2018-01-01

The Protective Effects Of Ferrostatin-1 (fer-1) In Response To Excitotoxicity In Mouse Hippocampal Slices

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THE PROTECTIVE EFFECTS OF FERROSTATIN-1 (FER-1) IN RESPONSE TO EXCITOTOXICITY IN MOUSE HIPPOCAMPAL SLICES

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Dedication

Dedicated to my mother Mireya Mendez Legarreta

THE PROTECTIVE EFFECTS OF FERROSTATIN-1 (FER-1) IN RESPONSE TO EXCITOTOXICITY IN CELL MOUSE HIPPOCAMPAL SLICES

By

MIREYA NAEL RAMIREZ, B.S.

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2018

Acknowledgements

Firstly, I would like to thank my P.I. Dr. Karine Fénelon for allowing me to join her lab and for giving me the confidence to be part of her research team. I would never be able to thank her enough for her guidance and support throughout these past years. She always pushed me to believe and to work hard to complete my goals. I deeply thank all the time she dedicated towards my project and to support me with her knowledge and her distinguishing enthusiasm.

I would also like to thank my thesis committee members: Dr. Giulio Francia and Dr. Rachid Skouta for their time and guidance throughout the completion of my Master's project. Additionally, I want to thank Dr. Arshad Khan and the BBRC core members for allowing me to use their equipment and facilities that helped me to conduct my research and produce the results presented here.

I am deeply grateful with my lab mates for their help, support and time they offered to me throughout my project: Jose Cano, Laura Montes, Luis Martinetti, Anahis Tena and our lab tech Carla Loyola. I would like to give special thanks to Vanessa Navarro and Eduardo Peru for their unconditional support and help in many of the techniques and experiments I performed during my project, as well as their absolute friendship in these past years.

Last but not least, I would like to thank the support from the people who helped me to reach my professional and personal goals: my parents, Mireya and Noel; my

brother, Noel Edmundo; and my loved one. To my family and friends who always showed me their support, motivation and love.

Abstract

The development of different neurological disorders has been associated with the accumulation of reactive oxygen species (ROS). Elevated ROS levels can disrupt the neuronal electrical activity. This phenomenon has been seen during epilepsy and neurodegenerative diseases like Parkinson's disease. Furthermore, the excess of ROS and development of such disorders have been linked to neuronal cell death partly due to excessive, non-physiological glutamate release. Ferroptosis is a recently defined iron-dependent cell death mechanism. Inducible by a small molecule called erastin, ferroptosis was first described in cancer cells. Erastin-induced ferroptosis differs from apoptosis, necrosis and autophagy, since it leads to different biochemical and morphological changes, compared to the other cell death mechanisms. It is characterized by the accumulation of iron-dependent lethal ROS. Interestingly, both ferroptosis and glutamate excitotoxicity are associated with an increase in ROS levels. Moreover, both erastin-induced ferroptosis in cancer cells and glutamate-induced cell death in neurons can be inhibited by Ferrostatin-1 (Fer-1), a small molecule bearing antioxidant properties. This suggest that both cell death mechanisms share a similar lethal pathway that can be rescued by Fer-1. To better understand the neuroprotective properties of Fer-1, the morphological and electrophysiological changes induced by glutamate excitotoxicity and erastin were examined and compared to conditions where Fer-1 is present. The proposed project employed cell cultures, immunostaining and *in vitro* electrophysiological experiments in hippocampal slices from mice.

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Chapter I. Introduction

I.1 Ferroptosis – an iron-dependent non-apoptotic cell death mechanism

Death is a word that often times alarms people, however at a cellular level, death is a mechanism that is needed in order to maintain homeostasis within many tissues and systems (Fuchs and Steller, 2011). Apoptosis is a physiological programmed cell death mechanism that has been extensively described. The regulation of apoptosis is critical to avoid the development of different developmental, neurodegenerative and hyperproliferative diseases such as cancers (Fuchs and Steller, 2011; Dixon et al., 2012;). This programmed cell death mechanism involves the activation of proteins like caspases, and physiological cell changes like nucleus condensation (which is a hallmark of this mechanism) without obvious cell membrane disruption. (Yuan and Kroemer, 2010; Fuchs and Steller, 2011). However, studies have shown that there are other types of cell death pathways that can be involved during the development of different pathological conditions (see Dixon et. al., 2012).

The development of many neurodegenerative disorders is associated with an impairment of neuronal activity, sometimes leading to cell death. As previously mentioned, cell death can be induced by different mechanisms. Previous studies have shown that induction of cell death by different small molecules like erastin and RSL3 involve iron (Cheah et al., 2006; Dixon et al., 2012; Skouta et al., 2014; Do Van et al., 2016). Based on recent in-vitro studies, a new form of iron-dependent non-apoptotic cell death called ferroptosis has been defined (Dixon et al., 2012; Skouta et al., 2014; Do Van et al., 2016; Cao and Dixon,

2016). Evidence show that ferroptosis differs from apoptosis since it shows no activation of caspases, release of mitochondrial cytochrome c and morphological changes like chromatin fragmentation (Dixon et al., 2012; Do Van et al., 2016; Cao and Dixon, 2016). In comparison to other cell death mechanisms like apoptosis, necrosis, and autophagy, ferroptosis seems to lead to morphological changes such as smaller mitochondria with a greater membrane thickness (Table 1) (Dolma et al., 2003; Cao and Dixon, 2016). Moreover, ferroptosis has been shown to increase the accumulation of intracellular reactive oxygen species (ROS) that affect cell function as well as to decrease the levels of glutathione (GSH), an important antioxidant (Mattson, 2000; Dixon et al., 2012; Cao and Dixon, 2016).

Table 1. Biochemical and morphological characteristic changes of different cell death mechanisms. (Modified from Cao and Dixon, 2016).

Type of cell death	Biochemical changes	Morphological changes
Apoptosis	Caspases activation, DNA fragmentation.	Rounded cell shape, decreased volume of cell, damaged nucleus (size and fragmentation) and chromatin condensation.
Necrosis	Reduced ATP levels and activation and hyperactivation of signaling pathways.	Disrupted plasma membrane, swelling of cytoplasm and organelles, and some chromatin condensation.
Autophagy	Substrate degradation.	Increase in doubled-membrane autophagic vacuoles and no chromatin condensation.
Ferroptosis	Reactive oxygen species (ROS) imbalance, iron accumulation, reduction of glutathione.	Rounded cell shape, smaller mitochondria and condensed membrane, regular nucleus and no chromatin condensation.

I.2 Erastin and excitotoxic glutamate levels as inducers of cell death

Ferroptosis was discovered while cell death induced by Ras-selective lethal (RSL) compounds was being studied (Yagoda et al., 2007; Dixon et al., 2012). In about 30% of different cancers, there is a mutation in the RAS GTPase superfamily (Dixon et al., 2012). The mutant Ras is a small GTPase protein that, under non-pathological conditions, is important for many signaling pathways like cell growth, migration and the programmed cell death mechanism – apoptosis (Fernández-Medarde and Santos, 2011; Cao and Dixon, 2016). However, mutations in RAS genes can lead to the production of permanently overactivated Ras proteins. Such RAS mutants are oncogenes and have been associated with the development of molecular abnormalities and cancer (Fernández-Medarde and Santos, 2011; Dixon et al., 2012). Previous studies have provided evidence that small molecules like erastin can induce cell death using RAS mutant cell lines. However, results indicate that the erastin-induced death does not show typical apoptotic features such as chromatin fragmentation (Cao and Dixon, 2016). In fact, erastin-induced death involves a unique set of morphological, biochemical, and genetic features suggesting that erastin act as an inducer of ferroptosis. Furthermore, the erastin-dependent cell death was shown to be inhibited by iron chelators and lipophilic antioxidants, which indicate that erastin-induced cell death depends on the iron-dependent accumulation of ROS (Weinreb et al., 2013; Cao and Dixon, 2016). Interestingly, the accumulation of ROS has a high impact on lipid membranes. Therefore, it has been suggested that ferroptosis, the erastin-induced

cell death pathway, includes ROS formation leading to the lipid peroxidation of cell membranes (Dixon et al, 2012; Do Van et al., 2016).

