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## Investigation of behavioral and cellular changes in the Maternal Immune Activation model of autism spectrum disorders

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*University of Nebraska Medical Center*

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**Investigation of behavioral and cellular changes in the  
Maternal Immune Activation model of autism spectrum  
disorders**

A THESIS

By

**Shreya Roy**

Presented to the Faculty of the University of Nebraska Graduate  
College in Partial Fulfillment of the Requirements for the  
Degree of Master of Science

Biochemistry and Molecular Biology  
Graduate Program

Under the supervision of Dr. Anna Dunaevsky

University of Nebraska Medical Center  
Omaha, Nebraska

May 2015

**Advisory Committee**

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Shreya Roy

May 1 2015

## ABSTRACT

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University of Nebraska, 2015

Advisor: Anna Dunaevsky, PhD.

Maternal infection during pregnancy, which leads to maternal immune activation (MIA), is an environmental risk factor for autism spectrum disorders (ASD). MIA can be induced in mice and their offspring exhibit behaviors that model the core symptoms of ASD. One of the core behavioral symptoms in ASD patients is presence of increased repetitive behavior, which is modeled by an increase in marble burying in MIA mice. It has been shown that the deficits seen in MIA mice are associated with the dysregulation of cytokine levels in the developing brain, specifically an increase in pro-inflammatory cytokines. In this thesis, I tested whether a reversal of this inflammatory state in the brains of MIA mice by administration of an anti-inflammatory drug, Ibudilast, can ameliorate the behavioral deficit. The results indicated that MIA mice exhibit increased marble burying which is normalized by prenatal administration of Ibudilast without significant effects on control mice. I also tried to establish an additional paradigm of repetitive behavior in the MIA mice, using the rotarod and found that MIA mice have a trend towards increased stereotyped motor routine as measured by an accelerating rotarod test.

Altered behaviors in MIA mice are thought to be mediated by impaired synaptic connectivity. Our lab recently showed structural and functional alterations in synapses in the MIA offspring. An important component of the synapse are astrocytes, a type of glial

cell, that regulate synapse formation and function. Whether astrocytic development and function are impaired in MIA offspring is not known. Because cytokines have been shown to modulate the release of transmitters from astrocytes it is possible that increase in inflammatory state in MIA offspring can affect the signaling in astrocytes. Based on these observations, I hypothesized that astrocytic calcium activity is altered in the brains of MIA offspring and proposed to study the same using in-vivo astrocytic calcium imaging.

## CONTENTS

ACKNOWLEDGEMENT .....	i
ABSTRACT.....	ii
CONTENTS.....	iv
Chapter 1- INTRODUCTION .....	1
1.1 Maternal infection during pregnancy: risk factor for ASD- epidemiologic evidence .....	1
1.2 Immune dysfunction in ASD .....	1
1.3 Rodent models of maternal immune activation .....	3
1.4 Effect of maternal immune activation on the synapse .....	6
1.5 Molecular mechanisms of synaptic deficits in MIA model .....	7
1.6 Role of astrocyte in synaptic development and function .....	9
1.7 Modeling behavioral deficits in ASD .....	11
Chapter 2- EXPERIMENTAL PROCEDURES- .....	14
2.1 Induction of MIA-.....	14
2.2 Marble burying- .....	14
2.3 Rotarod- .....	15
2.4 Breeding strategy for GCAMP3 transgenic mice- .....	15
2.5 GCAMP3 expression- .....	16
2.6 Craniotomy- .....	16
2.7 <i>In vivo</i> multiphoton imaging-.....	16
2.8 Image analysis- .....	17

2.9 Calculation of frequency, amplitude and duration of calcium transients- .....	17
Chapter 3- RESULTS AND DISCUSSION.....	18
3.1 Experiment 1- To test whether treatment with anti-inflammatory drug Ibudilast (AV-411) ameliorates marble burying deficits in MIA mice .....	18
3.1 a) Discussion-.....	20
3.2 Experiment 2- To establish a new behavioral paradigm for MIA offspring using the rotarod.....	23
3.2 a) Discussion.....	25
3.3 Experiment 3- To compare basal levels of astrocytic calcium activity in control and MIA offspring.....	27
3.3 a) Discussion.....	31
REFERENCES .....	33

## **Chapter 1- INTRODUCTION**

### **1.1 Maternal infection during pregnancy: risk factor for ASD- epidemiologic evidence**

Neural development is a highly orchestrated process, and any disturbance in the early development of the CNS has been linked to neurological disorders, later in life<sup>1</sup>. There is a dynamic interplay between genetic, epigenetic and environmental factors, which influences early neural development. One such environmental factor which has been implicated as a risk factor for autism spectrum disorders (ASD) is maternal infection<sup>2</sup>. Epidemiologic studies have shown an association of maternal viral or bacterial infection in the first and second trimester of pregnancy, respectively, to a diagnosis of ASD in the offspring<sup>2</sup>. An early environmental insult like this induces a response of the maternal immune system and is capable of affecting sensitive aspects of fetal brain development. Here, I review the existing literature about how prenatal maternal immune activation (MIA) can be a risk factor for ASD, what effects does MIA have on the synapse and what are the known molecular mechanisms mediating these effects.

### **1.2 Immune dysfunction in ASD**

Studies have demonstrated the presence of a neuroinflammatory state in autism patients. One of the initial studies in this field investigated post-mortem brain tissue of ASD patients; and revealed activation of neuroglia, a high prevalence of neuroglia-derived cytokines, and an increase in pro-inflammatory cytokines in the cerebrospinal fluid of autism patients<sup>3</sup>. This study was among the first to demonstrate immune dysfunction in

the pathogenesis of ASD. Later studies provided pathological evidence to show activated microglia and increased microglial density in the dorsolateral prefrontal cortex in autism<sup>4</sup>. Such activated microglia had enlarged soma, retracted and thicker processes and extending filopodia from the processes, which might play a role in the pathogenesis of the disease. Microglia are the resident immune cells (macrophages) of the brain and constitute the first line of active immunological defense in the CNS. Usually present in the resting state, they are activated upon an immune challenge or insult in the brain, such as disease or injury. They are recruited to the site of damage, where they phagocytose debris as well as dying or unwanted cells<sup>57</sup>. Apart from mediating immune responses to infection and trauma, microglia have been shown to play important roles in the patterning and wiring of the developing, healthy, CNS<sup>57</sup>.

They participate in programmed cell death or apoptosis during neural development, by phagocytosing excess neurons as well as mediating the process of apoptosis using molecular cues<sup>57</sup>. Using their processes, microglia have been shown to survey the functional state of the synapses<sup>57</sup> and they also participate actively in the formation of eye-specific territories by engulfing retinal ganglion cell synaptic inputs to dorsal lateral geniculate nucleus (visual thalamus) <sup>58</sup>. Microglia are also key players in synapse function and maturation. It has been shown that knockout of the fractalkine receptor present on microglia, CX3CR1, led to the reduction in functional connectivity between two different regions of the brain<sup>59</sup>. Abnormal synaptic pruning due to the disruption of neuron-microglial signaling, is thought to be the reason for this reduction in connectivity<sup>59</sup>.

