed on the system. Hereon we have demonstrated the aggregation of human insulin induced by the Zn (II) ions in the subphase.

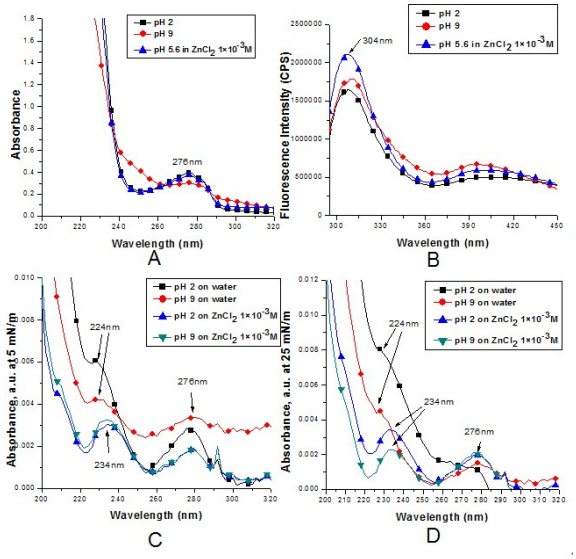


**Figure 2:** UV-vis and fluorescence (excitation at 270 nm, Raman at 297 nm) spectra of 10-4 M human insulin aqueous solutions (A,B) and UV-vis spectra of human insulin Langmuir monolayer at pH 2 and pH 9 in absence and presence of ZnCl2 (10-3 M) in the subphase at 5 mN/m (C) and at 25 mN/m (D).

Figure 3C shows the IRRAS spectra of human insulin Langmuir monolayer at 5 mN/m at various pH values in the absence and presence of Zn (II) ions. A peak at 1650 cm-1 that corresponds to α-helix was observed in all spectra. Through further analysis of this spectra we have seen that: (i) the spectra of HI with basic spreading solvent (lines 2 and 4 from top) showed different peak maxima and shape from the spectra with acidic spreading solvent (lines 1 and 3 from top), indicating a conformational and orientational difference of the human insulin Langmuir monolayer that exists at different pH levels; (ii) an increase in the quantity of β-strands on the solution in the presence of Zn (II) ions to the solution in absence of Zn (II) ions has been observed as a wider peak at around 1650 cm-1 as observed in the plot representative of the HI solution with Zn (II) ions. The new absorption peak at around 1720 cm-1 in the presence of zinc ions was assigned to β-turn structures; (iii) the insulin Langmuir monolayer with acidic and basic spreading solvent had unique absorption peaks at around 1525 and 1570 cm-1, respectively. This observation shows a significant difference between samples under acidic and basic conditions, implying that the human insulin Langmuir monolayers under different pH levels have different orientations at the air-water interface. Similar analysis also apply to the IRRAS spectra of human insulin Langmuir monolayer at 25 mN/m at various pH values in absence and presence of Zn (II) ions (Fig. 3D). We, therefore, assume that the major aggregated form of HI Langmuir monolayer is hexamer because different orientations of HI dimer have little effect on surface physical property.

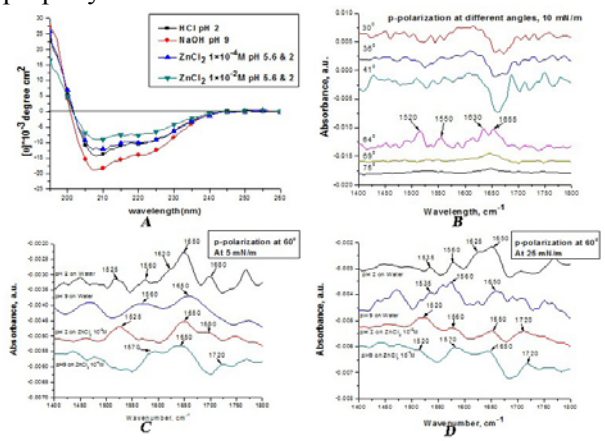


**Figure 3:** CD spectra of insulin aqueous solution with different conditions (A) and IRRAS spectra at different angles (B) and different surface pressure (C,D) with different conditions.

