FRAGILE X SYNDROME AND DIABETIC RETINOPATHY: PATHOLOGICAL CONDITIONS AND ASSOCIATE ALTERATION IN NEURONAL PROPERTIES

By

Hayyaf Saad Aldossary

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ABSTRACT

FRAGILE X SYNDROME AND DIABETIC RETINOPATHY:
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Fragile X syndrome (FXS) and diabetic retinopathy are poorly treated conditions that dramatically affect patient's and family members' life style. FXS is the most common known form of inherited mental retardation. FXS is caused by a genetic mutation leading to decreased fragile X mental retardation protein (FMRP) production. The absence of FMRP leads to alterations in synaptic plasticity, which are dependent on activation of metabotropic glutamate receptor (mGluR) activation. In this study, short term activation of group I and II mGluRs is not altered between an animal model of FXS, *Fmr1* knock out mice and wild-type mice. These negative findings suggest that short-lasting actions of mGluR activation in the neocortex may not contribute to the cognitive or sensory processing alterations associated with FXS.

Diabetic retinopathy is a common complication of diabetes and is the leading cause for blindness in US working age adults. In this experiment, intraocular injection of the proinflammatory cytokine, interleukin 1-β, was used to mimic inflammation similar to that which occurs during diabetic retinopathy. We used electrophysiological recording techniques to determine the impact of this manipulation on the excitability of thalamocortical neurons in the dorsal lateral geniculate nucleus. We found alterations in excitability, which could lead to altered visual processing as identified in diabetic retinopathy.

To my family

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KEY TO ABBREVIATIONS

ADHD: Attention defect hypersensitivity disorder

ASD: Autism spectrum disorder

BM: basement membrane

dLGN: Dorsal lateral geniculate nucleus

FMR1: Fragile X mental retardation gene

FMRP: Fragile X mental retardation protein

FXS: Fragile X syndrome

IGL: Intergeniculate nucleus

IL: Interleukin

IL-1β: Interleukin-1 beta

ILR: Interleukin receptor

KO: Knocked out

LGN: Lateral geniculate nucleus

LTD: Long-term depression

LTP: Long-term potentiation

mGluRs: Metabotropic glutamate receptors

MGN: Medial geniculate nucleus

mRNA: Messenger RNA

RGCs: Retinal ganglion cells

RMP: Resting membrane potential

R_{in}: Input resistance

SEM: Standard error of the mean

vLGN: Ventral lateral geniculate nucleus

VPN: Ventral posterior nucleus

WT: Wildtype

NPDR: Non-proliferative diabetic retinopathy

PDR: Proliferative diabetic retinopathy

Chapter 1

Literature Review

Fragile X Syndrome

Introduction

Fragile X syndrome (FXS) is one of the most common known causes of inherited mental retardation, and it is associated with many mental defects including cognitive, physical and biological alteration (Crawford et al., 2001). FXS is a genetic disorder in which there is a significant reduction of the fragile X mental retardation protein (FMRP), which results from alteration in the fragile x mental retardation (*FMR1*) gene (Morgan, 1911; Li et al., 2002; Ridaura-Ruiz et al., 2009). The decreased FMRP levels is responsible for the cognitive deficits, which can range from mild to severe alterations (Li et al., 2002; Ridaura-Ruiz et al., 2009).

FXS affects approximately 1 in 4000 males and 1 in 8000 females and the cognitive/behavioral alterations includes multiple disorders including attention-deficit disorder, anxiety disorder, epilepsy, and delays in speech and language development (Sabaratnam, 2006). The lack of FMRP expression plays a major role in altering the normal development of synaptic connectivity, synaptic transmission, as well as alterations in the intrinsic properties of neurons (Pfeiffer and Huber, 2009; Zhang et al., 2016; Wilson and Cox, 2007). In addition to neurological deficits, many FXS patients also have altered physical characteristics including long and narrow face, large forehead, and large ears (Ridaura-Ruiz et al., 2009).

FMR1 gene in Fragile X Syndrome

FXS is a disorder caused by genetic mutation. In FXS, the X chromosome specifically in the 5' untranslated region, there is an increase in the CGG repeat times; normally range from (1 to 50 CGG repeats), the permutation range is 50-200 CGG repeats, and when the number of CGG repeats exceeds 200, as seen in FXS, the *FMR1* gene will be silenced. This mutation leads to the loss of the FMRP production (Rousseau at el., 1994). The permutation in *FMR1* gene (50-200 repeats) results in abnormal FMRP production (Brouwer et al., 2009), and can lead to a couple of fragile X related conditions: fragile X associated primary ovarian insufficiency and fragile X-associated tremor syndrome. Fragile X associated primary ovarian insufficiency is a condition that can lead to infertility or in mild conditions cause a reduced fertility. On the other hand, fragile X-associated tremor syndrome is a condition that affects both men and women, and is characterized by tremors, memory loss, behavior changes and peripheral neuropathy and can be misinterpreted as Parkinson's disease (Sheridan et al., 2011).

The significance of FMRP loss in Fragile X Syndrome

It is generally thought that the reduction or absence of FMRP is the cause for the broad range of deficits/alterations associated with FXS (Abrams et al., 1997). FMRP is an RNA binding protein that influences a large number of mRNAs including many that are found in the brain (Darnell and Klann, 2013; Bardoni et al., 1999). FMRP plays a significant role in local protein synthesis at the postsynaptic site, it is also crucial for dendritic spine maturation (Schenck et al., 2001). FMRP could also regulate and maintain long-lasting changes in synaptic strength, due to its role in local protein synthesis (Kim et al., 2009). The loss of FMRP production will lead to the

reduction of synaptic plasticity and the developmental spine maturation, contributing in the cognitive defects and dendritic spines immature associated with FXS (Sidorov et al., 2013; Irwin et al., 2002). FMRP is also crucial for the translation and transportation of the mRNA, which makes its loss contributes to the alteration in the translation signaling pathway (Bassell and Warren, 2008). It has been showed that this alteration is leading to defects in synaptic function, which in turn causes behavioral phenotype in individuals with FXS (Bassell and Warren, 2008).

Behavioral phenotype in Fragile X Syndrome

The behavioral phenotype associated with FXS involves cognitive disability, learning and language difficulties, and social anxiety (Huddleston et al., 2014). Most patients with FXS present with developmental delay or intellectual disability, and considering FXS is associated with an X chromosome, male patients tend to have higher severity in the manifestation of behavioral defects (Gallagher A and Hallahan, 2012). It has also been shown that males have a lower IQ range (40-70), while heterozygotes females with FXS have an IQ range of (70-90; Hagerman et al., 1992; Huddleston et al., 2014).

Individuals with FXS typically show other behavioral disorders, including attention defect hypersensitivity disorder (ADHD), which is seen in approximately 75% of FXS patients. It also have been reported that FXS is associated with increased anxiety, which is displayed by behaviors such as isolation and shyness, is accounted for approximately 65% of FXS patients (Boyle and Kaufmann, 2010). Almost half of individuals with FXS were either diagnosed or treated for general hyperactivity, which is considered to be one of another common abnormal behaviors associated with FXS (Sullivan et al., 2006; Boyle and Kaufmann, 2010). Approximately

30% of individuals with FXS were also diagnosed with autism spectrum disorder (ASD; Boyle and Kaufmann, 2010). In addition, approximately 20% of FXS individuals are also diagnosed with a seizure disorder (Hagerman et al., 2009). These findings indicate that FXS is associated with many neurological defects that could play a significant role in the misleading of its diagnosis leading to late recognition of the condition.

Pathophysiology in Fragile X Syndrome

Thomas H. Morgan was first one to discover the sex-linked characteristics in 1911, when he saw the "white eye" phenomena affecting males only. He saw that in drosophila flies that have a red eye color originally, only males could have a white eyes color after breeding. Morgan then suggested that there could be genetic features that were sex-linked and thus would preferentially affect one sex (males) as opposed to females. Years after Morgan, studies showed that FXS occurrence is higher in males than females, 2:1 ratio (Turner et al., 1986; Pengarikano et al., 2007).

Fmr1 KO mice are FXS mice models that were genetically modified by The Dutch and Belgian group, this modification led to the inactivation of Fmr1 gene causing the prevention of FMRP production (Bakker et al., 1994). After modification, the *Fmr1* KO mice showed an increase in motor activity and in exploratory behaviors, which can also be seen in individuals with FXS (Bakker et al., 1994). This animal model provided an opportunity to study the underlying mechanisms that give rise to specific FXS phenotypes**.** By knowing more about the neurological alterations in *Fmr1* KO mice, we may be able to develop appropriate treatments for FXS patients.

Altered neuronal function associated with Fragile X Syndrome

In *Fmr1* KO mice, the dendritic spines are abnormally immature due to the loss of FMRP (Martín et al., 2010). Anatomical studies have reported that dendritic spines in both FXS patients and *Fmr1* KO mice look thinner and longer than normal (Martín et al., 2010). These changes in spine morphology are indicators for dendritic spine immaturity. These defects in dendritic spines lead to neuronal function abnormalities including synaptic plasticity alteration (Pfeiffer and Huber, 2009). Alterations in neuronal function associated with FXS have been studied in many brain regions. Here we will review alterations in hippocampal function that were found in *Fmr1* KO mice and subsequently gave rise to the mGluR theory.

Hippocampus (mGluR theory)

In the hippocampus, it has been shown that the magnitude of long-term depression (LTD), which is the activity dependent reduction in the efficacy of the neuronal synapse, is enhanced in *Fmr1* KO animals after the activation of group I mGluRs. The reason for this increase is that the absence of FMRP will lead to the exaggeration in group I mGluR dependent protein synthesis (Bear et al., 2004). The over-activation of the group I mGluR signaling might contributes to cognitive impairment, developmental delay and the loss of motor coordination in FXS (Bear et al., 2004).It has been reported by (Bear et al., 2004) that the increase in LTD in the hippocampus of FXS mice could slow the net synaptic maturation, by shifting the synaptic balance towards synaptic loss rather than synaptic gain, which will eventually lead to the cognitive defects and the development delay associated with FXS (Bear et al., 2004).

Neocortex

Altered intrinsic properties

The neocortex also exhibits alterations in neuronal function. In the somatosensory neocortex, FXS neurons show that the resting membrane potential (RMP) and the time constant (TC) did not change when comparing neonatal mice (postnatal age: 7 days) with older mice (postnatal age: 28 days), while when the same comparison was done on WT mice, both RMP and TC where altered. These alterations indicating that the loss of FMRP is slowing the neuronal maturation process, which could contribute in the defects associated with FXS (Zhang et al., 2016). It has also been reported that pyramidal cortical neurons from *Fmr1* KO mice exhibit more intrinsic properties excitability than WT mice, which may contribute to seizure and seizure-like neuronal activity in FXS (Contractor et al., 2015). Another neuronal alteration is an altered action potential firing rate; the discharge rate of cortical pyramidal neurons is higher in *Fmr1* KO neurons compared to WT neurons and the time for the first spike is shorter in *Fmr1* KO neurons compared to WT mice, indicating hyperactivity that could lead to altered sensory processing (Contractor et al., 2015). And when the persistent activity states, or UP state, which occur predominantly during slow-wave sleep, was studied by Hays et al., they showed that the UP state is ~50% longer in the *Fmr1* KO when compared with the WT mice. This finding supports that the *Fmr1* KO mice exhibit more excitatory circuits than the WT mice (Hays et al., 2011).

Altered synaptic plasticity

Synaptic plasticity is the ability of the neurons to change due to behavioral stimuli; i.e. strengthening (LTP) or weakening (LTD) changes (Berlucchi and Buchtel, 2009). In hippocampus, local protein synthesis in the presence of FMRP plays a significant role in synaptic plasticity, which is present in the dendritic spine, where synaptic transmission occurs (Pfeiffer and Huber, 2009). The absence of FMRP in *Fmr1* KO mice contributes to the alteration in plasticity in *Fmr1* KO neocortical neurons. It has been shown that in neocortex, *Fmr1* KO mice exhibit significant alterations in LTP, due to the loss of FMRP (Wilson and Cox, 2007). It has also been shown that in deep layer neocortex, LTP is primarily dependent on $mGluR_5$ activation (Wilson and Cox, 2007). Larson et al (2005) have suggested that in *Fmr1* KO mice, LTP alteration could be age dependent, they showed that there was no alteration in LTP in Fmr1 KO mice younger than 6 months, after that the LTP showed significant alteration, especially 12 months post-natal age.

