Agnieszka Tarasiewicz
tarasiewa@gmail.com

Education:

2020-2022 **Master of Science Cellular & Molecular Biomedical Sciences**
University of Maryland Baltimore, Baltimore Maryland
Visual Deficits in a Model of Gestational Hypothyroidism

2015-2017 **Bachelor of Science Biology**
Morgan State University, Baltimore, Maryland
(*magna cum laude*)

2008-2010 **Associate of Applied Science: Biotechnology**
Baltimore City Community College, Baltimore, Maryland
(Phi Theta Kappa Honors)

Professional Experience:

2021- Current **Research Associate - Gliknik INC.**
Baltimore, Maryland

- Works on protein purification on AVANT and ÄKTA pure systems: affinity, anion exchange, size exclusion chromatography
- Assess samples with high-performance liquid chromatography
- Contributes to drug development formulation and stability studies.
- Performs animal and human sample analysis for toxicology and pharmacokinetic studies

Spring 2021 **Laboratory Rotation in Dr. Banerjee**
University of Maryland School of Medicine, Baltimore, Maryland

- Maintain colon cancer cell lines HT29 and HCT15
- Work with parental colon stem cell lines
- Cell assays: ATP determination, Angiogenesis, MTT, Luciferase-based Reporter Assays.

2017-2020 **Laboratory technician Dr. Medina Laboratory**
University of Maryland School of Medicine, Baltimore, Maryland

- Conduct brain microsurgical experiments such as electrode implantation for visual evoked potentials, intracranial injections
- Managing mice breeding colonies consisting of multiple strains and associated record-keeping
- Sample/ tissue collection from mice and ferrets
- Trained in using different confocal microscopes
- Molecular biology skills such as DNA/ RNA extraction, genotyping, PCR
- Writing IACUC protocols and amendments

2016-2017 **Near-peer mentor**
A Student-Centered Entrepreneurship Development Program
Morgan State University, Baltimore, Maryland

- Led and helped to organize the work of the small group of students
- Helped students with writing proposals and assignments
- Helped lead mentor to prepare students for work in a laboratory

2010-2014 **Research Associate - Gliknik INC.**
Baltimore, Maryland

- Successfully contributed to an exclusive worldwide licensing agreement with Pfizer. Inc (press release Sept 30, 2013)
- Maintained different cell lines (CHO, 293F), performed transfection, and helped adapt the cell line for protein production.
- Conducted protein purification on AKTA and AVANT System: affinity purification, ion exchange (IEX) chromatography, size exclusion (GF) chromatography
- Life science laboratory skills such as Western Blots, ELISA, Octet system, endotoxin detection, and depyrogenation

Professional Associations:

- Research Society on Alcoholism

Language Spoken:

- English
- Polish

Posters and presentations:

- *Visual Deficits in a Model of Gestational Hypothyroidism*
  Agnieszka Tarasiewicz
  Presentation, Cellular Molecular Biomedical Science Program Retreat, UMB, May 6, 2022

- *Reduction of thyroid hormones during the third trimester equivalent of human gestation induces visual deficits in mice.*
  Agnieszka Tarasiewicz, Alexandre E Medina
  Poster, Department of Pediatrics Research Day SOM UMB, May 12, 2022

- *Impact of Novel Kinase Inhibitor on Metastatic Colon Cancer Cells*
  Agnieszka Tarasiewicz, Daniil Sokolov, Mikyung Kim, Aditi Banerjee
  Poster, Department of Pediatrics Research Day SOM UMB, May 13, 2021

- *Effects of Gestational Hypothyroidism on Activity-Dependent Neuronal Plasticity and Visual Acuity*
  A. Tarasiewicz, W.S. Rodrigues Junior, A. E; A.E. Medina
  Poster, Department of Pediatrics Research Day SOM UMB, May 9, 2019

- *Effects of Developmental Alcohol Exposure on AMPA Receptor Phosphorylation in the Cortex.*
  A. Tarasiewicz, N. Polimood, T.E. Krahe, A.E. Medina.
  Poster, Research Society on Alcoholism Annual Meeting, San Diego, Ca. June 2018

Publications:

- Sokolov D, Sharda N, Giri B, Hassan MS, Singh D, **Tarasiewicz A**, Lohr C, von Holzen U, Kristian T, Waddell J, Reiter RJ, Ahmed H, Banerjee A. Melatonin and andrographolide synergize to inhibit the colospheroid phenotype by targeting Wnt/beta-catenin signaling. J Pineal Res. 2022 May 26: e12808. doi: 10.1111/jpi.12808. Epub ahead of print. PMID: 35619550.

- Pulimood NS, Contreras M, Pruitt ME, **Tarasiewicz A**, Medina AE. Phosphorylation of CREB at Serine 142 and 143 Is Essential for Visual Cortex Plasticity. eNeuro. 2021 Oct 28;8(5): ENEURO.0217-21.2021. doi: 10.1523/ENEURO.0217-21.2021. PMID: 34607805; PMCID: PMC8555886.

Abstract

Visual Deficits in a Model of Gestational Hypothyroidism

Agnieszka Tarasiewicz, Master of Science, 2022

Thesis Directed by: Dr. Alexandre E Medina de Jesus, Associate Professor, Pediatric Department SOM UMB

Hypothyroidism prevalence among pregnant women is between 0.5 to 4% (Carney et al., 2014). Most studies look at the prolonged or severe reduction in thyroid hormone (TH) levels. We looked at how the reduced levels of TH during the third trimester of human gestation and the first weeks after birth in rodents impact the visual system. Additionally, we try to answer whether PTU treatment would affect neuronal plasticity in the visual cortex. We used the Visual Evoked Potential recordings to assess contrast sensitivity, spatial frequency acuity, and ocular dominance plasticity. In addition, we look into the expression of the photoreceptors in the retina. PTU exposure impacts the contrast sensitivity but not the spatial frequency acuity or ocular dominance plasticity. The expression level of the photoreceptor Opsin-M was also impacted. The reduced levels of the thyroid hormones during this crucial time have long-lasting consequences for the proper visual system processing.

Visual Deficits in a Model of Gestational Hypothyroidism

by
Agnieszka Tarasiewicz

Thesis submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
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List of Abbreviations

ATA	American Thyroid Association
CBI	contralateral bias index
CH	congenital hypothyroidism
cpd	cycle per degree
CREB	cAMP response element-binding protein
CS	contrast sensitivity
ETA	European Thyroid Association
GS	gestational
IACUC	Institutional Animal Care and Use Committee
IGN	Iodine Global Network
LGN	lateral geniculate nucleus
MD	monocular deprivation
ODP	ocular dominance plasticity
OH	overt hypothyroidism
P/N	postnatal
PTU	propylthiouracil
PVDF	polyvinylidene difluoride

SCH	subclinical hypothyroidism
SEM	standard error of mean
TH	thyroid hormone
TPO	thyroid peroxidase
TRH	thyrotropin-releasing hormone
TRs	thyroid hormone receptors
TSH	thyroid-stimulating hormone
VA	visual acuity
VEP	visual evoked potential
WB	Western Blot

Chapter 1

Introduction

The thyroid gland plays a significant role in body functioning at each step of an organism's life. This organ shaped like a butterfly is crucial in metabolism, growth, and development. Dysregulated thyroid (hypothyroidism as well as hyperthyroidism) are the most common endocrine disorders (Hypothyroidism (Underactive Thyroid) | NIDDK). One of the major causes of maternal hypothyroidism (followed by the autoimmune disease against the thyroid gland such as Hashimoto's thyroiditis) is access to the iodine, which is crucial in the thyroid hormone (TH) regulation. Hypothyroidism prevalence is 1 to 2%, and it is more common in women than men (Vanderpump, 2011). The incidence rate varies depending on the world region and diet of the expecting mother. According to the Iodine Global Network (IGN), in 2020, 21 countries out of 152 had insufficient iodine levels in their diets. The US was classified as a country with sufficient levels of iodine; however, it was reported that mild iodine deficiency appears among 55% of pregnant women (IGN - Annual Report 2020. pdf). During pregnancy, the TH requirements increase by 50% due to increased renal elimination of iodine and fetus demands. The fetus's thyroid gland is formed early during gestation, but it is inactive until the 12th-14th week of pregnancy, and even then, the fetus depends mainly on the maternal source until the 38th week. During this time, maternal TH is the only source of hormones required for the proper fetus development (Tingi et al., 2016). Pregnant women must increase iodine consumption accordingly to the increased demand for the TH. Daily iodine intake is 150 µg for adults, 220 µg for pregnant women, and 290 µg during lactation (Kuriti *et al.*, 2014). The

deficiency in iodine during this time may lead to hypothyroidism which can have a tremendous impact on the fetus.