The proposed model of HI hexamer Langmuir monolayer at different pH was shown in Figure 4. When HI monolayer is formed in acidic condition, the orientation of hexamer is more likely to have hydrophobic chain B (Fig. 4A, Red Curve) in contact with subphase. In this orientation, the hydrophilic chain A is not accessible to zinc ion from subphase so it is relatively more stable than the basic case. The limiting molecular area is expected to increase in this orientation, which corresponds to the surface pressure-area and surface potential-area isotherms in acidic condition (Fig 1). As shown in the model, chain A is partially submerged into the subphase in this orientation so that the

hydrophilic chain A is open to the attack of Zn (II) ions. The basic condition makes this attack easier to take place because the insulin in basic condition has a relatively loose structure due to the titration of the A1 alpha-amino group. When chain A of HI was attacked by Zn (II) ions, the hexamer is easy to disaggregate and the chain A immerges more into subphase. This destruction process is irreversible and lead to lower limiting molecular area, which well interprets the surface pressure-area and surface potential-area isotherms in basic condition. The proposed orientation also corresponded to the IRRAS spectra data.

**Table 1. Band Assignment of the FTIR and IRRAS Spectra Related to the Secondary Structure of Human Insulin in Aqueous Solution and as Langmuir Monolayer**

| band position, $cm^{-1}$ (surface pressure, incident angle, pH) | band assignment |
|---|---|
| 1630 (10 mN/m, 64°, pH 2) | β-sheet (amide I) |
| 1655 (aqueous solution and 10 mN/m, 64°, pH 2) | α-helix (amide I) |
| 1650 (5 and 25 mN/m, 60°, pH 2 and 9) | |
| 1620 (aqueous solution, pH 2) | β-strands |
| 1544 (aqueous solution, pH 2) | α-helix (amide II) |
| 1550 (10 mN/m, 64°, pH 2) | |
| 1525 (5 and 25 mN/m, 60°, pH 2) | |
| 1560 (5 and 25 mN/m, 60°, pH 2) | |
| 1570 (5 and 25 mN/m, 60°, pH 9) | β-sheet (amide II) |
| 1720 (5 and 25 mN/m, 60°, pH 9) | β-turn |

**Table 1:** Band Assignment of the FTIR and IRRAS Spectra

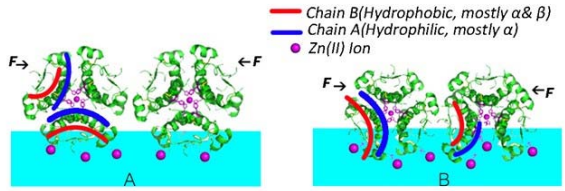


**Figure 4:** The proposed insulin hexamer orientation at the air-water interface with acidic (A) and basic human insulin samples (B).

Several studies on insulin aggregation have been done in the aqueous and crystalline phases. However, such research done at the air-water interface has been seldom. Based on the experimental data, we proposed a human insulin (HI) monolayer model of the insulin conformation and dynamics under acidic and basic conditions. Overall we have shown the contrasting aggregation dynamics that human insulin has on different pH at the air-water interface, shedding light for promising research on the effect that pH has on protein aggregation at cell membranes.


## Acknowledgement

I wish to thank my girlfriend Jinqing Huang, who contributed lots of innovative ideas regarding my experimental data. Her passion about chemistry inspired me to be dedicated to my research for the last two years.


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# Comparative analysis of synaptic vesicles present in the axon of hippocampal neurons in wild-type (WT) and synapsin triple knock-out (TKO) mice.

Julia White (Class of 2013)

*Major:* Neuroscience
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*Senior Thesis:* No

*The location and mobility of synaptic vesicles are important in synaptic organization. It is suggested that synapsin proteins play a significant role in vesicle pool turnover and have been shown to bind vesicle membranes to actin filamentous networks on the cytoskeleton, effectively tethering vesicles near the synaptic terminal in the active zone. In the following investigation, hippocampal neurons from synapsin triple knock-out mice were compared with neurons of wild-type mice using transmission electron microscopy. Analysis indicated a significant difference in the number of vesicles present in the axons of the two types of mice.*

