NEUROPROTECTION DURING ACUTE OXIDATIVE STRESS: ROLE OF THE PKG PATHWAY AND IDENTIFICATION OF NOVEL NEUROMODULATORY AGENTS USING *DROSOPHILA MELANOGASTER*

by

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This dissertation was prepared under the direction of the candidate's dissertation co-advisors, Dr. Ken Dawson-Scully and Dr. Sarah L. Milton, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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Title: Neuroprotection During Acute Oxidative Stress: Role of the PKG Pathway and Identification of Novel Neuromodulatory Agents Using *Drosophila Melanogaster*

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Oxidant stress and injury is inherent in many human diseases such as ischemic vascular and respiratory diseases, heart failure, myocardial infarction, stroke, perinatal and placental insufficiencies, diabetes, cancer, and numerous psychiatric and neurodegenerative disorders. Finding novel therapeutics to combat the deleterious effects of oxidative stress is critical to create better therapeutic strategies for many conditions that have few treatment options. This study used the anoxia-tolerant fruit fly, *Drosophila melanogaster*, to investigate endogenous cellular protection mechanisms and potential interactions to determine their ability to regulate synaptic functional tolerance and cell survival during acute oxidative stress. The *Drosophila* larval neuromuscular junction (NMJ) was used to analyze synaptic transmission and specific motor axon contributions. *Drosophila* Schneider 2 (S2) cells were used to assess viability. Acute oxidative stress

was induced using pharmacological paradigms that generate physiologically relevant oxidant species: mitochondrial superoxide production induced by sodium azide (NaN_3) and hydroxyl radical formation via hydrogen peroxide (H_2O_2). A combination of genetic and pharmacological approaches were used to explore the hypothesis that endogenous protection mechanisms control cellular responses to stress by manipulating ion channel conductance and neurotransmission. Furthermore, this study analyzed a group of marine natural products, pseudopterosins, to identify compounds capable of modulating synaptic transmission during acute oxidative stress and potential novel neuromodulatory agents.

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FIGURES

CHAPTER 1. INTRODUCTION

Cellular Stress

For most organisms, oxygen is essential to survive. It is required for development, growth and the functional integrity of cells and tissues. A natural by-product of cellular respiration is the generation of toxic partially reduced forms of oxygen, known as reactive oxygen species (ROS), during mitochondrial reduction of oxygen to water (Chance et al., 1979; Inoue et al., 2003). ROS is a general term used to describe small molecules containing oxygen that are free radicals, such as the superoxide anion, or oxidizing agents, such as hydrogen peroxide (H_2O_2), that are easily converted into radicals (Figure 1). ROS display a variety of different biological properties. For instance, they can be charged or uncharged and the site of their origin plays a key role in their biological properties. Superoxide radicals may disrupt iron-sulfur clusters or react with nitric oxide, while hydrogen peroxide is able to propagate hydroxyl radicals by reacting with transition metals (Halliwell, 2001). ROS serve important roles in cellular signaling pathways, such as apoptosis and gene expression, and are formed as necessary intermediates in many biological reactions by oxidoreductase enzymes and metal catalyzed oxidation (Hancock et al., 2001).

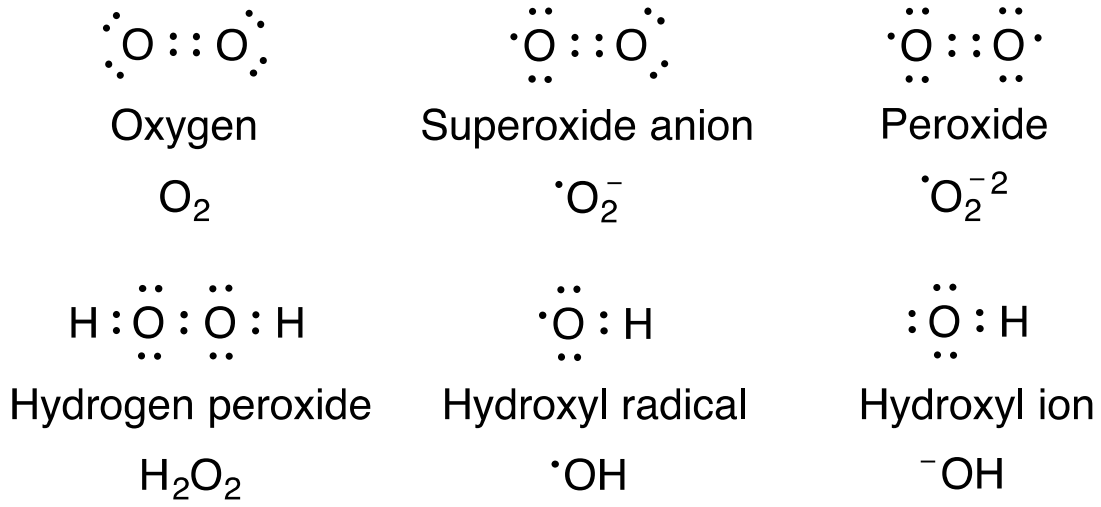

Figure 1. Types of reactive oxygen species (ROS). Reduction of oxygen leads to the formation of ROS in the following order: superoxide anion, peroxide, hydrogen peroxide, hydroxyl radical and hydroxyl ion. ROS are pictured above with their name, chemical formula, and Lewis structure.

However, when the concentration of ROS is too high within cells, these molecules can damage cells by affecting cellular membrane permeability and disrupting intracellular components such as DNA, lipids, and proteins (Marnett et al., 1985; Fraga et al., 1990; Stadtman et al., 1992). Within biological systems there is a homeostatic balance between the production of ROS and cellular defense mechanisms, such as ROS scavengers (superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), vitamins C, E) and DNA repair mechanisms that fix the damage. When this dynamic becomes unbalanced during traumatic stress insults, such as ischemia/reperfusion, stroke and neurodegeneration, it leads to a cellular condition termed oxidative stress (Lipton, 2004). Oxidative stress is inherent in the pathophysiology of an array of devastating human ailments including ischemic vascular diseases, heart failure, myocardial infarction, stroke, cancer, and numerous psychiatric and neurodegenerative disorders. Understanding the complicated cellular mechanisms that underlie the cellular ischemic cascade during disease progression is essential to enable the development of more effective therapies for many of these conditions.

There are many different types of environmental stressors such as high temperature or low oxygen that cause cellular damage; however, the physiological effects on organisms converge on one common theme, the elevated generation of ROS. It may be possible that the detrimental effects caused by numerous cellular stresses are mediated by the intracellular overload of ROS. The production of oxygen-containing free radicals within cells is increased during exposure to hyperthermic stress, which elevates the associated cellular damage (Flanagan et al., 1998). However, the mechanism(s) of how

increasing temperature mediates this process is not fully understood. Numerous events could potentially induce ROS formation during thermal stress such as increased mitochondria degradation and oxidation of xanthine dehydrogenase (Skibba et al., 1989; Salo et al., 1991). Another cellular stressor that increases intracellular ROS production in neuronal cells is oxygen deprivation (Liu et al., 2005), which is also known as hypoxia or anoxia. Additionally, an explosion of ROS occurs during ischemic injury and upon reperfusion when the oxygen supply is restored (Ferriero, 2001).

ROS formation occurs mostly in mammalian tissues from high altitude or pathological diseases such as ischemia-reperfusion, stroke, and myocardial infarction. Biologically relevant oxidants target a wide array of molecules including numerous types of ion channels, such as calcium (Ca^{2+}) channels and potassium (K^{+}) channels (Ueda and Wu, 2009). When ion channels become oxidized, functional modifications occur that can affect critical cellular signaling mechanisms in excitable cells such as neurons, cardiac myocytes, and skeletal muscle cells (Pallota and Wagoner, 1992). Increased ROS in the brain triggers numerous molecular cascades that lead to increased blood-brain barrier (BBB) permeability, alterations of brain morphology, neuroinflammation, synaptic failure, and neuronal death (Gu et al., 2011).

The mammalian brain is especially sensitive to ROS-induced oxidative stress because it has a lower antioxidant capacity and higher levels of fatty acids, iron, and ascorbate compared to other tissues and requires an elevated energy consumption to maintain neuronal ion homeostasis (Uttara et al., 2009; Halliwell, 2001). Oxidative stress

is a hallmark feature of many central nervous system conditions including cerebrovascular disorders, demyelinating diseases, psychiatric disorders, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Sorce and Krause, 2009). Understanding the pathological effects of oxidative stress and finding novel strategies to combat its pathomechanistic role in disease progression and cell death remains a relevant topic in neuroscience research.

In a broader aspect, the study of cellular stress and its effects is very important to discover the pathological mechanisms of disease progression and the recruitment of intracellular protection mechanisms (Douglas, 2001). Though interests are widespread across numerous fields and applications, the focus of cellular stress research can generally be categorized into basic groups with studies concentrating on individual responses, how adaptations to such stressors arise, and the distinct mechanisms that regulate cellular survival pathways (Haddad, 2000). There are many intracellular and extracellular events that shadow cellular stress events that can influence cellular function, instigate numerous cellular mechanisms involving large numbers of different molecules, and ultimately determine survival, depending on the severity of the impact (Haddad, 2000). Examining methods to protect organism function and/or survival during cellular stress will reveal insights into the complex physiological processes involved in organism activation, response and adaptation of endogenous cellular protection mechanisms.

Protection Mechanisms

Altered ROS production and regulation due to cellular stress insults and pathological diseases affect mammals such as humans very differently from other organisms that are tolerant to numerous types of cellular stress. Organisms respond differently to cellular stress and the basis for the known differences among species is still not fully understood. The diversity of physiological responses to cellular stress goes beyond organism differences to differences amongst organs within the same individual and even among cell types within that organ (Haddad, 2000). Numerous organisms have developed various strategies for dealing with cellular stress (Zhou, 2008) that involves processes such as feedback systems, dependence on inherent cellular properties and reliance on personal tolerance. For instance, seals respond to hypoxia by diving to extreme depths to reduce their heart rate, cardiac output, and respiration rate, while turtles rely on a cellular hypometabolic state to survive (Haddad, 2006). Additionally, differences in environmental stress responses exist between adolescent and mature mammals (Haddad, 1990) and different regions within the mammalian central nervous system, such as the neocortex, the hippocampus, and the brain stem, have been reported to respond differently to stress insults (Donnelly, 1992). Understanding the diversity of physiological stress responses involves characterizing the underlying complexity of the basis for organism tolerance and susceptibility to cellular stress.

Since most mammals are intolerant to environmental stresses, such as hyperthermia, anoxia, and oxidative stress, their use as a model system is extremely

limited. Along with this limitation, comes the additional complication that mammalian systems are physiologically complex and little is known about the underlying cellular mechanisms involved in their response to stress insults. Many organisms have evolved numerous ways to deal with cellular stress insults. Insects can survive extreme ecological conditions and thrive in almost every habitat on Earth. Insect stress resistance mechanisms are believed to have evolved very early and be highly conserved (O'Farrell, 2001). One universal mechanism that organisms use to deal with environmental conditions, such as hyperthermia, anoxia and oxidative stress, is the heat shock (HS) response. This well-characterized protection mechanism is regulated via transcription of heat shock proteins (HSPs) that act as molecular chaperones during stress events (Wu, 1995; Fujimoto and Nakai, 2010).

The basis of thermotolerance protection is demonstrated when an organism is exposed to a lethal temperature, it will not survive. However, exposure to a sub-lethal temperature followed by a recovery period before a lethal temperature elevation will result in organism survival (Figure 2). The HS response confers protection against numerous cellular stressors and pathologies (Zou et al., 2003; Venkatakrishnan et al., 2006; Brundel et al., 2006; Zhang et al., 2011; Arrigo, 1998) and produces long-lasting effects on neural function that alters neuronal properties. Sub-lethal hyperthermic stress induces behavioral and synaptic transmission failure before permanent thermally-induced damage (Tryba and Ramirez, 2004). In locusts, HS exposure produces a physiological change in outward K^+ currents, where fast transient K^+ conductance is evoked instead of the slow, delayed-rectified K^+ conductance observed in animals not exposed to a prior

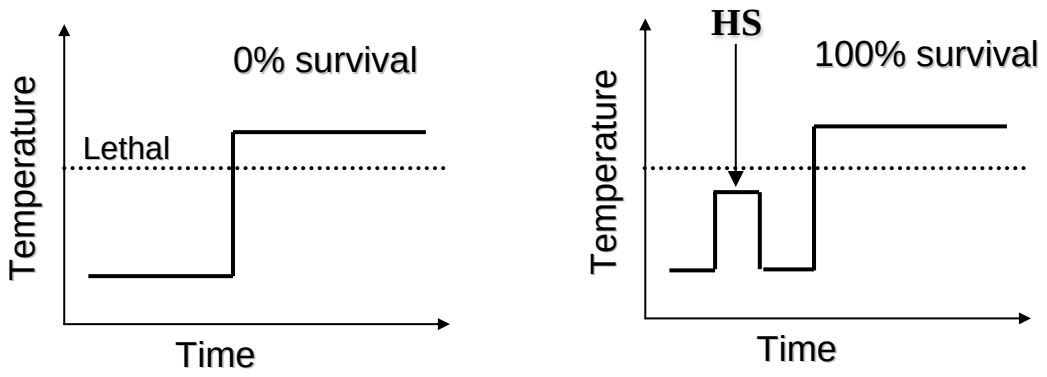

Figure 2. The heat shock (HS) response. HS response to cellular stress is a protection mechanism used by all organisms to cope with cellular stress. When an organism is exposed to a lethal temperature (dotted line), there will be no survival; however, exposure to a prior sub-lethal temperature (HS) followed by a recovery period allows for organism survival.

HS, and is believed to be an adaptive protection mechanism (Ramirez et al., 1999; Figure 3).

The fruit fly, *Drosophila melanogaster*, uses the cGMP-dependent protein kinase G (PKG) intracellular signaling pathway as an endogenous protection mechanism to cope with environmental stress (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). The PKG cascade (Figure 4) is highly conserved and an important part of numerous signaling mechanisms in diverse cell types (Hofmann et al., 2009). Pharmacologically or genetically manipulating this pathway leads to protection of neurological function during acute hyperthermic stress and this protection is conserved across species in the central circuit of *Locusta migratoria* (Dawson-Scully et al., 2007). Additionally, the PKG pathway modulates neural functional tolerance of fruit flies (Dawson-Scully et al., 2010), tadpoles (Robertson et al., 2010), zebrafish (Dawson-Scully and Gerlai, unpublished), and mice (Armstrong et al., 2010) during anoxic stress. Since PKG activity is linked to the regulation of neurological function during acute hypoxia and hyperthermia, the PKG pathway likely represents a conserved cellular mechanism that modulates the response to a number of acute physiological stressors.

In the fruit fly, PKG activity is encoded by the *foraging* (*for*) gene, a homolog of the human *PRKG1* gene, which modulates behavioral food-searching strategies (Sokolowski, 1980). In nature, fly populations exhibit two allelic variants of this gene, where 70% of flies exhibit the rover (for^{R}) allele with high PKG activity and 30% display the sitter (for^{S}) allele with low PKG activity (Osborne et al., 1997). From larvae to

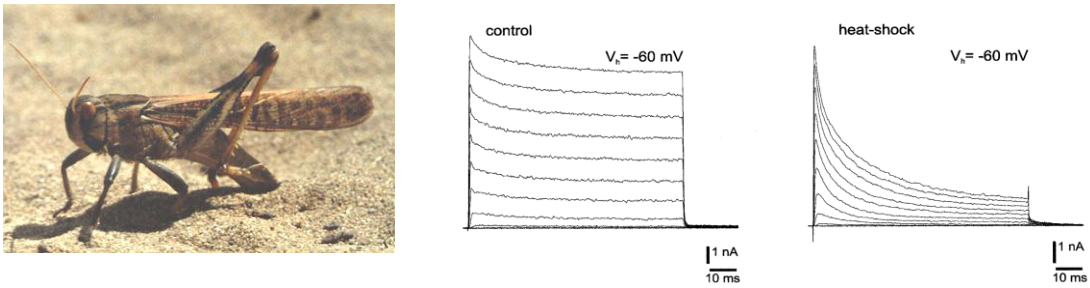

Figure 3. Voltage-clamp recordings from locust neurons. Locust neurons from control and heat-shocked slice preparations from the cell bodies at the ventral midline of the A1 neuromere were voltage-clamped to obtain whole cell patch recordings at different holding potentials (-60 mV to 40 mV). There is significant difference between the steady-state K^+ current in control and heat-shocked neurons. The outward current is rapidly inactivated in animals exposed to a prior HS (57%) when compared to control (15.5%) animals (adapted from Ramirez et al., 1999).

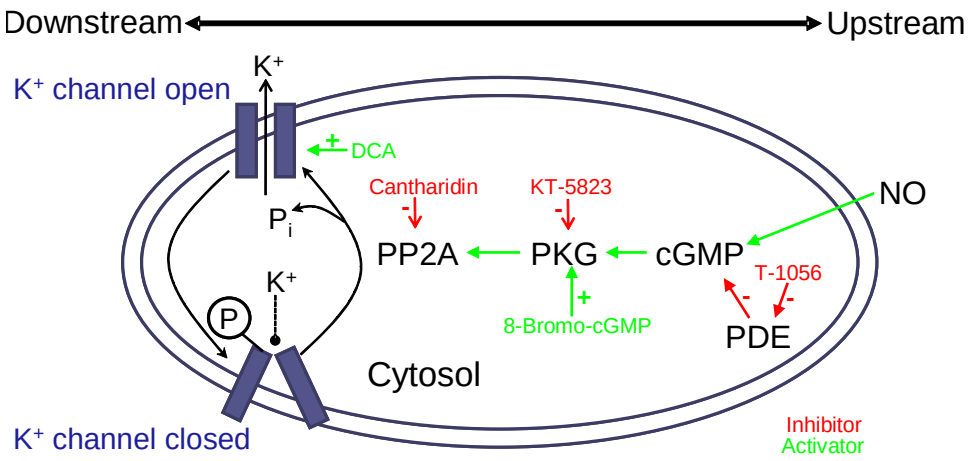

Figure 4. The PKG pathway. This signaling cascade is activated by nitric oxide (NO) that activates the 2nd messenger cyclic-guanylmonophosphate (cGMP), which activates protein kinase G (PKG) that activates protein phosphatase 2A (PP2A). This activation results in the K^+ channel being dephosphorylated and having increased conductance. This pathway is inhibited by phosphodiesterase (PDE) and pharmacological agents such as T-1056, KT-5823, and Cantharadin. This pathway is activated by pharmacological agents such as 8-Bromo-cGMP and dichloroacetate (DCA; adapted from Zhou et al., 1996).

adulthood, *for*^{R} are more active than *for*^{s} in the presence of food, however this distinct phenotypic difference disappears in the absence of food (de Belle et al., 1989; Pereira and Sokolowski, 1993). In addition to foraging strategies, *for* produces natural variation in other behaviors such as learning and memory (Mery et al., 2007) and variation in physiological properties such as neuronal morphology and connectivity (Renger et al., 1999). Natural and mutant *for*^{s} neurons display significantly lower voltage-activated, outward K^{+} currents than *for*^{R} with clear differences in both the peak and sustained components (Figure 5), suggesting a regulatory role for PKG activity in neuronal excitability and synaptic transmission via voltage-dependent K^{+} channel conductance (Renger et al., 1999). The PKG pathway affects neuronal excitability, neurotransmitter release, and neuroprotection during cellular stress and, like HS, represents another conserved endogenous protective mechanism used by organisms that are tolerant to cellular stress.