Activation of microglia is marked by morphological changes accompanied with release of various immunomodulatory signals. Cytokines and chemokines constitute a major component of these signals where they participate in regulating the immune response, and very importantly dictate the movement of leukocytes to the CNS, thereby contributing to neuroinflammation.

Evidence of involvement of cytokines in ASD, came from a study, which compared the levels of the cytokine tumor necrosis factor-alpha (TNF- $\alpha$ ) in the serum and cerebrospinal fluid of children with autism<sup>5</sup>. The pro-inflammatory cytokine TNF- $\alpha$  is released predominantly by the glia in the brain and has many physiological roles in normal brain functioning. An approximate 8-fold increase in the levels of TNF- $\alpha$  in the cerebrospinal fluid as compared to the serum was reported<sup>5</sup>. This suggested an isolated CNS immunological response in autism, independent of any systemic alterations. However, systemic alterations have also been observed. Elevation in the levels of chemokines like MCP-1, RANTES, eotaxin and non-chemokine cytokines like IL-6 have been observed in the plasma of children with ASD as compared to normally developing children<sup>6,7</sup>. Also, the elevations matched with the severity of the core behavioral symptoms of autism<sup>6,7</sup>. Although the mechanisms linking immune dysregulation and neural dysfunction had not completely been elucidated, these studies pointed towards a role of cytokines and chemokines in impaired neurodevelopment.

### **1.3 Rodent models of maternal immune activation**

The finding that maternal infection is a risk factor for autism necessitated suitable rodent models to study the mechanism of development of the disease. Infection in the mother was introduced through different mechanisms. Initial studies did respiratory infection of

pregnant mice with human influenza virus<sup>8</sup>. The offspring showed hyperanxiety in novel or stressful situations, which is common in autism patients<sup>8</sup>. In trying to investigate whether the behavioral alterations observed were due to the viral infection of the fetus or the maternal immune response, the investigators found that the effects of the viral injection could be achieved by intraperitoneal injection of the synthetic double-stranded RNA poly (I:C) (PIC) to the mother during pregnancy. This showed that the maternal immune response, even in the absence of the virus, was sufficient to cause the behavioral changes observed in the offspring<sup>8</sup>. The placental transfer of maternal antibodies against the viral mimic, PIC, possibly contributed to the behavioral deficits in the offspring. The pathogenicity of placentally transferred maternal antibodies was demonstrated in a later study, in which human IgG from mothers of children with autistic disorder (MCAD) were administered to pregnant mice. The offspring of these mothers showed altered behavior observed in autism such as hyperactivity and less sociability<sup>9</sup>. Behavioral patterns observed in the offspring of immune activated mothers were also critically dependent on the gestational age at which the infection was administered<sup>10</sup>.

Later, a well characterized model of maternal immune activation was established, which displayed the three core symptoms of autism- deficits in social interaction, language and the presence of repetitive/stereotyped behaviors in the offspring of mothers injected with PIC.<sup>12</sup>

A mechanism by which maternal immune activation causes alteration of fetal brain development was demonstrated in a study showing that elevation of the pro-inflammatory cytokine IL-6, was responsible for the behavioral deficits observed upon maternal immune activation<sup>11</sup>. A single maternal injection of IL-6 on embryonic day 12.5 of

mouse pregnancy was shown to cause prepulse inhibition (PPI) and latent inhibition (LI) deficits in the adult offspring, behaviors which model deficits in sensorimotor gating<sup>11</sup>. Upon co-administering an anti-IL-6 antibody in the PIC model of MIA the deficits caused by PIC were prevented<sup>11</sup>. Also, MIA in IL-6 knock-out mice did not result in several of the behavioral changes observed upon activating the maternal immune system<sup>11</sup>. These findings gave rise to the thought that the balance between pro- and anti-inflammatory influences was critical in fetal brain development. Further investigation into the mechanism by which IL-6 influenced fetal brain development showed that maternal immune activation led to an increase in both maternally derived IL-6 mRNA and protein in the placenta<sup>14</sup>. The placenta also showed an increase in various immune cells such as macrophages, granulocytes and uterine NK cells, which were indicative of early immune activation<sup>14</sup>.

A proposed model tries to explain the effects mediated by IL-6 in MIA on the placenta and thereby fetal development<sup>14</sup>. Upon injection of PIC to mother, the maternal immune system is activated via a TLR3- mediated anti-viral response<sup>14</sup>. Activated TLR3<sup>+</sup> cells secrete pro-inflammatory factors, such as IL-6, into the maternal bloodstream<sup>14</sup>. Circulation of maternal blood containing IL-6 through the placenta leads to increase in IL-6 in the decidua and spongiotrophoblast, which leads to activation of the resident immune cells in the decidua, and they propagate the inflammatory response by further cytokine release<sup>14</sup>. IL-6 release from decidual cells acts on target cells in the spongiotrophoblast layer by paracrine signaling<sup>14</sup>. IL-6 ligand upon binding to receptor IL6Ra and gp130 leads to JAK/STAT3 activation and downstream changes in gene expression leading to reduced GH (growth hormone), IGF-1 (insulin-like growth factor-

1) and IGFBP3 leading to disruption of the GH-IGF axis<sup>14</sup>. Also, there is alteration in production of placenta-specific prolactin like proteins (PLPs)<sup>14</sup>. These changes in endocrine factors in the placenta has effects on fetal development<sup>14</sup>.

The MIA model was utilized to study long-term changes in the brains of the offspring of MIA mice. Multiplex array analysis studies showed that MIA leads to age and region-specific changes in the levels of different cytokines in the brain and sera of the offspring<sup>13</sup>. These cytokines are both pro- and anti-inflammatory. This study provided further support to the importance of these molecules in the mechanism of disease development in ASD. Other abnormalities observed in the brains of MIA offspring include altered cerebellar pathology, similar to what is observed in the brains of autism patients<sup>15</sup>. In conclusion, these studies demonstrated how immune activation of the mother can have global and long-lasting effects on brains of the offspring.

#### **1.4 Effect of maternal immune activation on the synapse**

Maternal infection can affect any step in neural development, and synaptogenesis is one of the most critical steps in neural development. Current research in the field of understanding the mechanisms of development of ASD has a strong focus on the role of the synapse<sup>60</sup>.

A study of dendritic spines, the post-synaptic compartment of excitatory synapses, on cortical pyramidal cells revealed an increase in dendritic spine densities in ASD patients as compared to age-matched controls<sup>19</sup>. In fact, some of the genetic mutations which confer a risk for autism, are present in synaptic genes. e.g, point mutations in Shank3, which is a post-synaptic scaffolding protein, have been identified in ASD patients<sup>17,18</sup>.

Other examples of synaptic genes mutated in ASD patients are Tsc2, Fmr1, Neuroligin-3, Neurexin-1 etc. The interrelatedness of the functions of these proteins mean that the defect in any one of these proteins is capable of triggering a cascade of reactions affecting other synaptic proteins and therefore leading to synaptic network deficits.