T4 (thyroxine, also known as thyroxine) and T3 (triiodothyronine) are two major thyroid hormones. T_3 is an active form due to the higher binding affinity to the thyroid hormone receptors (TRs) (Schroeder and Privalsky, 2014). Interestingly, the majority (around 80%) of circulating T_3 is derived from T_4 (94% of secreted hormones by the thyroid gland) in the deiodination process (Kyriacou *et al.*, 2015). The hypothalamic-pituitary axis is the primary regulatory mechanism of the thyroid hormone's homeostasis. The increased secretion of the hypothalamic thyrotropin-releasing hormone (TRH) causes the release of the thyroid-stimulating hormone (TSH) located on the anterior pituitary gland. The increased levels of the TSH cause the synthesis and secretion of the T_4 and T_3 hormones by the thyroid gland. T_3 and T_4 regulate TRH and TSH in a negative feedback loop (Chiamolera and Wondisford, 2009). There are two mechanisms of TH action that impact body functions. Binding T3 to the nuclear TRs promotes up or downregulation of targeted genes on the genomic level. It can also act with other molecules and influence gene transcription by acting as a coactivator. The second mechanism is on the nongenomic level. TH interacts with the receptors located on the cell membrane and activates different pathways; for example, it can bind with serine-threonine kinase (MAP/ER), PI3K, and calcium (Senese *et al.*, 2014).

The thyroid hormone plays a crucial role in brain development and maturation states. The impact of the low levels of thyroid hormones on the developing brain is well documented. For instance, TH is involved in the regulation of myelination and neuronal

cell differentiation (Chen *et al.*, 2012; Thompson and Cline, 2016; Prezioso *et al.*, 2018a) Accordingly, a lack of the appropriate levels of TH can alter brain development.

Hypothyroidism is a condition characterized by underproductive thyroid. There are two subcategories of hypothyroidism present during the gestation period.

Subclinical hypothyroidism (SCH) is characterized by standard T_3 and T_4 and higher than the upper limit of the pregnancy-related levels of TSH in the serum (Hypothyroidism | American Thyroid Association). Symptoms of the SCH can easily be mistaken for typical pregnancy symptoms such as fatigue, constipation, and weight gain, resulting in undiagnosed issues with the thyroid hormone imbalance. The prevalence of this type of hypothyroidism in the general population is 3 to 12% (Kim and Park, 2014), and the prevalence among pregnant women is between 2 to 4% (Batistuzzo and Ribeiro, 2020). This type of hypothyroidism during pregnancy is associated with increased occurrence of hypertension, pre-eclampsia, pregnancy loss in the early stages, gestational diabetes, and possible cognitive impairment of the infants (inconsistent evidence) (Deshauer and Wyne, 2017). Elevated levels of the TSH are considered between 4 and 10 mU/L. So far, there is no clear guidance on approaching and treating SCH. The European Thyroid Association (ETA) recommends treatment with Levothyroxine which is a synthetic version of thyroxine, for each patient diagnosed with the SCH regardless of their level of thyroid peroxidase (TPO) antibody (Lazarus *et al.*, 2014). At this same time, American Thyroid Association (ATA) recommends the treatment only when the TPO antibody level is elevated (Thyroid Health - Management of Hypothyroidism During Pregnancy: When and how to treat?, 2020). In his study, Lazarus et al. concluded that

treatment of maternal hypothyroidism did not result in significant differences in cognitive development in children tested at three years of age (Lazarus *et al.*, 2012).

Overt hypothyroidism (OH; also called clinical hypothyroidism) is characterized by higher than 10 mU/L TSH levels and lower T_4 levels. The OH affects 0.3 to 0.5% of pregnant women (Batistuzzo and Ribeiro, 2020). Often the OH is the consequence of a preexisting condition such as autoimmune thyroiditis (Hashimoto's) or treatment of hypothyroidism or thyroid cancer with radioactive iodine (Tingi *et al.*, 2016). The OH is associated with (not limited to) preterm birth, low birth weight, anemia, fetal death, and cognitive developmental impacts (Prezioso *et al.*, 2018b). While in SCH, there is no clear statement on how to approach the treatment in the case of the patients diagnosed with the OH, treatment with levothyroxine is required to avoid cognitive impairments in the developing fetus because the T4 but not T_3 crosses the placenta. Low maternal thyroxine levels will result in insufficient hormone levels for the developing fetus (Ahmed, 2015).

Congenital hypothyroidism (CH) is classified as a deficiency of TH at birth. This condition is not a maternal hypothyroid condition per se, although it can result from maternal iodine deficiency or thyroid condition treatment. It is one of the most common preventable causes of cognitive and intellectual disabilities. CH affects 1 in 2000 newborns, and untreated conditions can have a devastating effect on neurocognitive development (Wassner, 2018). The causes underlying the CH can be transient (temporary) or permanent. The transient form of CH can result from exposure to maternal antithyroid medications, antibodies, and iodine diet deficiency (Kanike *et al.*, 2017). These effects do not last long, and usually, they are cleared in a matter of weeks (Rastogi and LaFranchi,

2010). The permanent causes of the CH can be a result of thyroid dysgenesis, a mutation in the TSH receptor gene, and mutations in the genes facilitating thyroid hormone metabolism (Peters *et al.*, 2018). This type of CH requires long-lasting treatment (Bowden and Goldis, 2022).

Visual deficits after gestational hypothyroidism

Visual acuity (VA) and contrast sensitivity (CS) are two aspects of visual function essential in our daily activities. VA is responsible for pattern recognition (applicable during activities such as reading). At the same time, the CS helps recognize the object against its background (used for mobility, and driving) (Xiong *et al.*, 2020). In his study, Mirabella shows that children with affected TH levels during the development (born to hypothyroid mothers, with CH, or born preterm) demonstrate contrast sensitivity and visual acuity changes. Depending on the cause of the decreased levels of TH, the severity of the changes varies. Children born to hypothyroid mothers and children with CH demonstrated the most significant deficit in contrast sensitivity at low spatial frequencies (Mirabella *et al.*, 2005a). Preterm-born babies show normal contrast sensitivity but weaker visual acuity. In typical instances, at the age of 3 months, children should reach the top of the contrast sensitivity. There was no recovery in the reduced contrast sensitivity at 3-6 months in the group of children with CH or born to hypothyroid mothers. All this suggests that proper TH levels are necessary for the first and third trimesters. Additionally, the decreased contrast sensitivity persists in cases with severe deficits, while others normalize with time (Mirabella *et al.*, 2005b).

In addition to VA and CS, assessing the visocognitive process such as visuoperceptual, visuospatial, and visuoconstructive abilities may help screen children with impacted TH levels. The visuoperceptual skills are involved in object recognition (analysis and identification of stimuli), visuospatial abilities allow observing spatial location (orientation, direction, distance), and visuoconstructive capabilities are needed when necessary to pieces together to form a whole object, picture, image. The regions responsible for mediating all of these processes are located in different brain regions (Strobl *et al.*, 2017). Looking closely at what and how is impacted will suggest which abilities are affected by diminished levels of TH during the development. The study conducted by Mirabella and colleagues showed that children with CH may experience particular issues in visocognition. For example, in the study, children had problems judging line orientation but not mental rotation. The construction of 3D block towers was problematic, but not 2D block design. Visuoconstructive abilities were impacted in the design matching and localizing of smaller parts within the bigger picture, but visual and feature discrimination was not affected. Problems with organizing small pieces into whole may explain why depending on the presentation view, the children sometimes have problems with face perception (¾ profile view recognition was lower, but in front view recognition groups did not vary). Additionally, they show no differences between CH children and the control group in visual or auditory tests. That suggests that memory and attention issues do not depend on visual dysfunctions (Simic *et al.*, 2013).