Drosophila as a Model System

Fruit flies have been classically used to study development and behavior, however, this emphasis has recently been shifted to studying biochemical and genetic processes behind disease progression (Farahani and Haddad, 2003). The *Drosophila* model system has been used to study numerous physiological questions concerning

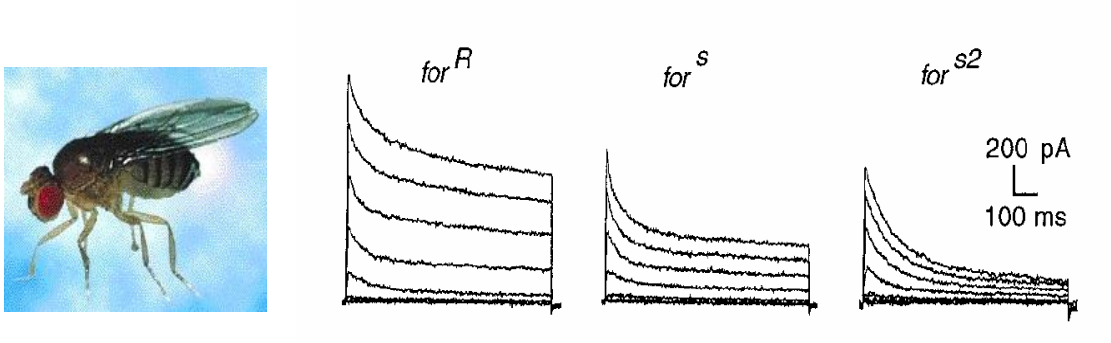

Figure 5. Whole-cell patch-clamp recording from *for* allelic variants. Both transient and sustained voltage-activated K^+ currents are reduced in natural (*for*^{s}) and mutant (*for*^{s2}) alleles compared to the natural *for*^{R} allele. The voltage-activated K^+ currents were evoked by depolarization steps (950 ms) in 20 mV increments from a holding potential of -80 mV to voltages between -60 mV and +60 mV (adapted from Renger et al., 1999).

genetic inheritance, organ development, ageing, memory, learning, circadian rhythm, alcohol intoxication, neurological disorders, and cancer development and progression (Haddad, 2006). Compared to other model systems, fruit flies are relatively easy to keep in supply, have a short life cycle (Figure 6), reproduce large offspring numbers quickly, and have a well-characterized and fully sequenced genome that is easy to manipulate (Feala, 2008). Furthermore, there are tens of thousands commercially available fly stocks from repositories such as the Bloomington *Drosophila* Stock Center at Indiana University. Fly stocks with genetic deficiencies and mutations, dominant steriles or lethals, balancers, genetic insertion lines, human disease models, special teaching stocks used for early development as well as transgenic constructs such as those for RNA interference (RNAi) expression can be purchased.

In addition to genetic tractability and the ready availability of fly stocks, a unique advantage of the *Drosophila* model system is the vast amount of conservation of mammalian biochemical pathways and homologous genes and transcription factors (Zhou, 2008; Arquier, 2006). With estimates that 75% of human disease-related genes have conserved functional fly orthologs, the fly is an attractive model system for probing molecular mechanisms of many human diseases (Pandey and Nichols, 2011). Like mammals, fruit flies are obligate aerobes, exhibit terminal branching similar to mammalian hypoxia-induced angiogenesis, and have homologous synaptic structures such as presynaptic cytosolic projections known as T-bars (Vigne, 2006; Arquier, 2006; Jiao et al., 2010). Invertebrates are also less complex than mammals with a smaller genome that facilitates an array of forward and reverse genetic approaches including

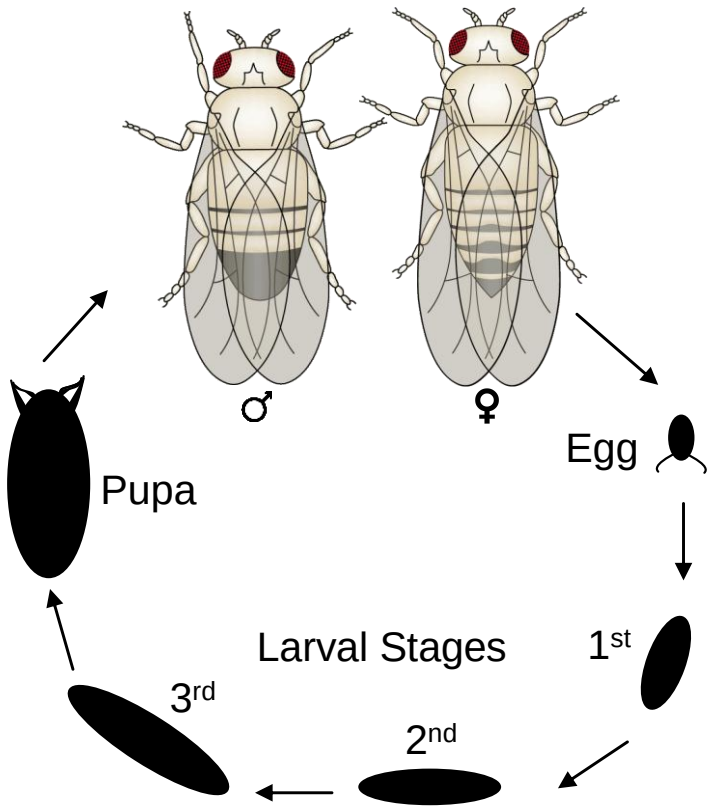

Figure 6. Life cycle of *Drosophila melanogaster*. The reproductive cycle of the fruit fly takes ~12 days at 25°C. Adult male (♂) and female (♀) flies mate and produce eggs that hatch into larvae. Larval stages progress with maturation from 1st instar, 2nd instar, to 3rd instar until pupation in two days. The pupae undergo metamorphosis and the adult fly ecloses from the pupa case one week later. Adult flies can begin the reproductive cycle within 8 - 12 hours following eclosion (reviewed in Nichols, 2006).

techniques such as cloning, mapping, and mutagenesis (Haddad, 2006).

The GAL4/UAS Expression System

The completely sequenced fly genome encodes for a little more than 14,000 genes on four chromosomes (Celniker and Rubin, 2003). Fruit flies have a very rapid life cycle and compact genome that make it relatively easy to create transgenic animals. These attributes combined with a variety of *Drosophila* genetic techniques make the fly a useful system for conducting complex genetic screens that would be cumbersome in other organisms such as traditional rodent models that produce only a handful of offspring every couple of months (Pandey and Nichols, 2011). The *Drosophila* GAL4/upstream activating sequence (UAS) system has been widely used for targeted gene expression in an abundance of cell and tissue specific patterns to study biological phenomena in *Drosophila* (Brand and Perrimon, 1993; Figure 7). This bipartite expression system works by carrying the GAL4 gene, encoding the yeast transcription activator protein GAL4, and the UAS-target gene, an enhancer that GAL4 specifically binds to activate gene transcription, separated in two different transgenic lines (Phelps and Brand, 1998). When these lines are crossed, the gene of interest is activated in a cell specific manner in the progeny. This system allows for spatially and temporally targeted gene expression in *Drosophila* (Kuo et al., 2012). Commercial availability of a large number of well-characterized Gal4 driver lines at the Bloomington *Drosophila* Stock Center makes it relatively easy to create transgenic animals with selective gene expression in a wide variety of tissues and temporal patterns in the fly compared to other organisms.

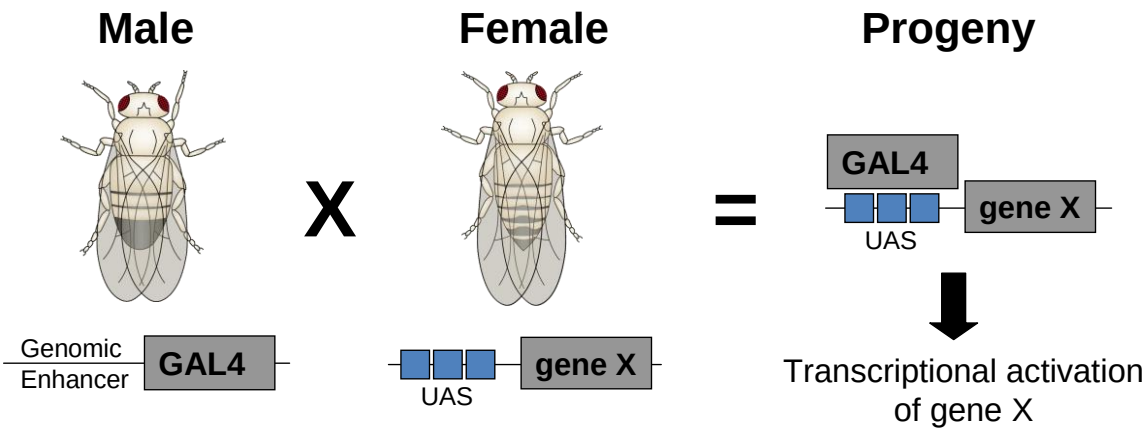

Figure 7. The *Drosophila* Gal4/UAS expression system. Developed by Brand and Perrimon in 1993, this genetic expression system allows for tissue-specific and temporal gene expression in *Drosophila*. This bipartite expression system works by carrying two genes (the Gal4 driver line and UAS-target gene) in separate fly lines that when crossed produces gene activation in the progeny. The Gal4 driver line contains an enhancer trap that encodes the yeast GAL4 transcription factor and the UAS-target gene line has a gene of interest (gene X) placed downstream of the GAL4 binding site. The progeny from this cross contain both the GAL4 driver and the UAS-target gene, which drives tissue- and temporal-specific expression of the gene of interest.

The Larval Neuromuscular Junction

Invertebrate model systems have helped pave the way to understanding key aspects of the mammalian central nervous system including synapse structure, function, and regulation. For instance, numerous studies investigating glutamate receptors at the neuromuscular junction (NMJ) of locusts and fruit flies has provided a better understanding of how these structures function in vertebrates (Anderson et al., 1976; Cull-Candy, 1983; Gration et al., 1981; Jan and Jan, 1976; Patlak et al., 1979). The *Drosophila* larval neuromuscular junction (NMJ) is a well-characterized model for studying the cellular mechanisms of synaptic development, physiology and neurotransmission (Jan and Jan, 1976).

Third instar larvae can be dissected to reveal the central nervous system (ventral nerve cord), segmental nerves, and the innervated abdominal muscles (Figure 8). The abdominal muscle pattern is a series of segmental repeats with a fixed set of 30 muscles in each bilaterally symmetric hemisegment (Cohen and Juergens, 1991). Each muscle fiber is a single, multinucleate cell that is formed by the fusion of neighboring myoblasts and has been assigned a number for ease of identification (Hoang and Chiba, 2001; Figure 9). Approximately thirty six motor neurons innervate these muscles in the following subgroups: four segmental nerves (SNa-d), an intersegmental nerve (ISN) and the transverse nerve (TN) fiber (Hoang and Chiba, 2001).

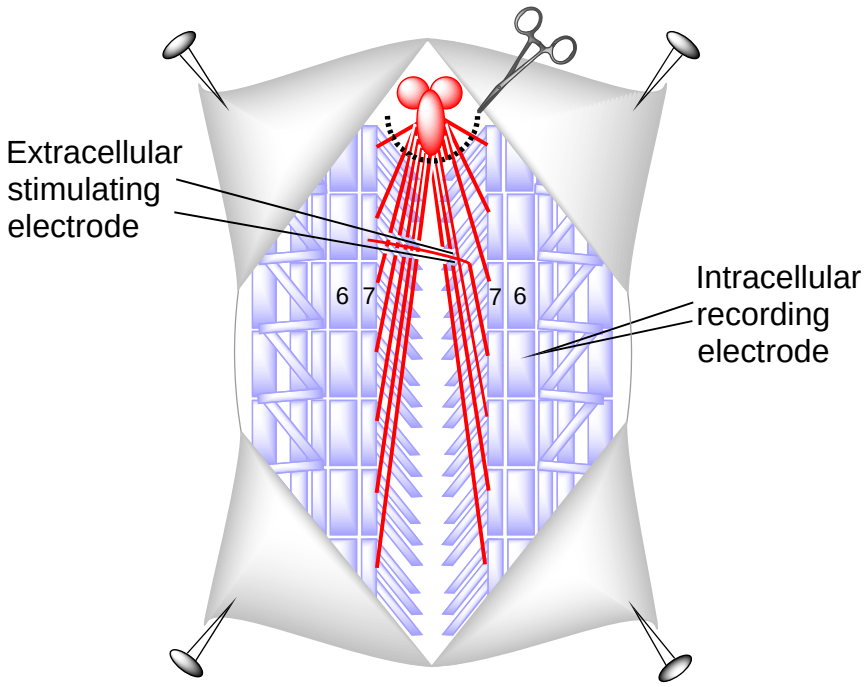

Figure 8. The *Drosophila* larval NMJ preparation. A diagram of the body wall muscles and innervating nerves in 3rd instar larvae. The larval NMJ dissection technique removes the central nervous system. A presynaptic nerve is suctioned into an extracellular stimulating electrode and the postsynaptic excitatory junction potential (EJP) is recorded from muscle 6/7 with an intracellular electrode.

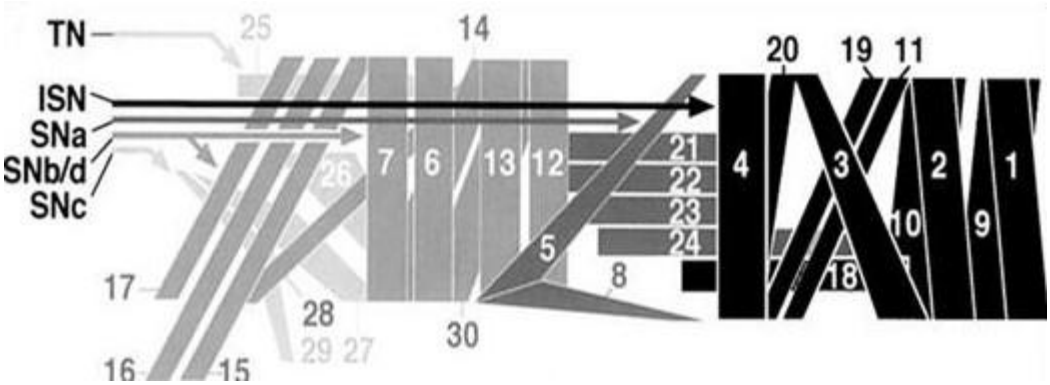

Figure 9. Numbering scheme of *Drosophila* larval body wall muscles. The schematic shows a dorsal view of a 3rd instar larval NMJ preparation. The abdominal muscle pattern is a series of segmental repeats with a fixed set of 30 muscles in each hemisegment that is innervated by the motor neurons through these six nerve branches: four segmental nerves (SNa-d), an intersegmental nerve (ISN) and the transverse nerve (TN) fiber (adapted from Hoang and Chiba, 2001).

The *Drosophila* larval NMJ has the advantage of uniquely identifiable neurons and muscles in a smaller system than vertebrate models of synaptic physiology, which eases physiological examination of synaptic properties and provides a simpler perspective. There are broad categories of nerve-muscle innervations with Type I having relatively large bouton endings (3 - 4 μm diameters), Type II having long, thin endings with small varicosities (1 - 2 μm diameter) that contain presynaptic vesicle machinery proteins and glutamate (Johansen et al., 1989; Landgraf et al., 2003), and Type III boutons (2 - 3 μm diameters) that co-release insulin and glutamate (Gorczyca et al., 1993; Hoang and Chiba, 2001; Figure 10). Type I boutons are further categorized into two classes (Atwood et al., 1993): Type Is (1 - 1.5 μm "small" diameter boutons) and Type Ib (3 - 5 μm "big" diameter boutons).

Contact points between presynaptic motor neurons and muscle fibers at the NMJ are called boutons. Each bouton has synapses where the motor neuron (presynaptic cell) communicates with the muscle (postsynaptic cell) by release of chemical signals called neurotransmitters that cause a depolarization in the muscle fiber (Figure 11). The *Drosophila* larval NMJ uses the neurotransmitter glutamate (Jan and Jan, 1976). Individual segmental nerves forming synaptic connections to these muscles can be stimulated with a sharp glass electrode and the corresponding post synaptic excitatory junction potential (EJP) from the muscle can be captured with a recording electrode (Figure 12). This technique, known as the *Drosophila* larval NMJ preparation, allows researchers to study synaptic functional properties in a diverse set of conditions including genetic and pharmacological manipulations.

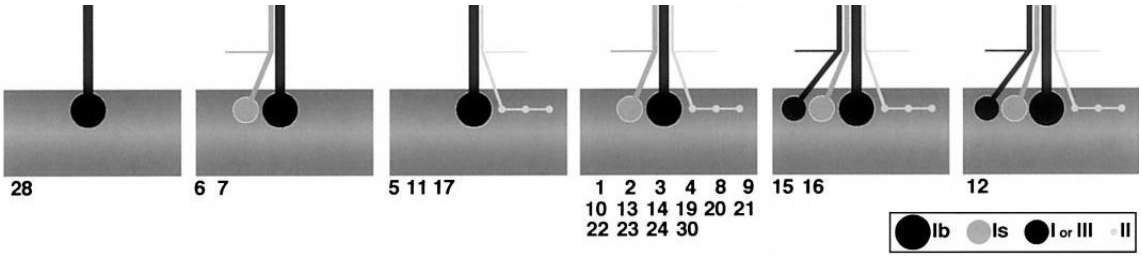

Figure 10. Motor neuron innervation of *Drosophila* larval body wall muscles. Illustration of the motor neuron innervation pattern in 3rd instar abdominal muscles (indicated by the numbers below). Type Ib boutons (3 - 5 μm diameters) are present on every larval muscle. Type Is boutons (1 – 1.5 μm diameters) and Type II boutons (1 - 2 μm diameter) are present on most larval muscles. Type III boutons (2 - 3 μm diameters) are found only on muscle 12 (adapted from Hoang and Chiba, 2001).

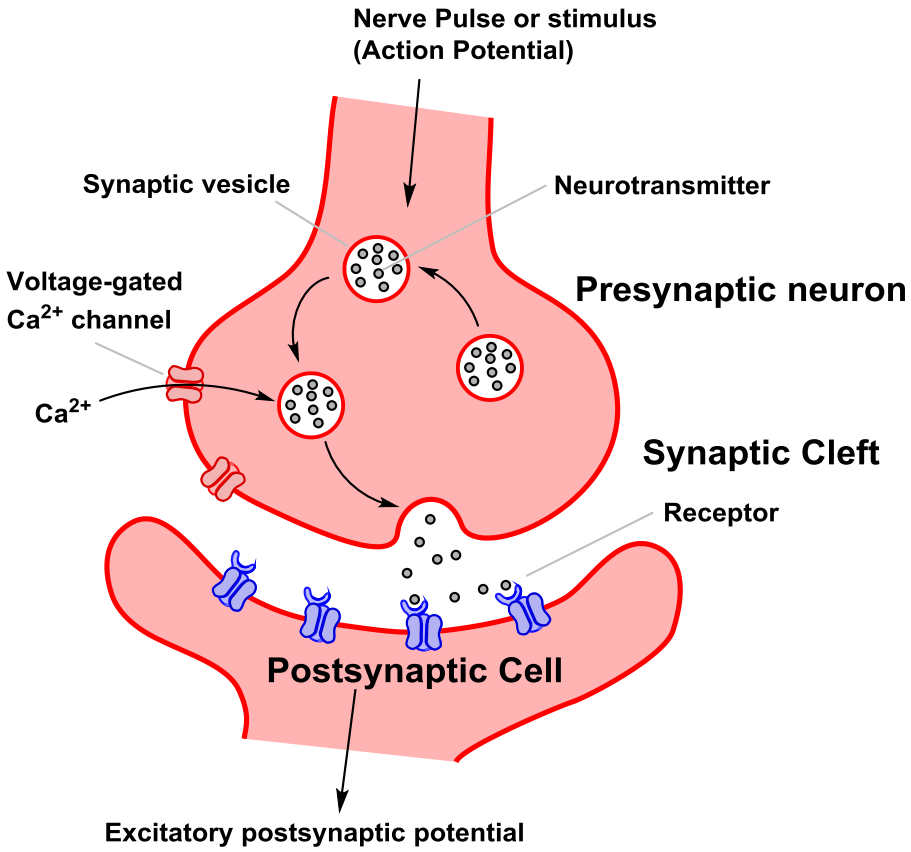

Figure 11. A synapse between two cells. A schematic of a synaptic cleft between two cells. As an action potential travels down the presynaptic cell, it causes a depolarization that causes an increase in intracellular calcium. Calcium assists in the docking of presynaptic vesicles to the membrane and release of neurotransmitters into the synaptic cleft. Once released, neurotransmitters travel to their binding sites on the postsynaptic cell (receptors), which causes a depolarization in the postsynaptic cell. Synapses occur between two neurons in the central nervous system and between nerve and muscle cells in the peripheral nervous system.