Another process that has been described to be iron-dependent is excitotoxic cell death in the nervous system. This oxidative, iron-dependent cell death is usually seen in strokes and epilepsy as well as in neurodegenerative diseases like Parkinson's disease characterized by the deterioration of the substantia nigra pars compacta (SNpc) (Choi, 1988; Linert et al., 2000; Dixon et al., 2012; Do Van et al., 2016). One of the most studied inducers of excitotoxicity is glutamate. At physiological levels, glutamate is very important for neuron-neuron communication at synapses, as well as for physiological processes like axonal and neuronal growth and guidance (Lai et al., 2014). Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Usually, under resting conditions, the extracellular levels of glutamate are kept low (nanomolar) by glutamate transporters and increase only during synaptic transmission (≈ 500-1000 μM) (Choi, 1988) when it is actively released. However, under pathological conditions, it has been demonstrated that high levels of glutamate (beyond physiological values; > 3 mM for example) can cause neuronal damage and loss, as seen in different neurological diseases (Choi, 1988; Dixon et al., 2012). Furthermore, high levels of glutamate are linked to the inhibition of the biosynthesis of intracellular glutathione, leading to the accumulation of lipid reactive oxygen species, similar to what is seen during ferroptosis (Dixon et al., 2012; Cao and Dixon, 2016). Given the neurotoxic nature of an excess of glutamate and the fact that it can lead to an oxidative iron-dependent cell death process,

it has been suggested that glutamate-induced lethal effects could be related to erastin-induced ferroptosis. Previous studies conducted by Dixon et. al. in 2012, demonstrated that the exposure of rat organotypic hippocampal slice cultures (OHSCs) to excitotoxic glutamate levels can induce cell death similar to the effects of erastin on oncogenic RAS-mutated cell culture (Dixon et al., 2012). Because the glutamate-induced cell death was rescued by iron chelation in OHSCs, it was concluded that glutamate-induced death in OHSCs and erastin-induced death in cancer cells share a common lethal mechanism of action that can be inhibited by iron chelation. Therefore, it is tempting to speculate that an excess of glutamate can kill neurons by a ferroptotic-like mechanism.

I.3 Inhibition of Ferroptosis by Ferrostatin-1

Ferroptosis can be induced by various molecules like erastin and, as suggested above, maybe also by cytotoxic levels of glutamate. Interestingly, both erastin-induced Ferroptosis and glutamate-induced cell death can be inhibited by another small molecule named Ferrostatin-1 (Fer-1) (Dixon et al., 2012; Skouta et al., 2014). Fer-1 is a small molecule with a radical scavenger activity that was recently synthesized and used to test its possible therapeutic effects on disease models such as stroke, Huntington's Disease and Epilepsy (Dixon et al., 2012; Skouta et al., 2014; Skouta, 2016; Do Van et al., 2016). Previous studies have provided evidence that Fer-1 can inhibit the effects of RSL-induced cell death. Furthermore, Fer-1 did not inhibit cell death induced by apoptotic agents or cell death induced by other oxidative lethal compounds. This suggests that Fer-1 specifically counteracts RSL-induced cell death, as was shown using erastin-induced cell

death in cancer cells (Dixon et al., 2012). Although the exact pathway by which iron contributes to ferroptosis and how Fer-1 can interfere in this process needs to be described in more details, it is clear that Fer-1 can inhibit the damage to membrane lipids and therefore it can prevent cell death induced by ferroptosis (Skouta et al., 2014; Do Van et al., 2016). Besides the findings of the role of ferroptosis in cancer, it has been suggested that ferroptosis inhibition can have a neuroprotective effect and can therefore prevent the development of different neurological disorders (Dixon et al., 2012; Skouta et al., 2014). Previous results hence strongly suggest that Fer-1 can have a protective effect on cells and on hippocampal tissue exposed to cytotoxic levels of glutamate, equivalent to pathological conditions such as epilepsy.

I.4 SH-SY5Y cells as a neuronal model

There are different cell lines that are widely used as models to study neurological diseases (Zhang et al., 2014). While conclusions obtained from data using cell cultures might not be directly translatable to neuronal tissue (Zhang et al., 2014; Do Van et al., 2016), many cell cultures like the human neuroblastoma SH-SY5Y cell line and the Lund human mesencephalic (LUHMES) are well established neuronal cell models which are widely used. The SH-SY5Y cells have the ability to produce Dopamine (DA) and noradrenaline (NA). Although when undifferentiated, this cell line cannot be considered as purely dopaminergic, SH-SY5Y cells have been extensively used as neuronal models to study dopamine-dependent neurodegenerative diseases (Xicoy et al., 2017). Remarkably, this cell line can be differentiated to have a neuronal-phenotype (Forster et

al., 2016). Previous protocols used Retinoic Acid (RA) to differentiate SH-SY5Y cells into neuron-like cells. Indeed, once differentiated, these cells showed prolonged extensions or neurites (Forster et al., 2016).

Moreover, recent studies used LUHMES cells to study neurological diseases and the effect of Erastin and other cell death inducers on the viability of these cells (Do van et al., 2016). Work done by Do Van et al., 2016, demonstrated that certain cells can be killed by erastin-induced ferroptosis. In that study, Do Van and collaborators tested three different culture cell lines, including SH-SY5Y cells. After incubation with Erastin (0 – 6μM), the viability of the three cell lines was tested by staining with the supravital dye propidium iodide (0.5μM), and the number of surviving cells was evaluated/quantified using flow cytometry. Their results showed that Erastin-induced ferroptosis killed LUHMES cells exclusively, since the SH-SY5Y cells and the motor neuron-like NSC-34 cells remained largely unaffected by the Erastin treatment **(Figure 1.1)**. Interestingly, their data also showed that all cell lines could be killed via an apoptotic mechanism, although the LUHMES cells were affected to a lesser extent than the other cell lines (Do Van et al., 2016). These results suggest that LUHMES and SH-SY5Y cells can be further used to understand in more details the mechanism of action used by of Erastin to induce ferroptosis.

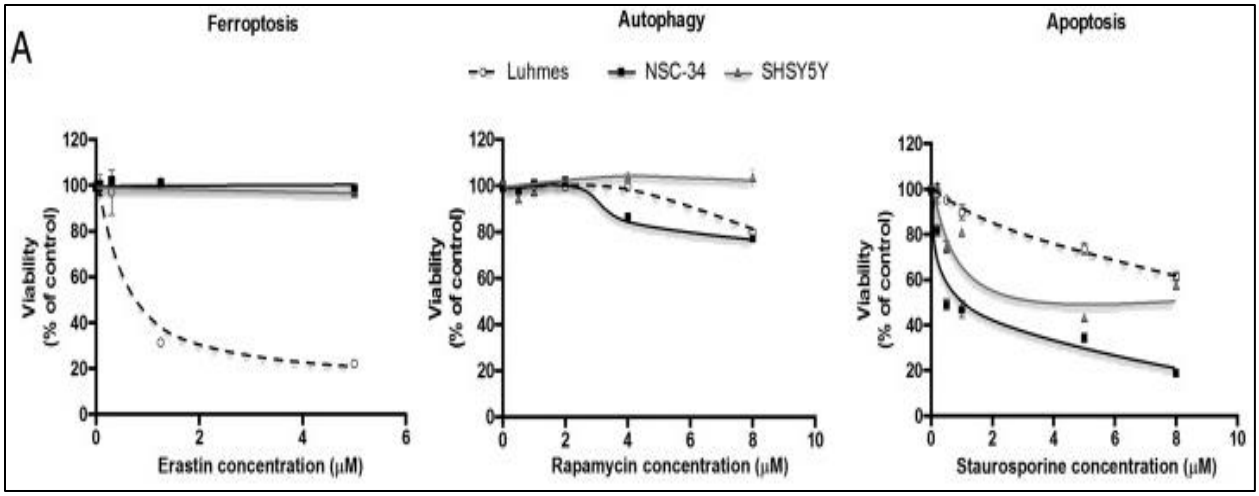

Figure 1.1. **The effect of various cell death inducers on LUHMES, NSC-34 and SHSY5Y cells.** The graphs show the cell viability as a function of treatment concentration on Luhmes, NSC-34 and SH-SY5Y cells. The treatments were as follows: Erastin to induce ferroptosis, Rapamycin to induce Autophagy and Staurosporine to induce Apoptosis. As seen on the first graph, Erastin only decreased the viability of Luhmes cells via ferroptosis, without affecting SH-SY5Y and NSC-34 cells, (Do Van et al., 2016).

Glutamate excitotoxicity has been linked to neuronal cell death similar to what has been observed during epilepsy (Murphy et al., 1989; Sundstrom et al., 2005; Dixon et al., 2012). In addition to these findings, studies performed by Dixon et al., 2012, suggest that OHSCs incubated with excitotoxic glutamate levels can be linked to ferroptosis-like effect. Therefore, OHSCs were co-incubated with glutamate (5mM) and Fer-1 (2μM) and other iron chelators to determine if glutamate-induced neurotoxicity could be rescued by a ferroptosis inhibitor. Results were analyzed on three different regions of the organotypic hippocampal slices, the dentate gyrus (DG), CA1 and CA3 fields. Results showed that glutamate excitotoxicity induced cell death in all the three regions analyzed. Interestingly, the co-incubation of an excess of glutamate with Fer-1 diminished the glutamate-induced cell death in all three hippocampal regions (Dixon et al., 2012) **(Figure 1.2)**.