These studies suggest that time and the severity of the diminished TH during development significantly affect children's everyday existence and task performance.

Role of thyroid hormones on the development of the visual system

The visual system is composed of the retina, optic nerve, optic chiasm, optic tracts, lateral geniculate nucleus (LGN), and the visual cortex (Esfahany *et al.*, 2018). The retina is composed of three nerve layers. The outer nuclear layer includes the photoreceptors. The inner layer is formed of bipolar, horizontal, and amacrine cells. The last layer consists of the ganglion cells. They are the output neurons that project received an impulse to other parts of the brain (Mahabadi and Al Khalili, 2022) (Figure 1).

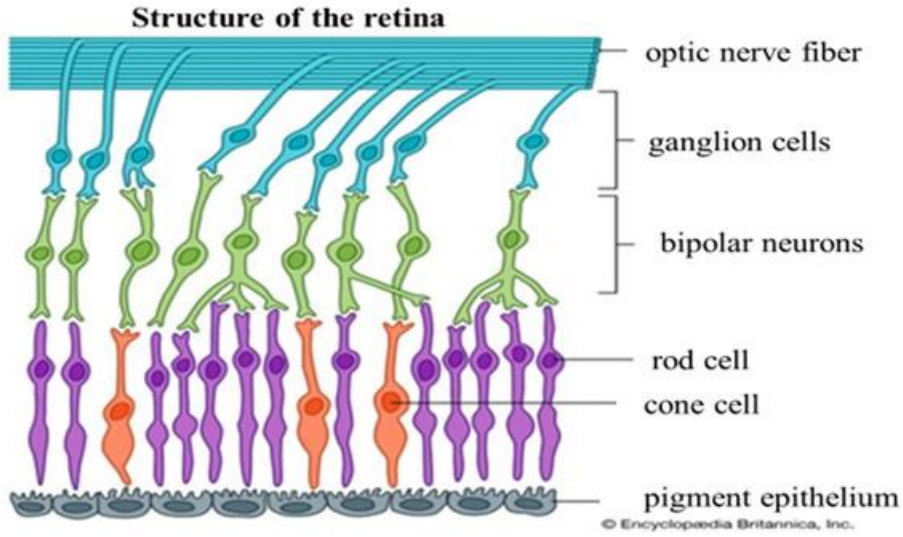

***Figure 1*: Schematic of the retina organization.**
Three layers of the retina. The photoreceptors rods and cones are located in the outermost first layer of the retina (human eye - The retina | Britannica).

The retina has two photoreceptors responsible for the image formation cones and rods. Both cell types include visual pigments, determining what light excites the cell. Rods express rhodopsin and are responsible for mediating vision in the dark, low light environment. The cons express opsins and are accountable for the light, color, and vision acuity (Xu *et al.*, 2022). There are three kinds of cone pigments. Opsins S (short-wavelength; “blue”), M (middle-wavelength; green) and L (Long-wave; “red”). Rodents

only have two types of pigment (S and M) (Fu and Yau, 2007). The spatial patterns of the cone expression vary between species. In the mice, the S-opsin is primarily expressed in the ventral retina shortly before birth, while M-opsin is expressed in the dorsal retina week after birth (Saponaro *et al.*, 2020).

The TH is not only crucial to the formation of the retina but also to other parts of the visual system. Studies suggest that TH levels, in cooperation with other hormones, regulate the expression of almost 200 genes which play an essential role in mood and behavior regulation (Morte *et al.*, 2018). On top, most of these genes are involved in forming psychological disorders such as schizophrenia (Bernal, 2000a). TH acts on the actin polymerization, and if low TH levels disrupt the process, suddenly, the microfilament organization and neuronal migration are affected. The deficiency of the TH prevents the laminin from binding to astrocytes (Leonard, 2008). TH also affects distribution connectivity and the number of GABAergic and parvalbumin neurons. The maturation of neurons and changes in dendritic spine number is also observed (Bernal, 2000b).

TH is essential to the proper brain and visual system development and function. Difficulties arise when studying a system as complicated as one that regulates the TH. Most studies are based on the design with severe alterations in the TH levels. To alter the TH levels, propylthiouracil (PTU) is often used. It is an anti-thyroid drug used to treat hyperthyroidism and Graves' disease. It inhibits the thyroid peroxidase, which is essential in the biosynthesis of TH (Le *et al.*, 2015). As a result, the administration of PTU prevents the production of the new TH (Amisha and Rehman, 2022). In their study, Boyes and colleagues' approach (Boyes *et al.*, 2018) was to mimic the impact of environmental contaminants, which often cause light to moderate dysregulation of the TH. They exposed

pregnant Long Evans rats to the different concentrations (0 to 3ppm) of PTU from gestational day (GS) 6 to postnatal day (PN) 21. Interestingly, they reported significant alterations in the visual system. In the retina, animals exposed to the highest concentration of PTU had a lower amplitude of electroretinograms with green light, indicating the reduced function of M-cones. The amplitudes for the S-cones did not differ. This suggests that M-cones but not S-cones are vulnerable to even slight changes in the TH during the development. The rods and M-cone photoreceptors are both derived from S-cone. In opposite to the rods, the M-cone formation is TH dependent. Due to the imbalance in TH, the differentiation of S-cones to M-cones was altered. That resulted in the increased function of rod photoreceptors. Changes in the retina were also observed in the changes in the cortical function. The amplitude and slope of the contrast visual evoked potentials (VEPs) in PTU exposed animals were lower dose-response than in the control animals. This indicates that an imbalance of TH during the development impacts the ability of the visual system to adapt to changing levels of stimulus. This study shows that moderate disruption of TH during development affects the optical system on many levels (Boyes *et al.*, 2018).

One limitation of the Boyes study was the prolonged exposure to PTU (gestational day 6 to postnatal day 21). This method would have affected many neurodevelopmental events such as neuronal differentiation and migration (Bernal, 2000a) that occur during the first and second trimesters of human gestation and prenatally in the rodent.

Here we want to focus our investigation on the effects of PTU during the time of cortical synaptogenesis and photoreceptors differentiation, which occur during the third trimester of human gestation and the first weeks after birth in rodents (Arango-Gonzalez

et al. 2010; Ebrahimi et al. 2014). So, our first goal is to evaluate whether restricting the PTU treatment to the first two postnatal weeks in mice would affect spatial frequency acuity, contrast sensitivity, and photoreceptor expression.

Another question remains unanswered is whether a PTU treatment would affect neuronal plasticity in the visual cortex. Neuronal plasticity can be described as the ability of the brain to adjust its activity in response to changes in the environment (Mateos-Aparicio and Rodríguez-Moreno, 2019). It can involve structural and functional adaptations that will help adapt to the new surroundings or new stimuli. This adaptation transformation change is essential in brain development, homeostasis, memory formation, learning, and recovery after brain injuries (Ganguly and Poo, 2013). In the visual cortex, the paradigm of ocular dominance plasticity has been extensively used to investigate the molecular mechanisms of neuronal plasticity in general (Hofer *et al.*, 2006; Lantz *et al.*, 2012; Sato and Stryker, 2008)

Ocular dominance plasticity

The axons of ganglion cells form the optic nerve, which projects to the lateral geniculate nucleus of the thalamus (LGN). Afferents from the right and left eye are segregated and connect to different LGN layers (Kerschensteiner and Guido, 2017). LGN afferents in turn project to the layer IV of the primary visual cortex (V1) in a segregated fashion as well. This segregation gives rise to columns in which each neuron has a specific ocular dominance (Fig 2). For instance, visual cortex neurons in the center of the blue and red columns in Figure 1, would respond only to stimulation of contra and ipsilateral,

respectively. This dominance decreases as you go to the border between columns where neurons are equally responsive to both eyes.

