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https://doi.org/10.33015/dominican.edu/2016.bio.02

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Evaluation of Seizure Threshold as an Early Behavioral Marker of Disease Progression in the Mouse Model of Mucopolysaccharidosis IIIA

By

Pamela Santiago

A culminating thesis submitted to the faculty of

Dominican University of California and

BioMarin Pharmaceutical Inc.

in partial fulfillment of the requirements

for the degree of

Master of Science in

Biology

San Rafael, CA

May, 2016

CERTIFICATION OF APPROVAL

This thesis, written under the direction of the candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

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ABBREVIATIONS

AED: anti-epileptic drugs

BBB: blood-brain barrier

BM: Barnes maze

CNS: central nervous system

ERT: enzyme-replacement therapy

GABA: gamma amino-butyric acid

GAG: glycosaminoglycan

HS: heparan sulfate

IACUC: Institutional Animal Care and Use Committee

IP: intraperitoneal

IV: intravenous

LSD: lysosomal storage disorder

MPS IIIA: mucopolysaccharidosis type A

MPS IIIB: mucopolysaccharidosis type B

MWM: Morris water maze

NAGLU: n-acetylglucosaminidase

NOR: novel object recognition

PK: pharmacokinetic

PTZ: pentylenetetrazol

RMS: rostral migratory stream

SC: subcutaneous

SGSH: N-sulfoglucosamine sulfohydrolase

SVZ: subventricular zone

VZ: ventricular zone

WT: wildtype

ABSTRACT

Mucopolysaccharidosis IIIA (MPS IIIA) is a lysosomal storage disease caused by a mutation in the gene that codes for the enzyme heparan sulfamidase. The decreased enzyme activity of heparan sulfamidase results in the accumulation of heparan sulfate (HS). HS accumulation in the brain causes severe central nervous system (CNS) complications, including learning and memory deficits and seizure. MPS IIIA patients have short life expectancies and there currently is no cure for the disease. This thesis work was aimed at identifying an early neurological phenotype in the mouse model of MPS IIIA. The data will aid in the design of an *in vivo* assay for CNS correction for drug discovery. The tests for learning and memory impairments using the Barnes maze and novel object recognition behavioral assays did not show a neurological defect earlier than 20 weeks. These results suggest an intact cognitive capacity for the diseased animals. To test whether seizure threshold is altered in diseased animals, mice were administered with the chemiconvulsant drug, pentylenetetrazol (PTZ), a GABA receptor inhibitor. The acute intravenous infusion with PTZ did not show threshold differences between the diseased and normal mice. However, chronic administration of a subconvulsive dose of PTZ, resulted in a difference in seizure sensitization or kindling, as early as 4 weeks. MPS IIIA mice showed significant resistance to kindling by 12 weeks. These seizure results not only demonstrate that we can record a CNS specific phenotype earlier than 20 weeks, it also suggests that neuroplasticity may be altered in diseased animals. Thus, this seizure assay may help understand the alterations that occur in the brain circuitry of MPS IIIA affected animals.

ACKNOWLEDGEMENT

I would like to acknowledge BioMarin Pharmaceutical Inc. for giving me the opportunity to further my education. In particular, I would like to acknowledge my advisor and supervisor, Dr. Manuel Lopez, for all his patience, support and scientific guidance throughout the entire time of my graduate career. All your efforts are greatly appreciated.

I would like to acknowledge my colleagues for their advices and insights from the time I decided to pursue graduate school. I thank you for listening to me when I stress out about work and school (endlessly!). I greatly value your professional friendship.

A big portion of this success I owe to my dad. Thank you for being there from start to finish. And to my friends, for picking up my phone calls and texts. Thank you for pushing me and reminding me that I can do it.

Finally, I dedicate this thesis to my wonderful children, Rameses and Mayumi. The last two years in the program would not have been bearable without your constant inspiration and love. I was thinking of you the whole time and I truly wanted to be successful for both of you. You guys are the reason I did this, and you are the reason I finished. Mommy made it! Know that I love you very much!

1. INTRODUCTION

1.1 Project Goal

The experiments detailed in this thesis were conducted to determine if a CNS phenotype can be detected before 20 weeks of age in the MPS IIIA mouse. An early and robust behavioral readout for CNS-related defects has not been reported in the MPS IIIA disease mouse model. Developing a robust functional assay of CNS progression in the mouse model can assist in testing efficacy of novel compounds *in vivo*. Here, we assessed learning and memory deficits and seizure in the MPS IIIA mice. To test memory, we used two behavioral assays, the Barnes maze (BM) and the novel object recognition (NOR). These tests have been reported to be sensitive in examining both acquired and natural behavioral responses in the mice. To test seizure, we provided a challenge to the animal's brain circuitry responses with PTZ, a gamma-Aminobutyric acid (GABA) receptor antagonist. Seizure induction with PTZ was used to evaluate the differences in seizure threshold between control and diseased mice. The data collected will aid in developing future assays to test therapies and further explore disease biology at earlier time points in the mouse model.

1.2 Background

MPS IIIA, also known as Sanfilippo syndrome, is a recessive autosomal lysosomal storage disease (LSD) caused by a loss-of-function mutation in the gene N-sulfoglucosamine sulfohydrolase (*SGSH*). *SGSH* is the gene that codes for the enzyme heparan N-sulfatase, or sulfamidase. The mutation in the *SGSH* leads to the impaired

synthesis of sulfamidase, an enzyme involved in the step-wise degradation of heparan sulfate (HS) (Esposito *et al.*, 2000; Valstar *et al.*, 2008). The reduction of sulfamidase activity, leads to HS accumulation in neurons primarily and other cell bodies, as a result, impairing the CNS in MPS IIIA (Bhaumik *et al.*, 1999; Fraldi *et al.*, 2007).

MPS IIIA has a global incidence of 1 in every 100,000 live births (Fedele, 2015). MPS IIIA, compared to other MPS III disorders, is the most severe form, has the earliest onset, fastest progression of symptoms and shortest survival rate (Bhaumik *et al.*, 1999; Meikle *et al.*, 1999; Valstar *et al.*, 2010). Clinical manifestations of the disease include severe learning disability, behavioral problems, sleep disorder and hyperactivity; with secondary disease complications of hearing loss, diarrhea and seizures (Meyer *et al.*, 2008; Buhrman *et al.*, 2014). Children born with MPS IIIA experience normal development at birth until they become symptomatic (Valstar *et al.*, 2010). Clinical abnormalities show between the ages of 2 and 6 years and neurological degeneration between 6 and 10 years of age (Esposito *et al.*, 2000). Severely affected children usually die by their mid to late teenage years (Van De Kamp *et al.*, 1981; Gliddon e Hopwood, 2004; Delgadillo *et al.*, 2013). Gene therapy (Lysogene, France; Abeora Therapeutics, Dallas, TX) as well as enzyme replacement therapy (ERT) (Shire Human Genetic Therapies Inc., Cambridge, MA) are in clinical trials but there is currently no cure for MPS IIIA.

A mouse model of MPS IIIA was established by Bhattacharyya in 2001 (Bhattacharyya *et al.*, 2001). The mouse contains a missense mutation in the murine *SGSH* gene, which replaces an aspartic acid with an asparagine (D31N) (Bhattacharyya *et al.*, 2001; Coutinho, Lacerda e Alves, 2012). This amino acid substitution reduces enzyme

activity to 3-4% of that of normal enzyme levels (Roberts *et al.*, 2007) and causes accumulation of HS and other cellular pathology similar to what is seen in human patients.

The clinical manifestations of the disease in the mouse closely resembles the human disease (Bhaumik *et al.*, 1999; Hemsley e Hopwood, 2005; Fraldi *et al.*, 2007). MPS IIIA diseased animals at birth are indistinguishable from non-diseased littermates. At 3 weeks of age, affected animals tend to be hyperactive (Fraldi *et al.*, 2007). By 6-7 months of age, significant differences in appearance and growth rates start to become apparent. Affected animals at this age begin to look scruffy, with the fur unkempt and greasy, and develop a hunch posture (Bhaumik *et al.*, 1999; Crawley *et al.*, 2006). By 10 months of age and toward the later stages of the disease, MPS IIIA mice display aggressive behavior and exhibit pronounced memory loss (Bhaumik *et al.*, 1999; Hemsley e Hopwood, 2005). By 12-14 months, animals exhibit hepatosplenomegaly (enlargement of the spleen and liver) and an expanded bladder containing 1-2 mL of turbid urine (Bhaumik *et al.*, 1999). Death usually occurs as a result of peripheral pathology prior to the increase in the severity of the CNS pathology.

CNS-related defects in the MPS IIIA mice have been characterized as early as 20 weeks in behavioral assays, such as in the Morris water maze (MWM), open field test or the negative geotaxis. The MWM assesses spatial learning and memory in mice. The test forces the animal to swim and learn the location of a submerged platform. MPS IIIA mice in the MWM, at 20 weeks of age, were reported to have a more difficult time in learning the location of the platform (Fraldi *et al.*, 2007). The open field test measures an animal's general movement, activity and exploratory behaviors. MPS IIIA mice at 22 weeks of age were reported to exhibit a decrease in exploratory behaviors and in distance traveled (Lau

et al., 2008; Sorrentino *et al.*, 2013). The negative geotaxis is a behavioral test that measures the ability of the animal for reorientation when facing downwards a sloping platform. Male MPS IIIA mice at 20 weeks of age showed a significant difference in their ability to reorient themselves (Hemsley e Hopwood, 2005). All these behavioral assessments demonstrated a difference in memory, general activity and motor functions in the MPS IIIA animals but nothing earlier than 20 weeks were reliably detected. A behavioral assay that is a direct readout of CNS activity in younger animals, might be useful in further characterizing the CNS disease progression in MPS IIIA mice. To the best of our knowledge, there is no published reports on the MPS IIIA mouse model being evaluated in a seizure assay.