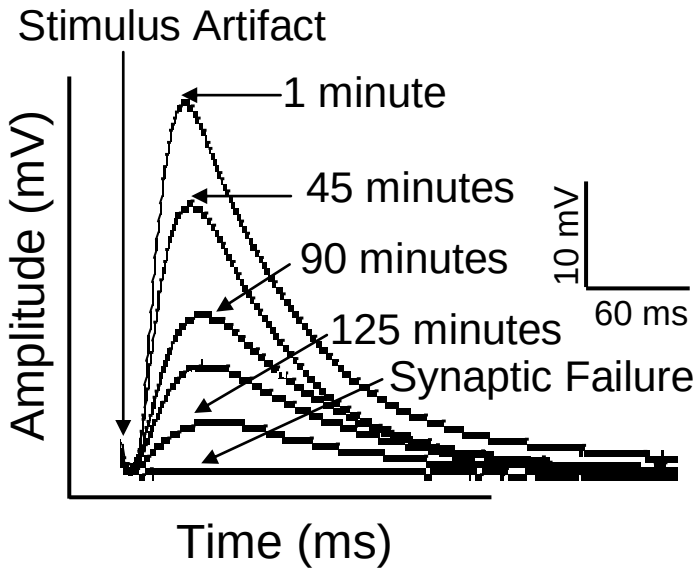

Figure 12. Excitatory Junction Potential (EJP) of the *Drosophila* larval NMJ. During physiological recordings from the *Drosophila* NMJ, a sharp glass electrode draws up a segmental nerve with gentle pressure and stimulation yields a postsynaptic response from the muscle. An intracellular recording electrode placed in the corresponding muscle innervated by the nerve ending will record the post synaptic excitatory junction potential (EJP). As time progresses, the amplitude will decay as a function of time to eventual synaptic failure (flat line of the EJP). Stress conditions such as hyperthermia, anoxia, and oxidative stress, accelerate this decline in synaptic function.

Physiological studies routinely examine the longitudinal abdominal muscles numbered 6, 7, 12, and 13; however, muscles 6 and 7 are preferentially selected because they are large and only innervated by Type I nerve endings, while muscles 12 and 13 are innervated by Type I and Type II nerve endings (Atwood et al., 1993; Johansen et al., 1989). An observed change in parameters of the EJP such as the amplitude, shape, or latency, can indicate potential alterations in synaptic function or structure and provides a powerful tool capable of being used in combination with an array of methodologies.

The *Drosophila* larval NMJ has many similarities to mammalian synapses including use of the neurotransmitter glutamate and the ability to remodel in response to activity (Jan and Jan, 1976; Collins and DiAntonio, 2007; Gramates and Budnik, 1999). Due to recent technical advances, the NMJ can be directly manipulated in a variety of different ways utilizing physiological, genetic, and microscopy tools (Bayat et al., 2011). For instance, studies utilizing combinations of *Drosophila* genetic and NMJ physiology tools have advanced the understanding of the roles of a number of proteins involved in numerous neurodegenerative diseases including Amyotrophic Lateral Sclerosis, Spinal Muscular Atrophy, Multiple Sclerosis, Hereditary Spastic Paraplegia, and Huntington's disease (Bayat et al., 2011). Studies utilizing the *Drosophila* larval NMJ have greatly enhanced our understanding of cellular stress responses and mechanisms underlying disease progression

Statement of Purpose

Unlike mammals, insects have evolved stress resistance mechanisms and can survive dramatic temperature and oxygen level oscillations without detrimental effects on cellular health or function (Chen and Walker, 1994; Feder, 1997; Haddad, 2006; Hoffmann et al., 2003; Misener et al., 2001; Wingrove and O'Farrell, 1999). The cellular basis for the difference between organism responses to celluar stress remains elusive. Oxidative stress plays an important role in the pathophysiology of an array of devastating human diseases and understanding the cellular ischemic cascade during disease progression is essential to enable the development of more effective therapies.

During extreme conditions, the stress tolerant fruit fly, *Drosophila melanogaster*, relies on a crucial intrinsic defense mechanism that involves the cGMP-dependent protein kinase G (PKG) pathway (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). The PKG pathway is conserved across species and is believed to regulate the tolerance of the nervous system tolerance during severe cellular predicaments (Dawson-Scully et al., 2007; Robertson and Sillar, 2009; Armstrong et al., 2010). PKG signaling is involved in many signal transduction processes in diverse cell types and has been implicated as a protective mechanism against ischemia-reperfusion injury in many animal models from insects to mammals (Dawson-Scully et al., 2010; Bolli, 2001; Garlid et al., 1997). The PKG pathway is a relevant neuroprotective mechanism that provides a potential avenue for creating novel strategies to combat a number of physiological stressors and neurological diseases with the added advantages of evolutionary conservation, diverse

model system applications, and the availability of both genetics and rapid pharmacological treatment interventions.

In addition to probing endogenous cellular protection mechanisms such as the PKG cascade, natural remedies have been used to treat disease and improve health and shaped traditional medicinal practices. Since the discovery and isolation of pure bioactive compounds derived from plants and animals or micro-organisms, these natural products have played an invaluable role within the drug discovery pipeline (Cragg et al., 2014). The untapped biological and chemical diversity of the world's oceans provide an enormous resource for the discovery of marine natural products that could eventually be developed into therapeutics to treat human diseases (Newman and Cragg, 2012). Finding novel therapeutics to combat the deleterious effects of oxidative stress and to better understand the cellular mechanisms that underlie dual role of oxidants in biological signaling mechanisms remains a relevant neuroscience research topic.

The present study aims to determine the regulatory role of the PKG pathway on nervous system function during acute oxidative stress if this mechanism occurs through downstream ion channel modification. A combination of genetics and pharmacological approaches were used to explore the hypothesis that the PKG pathway is involved in controlling cellular responses to acute physiological stress by modulating neurotransmission. In addition to the PKG pathway, this study also used the Drosophila model system to find novel structures capable of modulating synaptic function during oxidative stress and explore the potential for marine natural products as novel

neuromodulatory agents. A novel bioimaging-optogenetics approach that combines the use of genetically-encoded calcium indicator (GECI) and the light activated ion channel, channelrhodopsin-2 (ChR2), was used to simultaneously visualize and control neural activity in single cells. This optogenetic assay can be expanded for use with *Drosophila* primary neural cultures that will enable genetic targeting of specific neuronal populations to identify *in vivo* targets and/or site of action of natural products. Ultimately, this will develop the framework for early-stage discovery of natural products with neuromodulatory and/or neuroprotective properties.

CHAPTER 2. MATERIALS AND METHODS

Fly Strains

Drosophila melanogaster third instar wandering larvae (~110 hrs old) from four wild-type strains, Canton-S (*CS*), yellow-white (*yw*), rover (for^{R}), and sitter (for^{s}), and one mutant *foraging* strain (for^{s2}) were used in this study. The for^{R} and for^{s} strains are natural allelic variants of the *Drosophila for* gene. The mutant for^{s2} strain was previously generated on a for^{R} genetic background with a mutation in *for* that produces lower PKG activity/transcript levels than for^{s} (Pereira and Sokolowski, 1993) and was utilized to control for genetic background.

All *Drosophila* genetic mutants, GAL4 driver lines, and UAS lines were ordered from the Bloomington *Drosophila* Stock Center at Indiana University. All animals were raised within an equal population density (approximately 100 flies per bottle) on 50 mL of a standard Bloomington stock fly food in an incubator at 25°C under a 12 hr:12 hr light-dark cycle.

Electrophysiology

Individual *Drosophila* larvae were collected and placed in a glass-dissecting dish containing 2mL of Schneider's Insect Medium (Sigma, St. Louis, MO). Each larva was positioned with the dorsal side up on the dissecting dish using standard insect pins. Removal of the internal organs and central nervous system was achieved by making a longitudinal cut in the anteroposterior direction along the dorsal surface to expose the underlying segmental muscles and nerves. An extracellular glass suction electrode was used to stimulate segmental nerves in muscle segments. The postsynaptic excitatory junction potential (EJP) was recorded from muscle 6 abdominal segments A3-4 with a sharp intracellular glass recording electrode filled with 3M KAc (~40 MΩ).

The preparation medium was replaced with hemolymph-like (HL-3) saline [$CaCl_2$ (1.5 mM), $MgCl_2$ (20 mM), KCl (5 mM), NaCl(70 mM), $NaHCO_3$ (10 mM), BES (5 mM), Sucrose (115 mM), Trehalose·$2H_2O$ (5 mM)] made fresh daily (Macleod et al., 2002; Stewart et al., 1996). EJP recordings were viewed with an oscilloscope and digitally stored using the Scope program (AD Instruments, Colorado Springs, CO) for analysis.

Two stimulation protocols were used to evoke response from both axons or a single axon fiber in larval muscle 6: dual axon fiber stimulation where both axons elicited a response or single axon fiber stimulation where one axon elicited a response. Evoked EJPs from repetitive stimulation (0.3 ms pulses delivered suprathreshold with a 1 Hz

frequency) of both axons in larval muscle 6 were recorded in a stop-flow condition (referred to as "dual axon fiber stimulation"). Evoked EJPs from repetitive stimulation (0.3 ms pulses delivered at 5 mV with a 1 Hz frequency) of one axon in larval muscle 6 were recorded in a stop-flow condition (referred to as "single axon fiber stimulation"). EJP recordings were taken until synaptic transmission failure occurred (amplitude less than 1mV).

Intracellular recordings of the resting membrane potential (RMP) and input resistance were measured from larval muscle 6 with signals amplified by an IX1 intracellular preamplifier (Dagan, Minneapolis, MN). These measurements were taken as previously described (Zhang and Stewart, 2010). Briefly, RMP measurements were taken from animals if the initial potential stabilized between -60 to -70 mV. If the membrane potential was equal to -45 mV or more depolarized, the preparation was discarded. Input resistance was measured by injecting small current pulses of 2 nA (40 ms duration at 1 Hz frequency) applied continuously. Electrode resistance was canceled prior to measuring the input resistance by adjusting the bridge balance control. Resistance was calculated using Ohm's law and only muscle fibers with an initial input resistance > 5 or < 40 MΩ were assayed.

Pharmacological Manipulation

The same electrophysiology preparation assay described above was performed; however, animals were also exposed to acute oxidative stress, pharmacological agents

that affect the PKG pathway, or pseudopterosins. Acute oxidative stress was induced in larval preparations by adding hydrogen peroxide (H_2O_2, 1 or 2.25 mM) or sodium azide (NaN_3, 75 µM or 0.1 mM) to the HL-3 saline. Larvae subjected to acute oxidative injury and simultaneous PKG drug treatment included one of the following agents dissolved in dimethyl sulfoxide (DMSO; all chemicals obtained from Sigma, Saint Louis, MO): Guanosine 3',5'-cyclic monophosphate 8-bromo-sodium salt (8-Bromo-cGMP, 40 µM), a PKG activator (Ruth et al., 1991), KT-5823 (1µM), a relatively selective, competitive PKG inhibitor (Grider, 1993; Kase et al., 1987), or Rp-β-phenyl-1,N2-ethanoguanosine 3':5'-cyclic monophosphorothioate sodium salt hydrate (Rp-8Bromo-cGMP, 50 µM), a more selective PKG inhibitor that irreversibly binds to the kinase (Petrov et al., 2008; Wei et al., 1996). These concentrations were previously shown to alter PKG activity in insect and mammalian models (Dawson-Scully et al., 2007; Li et al., 2007).

Dose response curves on pseudopterosin analogues (0.5 - 50 µM) were performed to determine effective concentrations that would extend synaptic function at the *Drosophila* larval NMJ during acute oxidative injury. Animals subjected to acute oxidative stress and simultaneous pseudopterosin exposure included one of the following agents dissolved in DMSO: pseudopterosin A (PsA), pseudopterosin B (PsB), pseudopterosin C (PsC), pseudopterosin D (PsD), pseudopterosin K (PsK), isopseudopterosin A (IsoA), or isopseudopterosin D (IsoD). Trolox (5 µM), a water-soluble analog of vitamin E, was selected as a positive control due to its ability to protect against oxidative stress via biological oxidation-reduction reactions.

In a separate assay, pharmacological PKG agents were examined for adverse effects on the parameters of the EJP, where larvae were exposed to each drug separately and the EJP recordings were compared to controls for differences in amplitude, waveform/shape, and latency values. For drug combinations, larvae were exposed to acute oxidative stress (H_2O_2, 2.25 mM), a PKG agonist (8-Bromo-cGMP, 40 μM), and tetraethylammonium hydroxide (TEA, 250 μM), a non-selective potassium (K^+) channel inhibitor, or 5-Hydroxydecanoic acid sodium salt (5-HD, 100 μM). A dose response assay was performed to determine the concentration used. In the dose response assay it was found that TEA (250 μM) had no detrimental effect on the preparation, including no observable changes to the EJP. However, the larval NMJ preparation was compromised with increasing concentrations of TEA: 500 μM caused sporadic contractions ~15 minutes into the experiment, 1 mM caused sporadic contractions in ~5 minutes, and 2 mM caused contractions immediately upon application.

Cell Culture

The *Drosophila* Schneider 2 (S2) cell line (Invitrogen, Carlsbad, CA) was cultured as described by the supplier. Briefly, S2 cells were grown at room temperature in complete Schneider's *Drosophila* medium containing $NaHCO_3$ (Sigma Aldrich) and supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 50 units/ml penicillin and 50 μg/ml of streptomycin (Invitrogen) in a T-25 cm^2 or T-75 cm^2 tissue culture flasks. The cells were split in a 1:2 or 1:3 ratio every 2-5 days. Before conducting experiments, the cells were centrifuged at 1000 × g for three minutes

and the supernatant was exchanged for an appropriate volume of fresh complete *Drosophila* Schneider's medium to generate 0.5 – 1 x 10^6 cells/ml suspension. The cell suspension then was divided into 1 ml aliquots to perform pharmacological treatments.

To induce acute oxidative stress, tert-butyl hydroperoxide (tbH_2O_2, 11 mM) or hydrogen peroxide (H_2O_2, 2.25 - 24 mM) was added to the cell suspensions to generate a final concentration of 1 mM. During the acute stress, the PKG pathway was modulated by either direct kinase activation, 8-Bromo-cGMP (40µM), or inhibition, Rp-8Bromo-cGMP (50µM). For these experiments, PKG pharmacological treatments were performed immediately before addition of the acute oxidative stress agent. After addition of pharmacological compounds, the cells were incubated for one or four hours before cell injury assessment.

Cell Viability

Drosophila S2 cell viability was determined using the colorimetric MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] viability assay (96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) or the Trypan blue exclusion method. This MTS assay kit contains a novel tetrazolium compound (MTS) and an electron coupling reagent (phenazine ethosulfate; PES). The MTS assay measures the mitochondrial activity of viable cells by quantifying the conversion of the tetrazolium salt to its formazan product, which is measured by absorbance at 490nm and is directly proportional to the number of living cells in culture.

This assay was performed according to the manufacturer's instructions and absorbance was measured at 490 nm using a 96-well plate reader. The Trypan blue exclusion method is based on the principle of an intact cellular membrane excludes certain dyes, such as Trypan blue, whereas dead cells do not. S2 cell counting was conducted using a Bright-Line hemocytometer (Improved Neubauer, 0.100 mm deep) and a compound light microscope (Swift Instruments International S.A.). Approximately 400-600 cells were counted for each treatment and then averaged. Cell death indicated by stained cells was expressed as a percent of Trypan blue staining rate of cells divided by the total number of cells.

Bioimaging

Imaging of transgenic RGECO/ChR2 *Drosophila* lines was performed on an upright Olympus BX51WI microscope fitted with a 100X water immersion objective on a TMC® vibration isolation table. A Sutter Instrument DG4 fluorescence excitation system was used to select excitation wavelengths, while a Sutter Instrument Lambda 10-B filter wheel, placed before an Andor Technology EMCCD camera (DV887), was used to filter emitted fluorescence. The confocal imaging system was controlled via Nikon NIS-Elements software. The physiological preparations were visualized with the genetically-encoded RGECO calcium indicator (561 nm laser) and ChR2 activation (neural stimulation) with a 488 nm laser line scan across synaptic boutons. The change in fluorescence (intensity) due to calcium influx when ChR2 is activated was measured during 2 s line scans.

Statistics

All data was analyzed using SimgaPlot (Systat Software, San Jose, CA) statistical analysis software. A One-Way ANOVA test was used to compare significant differences across all treatment groups followed by a *post hoc* method to determine significance using multiple comparisons. Statistical significance ($p < 0.05$) was assigned using letter designations, where different letters show significant differences and the same letter assignments are not significant. The letter assignments begin with "A" representing the highest mean, "B" indicating the next highest, and so forth. All vertical bar charts are shown with the means ± SEM.

CHAPTER 3. PKG-INDUCED SYNAPTIC EFFECTS DURING ACUTE OXIDATIVE STRESS

Introduction

Unlike mammals, insects possess numerous strategies that enable survival during extreme ecological conditions. The fruit fly, *Drosophila melanogaster*, is capable of handling drastic fluctuations in temperature and oxygen levels, allowing survival without causing serious damage to cells and tissues (Chen and Walker, 1994; Feder, 1997; Haddad, 2006; Hoffmann et al., 2003; Misener et al., 2001; Wingrove and O'Farrell, 1999). Since fruit flies are able to endure these conditions, they are an ideal model system for studying endogenous protective mechanisms that facilitate tolerance of the nervous system to acute stress. In response to experiencing drastic environmental changes in atmospheric temperature and oxygen levels, this organism relies on a crucial intrinsic defense mechanism that involves the cGMP-dependent protein kinase G (PKG) pathway (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). Analogous protection from modulating PKG signaling during acute hyperthermia and hypoxia has also been demonstrated in other species, such as the locust (Dawson-Scully et al., 2007), tadpole (Robertson and Sillar, 2009), and mouse (Armstrong et al., 2010). Conservation of this pathway between insects and mammals implies its importance as a stress-mediated regulator that controls nervous system tolerance throughout severe cellular predicaments.

PKG activity is encoded by the *Drosophila foraging* (*for*) gene, a homolog of the human *PRKG1* gene, and is expressed as a polymorphism that modulates behavioral food-searching strategies (Sokolowski, 1980). Two variants of this gene exist in nature, where 70% of flies express the rover (for^R) allele with high PKG activity and 30% of flies express the sitter (for^s) allele with low PKG activity (Osborne et al., 1997). These natural fly populations display differential feeding behaviors, where rovers travel more than sitters in the presence of food, which remain throughout their entire life cycle (de Belle and Sokolowski, 1987; Pereira and Sokolowski, 1993). In addition to affecting foraging strategies, *for* influences other functions, such as learning and memory (Mery et al., 2007) and acute stress tolerance (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010), as well as fundamental neuronal properties including excitability, transmitter release (Feil and Kleppisch, 2008), synaptic plasticity (Kleppisch and Feil, 2009), and potassium channel conductance (Renger et al., 1999).

The present study analyzed synaptic transmission at the *Drosophila* larval neuromuscular junction (NMJ) glutamatergic synapse during acute oxidative stress. A combination of genetics and pharmacological approaches were used to explore the hypothesis that the PKG pathway is involved in controlling cellular responses to acute physiological stress by modulating neurotransmission. More specifically, this study investigated if altering the PKG signaling cascade can regulate synaptic tolerance during acute oxidative stress and if this mechanism potentially occurs through downstream ion channel modification. PKG activity was manipulated in the following ways: 1) naturally using rover (for^R) or sitter (for^s) larvae, 2) genetic modification with for^{s2} larvae that

possess a mutation, and 3) via pharmacological disruption with the PKG inhibitors KT-5823 and Rp-8Bromo-cGMP, the PKG activator 8-Bromo-cGMP, and the K^+ channel inhibitor tetraethylammonium hydroxide (TEA).