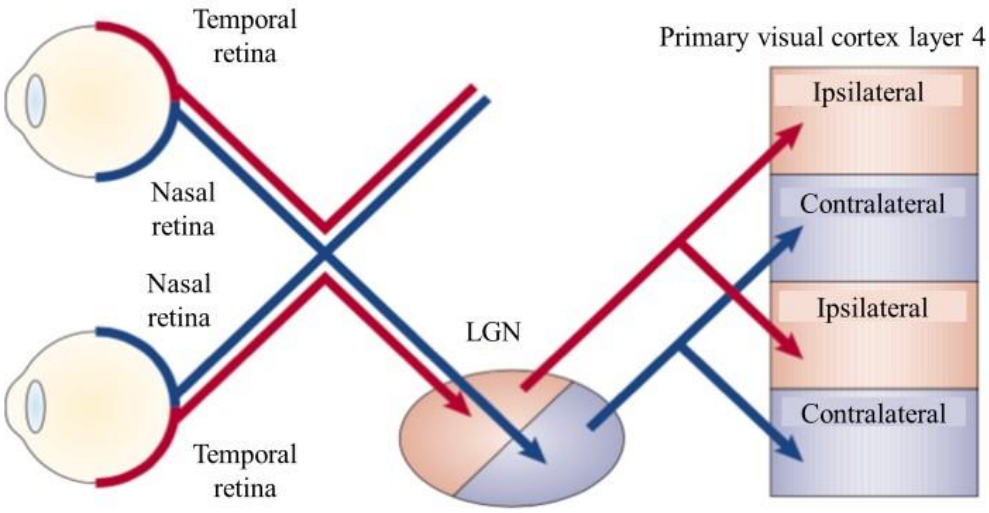

Figure 2: Anatomy of ocular dominance columns

(Katz and Crowley, 2002)

During a critical period of development, short deprivation of one eye leads to a reduction in the responses of visual cortical neurons to this eye and a matching increase in responses to the open eye (Katz and Crowley, 2002). Changes that accommodate the visual system to the new environment or visual stimuli strength can occur on a functional and structural level (Hubel and Wiesel, 1962). The critical period is defined as the time when the brain has the highest plasticity and adaptative capabilities. The taming and the duration of the critical period vary between species; in mice, the critical period is attributed to the time between the third and fifth postnatal week (Wong-Riley, 2021). Recently, evidence shows that the adult brain has some neuronal flexibility during adulthood (residual plastic potential) (Lunghi *et al.*, 2013). Additionally, recent studies suggest that CREB plays a

vital role in activating neuronal plasticity (Pham *et al.*, 2004; Pulimood *et al.*, 2017). The imbalance of TH during the development of the brain interferes with the activation of the CREB pathway (Zhang *et al.*, 2015). Therefore, it is possible that animals with reduced TH have deficits in ocular dominance plasticity.

There are many animal models used to study ocular dominance plasticity (ODP). Cats and monkeys were the first animal models used in the experiments (Mitchell and Duffy, 2014). In recent years, researchers have developed new tools to utilize the mouse as an animal model. Genetic and optogenetic open new possibilities to study mechanisms and relations between different systems. That is why the mouse has become widely used as a model for ODP. Studies suggest that a similar hierarchical organization characterizes the mouse visual cortex as primates. The significant difference is that mice brain does not undergo a gyrification process.

The organization of the mouse visual system starts at the retina. The photoreceptors process the stimuli, pushing the signal via the retina layers. When the impulse arrives at the retinal ganglion cells, it is later projected to different brain regions. The ganglion cell axons are bundled together and cross to the contralateral hemisphere at the optic chiasm (Figure 2) (Resulaj, 2021). Primary visual area 1 (V1) can be divided into two zones. The binocular zone receives the input from both the contra and ipsilateral eye, while the monocular zone receives information mainly from the contralateral eye (Scholl *et al.*, 2013). Different than in higher mammals, rodents do not have ocular dominance columns. Instead, neurons from different ocular dominance profiles are represented in the binocular zone in a “salt and pepper” fashion. There are a few techniques used to test the ODP in animal models. The widely used method is called visually evoked potentials (VEPs). This

technique is commonly used in clinical settings in diagnosing visual deficiencies in humans (Sharma *et al.*, 2015). This technique allows testing OPD in awake animals (a procedure described in detail in the methodology section).

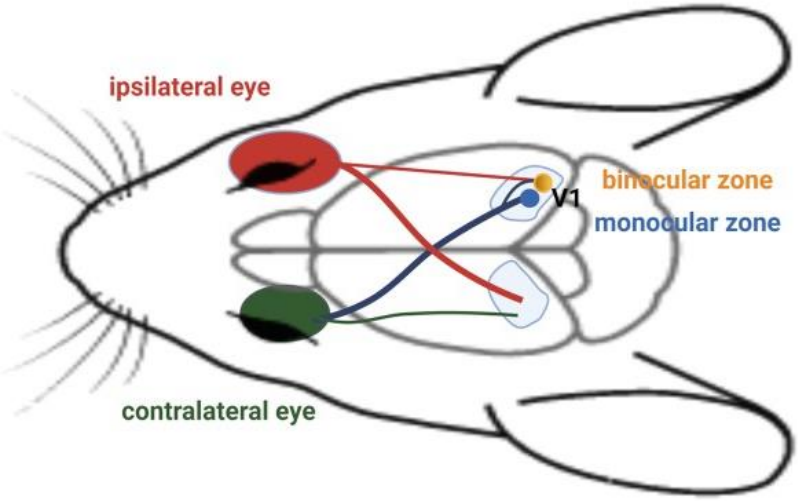

Figure 3: Schematic of the mouse visual system
The mouse visual system has two regions: binocular and monocular. The binocular (depicted in yellow) zone receives inputs from the contralateral (green) and ipsilateral eye (red), while the monocular region (shown in blue) receives inputs only from the contralateral eye. (Created with BioRender.com and SciDraw doi.org/10.5281/zenodo.3925903)

Chapter 2

Methods

Neonatal Hypothyroidism Animal Model: All procedures described were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, Baltimore. Pregnant C57/BL6 mice were singularly housed. The date of the birth of the pups was considered as P/N 0. Hypothyroidism was induced in the offspring by exposing the lactating dams to the thyroid hormone synthesis inhibitor propylthiouracil (PTU), this method has been extensively used (Ferreira *et al.*, 2007), (Kemkem *et al.*, 2020), (Khairinisa *et al.*, 2018). No later than P/N 1 dams from the experimental group received water containing 500ppm of PTU with cherry Kool-Aid (to mask the bitter taste of the drug). The water with PTU was available ad libitium till P/N 14. The dams in the control groups received water with Kool-Aid till P/N 14 starting no later than P/N1 . At P/N 14 PTU, the control group had experimental water pouches changed to regular water. To ensure that dams did consume the drug or Kool-Aid mixture, the water pouches were weighted at least twice a week.

Electrode implantation: Electrode implantation was done as previously described (Lantz *et al.*, 2014). After weaning (P/N 21) between P/N 22 and P/N 25, animals underwent electrode implantation surgery. An anesthetized animal (isoflurane maintained at 1-2%) was placed in the stereotaxic setup with the heating pad. As a pre-emptive analgesia animal received 0.1mg/kg of buprenorphine and 5 mg/kg of Carprofen. The animal's head was shaved and cleaned with betadine. An incision in the scalp was made, and the skin was

pulled back enough to expose the skull. Four burr holes were drilled. Two were created at 1.5 mm caudal from bregma and 2.0 mm lateral from the midline for reference electrodes (tungsten microelectrodes FHC, tip impedance 0.3-0,5 MΩ). The two other burr holes for recording electrodes were drilled at 3mm lateral midline and 0.5 of lambda at 0.45-0.5mm depth. All electrodes were secured with cyanoacrylate glue. At the anterior part of the skull, the nail was glued and later was used as an immobilizing post (Figure 3A). After surgery, the animal was monitored following the IACUC recommendations and returned to the colony after recovery.