Seizure is the uncoordinated electrical discharge of neurons in the brain. The brain's electrical signaling is maintained by the balance of the excitatory and inhibitory neurotransmitters, glutamate and gamma-Aminobutyric acid (GABA), respectively (Petroff, 2002). An imbalance in the activity of either of these neurotransmitters can cause seizure episodes. Seizures are defined behaviorally as involuntary muscle jerks or spasms. In the absence of a stimulus, MPS IIIA mice do not show visible signs of seizure. However, we hypothesized that in response to a stimulus, MPS IIIA mice can develop seizure and the change in seizure threshold can be scored. This seizure test can be developed into an assay to examine CNS alterations during disease progression or as a benefit of treatment.

Although diseased animals do not exhibit seizures, seizure is a common symptom in MPS IIIA human patients. A study on the natural history of the disease in Spain, reported that 45% of patients develop recurring generalized seizures at a median age of 8.7 years old (Delgadillo *et al.*, 2013; Buhrman *et al.*, 2014). In another study, 53 of the 80

patients (66%) in the study experienced seizures, where the first occurrence was observed around 11 years of age (Valstar *et al.*, 2010). An 18 year old woman from a conducted autopsy study, was only diagnosed with MPS IIIA after evidence of neurologic difficulty with seizures (Rush *et al.*, 2015).

Our attempt to understand seizure susceptibility is not solely based on clinical seizure symptoms in patients but also on an observation made by Bruyere in the MPS IIIB mouse model. MPS IIIB exhibits the same general disease characteristics as MPS IIIA. MPS IIIB is caused by a deficiency in the enzyme, N-acetylglucosaminidase (NAGLU), affecting the degradation of HS. Bruyere showed a disorganized cluster of neuronal precursors in the rostral migratory stream (RMS) of adult MPS IIIB mice brains (Bruyere *et al.*, 2015). These neuronal precursors actively migrate from the subventricular zone (SVZ) toward the olfactory bulb and other regions such as the cortex, where they differentiate into fully mature GABAergic interneurons. These mature interneurons are thought to play a role in regulating neuronal inhibition in the brain. This migratory defect is yet to be confirmed in MPS IIIA. But considering the similarities between MPS IIIA and MPS IIIB, it is possible that there may be a change in GABAergic response and it could be measured *in vivo* as changes in seizure thresholds.

Seizure threshold in mice is commonly examined using a chemical agent called pentylenetetrazol (PTZ), also known as pentetrazol, leptazol or metrazol. PTZ was discovered by Ladislas J. Meduna in 1934 as a circulatory and respiratory stimulant. When PTZ is given at high doses, it causes convulsions. It has been shown that the direct binding of PTZ to the benzodiazepine/GABA/chloride receptor complex *in vitro* correlates to its convulsive effect *in vivo* (Squires *et al.*, 1984). The exact mechanism of PTZ is not fully

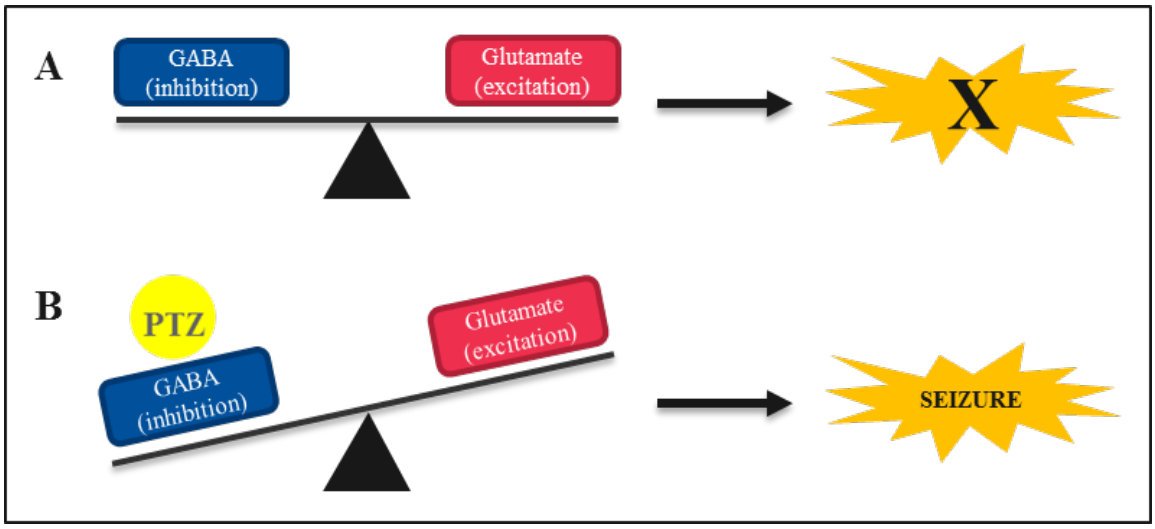


Figure 1 Mechanism of PTZ

(A) The brain's neuronal activity is maintained by the balance of GABA and Glutamate neurotransmitters. (B) PTZ is a GABA antagonists proposed to inhibit inhibitory functions of GABA. The increase in excitation produces seizures.

understood but evidence suggests that it stimulates the CNS by antagonizing the inhibitory transmission of the neurotransmitter GABA (Kaminska, Lukasiuk e Kaczmarek, 1994). PTZ inhibits the GABA-activated Cl-influx to increase depolarization in the cell, thus, eliciting seizures (Figure 1).

PTZ has been widely used for both acute and chronic induction of seizures to investigate efficacy of anti-epileptic drugs (AED) (Purpura Dp, 1972; Loscher, 2011; Dhir, 2012). The intravenous (IV) delivery of PTZ serves as an acute screen of seizure activity for AEDs and has been reported to be advantageous over a subcutaneous (SC) administration (Mandhane, Aavula e Rajamannar, 2007). The IV infusion, particularly, offers a more consistent and reliable measurement of seizure thresholds (Nutt, Taylor e Little, 1986). A more chronic method to determine seizure susceptibility is through kindling. Kindling is the gradual increase in cellular discharge from a sporadic low-intensity electrical or chemical stimulant to the limbic system that climaxes to a full-blown seizure episode (Racine, 1978). Kindling involves chronic administration of a sub-

convulsive dose, either SC or intraperitoneal (IP), until a decrease of seizure threshold is achieved (Da Silva, Pereira e Elisabetsky, 1998; Dhir, 2012). Both the IV infusion and kindling have been extensively studied for models of seizure development.

We conducted this thesis study to examine two aspects of CNS function in the MPS IIIA mouse model, learning and memory and seizure. First, we assessed the animal model's progressive memory deficits in the Barnes Maze (BM) and the Novel Object Recognition (NOR) behavioral tests. The BM was developed by Carol Barnes in 1979 as a dry-land maze, eliminating the stress of swimming in the MWM. The BM has long been used in mice to measure cognitive impairment (Sunyer *et al.*, 2007). The BM results will compliment data from MWM in MPS IIIA mice. The NOR is a task designed to particularly evaluate recognition memory. An animal's preference to novelty, in the absence of a positive or negative reinforcements, was first investigated by Ennaceur and Delacour in 1988 (Ennaceur e Delacour, 1988). This behavioral assay evaluates how the animal's innate exploratory behaviors could be affected by memory alterations. The NOR data will supplement findings in the BM. Finally, we determined if by challenging the CNS, we can characterize the seizure phenotype in diseased animals. Seizure susceptibility was evaluated by two methods: (a) PTZ-induced IV infusion and (b) PTZ-induced IP kindling. The BM and NOR tests did not seem to indicate an impaired cognitive ability in diseased animals. The seizure test, on the other hand, revealed a phenotype that we were able to score prior to 20 weeks of age in the MPS IIIA mouse.

2. MATERIALS AND METHODS

2.1 Animals

All animal procedures were performed in accordance to national standards of the Institutional Animal Care and Use Committee (IACUC) (protocol #A10109) at the Buck Institute for Research on Aging (Novato, CA). Mice carrying the sulfamidase mutation, $Sgsh^{mps3a}$, in the congenic C57Bl6/J background were obtained from Jackson Laboratory (Bar Harbor, ME). Heterozygote animals were mated to establish and maintain a colony. MPS IIIA (B6.Cg-$Sgsh^{mps3a}$/B6.Cg-$Sgsh^{mps3a}$), heterozygous (+/B6.Cg-$Sgsh^{mps3a}$) and wildtype (WT) ($Sgsh^{+/+)}$ mice were used for experiments. To determine genotype, animals were ear tagged (Fine Science Tools Foster City, CA) and ear samples were collected for analysis at Transnetyx, Inc (Memphis, TN). For initial assay development, male mice were used for all studies and aged matched C57Bl6/J mice, as an additional WT control comparison, were ordered from Jackson Laboratory. Animals were acclimated at least 3 days prior to start of the study. Mice were group housed in individually ventilated and temperature controlled polysulfone cages and provided with certified rodent diet (Teklad Global Diet #2018) and water ad libitum. Environmental controls for the animal room were set at 20-26 °C, with relative humidity of 30 to 70%, a minimum of 10 room air changes/hour, and a 12-hour light/12-hour dark cycle.