Results

Natural variation in PKG activity modulates synaptic tolerance

To investigate if the PKG pathway is able to modulate synaptic transmission tolerance to acute oxidative stress, H_2O_2 (2.25 mM) was exogenously applied to *Drosophila* larval NMJ preparations. Larvae of three different fly strains, for^R, for^s, and for^{s2}, with genetically different PKG activity levels were utilized to distinguish if any differences in neural communication during the acute stress existed and could be associated with differences in PKG activity. Wild-type for^R larvae express high PKG levels, wild-type for^s larvae possess low PKG levels, and mutant for^{s2} larvae have a for^R genetic background with a mutation in *for* that produces lower PKG levels comparable to for^s larvae.

Under acute stress conditions, the PKG allelic variants demonstrated differential synaptic transmission tolerances indicated by varying rates in the decline of the EJP amplitudes over time (Figure 13B). Animals with high PKG activity (for^R) had the quickest decline in EJP amplitude, while those with low PKG activity (for^s, for^{s2}) were slow to decline as time progressed, suggesting that the difference in the rate of amplitude

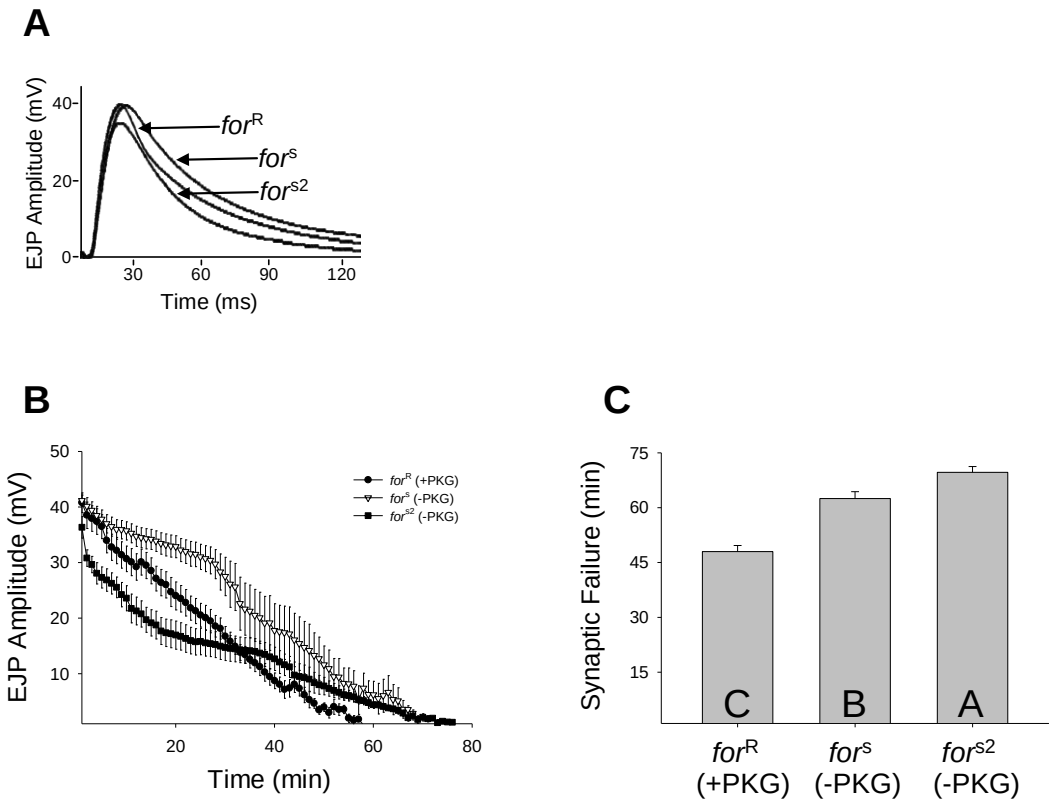

Figure 13. Genetic variation in PKG activity modulates synaptic function. A: representative EJP amplitudes from larval muscle 6 of *Drosophila* PKG allelic variants (*for*^{R}, *for*^{s}, *for*^{s2}) prior to H_2O_2 application. B: *Drosophila* larvae [*for*^{R} (+PKG), N = 8; *for*^{s} (-PKG), N = 8; *for*^{s2} (-PKG), N = 6] with varied PKG expression levels show different rates of evoked EJP amplitude decline as a function of time during H_2O_2 exposure (2.25 mM). C: time to NMJ synaptic transmission failure differed significantly among PKG variants during acute H_2O_2 treatment (Holm-Sidak, $p < 0.05$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

decay may be attributed to differences in PKG activity. Amplitudes of the evoked EJPs declined over time in the following order: $for^{R} > for^{s} > for^{s2}$. Mirroring EJP decay rates, variation in PKG expression also modulated the time until NMJ synaptic transmission failure (Figure 13C). On average, *Drosophila* for^{R} larvae displayed neuromuscular communication loss significantly earlier at 48 ± 2 minutes compared to for^{s} and for^{s2} larvae at 63 ± 2 and 70 ± 2 minutes, respectively (One-Way ANOVA, $F_{(2,17)} = 43.76$, $p < 0.001$).

Pharmacological agents do not disrupt EJP characteristics

The data indicated that genetically altered PKG expression modulates synaptic transmission tolerance to acute H_2O_2 exposure, but it did not demonstrate if wild-type *yw* *Drosophila* with chemically modulated PKG activity would exhibit similar differences in synaptic susceptibility to bath-applied H_2O_2. Before this question was addressed, whether pharmacological PKG modulators would adversely affect parameters of the NMJ postsynaptic response (i.e. the EJP latency time, shape, amplitude) was examined. Pharmacological agents that either activate (8-Bromo-cGMP, +PKG) or inhibit (KT-5823, -PKG; Rp-8Bromo-cGMP, -PKG) PKG activity were added to the HL-3 saline bath of larval preparations. Individual wild-type *yw* larvae were exposed to each PKG manipulator separately and recordings were taken one minute before and ten minutes after the application of each drug (Figure 14). There were no observable differences in EJP characteristics for each agent used in the pharmacology experiments, suggesting that these pharmacological PKG manipulators do not significantly affect the evoked response

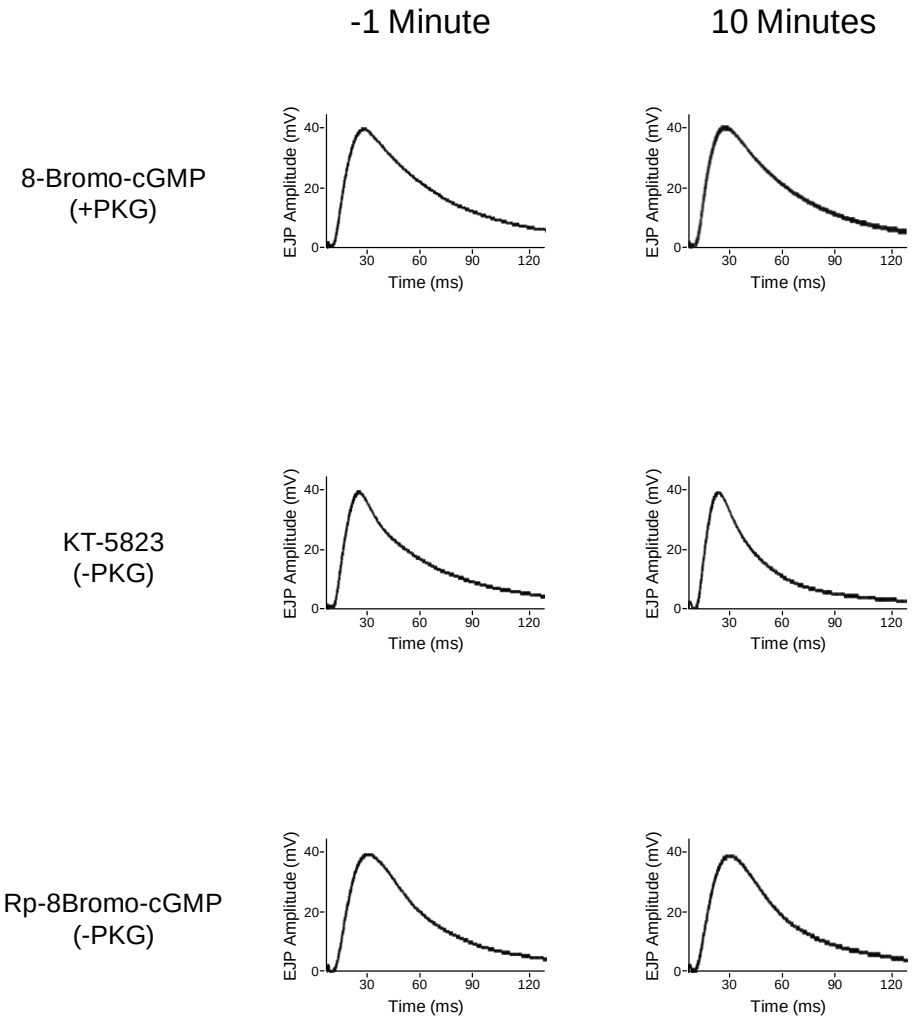

Figure 14. Pharmacological PKG agents do not affect distinctive EJP features. Differential drug treatments that manipulate PKG activity were tested for adverse effects on the parameters of the evoked EJP response from *yw Drosophila* larval muscle 6. Animals were bathed in 2 mL of HL-3 saline containing one of the following agents dissolved in DMSO: 8-Bromo-cGMP (+PKG, 40 μM), KT-5823 (-PKG, 1 μM), or Rp-8-Bromo-cGMP (-PKG, 50 μM). EJP recordings were taken 1 minute before addition of the drug/saline solution and 10 minutes after the application. No differences were observed for each drug tested.

from the NMJ glutamatergic synapse. Therefore, any differences among evoked EJPs from *yw* larvae during the acute stress were attributed to altered PKG activity from chemical manipulation of the signaling cascade.

Chemical PKG manipulation alters synaptic function

To demonstrate that the PKG pathway modulates synaptic transmission tolerance to acute oxidative stress, *yw* flies with chemically modified PKG activity were exposed to H_2O_2 (2.25 mM). This H_2O_2 concentration was chosen to create a standard timeframe to compare changes in NMJ synaptic failure times during pharmacological manipulation of PKG activity. Modification of the PKG pathway was achieved via pharmacological agents that directly activate (8-Bromo-cGMP, 40 μM) or inhibit (KT-5823, 1 μM; Rp-8Bromo-cGMP, 50 μM) PKG cellular function. When the EJP amplitude decay was plotted as a function of time, the differential drug treatments separate into clear patterns of varying synaptic transmission tolerance during the acute stress condition (Figure 15A). Control preparations not exposed to acute oxidative stress nor any drug displayed a maximal timeframe of 142 minutes before failure of synaptic transmission occurred in the stop-flow experimental assay. Activation of the PKG pathway during H_2O_2 exposure reduced the maximum time until synaptic breakdown from 52 minutes to 44 minutes. Conversely, inhibition of the PKG pathway during acute H_2O_2-induced stress extended the maximum time until synaptic transmission failure from 52 minutes to 67 or 84 minutes depending on the drug administered. These results suggest that during acute oxidative stress PKG inhibition extends the time until synaptic transmission failure, while

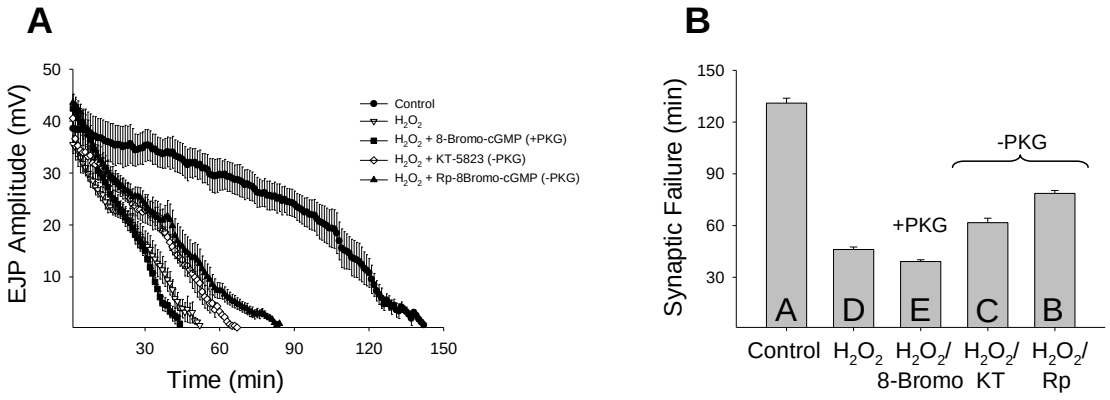

Figure 15. Chemical PKG pathway manipulation alters NMJ synaptic transmission. A: amplitude of evoked EJPs in *yw Drosophila* larvae reduced over time at different rates in control preparations (saline only, N = 6) and those exposed to H_2O_2 (2.25 mM, N = 6). The addition of either PKG inhibitor (KT-5823, 1 μM, N = 3; Rp-8Bromo-cGMP, 50 μM, N = 6) to the saline/H_2O_2 bath solution extended the time that synaptic transmission continued, while addition of a PKG activator (8-Bromo-cGMP, 40 μM, N = 6) reduced this time frame. B: time to synaptic transmission failure in *yw Drosophila* larvae is significantly affected by pharmacologically altering PKG activity during acute oxidative stress (Holm-Sidak, $p < 0.05$). PKG inhibition (-PKG) delayed the time until synaptic failure during H_2O_2 exposure, while activation (+PKG) accelerated this process. Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

PKG activation reduces this time frame.

Additionally, there were significant differences between the average synaptic failure times of all treatment groups (Control, H_2O_2, H_2O_2/+PKG, and H_2O_2/-PKG) depending on the drug administered (One-Way ANOVA, $F_{(4,22)} = 387.24$, $p < 0.001$; Figure 15B). Animals exposed to H_2O_2 displayed synaptic failure at an average of 46 ± 1 minutes compared to control preparations which occurred at an average of 131 ± 3 minutes. Elevated synaptic transmission tolerance during acute oxidative stress was observed in larvae treated with PKG inhibitors (-PKG). The addition of KT-5823 or Rp-8Bromo-cGMP increased the average synaptic failure time to 62 ± 3 minutes and 79 ± 2 minutes, respectively. In contrast, animals treated with the PKG activator 8-Bromo-cGMP (+PKG) showed a reduction in the average time until synaptic transmission failure occurring at 39 ± 1 minutes. These results indicate that pharmacological manipulation of the PKG pathway can either protect or sensitize synaptic transmission tolerance during acute oxidative stress.

Modulating K^+ channel kinetics affects neuromuscular transmission

To explore potential mechanisms responsible for PKG's effect on synaptic transmission during acute oxidative injury, an experiment that combined pharmacological manipulation of K^+ channel kinetics and the PKG pathway was performed. Individual wild-type *yw* larvae were exposed to acute oxidative stress (H_2O_2, 2.25 mM), a K^+ channel inhibitor (TEA, 250 μM), and a PKG activator (8-Bromo-cGMP, +PKG, 40

μM), both individually and concurrently. Similar to the paradigm described in Figure 14, the EJP waveform was examined before application of the pharmacological compounds and 10 minutes post application to determine that the compounds caused no immediate change in the waveform (Figure 16). The drug combination revealed that widespread K^+ channel inhibition slowed the average decay rate of EJP amplitudes decline over time not only in the presence of H_2O_2 but also during PKG pathway activation (Figure 17A). TEA extended the maximum time until synaptic transmission failure during the acute stress condition from 53 minutes to 73 minutes. Additionally, this reduction in the EJP amplitude decline rate continued in the presence of the PKG agonist with the maximum failure time occurring at 70 minutes.

Drosophila NMJ synaptic transmission failure times of all treatment groups (H_2O_2, H_2O_2/TEA, and H_2O_2/TEA/+PKG) were significantly altered by modifying K^+ channel kinetics during acute H_2O_2 exposure (One-Way ANOVA, $F_{(2, 15)} = 31$, $p < 0.001$; Figure 17B). Animals exposed to TEA and H_2O_2 exhibited an extended average synaptic transmission failure time occurring at 66 ± 2 minutes. This functional protection persisted during simultaneous PKG pathway activation with the average synaptic failure time occurring at 61 ± 2 minutes. These drug combinations suggest that reducing overall K^+ conductance during acute oxidative stress protects synaptic function and this protection overrides PKG manipulations, implicating these channels as potential downstream effectors of PKG activity in the signaling mechanism.

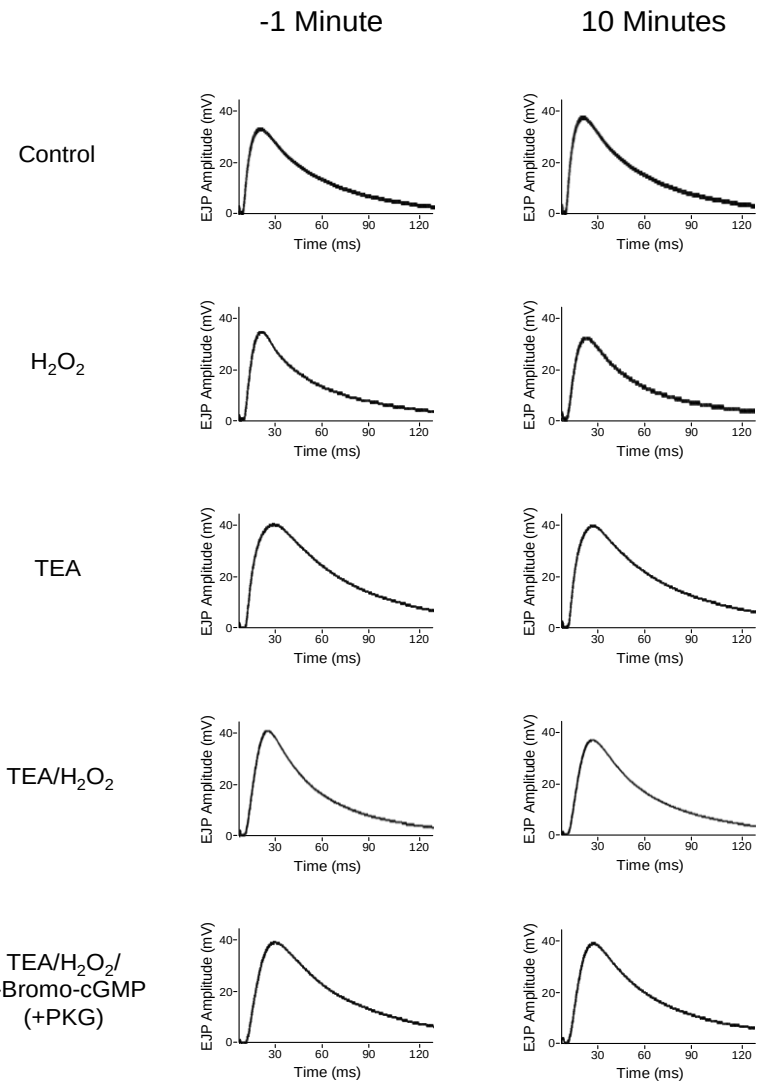

Figure 16. TEA at 250µM does not affect distinctive EJP features. Representative traces of the evoked EJP response from *yw Drosophila* larval muscle 6 after differential drug treatments. Animals were bathed in 2 mL of HL-3 saline containing one of the following exposure conditions: control (saline only), H_2O_2 (2.25 mM), TEA (250 µM), TEA (250 µM)/H_2O_2 (2.25 mM), TEA (250 µM)/ H_2O_2 (2.25 mM)/8-Bromo-cGMP (+PKG, 40 µM). EJP recordings were taken 1 minute before addition of the drug/saline solution and 10 minutes after the application.