The proteins under investigation, the synapsins, are three similar phosphoproteins present in neural presynaptic terminals in the central and peripheral nervous systems of mammals [5]. In order for the active zone to function properly during neurotransmitter transmission, vesicles must be controlled presynaptically and made available for release via exocytosis [9]. Chi and others have shown that synapsin proteins dissociate from presynaptic vesicles into the axon as they are phosphorylated during action potentials in hippocampal terminals [4]. After the transmission is complete, synapsin proteins return and bind with returning vesicles, essentially controlling "vesicle pool turnover" [4]. This tethering may be specifically accounted for by Synapsin I because of its ability to attach vesicles reversibly to actin filaments (cytoskeletal proteins) [3]. Mice genetically deficient in either Synapsin I or II (or both) showed almost a complete absence of vesicle clustering and a reduction in the number of synaptic vesicles [2].

Primary cultures of hippocampal neurons from synapsin triple knock-out mice (TKO) and control wild-type (WT) mice were subjected to electron microscopy, resulting in hundreds of images that were aligned via Xara Designer Pro 6 and ImageJ software. Appropriate neural processes were selected from each condition and an active zone (denoted by a green line in figure 1) was defined. view.

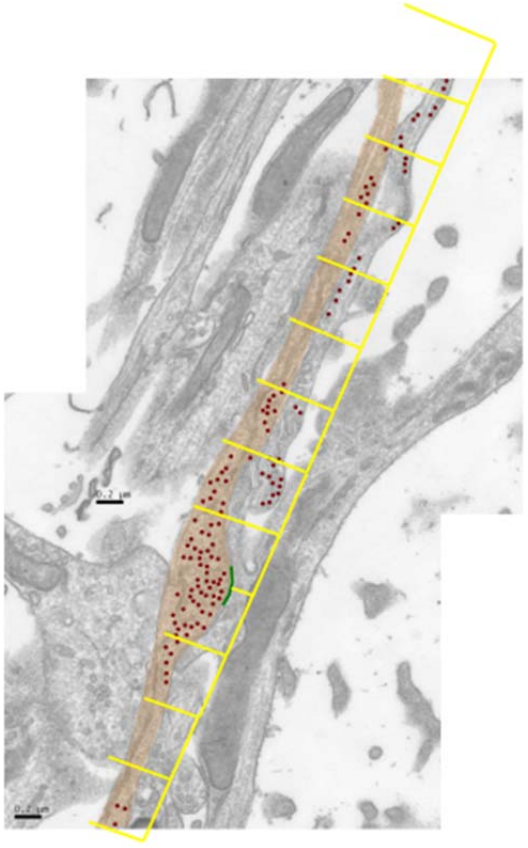
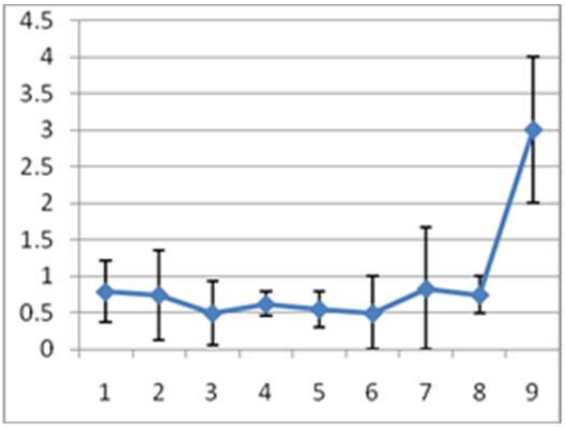


**Figure 1:** Example analysis of a TKO axon process. The yellow grid represents ½ micron bins; the green line indicates the active zone; only the vesicles in the orange-highlighted process were counted.

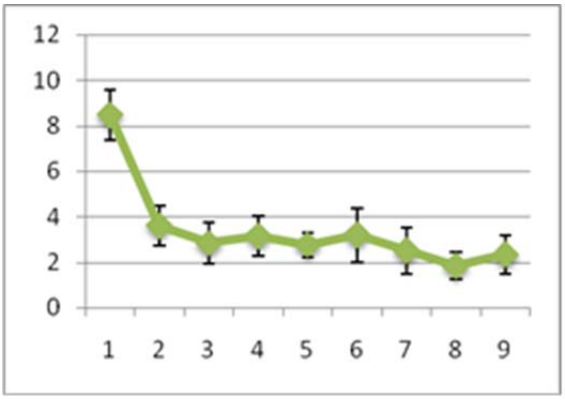
The samples were then blindly analyzed to determine the extent of vesicle scatter. Bins were standardized by the image scale at ½ µm. The number of bins varied for each process analyzed due to the unique variation of each cell. The analysis was unidirectional to avoid overlap.