Visual Evoked Potential Recordings: Animals were habituated to the experimental setup a day before the recording for 30 min. VEPs were recorded using XCell-3 amplifiers (low cut-off at 0.1Hz and high cut-off of 100Hz), 50/60 Noise Eliminator (Quest Scientific, Vancouver, Canada), 1401 digitizer, and Spike 2 software (CED, Cambridge, England). The animal was placed in the setup 18 cm from the monitor, and a visual stimulus was presented to each eye separately (Figure 3B). During the visual stimuli presentation, the eye patch obstructed the other (nonrecording eye). The stimulus design was sine wave reversing gratings at 0.05 Hz with 100% contrast and was controlled by the MATLAB program (MathWorks, Natick, MA). The presentation before and after monocular deprivation were different (45°and 135°) (Figure 3C) to avoid stimulus-selective response potentiation (Cooke and Bear, 2010). The result was an average of at least 100 presentations amplitudes from peak to through. The calculated contralateral bias index (CBI, contralateral/ipsilateral response) is presented as average CBI with the standard error of the mean (SEM), analyzed using a two-tailed paired t-test.

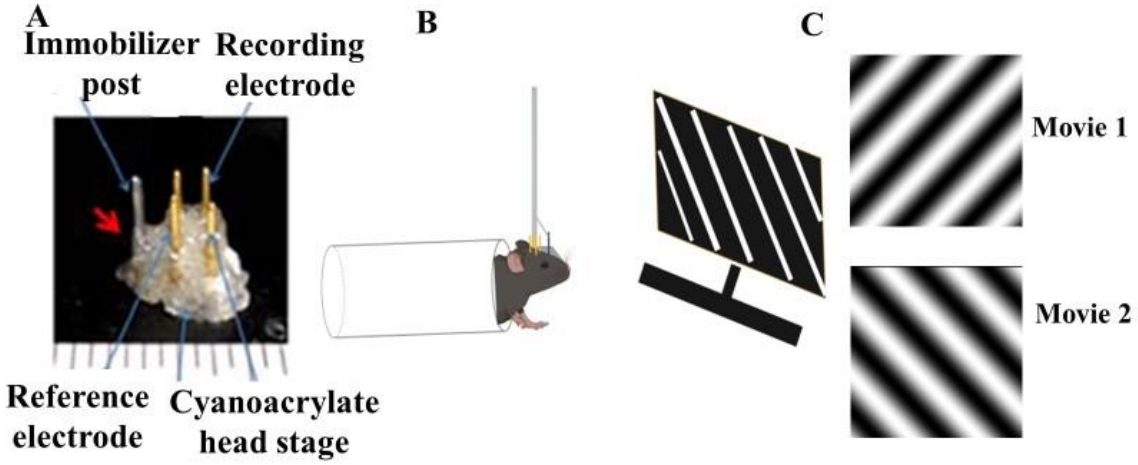

Figure 4: Head stage and VEP setup.
A) Head stage for VEPs recording. Recording electrodes were placed 3mm lateral of midline and 0.5 of lambda at 0.45-0.5mm depth. The reference electrodes were placed at 1.5 mm caudal from bregma and 2.0 mm lateral from the midline. The immobilizer post was placed at the anterior part of the skull. The setup was secured with cyanoacrylate glue. B) Set up for the VEPs recording. Awake animal in the tube in front of the computer monitor. The animal is restricted only by the immobilizing post (Created with BioRender.com). C) An example of the visual stimulus presented to the animal before and after monocular deprivation.

Monocular Deprivation (MD): After the reference (basal) VEPs recordings animal was anesthetized with isoflurane. The ophthalmic proparacaine was applied to ensure the animal would not feel any pain. The fur and a small portion of lower and upper eyelids were trimmed. The eyelids were sutured together (7.0 prolane Ethicon suture) and sealed with a thin layer of tissue glue (Abbott Laboratories, Chicago). After the surgery, animals received post-surgical care following IACUC guidelines. Animals were monocularly deprived for five days, and any animal with an eye opened during this time was not used for further study. After this time, sutures were removed under light anesthesia, and post-MD VEPs were recorded. During the post-MD testing, the ipsilateral eye was recorded first, and then after removing the suture, the contralateral eye was recorded.

Contrast Sensitivity Recordings: Animals underwent this same PTU exposure and electrode surgery as animals for ODP testing. The stimuli for contrast sensitivity were randomly presented to the animals and consisted of six full-field reversing sine-wave gratings of 0.5 cpd and contrast from 0% to 100%. The amplitude was calculated from at least 100 presentations and was measured from the peak-to-through. Results are presented as normalized VEP amplitude with the SEM.

Spatial Frequency Acuity Recordings: Animals went under this same PTU exposure and electrode surgery as animals for ODP testing. The stimuli for spatial frequency acuity were randomly presented to the animals. They consisted of six full-field reversing sine-wave gratings from 0.02 to 0.5 cpd and an equal luminance gray screen. The amplitude was calculated from at least 100 presentations and was measured from peak to through. Results are presented as normalized VEP amplitude with the SEM.

Assessments of T4 levels: We used a subsample of animals to analyze thyroid hormone levels in the blood. Animals were euthanized between P30 to P45, and blood collected by cardiac puncture. T4 levels were measured through an in vitro diagnostic test for the quantitative measurement of FT4 in serum plasma on the Dimension Vista® System. The PTU group was compared to a control group composed by naive and CT animals. Blood was collected into BD vacutainer serum tubes and left at room temperature for 30 minutes. Then, samples were centrifuged for 10 min at 2000g at 4°C. Serum (supernatant) was transferred into new tubes and used in the assay. The system used to test T4 levels is based on the luminescent oxygen channeling immunoassay (LOCI).

Western Blot (WB)**:** Retinas from control and PTU animals were collected and placed and homogenized in RIPA lysis buffer (Millipore, 20-188) with protease (Roche cOmplete Mini 11836153001) and phosphatase (Roche, PhosSTOP 04906837001) inhibitors. The Bradford assay determined protein concentration. Samples were run on 4-15% (gel specifications) and transferred to polyvinylidene difluoride (PVDF) membrane using Bio-Rad Trans-blot system. Membranes were blocked for two hours at room temperature in 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad #1706404XTU) in 1X Tris Buffered Saline with 0.1% Tween. Primary antibodies Anti-S-Opsin (Millipore, ABN1660) and Opsin-1 (Novus Biologicals, NB110-74730, detects Opsin-M) were diluted in the blocking solution at 1:1000 and incubated at 4°C overnight. Then, membranes were washed and incubated for two hours at RT in horseradish peroxidase-conjugated anti-rabbit IgG (Cell signaling Technology, #7074) at 1:3000 dilution. Samples were visualized using the ECL (Bio-Rad Clarity Western ECL Substrate, #1705060) on the G Box imaging system (Syngene). Densitometry and OD values were analyzed using the ImageJ (RRID: SCR_003070). All values were normalized to Cyclophilin B (Thermo Fisher Scientific, #PA1-027A), used as a loading control.

Data analysis: Ocular dominance plasticity is assessed by comparing the contralateral bias index (CBI) of a given animal before and after monocular deprivation. Contralateral Bias Index is a ratio of the contralateral VEP amplitude over ipsilateral VEP amplitude. The CBIs were calculated for each animal using a directional paired t-test. In contrast, sensitivity VEP amplitudes were analyzed using Mann-Whitney U and two-sample assuming unequal variance t-test. Western blots were analyzed using a two-tail t-test.

Statistical tests were performed on IBM SPSS (version 28), marked by an asterisk (*) significance at $p \leq 0.05$.

Chapter 3

Results

To confirm the efficacy of the PTU treatment in reducing THs we assessed T4 levels in a subset of animals. As expected, the PTU treatment resulted in a decrease in T4 levels still detected at P40, time of the blood collection. Controls showed an average of 6.8±0.2 (n=11) whereas PTU treated animals only 4.7±0.4 (n=5). This different was significant (t=5.4; p<0.0001) (Figure5).

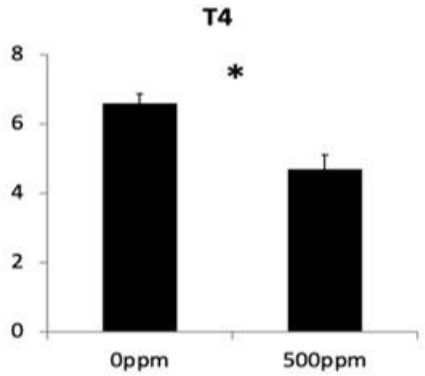

Figure 5: T4 levels (in µg/dL) after the PTU exposure.
Blood was collected for the assay between P30 to P40. Immunoassay was run and analyzed on the Dimension Vista® System.