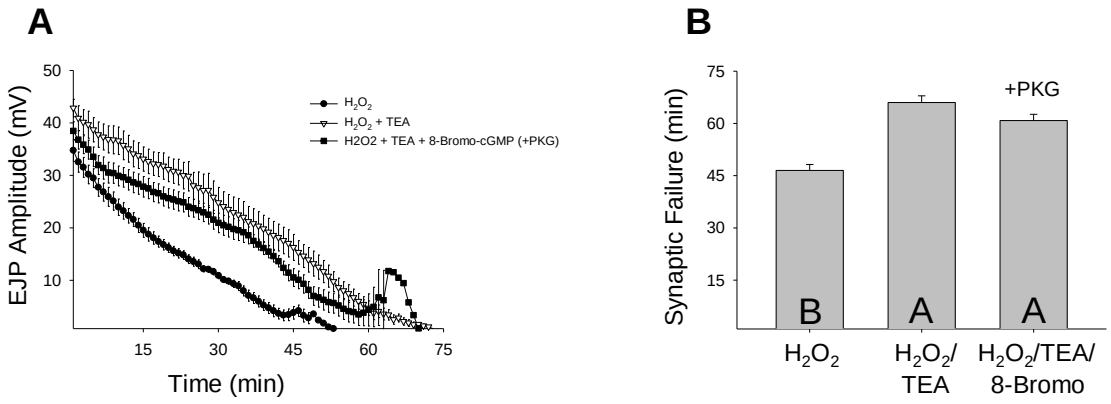

Figure 17. K^+ channel inhibition protects synaptic function. A: decay rate of evoked EJP amplitudes of *yw Drosophila* larvae during H_2O_2 treatment (2.25 mM, N = 6) is reduced by pharmacologically blocking K^+ channels. The addition of TEA (250 µM, N = 6) extended the maximum timeframe until synaptic failure occurred and this continued in the simultaneous presence of the PKG agonist, 8-Bromo-cGMP (40 µM, N = 6). B: time to NMJ synaptic transmission failure of *yw Drosophila* larvae during acute oxidative stress is significantly extended by nonselective K^+ channel inhibition with TEA solely and in the presence of the PKG activator (Holm-Sidak, $p < 0.05$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

Characterizing mechanisms of synaptic failure from acute oxidative stress

To determine if synaptic failure during acute oxidative stress occurs as a result of presynaptic or postsynaptic mechanisms, the resting membrane potential (RMP) and input resistance of the postsynaptic cell was measured over a prolonged period of time (Figure 18). This was done under three pharmacological conditions (H_2O_2, H_2O_2/+PKG, and H_2O_2/-PKG) in separate experiments. During H_2O_2 treatment (2.25mM) the RMP begins to depolarize at ~30 minutes, and this timeframe is altered by PKG manipulation (Figure 18A). During PKG activation, the membrane depolarizes at a faster rate (~20 minutes) while PKG inhibition extends this timeframe until ~60 minutes. The input resistance of the larval muscle approached zero for all treatments, however, it did so at different rates of decline (Figure 18B). PKG activation displayed the steepest decay rate beginning at ~20 minutes, while this decline in resistance is delayed until ~30 and ~50 minutes during H_2O_2 application and PKG inhibition, respectively.

Discussion

The main findings from the present study show that PKG pathway manipulation, whether genetically or pharmacologically induced, modifies synaptic tolerance during H_2O_2 exposure and results in protection against or vulnerability to neuromuscular communication breakdown. Synaptic transmission was prolonged upon inhibition of this signaling cascade, while failure occurred more rapidly following activation. Furthermore, shunting K^+ channel kinetics during acute oxidative stress extends neurotransmission and

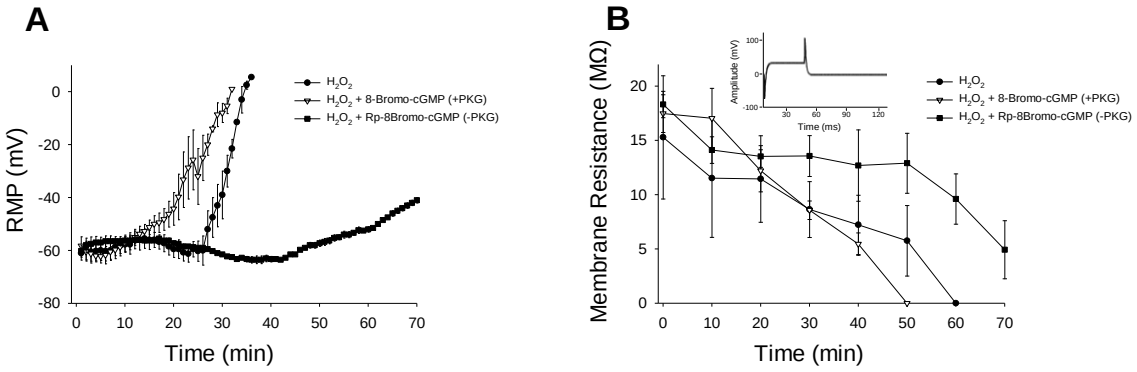

Figure 18. PKG pathway modulation alters Drosophila muscle membrane integrity. A: postsynaptic RMP from *yw Drosophila* larval muscle 6 was recorded during H_2O_2 exposure in the following conditions (N = 4 for each group): H_2O_2 only (2.25 mM), 8-Bromo-cGMP (+PKG, 40 μM), and Rp-8Bromo-cGMP (-PKG, 50μM). B: input resistance from muscle 6 was measured during H_2O_2 exposure in the following conditions: H_2O_2 only (2.25 mM, N = 4), 8-Bromo-cGMP (+PKG, 40 μM, N = 5), and Rp-8Bromo-cGMP (-PKG, 50 μM, N = 5). The membrane resistance decays at a rate similar to the RMP depolarization and follows the same pattern where PKG activation exacerbates and inhibition reduces the loss of resistance.

this protection continues in the presence of a PKG agonist. These data suggest that the PKG pathway may contribute to an endogenous neuroprotective scheme that regulates neurological function in the presence of acute trauma. In favor of this hypothesis, PKG activity has been implicated in regulating the function of neural circuits, behavior, and susceptibility to stress in insect and vertebrate model systems, highlighting its potential as a novel target to rapidly relieve neural failure associated with traumatic brain insults (Armstrong et al., 2010; Dawson-Scully et al., 2010; Robertson and Sillar, 2009).

Nitric oxide (NO) is a short-lived signaling molecule that readily diffuses across membranes and has been implicated as a neuromodulator in the central nervous system (Boehning and Snyder, 2003; Garthwaite, 2008; Snyder and Bredt, 1991). NO is generated by NO synthases and NO-sensitive guanylyl cyclases (NO-GCs) act as receptor molecules (Mergia et al., 2009). NO-GCs up-regulate the amount of cyclic guanosine monophosphate (cGMP), which activate targets such as PKG (Hofmann et al., 2006). Properties of NO synthase have been characterized in numerous model organisms including *Drosophila* (Muller, 1994). NO/cGMP signaling has been identified during post-embryonic insect development at the fruit fly NMJ with NO most likely acting as the presynaptic retrograde messenger (Wildemann and Bicker, 1999a; b), mirroring its organization in mammalian systems (Hopkins et al., 1996; Southam and Garthwaite, 1993). Therefore, the larval NMJ provides a good model to study how synaptic transmission is modulated by PKG activity during acute oxidative stress.

Organisms respond differently to cellular stress and the basis for differences among species is still not fully understood. There are many different types of environmental stressors that cause cellular damage; however, the physiological effects on organisms converge on one common theme, the elevated generation of reactive oxygen species (ROS). Numerous stress events are associated with the increased intracellular production of ROS, such as hyperthermia (Flanagan et al., 1998), hypoxia (Waypa et al., 2001), oxygen deprivation (Liu et al., 2005), and reperfusion following an ischemic event (Ferriero, 2001).

Experimental use of H_2O_2 as a model for acute oxidative stress has been employed to imitate the pathology of numerous neurological disorders including cerebral ischemia (Methy et al., 2008; Wei et al., 2011), Amyotrophic lateral sclerosis (Nani et al., 2010), Parkinson's disease (Jenner, 2003), Friedreich's ataxia (Anderson et al., 2008), and Alzheimer's disease (Behl et al., 1994; Miyata and Smith, 1996). In addition to being a classic oxidative stress paradigm, H_2O_2 has been shown to modulate neuromuscular activity in vertebrates (Giniatullin and Giniatullin, 2003) and manipulate neuronal signal transduction in mammalian cell culture (Chai and Lin, 2010). This study exposed *Drosophila* larval NMJ preparations to H_2O_2 (2.25 mM) and demonstrates that PKG manipulation alters synaptic transmission tolerance to acute oxidative toxicity. This H_2O_2 model provides a unique avenue for finding novel PKG signaling targets that may promote cell function and survival during acute stress insults.

We took advantage of the polymorphic expression of *for* to investigate if the PKG pathway would demonstrate similar command over acute oxidative stress tolerance as it has been shown to modulate neurological function and promote behavioral tolerance to other types of physiological stressors. Our data reveal that this signaling mechanism manipulates synaptic function during H_2O_2-mediated cellular stress. Animals with inherently lower PKG activity (for^s) maintain synaptic performance longer than those with higher PKG activity (for^R) during acute stress exposure. The mutant for^{s2} larvae, which express the for^R background, respond similarly to neural stimulation as for^R larvae, but exhibit failure during acute oxidative stress significantly later than for^R and for^s larvae (Figure 13). This suggests that the time to synaptic breakdown during acute H_2O_2 treatment can be manipulated by genetic alteration of PKG pathway function. Further investigations are required to determine the direct mechanism by which PKG signaling promotes protection of neurological function during extreme cellular insults, with a specific emphasis on determining novel molecular interactions, cellular localization(s), and cell expression patterns (Sokolowski and Riedl, 1999).

The PKG pathway can not only be genetically manipulated, but also chemically modified via numerous commercially available agents that alter its functional ability by targeting upstream and downstream components. Some examples of such drugs, besides the specific protein kinase effectors used in this study, include T-0156, a potent phosphodiesterase-5 inhibitor (Dawson-Scully et al., 2010; Kikkawa et al., 2001; Mochida et al., 2002), LY83583, a cGMP inhibitor (Schmidt et al., 1985), and Cantharidin, a protein phosphatase 2A inhibitor (Dawson-Scully et al., 2010; Li and

Casida, 1992). The PKG agonist (8-Bromo-cGMP) and antagonists (KT-5823 and Rp-8Bromo-cGMP) used in this study did not solely alter characteristics of the EJP waveform (Figure 2). Our pharmacology data further indicate that modifying PKG activity during acute H_2O_2 exposure regulates synaptic transmission in the same manner exhibited by genetic manipulation of this protein kinase. PKG inhibition increased the time until loss of neuromuscular communication was observed, while activation decreased synaptic tolerance to the acute stress by reducing this timeframe (Figure 15).

The modest concentrations of PKG modulators used in this study reinforce the involvement of PKG, as opposed to other protein kinases such as PKA or PKC. Studies using cyclic nucleotide analogs, such as 8-Bromo-cGMP, have demonstrated its preference for cGMP-dependent protein kinases compared to cAMP-dependent protein kinases (Francis et al., 1988). Since KT-5823 is known to be not only a potent PKG antagonist (*in vitro* IC_{50} = 234 nM), but also a relatively weak inhibitor of both PKC (K_i = 4 µM) and PKA (K_i > 10 µM) (Hidaka and Kobayashi, 1992), we included the more selective inhibitor, Rp-8Bromo-cGMP, in the study. This metabolically stable analog has been shown to inhibit both PKGI (K_i = 30 nM) and PKGII and block mammalian cGMP-gated ion channels (IC_{50} = 25 µM) in excised *Xenopus* rod photoreceptors (Wei et al., 1996). The observed effects on synaptic transmission during acute oxidative exposure most likely reflect the actions of PKG.

The distinct mechanisms underlying the PKG pathway's potential for modulating neuronal function and ultimately neuroprotection during acute trauma remain only

partially understood. Previous published data indicate a link between K^+ conductance and PKG activity (Chai and Lin, 2010; Dawson-Scully et al., 2010; Renger et al., 1999; White et al., 1993), which leads to speculation that these ion channels are potential downstream targets of this intracellular signaling mechanism. However, which specific K^+ channel(s) mediate PKG pathway effects is still a matter of debate. Our drug combination data indicate the likely downstream involvement of K^+ channels during PKG manipulation and acute oxidative stress (Figure 17). This is in agreement with the proposed model of upstream and downstream components involved in the PKG signaling cascade (Zhou et al., 1996).

Furthermore, our data agree with earlier findings that simultaneous pharmacological intervention with different compounds that effect diverse targets within the PKG pathway results with further downstream component dominating the overall effect (Dawson-Scully et al., 2010). We found that exposure to a K^+ channel blocker (TEA) and PKG agonist (8-Bromo-cGMP) during acute oxidative stress protects synaptic function by increasing the time to failure (Figure 17B). These results mimic what Dawson-Scully et al. reported in 2010, except that we employed K^+ channel inhibition rather than activation. These results are not, however, surprising since Renger et al. (1999) conclusively demonstrated that K^+ conductance in the +PKG and –PKG alleles of the *for* gene in *Drosophila* embryos resulted in differing whole cell K^+ conductance, which was reinforced through the use of pharmacological PKG modulators in this system (Renger et al., 1999). Interestingly, the simultaneous application of PKG activator combined with TEA resulted in a lower but not significant synaptic failure time than TEA

alone (Figure 17A). It is possible that TEA and the PKG activator may operate through competing pathways, but we believe this is unlikely due to a number of factors: 1) due to TEA's disruptive nature in the preparation, very low dosages are used (250 μM) likely resulting in a reduced effect, 2) previous work using activators demonstrates the same pattern of the dominant effect of K^+ modulation combined with PKG modulation during anoxic stress (Dawson-Scully et al., 2010), and finally it was conclusively demonstrated that K^+ channel conductance is modulated not only by PKG expression and activity levels in *Drosophila* embryos but also by pharmacological PKG modulation (Renger et al., 1999). However, since TEA results in global K^+ inhibition, future work using more specific K^+ channel modulators and *Drosophila* K^+ channel mutants will be required to identify the specific type and cellular localization of K^+ channel(s) responsible for modulating PKG's effect on neurological function during acute oxidative injury.

Interestingly, we also demonstrate that input resistance declines simultaneously with the depolarization of the postsynaptic cell RMP in a PKG-dependent manner (Figure 18). PKG activation shows faster onset of depolarization of the postsynaptic RMP than that of PKG inhibition, where no drug is intermediate (Figure 18A). Further, PKG activation causes the loss of postsynaptic input resistance sooner than that of PKG inhibition, where again no drug is intermediate (Figure 18B). These data demonstrate that modulation of the PKG pathway can alter membrane integrity, and this may be through the inhibition of ionic conductances such as K^+. The finding that resting membrane potential depolarizes along with the reduction of input resistance during this oxidative stress suggests that, at least postsynaptically, there is an overwhelming influx of ions

such as Ca^{2+} compared to the potential efflux of K^{+}, where one would expect a significant hyperpolarization of the RMP over time. H_2O_2 has been shown to increase intracellular Ca^{2+} concentration in cell culture (Herson et al., 1999) and these data are similar to H_2O_2 effects in medium spiny neurons, where it also causes membrane depolarization and decreased input resistance (Bao et al., 2005). These effects are likely caused by the influx of positive ions such as Ca^{2+} and indicate that future work could examine low doses of Ca^{2+}, Na^{+}, or Cl^{-} channel blockers to determine their protective effects during acute oxidative stress.

These data provide a compelling comparison between genetically and chemically manipulated animals and suggest that PKG activity controls synaptic tolerance to acute oxidative stress in a manner similar to previous findings investigating thermotolerance and anoxic tolerance (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). In addition to protection from abiotic stress, PKG pathway inhibition has been shown to rescue the occurrence and severity of spreading depression (SD) events in the locust metathoracic ganglion (Armstrong et al., 2009). SD is associated with numerous mammalian central nervous system disorders including migraine, stroke, and acute brain injury. Therefore, the PKG pathway is a relevant neuroprotective mechanism that provides a potential avenue for creating novel strategies to combat a number of physiological stressors and neurological diseases with the added advantages of evolutionary conservation, diverse model system applications, and the availability of both genetics and rapid pharmacological treatment interventions.

CHAPTER 4. PROTECTION OF AXONAL FUNCTION DURING ACUTE OXIDATIVE STRESS

Introduction

Oxygen is essential for the survival of most organisms. A natural by-product of cellular respiration is the generation of toxic partially reduced forms of oxygen, known as reactive oxygen species (ROS; Inoue et al., 2003). ROS is a general term used to describe small molecules containing oxygen that are free radicals or oxidizing agents that are easily converted into radicals. ROS display a variety of biological properties and are known for their dual nature as being both beneficial and deleterious species (Kohen and Nyska, 2002). ROS serve important roles in cellular signaling pathways, modulate synaptic transmission and plasticity (Auerbach and Segal, 1997) and contribute to cardioprotection resulting from ischemic preconditioning (Vanden Hoek et al., 1998). However, when the concentration of ROS is too high within cells, these molecules can damage cells by disrupting intracellular components such as DNA (Fraga et al., 1990), lipids (Marnett et al., 1985), and proteins (Stadtman et al., 1992), affect cellular membrane permeability, and contribute to the physiology of age-associated disorders (Wicks et al., 2009). Within biological systems there is a homeostatic balance between the production of ROS and cellular antioxidant defense mechanisms and DNA repair mechanisms that fix the damage. When this dynamic becomes unbalanced and there is an

overabundance of ROS, it leads to a cellular condition termed oxidative stress.

In vitro models that experimentally mimic pathological conditions such as ischemia/reperfusion, cerebral stroke, and numerous neurodegenerative disorders provide a convenient, quick, and economical method to screen potentially protective compounds and study their mechanism of action (Sbrenna et al., 1998). The compounds sodium azide (NaN_3) and hydrogen peroxide (H_2O_2) chemically induce neuronal damage via ischemic and oxidative stress (Varming et al., 1996; Miguel et al., 2009). NaN_3 induces ischemic stress by inhibiting cytochrome c oxidase (Bennett et al., 1996), ATP production (Noumi et al., 1987), superoxide dismutase (Misra and Fridovich, 1978), DNA synthesis and cell division (Ciesla et al., 1974). H_2O_2, a strong oxidizing agent that is easily converted into the hydroxyl radical, induces oxidative stress that reduces aconitase and antioxidant enzyme functions (Miguel et al., 2009). In addition to being classic oxidative stress paradigms, NaN_3 and H_2O_2 affect synaptic function, where NaN_3 induces synaptic fatigue in crayfish motor neurons (Nguyen and Atwood, 1994) and H_2O_2 induces synaptic depression by reducing neuromuscular activity in vertebrates (Giniatullin and Giniatullin, 2003) and manipulates neuronal signal transduction in mammalian cell culture (Chai and Lin, 2010).

Organisms have evolved numerous ways to deal with cellular stress insults (Zhou, 2008). The heat shock (HS) response is a universal mechanism used by prokaryotes and eukaryotes to deal with environmental stress such as hyperthermia, anoxia and oxidative stress (Lindquist, 1986). This well characterized protection mechanism is regulated via

heat shock factor (HSF) transcription of heat shock proteins (HSPs) in response to cellular stress events (Wu, 1995). HSPs act as molecular chaperones that facilitate proper protein folding and suppress aggregate formation (Fujimoto and Nakai, 2010). The HS response confers protection against numerous cellular stressors and pathologies including ischemia-reperfusion injury, cardiomyopathy, atrial fibrillation, H_2O_2-induced oxidative stress, hyperthermia, and anoxia (Zhang et al., 2011; Venkatakrishnan et al., 2006; Brundel et al., 2006; Zou et al., 2003; Arrigo, 1998).