2.2 Barnes Maze

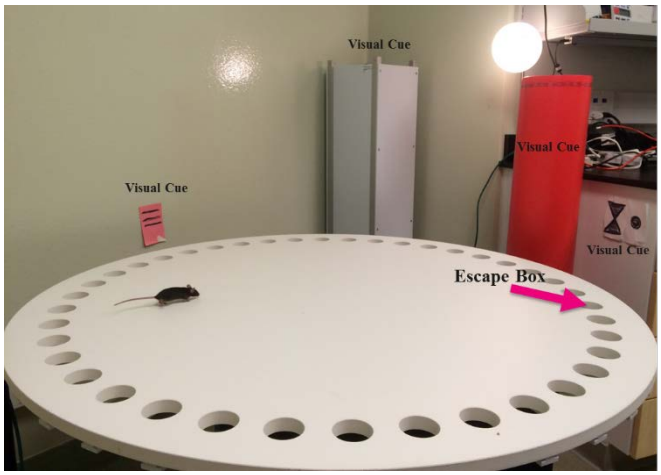


Figure 2 Barnes Maze Experimental Set-up
Mice laboratory experiment to measure spatial learning and memory in the Barnes maze. Visual cues of varied sizes and color are permanently situated around the experimental room. This behavior assay records time latency on how fast the animal can locate an escape box.

The Barnes Maze test was used to examine cognitive defects in spatial learning and memory in MPS IIIA mice compared to the C57Bl6/J control group at 4, 12 and 24 weeks (Figure 2). The maze is an elevated circular platform with 40 equally spaced holes running along the perimeter. All of the holes are blank-bottomed except for one that leads to an "escape box". The escape box is provided with bedding and food pellets, which were changed after every single animal to eliminate odor biases between animals. Several stationary visual cues were placed in the room as the animal's reference point for locating the escape box, and a stimulus (noise) was used to motivate an escape response. Prior to start of any trial, the animal was habituated to the escape box for 3 minutes. After habituation, the mouse was placed in the middle of the maze under a box. The trial starts upon lifting the box, the stimulus (a steady noise generated by a metronome) turned on and the animal released to find the escape box. During the trials, the animal was given up to 3 minutes on the first trial and 1 minute on subsequent trials. An additional aversive stimulus (clapping) was given if the animal failed to locate the escape box in 1 minute. Once the animal was in the escape box, the noise stimulus was turned off and the animal was allowed to stay in the escape box for another minute. Time latencies were manually recorded at

every trial. The maze was sprayed with 70% ethyl alcohol and wiped dry to minimize olfactory cues after every trial. An overhead camera was positioned above the maze for video recordings, this will allow for a variety of data (speed/velocity, time latencies, distance traveled or heat maps) to be analyzed with an automatic video tracking software (EthoVision XT 11).

The BM was used in two different experimental designs. The first experimental protocol was an acute 1-day acquisition phase. The acquisition phase is where the animal initially learns to find the escape box. During the acquisition phase, the animals received 8-10 learning trials until the mice gave a time latency of less than 1 minute, in locating the escape box, on 3 consecutive trials. The animals were tested for memory at 24 hour and 48 hour consisting of 3 trials, post the acquisition trials. The second protocol extended the acquisition phase over 4 days and consisted of only 2 trails per day. The animals were tested for memory on day 5 and 15.

2.3 Novel Object Recognition

Figure 3 NOR Experimental Objects
The objects used in NOR were odorless; similar in height and texture; different in shape and color. Objects were washed and cleaned after every animal to remove possible olfactory cues.

The NOR test was administered to assess recognition memory deficits in MPS IIIA mice at 4, 12 and 24 weeks. The test was conducted in an opaque shoebox cage arena that measured 18 cm x 28 cm x 4.5 cm high. The objects, as shown on Figure 2, are plastic that varied in shape and color (width=3.4-4.0 cm; height=4.0 cm) and were placed 13 cm apart to the floor of the cage. The cage did not contain bedding to minimize any distractive elements and the objects were secured with tape to prevent the

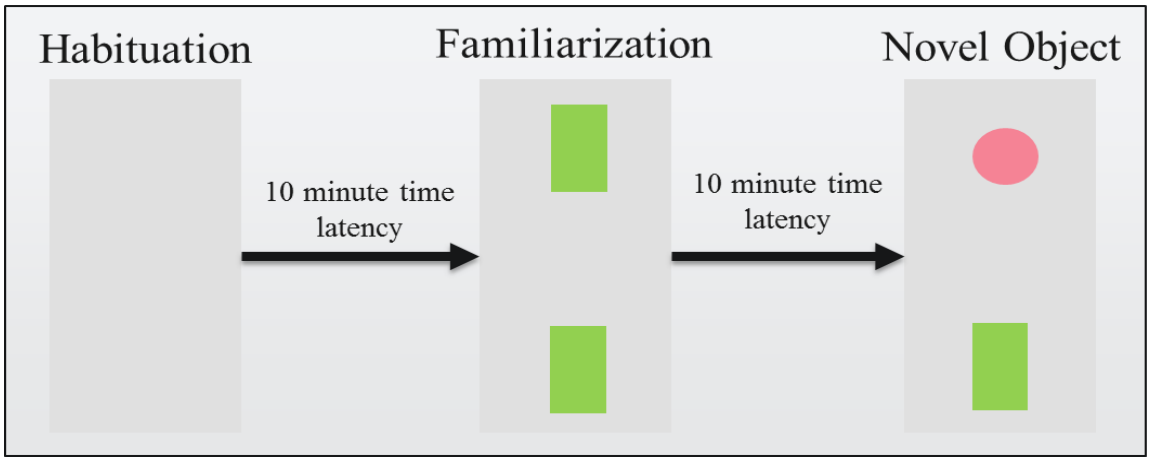


Figure 4 Schematic of NOR Experimental Procedure

Animals were placed in an empty cage for 10 minutes during habituation. 2 identical objects are placed in the cage and mice were allowed to explore them. To test recognition memory, one of the objects are replaced for a novel one. Animals explored the objects for 10 minutes at each of the phases.

mouse from displacing them**.** An overhead camera and fluorescent lighting were mounted above the box cage for video recording and illumination.

The NOR test consisted of three phases (Figure 4): habituation, familiarization and test phase. During the habituation phase, a single mouse is allowed to freely explore the empty arena. The animal was then removed and placed back to its home cage for a time delay of 10 minutes. In the 10 minute familiarization phase, two identical objects were introduced and animals were released on the north-east side of the cage. Animals were then returned to their home cages for 10 minutes. During the test phase, the animal was reintroduced back to the arena where one of the objects was replaced with a novel one and observed for 10 minutes. Animal exploration was manually measured as nose pokes towards the objects and verified by an automatic video tracking (EthoVision XL 11) to analyze the animal's interest towards the novel object.

2.4 PTZ-Induced Seizure Test

The PTZ induced seizures either via an IV continuous infusion or IP kindling was done to assess seizure thresholds in MPS IIIA mice when compared to C57Bl6/J control group at 4, 12 and 24 weeks.

2.4.1 Drug

PTZ (Sigma Aldrich, St. Louis, MO) is kept in -20°C and brought up to room temperature prior to formulation. PTZ was weighed into a glass vial and sterile 0.9% saline was added to achieve designated concentrations. All formulations were prepared fresh on the day of each experiment.

2.4.2 Seizure stages with PTZ induction

PTZ has been shown to elicit distinct individual seizure stages (Da Silva, Pereira e Elisabetsky, 1998; Dhir, 2012). Animals received a single IP administration of PTZ at a convulsive dose of 60 mg/kg and seizure activities were observed to ascertain that PTZ can produce seizures that we are able to score in MPS IIIA.

PTZ is a fast acting drug that was able to elicit a full blown seizure within 5 minutes post IP injection. Seizure activity progresses through three distinct stages (Figure 5). Stage 0 decreased the animal's activity but did not produce seizures. Stage 1 showed involuntary muscle jerks. Stage 2 is often referred to as Straub's tail (Dhir, 2012), where the tail erects and is perpendicular to the body. Stage 3 is a full blown body muscle contraction, sometimes accompanied with vocalization, lasting from 7 to 30 seconds.

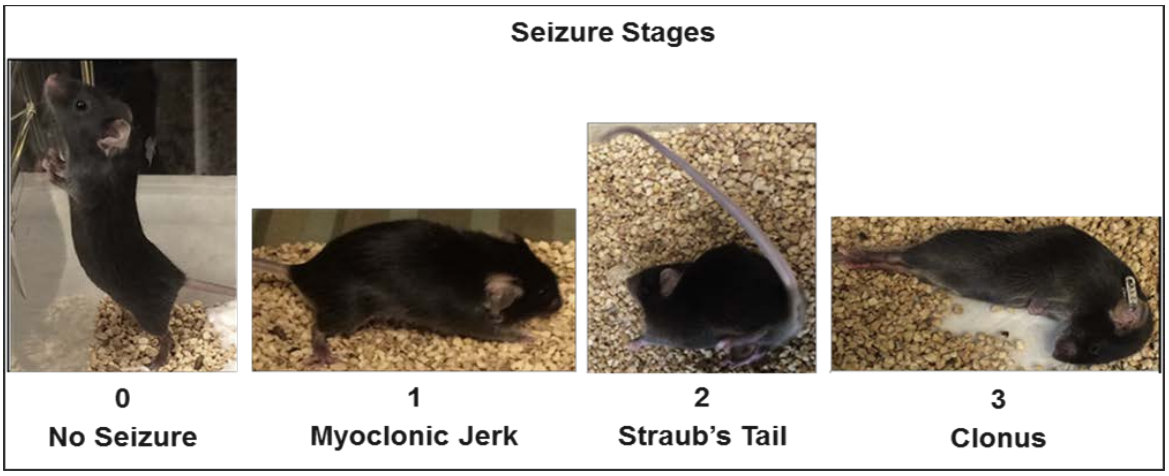


Figure 5 Seizure Stages Progression with PTZ Treatment

PTZ treatment produced a well define seizure progression, that can be separated into stages. Stage 0 - animal activity decreases with no sign of seizure; Stage 1: Myoclonic jerks - involuntary muscle jerks; Stage 2: Straub's tail - rigidity with tail erection perpendicular to body; Stage 3 Clonus - full body muscle contraction with clonic episodes.