The only study to have directly looked at the effect of MIA on synapses, showed a reduction in synaptic density in cultured cortical neurons from MIA neonatal offspring<sup>25</sup>. In other studies which examined the effect of MIA on synaptic function, induction of inflammation, prenatally, using lipopolysaccharide (LPS), was shown to give rise to altered glutamatergic transmission in the adult offspring<sup>20</sup>. This effect was shown to be mediated by activated microglia at birth, due to prenatal inflammation<sup>20</sup>. Also, examination of synaptic properties in hippocampal CA1 pyramidal neurons of the offspring of PIC-treated mothers showed reduced frequency and increased amplitude of miniature excitatory postsynaptic currents<sup>21</sup>. Very recently, it has also been shown that the adult offsprings of MIA mothers have shown deficits in hippocampal long term potentiation<sup>27</sup>, a cellular mechanism of synaptic strengthening. All these examples give evidence of the fact that maternal immune activation is indeed capable of affecting synaptic structure and function. Also, these findings lend credence to the school of thought that synaptic deficits may be the starting point of neural dysfunction in ASD.

### **1.5 Molecular mechanisms of synaptic deficits in MIA model**

The elucidation of the link between prenatal inflammation and synaptic impairments leading to cognitive dysfunction is critical for understanding the mechanisms of development of ASD and also for identification of therapeutic targets. Immune molecules, which have been shown to be dysregulated in the MIA offspring, are now

known to play essential roles in the healthy brain such as establishment, function and alterations of synaptic networks. These include cytokines like IL-6 and TNF- $\alpha$ , complement proteins such as C1q and C3 and MHCI (Major Histocompatibility Complex I) family members.

It is now known that MHCI expression in the normal, developing brain plays a critical role in regulating synaptogenesis. In early post-natal brain development, MHCI was shown to negatively regulate the density and function of cortical synapses, during their initial establishment<sup>24</sup>. Also, expression of MHCI was needed for refinement of synaptic inputs to Lateral geniculate nucleus (LGN) neurons, so that eye-specific separation of connections is achieved<sup>22,23</sup>. These findings showed the role of MHCI in regulating structural and functional synaptic plasticity. Hence, it can be expected that disruption or alteration of MHCI expression can lead to synaptic deficits thereby leading to cognitive dysfunction such as ASD. Evidence of immunological dysfunction in ASD lends support to this argument. Investigation into how MHCI exerts its effect on synapses, has shown that MHCI, in an activity-dependent manner, activates a pathway that also contains the transcription factor, MEF2, and this pathway plays a role in negatively regulating synapse density<sup>25</sup>. In fact, cultured cortical neurons from MIA neonatal offspring show a reduction in synaptic density, and an increase in the levels of MHCI and MEF2<sup>25</sup>. This deficit in MIA offspring requires increased MHCI-MEF2 signaling<sup>25</sup>. This study elucidated the mechanism by which MHCI plays an important role in the early post-natal brain development of MIA offspring.

The initiator of the complement cascade pathway, C1q, has been shown to be expressed in developing brains and participate in synaptic elimination, thereby fine-tuning the

synaptic connections<sup>26</sup>. This finding is further supported by the observation of enhanced synaptic connectivity and epilepsy in C1q knockout mice<sup>27</sup>. However, the role of C1q mediated synaptic pruning in MIA has not yet been explored, but it is possible that it may also have a role.

Further investigations into the molecular mechanisms of synaptic deficits, which utilize the MIA model can shed light upon how critical synaptic deficits are in the development of the disease, and can also provide targets for reversal for these deficits, to test whether reversal of synaptic deficits can take away any of the symptoms associated with ASD.

### **1.6 Role of astrocyte in synaptic development and function**

In the CNS, the synapses are ensheathed by astrocytes, which are glial cells. The intimate association, both structural and functional, of the perisynaptic astrocytes with the synapse, has led to the concept of the ‘tripartite synapse’<sup>32</sup>. In this, synapses are defined as being composed of the presynaptic and postsynaptic specializations of the neurons, and the astrocytic processes ensheathing them<sup>32</sup>. Functionally, besides supporting neuronal function by regulating blood flow, providing energy to neurons and supplying the building blocks of neurotransmitters, astrocytes also express receptors for neurotransmitters, which allow them to sense neuronal activity and respond to it<sup>33</sup>. Astrocytes respond to neuronal activity by making changes in intracellular calcium ion concentrations<sup>33</sup>. Fluctuations in intracellular calcium ion concentrations are a manifestation of astrocyte excitability, although they are electrically non-excitable, unlike neurons. Besides neuron-dependent excitation, astrocytes can also exhibit spontaneous  $\text{Ca}^{2+}$  oscillations, which are independent of any neuronal input<sup>36</sup>. Closely linked with these fluctuations in calcium levels are the release of various neurologically active

substances or gliotransmitters, such as ATP and D-serine<sup>33</sup>. By the release of these factors, the astrocytes are capable of modulating synapses such as controlling synapse formation, elimination and synaptic connectivity<sup>33</sup>. There are other mechanisms by which astrocytes are capable of modulating synapses, which are dependent on  $\text{Ca}^{2+}$  elevations in astrocytes. It has been shown that stimuli which induce synaptic LTP in the hippocampus, also enhance the motility of astrocytic processes associated with these synapses<sup>61</sup>. This motility is dependent on  $\text{Ca}^{2+}$  elevations in astrocytes and also leads to changes in the ability of astrocytes to regulate synaptic transmission<sup>61</sup>. These were shown in hippocampal slice preparations. Similar observations were also made in vivo, where sensory stimuli which led to increases in astrocyte  $\text{Ca}^{2+}$ , induced structural plasticity in astrocytic processes and dendritic spines in the mouse somatosensory cortex<sup>61</sup>. The structural plasticity of astrocytic processes in response to  $\text{Ca}^{2+}$  elevations was also correlated with spine coverage and stability<sup>62</sup>.

Cytokines have been shown to have an effect on gliotransmission. Glutamate release from astrocytes has been shown to be controlled by inflammatory molecules such as  $\text{TNF-}\alpha$  and prostaglandins, by a calcium dependent mechanism<sup>38,39</sup>. These results suggest that production of these mediators in pathological conditions, may affect gliotransmission and astrocyte excitability.

The metabolic coupling between neurons and astrocytes has been shown to be affected in ASD. Astrocytes communicate within multicellular syncytium of astrocytes and neurons using connexin-based gap junctions (specifically Cx43)<sup>40</sup>. It has been shown that in ASD patients, there is an elevation of Cx43 expression in astrocytes, which will affect the astrocytic syncytia<sup>41</sup>. Also, astrocytes are an important component for the glutamate-

glutamine shuttle, which is critical for excitatory glutamatergic signaling in neurons. Glutamatergic signaling has been strongly involved in the pathology of ASD because alterations in excitatory signaling are critical for cognitive functions such as memory and learning, which are impaired in ASD patients<sup>40</sup>. Alterations in synaptic glutamate concentrations have been shown in a model of autism<sup>42</sup>, and patients with ASD have been shown to have abnormalities in the glutamate transporters in the cerebellum<sup>43</sup>.

However, the contribution of astrocytes in the MIA model remains unexplored. Here, I have hypothesized that MIA results in altered astrocytic  $\text{Ca}^{2+}$  signaling resulting in altered synaptic development.

### **1.7 Modeling behavioral deficits in ASD**

ASD is diagnosed using behavioral symptoms, and there is a great degree of diversity of symptoms in ASD patients. The diagnostic manual of the American Psychiatric Association, DSM-V, outlines the criteria used for diagnosis of ASDs, which includes persistent deficits in social communication and interaction, restricted and repetitive patterns of behavior, interests and activities and requires that these symptoms be present in the early developmental period, causing significant impairments in social, occupational or other areas of functioning.