Metabotropic Glutamate Receptors

Introduction

Most excitatory neurons in the central nervous system use glutamate as their primary neurotransmitter (Tanabe et al., 1992). Glutamate receptors play significant roles in synaptic transmission, including synaptic plasticity, which is altered in *Fmr1* KO mice (Monaghan et al., 1989). There are two families of glutamate receptors: ionotropic glutamate receptors, which are ligand-gated ion channels that mediate a fast synaptic transmission consisting of NMDA, AMPA and kainate receptors (Wisden and Seeburg, 1993). The other glutamate receptor family is the mGluRs, multiple subtypes of G-protein coupled receptors that mediate slow synaptic transmission and serve as neuromodulators (Ferraguti and Shigemoto, 2006).

mGluRs subtypes and Signaling pathways

There are multiple subtypes of mGluRs based on their function in the central nervous system (Conn and Pin, 1997). Based on their amino acid sequence, eight distinct mGluRs ($mGluRs_{1\text{-}8}$) have been identified and these have been divided into three groups; group I mGluRs (including $mGluR_1$ and $mGluR_5$), group II mGluRs (including $mGluR_2$ and $mGluR_3$) and group III mGluRs (including $mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$), (Jeffrey and Pin, 1997).

Group I mGluR: $mGluR_1$ and $mGluR_5$ are typically located postsynaptically. The activation of group I mGluRs will activate phospholipase C, resulting in the generation of IP_3. This pathway will lead to the mobilization of calcium from the endoplasmic reticulum, which in turn cause a

depolarization response as well as the activation of protein kinase C (PKC; Niswender and Conn, 2010).

Group II mGluR: $mGluR_2$ and $mGluR_3$, are located pre- and post-synaptically. The presynaptic activation of group II mGluRs will result in the inhibition of adenylyl cyclase and subsequent inhibition of cAMP as well as the inhibition of calcium channels (Bellonea et al., 2008; Niswender and Conn, 2010). On the other hand, the postsynaptic activation of group II mGluRs will result in the activation of the G-protein-coupled inward rectifying (GIRK) channels (Bellone et al., 2008). The activation of both pre and postsynaptic sites of group II mGluRs will lead to an inhibitory effect, which in turn will result in neuronal hyperpolarization (Niswender and Conn, 2010). One of their most selective agonist is 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC; Niswender and Conn, 2010).

Group III mGluR: $mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$, in group III mGluR, $mGluR_4$, $mGluR_7$ and $mGluR_8$ are expressed in neurons, while $mGluR_6$ is expressed in retinal bipolar cells (Nakajima et al., 1993). $mGluR_4$, $mGluR_7$ and $mGluR_8$ are located presynaptically, while $mGluR_6$ is located postsynaptically in retinal cells (Coutinho and Knöpfel, 2002) (Coutinho and Knöpfel, 2002). Signaling of $mGluR_4$, $mGluR_7$ and $mGluR_8$ occurs through the inhibition of adenylyl cyclase, inhibition of cAMP, the activation of potassium channels, and the inhibition of calcium channels, while $mGluR_6$ signaling occurs through the stimulation of cGMP phosphodiesterase, leading to neuronal hyperpolarization and inhibitory effect. (Niswender and Conn, 2010).

mGluRs and their role in Fragile X Syndrome

mGluRs are involved in many impactful elements of brain function, such as neuronal plasticity, synaptic transmission and neuronal development (Coutinho and Knöpfel, 2002). Learning and memory is considered to be the most researched brain function and mGluRs play a significant role, while mGluR induced plasticity is crucial for cognitive functions (Riedel et al., 2003). One function of FMRP is to regulate mRNA translation and transportation; both are critical for synaptic maturation and function. (Pfeiffer and Huber, 2009). It has been shown by Antar et al. that in hippocampus, mGluRs activation increases FMRP localization in the dendritic spines, which is significant for synaptic plasticity, and the use of group I and II mGluR antagonist was sufficient to prevent this FMRP localization (Antar et al., 2004).

Multiple subtypes of mGluRs play a significant role in the neocortex, where many brain functions including sensory perception and motor commands take place. Neuronal plasticity is being influenced by mGluRs; the activation of group I mGluRs contribute in LTP induction, which is attenuated in *Fmr1* KO mice (Wilson and Cox, 2007). The absence of mGluR function in the neocortex would lead to neurological defects in plasticity after brain stimulation (Wilson and Cox, 2007). The involvement of LTP with the activation of mGluRs could contribute in the impairment in cognitive functions associated with FXS (Desai et al., 2006).

Diabetic Retinopathy

Introduction

Diabetic retinopathy is the leading cause of blindness in working-age adults and it is considered to be one of the more serious complications associated with diabetes mellitus (Kempen et al., 2004). The pathogenesis of diabetic retinopathy involves vascular, neuronal, and inflammatory processes (Qiu et al., 2016). In diabetic retinopathy, the inflammatory response will increase proinflammatory cytokine levels, including interleukin (IL)-1β, IL-6 and IL-8, in the ocular fluid (Qiu et al., 2016). These inflammatory components will lead to retinal neovascularization, blood-retinal barrier breakdown, and eventually retinal ganglion apoptosis (Kempen et al., 2004; Qiu et al., 2016; Zhang et al., 2017).

The early stage of the disease is the leading cause for visual defects and the late stage is the leading cause of blindness in working age adults (Zhang et al., 2017). Excitotoxicity of glutamate, the reduction of nerve growth factors, and inflammatory factor release are associated with diabetic retinopathy (Zhang et al., 2017). Retinal ganglion cell protection could be an effective way to prevent the pathogenesis of diabetic retinopathy (Zhang et al., 2017).

In diabetic retinopathy, the retinal function is gradually reduced; it usually starts with a reduction in night vision and might continue worsening to a total vision loss (Caldwell et al., 2003). When diabetic retinopathy starts, alterations in retinal blood flow and RGC function will occur, as well as thickening of the basement membrane and an increase in vascular permeability (Caldwell et al., 2003).

Stages of diabetic retinopathy

Non-Proliferative Diabetic Retinopathy

Non-proliferative diabetic retinopathy is the early stage of diabetic retinopathy and is associated with vasodegeneration lesions in the retinal microvascular bed (Gardiner et al., 2007). This stage of diabetic retinopathy is characterized by the thickening of the capillary basement membrane (BM), vascular smooth muscle dropout, microanyorisms, and capillary occlusion (Gardiner et al., 2007). The first stage of diabetic retinopathy is associated with visual defects, such as dark spots vision and blindness is not a risk factor in the early stage, but if untreated will eventually lead to the advanced stage (Curtis et al., 2009). Since diabetic retinopathy mainly affects the retina, the most common symptom of diabetic retinopathy is retina capillary cell loss, which is caused by an increase in the number of microaneurysms over time. This loss in capillary cells will lead to the diabetic retinopathy visual associated defects (Cai and Boulton, 2002). The increase in vascular permeability, and the symptom associated with this stage will lead to the reduction in central accuracy caused by retinal trephining and edema (Antonetti et al., 2006).

Proliferative Diabetic Retinopathy

Proliferative diabetic retinopathy occurs when new vessels and connective tissues start to grow on the outer layer of the retina or the optic nerve (Kroll et al. 2007). This new growth is an indicator of retinal damage and it starts many years after the initiation of the non-proliferative stage (Kroll et al., 2007). In this advanced stage of diabetic retinopathy, retinal capillary aneurysms and the loss of perfusion of capillaries and arterioles take place (Engerman,

1989). There are some associated damage in the retina, such as vessel leakage and hemorrhage, which in turn will activate Muller cells and microglial cells and initiate an inflammatory process (Engerman, 1989). In this stage, neuronal impairments are the primary symptom, where the reduction in night and color vision, caused by nerve defects (Antonetti et al., 2006). Eventually, if not treated, diabetic retinopathy has a very high probability of causing blindness (Antonetti et al., 2006; Cai and Boulton, 2002).

The role of inflammation in Diabetic Retinopathy

Diabetic retinopathy is classified as an inflammatory disease, because of the increased number of leukocytes present at the retinal vasculature in diabetic patients after retinal vascular leakage, capillary non-perfusion, and endothelial cell damage (Joussen et al., 2004). The activation of Muller and microglia cells will trigger the inflammatory process; many pathways are activated including interleukin-1 beta (IL-1β), which induces the expression of proinflammatory proteins (Tang and Kern, 2011). The increase expression of proinflammatory proteins (IL-1β and others) will lead to the synthesis of more cytokines and chemokines (Tang and Kern, 2011). Most of the inflammatory changes such as the activation of Mullar cells and its downstream effect, are keys for diabetic retinopathy development, without these inflammatory changes, many characteristics of diabetic retinopathy will be blocked (Joussen et al., 2004).

Interleukins are a large group of immunomodulatory proteins, which have many responses in cells and tissues (Akdis et al., 2011). They initiate immune responses by binding to high-affinity receptors on the cell surface (Mizel, 1989). Interleukins have many complex

functions including cell proliferation, maturation, migration, and adhesion (Akdis et al., 2011). The interleukins have many subtypes, where each one has its specific role and pathway and all of them work as a part of the immune system (Akdis et al., 2011; Mizel, 1989).

The interleukin family consists of more than 35 subtypes and their structure is dimer, heterodimer, or monomer (Akdis et al., 2011). IL-1 and IL-17 are proinflammatory cytokines, while IL-23 stimulates the production of IL-17 (Akdis et al., 2011).

IL-1 has two subtypes IL-1α and IL-1β and it has two receptors IL-1RI and IL-1RII and macrophages are their major source (Akdis et al., 2011). On the other hand, IL-17 has one receptor IL-17R and its main source is the T helper 17 cells (Akdis et al., 2011).

Interleukin 1 Beta (IL-1β) Pathway

The activation of IL-1 would contribute in many inflammatory conditions leading to several medical situations (Dinarello, 1996).

IL-1β is activated by the caspase 1 gene, which is highly involved in cell proliferation, differentiation, and apoptosis processes and its activity could be influenced by gene expression modification (Dinarello, 1988). Activation of IL-1β will lead to activation of cyclooxygenase inducing inflammatory hypersensitivity (Dinarello, 1988; Weber et al., 2010). During systemic inflammation, an overexpression of IL-1β gene will occur in the central nervous system, due to its significance as a neuroregulator (Wong et al., 1997).

In general, IL-1 plays a significant role in inflammatory process; IL-1α and IL-1β are synthesized as pro IL-1α and pro IL-1β, which can be cleaved to generate mature forms (Gabay

et al., 2010). Pro IL-1α can be found as a membrane-associated protein at many cell-type surfaces, which is involved in cell-to-cell signaling (Claudia, 2009). Pro IL-1β is inactive and it needs to be activated to function, its activation requires the activation of caspase 1, gram positive and gram negative bacteria could activate caspase 1 (Claudia, 2009). IL-1β release requires two signals from the primary microphages; the first signal induces transcription and translation, while the second signal activates caspase 1, these two signals will result in the production of active IL-1β (Gabay et al., 2010).

Interleukin cytokines are associated with infection and inflammation, some cytokines promote inflammation, such as the proinflammatory cytokines, others suppress the activity of the proinflammatory type, which are the anti-inflammatory cytokines (Dinarello, 2000). Their function is based on gene coding and it is likely that the proinflammatory cytokines will not be produced in a healthy individual. These genes are targeted in conditions where immunity is already compromised, during infection or trauma for example (Dinarello, 2000). When the inflammatory cascade is triggered by the proinflammatory cytokines, it will induce nitric oxide synthesis, leading eventually to the activation of neutrophils and this is when inflammatory tissue destruction and loss of function occur.