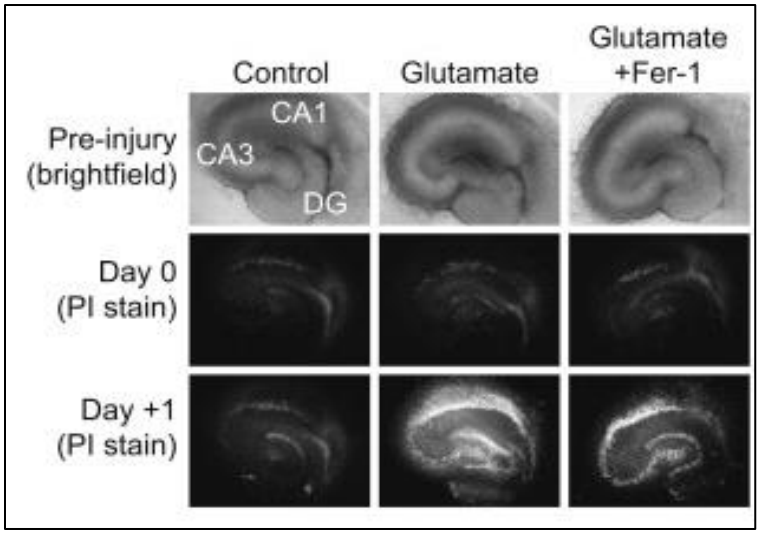

Figure 1.2. **Glutamate effects on Organotypic hippocampal slice cultures (OHSCs).** Images showing OHSCs exposed to 5 mM glutamate and glutamate + 2 μM Fer-1. OHSC were stained with propidium iodide (PI) to indicate cell death (Dixon et al., 2012).

I.5 ROS accumulation and its correlation to neurological disorders

Many neurological diseases involve an abnormal accumulation of ROS (Dixon et al., 2012; Brožíčková and Otáhal, 2013). Iron-dependent lipid radical species accumulation has been implicated in Parkinson's Disease, strokes and epilepsy (Choi, 1988; Morrison et al., 2002; Brožíčková and Otáhal, 2013; Do Van et al., 2016). At physiological concentrations, radical species like Nitric Oxide (NO) contribute to neuronal activity. NO is a physiological regulator of synaptic transmission and plasticity (Garthwaite et al., 1998; Brožíčková and Otáhal, 2013), however at higher concentrations, NO can also harm neuronal network activity due to its reactivity. In fact, NO imbalances have been involved in different neurodegenerative diseases and in epilepsy (Giux et al., 2005; Brožíčková and Otáhal, 2013). During epilepsy-related seizures, the levels of NO are increased and the electrical activity of neurons in brain regions like the hippocampus

is affected (Brožíčková and Otáhal, 2013). Epileptic-like activity can be elicited in vitro in rodent hippocampal slices using an excess of glutamate (Do Van et al., 2016). Therefore, since: 1- glutamate excitotoxicity can lead to NO imbalances and abnormal electrical activity in hippocampal neurons; and 2- the effects of glutamate excitotoxicity can be rescued by the ROS-dependent ferroptotic antagonist Fer-1, it was suggested that the cellular mechanisms underlying neuronal epileptiform activity might be linked to ferroptosis induction (Cater et a., 2007; Dixon et al.,2012).

I.6 Specific Aims

Therefore here, we used cells in culture and mouse hippocampal slices to further understand the effects of erastin and glutamate and the neuroprotective properties of Ferrostatin-1. We tested our hypothesis that glutamate excitotoxicity share ferroptotic properties by disrupting neuronal cell structure and function in a similar way. To test our hypothesis, we designed the following specific aims:

Specific Aim 1: Determine whether glutamate-dependent excitotoxicity and erastin-induced ferroptosis lead to similar morphological changes. The "control" SH-SY5Y neuronal-like cell line was cultured and differentiated and treated with erastin. In addition, mouse hippocampal slices were incubated with either glutamate or erastin, in the absence and presence of Fer-1. Subsequently, immunocytochemistry was used to analyze the morphological changes of culture cells and hippocampal neurons induced by the different treatments.

Specific Aim 2: Determine whether Fer-1 can rescue the abnormal *in vitro* electrophysiological activity of hippocampal neurons induced by glutamate excitotoxicity. We performed extracellular field electrophysiological recordings on acute hippocampal brain slices from WT mice. During these *in vitro* recordings, high levels of glutamate were first added to the perfusing solution to elicit epileptiform activity in CA3 hippocampal neurons. Then, the ferroptotic antagonist Fer-1 was bath applied to determine whether this small antioxidant molecule can restore the abnormal electrical activity of hippocampal neurons towards normal values.

Chapter II. Experimental Approach

II.1 Specific Aim 1: Determine whether glutamate-dependent excitotoxicity and ferroptosis lead to similar morphological changes in cell culture and hippocampal neurons.

II.1.1 Objective and Overview

As mentioned above, erastin can induce ferroptosis in different cell cultures. Indeed, LUHMES cells incubated with erastin showed a decreased viability, whereas SH-SY5Y cell line were unaffected by erastin **(Figure 1.1)** (Do Van et al., 2016). The rationale for using the SH-SY-5Y cells line relies here was to use them as a control and confirm that we could replicate published data. Interestingly, the morphological changes consequent to erastin were not investigated in differentiated SH-SY5Y cells. Moreover, comparing effect of Fer-1 on cytotoxic glutamate or erastin exposure using mouse hippocampal neurons *in vitro* remained to be done. Therefore, the main goal of this aim was to identify the morphological changes induced by erastin or an excess of glutamate and to determine whether such morphological changes could be prevented (or reversed) by Fer-1. To address this aim, I first used the SH-SY5Y cell line. These cells were differentiated and incubated with erastin. I used these cells as "control" since we expected to see no erastin-induced changes on their morphology, according to previous reports. I then performed immunocytochemical experiments to label and quantify morphological features such as, neurite and nucleus morphology under the different experimental conditions (i.e., drug treatments). Second, I used mouse hippocampal slices either under control conditions or

incubated either with glutamate or erastin, In the absence and presence of Fer-1. Then I used confocal microscopy and Immunohistochemistry to examine the soma of these hippocampal neurons under the different experimental conditions

II.1.2 Materials and Methods

Cell culture: SH-SY5Y (donated by Dr. Rachid Skouta's laboratory, Chemistry department, The University of Texas at El Paso, USA) cells were grown on T75 flasks (Corning, USA) in Dulbecco's modified Eagle's Medium (DMEM) (Corning, USA), supplemented with 10% fetal bovine serum and 1 % streptomycin in in 5% CO2/95% air at 37°C. During cell culture, complete medium was changed every three to four days and cells passaged before 90% confluency.

Cell differentiation: For cell differentiation, about $8X10^5$ cells were seeded onto glass coverslips (Fisher, USA, Cat # 12-545-100) in a six-wells plate (VWR, USA, cat. #10062-892) in complete medium. After twenty-four hours, cells were treated with differentiation medium containing 10% fetal bovine serum, 1 % streptomycin and 0.1% Retinoic acid (RA) (Sigma, USA, cat. #R2625). Medium containing RA was changed every two days for five consecutive days in order to induce a neuronal phenotype. A negative and a positive control were always included on each experiment. Our negative control consisted of undifferentiated cells; the positive control was the differentiated cells treated with Retinoic Acid.

Treatments: 10μM erastin was applied to the cells either before the end of the cell differentiation (i.e., co-incubation) OR after the cell differentiation period (i.e., post-incubation)

a) During the co-incubation condition, cells were treated with differentiation medium along with erastin. The incubation was interrupted on day 6 to perform immunocytochemistry. This protocol was identified as "co-incubation treatment". Here, medium was renewed every two days.

b) In a subset of experiments, erastin was added to the cells after complete differentiation was obtained. Some cells were co-treated with Fer-1. Cells were incubated for 24 hours with the treatment and then incubation was stopped to proceed with fixation and immunohistochemical staining; this protocol was identified as "post-incubation treatment". Differentiation media was changed every 2 days.