In addition to organism tolerance to cellular stress, a HS treatment produces long-lasting effects on neural function that alters neuronal properties. In locusts, a heat shock pretreatment allows for an expanded thermal range of neuronal circuitry operations such as wing beat and ventilation motor patterns (Newman et al., 2003; Robertson et al., 1996). Additionally, HS exposure protects neurological function by producing changes in outward K^+ currents and is believed to be an adaptive mechanism that protects the locust from loss of cellular K^+ homeostasis during hyperthermia (Ramirez et al., 1999). This HS-mediated change in K^+ conductance is likely important for cellular signaling mechanisms during cellular stress insults and may extend to other organisms. In addition, this effect on neuronal integrity and maintenance of ionic homeostasis via modified K^+ conductance could be a direct effect of HSPs or an indirect effect caused by interaction with intracellular signaling mechanisms.

Maintaining homeostasis is very important for cellular function, especially during environmental stresses such as oxidative stress, anoxia, and hyperthermia. The fruit fly,

Drosophila melanogaster, uses the cGMP-dependent protein kinase G (PKG) pathway as an endogenous protection mechanism to cope with environmental stress (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). Manipulating this intracellular signaling cascade leads to protection of neurological function during acute hyperthermia and anoxia in fruit flies as well as the locust (Dawson-Scully et al., 2007), tadpole (Robertson and Sillar, 2009), and mouse (Armstrong et al., 2010), indicating that this neuroprotective pathway likely represents a conserved cellular mechanism that modulates the response to a number of acute physiological stressors. In addition to modulating behavioral and neurological functions, PKG activity produces variation in physiological properties, such as neuronal morphology and connectivity, and *Drosophila* voltage-activated K^+ currents can be manipulated with pharmacological PKG modulators, suggesting a potential regulatory role for PKG activity in neuronal excitability and synaptic transmission via voltage-dependent K^+ channel conductance (Renger et al., 1999).

The present study analyzed the protective effects of a HS pretreatment, pharmacological and genetic PKG pathway manipulation and direct K^+ channel modulation on axonal function in muscle 6 at the *Drosophila* larval neuromuscular junction (NMJ) during acute oxidative stress induced via two pharmacological paradigms that generate physiologically relevant oxidant species: mitochondrial superoxide production induced by sodium azide (NaN_3) and hydroxyl radical formation via hydrogen peroxide (H_2O_2). Potential interactions between the HS and PKG pathways were investigated to determine if the overall level of functional protection could be additively increased by combining these mechanisms. Furthermore, this study compares the

differences between stimulating a single axon versus both axonal fibers in muscle 6 at the *Drosophila* larval NMJ.

Results

Acute oxidative stress reduces axonal function at the *Drosophila* larval NMJ

Previous work demonstrated that acute oxidative stress rapidly reduces synaptic function (Chapter 3), but these experiments used a stimulation protocol well above threshold and did not investigate effects on axonal function. To address this question, *yw Drosophila* larvae were exposed to two paradigms of chemically-induced oxidative stress: the mitochondrial inhibitor NaN_3 (0.1 mM) or oxidative overload via H_2O_2 (2.25 mM). Axon fibers were targeted using a stimulation protocol with repetitive voltage delivered in 0.3 ms pulses at 5 mV with a 1 Hz frequency for each larval preparation (complete protocol is described in Chapter 2). When the EJP decline was plotted as a function of time, acute oxidative stress rapidly reduced axonal function (Figure 19A). Control preparations not exposed to acute oxidative stress functioned for a maximum of 57 minutes before failure, while acute stress exposure reduced this time frame to 31 and 27 minutes during NaN_3 and H_2O_2 treatment, respectively. Additionally, there were significant differences between average axonal failure times during acute oxidative stress treatments and controls (One-Way ANOVA, $F_{(2,24)} = 80.78$, $p < 0.001$; Figure 19B). Average axonal failure occurred at 52 ± 1 minute for control preparations and this was

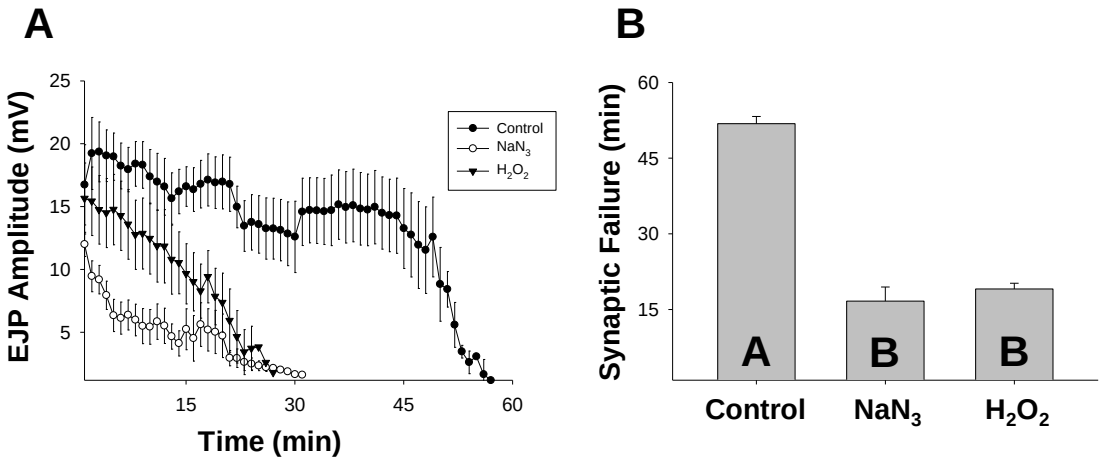

Figure 19. Acute oxidative stress reduces the time until synaptic transmission failure. A: *yw Drosophila* EJP amplitude decline during control (N = 6), NaN_3 (0.1mM, N = 9), and H_2O_2 (2.25 mM, N = 12) conditions. B: average NMJ synaptic failure times significantly differ among groups (One-Way ANOVA, $F_{(2,24)} = 80.78$, $p < 0.001$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

reduced to 17 ± 3 minutes and 19 ± 1 minute during NaN_3 and H_2O_2 exposure, respectively.

Pharmacological PKG modulators do not disrupt EJP characteristics

To investigate endogenous cellular protection mechanisms against acute oxidative stress, the present study analyzed whether PKG pathway manipulation could protect axonal function at the *Drosophila* larval NMJ. Before addressing this question, pharmacological PKG modulators were examined for adverse effects on the NMJ postsynaptic response (i.e. the EJP latency time, shape, amplitude). Pharmacological agents that either activate (8-Bromo-cGMP, +PKG) or inhibit (KT-5823, -PKG; Rp-8Bromo-cGMP, -PKG) PKG activity were added to the HL-3 saline bath of larval preparations. Individual wild-type *yw* larvae were exposed to each PKG manipulator separately and recordings were taken one minute before and ten minutes after the application of each drug (Figure 20). There were no observable differences in the EJP characteristics for each treatment, suggesting that these agents do not significantly affect the evoked response from axons in muscle 6 at the *Drosophila* larval NMJ.

NaN_3-induced axonal failure is not affected by PKG activity

To determine if PKG pathway manipulation can alter axonal failure during acute oxidative stress, *yw* flies with chemically modified PKG activity were exposed to NaN_3 (0.1 mM). This concentration was chosen to create a standard timeframe to compare

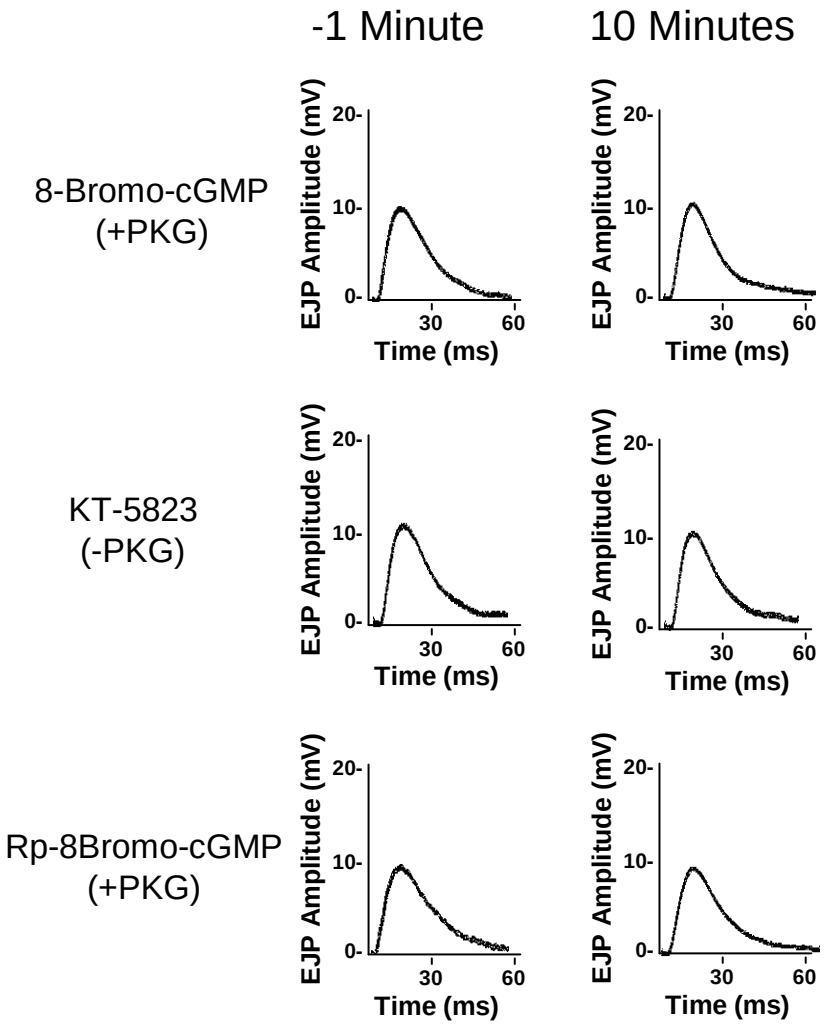

Figure 20. Pharmacological PKG agents do not affect distinctive EJP features. Differential drug treatments that modulate PKG activity were tested for adverse affects on the parameters of the evoked EJP response from *yw Drosophila* larval muscle 6. Animals were bathed in 2 mL of HL-3 saline containing one of the following agents dissolved in DMSO: 8-Bromo-cGMP (+PKG, 40 μM), KT-5823 (-PKG, 1 μM), or Rp-8-Bromo-cGMP (-PKG, 50 μM). EJP recordings were taken 1 minute before addition of the drug/saline solution and 10 minutes after the application. No differences were observed for each drug tested.

changes in NMJ axonal failure times during pharmacological manipulation of PKG activity. Modification of the PKG pathway was achieved via pharmacological agents that directly activate (8-Bromo-cGMP, +PKG, 40 μM) or inhibit (KT-5823, -PKG, 1 μM) PKG cellular function. Animals exposed to NaN_3 displayed axonal failure at an average of 17 ± 3 minutes compared to control preparations which occurred at an average of 52 ± 1 minute (Figure 21). There were no significant differences between the average synaptic failure times of the PKG treatment groups (Student's t-test, $p < 0.05$). Average axonal failure times were 34 ± 7 minutes and 30 ± 10 minutes during PKG activation and inhibition, respectively.

Pharmacological PKG manipulation alters H_2O_2-induced axonal failure

The data indicated that pharmacological PKG manipulation did not alter axonal function during NaN_3-induced acute oxidative stress, but it did not demonstrate if wild-type *yw Drosophila* with chemically modified PKG activity would exhibit differences in axonal function during other types of induced stress. To address this question, *yw* larval NMJ preparations were exposed to H_2O_2 (2.25 mM) and agents that directly activate (8-Bromo-cGMP, 40 μM) or inhibit (KT-5823, 1 μM; Rp-8Bromo-cGMP, 50 μM) PKG activity. When the EJP amplitude decay was plotted as a function of time, the differential drug treatments separate into clear patterns of varying axonal functional tolerance during

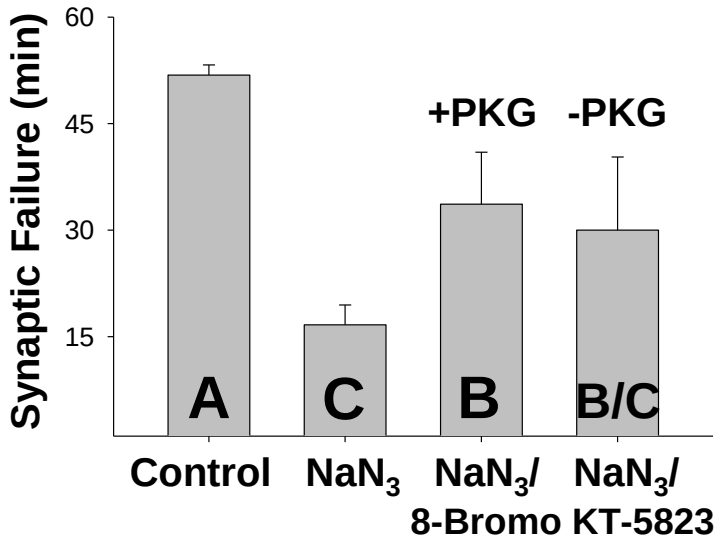

Figure 21. NMJ synaptic failure caused by NaN_3 is not attenuated via PKG inhibition. Average synaptic failure times of *yw Drosophila* larvae during the following conditions: control (N = 6), NaN_3 (0.1 mM, N = 9), NaN_3/8-Bromo-cGMP (0.1 mM/40 μM, N = 3), and NaN_3/KT-5823 (0.1 mM/1 μM, N = 5). NMJ failure time is significantly extended by exposure to either PKG activation (8-Bromo-cGMP, +PKG) or inhibition (KT-5823, -PKG) during NaN_3 exposure (Student's t-test, $p < 0.05$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

the acute stress (Figure 22A). Control preparations not exposed to acute oxidative stress nor any drug functioned for a maximal time of 57 minutes before failure occurred. During H_2O_2 exposure function continued for a maximum of 19 minutes and PKG activation reduced this timeframe to 16 minutes, while inhibition extended this timeframe to 45 minutes (KT-5823) or 55 minutes (Rp-8Bromo-cGMP) depending on the drug administered. These results suggest that during H_2O_2-induced stress PKG inhibition extends axonal function, while activation reduces this time frame.

Additionally, there were significant differences between the average axonal failure times of all treatment groups (Control, H_2O_2, H_2O_2/+PKG, and H_2O_2/-PKG) depending on the drug administered (One-Way ANOVA, $F_{(4,36)} = 222.65$, $p < 0.001$; Figure 22B). Animals exposed to H_2O_2 displayed failure at an average of 19 ± 1 minute compared to control preparations which occurred at an average of 52 ± 1 minute. Elevated functional tolerance during H_2O_2-induced stress was observed in larvae treated with PKG inhibitors (-PKG). The addition of KT-5823 or Rp-8Bromo-cGMP increased average axonal failure times to 37 ± 2 minutes and 50 ± 1 minute, respectively. In contrast, animals treated with the PKG activator 8-Bromo-cGMP (+PKG) showed a reduction in the time until axonal failure occurring at an average of 14 ± 1 minutes. These results indicate that pharmacological manipulation of the PKG pathway can either protect or sensitize axonal function during acute oxidative stress.

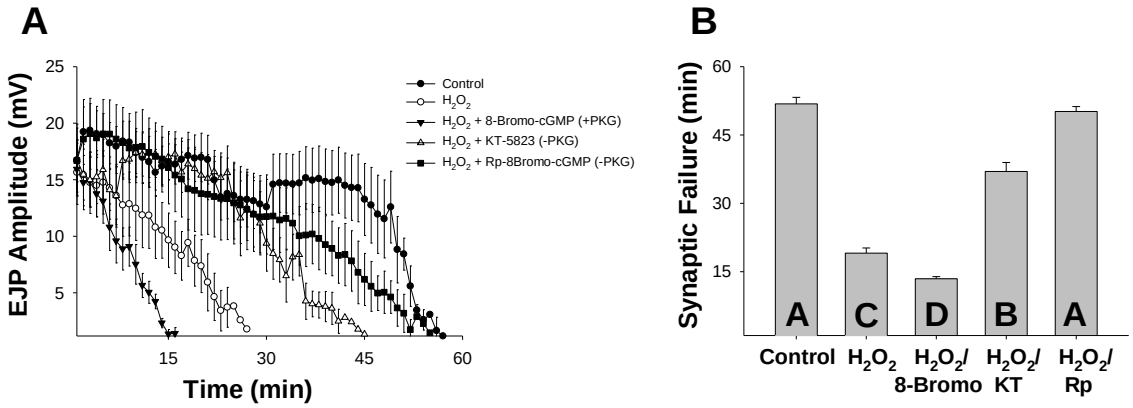

Figure 22. PKG pathway manipulation alters NMJ synaptic transmission failure during H_2O_2 exposure. A) *yw Drosophila* EJP amplitude decline as a function of time. B) average synaptic failure times significantly differ across groups (One-Way ANOVA, $F_{(4,36)} = 222.65$, $p < 0.001$). PKG inhibition with KT-5823 (1 μM, N=6) or Rp-8Bromo-cGMP (50 μM, N = 6) extended the time until synaptic failure, while activation with 8-Bromo-cGMP (40 μM, N = 11) reduced this period. Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

Genetic PKG manipulation alters H_2O_2-induced axonal failure

To investigate if the PKG pathway is able to modulate axonal functional tolerance to acute oxidative stress, H_2O_2 (2.25 mM) was exogenously applied to *Drosophila* larval NMJ preparations. Larvae of three different fly strains, for^R, for^s, and for^{s2}, with genetically different PKG activity levels were used to distinguish if any differences existed and could be associated with differences in PKG activity. During acute stress, the PKG allelic variants demonstrated differential axonal functional tolerances indicated by varying rates in the decline of the EJP amplitudes over time (Figure 23A). Animals with high PKG activity (for^R) had the quickest EJP amplitude decline and those with low PKG activity (for^s, for^{s2}) declined slower, suggesting that the difference in the rate of amplitude decay may be attributed to differences in PKG activity. Evoked EJP amplitudes declined over time in the following order: $for^R > for^s > for^{s2}$. Mirroring EJP decay rates, variation in PKG expression also modulated the time until NMJ synaptic transmission failure (Figure 23B). On average, *Drosophila* for^R larvae displayed axonal failure significantly earlier at 21 ± 1 minute compared to for^s and for^{s2} larvae at 39 ± 2 minutes and 46 ± 2 minutes, respectively (Student-Newman-Keuls, $p < 0.05$).

A prior HS protects axonal function during acute oxidative stress

To explore other endogenous cellular protection mechanisms, we investigated whether a prior HS before acute oxidative stress induced by H_2O_2 (2.25 mM) could protect axonal function at the *Drosophila* larval NMJ. Larvae were exposed to a one hour

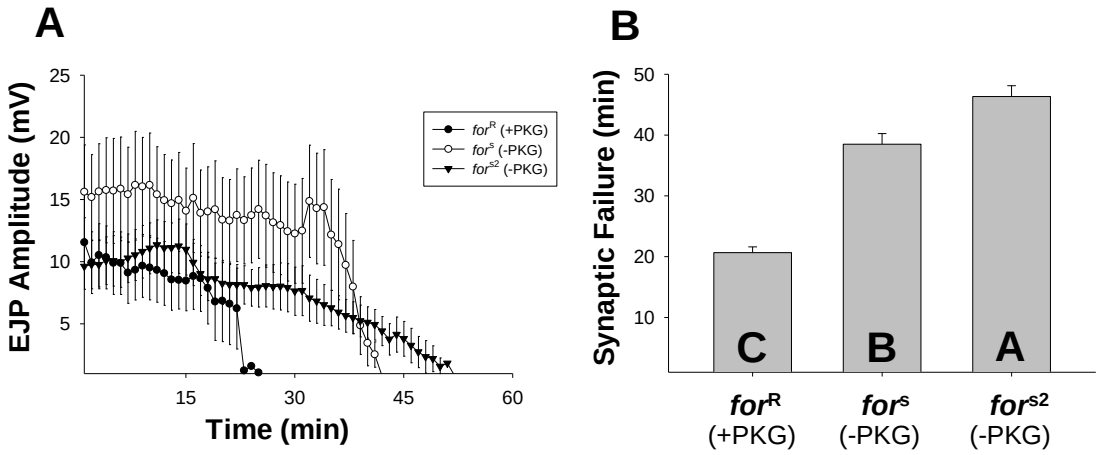

Figure 23. Genetic variation in PKG activity modulates synaptic function. A: rates of evoked EJP amplitude decline as a function of time between *foraging* allelic variants with high (+PKG) and low (-PKG) PKG activity during acute oxidative stress (H_2O_2, 2.25 mM). B: NMJ synaptic failure times significantly differ among PKG variants during H_2O_2 treatment (Student-Newman-Keuls, $p < 0.05$; N = 6 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

HS at 37°C followed by a thirty minute recovery period before dissection and exposure to acute oxidative stress. When the evoked EJP amplitude decay was plotted as a function of time, the differential treatments separate into clear patterns of varying axonal functional tolerance during the acute stress (Figure 24A). Control preparations functioned for a maximal time of 57 minutes before failure occurred and acute oxidative stress reduced this timeframe to 27 minutes. A prior HS extended this time frame to a maximum of 48 minutes. Additionally, HS pretreatment before H_2O_2 exposure significantly extended the average axonal failure time from 19 ± 1 minute to 42 ± 4 minutes, which is comparable to the control group at 52 ± 1 minute (Dunn's Method, $p < 0.05$; Figure 24B). These data demonstrate that a prior HS protects axonal function during acute oxidative stress.