2.4.3 PTZ-induced IV Infusion

Animals were weighed prior to study start. A tail vein cannula (extended tubing attached with a 27G ¾ needle) for an IV administration was connected to a 5 mL syringe preloaded with PTZ. Animals were restrained and the needle inserted to the tail and secured by tape. A Hamilton programmable infusion pump was used to deliver 10 mg/mL of PTZ at a constant infusion rate of 50 uL/min. Time latencies from start of infusion to the appearance of first clonus (Stage 1) and subsequent tonic forelimb and/or hindlimb extension lasting longer than 5 seconds (Stage 3) were recorded. Infusion was stopped at the appearance of a Stage 3 seizure. The threshold dose in mg/kg for appearance of stage 1 and stage 3 seizures was calculated using the formula: [Infusion rate of infusion (ml/min) * time for onset of seizure (s) * concentration of PTZ (mg/mL) * 1000] / [60 * body weight of animal (g)]. At the end of infusion and observation of seizure episodes, surviving

animals were anesthetized using isoflurane (4% for induction and 1.5% for maintenance) and euthanized by cervical dislocation.

2.4.4 PTZ-induced IP Kindling

Animals were weighed for dose volume determination prior to every treatment day and received a single IP administration of PTZ at 40 mg/kg every other day. After PTZ injection, the animals were placed in individual clear cages for 30 minutes to observe seizure activity. Seizure activity (Figure 5) was rated in stages as 0-no appearance of seizure, 1-myoclonic jerk, 2-straub's tail and 3-generalized convulsions with tonic extensions. Seizure stages and time of onset were recorded, with animals considered kindled if the animal produce a stage 3 seizure over 3 consecutive treatment days. Animals who died prior to the third stage 3 are considered kindled.

2.4.5 PTZ pharmacokinetic (PK) evaluation

For brain and plasma PTZ levels, animals were assigned to study groups as described on Table 1. Animals received a single IP administration of PTZ and were euthanized at specified time points. Animals were anesthetized using isoflurane and blood sample was collected via cardiac puncture into K3 EDTA anticoagulant tubes which were kept on ice until centrifugation at 10,000 RPM for 5 min at 4°C. Animals were decapitated to remove the brain. The brain was separated into 2 hemispheres, right and left, and placed into two different 2 mL Eppendorf tubes. The plasma was aliquoted into two 1.5 mL Eppendorf tubes. Plasma and brain samples were stored in a -80°C freezer until submission. Samples were sent to Climax Laboratories Inc. (San Jose, CA) for PTZ brain and plasma measurements by mass spectrometry analysis.

Table 1 Group designation for PK profile

Group[1, 2]	Genotype	Age (weeks)	Timepoint (minutes)
1	MPS IIIA	4	5
2	C57Bl6/J	4	5
3	MPS IIIA	12	5
4	C57Bl6/J	12	5
5	MPS IIIA	24	5
6	C57Bl6/J	24	5
7	MPS IIIA	4	15
8	C57Bl6/J	4	15
9	MPS IIIA	12	15
10	C57Bl6/J	12	15
11	MPS IIIA	24	15
12	C57Bl6/J	24	15
13	MPS IIIA	4	30
14	C57Bl6/J	4	30
15	MPS IIIA	12	30
16	C57Bl6/J	12	30
17	MPS IIIA	24	30
18	C57Bl6/J	24	30

[1] n=3 animals per group; PTZ at 40 mg/kg, 4 mg/mL, 10 mL/kg.
[2] 12 week samples at 5 and 15 minutes were the only data presented.

3. RESULTS

MPS IIIA is marked by neurological consequences, such as cognitive impairment and seizure. In this thesis, we assessed and demonstrate the cognition and seizure performance of MPS IIIA mice in 1) the Barnes Maze and Novel Objection Recognition assays to evaluate learning and memory and 2) by PTZ seizure induction experiments to measure seizure susceptibility.

3.1 Behavioral Tests

3.1.1 The Barnes maze does not detect early spatial memory deficits in the MPS IIIA mice

Spatial memory and learning deficits in the MPS IIIA mice have been reported at 20 weeks using the MWM (Fraldi *et al.*, 2007) . Here we use a similar behavioral test, the Barnes maze. The BM has been shown to be sensitive to changes in spatial learning related to neurodegeneration (Kennard, 2012). To determine whether we can detect a deficit in spatial learning and memory earlier than the 20 weeks reported with MWM, we tested MPS IIIA animals in the BM.

The data from the 1-day learning and memory method suggests that diseased animals have similar spatial learning and memory abilities as in the normal mice. The 1-day BM learning method was designed to assess acute learning and memory using 9-10 consecutive trials followed by a 24 and 48 hour memory tests. As shown on Figure 6, there was evidence of normal learning across all the ages tested as measured by performance in each of the training trials (A-C). The average time to find the escape box during trial 1 was similar between the controls (4 weeks: 159 secs; 12 weeks: 185 secs; 24 weeks: 204

secs) and the diseased animals (4 weeks: 98 secs; 12 weeks: 171 secs; 24 weeks: 180 secs). On succeeding trials, the time latencies to locate the escape box decreased for all animals. The animal's ability to locate the escape box seemed to be solidified by the third trial, with a mean time of 63.02 seconds (C57Bl6/J) and 51.05 seconds (MPS IIIA). To test if the memory was retained, animals were tested at 24 and 48 hours. All animals demonstrated a low time latency in finding escape at 39.4 and 37.40 seconds for controls and MPS IIIA mice respectively. Performance between the animals was not significantly different ($p<05$).

The quick succession of learning trials in the 1-day protocol may have strongly reinforced learning and memory to be able to see any differences in behavior. To determine whether with less trials and a prolonged learning phase with BM can better assess differences in performance, we extended the training trials over 4 days at 2 trials per day. To provide a greater memory challenge, the memory test was conducted at day 5 (24 hours) and then delayed until day 15.

The 4-day learning method in Figure 7 showed similar learning performance between diseased and normal animals. The time latencies to reach the escape box decreases over the 4 days and seemed to be established by the third day (A-B). The memory tests on day 5 and 15 show an average time of 85.85 seconds for the controls and 94.22 seconds for MPS IIIA. Overall, our BM results in both the 1-day and 4-day learning protocols do not suggest a decline in learning and memory even by 24 weeks. This data suggests that even under a variety of reinforcement conditions, 1-day with multiple trials or gradual training over time, MPS IIIA animals in the BM will display the same learning and memory capacity as in the normal mice.

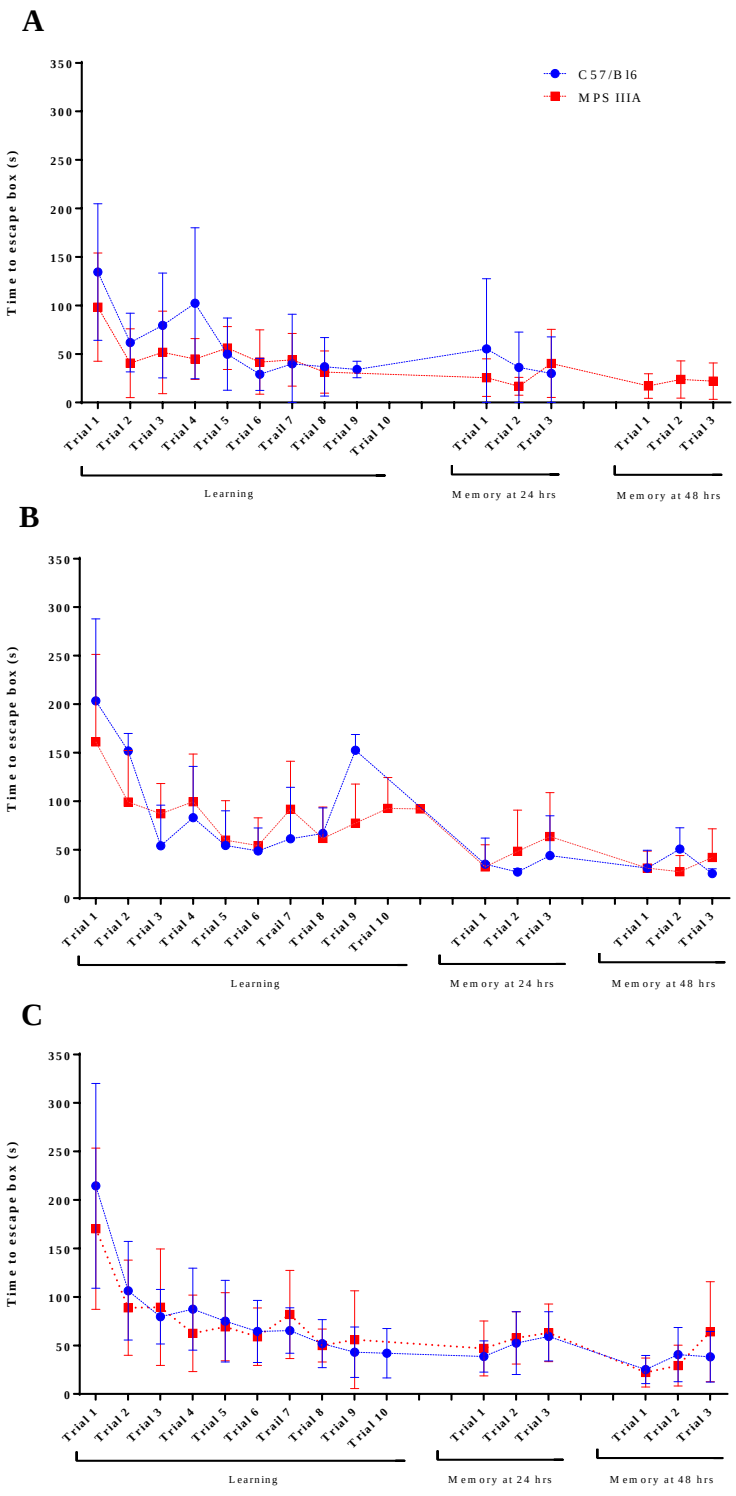


Figure 6 Barnes Maze 1-day Learning and Memory Curves

Mean (± SEM) time to locate escape box in seconds (s) for MPS IIIA animals 4 (A), 12 (B) and 24 (C) weeks. Animals received a succession of 9-10 trials to remember the specific location of an escape box. Animals were then tested for memory at 24 hour and 48 hour. MPS IIIA animals showed learning ability during the training trials. The memory tests showed that animals are able to remember a previously learned behavior. *No 4 week control data available at 48 hour memory test.