IL-1β and Diabetic Retinopathy

Many members of the interleukin family, including IL-1β, were detected in the vitreous fluid of patients with diabetic retinopathy and the expression of IL-1β is upregulated in retinal ischemic conditions (Kowluru and Odenbach, 2004). This indicates that the involvement of IL-1β cytokine may play a significant role in the pathogenesis of diabetic retinopathy. It was shown by Kowluru and Odenbach, 2004, that the increase of IL-1β expression in the retina showed 70% raise in cell death as well as the acceleration of retinal cell death processes.

The expression of IL-1β is increased when retinal endothelial cells were exposed to high glucose, this increase in expression will lead to a dramatic increase in nitric oxide levels (Kowluru and Odenbach, 2004). The increase in IL-1β levels will significantly increase endothelial cells apoptosis (Kowluru and Odenbach, 2004; Abu El-Asrar, 2012). In conclusion, the continued hyperglycemia circulation is the main reason of keeping this positive feedback cycle going, leading to more activation of IL-1β, which in turn cause the development of retinopathy (Kowluru and Odenbach, 2004).

Alteration in neuronal function in diabetic retinopathy

The advanced stage of diabetic retinopathy is associated with blindness; this blindness is mainly due to the increase in retinal ganglion cell (RGC) apoptosis (Vujosevic and Midena, 2013). It has been shown that diabetic retinopathy is not only a vasculature disease, it also involves neurons and glial cells in the retina (Vujosevic and Midena, 2013; Whitmire et al., 2011). After an acute retinal injury, glial cells will be activated to protect retinal neurons (Whitmire et al., 2011). The activation of glial cells will result in growth factors release, which will promote either cell survival or cell death (Whitmire et al., 2011). Retinal neurodegeneration occurs before the development of clinically detectable microvascular damage, indicating that retinal neurodegeneration plays a major role in the microvascular changes seen in diabetic retinopathy (Vujosevic and Midena, 2013). In diabetic retinopathy conditions, apoptosis will occur in different retinal layers and from different types of neurons, as well as the reduction in retinal thickness due to ganglion cells loss (Vujosevic and Midena, 2013; Whitmire et al., 2011). All of these alterations in the retina and RGCs could lead to alterations in its signaling recipient, which is the dLGN.

The Thalamus

Introduction

The thalamus is a midbrain structure that is often referred to as the major relay station to the neocortex. With regards to sensory systems, most peripheral sensory information passes through the thalamus in route to the neocortex. The thalamus is divided into three groups; specific, associated and non-specific nuclei (Herrero et al., 2002). The specific nuclei, which are connected to specific areas in the neocortex, are composed of three nuclei; the medial geniculate nucleus (MGN), the dorsal lateral geniculate nucleic (dLGN) and the ventral posterior nucleus (VPN) (Ward, 2013; Zhang, 1988). The MGN is the relay nucleus for the auditory system; it receives auditory input from brain stem auditory nuclei and sends signals to the auditory cortex (Ward, 2013). The dLGN is the visual sensory relay nucleus. It receives retinal output and sends visual information to the visual cortex (Ward, 2013), while the VPN is the somatosensory relay nucleus; it receives its input from cerebellum and projects to layer IV somatosensory cortex (Zhang, 1988). The non-specific nuclei, projects to non-specific areas and receives inputs from both the cortex and the thalamus, (Pinault, 2004; Swenson, 2006). It mainly projects to thalamic nuclei; its function plays a significant role in the regulation of thalamic input, which in turn will regulate thalamic output. The non-specific nuclei participate in the regulation of many high order functions in the brain (Pinault, 2004; Swenson, 2006).

The lateral geniculate nucleus and vision

The lateral geniculate nucleus is divided functionally into three subdivisions; dorsal (dLGN), ventral (vLGN) and intergeniculate (IGL) (Harrington, 1997). The role of dLGN is to relay visual information from the retinal ganglion cells (RGCs) to primary visual cortex (Essen and Anderson, 1995; Dhande and Huberman, 2014). The function of the vLGN is not precisely known, but it has been suggested that it has roles in both brightness discrimination as well as the regulation of circadian rhythms (Harrington, 1997). The third division of the LGN is the IGL, which plays a significant role in the modification of circadian rhythms, it is also suggested that IGL contributes in the regulation of sleep and arousal and visuomotor functions (Harrington, 1997).

In visual processing, and when visual information gets to the RGCs in the retina, it will be projected to the relay neurons of the dLGN. The dLGN in turn relays the visual information to the visual cortex (van Essen and Anderson, 1995; Huberman and Niell, 2011). Historically, the dLGN was thought to serve as a passive relay station, but over the last several decades it appears that there is significant processing and modification of visual information prior to going to visual cortex.

LGN Structure and cell types

The dLGN is the gateway between the retina and the visual cortex. In the dLGN, less than 10% of synapses come from the retina as a driving input, while the rest is an inhibitory input from visual cortex as well as the brain stem (Sherman and Guillery, 2002). The inhibitory influence in the dLGN controls many retinogeniculate transmissions, which in turn leads to the prevention

of hyperexcitation conditions manifested by burst and tonic mode in the relay neurons, which are two firing modes in the thalamic relay neurons (Sherman and Guillery, 2002). These two firing modes depend on the activation of T type calcium channels, when the T channels are inactivated due to depolarization; the tonic mode will occur (Sherman, 2001). On the other hand, hyperpolarization will activate T channels leading to the burst firing mode (Sherman, 2001). The dLGN relay neurons are divided into three groups; X (biconical), Y (symmetrical) and W (hemispherical), based on their neuronal properties and function (Sherman, 1985; Krahe et al., 2011; Tang et al., 2016). dLGN cell types function were described by Sherman in 1985 as: the Y cell type is responsible for the analysis of basic visual information, the X cell type will provide high resolution for the analyzed input, while the W cell type plays a role in conscious perception of visual patterns (Sherman, 1985). In the dLGN there are relay neurons and interneurons; the relay neurons are responsible for the thalamocortical communication (Dhande and Huberman, 2014), while the interneurons, which account for ~30% of dLGN neurons, function is to inhibit the thalamocortical neurons and modulate the signal transmission in the dLGN (Leist et al., 2016).

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Chapter 2

Postsynaptic actions of mGluR activation in neocortical neurons are unaltered in *Fmr1* KO mice

Abstract

Fragile X Syndrome (FXS) is one of the most common forms of inherited mental retardation. In FXS, there is a mutation in the fragile X mental retardation 1 (*Fmr1)* gene resulting in a lack of FMRP production, which serves as an important element in many neuronal processes. Previous studies have indicated that FXS patients as well as an animal model of FXS have altered neuroanatomical characteristics. FXS is associated with neuronal defects; previous studies have also found alterations in synaptic plasticity that is dependent on the activation of metabotropic glutamate receptors in the *Fmr1* KO mice, a fragile X mice model. In the neocortex, mGluR-dependent long-term potentiation is significantly attenuated in *Fmr1* KO animals. In this study, we determined if mGluR-dependent regulation of neuronal excitability is also altered in *Fmr1* KO animals. Changes in mGluR-dependent functions could serve as potential mechanisms for the dampening of cognitive abilities associated with FXS. We tested the effects of selective mGluR agonists on the excitability of deep layer neocortical pyramidal neurons in *Fmr1* KO and wild type (WT) mice. We found that postsynaptic actions of mGluR activation on membrane potentials were unaltered in *Fmr1* KO mice, leading to the conclusion that short term membrane potential changes produced by mGluR activation are probably not involved in the neurological conditions observed in FXS.

Introduction

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation. This condition results from the loss of fragile X mental retardation protein (FMRP; Song et al., 2003). In addition to cognitive deficits, FXS patients may display a wide range of other conditions including anxiety, compulsive behaviors, epilepsy, autism spectrum disorder, and poor motor coordination (Bear et al., 2004). The current pharmacological treatment for FXS patients is symptomatic, with mixed results, which suggest that there are likely multiple mechanisms that underlie the neurological alterations. This is further supported in that the magnitude of cognitive attenuation varies from mild to severe conditions.

Alterations in metabotropic glutamate receptor (mGluR) function appear to play a crucial role in the defects associated with FXS. In *Fmr1* KO mice, group I mGluRs are overactivated, due to the absence of FMRP; this absence will lead to the increase of mRNA translation (Chuang et al., 2005; Huber et al., 2002). In the hippocampus, *Fmr1* KO mice showed that protein synthesis occurs after group I mGluR activation, leading to the enhancement of long term depression (LTD; Bear et al., 2004). It has been also hypothesized that FMRP, which is found in dendritic spines after being encoded by the *Fmr1* gene, has a major influence in slowing down local protein syntheses at synapses as a response of metabotropic glutamate receptors activation (Bassell and Gross, 2008; Weiler and Greenough, 1999).

An early finding in both FXS patients and *Fmr1* KO mice was an alteration of dendritic spine density and morphology in the neocortex and hippocampus. There is an increased proportion of immature-appearing spines within the FXS condition, and thus led to the speculation that these abnormalities may be related to the cognitive dampening in FXS (Comeryet al., 1997; Levenga et al., 2011). While the functional consequence of these immature spines is unclear, these changes could impact synaptic connectivity and subsequent synaptic efficacy. Multiple forms of synaptic plasticity in *Fmr1* KO mice have been broadly studied in many brain regions. In neocortex, long term potentiation (LTP) that is dependent on mGluR5 activation is significantly attenuated in deep layers of the *Fmr1* KO mice (Wilson and Cox, 2007). Similarly, mGluR-dependent LTP in the lateral amygdala is impaired in the *Fmr1* KO mice (Suvrathana et al., 2010).

In addition to alterations in synaptic plasticity, certain intrinsic properties of neurons are altered in *Fmr1* KO mice. The *Fmr1* KO mice excitatory neurons exhibit more neuronal excitability and this excitement is driven by the excessive activation mediated by group I mGluR (Hays et al., 2011). In pyramidal neurons from somatosensory cortex, Zhang et al. (2016) found that *Fmr1* KO mice have an increased neuronal excitability, which can be seen in the abnormally higher action potential firing frequency when compared with a WT population. It is hypothesized that cognitive and behavioral defects associated with FXS is due to changes in neocortical excitability and functions (Gibson et al., 2008; Till et al., 2012). These increases in intrinsic excitability of excitatory neurons tend to push the system to a hyperexcitable state which could account for the circuit hyperexcitability observed in the neocortical excitatory neurons (Pfeiffer and Huber, 2009). It was also reported by Gibson et al., 2008 that the increase

in excitability in *Fmr1* KO mice might be due to the higher input resistance and reduced membrane capacitance of layer 4 excitatory somatosensory cortical neurons. The synaptic plasticity alteration as well as neuronal hyperexcitability associated with FXS are significant elements behind the phenotypes seen in FXS.

The activation of mGluRs has been associated with many brain functions including learning and memory, anxiety, pain, epilepsy, and cognitive development (Niswender and Conn, 2010). Clearly there appears to be differential effects on mGluR-dependent functions in the *Fmr1* KO animals. These finding indicate that the lack of FMRP will result in synaptic plasticity alterations, and it is suggested that these modifications could be region specific (Hays et al., 2011; Gibson et al., 2008; Wilson and Cox, 2007).

Considering earlier work from our laboratory, where we found a decreased mGluR-mediated plasticity in the neocortex (Wilson and Cox, 2007), we wanted to follow up on this finding and determine if the neuronal responsiveness to mGluR activation is altered in *Fmr1* KO mice. We previously found that mGluR-dependent LTP is reduced in *Fmr1* KO, and predict that there may be decreased levels of group I mGluRs in the neocortex, and thus postsynaptic responses to mGluR activation may be attenuated in *Fmr1* KO mice. In this experiment, we will determine if selective mGluR agonists differentially alter membrane potential of deep layer (V/VI) pyramidal neurons from WT and *Fmr1* KO mice.