Combining HS and PKG modulation does not increase functional protection

Taken together, the data indicated that PKG inhibition and HS pretreatment protect axonal function at the *Drosophila* larval NMJ during acute oxidative stress. Since these are both endogenous protection mechanisms against stress, the next experiments analyzed the potential for interaction between these mechanisms and if synaptic function could be further extended during acute oxidative stress by combining these protective treatments. *Yw Drosophila* larvae were exposed to the same HS protocol (one hour HS at 37°C followed by a thirty minute recovery period) and then dissections were exposed to H_2O_2 (2.25 mM). Average axonal failure times significantly differed between groups exposed and not exposed to a prior HS (Two-Way ANOVA, $F_{(2,42)} = 164.427$, $p < 0.001$; Figure 25). HS pretreatment extended synaptic function from an average of 19 ± 1 minute

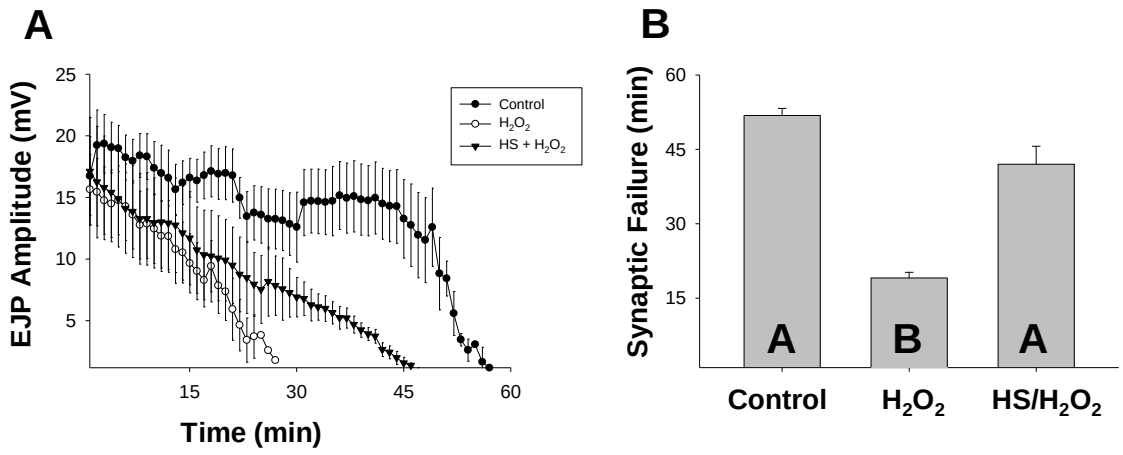

Figure 24. A prior heat shock (HS) protects synaptic transmission during acute oxidative stress. A: *yw Drosophila* EJP amplitude decline over time during control, H_2O_2 (2.25 mM), and HS/H_2O_2 (2.25 mM). B: average time to NMJ synaptic transmission failure is significantly extended by a one hour HS followed by a thirty minute recovery period before H_2O_2 exposure (Dunn's Method, $p < 0.05$; N = 6 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

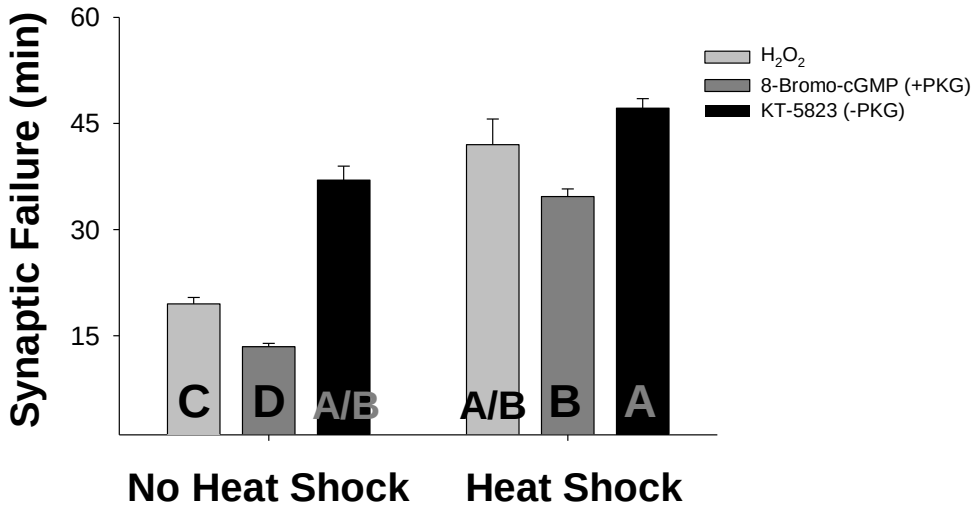

Figure 25. Protection from a prior HS and PKG inhibition does not increase. Average NMJ synaptic failure times of *yw Drosophila* larvae during H_2O_2 exposure (2.25 mM) significantly differ between groups exposed and not exposed to a one hour HS followed by a 30 minute recovery period (Two-Way ANOVA, $F_{(2,42)} = 164.427$, $p < 0.001$; N = 6 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

to 42 ± 3 minutes during acute oxidative stress and this HS-induced protection persisted during simultaneous PKG activation with the average time until failure increasing from 14 ± 1 minute to 35 ± 1 minute. A prior HS before H_2O_2 exposure provided functional protection at similar levels as pharmacological PKG inhibition; however, the overall level of functional protections did not additively increase during simultaneous PKG inhibition.

Modulating K^+ channel kinetics affects axonal function during acute stress

To explore potential mechanisms responsible for PKG's effect on synaptic function during acute oxidative injury, an experiment that combined pharmacological manipulation of K^+ channel kinetics and the PKG pathway was performed. Individual wild-type *yw* larvae were exposed to acute oxidative stress (H_2O_2, 2.25 mM), a general K^+ channel inhibitor (TEA, 250 µM), and a PKG activator (8-Bromo-cGMP, +PKG, 40 µM), both individually and concurrently. The drug combination revealed that widespread K^+ channel inhibition slowed the average decay rate of EJP amplitudes decline over time not only in the presence of H_2O_2 but also during simultaneous PKG activation (Figure 26A). TEA extended the maximum time until functional failure during the acute stress from 27 to 46 minutes. Additionally, this reduction in the EJP amplitude decline rate continued in the presence of the PKG agonist with the maximum failure time occurring at 41 minutes.

Drosophila NMJ synaptic failure times were significantly altered by modifying K^+ channel kinetics during H_2O_2 exposure (One-Way ANOVA, $F_{(2,17)} = 34.88$, $p < 0.001$;

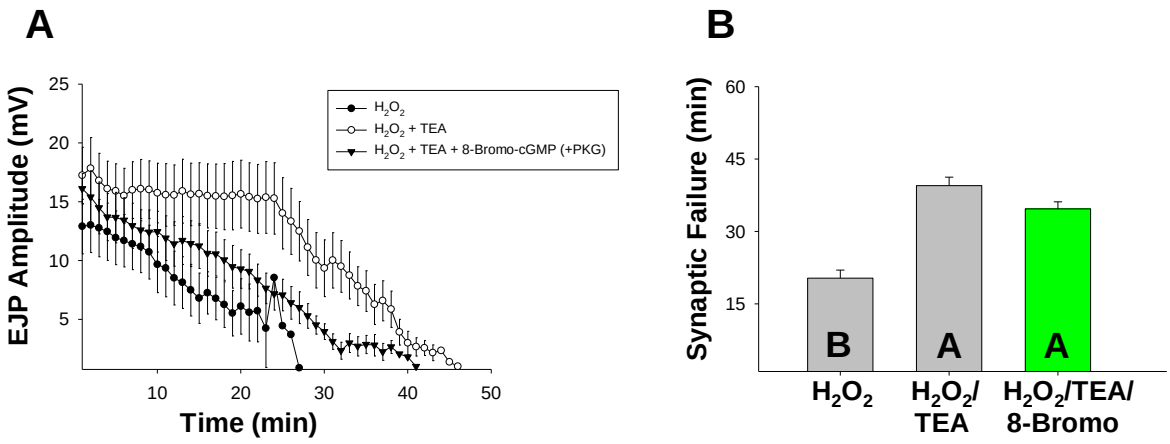

Figure 26. K^+ channel inhibition protects NMJ synaptic transmission. A: EJP amplitude decline of *yw Drosophila* larvae as a function of time during the following conditions: H_2O_2 (2.25 mM, N = 6), H_2O_2/TEA (2.25 mM/250 µM, N = 8), H_2O_2/TEA/8-Bromo-cGMP (2.25 mM/250 µM/40 µM, N = 6). B: NMJ synaptic transmission failure times significantly differ among all treatment groups (One-Way ANOVA, $F_{(2,17)} = 34.88$, $p < 0.001$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

Figure 26B). Animals exposed to TEA and H_2O_2 exhibited an extended average synaptic transmission failure time occurring at 40 ± 2 minutes compared to 20 ± 2 minutes during H_2O_2 treatment. This functional protection persisted during simultaneous PKG pathway activation with the average synaptic failure time occurring at 35 ± 2 minutes. These drug combinations suggest that reducing overall K^+ conductance during acute oxidative stress protects synaptic function and this protection overrides PKG manipulations. These data further implicate K^+ channels as downstream effectors of PKG activity.

The next experiments investigated whether PKG-induced synaptic effects during acute oxidative stress were mediated via mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}), which have been implicated as a target of the PKG pathway in neurons (Chai and Lin, 2010). Pharmacological blockade of these ion channels was achieved using 5-HD (Jaburek et al., 1998) during H_2O_2 exposure (2.25 mM). These channels were expected to extend axonal function if they were indeed downstream mediators of the PKG pathway; however, 5-HD (100 µM) did not considerably extend the time until axonal failure with the average occurring at 34 ± 5 minutes, suggesting that these channels do not mediate PKG-induced synaptic effects during H_2O_2 exposure (Figure 27).

Comparison of EJP amplitude decay using different stimulation protocols

To determine if PKG-induced synaptic transmission tolerance during acute oxidative stress acts in a gene-specific manner, the next experiments analyzed the EJP amplitude declines of PKG genetic variants and animals with chemically altered PKG

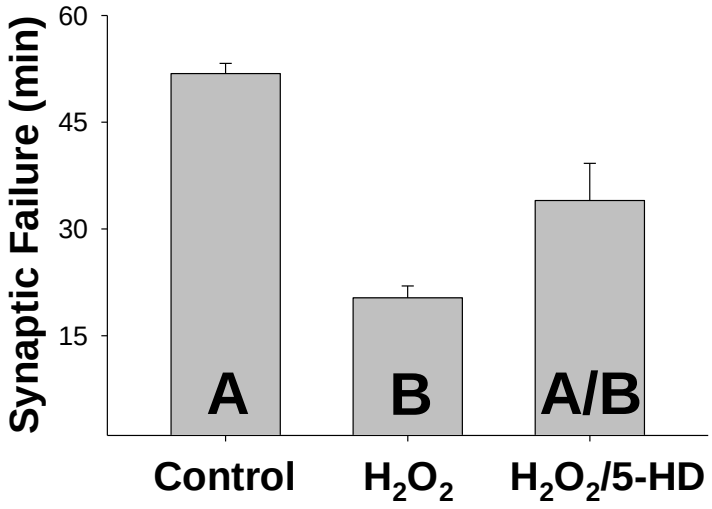

Figure 27. Specific blockade of mitoK_{ATP} channels does not protect synaptic function. NMJ synaptic failure time of *yw Drosophila* is not significantly extended by exposure to the mitochondrial ATP-sensitive K^+ channel inhibitor 5-HD (100 μM) during H_2O_2 (2.25 mM) exposure (Tukey Test, $p < 0.05$; N = 6 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

activity during dual axon and single axon fiber stimulation of muscle 6. Both stimulation protocols were previously described (Chapter 2). Briefly, the dual axon fiber protocol used repetitive stimulation (0.3 ms pulses delivered suprathreshold with a 1 Hz frequency) of both axons in larval muscle 6, qualitatively determined by EJP size (amplitude > 30mV). The single fiber axon protocol used repetitive stimulation (0.3 ms pulses delivered at 5mV with a 1 Hz frequency) of one axon in larval muscle 6, qualitatively determined by EJP size (amplitude < 30mV).

Normalized EJP amplitude declines of PKG genetic variants (for^{R}, for^{s}, for^{s2}) demonstrate a clear difference between single and dual fiber stimulation protocols, where animals with high PKG activity (for^{R}) and animals with low PKG activity (for^{s}) show an abrupt decline in EJP amplitudes at ~20 and ~40 minutes, respectively, during single axon fiber stimulation of muscle 6 (Figure 28A). The EJP amplitude decline in mutant for^{s2} larvae, which has a for^{R} genetic background with a mutation in *for* that produces lower PKG levels comparable to for^{s} larvae, did not rapidly fall to zero until ~50 minutes. These time points likely indicate when the axon lost its ability to function, thus the amplitude plummeted to zero. Normalized EJP amplitudes decayed more gradually during dual axon fiber stimulation at the *Drosophila* larval NMJ, where decline rates did not change until later time points and were not as abrupt as those during single axon fiber stimulation (Figure 28B). The EJP amplitudes declined to zero in the following order: for^{R} at 55 minutes < for^{s} at 65 minutes < for^{s2} at 72 minutes. It's possible that when one axon loses its ability to function during acute oxidative stress the other axon could still be

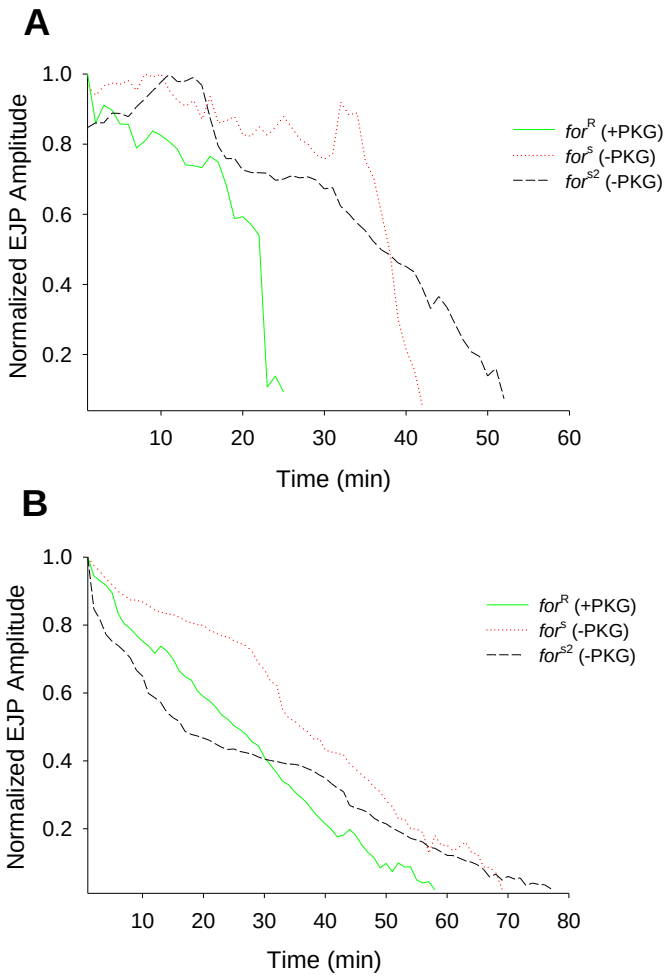

Figure 28. EJP amplitude decline of PKG genetic variants. A: *Drosophila* larvae with varied PKG expression levels [for^{R} (+PKG); for^{s} (-PKG); for^{s2} (-PKG); N = 6 for all groups] show different rates of evoked EJP amplitude decline as a function of time during H_2O_2 exposure (2.25 mM) and single axon fiber stimulation of muscle 6 (0.3 ms, 5 mV pulses delivered at 1 Hz). B: evoked EJP amplitude decline of PKG genetic variants [for^{R} (+PKG, N = 8); for^{s} (-PKG, N = 6); for^{s2} (-PKG, N = 6)] during H_2O_2 exposure and dual axon fiber stimulation of muscle 6 (0.3 ms pulses delivered suprathreshold at 1 Hz).

functional, which would make the summed EJP amplitude decline more gradual compared to that of a single axon.

A similar trend is observed for the EJP amplitude decline in larvae with pharmacologically altered PKG activity, where single axon fiber stimulation shows steeper declines than dual axon fiber stimulation. During single axon fiber stimulation of muscle 6 at the *Drosophila* larval NMJ, EJP amplitudes declined exponentially to zero in the following order: 8-Bromo-cGMP (+PKG) at 10 minutes < H_2O_2 at 20 minutes < KT-5823 (-PKG) at 40 minutes < Rp-8Bromo-cGMP (-PKG) at 50 minutes < control at 55 minutes (Figure 29A). Similar to PKG genetic variants, animals with increased PKG activity demonstrated faster EJP amplitude decay rates than animals with lowered PKG activity. During dual axon fiber stimulation of muscle 6, normalized EJP amplitudes declined more gradually to zero at later times (Figure 29B). EJP amplitudes declined in the following order: 8-Bromo-cGMP (+PKG) at 38 minutes < H_2O_2 at 48 minutes < KT-5823 (-PKG) at 60 minutes < Rp-8Bromo-cGMP (-PKG) at 80 minutes < control at 140 minutes.