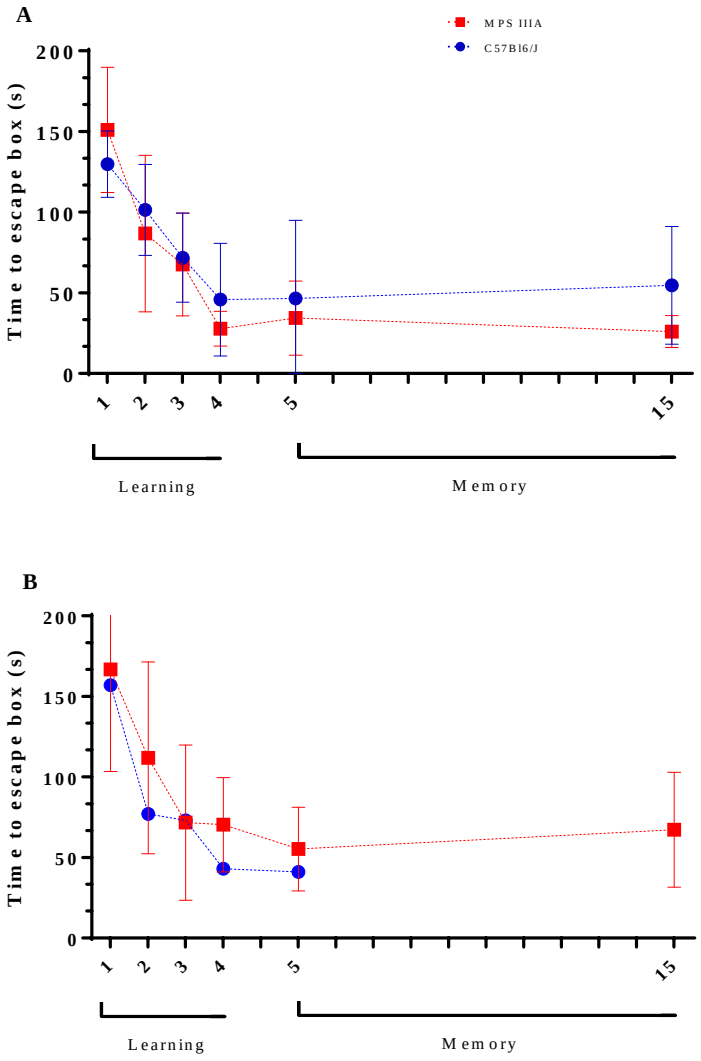


Figure 7 Barnes Maze 4-day Learning and Memory Curves

Mean (±SEM) time to locate escape box in seconds (s) for MPS IIIA mice at 12 weeks (A) and 24 weeks (B). The 4-day acquisition method extended the trials to 4 days, consisting of 2 trials per day. The animals were then tested for memory at 24 hour and 15 days. MPS IIIA showed learning ability during the training trials and showed retention of memory at the 24 hour and 15 day tests. *There is no 4 week data presented due to animal availability and time constraints. *No 24 hour controls presented for 15 day memory test.

3.1.2 Novel object recognition showed decreased exploratory behaviors

The NOR test, unlike the BM or MWM, relies on the rodent's spontaneous exploratory behavior with no positive or negative reinforcements, such as a reward or punishment. The animals were tested using the NOR assay to determine if the natural tendency of an animal to explore a novel object can be used to detect alterations in recognition memory.

The NOR data are presented on Figure 6. In the familiarization session (A-C), all the animals showed similar number of exploration pokes between the two identical objects. The diseased animals seem to exhibit lower number of nose pokes as compared to controls but the difference was not significant. The mean numbers of nose pokes for controls were 30.51 at 4 weeks, 50.67 at 12 weeks and 33.83 at 24 weeks. The mean number of nose pokes for MPS IIIA were 24.25 at 4 weeks, 36.75 at 12 weeks and 21.59 at 24 weeks. The number of nose poke for MPS IIIA at 24 week MPS IIIA (C) decreases as compared to the controls. The difference however, was not significant.

When the novel object was presented to the animals (D-F), the control animals showed a significant increase in nose pokes towards the new object (4 wks: $p<0.0001$; 12 wks: $p=0.0126$; 24 wks: $p=0.0051$). In contrast, the diseased animals did not seem to show recognition interest for the novel object (4 wks: $p=0.4699$; 12 wks: $p=0.7360$; 24 wks: $p=0.4286$). MPS IIIA mice at 24 weeks consistently do not explore the objects (F) as much as the controls.

This NOR data indicates that although the assay can detect differences between control and MPS IIIA animals, there is a decrease in exploratory behavior in older MPS

IIIA mice that is similar to previous reports (Lau *et al.*, 2008; Sorrentino *et al.*, 2013). The magnitude of difference between the normal and diseased animals was not within a significant range. Furthermore, diseased animals presented a generally low interest to the objects. This overall lack of object interest can confound results obtained with the NOR assay.

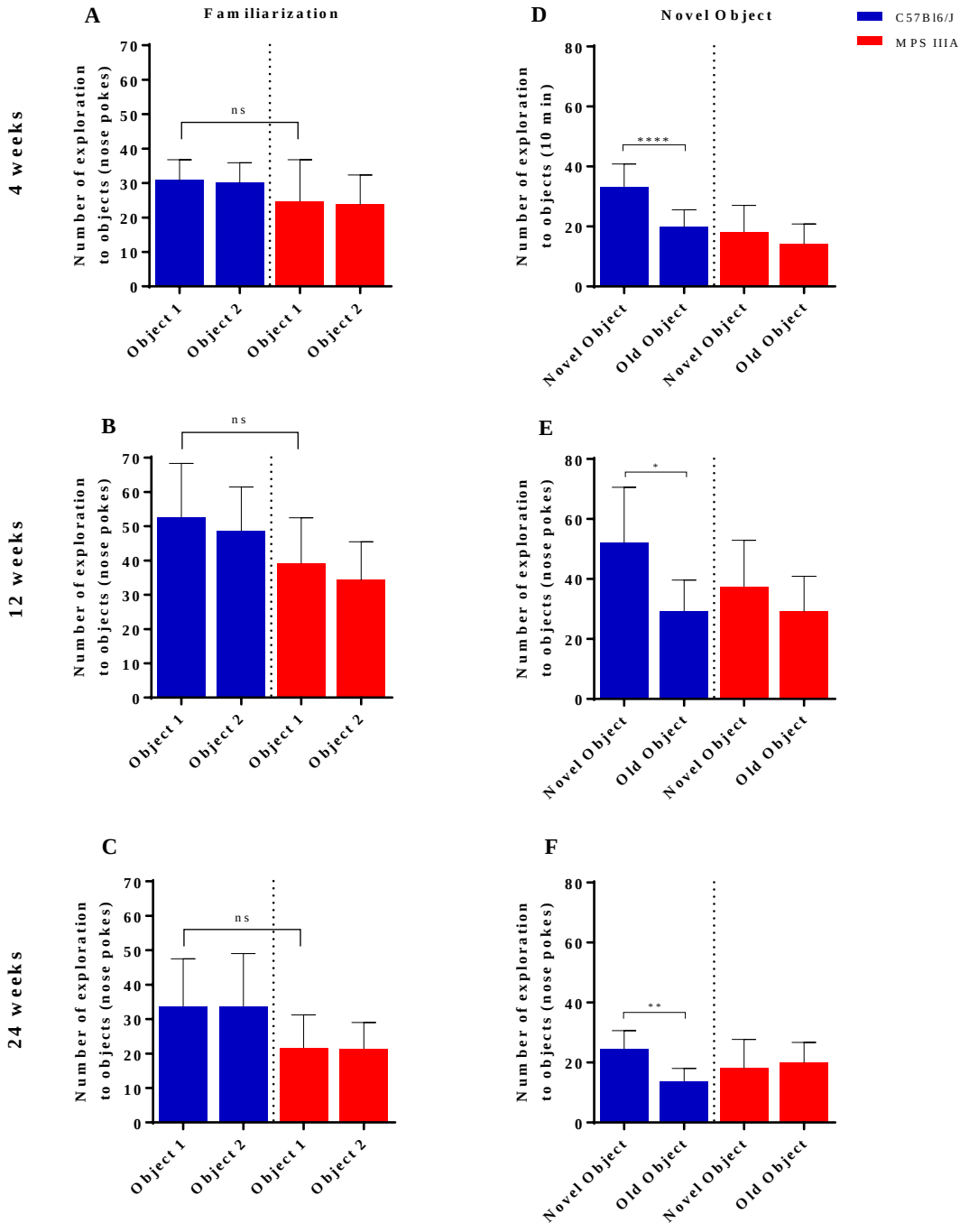


Figure 8 10 Minute Novel Object Exploration Scores

Exploration scores were measured as individual nose pokes that were manuallyscored for a duration of 10 minutes in MPS IIIA animals at 4, 12 and 24 weeks. (A-C) Animals showed the same number of nose pokes when presented with two identical objects. (D-F) Control animals showed a significant increase in the number of nose pokes towards the novel object, as compared to MPS IIIA mice. There was a decrease in exploration behaviors in MPS IIIA during familiarization and introduction of the novel object. Bars represent mean ± SEM; *p=0.0126, **p=0.0051 and ****p<0.0051.