Materials and Methods

All animal care and experimental procedures used in this study were approved by the Michigan State University Institutional Animal Care and Use Committee. Both *Fmr1* KO and WT mice used in these experiments were maintained on a C57Bl/6J background (Bakker et al., 1994). Mice of either sex, ages 16 to 21 days, were deeply anaesthetized with 2-4% isoflurane. The animal was decapitated, and the brain was quickly removed and placed in a cold (<4 °C), oxygenated slicing solution containing (in mM): 2.5 KCl, 1.25 NaH_2PO_4, 10.0 $MgSO_4$, 0.5 $CaCl_2$, 234.0 sucrose, 10.0 glucose, and 26.0 $NaHCO_3$. Slices (300 μm thick) were cut on a vibrating tissue slicer in the coronal plane, and then incubated in a heated holding chamber (35 °C) containing oxygenated physiological saline containing (in mM): 26.0 $NaHCO_3$, 2.5 KCl, 10.0 glucose, 126.0 NaCl, 1.25 NaH_2PO_4, 2.0 $MgCl_2$, and 2.0 $CaCl_2$ for at least 30 minutes and then maintained at room temperature until used. Individual slices were then moved to a submersion recording chamber, and continuously super fused with oxygenated physiological saline (2-3 ml/min) and maintained at 32 $\pm$ 1 °C.

Whole cell recordings were obtained from layer V/VI pyramidal neurons in somatosensory cortex. Pipettes had tip resistances of 2-5 MΩ when filled with internal solution containing (in mM): 117.0 K-gluconate, 13.0 KCl, 1.0 $MgCl_2$, 0.07 $CaCl_2$, 0.1 EGTA, and 10.0 HEPES, with a pH of 7.30 and an osmolarity of 290. Data were acquired using Multiclamp 700B amplifier, signals were digitized (10 kHz) and filtered (10 kHz) and subsequent analyses were done using pClamp software (Molecular Devices, Sunnyvale, CA). The liquid junction potential (10 mV) was corrected for in all recordings.

Agonists were bath applied by infusion into the bath via a syringe pump (Paul and Cox, 2010). The following selective mGluR agonists were used: group I mGluR: (S)-3,5-dihydroxyphenylglycine hydrate (DHPG) and group II mGluR: 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC). The sodium channel blocker tetrodotoxin (TTX) was added to the bath prior to mGluR agonist application to prevent action potentials in both pre- and post-synaptic neurons. All chemicals were purchased from Tocris (St Louis, MO, USA) and Sigma (St. Louis, MO, USA). All data statistical analysis were done using ANOVA test, where the statistical significance was $p<0.05$. All error bars in figures represent the SME.

Results

Recordings were obtained from 30 WT and 23 *Fmr1* KO layer V/VI pyramidal neurons of somatosensory cortex. In WT neurons, the average resting membrane potential (RMP) was -72.1 ± 3.9 mV (n=30) and apparent input resistance (R_{in}) averaged 266.4 ± 141.8 MΩ (n=30). In neurons from *Fmr1* KO animals, the resting membrane potential averaged -74.4 ± 4.6 mV (n=23) and the apparent input resistance averaged 384.9 ± 112.0 MΩ (n=23). The membrane potential did not significantly differ from between WT and *Fmr1* KO neurons (RMP: p=0.06, t-test); however, the input resistance of neurons from *Fmr1* KO animals was significantly greater that those from WT animals (Rin: p= 0.001, t-test).

	WT	***Fmr1* KO**	
RMP (mV)	-72.1 ± 3.9 (n=30)	-74.4 ± 4.6 (n=23)	p= 0.060, t-test
Rin (MΩ)	266.4 ± 141.8 (n=30)	384.9 ± 112.0 (n=23)	*p= 0.001, t-test

Table 2.1. Intrinsic properties of layer V/VI pyramidal neurons from WT and *Fmr1* KO mice.

Group I mGluR

We tested the effect of the selective group I mGluR agonist DHPG on the membrane potential of somatosensory pyramidal neurons. After establishing a stable baseline, DHPG was briefly bath applied for 60 seconds. At the lowest concentration tested, DHPG (5 μM) produced a small depolarization in WT neurons that averaged 0.6 ± 0.2 mV (n=9). At higher concentrations, DHPG produced a larger membrane depolarization (Figure 2.1, 25 μM: 3.4 ± 2.1 mV, n=17; 100 μM: 4.2 ± 2.7 mV, n=15). In neurons from *Fmr1* KO animals, DHPG also produced a dose dependent increase in membrane potential depolarization (Figure 2.1, 5 μM: 0.5 ± 0.2 mV, n=8; 25 μM: 3.3 ± 1.8 mV, n=15; 100 μM: 3.6 ± 2.1 mV, n=15). The magnitude of the membrane depolarization produced by DHPG did not differ between WT and *Fmr1* KO neurons at each concentration tested, (p=0.062, ANOVA test).

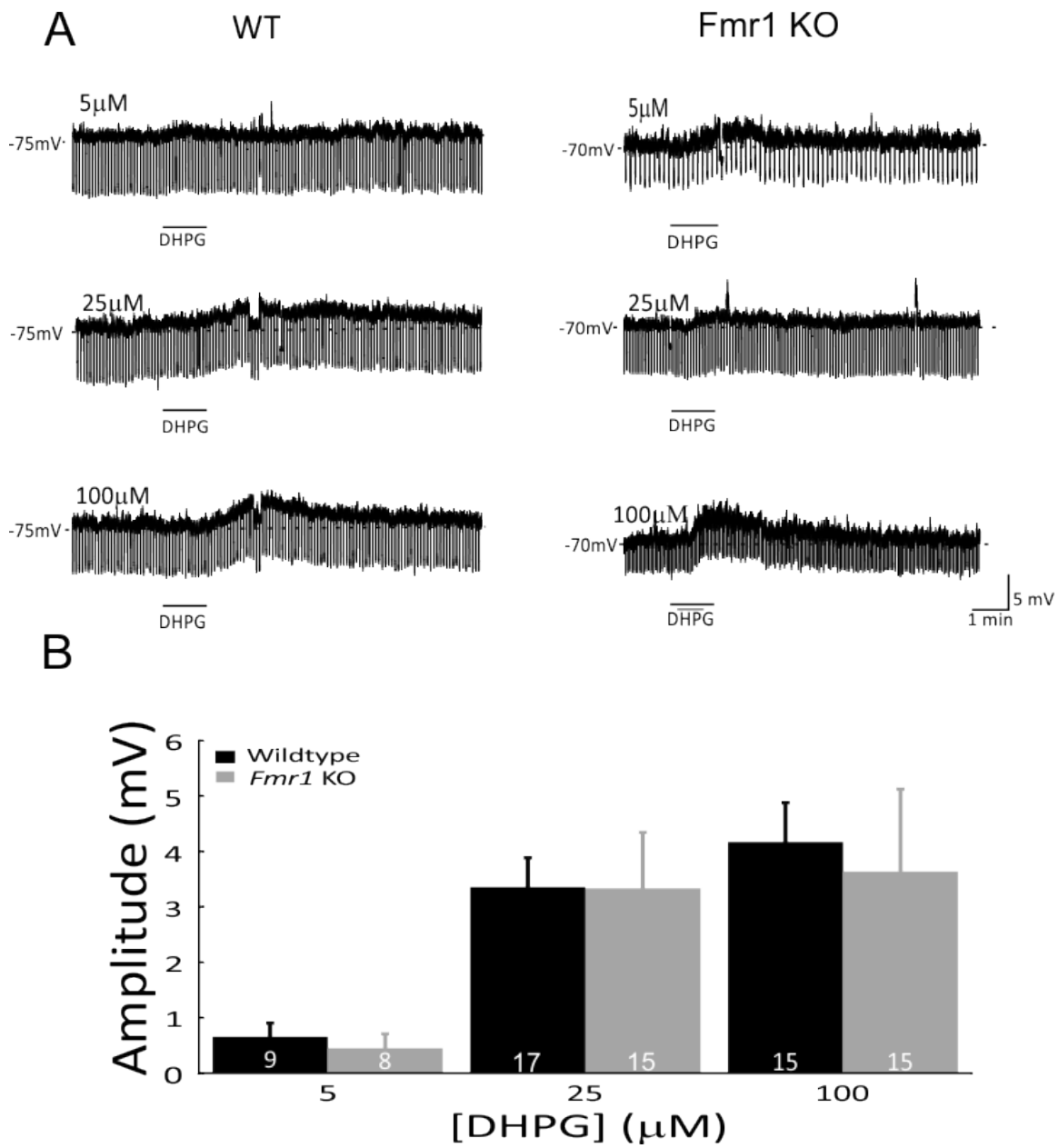

Figure 2.1. Group I mGluR agonist DHPG produces similar membrane depolarizations in pyramidal neurons from WT and *Fmr1* KO animals. A. Voltage recordings from representative WT and *Fmr1* KO neurons. DHPG produced a concentration dependent increase in amplitude in both types of neurons. B. Summary of population data (mean ± SEM).

Group II mGluR

In WT neurons, the group II mGluR agonist APDC (10 μM), produced a small membrane hyperpolarization (Figure 2.2, 1.0 ± 0.5 mV, n=6). At higher concentrations, the hyperpolarization was larger (75 μM: 1.9 ± 0.8 mV, n=11; 150 μM: 1.7 ± 0.9 mV, n=13). In neurons from *Fmr1* KO animals, APDC produced a dose dependent increase in membrane hyperpolarization (10 μM: 0.6 ± 0.4 mV, n=8; 75 μM: 1.2 ± 0.5 mV, n=8; 150 μM: 1.4 ± 0.9 mV, n=13). The membrane depolarization produced by APDC did not differ between WT and *Fmr1* KO neurons at each concentration tested, (p= 0.24, ANOVA test).