Behavioral deficits in rodent models are used as an important marker for the manifestation of ASD. “ASD-like behavior” has been used extensively for the characterization of rodent models in the case of many genetic models of ASD as well the MIA environmental model of autism. However, it is indeed challenging to design mouse or rat behavioral tasks that are relevant to the symptoms seen in human patients. Some of

the most commonly used assays for ASD in rodent models such as mouse models observe for abnormalities in social interactions, communication deficits and repetitive behaviors<sup>31</sup>.

The parameters evaluated in mice to assay social interactions look at sniffing, following, pushing and crawling over each other with physical contact, chasing, mounting and wrestling<sup>31</sup>. One commonly used experiment measures the time spent with an inanimate object versus a novel mouse. A normal mouse would spend more time in the chamber containing the novel mouse as compared to mice having ASD related mutations<sup>31</sup>. Other tests evaluate social memory, recognition and affiliation and also social transmission of food preference<sup>31</sup>. Communication deficits are assayed by measuring the interest in urinary scents from other mice, olfactory habituation to social odors, preference for social odors over non-social odors, and measurement of ultrasonic vocalizations<sup>31</sup>. Repetitive behavior is measured by scoring motor stereotypies, repetitive self-grooming, insistence on sameness and restricted interests such as restricted exploratory behavior in a novel environment<sup>31</sup>. A test for repetitive behavior, that has been used in the characterization of deficits in the MIA offspring is marble burying, a repetitive digging behavior<sup>12</sup>. This test presents a novel situation and is capable of evoking a highly repetitive and stereotyped response, which is scored by counting the number of unburied marbles<sup>12</sup>. The MIA offspring have been shown to bury 2.8 fold more marbles as compared to controls<sup>12</sup>.

Few studies have looked at the links between synaptic changes, circuit dysfunction and behavioral abnormalities. However, mutation in NL3 gene, which is a post-synaptic cell adhesion molecule, has been shown to cause acquisition of repetitive motor routines, using the rotarod<sup>54</sup>. The rotarod test is typically used for evaluating motor skill learning,

However, in this study, increased time spent on the rotarod was interpreted as being due to repetitive movements of mice having mutations in the NL3 gene<sup>54</sup>. This increased learning was attributed to a synaptic impairment in the nucleus accumbens<sup>54</sup>.

In this thesis, I have tried to explore whether any differences are observed in MIA mice as compared to controls, by comparing the time spent running on the rotarod, due to increase in repetitive movements. Any differences observed would point towards possible synaptic alterations in the MIA mice.

## **Chapter 2- EXPERIMENTAL PROCEDURES-**

### **2.1 Induction of MIA-**

MIA is induced in the mother by intraperitoneal injection of synthetic dsRNA, Poly I:C potassium salt (Sigma Aldrich; St. Louis. MO) at a dose of 20 mg/kg at embryonic day 12.5 (E12.5)<sup>12</sup>. The control mother is injected with Saline at the same time E12.5

### **2.2 Marble burying-**

Mice are tested at P60 (Postnatal day 60). There are two cages that are needed to test one litter of animals- the acclimatization cage and the testing cage. Both the cages have the same bedding set up. Corn cob bedding upto a depth of 3.5 cm and Tek-fresh bedding with total weight of 200g are thoroughly mixed and distributed evenly throughout the cage. 24 hours prior to testing, the mice are placed in the new cage and allowed to acclimatize overnight.

On the day of the testing, the weights and sex of the animals are recorded. Testing is done from 12pm to 5pm. Prior to testing, the animals are acclimatized for 30 minutes in the testing cage, which does not have any food and water. For testing, 30 marbles are added to the same cage in a 5X6 arrangement. One mouse at a time is placed in the cage with marbles, and allowed to be in the cage for 30 minutes. Periodically, the mouse is checked for signs of activity, to ensure that the mouse is not sleeping. After 30 minutes, the mouse is removed in such a way, so as to not disturb the arrangement of marbles in the cage.

The number of marbles which are greater than  $2/3^{\text{rd}}$  buried in the bedding are counted and recorded as the number of buried marbles. The percentage of marbles buried is recorded.

### **2.3 Rotarod-**

6-week old mice are used for this task. Rotamex4/8 4-lane machine (Columbus Instruments) is used for testing.

#### Habituation-

Prior to testing, the mice are habituated on the rotarod at a constant speed of 4 rpm for 3 minutes for Day 1 and 2 and at constant speed of 8 rpm for 3 minutes for Day 3. If the mice fall off during the course of the 3 minutes, they are again put back on the rod till they complete a total period of 3 minutes on the rod. They are then given a break of 10 minutes.

#### Testing-

Testing consists of 3 trials in a day separated by an interval of 10 minutes each, for 3 days. The paradigm- 4-40 rpm for 5 minutes for Day 1 and Day 2, 8-79 rpm for 5 minutes for Day 3

The mice are placed on the rotating rod and allowed to run on the rod. When the mice are unable to keep up with the speed, they fall down. The rod stops rotating when the mice fall, and the total duration for which they stay on the rod is displayed. This is noted as the latency. The latency (in seconds) is plotted against trial number, for a total of 12 trials.

### **2.4 Breeding strategy for GCAMP3 transgenic mice-**

Transgenic mice expressing the genetically encoded calcium indicator, GCAMP3 in astrocytes, are used. Two mouse lines are crossed: one that expresses the CreER recombinase under the control of an astrocyte specific promoter GLAST, and the other

line carrying loxP sites flanking a STOP codon upstream of the gene of interest, GCAMP3 (GCAMP3<sup>fl/fl</sup> mice).

### **2.5 GCAMP3 expression-**

4-Hydroxytamoxifen (Sigma-Aldrich Inc., St Louis, MO) is injected intraperitoneally into the pups that are Cre<sup>+</sup> flox<sup>+</sup>, for the expression of GCAMP3 in the astrocytes. The injections are on P5 and P7. Each pup receives a total dose of 0.5 mg per injection.

### **2.6 Craniotomy-**

Animals between the ages of P18-P21 are used. To perform imaging in the brain, a craniotomy is performed in the somatosensory cortex by removing a 1-2 mm wide portion of the skull in both the hemispheres. The surgery is performed under 1-1.5% isoflurane. After the cortex is exposed, SR101 dye (Sigma- Aldrich Inc., St Louis, MO) at a final concentration of 100  $\mu$ M (prepared by adding 1  $\mu$ l of 5mM stock of SR-101 to 50  $\mu$ l of ACSF solution) is loaded on the cortex for 20 minutes, and washed out for half an hour. This is done to label the astrocytes, so that they can be used as a structural marker to locate the astrocytes and check for GCAMP3 expression.