3.2 PTZ-induced Seizure Tests

The results from BM did not suggest cognitive decline even at 24 weeks. The results with NOR also did not demonstrate a conclusive difference between normal and MPS IIIA animals. Perhaps stimulation of the CNS might bring about a phenotype that is CNS specific and can be measured prior to 20 weeks. To determine if seizure susceptibility is altered in MPS IIIA mice, we administered PTZ via the IV infusion and IP kindling experimental methods.

3.2.1 There is no significant difference in seizure thresholds with PTZ-induced seizure via the IV infusion

The time latency for the onset of a full blown stage 3 seizure is presented in Figure 9A. Animals were administered with 10 mg/mL of PTZ at a slow constant infusion rate of 50 uL/min and the appearance of seizure stages were observed and recorded and the seizure threshold dose were calculated. There were no significant difference ($p=0.58$) in the onset of stage 3 seizures between the control and diseased animals. There was also no significant difference in seizure threshold as presented in Figure 9B. Similar threshold doses elicited stage 3 seizures for all animals (4 weeks: 69.7 ± 13.1 mg/kg C57; 66.3 ± 20 mg/kg MPS IIIA, 12 weeks: 53.6 ± 3.9 mg/kg C57; 62.4 ± 22.3 mg/kg MPS IIIA and 24 weeks: 68.9 ± 5.9 mg/kg C57; 62.6 ± 15.6 mg/kg MPS IIIA).

The data on the induction of seizure via an acute IV infusion collectively indicates that there was no detectable difference on seizure onset and threshold between normal and diseased animals via this method.

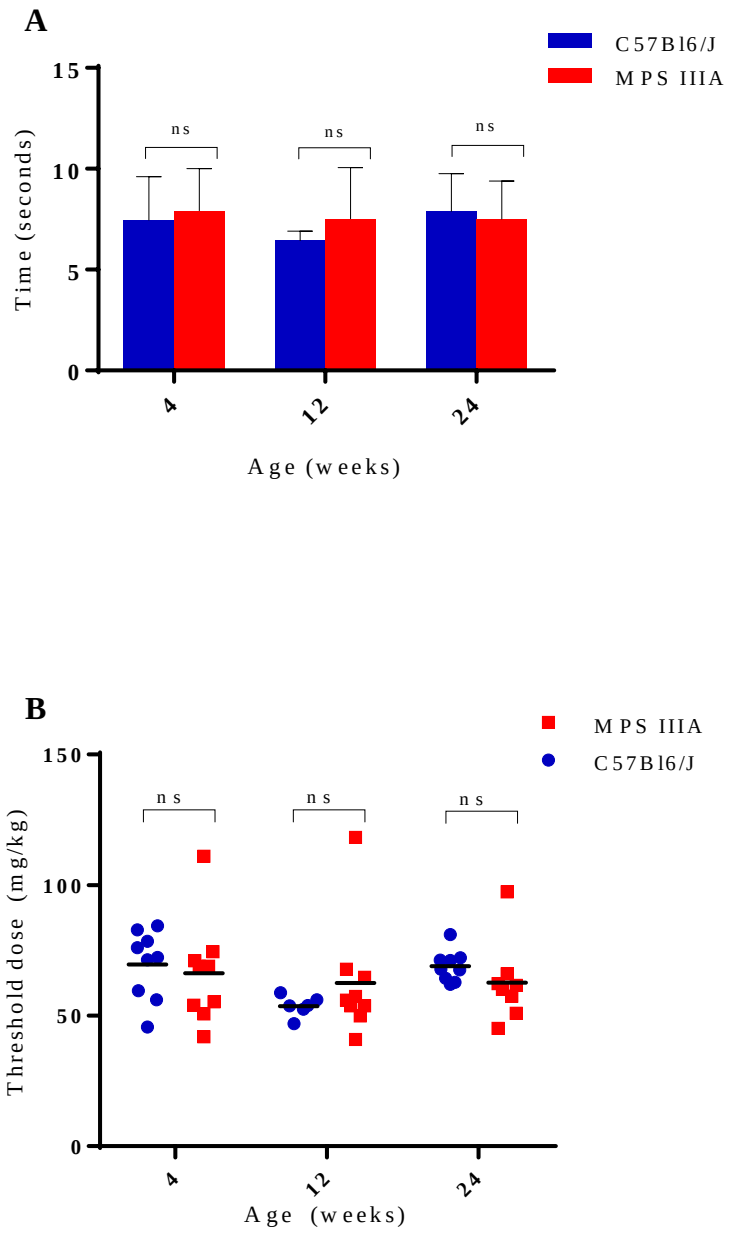


Figure 9 PTZ-induced Seizures via IV Infusion

PTZ was administered at 10 mg/mL at a constant rate of 50 uL/min. The onset of the first sign of seizure and a full blown epileptic episode was recorded. Threshold doses at mg/kg was calculated based on animal body weight, PTZ concentration, the IV infusion rate and seizure onset (seconds). (A) Seizure thresholds dose did not show a significant difference at 4, 12 and 24 weeks. The same dose elicit similar seizures in the animals (B) The time latency (s) for the onset of stage 3 seizure was not significant, p=0.58. This data was not normalized to animal body weight.

3.2.2 There was a delay in the onset of PTZ-induced IP kindling

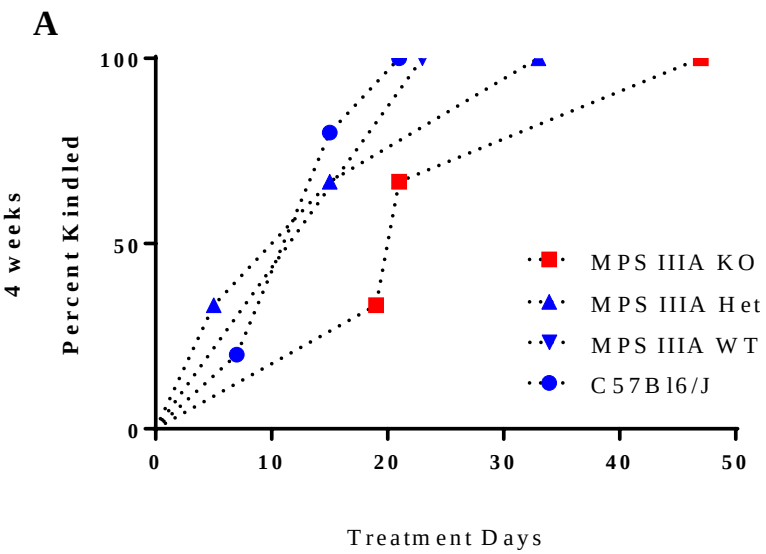
An acute method of inducing seizure with PTZ was not able to show differences in seizure thresholds. We then examined if by using a chronic kindling method, we can see a defect in the adaptive changes in seizure susceptibility in MPS IIIA mice as compared to normal mice. Animals were administered with a subconvulsive dose of PTZ at 40 mg/kg every other day and observed for seizure onset, stages and time to kindle.

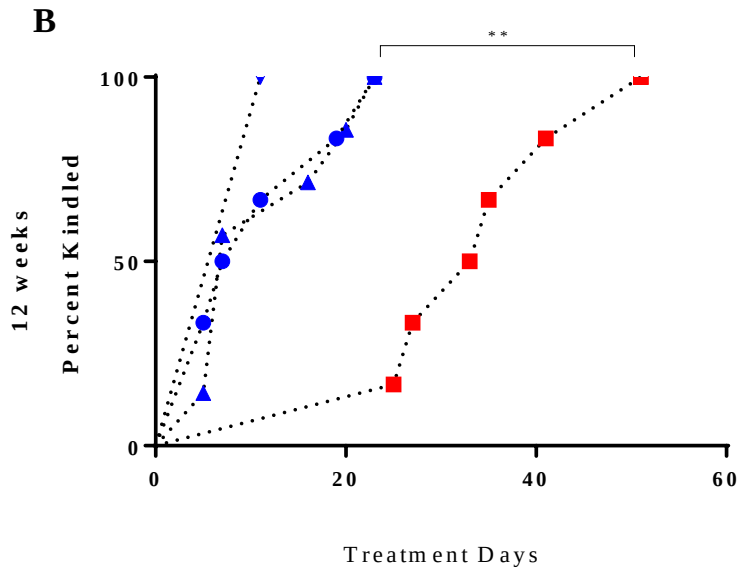
The kindling results are presented in Figure 10. Unexpectedly, there was a consistent delay in the kindling behavior of MPS IIIA mice starting at 4 weeks. On average, 72% of all the animals tested kindled. For MPS IIIA mice: 60% of the 4 week old mice kindled after 22 treatments, 67% after of the 12 week old mice kindled after 24 treatments and 60% of the 24 week old mice kindled after 18 treatments. For C57Bl6/J mice: 83% of the 4 week old kindled after 10 treatments, 100% of the 12 week old mice kindled after 11 treatments and 60% of the 24 week old mice kindled after 17 treatments. For MPS IIIA heterozygous mice: 40% of the 4 week old mice kindled after 16 treatments, 88% of 12 week old mice kindled after 12 treatments, 57% of 24 week old mice kindled after 5 treatments. For MPS IIIA WT mice: 67% of 4 week old mice kindled after 10 treatments, 67% of 12 week old mice kindled after 11 treatments and 50% of 24 week old mice kindled after 4 treatments. The delay in kindling was found to be significant at 12 weeks, $p=0.0015$, and 24 weeks, $p=0.0027$. Our findings with the chronic IP kindling seem to indicate that MPS IIIA mice are resistant to repeated subconvulsive PTZ stimulation.