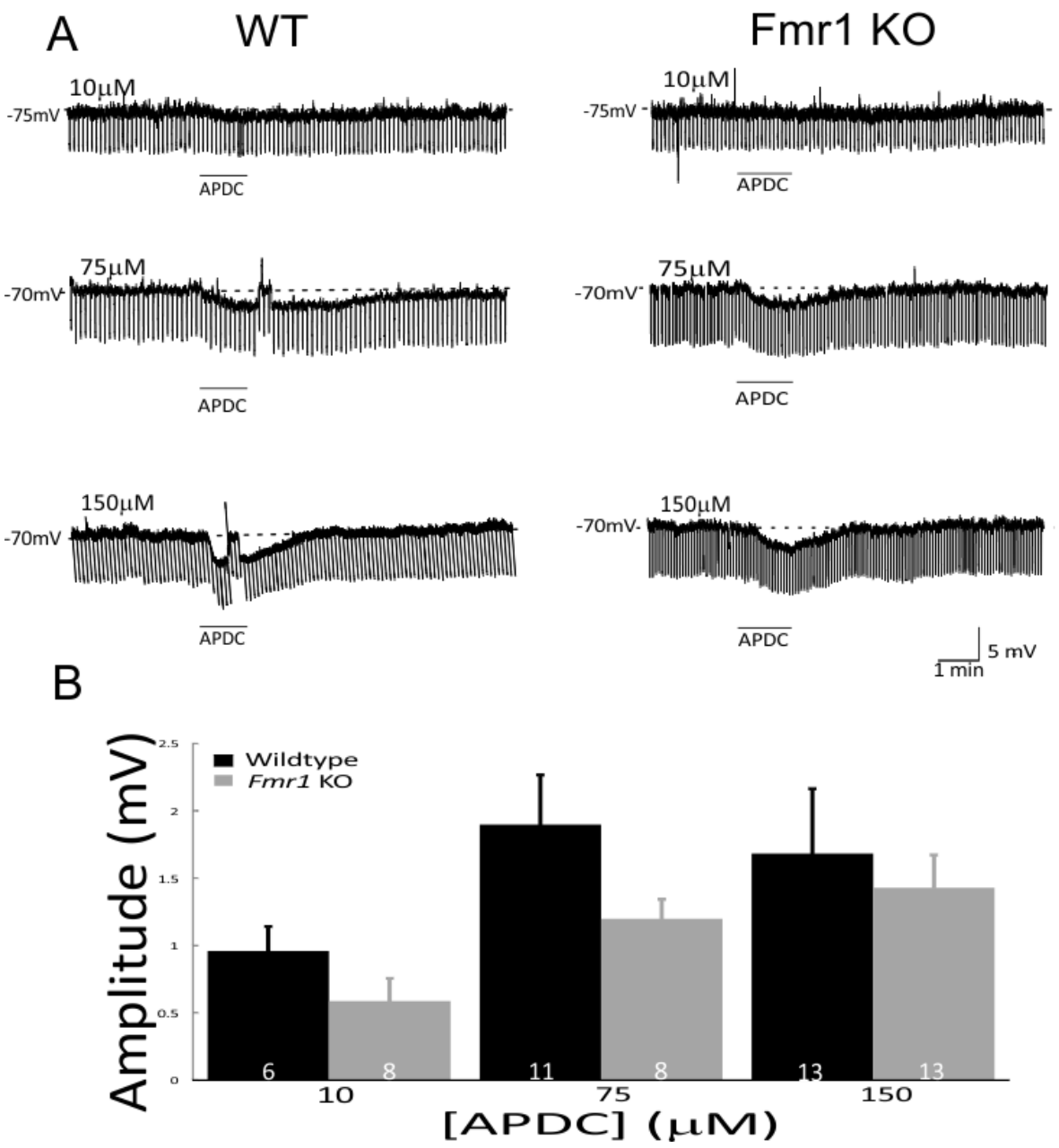

Figure 2.2. Group II mGluR agonist APDC produces similar membrane hyperpolarizations in pyramidal neurons from WT and *Fmr1* KO animals. A. Voltage recordings from representative WT and *Fmr1* KO neurons. APDC produced a concentration dependent increase in amplitude. B. Summary of population data (mean ± SEM).

Discussion

In this study, we found that the postsynaptic membrane changes produced by activation of either group I or II mGluRs in layer V/VI pyramidal neurons did not differ between WT and *Fmr1* KO mice, indicating that the loss of FMRP is not affecting the membrane potential changes produced by short term activation of type I and II mGluRs.

Previous studies suggest that stimulation of mGluR can lead to increases in local protein synthesis (Bear and Huber, 2004; Dölen and Bear, 2008), and in *Fmr1* KO, this protein synthesis is dampened (Bear et al., 2004). Numerous studies report that protein synthesis can occur within dendritic spines (Steward and Schuman, 2001). Such intracellular alterations by mGluR activation do not seem to impact the membrane potential changes. In this work, the lack of quantitative differences between *Fmr1* KO and WT mice when tested with selective group I and II mGluR agonists indicates that the defects associated with mGluR activation are primarily associated with long term plasticity (Zhang and Alger, 2010).

We have previously shown that LTP in deep layer neocortex is strongly reduced in *Fmr1* KO mice, and this reduction is due to a decrease in $mGluR_5$-dependent plasticity (Wilson and Cox, 2007). From that study, we concluded that there was a reduction in mGluR5-mediated activity in the *Fmr1* KO mice compared with the WT mice. Our current results show no significant differences between the membrane response to group I and II mGluR activation in WT and *Fmr1* KO mice. We would predict that the membrane depolarization depends on mGluR1 activation (Martín et al., 2010; Desai et al., 2006), or alternatively is likely due to activation of $mGluR_1$, and that $mGluR_5$ activation is tied to LTP induction in the deep layer

neocortex.

In this study, we show that even with the lack of FMRP in the *Fmr1* KO mice, layer V/VI somatosensory neurons are still able to produce an intact short term membrane potential changes, short term depolarization or short term hyperpolarization. It might be that the binding affinity of the mGluRs is affected in the absence of FMRP. When the plasticity was measured in our previous study, the *Fmr1* KO mice potentiated only for 20 minutes, while the WT mice showed a potentiation to up to 60 minutes after tetanic stimulation in the neocortex. In this study, the lack of alteration could be due to intact mGluR function in *Fmr1* KO mice for that limited time, and when this time is exceeded, their function will start to deteriorate, which could be directly linked to the mGluR binding ability alteration in the *Fmr1* KO. If this is the case with *Fmr1* KO mice, we could pharmacologically increase the mGluR binding ability and restore its function.

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Chapter 3

Intraocular injection of Interleukin 1-β alters intrinsic properties of dorsal lateral geniculate nucleus relay neurons

Abstract

Diabetic retinopathy is the most common diabetic related condition and it is considered to be the leading cause of blindness in working age adults. The circulation of hyperglycemic blood in retinal blood vessels lead to swelling of the vessels and eventually leakage of blood content, which will activate Muller as well as microglia cell. This activation will lead to the initiation of inflammatory response, which in turn will lead to the activation of many proinflammatory cytokines, including interleukin 1-β (IL-1β). The activation of Muller and microglia cells will also lead to the excess production of glutamate, which can directly lead to the over-excitation of retinal ganglion cells (RGCs) leading to their death. After mimicking the inflammatory response associated with diabetic retinopathy, we show that the IL-1β injection alters the lateral geniculate nucleus relay neurons. After inducing inflammation by injecting a pro-inflammatory cytokine IL-1β into the intraocular fluid, we studied its effect on the dorsal lateral geniculate nucleus (dLGN) relay neurons and we found that after 72 hours post IL-1β injection, the resting membrane potential was hyperpolarized, indicating more inhibition of relay neurons. Further, two weeks after injection, the average maximum firing rate was higher. It was also noted that dLGN relay neurons did not show any changes directly after the injection, indicating that the inflammatory response led to retinal cells alteration, which in turn influenced dLGN neurons.

Introduction

Diabetic retinopathy is a common diabetes related complication, and it is the leading cause of blindness in US adults (Williams et al., 2004). When hyperglycemic blood circulates in the retina, it causes abnormalities in blood vessels, including the presence of nonperfused capillaries and thickening of the basement membrane (Vincent and Mohr, 2007), which in turn produces an inflammatory response leading to early stages of diabetic retinopathy (Sheetz and King 2002; Kohner et al., 1995). There are two main stages in the development of diabetic retinopathy: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). In the NPDR, the initiation of inflammatory cascades caused by the hyperglycemic blood will lead to blood vessels leak and cause retina swelling; this initiation of the inflammatory response will be associated with the activation of caspase 1, glia and microglia cells. The activation of caspase 1 will lead to the activation of proinflammatory cytokines as well as the acceleration in apoptotic cell death (Vincent and Mohr, 2007). On the other hand, the activation of glia and microglia cells will lead to the production of glutamate; this glutamate production will eventually lead to RGC excitotoxicity (Vincent and Mohr, 2007; Kempen et al., 2004; Penn et al., 2008). The advance stage of diabetic retinopathy PDR, in which new blood vessels grow within the retina, and these new vessels are more fragile and can rupture more easily leading to chronic inflammation (Penn et al., 2008). Chronic inflammation is usually associated with an increase in vascular permeability, inflammatory cell infiltration, edema, tissue destruction, and neovascularization, which most can be seen in diabetic retinopathy, and these inflammatory responses further maintain the inflammatory cycle as a positive feedback (Adamis and Adrienne, 2008). This advanced stage of diabetic retinopathy

can lead to dark spot vision or can even result in complete blindness when the edema in the blood vessels increase to a level where no blood supply reaches the retina, causing an ischemic retina (Vincent and Mohr, 2007; Ciulla et al., 2003; Kempen et al., 2004).

IL-1β is a pro-inflammatory cytokine, which is expressed in many cells in the immune system (Eskan et al., 2008). Studies have shown that IL-1β expression is increased during inflammatory responses, such as diabetic retinopathy (Michelle and Akpek, 2014; Antonetti et al., 2006). Many studies have reported that diabetic patients tend to have a higher rate of RGC apoptosis; this was also seen in a diabetic mouse model (Kern and Barber, 2008). It was also reported that after the initiation of the inflammatory cascade, the activation of inflammatory factors, including IL-1β, will lead to the thinning of the inner plexiform layer, where RGC dendrites are located, causing the loss of RGCs function and contributes in their death. (Kern and Barber, 2008; Lieth et al., 2000).

The dLGN is the visual relay nucleus of the thalamus, where sensory input is received by the retina and sent to the visual cortex (Wong, 1999). In mice, RGCs show structural changes, including swelling of the axons after 3 months of diabetes (Kern and Barber, 2008). Alterations in RGC function could directly affect dLGN neurons. During diabetic retinopathy, RGCs and glial cells undergo many inflammatory responses including increases in inflammatory factors, which would enhance apoptosis (Gardner et al., 2002**;** Barber et al., 1998). In mice, the peak of RGC apoptosis will occur 22 weeks after hyperglycemia (Kern and Barber, 2008). Cell death in the retina can lead to elevated levels of glutamate, which can lead to hyperexcitability or excitotoxicity of the RGCs (Barber et al., 1998).

In general, the pathogenesis of diabetic retinopathy is chronic, occurs over long period of time, months or even years. Here we hypothesize that by the intraocular injection of IL-1β, we will be able to induce an acute inflammatory response, which in turn allows us to test its downstream effect on the dLGN in shorter time scale.

In this study, we will determine whether an inflammatory response after intraocular injections of IL-1β leads to a downstream alteration in dLGN relay neurons. We will record dLGN relay neurons' intrinsic properties at various time points following IL-1β eye injections. We will measure the resting membrane potential, input resistance, time constant, average maximum firing rate, spike frequency adaptation, and rheobase to test whether the proinflammatory injection into the vitreous will alter dLGN relay neurons' intrinsic properties. We hypothesize that injecting IL-1β will lead to modification of the intrinsic properties of dLGN relay neurons.

Materials and Methods

All animal care and experimental procedures used in this study were approved by the MSU Institutional Animal Care and Use Committee. C57Bl/6 mice of either sex were used.

Mice (postnatal day 22-31) were deeply anesthetized using 2-4% isoflurane and kept on a heated pad during the procedure. The sclera and cornea were punctured using a 30 gauge needle and the vitreous was drained to reduce the intraocular pressure. Each eye was injected with 2 µl of IL-1β (10 ng/ml) by using a Hamilton syringe. After the surgery, animals recovered and brain slice recordings were obtained at 24 hours, 48 hours, 72 hours, 1 week, or 2 weeks post injection.

Mice were deeply anaesthetized with 2-4% isoflurane, decapitated, and the brain was quickly removed and placed in a cold (<4°C) oxygenated (95 % O_2/5 % CO_2) slicing solution containing KCl 2.5 mM, NaH_2PO_4 1.25 mM, $MgSO_4$ 10.0 mM, $CaCl_2$ 0.5 mM, sucrose 234.0 mM, glucose 10.0 mM and $NaHCO_3$ 26.0 mM. Slices (300 µm thick) were cut on a vibrating tissue slicer in the coronal plane. Slices were then incubated in a heated holding chamber (~35°C) containing artificial cerebrospinal fluid solution (ACSF) composed of $NaHCO_3$ 26.0 mM, KCl 2.5 mM, glucose 10.0 mM, NaCl 126.0 mM, NaH_2PO_4 1.25 mM, $MgCl_2$ 2.0 mM and $CaCl_2$ 2.0 mM for at least 30 minutes and then maintained at room temperature until use. Individual slices were transferred to a submersion recording chamber, perfused with oxygenated ACSF solution at a rate of 3 ml/min and maintained at 32 ± 1°C.