Immunocytochemistry of SH-SY5Y cells: Once attached to glass coverslips, the cells treated under the different conditions mentioned above, were fixed with 4% paraformaldehyde (PFA) for 20 minutes at 4°C, followed by incubation with permeabilization buffer (Phosphate Buffered Saline (PBS), Bovine serum albumin (BSA) and detergent) for 10 minutes at room temperature. Cells were then blocked with blocking solution (10% normal goat serum (NGS) (abcam, USA, cat. #ab138478), 1% BSA and 0.15% saponin in PBS) for 1 hour at 4°C. Subsequently, primary antibodies were added and incubated overnight at 4°C followed by incubation with secondary antibodies for 1 hour at room temperature in the dark. The coverslips were then washed and

mounted on glass slides with Pro-Long Gold antifade reagent with DAPI (Invitrogen, USA, cat. #P36931).

Confocal microscopy: LSM 700 Confocal Zeiss microscope system was used to obtain and analyze the images of the stained SH-SY5Y cells. For the length measurements of neuronal processes, the processes were traced and measured using the 2009 ZEN software (Zeiss). A cell was considered as neurite-bearing if it contained at least one neuronal process that was longer than the soma of the cell.

Slice preparation: Mice aged between 1 – 3 months were anesthetized with 1.5% isoflurane (Vedco, Saint Joseph, Missouri) followed by decapitation. Brains were cautiously extracted and transferred to cold oxygenated artificial cerebrospinal fluid (aCSF). Acute horizontal hippocampal slices were cut using a vibratome (Leica Instruments). Slices were cut at a 150μm thickness and at speed of 0.07m/s. Slices were then transferred to a beaker containing oxygenated aCSF at a temperature of ~28°C. Slices were then allowed to recover during a 2-hour incubation period in oxygenated aCSF.

Glutamate, erastin and Fer-1 incubation of hippocampal slices: Following the 2-hr recovery period, hippocampal slices were transferred to beakers containing the following drugs dissolved in aCSF: glutamate (7mM), erastin (10μM), glutamate (7mM) + Fer-1 (100 μM) and erastin (10μM) + Fer-1 (100 μM), all final concentrations. A subset of slice were incubated in aCSF only, as a control condition. The incubation time for each experimental condition was about an hour, this to mimic the conditions of the field electrophysiological recordings experiments (see Aim 2 below, for more details).

Immunohistochemistry: Following the 1-hr incubation, all hippocampal slices were fixed with 4% PFA for three days at 4°C. Then, the 4% PFA or cryoprotectant solution was rinsed with Tris-buffered saline (TBS; pH 7.4), 5 times (5 minutes each time), at room temperature. Slices were then incubated with blocking solution 10X TBS, (NDS) (normal donkey serum; EMD-Millipore, Billerica, MA; catalog #S30-100ML; lot NG1827420) and Triton X-100; Sigma-Aldrich, St. Louis, MO; catalog #T8532 in TBS) for 1 hour at room temperature. Slices were incubated with nissil green neurotrace for ~1 hour in the dark at room temperature. Slices were then mounted on superfrost slides containing 50% glycerol and 50% 10X TBS and covered with a coverslip.

Confocal microscopy: LSM 700 Confocal Zeiss microscope system was used to visualize the slides and acquire/analyze the images. The area of interest was the CA3 regions of the hippocampus, where the epileptic-like activity was recorded during the extracellular field electrophysiological experiments. The soma of neurons of subfields within the CA3 region was measured and quantified using statistical analysis.

II.2 Specific Aim 2: Determine whether Fer-1 can rescue the abnormal *in vitro* electrophysiological activity of hippocampal neurons induced by glutamate excitotoxicity

II.2.1 Objective and Overview

Glutamate excitotoxicity has been extensively studied and linked to neuronal injury or cell death (Dixon et al., 2012; Do Van et al., 2016; Kandeda et al., 2017). Subsequent to excessive glutamate receptor activation, calcium influx rises, which is in part responsible

for cell injury (Emerit et al., 2004; Kandeda et al., 2017). Glutamate excitotoxicity can be associated to epileptogenesis (Kandeda et al., 2017) and the overproduction of reactive oxygen species (Azam et al., 2010). Because of this, glutamate excitotoxicity has been previously used to induce epilepsy or cell death in *in vitro* models (Dixon et al., 2012; Do Van et al., 2016; Kandeda et al., 2017). The overproduction of reactive oxygen species has been established as a hallmark of ferroptosis (Dixon et al., 2012; Skouta et al., 2014; Kabiraj et al., 2015; Skouta, 2016; Cao and Dixon 2016). Therefore, it has been suggested that there is a link between ferroptosis, glutamate excitotoxicity and epilepsy (Dixon et al., 2012; Do Van et al., 2016). Studies performed by Dixon et al., 2012 showed that glutamate can induce cell death in organotypic hippocampal culture slices, more specifically in the DG, CA1 and CA3 regions. Furthermore, it has been shown that Fer-1 can alleviate the effects of high glutamate levels in OHSC (Dixon et al., 2012). However, the effects of Fer-1 on the electrical activity of hippocampal brain slices exposed to high levels of glutamate had not been yet established. Therefore, the goal of this aim was to further understand whether the aberrant electrical activity induced by cytotoxic glutamate levels could be rescued by Fer-1 in hippocampal neurons. To do so, we used extracellular field electrophysiological recordings in acute hippocampal slices from WT mice. Glutamate-induced epileptiform activity was evaluated in the absence and the presence of 100 μM Fer-1. Results from this aim should help understanding the mechanisms underlying the neuroprotective effects of Fer-1. Ideally, our results could also contribute to the

development of new therapies for disorders involving ferroptosis-dependent mechanisms.

II.2.2 Materials and Methods

Animals: C57BL/6 female and male mice aged between 2-3 months were obtained from Jacksons Laboratories. The animals were accommodated at The University of Texas at El Paso (UTEP). Mice were kept in the UTEP Bioscience Research Bldg Vivarium under conditions according to UTEP Institutional Animal Care and Use Committee (IACUC) protocols and maintained on a 12-hour light and dark cycle.

Hippocampal slice preparation: Mice were anesthetized with 1.5% isoflurane (Vedco, Saint Joseph, Missouri) followed by decapitation. Brains were extracted and transferred to cold oxygenated artificial cerebrospinal fluid (ACSF). Acute horizontal hippocampal slices were obtained using a vibratome (Leica Instruments). Slices were cut at a 300µm thickness and at a speed of 0.07m/s. Slices were then re-sized to isolate the hippocampus section, and then transferred to a perfusing chamber that continuously perfused hippocampal slices with oxygenated aCSF. Slices were allowed to recover during a 2-hour incubation period during which they were maintained at a temperature of approx. 30°C. After the recovery period, slices

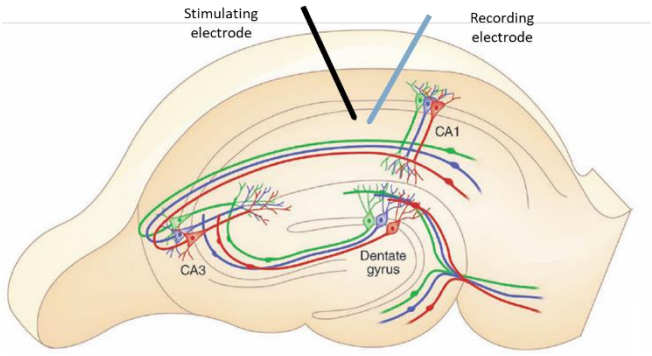

Figure 2.1. **Viability test in the CA1 region of the hippocampus.** Cell viability is measured by placing a stimulating electrode onto CA3 neuronal fibers and a recording electrode in the CA1 region (stratum radiatum) of the hippocampus.

were tested for viability by placing a concentric bipolar platinum radium electrode on the CA1 region of the hippocampus and then applying an electric shock to the CA1 afferent fibers. The recordings were performed by placing a pulled glass micropipette electrode on the CA3 region.

Extracellular field recordings: Following a two-hour recovery period and during the recordings, hippocampal slices were continuously perfused with oxygenated aCSF. An initial electrical test stimulation was performed by placing a concentric bipolar platinum radium electrode on the CA3 fibers and recording with a pulled glass micropipette electrode place within the CA1 region (**Figure 2.1)**. This test confirmed the viability of the brain tissue prior to the induction of the epileptiform activity. Then, 7mM glutamate was added to the perfusing solution to induce epilepsy-like events. Subsequently, once the glutamate-induced epileptic-like events were consistent and stable, 100 μM Fer-1 was added to the glutamate-containing perfusing solution, in an attempt to neuro-protect the slice from cytotoxicity. All the recordings were acquired and saved using the pClamp 10 software and the analysis of the events was performed

using the Clampfit program.