### **2.7 *In vivo* multiphoton imaging-**

Imaging is performed with a multiphoton microscope (Moving Objective Microscope (MOM), Sutter), using a Ti:sapphire laser (Chameleon Vision II, Coherent) tuned to 920 nm. Images are collected with a Nikon water immersion objective (60X, 1.0 NA). For imaging, ScanImage software (Pologruto et al., 2003) written in MATLAB (MathWorks) is used. All imaged astrocytes are in layer 1 (within the first 100  $\mu$ m below the dura mater) within the somatosensory cortex. Time lapse imaging is done at a single focal

plane for 5 minutes and images are acquired every 2s. A total of 150 images are collected in a span of 5 minutes. Image acquisition is done at zoom 3 on two different channels- the red channel (610/75) for the SR101 labeled astrocytes and the green channel (535/50) for the GCAMP3 labeled astrocytes. Each image is collected at 256 X 256 pixels, 0.36  $\mu\text{m}/\text{pixel}$ .

## **2.8 Image analysis-**

Images are analysed using ImageJ software. Images are first corrected for Z- drift and X-Y drift. The SR101 labeled cells are Z-projected and regions of interest are put around the soma and processes of the cell. The fluorescence intensity is calculated for each region of interest and the background is subtracted. Baseline fluorescence values are calculated and  $\Delta F/F$  percentage values are calculated where  $\Delta F$  is the difference from the baseline fluorescence values.<sup>35</sup> The transients that have fluorescence values higher than three standard deviations are counted as transients showing calcium activity. Average of the number of calcium transients will be taken for each Region of Interest (ROI) to give the number of transients per cell.

## **2.9 Calculation of frequency, amplitude and duration of calcium transients-**

The average of number of transients per cell is averaged for all the cells analysed per animal to give the frequency of calcium transients per animal. The amplitude of the transients are calculated and the duration is calculated at 50% of the amplitude. These values are then averaged per cell, to give one value per animal.

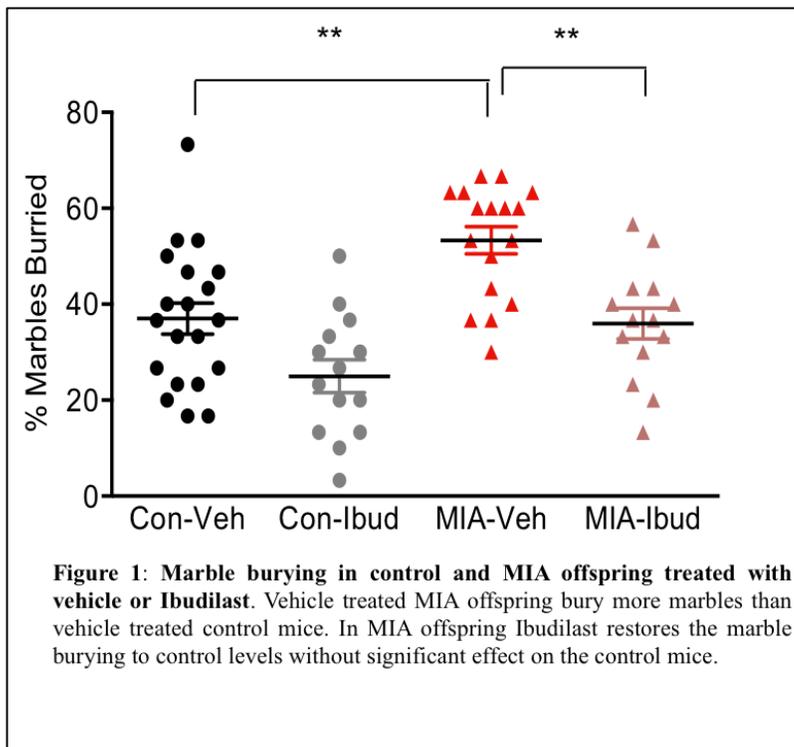
## Chapter 3- RESULTS AND DISCUSSION

### **3.1 Experiment 1- To test whether treatment with anti-inflammatory drug Ibudilast (AV-411) ameliorates marble burying deficits in MIA mice**

Marble burying is used as a test for evaluating repetitive and stereotypic behavior in small rodents<sup>46</sup>. Previously, it has been demonstrated that C57BL6 MIA offspring display increased marble burying as compared to control mice<sup>12</sup>. Since, it has been shown that most of the behavioral deficits observed in MIA are mediated by increase in the levels of inflammatory cytokines like IL-6<sup>11</sup>, we wanted to test whether treatment with an anti-inflammatory drug can reverse the increase observed in marble burying. Ibudilast (AV-411) is an anti-inflammatory drug, which is a type- 4 phosphodiesterase inhibitor and can cross the blood-brain barrier. It has been shown to suppress the production of Nitric Oxide (NO), Reactive Oxygen Species and pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF $\alpha$  from activated microglia as well as enhance the production of anti-inflammatory cytokines such as IL-10 and other neurotrophic factors such as NGF, GDNF and NT-4 in activated microglia, in a dose-dependent manner<sup>47,48</sup>. By doing so, it exerts a neuroprotective effect and promotes neuronal survival in the presence of activated microglia.

Since published work<sup>25</sup> and data from our lab indicate that MIA results in altered synaptic formation in the first few postnatal weeks, we wanted to reverse any potential inflammatory-induced synaptic changes early in development. To avoid handling and potentially stressing the young MIA pups, the lactating mothers were injected with 30

mg/kg Ibudilast or vehicle daily for two weeks starting one day after parturition. The MIA and control offspring were tested for marble burying on P60 (Post natal day 60). After the initial pilot study showing that MIA increases marble burying and that Ibudilast reduces it, I investigated if Ibudilast also affects marble burying in control mice. To keep me blinded additional litters from other groups were also included.



It was observed that MIA offspring treated with vehicle buried 44% more marbles as compared to control mice treated with vehicle (Con+Veh: 37±3.2%, n=20 mice; MIA+Veh: 53±2.8%, n=17 mice, Two- way ANOVA, post-hoc Tukey's test, P=0.002). Also, treatment of MIA offspring with Ibudilast during the first 2 postnatal weeks normalized the marble burying behavior (MIA+Ibud: 36±3.2%, n=14, P=0.003). In the control, drug treated mice there was a trend but not a significant reduction in marble burying (Con+Ibud: 28.66±3.59%, n=14, P=0.06).

The anti-inflammatory drug, Ibudilast was able to rescue the behavioral deficits observed in MIA offspring. These results are consistent with the interpretation that the inflammatory state in the developing brains of MIA mice is responsible for the observed behavioral deficit in marble burying.

### **3.1 a) Discussion-**

The drug, Ibudilast, mediates its neuroprotective effects by inhibiting the production of pro-inflammatory cytokines such as IL-6 and upregulating the production of anti-inflammatory cytokines such as IL-10 and neurotrophic factors from activated microglia<sup>47,48</sup>. Prenatal intraperitoneal injection of IL-6 to the mother has been shown to cause ASD- relevant behavioral deficits in the offspring<sup>11,13</sup>. Drawing from these observations, it can be hypothesized that the reversal in the marble burying behavior upon administration of Ibudilast, could be due to the restoration in the balance of pro and anti-inflammatory cytokines in the brains of MIA offspring. However, it has been reported that there is no increase in the density of microglia or any qualitative changes in microglial morphology, which might be indicative of microglial activation, in the brains of MIA offspring<sup>13</sup>. Therefore, the exact mechanism by which Ibudilast causes reversal of behavioral deficits in MIA offspring, is still unclear. This also brings ambiguity about whether the effects of the drug are being mediated by acting upon the brain or it has other systemic effects. To check whether pro-inflammatory cytokines like IL-6 are reduced upon administration of Ibudilast, brain lysates from mice before and after administration of the drug can be collected, and checked for levels of different pro-inflammatory cytokines using ELISA. This will also give a better idea about whether the effects of the drug are being mediated by acting upon the brain or is it due to other systemic effects that

the drug has. It has been shown that some behavioral abnormalities of MIA mice can be corrected by irradiating them and transplanting them with bone marrow from wild-type mice<sup>53</sup>. Also, examination of cytokine production from bone-marrow derived macrophages in adult MIA offspring revealed increased production of the pro-inflammatory cytokine IL-12 and the chemokine, CCL3<sup>63</sup>. This suggested the increase of M1 polarization in macrophages, which results in increased production of the natural killer cell and T<sub>H</sub>1 cell activating cytokine IL-12 and very low levels of anti-inflammatory cytokine IL-10<sup>63</sup>. These observations imply that the reversal in increased marble burying could be due to other mediators, outside the brain too.