Recordings were obtained from dLGN thalamocortical relay neurons. Researchers were blind to the condition (control or IL-1β injections) during recordings. All recordings were

performed using the whole cell configuration, and the liquid junction potential (10 mV) was corrected for all measurements. Recording micropipettes had tip resistances of 3-5 MΩ when filled with internal solution containing K-gluconate 117.0 mM, KCl 13.0 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 0.07 mM, EGTA 0.1 mM and HEPES 10.0 mM. Data were acquired using a Multiclamp 700B amplifier (Molecular Devices, Union City, CA) in current-clamp mode. Data were filtered at 10 kHz and digitized at 10 kHz using a Digidata 1550 digitizer, and collected using pClamp software (Molecular Devices, Union City, CA).

Resting membrane potential (RMP) and input resistance (Rin) were calculated; the RMP was measured befor the hyperpolarization current step injection occurs, when the membrane is at rest (fig 3.1). On the other hand, the Rin was measured from the linear slope of the voltage–current relationship obtained by applying constant current pulses ranging from -200 to +200 pA (1 s duration; fig 3.2). The time constant was measured after injecting a fast (100 ms) and small (-10 pA) current step 20 times, we averaged the data and fit a single order exponential curve to obtain the time constant (fig 3.3). Time constant is the time at which the hyperpolarization current step reaches 63% of the maximum response. An average action potential discharge rate (over 1 s) versus current injection graph was generated for each neuron. To measure spike frequency adaption (SFA), we identified the current step that elicited half the maximum average action discharge rate for each neuron. Using that sweep, we averaged the first three and last three instantaneous action potential frequencies (1/interspike interval) and generated an action potential adaptation ratio (mean last/mean first; fig 3.5). The firing characteristics were measured by using (Clampfit 10.7) analysis software. All data are presented as mean ±

standard error of the mean (SEM). All data statistical analysis were done using ANOVA test, where the statistical significance was $p<0.05$.

Results

Recordings were obtained from 54 control (non-injected mice) relay neurons, 14 relay neurons after 24 hours following IL-1β injection, 12 neurons after 48 hours, 11 neurons after 72 hours, 8 neurons after 1 week, and 2 neurons after 2 weeks following IL-1β injection. First, we compared the resting membrane potential of dLGN relay neurons between all groups. In the control group, relay neurons had an average resting membrane potential of -69.7 $\pm$ 2.5 mV (n=54). While 24 hours following IL-1β injection, relay neurons had an average resting membrane potential of -71.4 $\pm$ 2.0 mV (n=14). 48 hours following IL-1β injection, relay neurons had an average resting membrane potential of -70.6 $\pm$ 1.0 mV (n=12). 72 hours following IL-1β injection, relay neurons had an average resting membrane potential of -71.9 $\pm$ 1.7 mV (n= 11). 1 week following IL-1β injection, relay neurons had an average resting membrane potential of -71.5 $\pm$ 1.1 mV (n=8). While 2 weeks following IL-1β injection, relay neurons had an average resting membrane potential of -69.5 $\pm$ 2.1 mV (n=2). After running an analysis of variance (ANOVA) with post-hoc Tukey Honestly Significant Difference (HSD) to compare means, the only significant difference was seen between the control and the 72 hours following IL-1β injection ($p<0.05$, F value: 3.08). This indicates that the resting membrane potential of relay neurons was more hyperpolarized 3 days following IL-1β injection (Fig. 1).

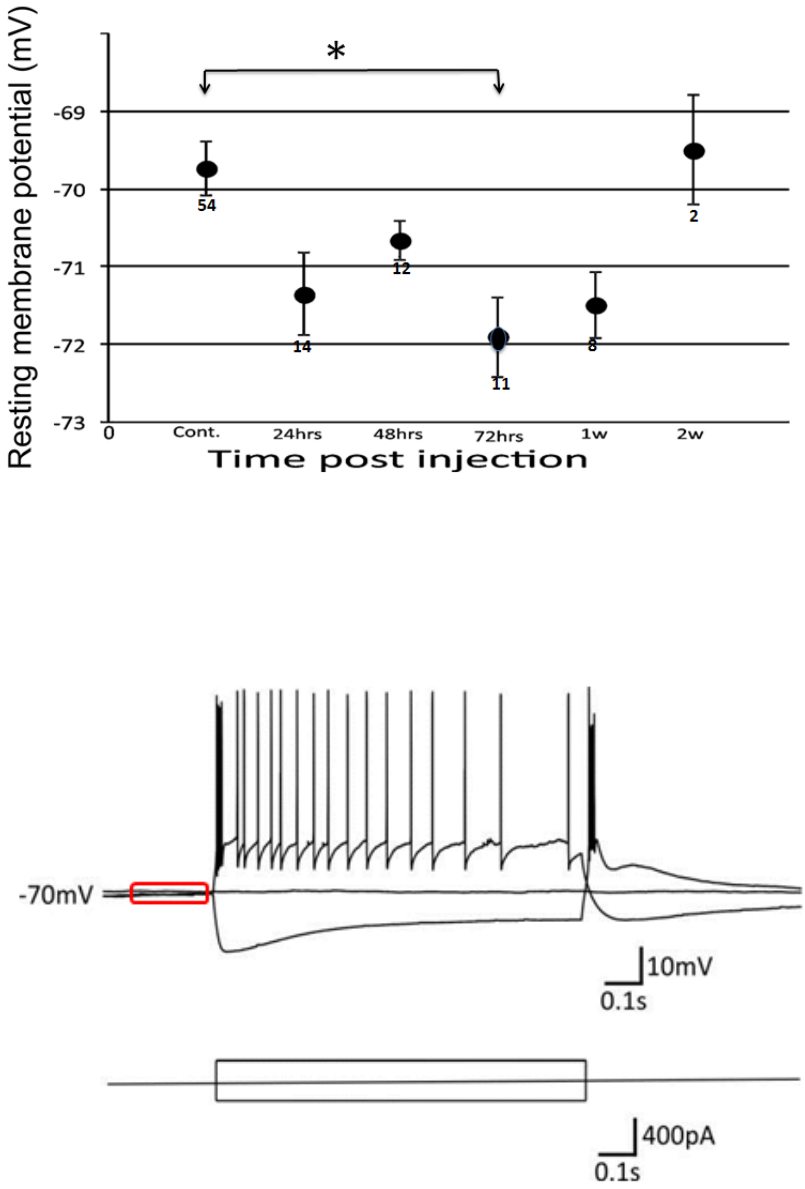

Figure 3.1. The relationship between intraocular IL-1β injection and resting membrane potential of dLGN relay neurons. (Top) Population graph of the average resting membrane potentials measured at various time points following IL-1β injection. 72 hours post-injection had a significantly hyperpolarized average resting membrane potential when compared to control mice (Cont.). No other time points tested were significantly different from control values. (Bottom) A representative trace showing where RMP was measured. *$p<0.05$

The average input resistance for the control population was 202.3 ± 114.2 MΩ (n=54), the 24 hours following IL-1β population was 135.3 ± 39.3 MΩ (n=14), the 48 hours following injection population was 162.5 ± 67.6 MΩ (n=12), the 72 hours following injection population was 166.3 ± 91.1 MΩ (n=11), the 1 week following injection population was 163.8 ± 73.6 MΩ (n=8), and the 2 weeks following injection population was 199.3 ± 46 MΩ (n=2). When input resistances were compared between the control population and each post-injection population, they showed no significant differences (ANOVA with post-hoc Tukey HSD, $p>0.05$, F value: 0.24), indicating that intraocular IL-1β injection does not affect input resistances of dLGN relay neuron within the time periods tested (Fig2).

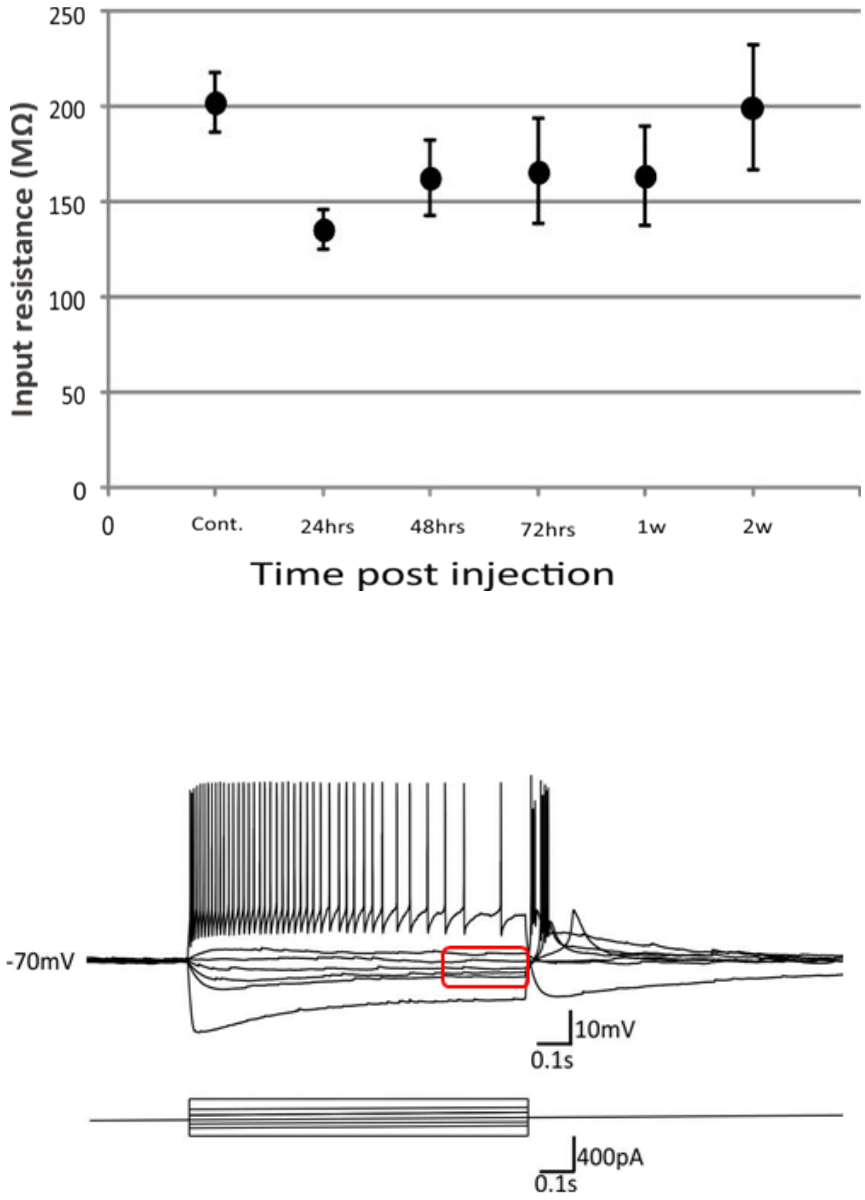

Figure 3.2. The relationship between intraocular IL-1β injection and input resistance of dLGN relay neurons. (Top) Population graph of the average input resistance measured at various time points following IL-1β injection. No significant different was shown between time points tested and control values. (Bottom) A representative trace showing where Rin was measured.

We next measured the membrane time constant to test if there are any differences following IL-1β injection. In control neurons, the membrane time constant averaged 27.4 ± 15.4 ms (n= 54), the 24 hours post-injection population averaged 17.2 ± 8.3 ms (n= 14), the 48 hours post-injection populations averaged 18.0 ± 6.0 ms (n= 12), the 72 hours post-injection population averaged 24.1 ± 11.2 ms (n= 11), the 1 week post-injection population averaged 21.1 ± 10.5 ms (n= 8), and the 2 weeks post-injection population averaged 24.0 ± 1.4 ms (n=2). There were no significant changes in the membrane time constant following injection of IL-1β (ANOVA with post-hoc Tukey HSD, $p>0.05$, F value 0.07), indicating that the time constant of dLGN neurons is unaltered in the time periods tested following intraocular injection of IL-1β (Fig 3).