Glutamate and Fer-1 treatments: Glutamic acid (7mMfinal concentration) was dissolved in aCSF to induce epileptic-like events as previously published (Dixon et al., 2012; Kandeda et al., 2017). The induction of the epileptic-like events was recorded for 5 minutes in the pyramidal cell layer of the hippocampus CA3 region (**Figure 2.2)**. After

this first recording, 100 µM Fer-1 was be added to the slice perfusing solution for 20 minutes followed by a

recording period of 10 minutes. Finally, Fer-1 will was washed out to revert back to the "pre" Fer-1 conditions, and therefore, confirm any Fer-1 effect. All perfusing solutions were kept fully oxygenated during the entire extent of the experiments.

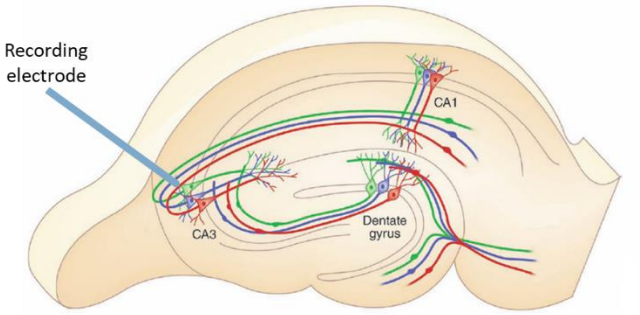

***Figure* 2.2. *In vitro*Electrophysiological recordings.** The glutamate-induced epileptic-like events were recorded using a pulled glass microelectrode on the CA3 region of the hippocampus in the absence and in the presence of Fer-1.

Chapter III. Results

The understanding of the excitotoxic effects of both glutamate and erastin and the neuroprotective properties of Fer-1 on hippocampal neurons requires both morphological and electrophysiological analysis. In order to understand the morphological changes induced by erastin and glutamate, immunostaining was used on cell culture and hippocampal tissue from mice. Furthermore, to examine the electrical activity of hippocampal neurons under the exposure of these compounds, *in vitro* extracellular field recordings were performed in mouse hippocampal slices. The results from these experiments are described below.

III.1. Effect of erastin on SH-SY5Y cells

To understand the morphological effects induced by erastin on cell culture, immunocytochemistry was performed on the SH-SY5Y cell line. Cells were induced to a neuronal phenotype by exposure to Retinoic Acid (RA), this to better understand the effects of erastin on mature cells. A negative control consisting of undifferentiated SH-SY5Y cells was included as well (**Figure 3.1)** The treatments were divided into two different conditions: co-incubation and post-incubation conditions. Cells were then exposed to erastin and processed for immunocytochemistry. Anti-beta-3-tubulin antibody was used to visualize the major structures of the cells as well as DAPI to visualize the nuclei of the same cells.

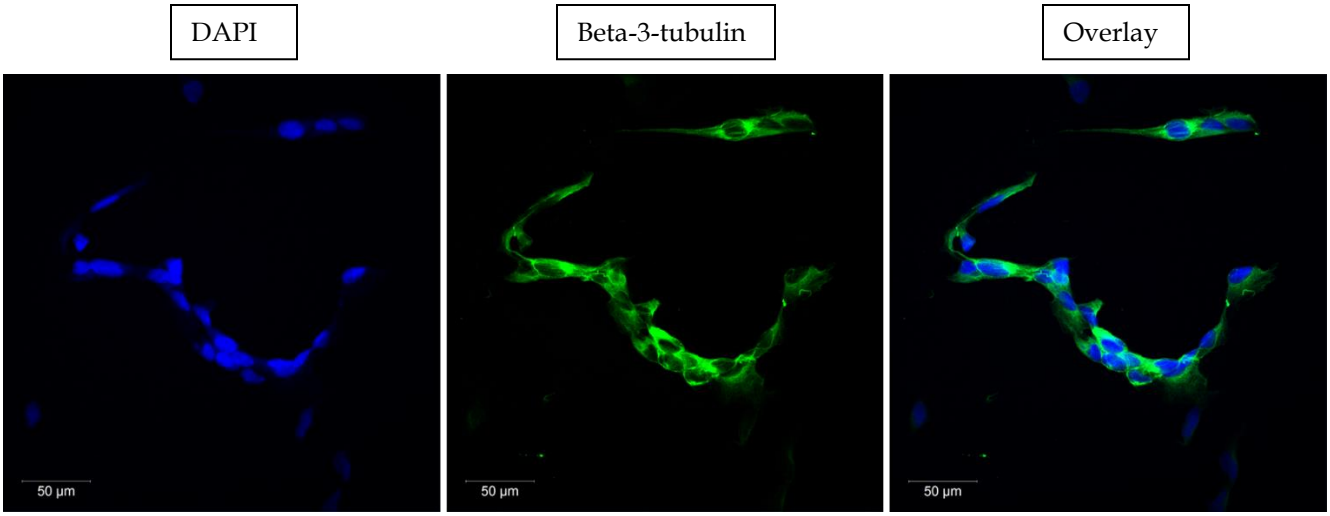

Figure 3.1. **Untreated SH-SY5Y cells.** Cells not exposed to the RA treatment were cultured, fixed and labeled with DAPI (blue) and beta-3-tubulin. These cells were included as negative control.

III.1.1. Post-incubation with erastin

About $8X10^5$ cells were seeded onto glass coverslips in a six-wells plate for each experiment. SH-SY5Y cells were first incubated with RA in order to induce a mature phenotype **(Figure 3.2)**. After six days of incubation with RA, 10µM erastin was added to the cells and incubated for 24 hours. Cells were then processed for immunocytochemistry and slides were viewed under a confocal microscope. Measurements of the cell structure were taken. About ten regions from each slide were analyzed. As expected from previous results (from Do Van et al., 2016) where no effect on cell viability was shown after exposure to erastin, SH-SY5Y cells were not significantly affected morphologically. The general shape of the cells remained the same for the positive control i.e., the untreated differentiated cells, similar to the differentiated cells exposed to erastin **(Figure 3.3)**.

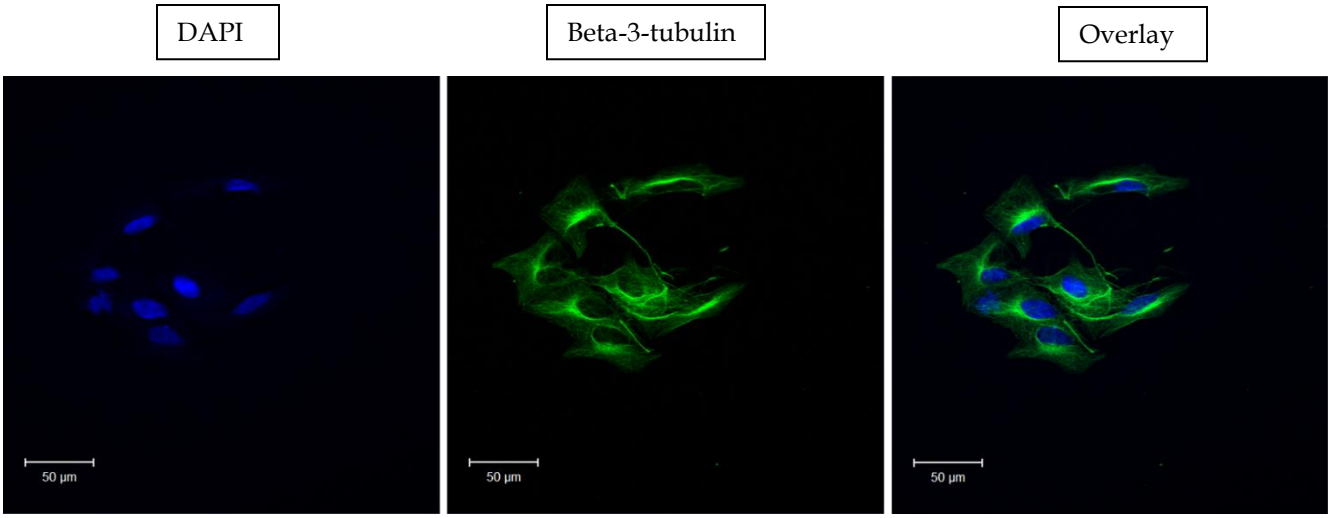

Figure 3.2. **SH-SY5Y cells incubated with RA.** SH-SY5Y were differentiated using RA and showed a neuronal phenotype. Cells were then fixed and labeled with beta-3-tubulin (green) and DAPI (blue).