3.2.4 Similar PTZ levels at a single dose of 40 mg/kg

To confirm that the PK of PTZ is not altered in MPS IIIA mice and that the kindling effect was not due to changes in PTZ brain penetration, we measured brain and plasma PTZ levels at 5 and 15 minutes following a subconvulsive dose of 40 mg/kg.

As shown in Figure 11, PTZ brain concentrations for MPS IIIA were comparable to normal controls (wildtype and heterozygous) at both time points. At 5 and 15 minutes, the PK trends the same with that of reported PTZ curves at 55 and 85 mg/kg doses (Yonekawa, Kupferberg e Woodbury, 1980). Furthermore, plasma concentrations (Figure 11B insert) indicated good peripheral distribution for PTZ. This data agrees with prior work showing that PTZ biodistributes readily and its availability is only limited by the dose levels (Mandhane, Aavula e Rajamannar, 2007).





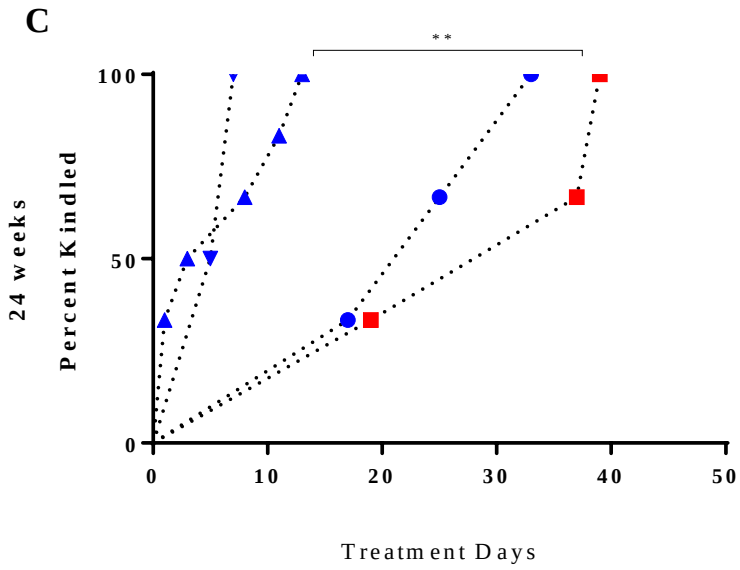


Figure 10 PTZ-induced Seizures via IP Kindling

Male 4, 12 and 24 week MPS IIIA mice received a single IP dose of PTZ at 40 mg/kg every other day until they kindled. Kindling was achieved after 3 consecutive PTZ dose produced a stage 3 seizure. MPS IIIA WT and Heterozygous animals were added as controls. (A) There was a delay in kindling starting at 4 weeks. (B-C) A significant difference in onset of kindling is seen at 12 weeks, **p=0.0015, and at 24 weeks with the **, p=0.0027.

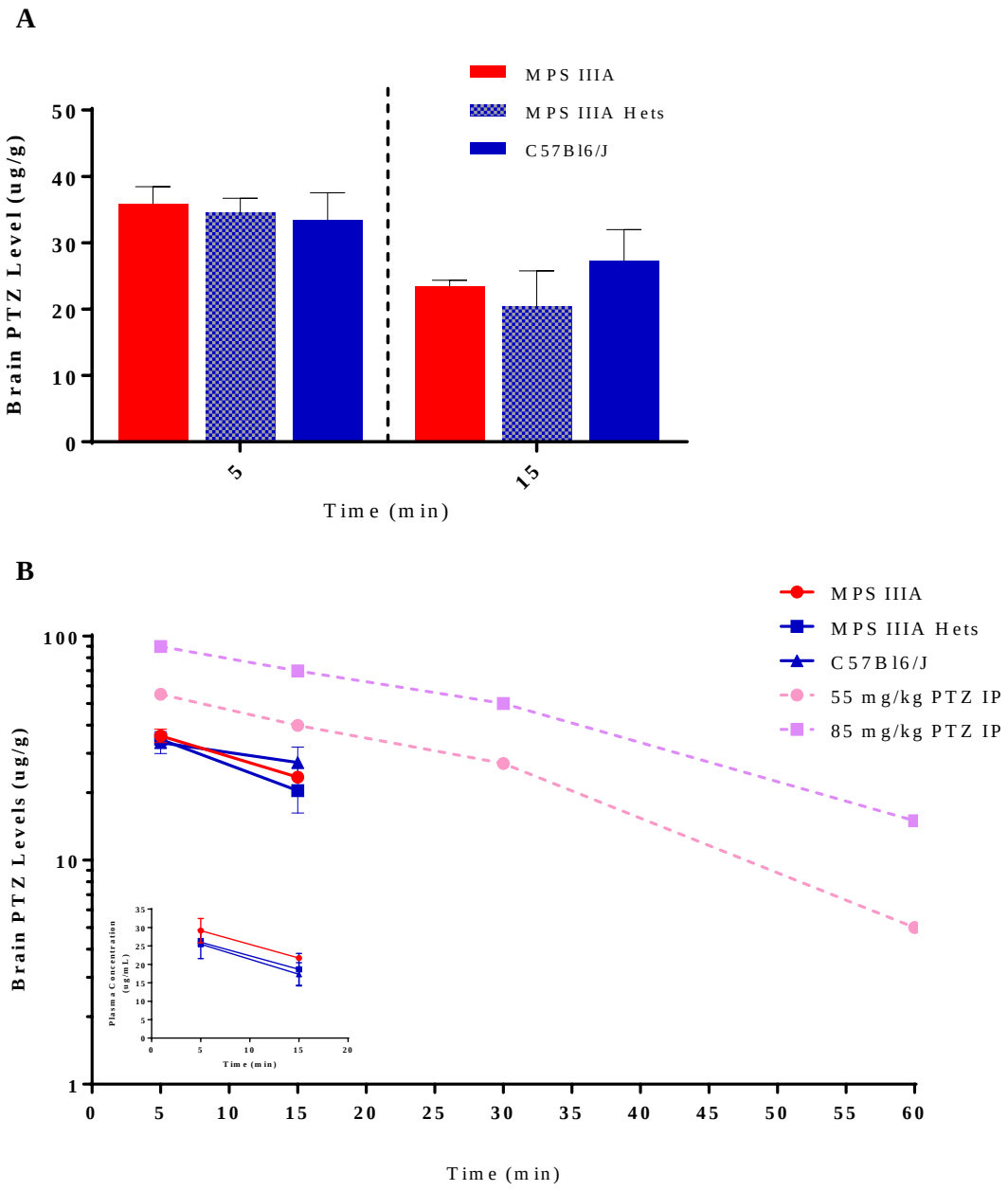


Figure 11 PTZ Brain Levels

Animals received a single IP dose of PTZ at 40 mg/kg. Brain and plasma (insert) were collected at 5 and 15 minutes. Solid colored lines are PTZ levels from in-house study. Lighter broken lines are published PTZ levels. (A) Brain PTZ levels were the same for all animals tested at 5 and 15 minutes. (B) The PK curve of PTZ seems to follow what has been reported for PTZ PK levels at 55and 85 mg/kg (Yonekawa, Kupferberg e Woodbury, 1980).

4. DISCUSSION

In this study, we investigated potential early CNS phenotypes in the MPS IIIA mice: spatial learning and memory and seizure threshold. In general, we found that both the Barnes maze and novel object recognition assays do not detect a robust phenotype earlier than 20 weeks. But, with the PTZ seizure assay, CNS stimulation showed a significant delay in kindling response by 12 weeks, with a slight trend as early as 4 weeks. Based on published results, animal work with the MPS IIIA mouse model has not been able to consistently demonstrate CNS-related defects earlier than 20 weeks of age. The seizure sensitivity data in diseased animals can be exploited to advance the understanding of disease biology. Furthermore, a seizure assay can be developed to screen for viable therapies.

4.1 Barnes Maze fails to detect cognitive defects in MPS IIIA mice

The Barnes maze was used to test spatial learning and memory in MPS IIIA mice. Prior work has shown that BM can measure CNS-related cognitive impairments. Our analysis however, did not show a performance difference between normal and MPS IIIA mice at 4, 12 and 24 weeks of age. Learning abilities appeared normal as both the control and diseased animals consistently learned to locate the escape box with the same amount of trials. Memory also appeared intact as the mouse was able to locate the escape box at 24 hours, 48 hours and even 15 days after having learned the task. This data suggests that there may not be a significant cognitive impairment in MPS IIIA animals before 24 weeks of age in the BM.

The results of the BM conflict with reported results using MWM. In MWM, a difference in spatial learning and memory has been reported in various publications between normal and MPS IIIA mice (Crawley *et al.*, 2006; Fraldi *et al.*, 2007). A possible confounding factor between these two assays is stress. Stress may have increased the sensitivity to cognitive defects in MWM. Mice that were subjected to both MWM and BM assays were tested for the stress hormone, corticosterone. Corticosterone levels were higher in animals subjected to MWM compared to BM tests (Chu *et al.*, 2003; Harrison, Hosseini e Mcdonald, 2009). BM-tested animals, after corticosterone injection, showed greater impaired memory, confirming that stress can directly impact performance (Harrison, Hosseini e Mcdonald, 2009).

Performance can also be altered by other stress-induced behaviors that are different between BM and MWM, such as thigmotaxis and passivity. Thigmotaxis is when an animal swims closer to the wall, "wall-hugging", and passivity is the reduction in swimming speed or frequent floating. Both are behaviors exhibited by the animals when using the MWM that occur in response to a stressful environment. These behaviors can affect the results of a MWM experiment. As the animal's anxiety increases, the time latency for escape may also increases. There may be a difference in stress response between normal and MPS IIIA animals that can confound the analysis of the maze results. The data can be misinterpreted as a decrease in spatial learning and memory specifically, when in fact there was minimal or no defect at all. Thigmotaxis and passivity are not noted in the Barnes maze.