After performing a student t-test, a significant difference between the numbers of vesicles in TKO mice compared to WT mice was found in the first five bins. The overall average number of vesicles in the axon in WT mice (4.53) was significantly less than that of TKO mice (22.09). In the WT condition, less than one vesicle on average was in the axon for the first eight bins away from the active zone (graph 1). However, there is large spike of vesicles in the axon occurring in distal bins, perhaps due to a synapse occurring later in the process but obscured from

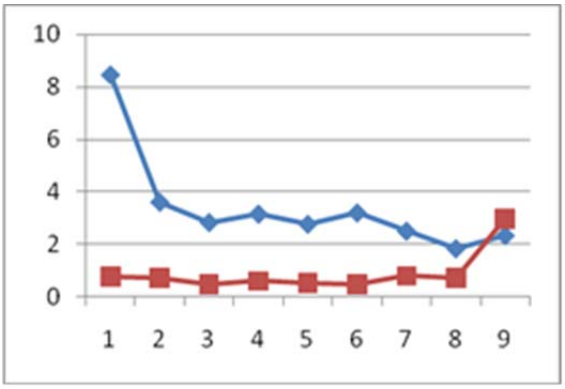
In the TKO condition, many vesicles are present in the bins closest to the defined active zone (graph 2). The number gradually decreases suggesting that fewer vesicles drift long distances within the axon. There was a significant difference in overall drift between the first five bins. The average number of vesicles in the active zone in WT mice was found to be 50.58. The TKO mice neurons had an average of 22.09 vesicles in the axon (not in the active zone). In TKO mice, 43.68% of vesicles in the active zone drifted in the absence of the synapsin proteins. It was shown that synapsin proteins do play a role in the cytomatrix and the tethering of vesicles near the active zone (graph 3). However, it is difficult to determine the extent of their role in transmission.




**Graph 1:** Number of synaptic vesicles in the axon/bin in WT condition; standard error on the mean is represented by error bars; vertical axis represents the number of vesicles; the horizontal axis represents the number of bins away from the active zone.




**Graph 2:** Number of synaptic vesicles in the axon/bin in TKO condition; standard error on the mean is represented by error bars; vertical axis represents the number of vesicles; the horizontal axis represents the number of bins away from the active zone.




**Graph 3:** Comparison of the WT and TKO conditions with $P < 0.05$ significance only in bins 1 through 5.

Although synapsins have been linked to recruiting vesicles and stimulating membrane fusion, their function is still not entirely clear. The absence of various synapsins in TKO mice does not cease all transmission; the effects can vary with synapse type (excitatory or inhibitory). It is suggested that one synapsin protein may compensate for the loss of the others [6]. Synapsin proteins may have other specialized roles (functions involving balance and spatial learning) but TKO mice maintain some normal vesicles and can function viably, though slightly impaired in the absence of it [8].

In some instances, the standardized grid had to be rotated to fit the processes, which may have influenced vesicle placement. Also, the 1 μm-long active zone might have been small; vesicles in the first bin may actually have been in the active zone. Vesicles present in the most

distal bins may have approached other active zones not observable (graph 2). The limited number of useable sections also restricted the analysis for this project.

Okadaic acid has been found to accentuate phosphorylation in certain cellular proteins, [7] which would cause vesicle drift. Thus, okadaic acid may naturally play a role in vesicle turnover. Brain-derived neurotrophic factor (BDNF) has been reported to increase synaptic transmission; mice with low levels showed far fewer vesicles "docked" at synapses and those lacking the BDNF receptor had fewer vesicles per synapse and overall less synapses [1]. Future studies might investigate these and other influences on tethering. Overall, synapsin proteins do play a substantial role in vesicle tethering and cytomatrix construction but their presence is not vital to the synapse integrity.

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