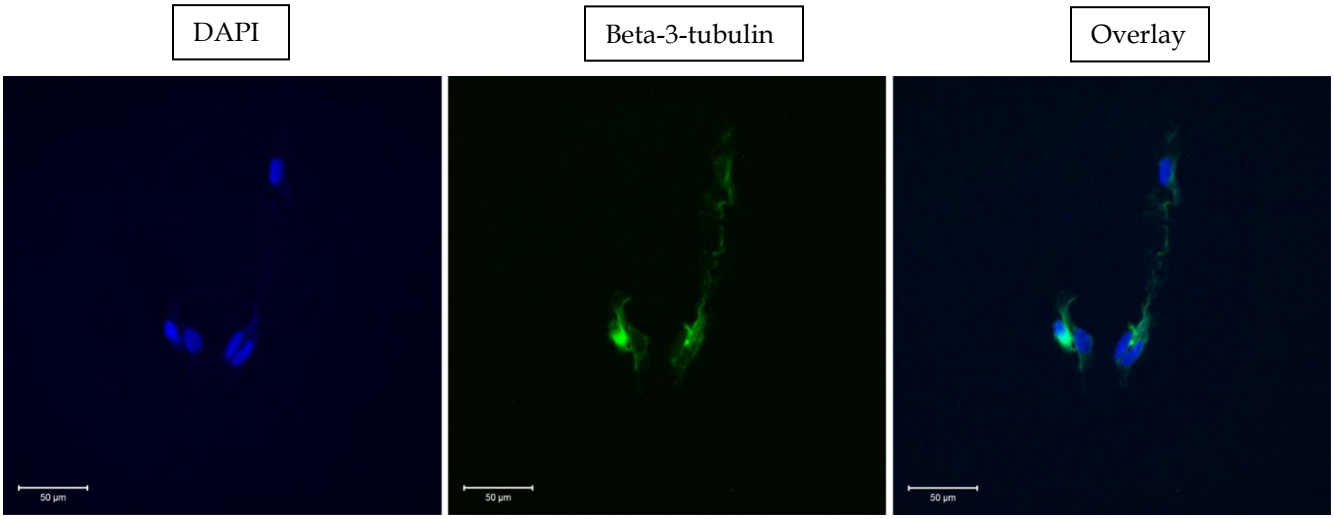

Figure 3.3. **SH-SY5Y cells post-incubated with erastin.** Cells were first induced to a neuronal phenotype by incubating with RA, followed by 24 hr treatment with erastin. Cells are labeled with DAPI in blue for the nucleus and beta-3-tubulin in green.

III.1.2. Co-incubation with erastin

To understand the effect of erastin on differentiated SH-SY5Y cells, these cells were co-incubated with erastin. About $8X10^5$ cells were seeded onto glass coverslips in a six-wells plate for each experiment. Cells were incubated with both RA and 10µM erastin

for six days. Media was renewed every two days. After the incubation period, cells were fixed and processed for immunocytochemistry. After visualizing the cells under the microscope, I found that, similarly to post-incubated treated cells, there was no change in the overall morphology on the cells as seen on **(Figure 3.4)**.

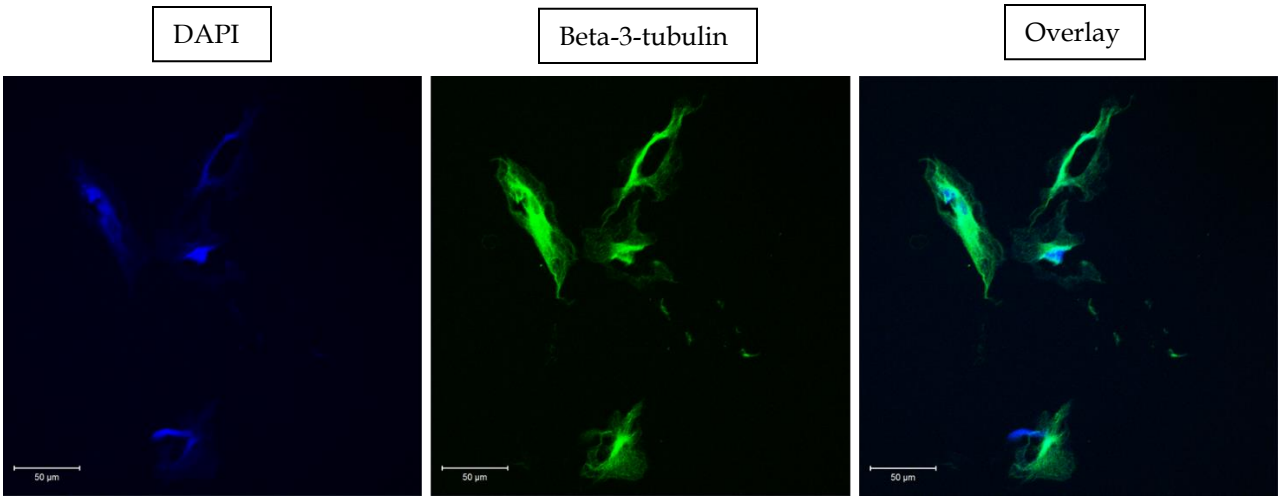

Figure 3.4. **SH-SY5Y cells post-incubated with erastin.** Cells were co-incubated with RA and erastin for 6 days. Cells were then fixed and stained with DAPI (blue) and anti-beta-3-tubulin.

III.1.3. Soma analysis of hippocampal neurons exposed to glutamate, erastin and Fer-1

In order to mimic the field electrophysiological experiments that were performed with glutamate and Fer-1, mouse hippocampal slices were incubated using similar experimental conditions **(Figure 3.5)**. The purpose of these experiments was to analyze whether morphological changes were associated with the perfusion of these drugs. During the electrophysiological field experiments (described in the next section, below), brain tissue was exposed to glutamate for about 1 hour and then to glutamate + Fer-1 for around 1 hour as well. Similar conditions were used here: hippocampal slices, that would be visualized for neuronal morphological changes, were exposed to glutamate, or erastin

in the absence and presence of Fer-1. This was done to compare the effects on cells' soma size by the different conditions. A total of 3 brains were obtained, cut and incubated with the corresponding treatments. After the incubation time, slices were fixed and processed for immunohistochemistry **(Figure 3.5)**. Slices were observed under a confocal microscope. The CA3 region of the hippocampus was identified with a magnification of 5X and 20X, and then cells from the same region were focused and measured with a magnification of 40X. Different areas from the CA3 region were captured and soma of neurons were measured using the tools from the Zeiss ZEN software. 50 neurons were measured from each slice, having a total of 150 soma measurements from each condition. My results show glutamate exposure increased the soma size of hippocampal neurons, and Fer-1 was able to reduce this increase in soma size, back to normal, control values **(Figure 3.5; ANOVA, $p<0.05$)**. Erastin and Erasting+Fer-1 solutions had no effects on Fer-1, similar to the SH-SY5Y cell results.

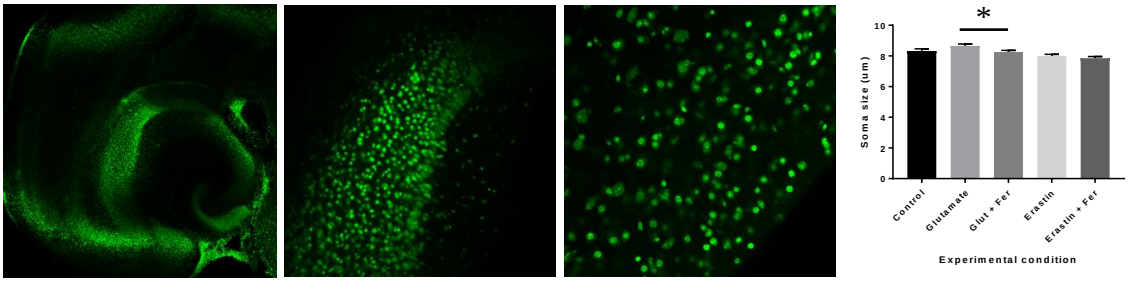

Figure 3.5. **Hippocampal slices.** Slices were stained and visualized under a confocal microscope at different magnifications (from left to right: 5X, 20X and 40X). Graph showing the quantification of the soma size measurements under the different experimental conditions.