Spatial frequency and contrast sensitivity

Increasing either stimulus spatial frequency (Figure. 6) or contrast (Figure. 7) led to the expected reduction of normalized visual evoked potentials. An ANOVA with repetitions showed a significant effect of both spatial frequency (f=32.2; p<0.001) and contrast sensitivity (f=62.7; p < 0.001) in all groups. However, there was no between groups difference.

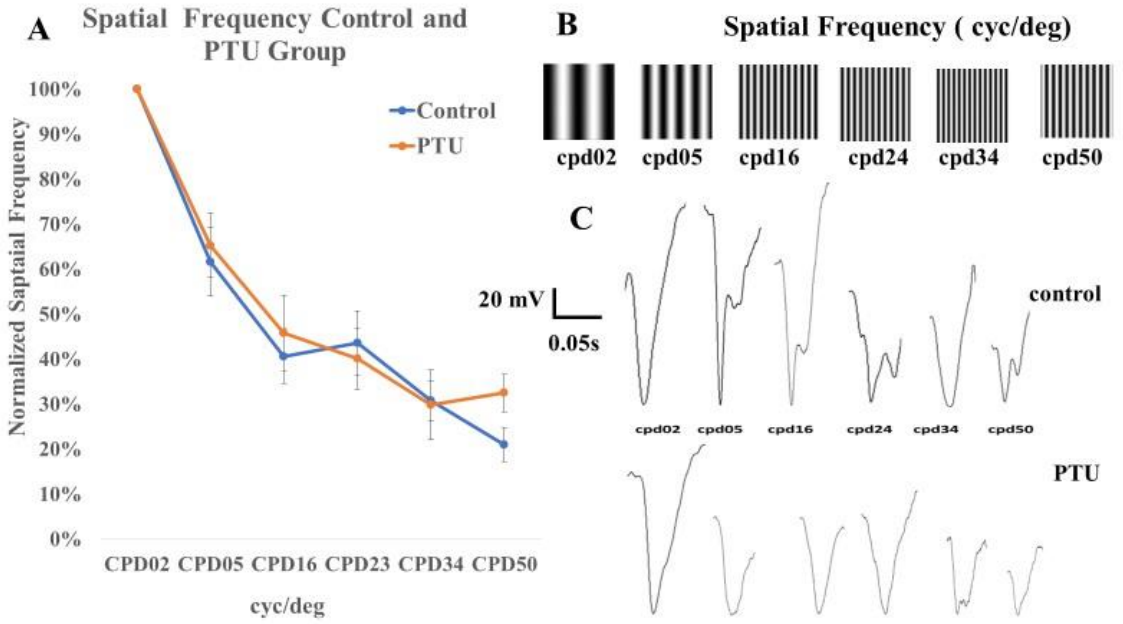

Figure 6: Spatial frequency recordings.

A) Normalized spatial frequency control and PTU group; B) visual stimuli progression from the low to high spatial frequency (left to the right) randomly presented to the animals; C) representative VEPs for control and PTU animals (Created with BioRender.com)

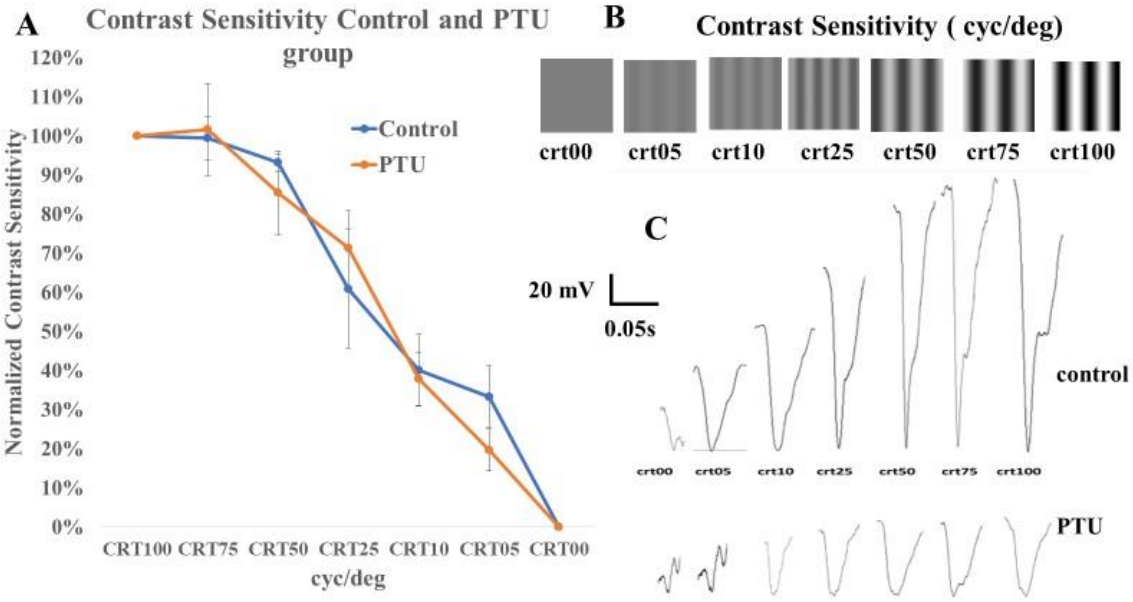

Figure 7: Contrast sensitivity recordings.

A) Normalized spatial frequency control and PTU group; B) visual stimuli progression from the low to high spatial frequency (left to the right) randomly presented to the animals; C) representative VEPs for control and PTU animals (Created with BioRender.com).

While the spatial frequency acuity and contrast sensitivity functions were similar between groups, analysis of the amplitude of the VEPs was reduced in the PTU group in both instances. A multivariate ANOVA shown a reduced amplitude in the PTU group at 5 cycles per degree (p=0.036). Regarding contrast sensitivity a reduced amplitude was observed at different contrasts: 100% (p=0.03); 75% (p=0.015); 50% (p=0.011); 15% (p=0.028); 5% (p=0.004).

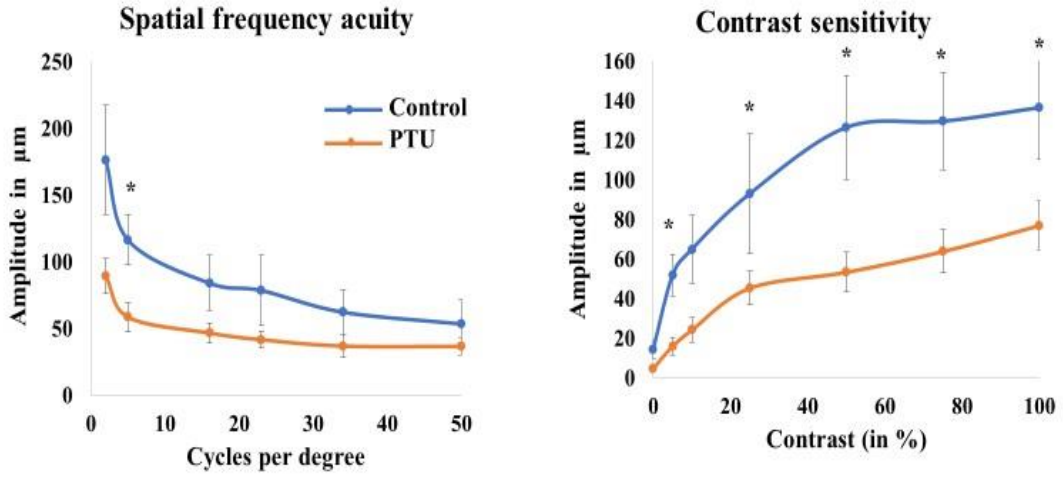

Figure 8: Amplitude of spatial frequency and contrast sensitivity in control and PTU group. *A) Spatial frequency analysis shows a similar trend between groups; the PTU exposed group amplitudes were much lower than control animals. B) Contrast sensitivity shows a similar trend in both groups with higher amplitudes in control groups.*

As mentioned earlier, the decreased TH levels may impact the retina composition. We look at the photoreceptors (cones and rods) to see how they are affected in our animal model. The levels of the rhodopsin pigment in both groups (in monomer and dimer form) were not significantly different between both groups (p=0.45 for monomer and p=0.65 for dimer). The changes in the levels of the Opsin-S between the groups were not significant, with p= 0.44. However, Opsin-1 (detects Opsin-M) was significantly decreased in the PTU

group with p=0.002 (Figure 9B). Our Western Blot data confirm that TH is crucial in expressing the M-cones but not S-cones or rhodopsin.