This is the reason why it is important to determine how much of the drug, which is being administered intraperitoneally, is reaching the brain. A full pharmacokinetic study of Ibudilast has not been performed yet, but preliminary LC-MS measurements 4 hours after injection of the drug to the mother has revealed presence of the drug in serum and brain of the pups.

Here, it is also important to take into account the neural circuits that are implicated in repetitive behavior. Historically, basal ganglia has been the most prominent candidate for explaining repetitive behavior, very specifically, motor stereotypies<sup>49</sup>.

Refinement of earlier understanding has identified three “macro-circuits” which are defined by the cortical output to the striatum. These are the sensorimotor (movement), associative (cognitive functions) and limbic (emotional-motivational behavioral) circuits<sup>50</sup>. These cortical inputs are partially overlapping and a disruption of coordination within the basal ganglia or between cortical and striatal structures may reflect in the development of repetitive behavior<sup>51</sup>.

It has been suggested that the motor loop is mainly involved in stereotypical motor behavior, specifically while repeating identical movements without a goal, an example of which is marble burying<sup>52</sup>.

However, it is unknown how dysregulation of inflammatory molecules affects these circuits. Unpublished data from the lab has shown that there are structural and functional synaptic deficits in the form of reduction in the density of dendritic spines in the cortex of MIA offspring, accompanied by alterations in excitatory and inhibitory synaptic transmission. Some synaptic impairments are prevented upon treatment with Ibudilast. Hence, it is clear that synaptic networks are affected in the brains of MIA mice, which is probably reflected in the altered behavior. The correlation between the synaptic and behavioral deficits becomes clear from the fact that both the deficits are reversed by treatment with Ibudilast. However, the exact molecular mechanism, which is the “trigger” for these changes, remains unknown. It can be speculated that the disbalance in the cytokine levels in the brain could be a possible trigger for these changes, setting into motion a cascade of events, which eventually culminates into synaptic and behavioral deficits observed in MIA offspring.

### **3.2 Experiment 2- To establish a new behavioral paradigm for MIA offspring using the rotarod.**

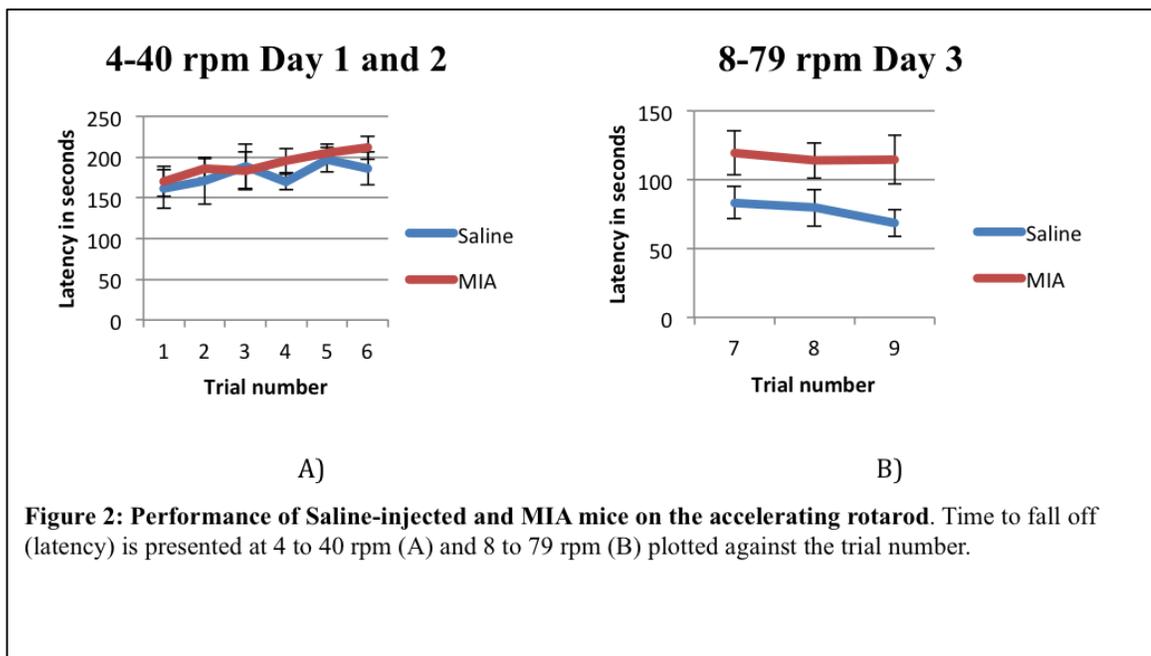
To better model human ASD behaviors in mice it is desirable to test different paradigms. Having established that MIA offspring have an impairment in a behavior that models repetitive behavior in ASD, we set out to determine if a different recently developed paradigm<sup>54</sup> for repetitive behaviors will detect impairments in MIA mice. This behavior also identified the specific synaptic circuit (including the neurons that the synapse project onto)<sup>54</sup> that was impaired in this behavior, which would give us some insights into the synaptic circuits that might be impaired in MIA, if any deficits were observed.

Motor coordination and learning in the offspring of mothers who were administered a respiratory system infection using influenza virus<sup>8</sup> and from mothers injected with PIC<sup>27</sup> had been tested using a paradigm in which the rod accelerates from 4-40 rpm in a span of 5 minutes. Such a paradigm, tests gross motor function over a period of days and is measured by increased time spent by the mice running on the rod, before they are unable to keep up with the accelerating speed and fall down<sup>8,27</sup>.

A new test of motor coordination, was able to show significant differences between control animals and animals which had mutations in the autism-associated gene NL3<sup>54</sup>. This test was for 4 days and consisted of two parts-the first part was for the first two days, and the acceleration of the rod was from 4-40rpm within 5 minutes. The second part was for Day 3 and 4 where the rod accelerated from 8-80 rpm within 5 minutes. The differences observed were more pronounced in the second part of the test. The increased time spent running on the rod was attributed predominantly to enhanced acquisition of repetitive motor routines due to mutations in NL3 gene.

Since repetitive behavior has also been shown in the MIA model, I wanted to establish if in this paradigm we can also detect increased repetitive behavior in the MIA offspring. In the paradigm I used, on the first two days the mice were allowed to run on the rod while it accelerated from 4-40 rpm in 5 minutes. On the last day, the mice were tested on the rod, while it accelerated from 8-79 rpm within 5 minutes.