III.2. Electrophysiological characterization of hippocampal neurons induced to excitotoxicity

To investigate whether Fer-1 can rescue the abnormal *in vitro* activity of hippocampal neurons induced by glutamate excitotoxicity, extracellular field electrophysiological recordings were performed. Mice brains were obtained and 300µM acute horizontal slices containing the hippocampus were cut **(Figure 3.6)** (n=8). An initial electrical stimulation was applied by placing a concentric bipolar platinum radium electrode and recorded with a pulled glass micropipette electrode to record on the CA1 region of the hippocampus **(Figure 3.7)**. To induce epileptiform activity, the brain tissue was perfused with 7mM glutamate dissolved in oxygenated aCSF. The time to induce epileptic-like events ranged from 20 minutes to 1 hour. Once epileptiform activity was induced, recordings were made on the CA3 region of the hippocampus, followed by the perfusion of glutamate + Fer-1 for another 30 minutes. To confirm the effect of Fer-1, a glutamate washout was performed. The recorded epileptiform activity was analyzed using two parameters: the frequency of the events and the amplitude of individual events.

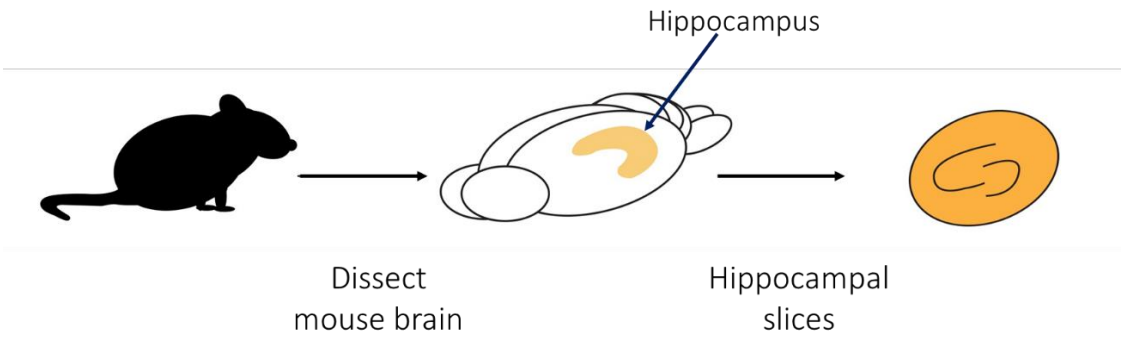

Figure 3.6. **Illustration of mouse hippocampal slice acquisition.**

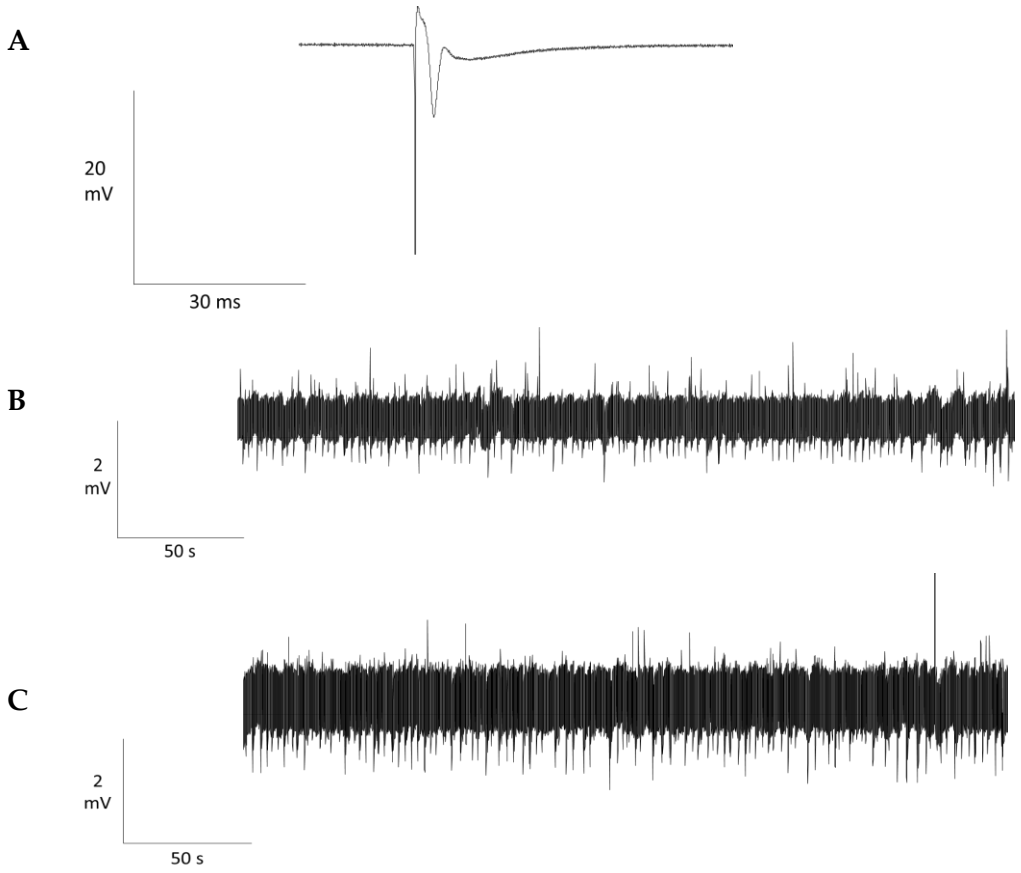

Figure 3.7. **Extracellular field experiments in the hippocampus.** (A) Initial electrical response after stimulation on the CA1 region. (B) Glutamate-induced epileptic-like events were recorded in the absence (C) and in the presence of Fer-1. The Effect of Fer-1 (100μM) in the presence of glutamate (7mM) were quantified by measuring the frequency and the amplitude of each epileptic-like event under the different experimental conditions in the CA3 region.

To assess the field electrophysiological recordings, we analyzed the amplitude and the frequency of the events of the last best minute on each condition. By combining all animals (N = 8), we observed a significant decrease in the frequency of epileptic-like events in the presence of Fer-1 **(Figure 3.8; ANOVA; $p<0.05$)**. Interestingly, the amplitude of the events remained unaffected.

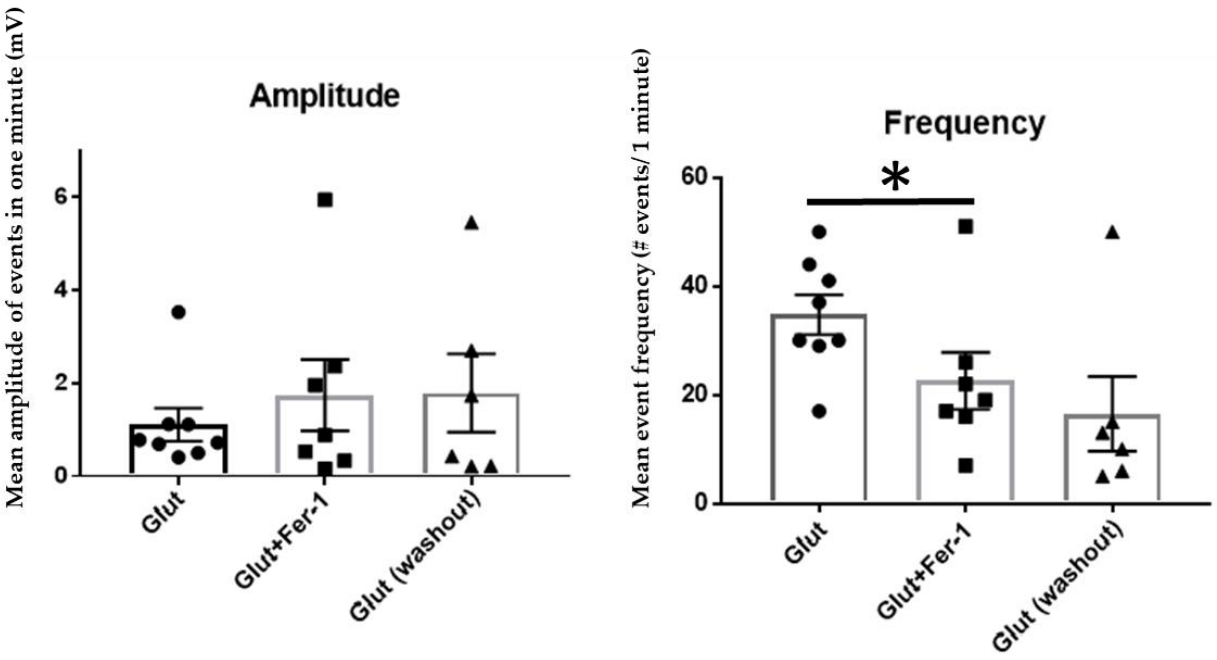

Figure 3.8. **The effect of Fer-1 on the amplitude and frequency of glutamate-induced epileptic-like events in the hippocampus.** Graphs showing the effect of Fer-1 on the amplitude (left) and frequency (right) of glutamate-induced epileptiform activity on the CA3 region of the hippocampus. N = 8 animals. Means ± SEM.