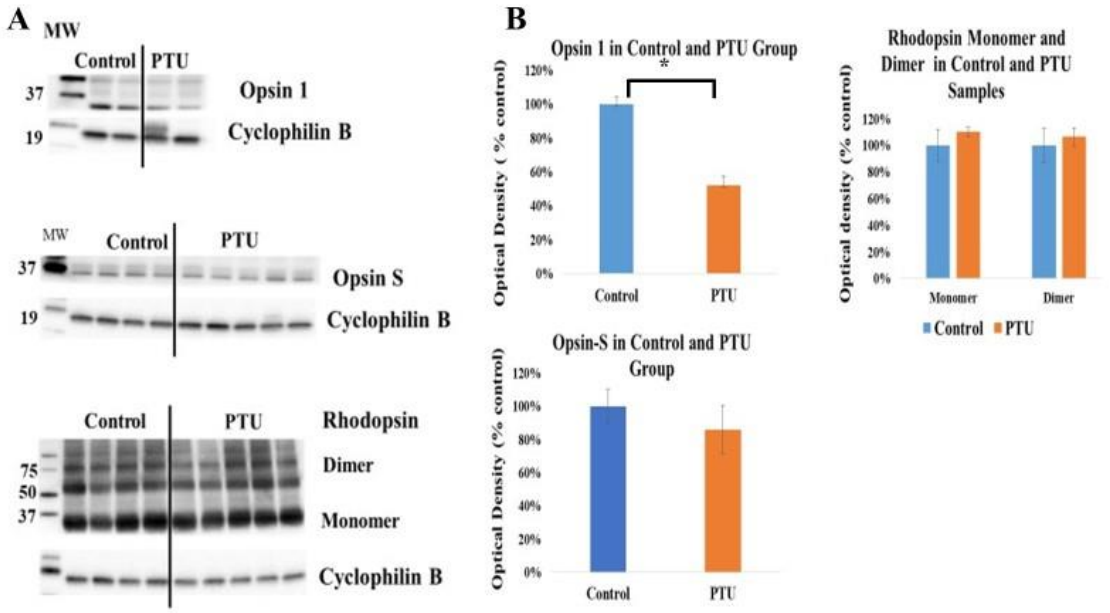

Figure 9: Expression of Opsin 1, Opsin S, and Rhodopsin in the retina of control and PTU animals.

*A) Representative Western blot of proteins. B) Densitometry data for the Opsin 1, Opsin S, and Rhodopsin in the control and PTU group. There is no significant difference in rhodopsin and Opsin S levels between both groups. Opsin-1 was significantly lower in the PTU-exposed group than in the control group. Data averaged and normalized to the Cyclophilin B. * significant with $p<0.05$.*

Ocular Dominance Plasticity

Mice pups were exposed to the PTU via mothers' milk from birth until P14. After the electrode implantation surgery and initial VEPs recordings, the animals underwent 5 days of monocular deprivation. After five days of MD the deprived eye was opened, and post-MD VEPs were recorded. The amplitude of VEP was measured from the peak to through. The shape of the VEPs between the two groups did not vary significantly. The amplitude of the VEPs was higher in the PTU group; however, the shift from the dominant eye to the ipsilateral eye was more defined in the control group (Figure 10A 10B).

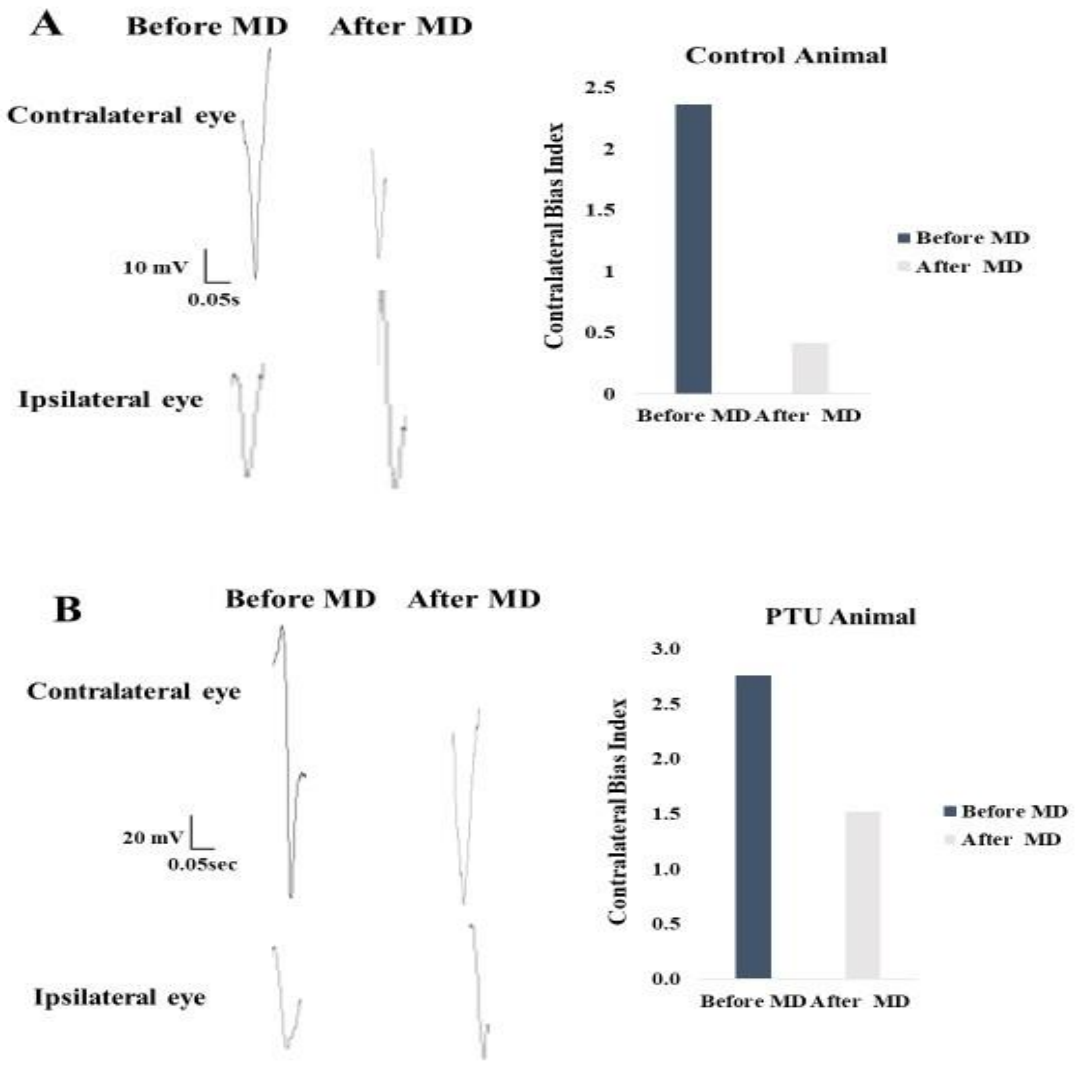

Figure 10: Representative VEP traces from control and PTU animal before and after MD and contralateral bias index of visually evoked potentials.