Each day consisted of 3 trials separated by a time interval of 10 minutes each. The time spent by each animal running on the rod was plotted against the trial number.



The saline group consisted of 6 animals (n=6) from 1 litter and the MIA group consisted of 10 animals (n=10) from 3 litters. In the first part of the test (4-40 rpm, Day 1 and 2), there is increase in latency over trials in both the groups, although insignificant (Repeated measures ANOVA,  $P=0.2436$ ). However, there is no difference between the two groups ( $P=0.4530$ ). In the second part of the test (8-79 rpm, Day 3), there is no difference in latency over trials in both the groups ( $P=0.4110$ ), however, there is a trend towards

higher latencies in the MIA group ( $P=0.0790$ ), suggesting a better repetitive motor routine in MIA offspring.

### **3.2 a) Discussion**

Our results have demonstrated that MIA offspring do not have impairment in basic motor coordination as is tested by the 4-40 rpm paradigm. This result is consistent with earlier reports where no differences in motor function were observed in the offspring of mothers who were administered a respiratory system infection using influenza virus<sup>8</sup> and from mothers injected with PIC<sup>27</sup>, and tested using the rotarod.

The rotarod is traditionally used as a test for motor learning and coordination. However, in a recent study of NL3 mutation<sup>54</sup>, it was used as a proxy for testing enhanced repetitive behavior. The act of running on the rod for a longer period of time was interpreted as being due to enhancement in the learning of repetitive motor routines<sup>54</sup>. Such a behavior can be classified as goal-directed repetitive behavior, which is thought to be controlled by the loop receiving inputs from the prefrontal cortex into the striatum.<sup>55</sup>

It was shown that mutations in NL3 impaired specific synaptic inputs in the nucleus accumbens<sup>54</sup>, which is a part of the ventral striatum, causing enhancement in learning of repetitive motor routines.

Although other forms of motor stereotypies have been demonstrated in the MIA model such as the marble burying, this test is unique in the way it ties repetitive behavior to motor coordination. The fact that the MIA mice have a trend towards achieving higher latencies as compared to Saline mice, despite not learning, could be due to enhanced motor coordination, which is attributed to presence of repetitive behavior. This points

towards involvement of multiple overlapping brain circuits in ASD, beyond cerebellum and dorsal striatum. Also, it gives a starting point to look for specific synaptic impairments linked to behavior in the MIA model. Unlike the previously characterized repetitive behavior test, marble burying, this test involves the whole body. The unique 8-79 rpm paradigm brings out the differences between the two groups, by creating more difficult conditions for the mice to keep up with and pushing them harder. Although, the difference between the two groups is not significant, there is a trend, and it would be interesting to see whether it persists and becomes significant upon addition of more animals.

### **3.3 Experiment 3- To compare basal levels of astrocytic calcium activity in control and MIA offspring.**

Cytokines have been shown to have an effect on gliotransmission. Glutamate release from astrocytes has been shown to be controlled by inflammatory molecules such as TNF- $\alpha$  and prostaglandins, by a calcium dependent mechanism<sup>38,39</sup>. These results suggest that dysregulation in the production of these mediators in conditions such as those which are thought to exist in the brains of MIA mice<sup>13</sup>, may affect gliotransmission and astrocyte excitability. Astrocyte excitability can be assayed by measuring intracellular calcium ( $\text{Ca}^{2+}$ ) signals<sup>36</sup>. Thus, it was hypothesized that the astrocytic  $\text{Ca}^{2+}$  activity is altered in the brains of the MIA offspring. Astrocytic  $\text{Ca}^{2+}$  elevations have been also shown to modulate synaptic structure, function and stability<sup>61,62</sup>. Hence, we wanted to image astrocytic calcium during spine formation and stabilization in MIA offspring.

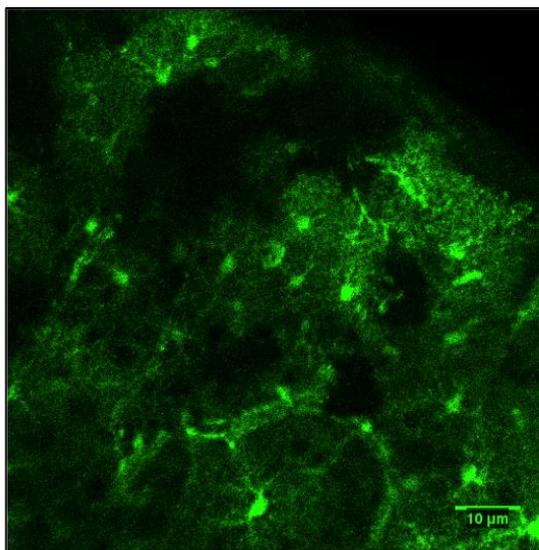
Astrocytic  $\text{Ca}^{2+}$  signals are representative of excitability due to both neuronal inputs as well as spontaneous changes in intracellular  $\text{Ca}^{2+}$  levels<sup>36</sup>. Most importantly, the  $\text{Ca}^{2+}$  dynamics represent encoded signals in both neuron-to-astrocyte as well as astrocyte-to-neuron signaling<sup>56</sup>. In the absence of any external neuronal stimulation such as sensory stimulation, the astrocytic  $\text{Ca}^{2+}$  levels represent the basal level of astrocytic  $\text{Ca}^{2+}$  levels.

Genetically encoded calcium indicators (GECIs) are an effective tool to image intracellular  $[\text{Ca}^{2+}]$  in astrocytes<sup>56</sup>. These can be expressed in genetically-defined cell populations and allow the monitoring of  $\text{Ca}^{2+}$  activity in the fine astrocytic processes such as branches and endfeet, besides the soma, which makes it a more desirable technique than bulk-loading and patch dialysis of  $\text{Ca}^{2+}$  indicator dyes<sup>56</sup>. Other relevant characteristics are signal-to-noise ratio (SNR),  $\text{Ca}^{2+}$  affinity, rise and decay kinetics,

dynamic range, photostability, compatibility with long-term imaging, and targeting to specified locations and fusion proteins<sup>56</sup>.

GCaMP is a genetically encoded calcium indicator, created from a fusion of GFP, Calmodulin and M13, a peptide sequence from myosin light chain kinase. Upon  $\text{Ca}^{2+}$  binding to Calmodulin, there is a conformational change in the fluorescent protein, resulting in bright fluorescence<sup>56</sup>.

For our experiments, we used GCAMP3 transgenic mice that were crossed to GLAST-Cre-ER. The pregnant female was injected with PIC at E12.5, to induce MIA. The offspring that expressed both CreER and loxP were injected with 4-Hydroxytamoxifen twice between the ages of P5 and P10. This leads to translocation of CreER into the astrocytic nuclei, excision of the STOP codon, and expression of GCAMP3 in the astrocytes. Surgery and imaging was done between P18 to P25 because previous studies in the lab have detected significant synaptic impairments at P17.