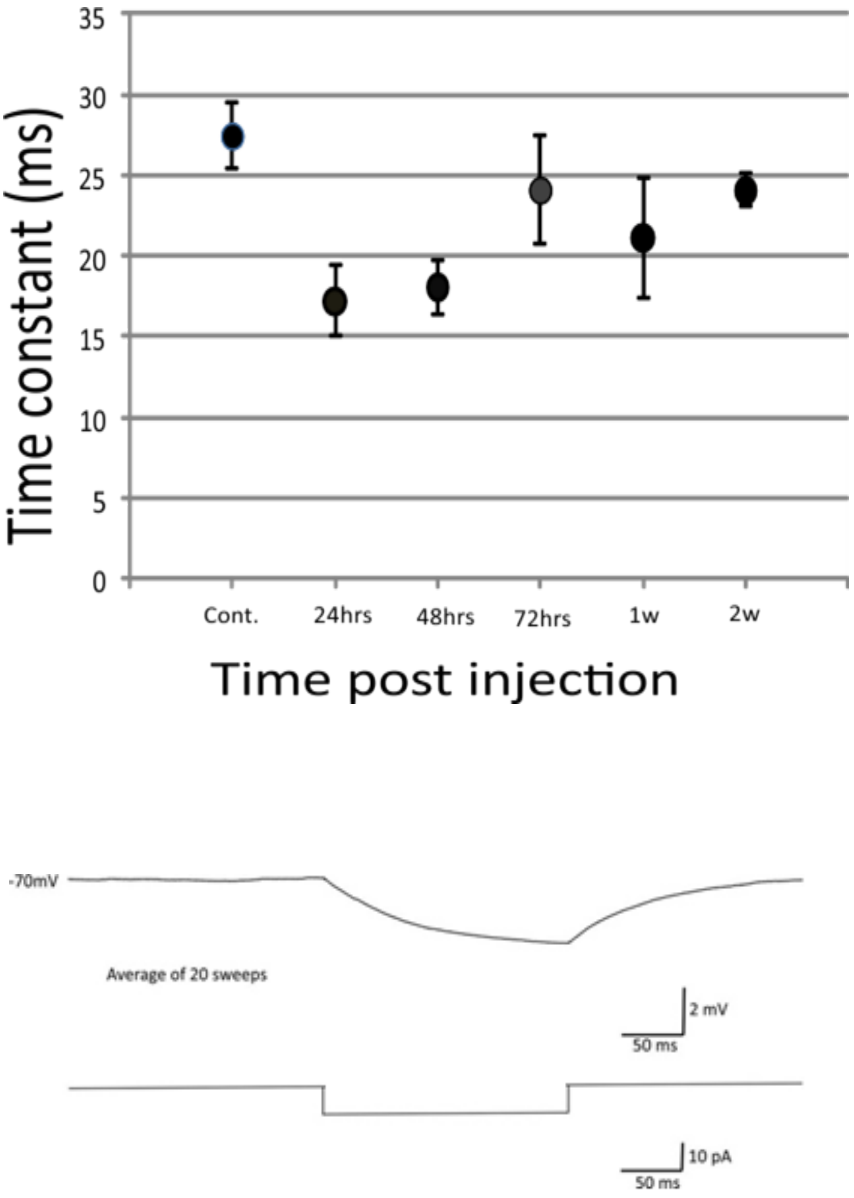

Figure 3.3. The relationship between intraocular IL-1β injection and membrane time constant of dLGN relay neurons. (Top) Population graph of the average time constant measured at various time points following IL-1β injection. No significant alteration was shown between time points tested and control values. (Bottom) A representative trace showing how TC was measured.

To further examine neuronal excitability, we determined the average maximum discharge rate. In control neurons, the average maximum discharge rate (over a 1 second current pulse) averaged 75 $\pm$ 23 spikes (n=54), the 24 hours population, with an average of 95 $\pm$ 49 spikes per second (n=14), the 48 hours populations, with an average of 91 $\pm$ 40 spikes per second (n=12), the 72 hours population with an average of 93 $\pm$ 40 spikes per second (n=11), the 1 week population with an average of 83 $\pm$ 31 spikes per second (n=8), and the 2 weeks population with an average of 174 $\pm$ 34 spikes per second (n=2). After analyzing these data using (ANOVA with post-hoc Tukey HSD), the 2 weeks population showed a significant increase in average maximum discharge rate compared with all other populations ($p<0.05$, F value 0.0016), however it is important to note only two samples were tested (Fig. 4).

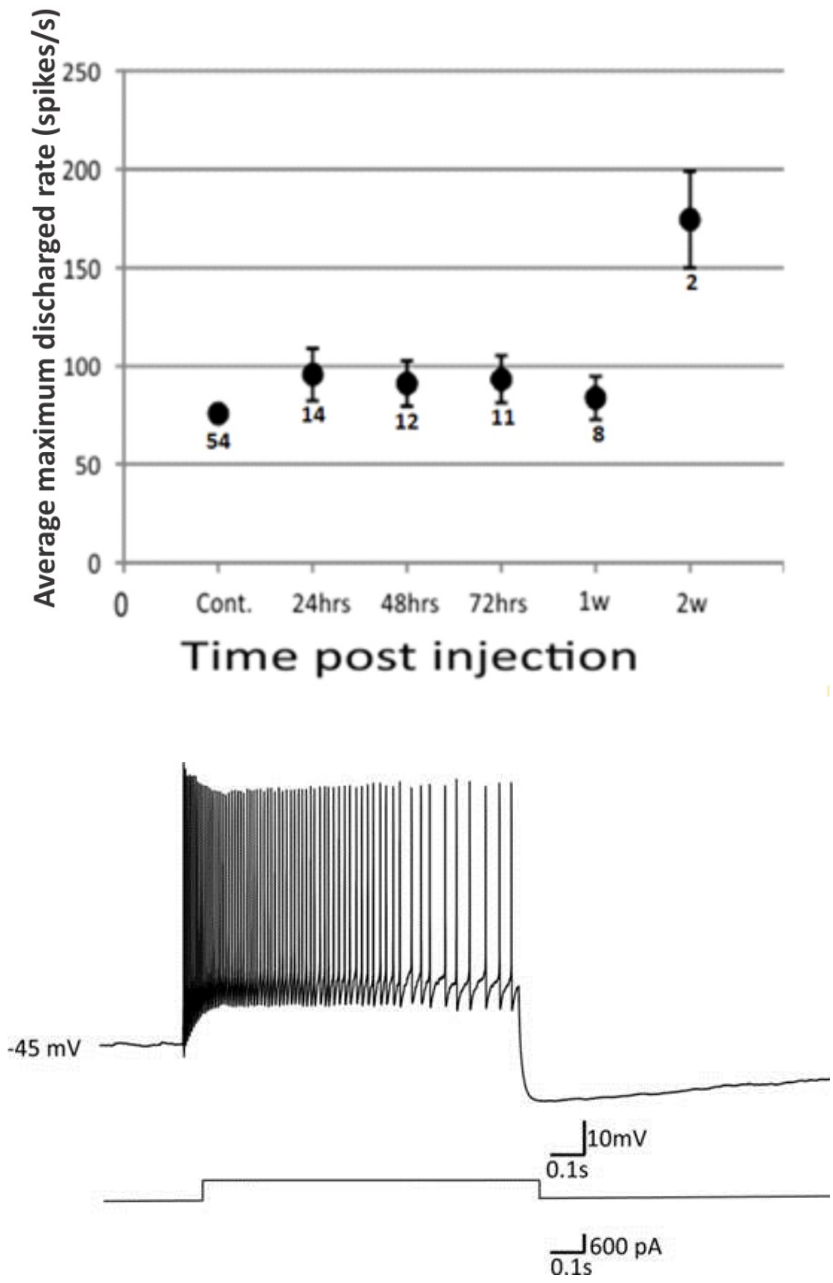

Figure 3.4. The relationship between intraocular IL-1β injection and the average action potential discharge rate of dLGN relay neurons per second. (Top) Population graph of the average action potential discharge rate measured at various time points following IL-1β injection. No significant alterations were shown between time points tested and control values. (Bottom) A representative trace showing how the average action potential discharge rate was measured.

In order to quantify the magnitude of action potential frequency adaptation, we measured the adaptation ratio (see methods) and compared them across populations. The control population ratio averaged 0.34 $\pm$ 0.22 (n=54), 24 hours post IL-1β injection ratio averaged 0.21 $\pm$ 0.09 (n=14), 48 hours post injection group had an averaged ratio of 0.28 $\pm$ 0.17 (n=12), 72 hours post injection group had an averaged ratio of 0.25 $\pm$ 0.11 (n=11), the 1 week post injection group had an averaged ratio of 0.39 $\pm$ 0.21 (n=8) and the 2 week post injection group had an averaged ratio of 0.26 $\pm$ 0.06 (n=2). No significant changes were seen between the control and post injection time groups (ANOVA with post-hoc Tukey HSD, $p>0.05$, F value: 0.15), indicating that spike frequency adaptation is unaltered in the time points tested following IL-1β injections (Fig. 5).

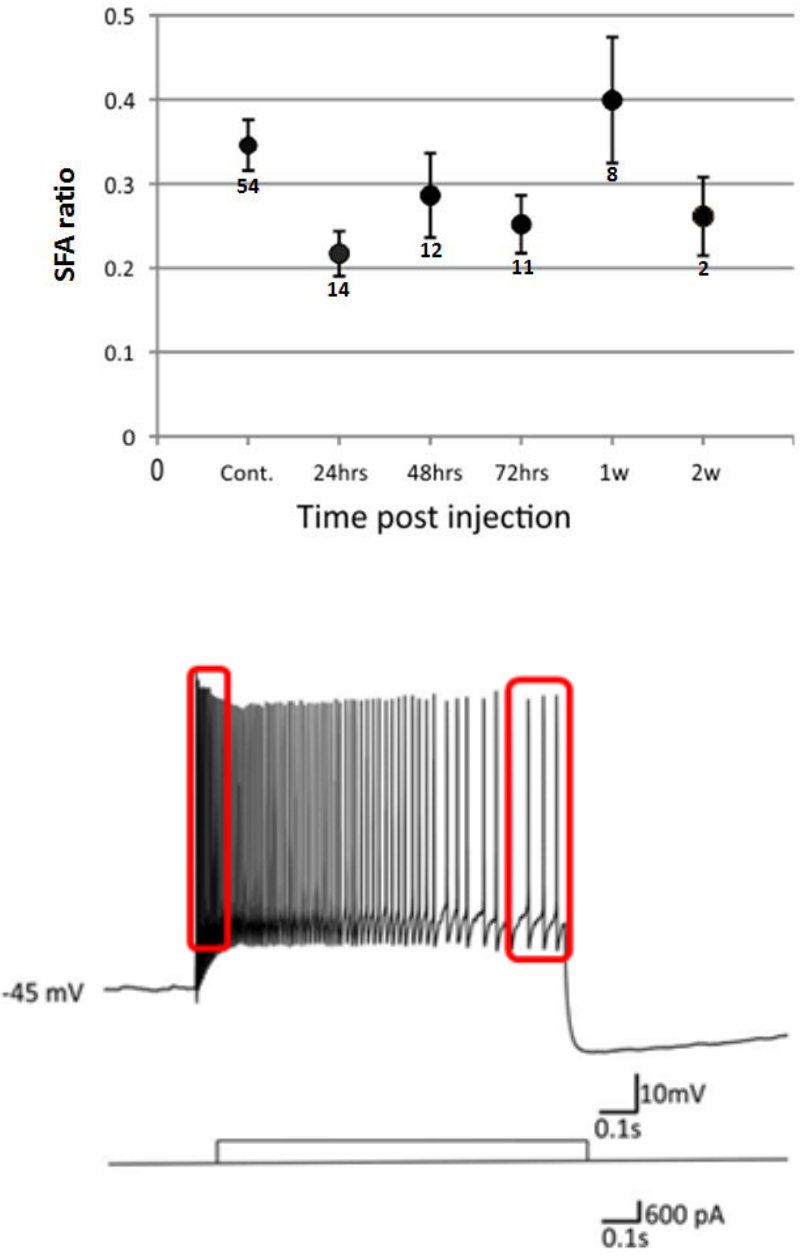

Figure 3.5. The relationship between intraocular IL-1β injection and spike frequency adaptation (SFA) of dLGN relay neurons. (Top) Population graph of the average SFA measured at various time points following IL-1β injection. No significant alteration was shown between time points tested and control values. (Bottom) A representative trace showing how SFA was measured.