A) Representative VEP traces from the naive animal before and after MD with indicated amplitude (mV). CBI for contralateral and ipsilateral eye in the control animal, which shows a downward shift. B) Representative VEP traces from the PTU animal before and after MD with indicated amplitude (mV). CBI for contralateral and ipsilateral eyes in the PTU animal shows a downward shift but a lesser magnitude than in the control group

The contralateral bias index (contralateral/ipsilateral) was calculated for each animal. The data is presented as an average CBI per group before and after monocular deprivation. The control animals significantly decrease the index value from 1.879 ± 0.166 to 0.657 ± 0.197 ($p=0.08$, $n=7$) (Figure 11A). The PTU exposed animals show a still significant but

more minor decrease in CBI from 1.691 ± 0.172 to 1.383 ± 0.334 (p=0.001, n=7) (Figure 11B). The percent reduction in the CBI in the control animals was 61%, while in the PTU exposed animals, 54%, and it was not significant at p<0.05 with p=0.3The change in the control group was more evident than in the PTU group. Looking at each animal within the groups, we can observe that the shift in CBI in the control group had a similar trend, while in the PTU group, the direction was not as defined. One animal had a paradoxical shift and was eliminated from the analysis.

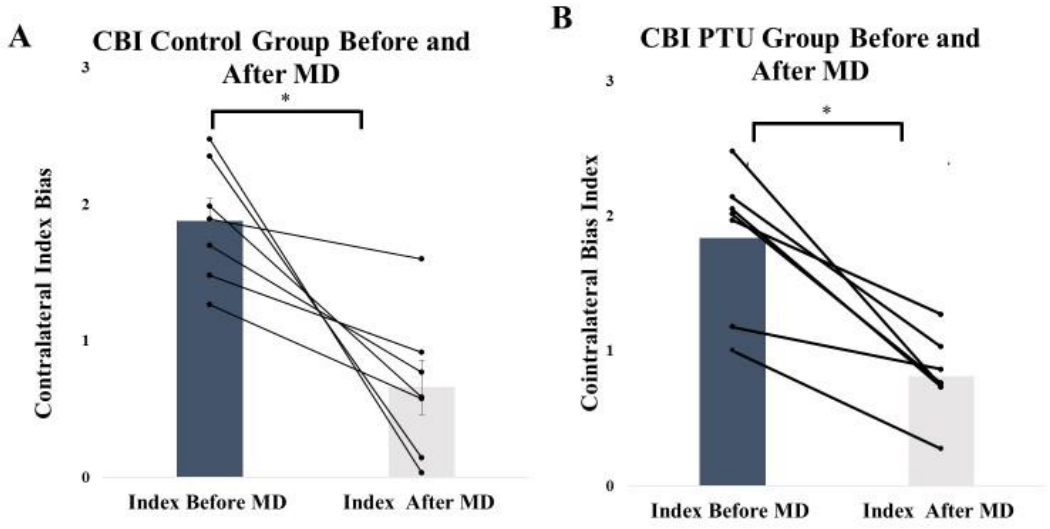

Figure 11: Shift in contralateral bias index of visually evoked potentials before and after monocular deprivation.

*The shift in the CBI in A) control group; B) PTU group. Each line represents one animal. Both groups control and PTU show a significant decrease in CBI within the group. The Control group changed from 1.879 ±0.165 to 0.657±0.197 n=7, and the PTU group changed from 1.691 ±0.172 to 1.383 ±0.334 n=8. The change in the CBI in the control group was 61%, while in PTU animals decrease was 54%. The percent decrease between the groups was not significant. Paired t-test with error bars indicating SEM and *p <0.05.*

Our results suggest that the decreased TH levels in our animal model were insufficient to impact the ocular dominance plasticity. Any deficiency in processing of the visual stimuli does not originate in the visual cortex but rather in other regions of the visual system, such as the retina.

Chapter 4

Discussion

Pregnancy is a demanding and fragile state in which an imbalance of hormones and micro and macro elements can have a crucial impact on the mother's health and the developing fetus. During gestation, the requirements for the TH hormones are increased up to 50 % (Ahmed, 2015). The female body has to support its own needs and the needs of the developing fetus. The thyroid hormone imbalance during pregnancy is one of many markers that must be closely monitored, even in patients who do not present thyroid problems before pregnancy. This study shows that gestational hypothyroidism can significantly affect contrast sensitivity. The VEP recordings for the spatial frequency did not differ substantially in how the animals processed the stimuli. However, there was a significant difference in contrast sensitivity between both groups. A significant difference was noted in the high contrasts, which indicated that animals had difficulties with pattern recognition. Contrast sensitivity is used to recognize the foreground and background, especially in a low-light environment (Pateras and Karioti, 2020). People with impacted contrast sensitivity may have issues in daily activities such as driving at night and recognizing changes in the walking pattern (curbs). Face recognition may also be affected due to the inability to identify facial features.

Additionally, the western blot data for the rhodopsin levels (monomer and dimer form) were not entirely different between the groups. The study suggests that lower TH levels change the opsin expression pattern in other regions of the retina, depending on the TH level, the opsins expression in the dorsal and ventral retina shifts. Additionally, studies

suggest that the normalized TH levels may restore, with time, the proper levels of the photoreceptor's expression (Cheng et al., 2009). This study did not distinguish between different regions of the retina, and whole retina tissue was used for the western blotting. We were able to show that animals exposed to PTU had a change in opsin expression. The TH impacted the expression of the M-cones but not the S-cones. The time of the exposure (first two post-natal weeks) to PTU in our animal model overlaps the time of the M-cones. This proves that TH is necessary for the M-cones expression. The levels of T4 in the PTU exposed animals show that changes that occurred during the exposure time (the equivalent of the human third trimester) can be detected during adulthood. During the development, the imbalance of the thyroid hormones can lead to hypothyroidism later in life. This suggests that close monitoring of TH levels during such a crucial time as pregnancy is necessary; also, continuous care into adulthood is recommended.

The animals exposed to the PTU during the study did not show as large of a shift as the control animals in CBI. During the monocular deprivation in the PTU group, the ipsilateral eye was not able to "pick up" and process all the visual stimuli even when the testing of the ocular dominance plasticity occurred during the critical period, a time when the brain still has a sizeable regenerative ability (Benoit *et al.*, 2015). Presented data shows that lower levels of thyroid hormones may not impact ocular dominance plasticity.

This study shows that maternal hypothyroidism significantly impacts retina composition but not visual cortex neuronal wiring. How our brain receives, and processes visual stimuli is essential in its development and a better understanding of our surroundings and even learning abilities. The better we understand what can have a detrimental impact on the developing brain, the better intervention and care we will be able to provide.

Chapter 5

Future Directions

Our study shows that gestational maternal hypothyroidism influences the proper visual system development in the offspring. There are a few aspects that should be further investigated. This study's main time focus was equivalent to the third trimester, a time of intense brain network refinement. Future studies could explore this issue further by targeting the changes that occurred to the visual system due to maternal hypothyroidism during each trimester. Testing where the modifications occur in the optical system can help mediate health issues. For example, if the levels of the expressed photoreceptors cause the changes, treatment will be different than in the case where neuronal connections are changed. Often, women are not aware of the pregnancy for the first few weeks. This is when suddenly the demand for the TH increases and the need is not met. That short period can have an impact on visual system development.

Studies show that the incident level of maternal hypothyroidism varies depending on the geographical region and diet. Racial and ethnicity are major health factors too. Being a part of the medical school, it will be interesting to conduct a long-term study following the pregnant patients to see how crucial these factors are regarding hypothyroidism and potential visual system complications.

We aimed to test how lower TH during pregnancy influences contrast sensitivity, visual acuity, and ocular dominance plasticity. We did not test the orientation selectivity. It will be interesting to see if TH modifies that aspect. Using in vivo electrophysiology, we could test if retina modification impacts orientation selectivity.

In our study, dams were exposed to one concentration of PTU. We could follow the Boyes and colleagues' research and expose animals to different medication concentrations as a next step. That could answer when and at what range we start seeing the negative impact of low TH on the visual system. In the presented experiment, the lower TH did not change the ocular dominance plasticity in the exposed animals. However, having different concentration groups could show us what level of low TH alters the ocular dominance plasticity.

Additionally, by increasing the number of animals and utilizing possible data from human studies, we could look at the sex differences. Many disorders and conditions have different manifestations depending on sex. Also, we could study what sex is more prone to the changes in their visual system. One more question that could be possibly investigated is if both sexes experience this same detrimental effect of lower TH during the development.

Vision and analysis of visual stimuli are essential in everyday life. As researchers, we should not stop finding the answers to the questions that could improve many people's lives. This study and others that will follow are part of the effort to show how vital thyroid hormones are during the visual system's development.

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