The differences in the mazes likely affect the kind of data being generated. The MWM do not report a phenotype earlier than 20 weeks. It's possible that the stress factor

present allowed some distortion of the parameters being measured. This fact about the MWM brings into question the robustness of the maze. The BM being a potentially less stressful version of MWM did not reveal any differences even by 24 weeks. Although the BM has been established for memory assessment, it's possible that it is not sensitive enough to identify phenotypic differences in the MPS IIIA mice. To further determine if there are learning and memory deficits in the diseased mice before 24 weeks when some of the problems in the mazes are addressed, we employed the novel object recognition assay.

4.2 Poor recognition and general lack of interest in objects for MPS IIIA mice

The NOR is an attractive test to use as it does not require long animal training and offers no external motivation, positive reinforcement or punishment as compared to BM or MWM (Antunes e Biala, 2012). Furthermore, novelty recognition requires a higher cognitive skill from the mice since the memory is only presented once, relative to repetitive training of the behavior in BM (Ennaceur e Delacour, 1988). The NOR is an assay for a more natural cognitive behavior in the animal.

Our data showed recognition memory deficits by 24 weeks. This agrees with prior work showing altered cognitive behavior at this age (Hemsley e Hopwood, 2005; Fraldi *et al.*, 2007; Lau *et al.*, 2008; Sorrentino *et al.*, 2013). But data from these other assays (water maze, open field or negative geotaxis) was able to discern a significant decrease in performance for the MPS IIIA animals. The range of difference in the NOR was small. In addition, it appears that the MPS IIIA mice had an overall reduced interest in exploring objects. Thus, it is difficult to interpret whether the loss of novel object recognition should be attributed to the inability to remember a familiar object or to the lack of interest for

objects in general. This decrease in exploratory behavior, even at the initial presentation of the objects, could have reduced the resolution of the data.

While NOR and MWM or BM are able to track CNS disease progression in MPS IIIA mice with greater sensitivity, they are not direct measurements of CNS activity. An assay that can challenge the CNS directly in order to elicit a specific neurological reaction can perhaps measure an early CNS phenotype in MPS IIIA mice.

4.3 A significant effect in PTZ chronic kindling in MPS IIIA mice

Although seizure is prevalent in MPS IIIA patients, it has not been reported in the mouse model. We chose to induce seizure using a convulsive drug for the following reasons. First, drug-induced seizures produce brain related phenotypes, as the compound we used, in this case PTZ, targets neurotransmitter receptors in the CNS. Second, drug levels and subsequent seizure induction can be controlled to accurately measure changes in intensity and threshold of seizure. Lastly, Seizure induced by PTZ is a well-known assay to quantify seizure onset and has been used to determine drug efficacy for AEDs (Orloff, Williams e Pfeiffer, 1949; Mandhane, Aavula e Rajamannar, 2007).

We utilized two seizure assays to test different aspects of seizure threshold, the IV infusion and IP kindling. The IV infusion has been reported to be an accurate technique to record seizure threshold doses in mice (Mandhane, Aavula e Rajamannar, 2007). The IV infusion conceivably can detect neurotransmitter defects that are present in the CNS. Initially anticipating that there may not be a major difference in neurotransmitter balance or GABA receptor activity in MPS IIIA mice but there may be a difference in how the CNS adapts to PTZ stimulation, a repeated dose of PTZ was administered. The repeated administration of a sub convulsive dose of PTZ elicits an imbalance in neuronal activity

over time (Da Silva, Pereira e Elisabetsky, 1998; Dhir, 2012). This is defined as kindling. Similar to published reports, we were able to score the stages and timing of seizure development in both the IV infusion and IP kindling methods (Da Silva, Pereira e Elisabetsky, 1998; Dhir, 2012).

PTZ via an IV infusion elicited the same seizure effect in both normal and MPS IIIA animals. The fact that a similar PTZ dose elicited the same seizure excitation in the animals (Figure 12A and 12B) may mean that there is no defect in GABA receptor or neurotransmitter imbalance in MPS IIIA mice. The infusion data here supports the BM results obtained, where there was no difference between the WT and MPS IIIA at any age.

Surprisingly, our data revealed a delay in seizure kindling when MPS IIIA mice were repeatedly stimulated (Figure 12D). This result is interesting because based on human clinical data we hypothesized that MPS IIIA mice would be more sensitive to seizure induction and would have a reduced threshold to PTZ compared to control mice. Instead, MPS IIIA mice appear to be more resistant to PTZ. When we measure the PTZ levels in the brain, the concentrations were the same in MPS IIIA and normal mice. This demonstrates that the distribution of PTZ is similar between mice and thus, validates the seizure phenotype.

As unexpected as our initial seizure data is, it opens new avenues for opportunities to fully understand the nuerological consequences of lysosomal function impairement to maintaining homeostatis in the brain. In order to expand our understanding of how brain mechanisms adjust in the presence of an insult, glutamate and GABA activity can be measured to determine the stability of these neurotransmitters with chronic PTZ dosing. This will begin to elucidate the molecular mechanism involved. In addition, rescue of the

disease with gene therapy as has been reported (Fraldi *et al.*, 2007; Aronovich e Hackett, 2015). Future experiments attempting to genetically correct the disease in the CNS of young animals can determine whether seizure is a reliable phenotype to measure for an assay.

PTZ induction produced the earliest CNS specific phenotype recorded. Kindling difference was statistically significant at 12 weeks with a large range for assay development. The subsequent normalization of the seizure threshold will validate PTZ kindling as a method for assessing the benefit of novel drug therapies for MPS IIIA.

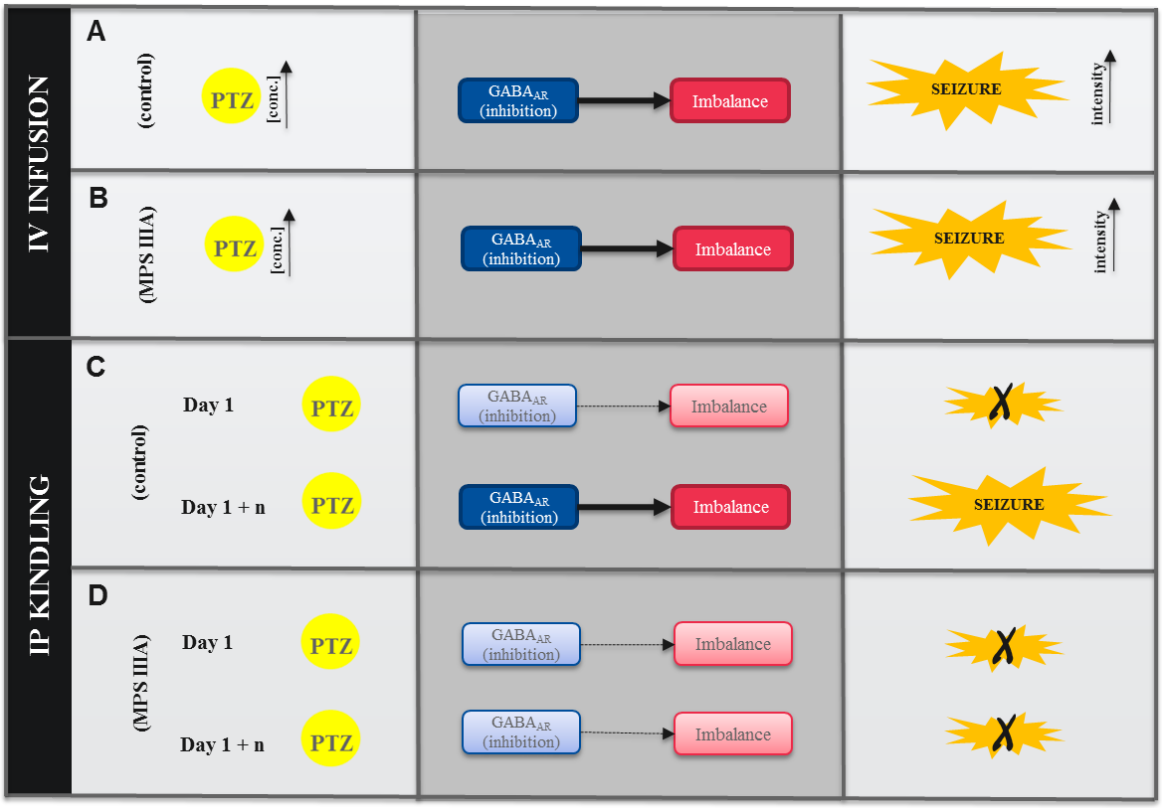


Figure 12 Depiction of PTZ-induced Seizure Responses in MPS IIIA mice

Shaded middle area indicates a possible mechanism of action starting with GABA receptor inhibition by PTZ and resulting in an imbalance of brain neurotransmitters to cause seizures. (A) In a normal animal, increasing concentration of PTZ by way of IV infusion leads to increase in seizure intensity. (B) In a MPS IIIA diseased animal, increasing PTZ also increases seizure intensity. (C) During the kindling experiments, a sub-convulsive dose of PTZ is delivered by IP injection. On day 1, no seizure is observed. Subsequent doses of the same amount of PTZ eventually sensitizes the animal leading to seizure. (D) The same frequency of sub-convulsive PTZ dosing does not appear to affect MPS IIIA mice.

5. CONCLUSION

This thesis project presents findings on an early CNS behavior phenotype in MPS IIIA mice. We are the first to characterize seizures across three age groups of MPS IIIA animals. In contrast to the data obtained with the learning and memory assays, PTZ-induced seizures yielded a significant difference at 12 weeks. The delayed onset of kindling seizure was unexpected but nonetheless, this was an early phenotypic change that appears to have a wider range of difference between control animals to warrant further investigation and assay development. Our results can therefore help establish a working *in vivo* assay, where seizure is a possible endpoint in the MPS IIIA disease model.

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