We next measured the rheobase to test whether the inflammatory response is changing the amount of current necessary to produce action potential discharge in the relay neurons. The average current that elicited action potential discharge for the control population was 54 $\pm$ 34 pA (n=54). The average rheobase for 24 hours post-injection population was 55 $\pm$ 25 pA (n=14), the average rheobase for 48 hours post-injection population was 67 $\pm$ 52 pA (n=12), the average rheobase for 72 hours post-injection population was 57 $\pm$ 34 pA (n=11), the average rheobase for 1 week post-injection population was 56 $\pm$ 19 pA (n=8) and the average rheobase for 2 weeks post-injection population was 90 $\pm$ 42 pA (n=2). No significant changes were noted (ANOVA with post-hoc Tukey HSD, $p>0.05$, F value: 0.67), indicating that IL-1β injection does not alter the rheobase in the time periods tested. (Fig. 6).

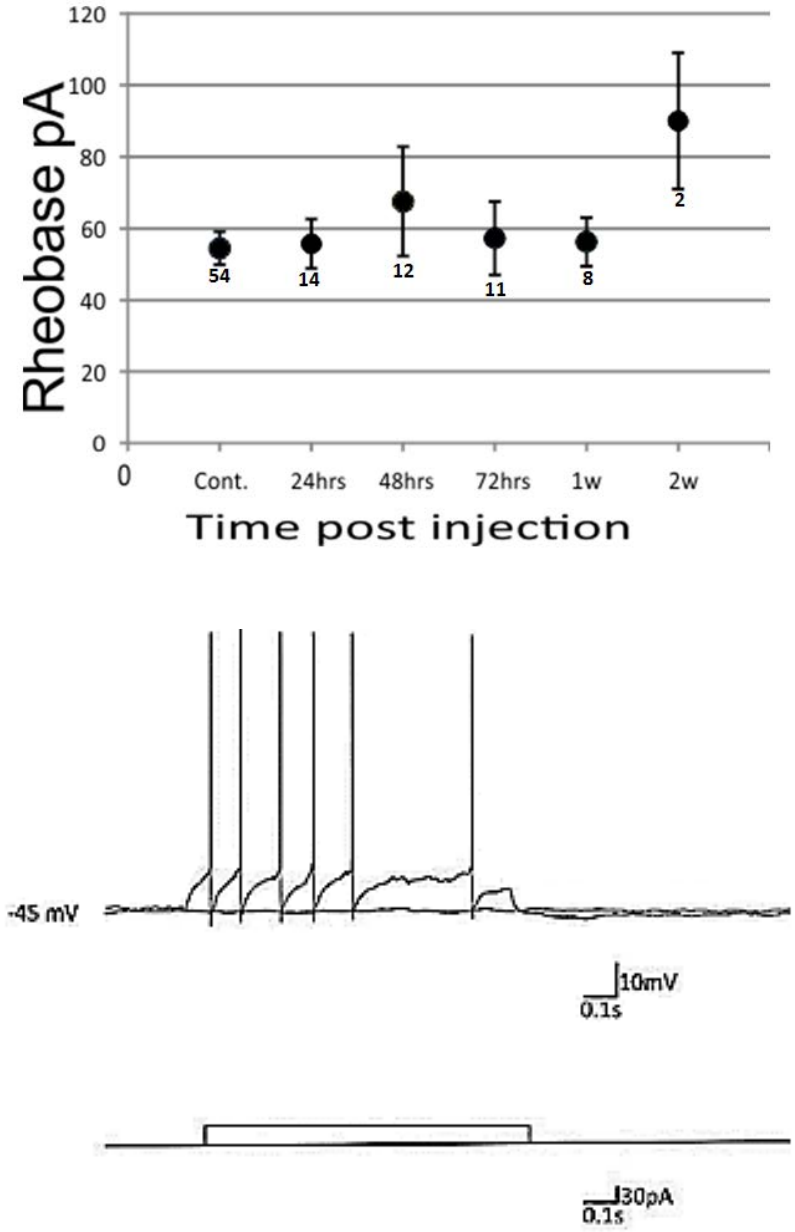

Figure 3.6. The relationship between intraocular IL-1β injection and rheobase of dLGN relay neurons. (Top) Population graph of the average rheobase measured at various time points following IL-1β injection. No significant alteration was shown between time points tested and control values. (Bottom) A representative trace showing how rheobase was measured.

Discussion

In this study we have shown that intraocular injections of IL-1β alter intrinsic properties of dLGN relay neurons. These alterations in the intrinsic properties of relay neurons occur at 72 hours and 2 weeks were the 2 weeks have only 2 cells, after IL-1β injection. These findings indicate that after the injections of IL-1β, the inflammatory response might lead to changes in the RGCs, three days after injection, it hyperpolarized the resting membrane potential of dLGN neurons. It also indicates that there might be a chronic inflammation affecting average maximum firing frequency after 2 weeks post injection, but with the number of cells analyzed it not enough to make a definite conclusion, this alteration might be related to the hyperexcitability due to either the excitotoxicity of RGC or the RGC apoptosis process.

The Hyperexcitability findings obtained two weeks after IL-β injection might suggest that glutamate released during RGCs apoptosis process, could lead to the hyperexcitability of the dLGN neurons. This neuronal hyperexcitability might be the key for visual problems associated with diabetic retinopathy.

These findings could direct us in many directions; first we need to collect more data two weeks post injection, to confirm our outcome. Then we could lengthen the time between injections and recordings following IL-1β, to test whether the excitability seen in this study will continue to increase and at what point does it return to control levels. We also could use higher dosages, which would accelerate RGC cell death and in turn increase the influence on dLGN. We also can test whether the changes in intrinsic properties are dose dependent or not. Since it is known that diabetic retinopathy is a chronic condition, we can add more than one set of

injections, meaning that we give 3 to 4 injections with a set time between injections to extend the inflammatory response period, and test if there will be any differences.

We also could do the same experiment and study IL-1β injection effect on dLGN interneurons, since they are deeply involved in the regulation of retinal output to the dLGN, and test whether it will alter its intrinsic property.

From this experiment, we conclude that intraocular injection of pro-inflammatory cytokine IL-1β, can alter dLGN neurons intrinsic properties. Studies have shown that retinal cell death, which could alert RGC output, could be prevented with the combination of many therapeutic strategies, such as the use of anti-inflammatory agents, which could significantly decrease the apoptosis rate, another way to prevent retinal cell death is by inhibiting glutamate, by using NMDA receptor antagonist, and one of the useful methods is the use of cannabidiol as neuronal protective therapy to prevent retinal cell death (Kern and Barber, 2008).

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Chapter 4

Summary and future direction

Summary

The brain is the organ that controls all other body functions, it controls every single movement and every single thought we do, and its regions are well connected. Both FXS and diabetic retinopathy are pathological conditions that have significant influences in the brain. In diabetic retinopathy, defect in the retinal RGCs could have an effect on the thalamus relay neurons and it even might have a downstream effect on the cortical pyramidal neurons. On the other hand, individuals with FXS, which is manifested in the brain, are experiencing many neurological and behavioral defects. Up to date there is no definite treatment to prevent either condition. The lack of cure as well as the expansion in their influences gave both FXS and diabetic retinopathy the attention of scientist to fully understand those conditions and to participate to find their cures.

FXS is associated with many cognitive, physical and biological defects. It occurs in a ratio of 1:4000 in males and 1:8000 in females making it the most common form of mental retardation. A genetic correction of the hypermethylation in the *Fmr1* gene could be possible someday, leading to a normal production of FMRP. Also, treating the mGluRs influence on the disease might be a solution in the near future, by finding the right pharmacological agent that can prevents the overactivation of group I mGluR, leading to the reduction of excitation associated with FXS, without affecting the numerous functions of mGluR. Finding such treatments might lead to the regulation of synaptic protein synthesis, which will solve many

hyperexcitability issues associated with FXS. Finding a treatment for FXS is the dream of many scientists, who spent their lives trying to make a miracle.

On the other hand, with our modern lifestyle, and with the fast and uncontrolled rise in diabetic patients, the occurrence of diabetic associated blindness will eventually rise. This increase will have an economic as well as productivity influences. When blindness increases, affected people may require more care and may hospitalization.

Diabetic retinopathy could be controlled when detected in the early stage, to slow its prognosis, but not to revers it, and it is untreatable, up to date, when it gets to its advanced stage. Continues vision monitoring is critical for diabetic patients, and any visual changes could be a sign for developing diabetic retinopathy.

The main reason for diabetic retinopathy development is its association in the RGCs death, preventing the death of RGCs could significantly limit the defects in diabetic retinopathy. RGCs death is the result of glia and microglia cells activation, which leads to the activation of caspase 1 as well as production of many inflammatory cytokines and the production of glutamate. The excess glutamate production as well as the activation of caspase 1 will lead to the RGCs death.

With more research and work in both FXS and diabetic retinopathy, we will eventually be able to treat them and prevent their influence on humanity.

Future directions after the postsynaptic actions of mGluR in *Fmr1* KO mice study

Since the activation of mGluRs is inducing alterations in plasticity in different brain regions, and after our results that showed no alterations in short-term induction of both group I and II mGluR agonist, we could try the induction of a general mGluR agonist for longer than (60s) and test its effect on *Fmr1* KO mice and compare it to WT. This might show that the binding affinity of the mGluR is altered in the *Fmr1* KO mice after a set time, and this alteration could be shown by increasing the drug application time.

We also could record from interneurons instead of excitatory pyramidal; this alteration in excitability could be driven by an exaggerated inhibition. We might find that the *Fmr1* KO mice are having significant reduction in the inhibitory circuit, leading to the hyperexcitability manifestation seen in FXS.

On the other hand, we could record from excitatory neurons in deep layers visual cortex, where we previously showed the alteration in plasticity, to support either outcome. We also could record from inhibitory interneurons in visual cortex, to test wither the hyperexcitability is due to reduced inhibition.

The conformation of the defects in mGluR would be significantly helpful for the full understanding of FXS and it could lead to the production of suitable pharmacological agents that could preserve the crucial functions of mGluR in FXS. With the huge amount of work and publications in FXS, and with the full understanding of the condition will make the prevention of FXS become possible.

Future direction after the IL-1β intraocular injection and its alteration on the dorsal lateral geniculate nucleus relay neurons

Based on the potential changes two weeks after intraocular injection of IL-1β, it is critical to lengthen the time between injection and recording in order to confirm the alteration seen in the average maximum firing discharge as well as increase the number of data in the two weeks post injection group.

It is also beneficial to start recording from the RGCs and determine the impact of intraocular injections of IL-1β on the intrinsic activity of RGCs. When recording directly from the RGCs, we could determine whether they are altered, and if they are, what the time point of RGC alteration, which can influence neurons of the dLGN.

Currently we are testing the influence of an acute inflammatory condition, after the outcome of this study, it may be more affective to use multiple injections (3-4 instead of one) to produce a chronic condition rather than an acute one, since diabetic retinopathy is a chronic disease. We then could use the 3 or 4 week post injection mice to test the influence of the chronic inflammatory response on the RGCs as well as the neurons of the dLGN.

Using higher IL-β concentration is going to be helpful to determine the influence of IL-1β on the RGC health. By injecting higher concentration, we are accelerating the RGC cell death, which could allow us to clearly identify the downstream effect of that on the dLGN relay neurons.

In this study, we have collected all eyes after mice decapitation and they were kept in … for farther assessment, it is significant to look at these eyes and assess the degree of damage to

the RGCs in each post-injection population, if there are any damages, which would be beneficial to identify the affect associated with the dosage we used and gives us a window for IL-1β concentration manipulation.