**Figure 3: Expression of GCAMP3 in astrocytes**

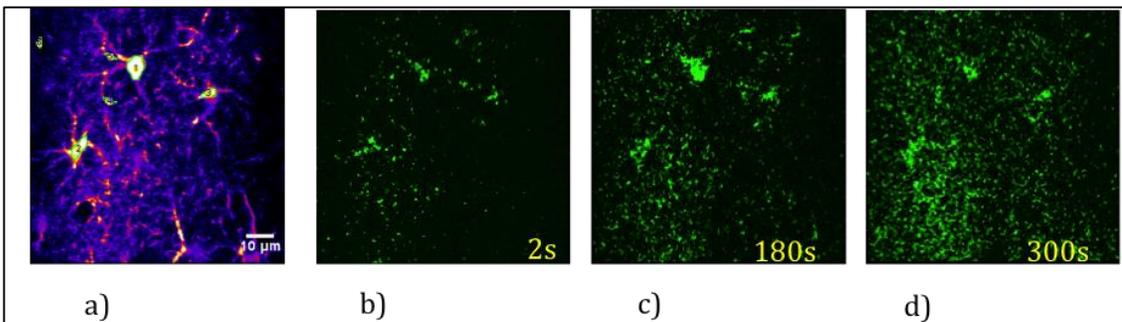
A  $\text{Cre}^+/\text{GCAMP3}^{\text{fl}}$  mouse was injected with 4-Hydroxytamoxifen (4-HT) at P5 and P8. The brain was fixed, sectioned and immunostained with antibody against GFP(GCaMP3). Image was collected using a confocal microscope.

Craniotomy was performed on the mice, which had been injected with 4-HT, and topical application of SR-101, a morphological marker of astrocytes was performed.



**Figure 4: View of a cranial window.** The skull has been removed revealing the surface of the brain and the vasculature.

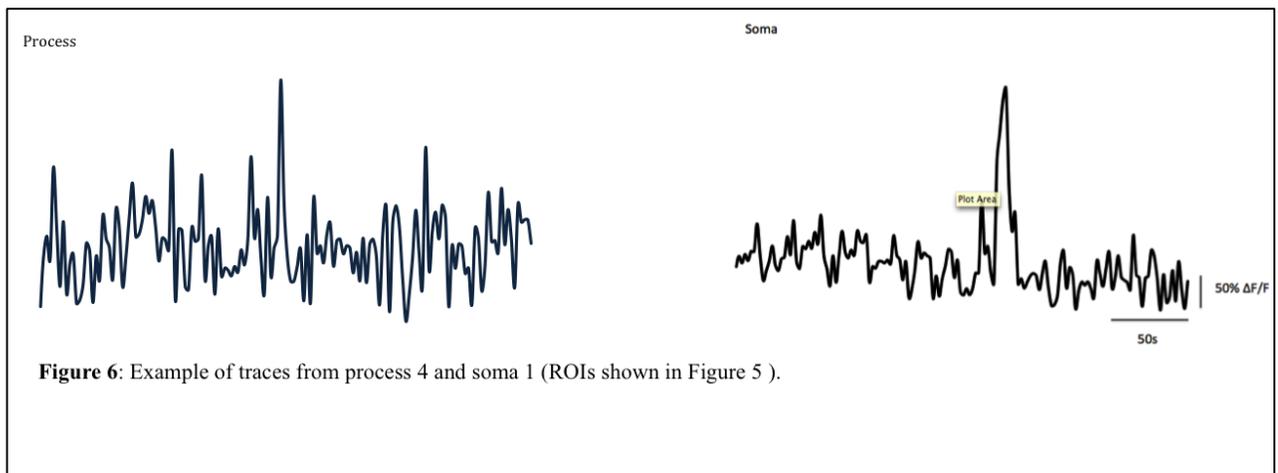
The mice were allowed to wake up after the surgery and multiphoton microscope was used to image different fields of view containing astrocytes labeled with GCAMP3 and the morphological marker SR-101, in awake mice. Images were acquired over a time frame of 5 minutes, every two seconds.



**Figure 5: Calcium activity in astrocytes expressing GCAMP3, labeled with SR-101 as a morphological marker.** a) Astrocytes in a field of view labeled with SR-101. Regions of interest on the soma, process and background are marked. GCAMP3 channel showing three frames at the start b), middle c) and end d) of a 5 minute acquisition period in the same field of view. Times are indicated in seconds.

In Figure 5b) At the beginning of the acquisition period, all the three GCAMP3 labeled astrocytes appear silent. In the middle, the cell 1 lights up, showing the occurrence of a somal transient. At the end of the acquisition period, there is activity in the processes of the other two cell 2 and cell 3.

For quantification of these transients, ImageJ is used. Regions of interest are marked on the cell body and processes, using SR-101 labeling as a marker. Fluorescence intensity in each of the marked ROIs is measured, background subtracted, and the difference from the basal fluorescence intensity is calculated as a percentage. Qualification as a transient requires that it should be greater than two standard deviations. Amplitude of a transient is calculated and the duration is calculated at 50% of the amplitude.



The figure shows two traces from a process and a soma respectively. The somal trace and the process trace show one transient each.

A total of 16 cells (astrocytes) from 16 different fields of view were analysed. These 16 different fields of view were from 5 different animals, 3 Saline and 2 MIA. However, no result could be obtained from these experiments because there was very little activity

observed in my experiments despite the fact that these animals were imaged under awake conditions. This indicated that the experiments were not working in the correct manner.

### **3.3 a) Discussion**

We had hypothesized that the astrocytic calcium activity is altered in the brains of MIA offspring. Although GCAMP3 expressing mice had been successfully used to image astrocytes in vivo in older mice at age P30-P60, I was unable to test this hypothesis in the young GCAMP3 mice, due to inability to reliably measure astrocytic calcium activity. Also, the GCAMP3 labeling in astrocytes was sparse in most of my experiments.

It was concluded that the best GCAMP3 expression was attained when imaging was done around 3 weeks after the first 4-HT injection. Hence, the best time to inject animals would be P5 and P7. This would allow sufficient time for effective recombination and good expression of GCAMP3 in astrocytes, which is critical for performing these experiments and to be able to differentiate between a signal and noise during analysis.

The second critical factor governing GCAMP3 expression is a good cranial window for imaging. A good quality cranial window will have absence of any bleeding during or after surgery. SR-101 labeling is a good marker for the quality of a cranial window. Clear and crisp SR-101 labeling indicates absence of any bleeding in the window, and also indicates that most of the cells have taken up the dye. Poor quality of the window may result in absence of any activity in the GCAMP3 channel. Also, poor SR-101 labeling limits the number of ROIs that can be marked, therefore resulting in loss of information. Such windows should not be imaged, and if imaged, should not be considered for analysis.

If anesthesia during surgery, has affected the health of the animal, it will get reflected in labored breathing, failure to revert back to a completely awake state, and often death of the animal. The images acquired from such an animal are not considered valid and so the health of the animal should be monitored closely throughout surgery and imaging.

It was decided that the induction would be done using 3% isoflurane and maintenance at 1.5-2% isoflurane, with 0.5 L/min oxygen throughout induction and maintenance.

Therefore it was concluded that the primary factor governing valid acquisition of images is the presence of calcium activity. Activity can only be observed if there is GCAMP3 expression, which is in turn governed by the ages at which 4-Hydroxytamoxifen was administered, age at which surgery and imaging was performed, quality of cranial window, and imaging conditions.

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