We also analyzed the frequency and amplitude of induced epileptiform activity by separating males and females. We used a total of four females and four males during the field recording experiments. Interestingly, the amplitude of the events was greater in females compared to males, however, for both genders, the amplitude of the events remained unaffected by Fer-1. In contrast, we observed a significant decrease in the frequency of the events in the presence of Fer-1. First, in females, the perfusion of Fer-1 significantly decreased the frequency of the events **(Figure 3.9; ANOVA, $p<0.05$)**. Similarly in males, the effect of Fer-1 seemed to have taken longer to occur, as the significant decrease in the amplitude of the events occurred during the "washout" period, as the Glutamate+Fer-1 solution was being replaced by a "Glutamate only" solution (**Figure 3.9)**

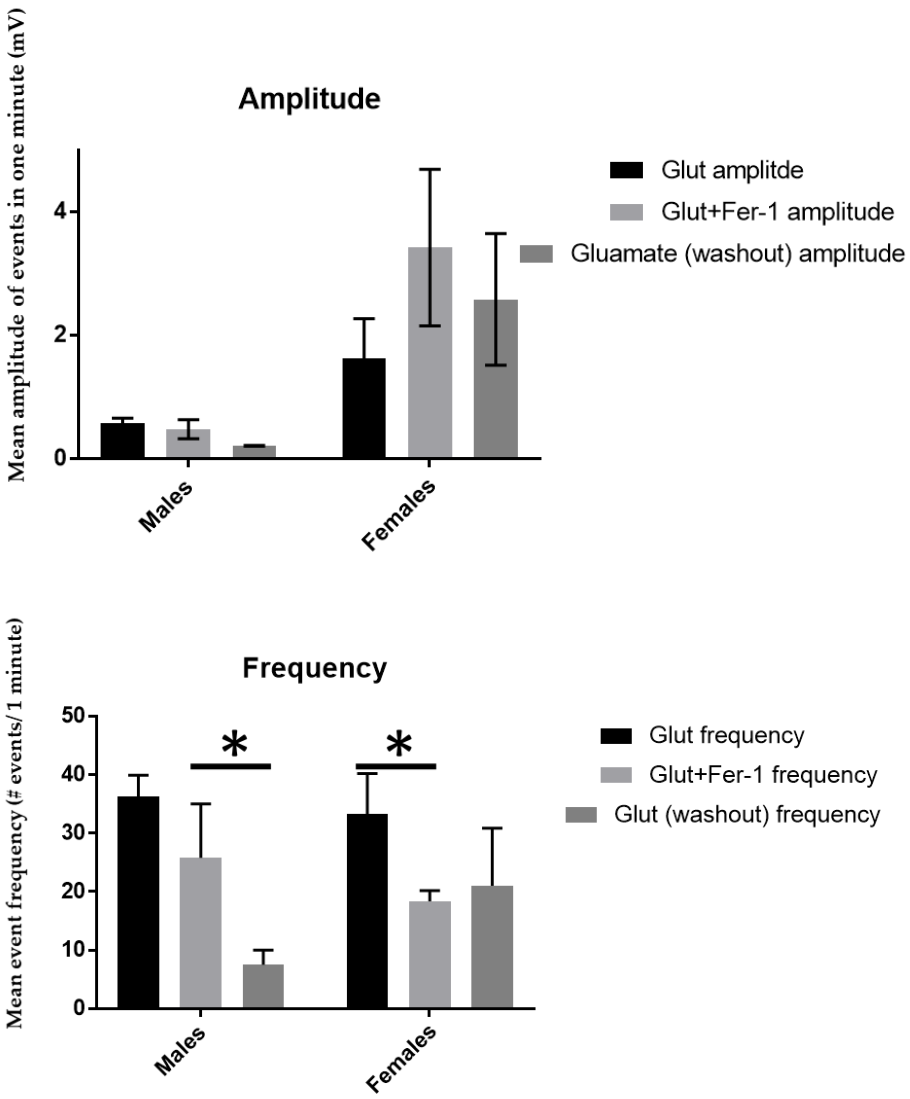

Figure 3.9. **The effect of Fer-1 on the electrical activity of hippocampal neurons on males and females.** Graphs showing the effects of Fer-1 on the mean amplitude and frequency of the glutamate-induced epileptiform activity in the hippocampus of male and female mice.

Chapter IV. Discussion

The goal of our research was to first examine the morphological effect of erastin and glutamate on cell culture and mouse hippocampal slices as well as the potential rescue by Fer-1. Second, we wanted to understand the effect of Fer-1 on the electrical activity in the hippocampus induced by a toxic level of glutamate to mimic the excitotoxicity associated with several disease states. Immunostaining experiments and electrophysiological field experiments were performed to accomplish these aims. First, the SH-SY5Y cell line was used to assess whether erastin had an effect on the overall shape of cells. Immunocytochemistry and confocal microscopy were used for analysis. Previous studies have shown that erastin has no effect on the cell viability of these cells, however there are no studies showing the effect of erastin on cell shape (Do Van et al., 2016). Our data show that similar to the effect on cell viability, erastin does not affect cell morphology. Next, to investigate the effect of these molecules, mouse brain slices were used for immunohistochemistry. The soma of neurons at the CA3 region of the hippocampus were measured under the different experimental conditions. Finally, extracellular field electrophysiological experiments were used to assess the neuroprotective effect of Fer-1 on hippocampal epileptiform activity induced by glutamate. My data from these experiments show that in the presence of Fer-1, the frequency of events was significantly decreased, compared to the "glutamate-only" condition.

IV.1. Immunostaining experiments

IV.1.1 Observing effect of erastin on SH-SY5Y cells

Erastin, a ferroptosis inducer, was used on SH-SY5Y cells. It has been shown that erastin has no effect on the cell viability of this cell line (Do Van et al., 2016). Based on this background we expected no change on the morphology of these cells after exposure to erastin. We analyzed the effects on both post-and-co incubation experiments. Since we wanted to understand the effect on mature cells we sought to quantify the extensions of the neuronal-induced cells. I found no significant changes in the extensions of these cells under both conditions.

IV.1.2. Quantifying effect of erastin, glutamate and Fer-1 on hippocampal neurons

Horizontal mice brain slices were Incubated with glutamate or erastin, in the absence or presence of Fer-1. The incubation time with each treatment sought to mimic the conditions by which slices were exposed during extracellular field experiments. A total of 750 soma size from the five groups described above. Similar to the results obtained on the SH-SY5Y cells, there was no change in the soma size of neurons incubated with erastin. The glutamate-exposed hippocampal neurons were found to have an increase in the soma size compared to the control. Interestingly, the soma size of neurons exposed to glutamate + Fer-1 showed to be similar to the size of the control group.

IV.2 Extracellular electrophysiological field experiments

In vitro extracellular electrophysiological field recordings were performed on acute horizontal hippocampal slices. Recordings of glutamate-induced epileptic-like events were taken, in the presence or absence of Fer-1. The two parameters used were the frequency and amplitude of events. These experiments can help determine the neuroprotective effects of Fer-1 on glutamate excitotoxicity.

IV.3. Future studies

Extracellular field recording helped us to speculate that Fer-1 might be able to restore the electrical activity induced by glutamate. The number of experiments needs be increased to establish a neuroprotective effect. Ideally, our work would form the basis for further functional investigation using *in vivo* models.

IV.4. Conclusions

Glutamate excitotoxicity and ferroptosis are two phenomenon that have been shown to induce cell death in neurons. Furthermore, glutamate excitotoxicity is known to be associated to abnormal electrical activity as in seen in epilepsy. Fer-1 has been reported to ameliorate the effects induced by both erastin-induced ferroptosis and glutamate excitotoxity. However, the effect of Fer-1 on the morphology and electrical activity of hippocampal neurons is not well known.

Our objective was to investigate the neuroprotective effects of Fer-1 on both the morphology and electrophysiological properties of hippocampal neurons induced to glutamate excitotoxicity. To test our hypothesis, we used immunochemical staining of hippocampal neurons. Soma size of neurons exposed to glutamate showed to have an increase in comparison to the control neurons. Glutamate + Fer-1 exposed neurons seemed to have a size similar to the control group. Our preliminary data from *in vitro* extracellular field electrophysiological recordings showed that Fer-1 has an effect on the frequency of epileptic-like events. This data, might suggest that Fer-1 has neuroprotective effects that can help to restore the abnormal electrical activity induced by glutamate. Future work could help to better establish the neuroprotective properties of Fer-1.

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Curriculum Vita

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