Comparison of EJP latency duration using different stimulation protocols

To determine if PKG-induced synaptic transmission tolerance during acute oxidative stress acts in a gene-specific manner, the next experiments analyzed the latency times of PKG genetic variants and animals with chemically altered PKG activity during single axon and dual axon fiber stimulation of muscle 6. The latency times of the evoked

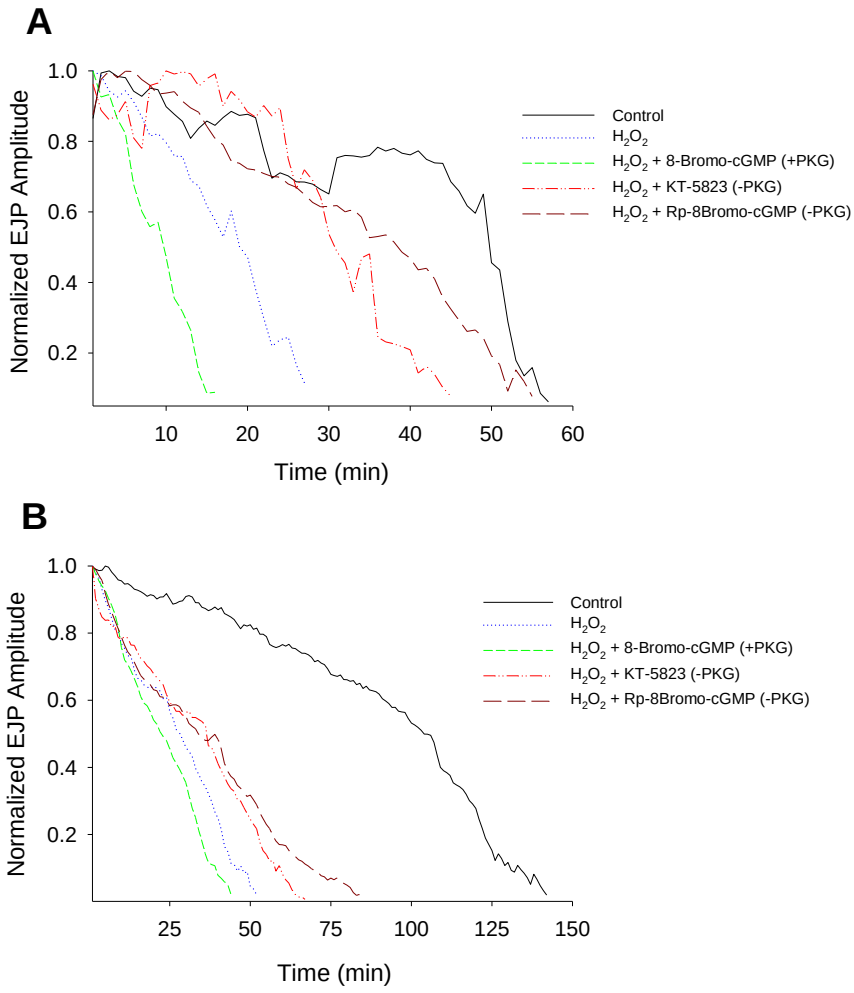

Figure 29. EJP amplitude decline of larvae with chemically altered PKG activity. A: yw *Drosophila* larvae [control (N = 6), H_2O_2 (2.25 mM, N = 12), H_2O_2/8-Bromo-cGMP (2.25 mM/40 μM, N = 11), H_2O_2/KT-5823 (2.25 mM/1 μM, N = 6), H_2O_2/Rp-8Bromo-cGMP (2.25 mM/50 μM, N = 6)] rates of EJP amplitude decline as a function of time during single axon fiber stimulation of muscle 6 (0.3 ms, 5 mV pulses delivered at 1 Hz). B: EJP amplitude decline [N = 6 for all groups, except KT-5823 (N = 3)] during dual axon fiber stimulation of muscle 6 (0.3 ms pulses delivered suprathreshold at 1 Hz).

EJP responses of PKG genetic variants (*for*^{R}, *for*^{s}, *for*^{s2}) increases as the larval NMJ preparations approach synaptic failure during single and dual axon fiber stimulation (Figure 30).

Interestingly, the latency increase was lower for single fiber versus dual fiber axon stimulation but *for*^{R} and *for*^{s2} animals displayed an overall lower latency time increase than *for*^{s} animals during both stimulation protocols. This observation may be attributed to differences in the two genetic backgrounds of the three fly groups. The same trend in latency times of the evoked EJP responses increasing as the preparations approached synaptic failure was also observed in animals with pharmacologically manipulated PKG activity (Figure 31). In these animals, PKG inhibition slowed the latency time increase during acute oxidative stress (H_2O_2), while the latency increase during PKG activation was similar to that of H_2O_2 treated animals and slightly accelerated during dual axon fiber stimulation. Similar to the PKG genetic variants, the increase in latency duration overall was lower for single versus dual fiber axon stimulation. Control animals had similar latency values during both stimulation protocols.

Discussion

The main findings from the present study show that PKG pathway manipulation during H_2O_2 exposure modifies synaptic tolerance and results in protection against or vulnerability to failure during single axon fiber stimulation of muscle 6 at the *Drosophila* larval NMJ. PKG-induced synaptic effects were not observed during NaN_3 exposure.

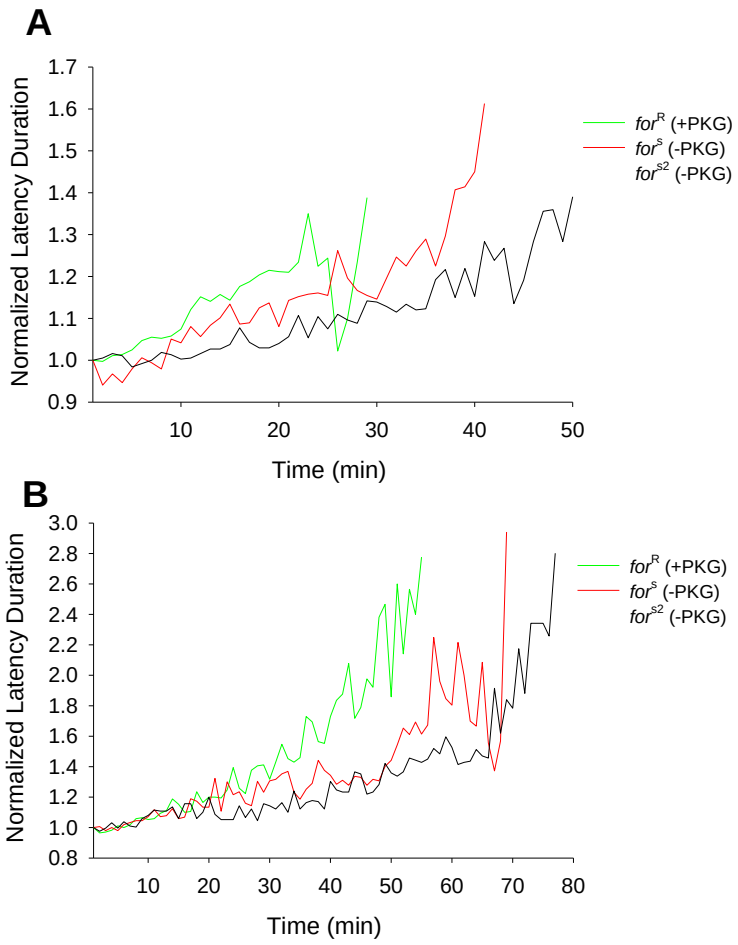

Figure 30. Latency duration of PKG genetic variants. A: *Drosophila* larvae with varied PKG expression levels [for^R (+PKG); for^S (-PKG); for^{S2} (-PKG); N = 6 for all groups] show different rates of latency duration as a function of time during H_2O_2 exposure (2.25 mM) and single axon fiber stimulation of muscle 6 (0.3 ms, 5 mV pulses delivered at 1 Hz). B: latency duration of PKG genetic variants [for^R (+PKG, N = 8); for^S (-PKG, N = 6); for^{S2} (-PKG, N = 6] during H_2O_2 exposure and dual axon fiber stimulation of muscle 6 (0.3 ms pulses delivered suprathreshold at 1 Hz).

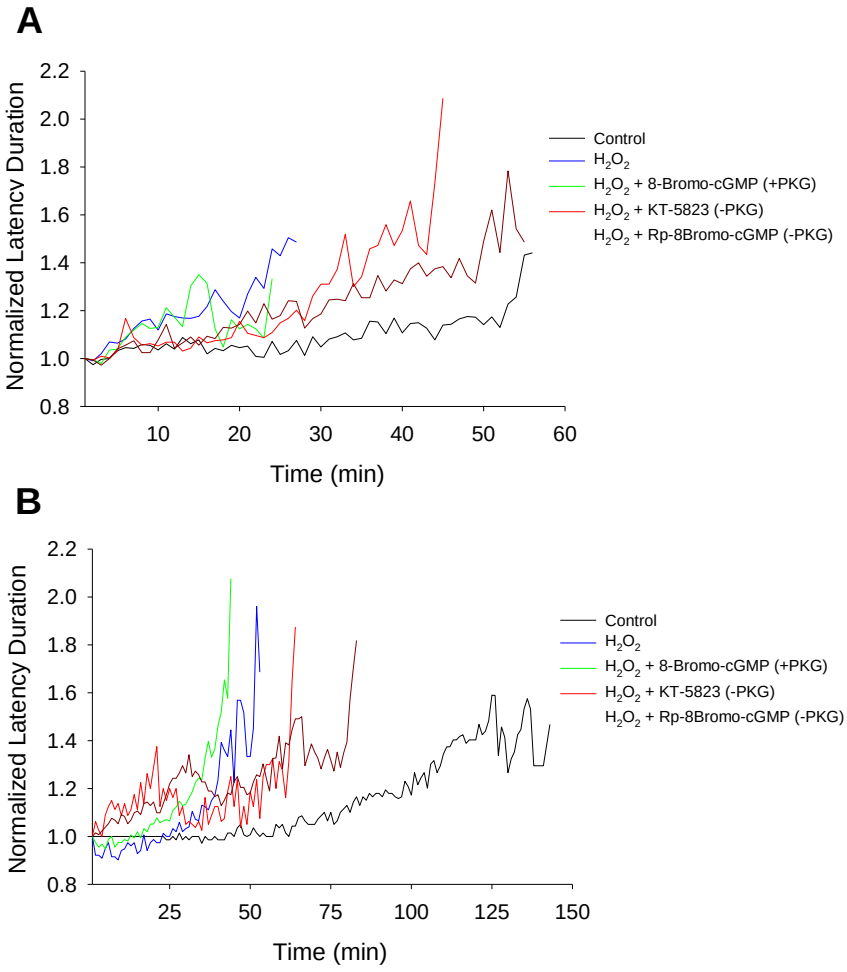

Figure 31. Latency duration of larvae with chemically altered PKG activity. A: *yw* *Drosophila* larvae [control (N = 6), H_2O_2 (2.25 mM, N = 12), H_2O_2/8-Bromo-cGMP (2.25 mM/40 μM, N = 11), H_2O_2/KT-5823 (2.25 mM/1 μM, N = 6), H_2O_2/Rp-8Bromo-cGMP (2.25 mM/50 μM, N = 6)] latency duration as a function of time during single axon fiber stimulation of muscle 6 (0.3 ms, 5 mV pulses delivered at 1 Hz). B: latency duration [N = 6 for all groups, except KT-5823 (N = 3)] during dual axon fiber stimulation of muscle 6 (0.3 ms pulses delivered suprathreshold at 1 Hz).

Similar to previous results, blocking K^+ channel kinetics during H_2O_2 exposure extends neurotransmission and this protection continues in the presence of a PKG agonist. Furthermore, a prior HS was also shown to be protective against acute oxidative injury induced via H_2O_2 and this effect cannot be additively increased with simultaneous PKG inhibition. These data suggest that, like HS, the PKG pathway may contribute to an endogenous neuroprotective scheme that regulates neurological function in the presence of acute stress. Finally, comparison of the two stimulation protocols used to target a single or both axons of larval muscle 6 demonstrated similar PKG-induced synaptic effects during acute oxidative stress, where low activity reduced the rate of EJP amplitude decline time and the overall latency duration versus high activity.

These data demonstrate that acute oxidative stress induced via NaN_3 or H_2O_2 exposure exhibit similar synaptic effects and rapidly reduce axonal function at the *Drosophila* larval NMJ (Figure 19). Pharmacological manipulation of the PKG pathway during H_2O_2-induced stress altered axonal function, where inhibition prolonged synaptic activity while activation of this signaling cascade caused failure to occur more rapidly (Figure 22). The same paradigm was also observed in PKG genetic variants (for^R, for^s, for^{s2}), where high activity (for^R) animals displayed steeper EJP amplitude declines and faster synaptic failure times than low activity (for^s, for^{s2}) animals (Figure 23). However, these PKG-induced effects on axonal function were not observed during NaN_3-induced stress, where activation and inhibition of PKG had similar effects (Figure 21).

When considering the role of NaN_3 in nervous tissue, where it specifically activates guanylate cyclase and increases intracellular cGMP concentrations in intact cells as well as cell-free systems (Murad et al., 1978; Ando et al., 1983; Murad, 1997), these data are not surprising. Since NaN_3-induced cellular effects ultimately lead to increased cGMP, which activates PKG (Figure 4), inhibiting PKG activity would not provide protection against NaN_3- induced stress; it would negate its action. Furthermore, acute NaN_3- induced toxicity could also be mediated via its conversion to nitric oxide by heme proteins (Murad, 1997). Elevated levels of nitric oxide could not only increase intracellular cGMP levels and activate the PKG pathway (Figure 4) but also induce additional cellular damage by generating reactive nitrogen species, impairing protein function and inhibiting mitochondrial respiration by binding to cytochrome c oxidase (Lam et al., 2009).

It is well known that a prior HS protects organisms from a variety of stresses and this assay provides a quantifiable measure of how organisms use intracellular mechanisms to protect themselves against cellular stress insults. Our data demonstrates that HS protects axonal function during acute oxidative stress and extends synaptic activity (Figure 24). These results are similar to other findings that a prior anoxic shock protects synaptic function during hyperthermia in locust muscle (Klose and Robertson, 2004), except our assay used a prior HS to induce protection against acute oxidative stress. Since HS and PKG pathways are both endogenous mechanisms that confer protection against a variety of cellular stressors and both also affect physiological properties of neurons, such as modifications in outward K^+ currents (Renger et al., 1999;

Ramirez et al., 1999), we investigated if these mechanisms interact and their protective effects could be enhanced by combining their activity during acute oxidative stress. While both pathways extended neural function during H_2O_2 exposure at the *Drosophila* larval NMJ, PKG inhibition following a prior HS did not additively increase synaptic function suggesting that these protective mechanisms act in distinct pathways (Figure 25). It is possible that there is a maximal level of protection that can be achieved, where modulating PKG activity following a HS pretreatment cannot provide a significantly higher level of protection.

Exploring potential mechanisms for PKG-induced synaptic effects during acute oxidative stress, we found that simultaneous exposure to a general K^+ channel blocker (TEA) and PKG agonist (8-Bromo-cGMP) protects axonal function by increasing the time to failure (Figure 26). These data demonstrate that general blockade of K^+ channels during acute oxidative stress can protect axonal function in larval muscle 6 whether one or both axons are stimulated. Furthermore, these data are similar to what Dawson-Scully et al. reported in 2010, where pharmacological intervention with multiple compounds that affect different targets in the PKG pathway results in the further downstream component dominating the overall effect. Since TEA results in global K^+ inhibition, additional work using more specific K^+ channel modulators will be necessary to identify which K^+ channel(s) are responsible for PKG-induced synaptic effects during acute oxidative injury.

Nitric oxide (NO) mediates many physiological processes within the body including vasodilation (Bian and Murad, 2007), regulation of the immune response (Bredt, 1999), neuronal excitability (Moroz and Gillette, 1995), learning and memory (Edwards and Rickard, 2007), circadian rhythms (Ding et al., 1994), and reproduction (Musicki and Burnett, 2006). Soluble guanylate cyclase is a key enzyme in the NO signaling pathway that leads to the formation of cGMP, which in turn, leads to activation of cGMP-dependent protein kinase (PKG) G (Denninger and Marletta, 1999). It is well known that the PKG pathway plays a pivotal role in protecting the brain against ischemia (Ardehali and O'Rourke, 2005; Chai and Lin, 2010), Parkinson's disease (Tai et al., 2003), and traumatic brain injury (Jiang et al., 2013). This protective effect on neurons is believed to be mediated via the opening of mitoK_{ATP} channels (Zhou et al., 2008; Yang et al., 2006). While NO and K_{ATP} channels have been implicated in protecting neurons against metabolic stress (Seino and Miki, 2003), the intracellular signaling mechanism responsible for this remains largely unknown.

MitoK_{ATP} channels have been shown to protect several types of neurons following ischemia-reperfusion injury (Wang et al., 2011) or lethal stress (Busija et al., 2008). Similar to what has been observed in mammalian systems; mitochondrial K_{ATP} channels have also been implicated in protecting the fruit fly during hypoxic and tachycardic stress (Akasaka et al., 2006). Therefore, we investigated whether these channels mediate the protective effects of PKG inhibition on synaptic function at the *Drosophila* larval NMJ during acute oxidative stress induced by H_2O_2 (Figure 27). 5-HD has been routinely used in many investigations of protective mechanisms against ischemic injury (Chai and Lin,

2010; Zhang et al., 2014); therefore it was employed to specifically block mitoK_{ATP} channels. NMJ synaptic failure during acute oxidative injury was not attenuated by 5-HD, suggesting that these channels do not mediate PKG-induced protective effects during H_2O_2 exposure. It is possible there is an additional toxic effect of calcium overload during H_2O_2 exposure as it has been shown to cause unregulated calcium entry and cell death (Herson et al., 1999). Increased intracellular calcium overload in combination with increased ROS production during H_2O_2-induced acute oxidative stress may cause further cell damage such as ROS-induced ROS release, modulation of calcium sensitive regulatory proteins, or activation of phospholipases and calpain (Gottlieb, 2011).

The *Drosophila* larval NMJ is a well-established model used to study cellular mechanisms of synaptic development and neurotransmission (Figure 8). The longitudinal abdominal muscles 6 and 7 are routinely used in physiological examinations and are innervated by two motor neurons (Atwood et al., 1993; Johansen et al., 1989). Since an evoked EJP at the muscle 6 and 7 synapse is the combined response of these two motor neurons, these experiments used a stimulation protocol designed to target only one of these motor neurons using a lower stimulus intensity and allows the comparison of the contributions of one versus both axons. Recruiting a desired evoked EJP response at the *Drosophila* larval NMJ has been done previously by stimulating the segmental nerve at a low intensity (Kurdyak et al., 1994). The EJP amplitude decline of PKG manipulated animals during single and dual axon fiber stimulation revealed that there is an abrupt exponential decay during single fiber stimulation compared to a more gradual reduction during dual fiber stimulation (Figures 28 and 29), suggesting that these motor neurons

exhibit failure at different times represented by the immediate drop. Sudden changes in evoked EJPs at the Drosophila larval NMJ have been previously observed and this effect is believed to be a "dropping out" of one of the axons (Zhong and Wu, 1991). The observable differences in the EJP amplitude declines between single and dual axon fiber stimulation can likely be attributed to sudden axonal failure. Since dual axon fiber stimulation recruits both axons, the evoked EJP reduces at a more gradual rate.

Comparison of the latency durations of evoked EJP responses during single and dual axon fiber stimulation demonstrated a difference between PKG genetic variants during single fiber stimulation, where for^{R} and for^{s2} animals had considerably lower latency durations at synaptic failure than for^{s} animals (Figure 30).This difference in latency duration at synaptic failure was not observed during dual axon fiber stimulation. Furthermore, comparison of the latency durations of evoked EJP responses of animals with chemically modulated PKG activity during single and dual axon fiber stimulation demonstrated a difference, where animals with low PKG activity (-PKG) had lower latency durations at synaptic failure than animals with high PKG activity (+PKG) during dual axon fiber stimulation (Figure 31). This difference was not observed during single axon fiber stimulation, however, the trend between the two groups was similar in that PKG inhibition caused a slower increase in the latency duration as the preparations approached synaptic failure. These data suggest that manipulating the PKG pathway, which modulates K^{+} channel conductance, may impact the cell's ability to reach threshold where PKG inhibition, which lowers K^{+} efflux, causes a shorter latency duration (more excitable) while activation causes the latency to increase at a faster rate (less excitable).

CHAPTER 5. PROTECTION OF *DROSOPHILA* S2 CELLS AGAINST ACUTE OXIDATIVE INJURY

Introduction

The fruit fly, *Drosophila melanogaster*, is an anoxia-tolerant organism that can withstand drastic oxygen fluctuations and survive in hypoxia (low oxygen) and anoxia (no oxygen) for hours without pathology (Haddad et al., 1997). While anoxic insults of this magnitude cause irreversible injury and cell death in mammals, fruit flies display no apparent signs of damage after oxygen deprivation (Krishnan et al., 1997). Therefore, due to their apparent lack of pathology following acute or prolonged hypoxia/anoxia exposure, *Drosophila* is routinely used as a model organism for investigations studying the molecular mechanisms that underlie hypoxic survival (Zhou and Haddad, 2013) and oxidative stress tolerance (Zhao and Haddad, 2011).

The cGMP-dependent protein kinase G (PKG) signaling cascade has been identified as a critical biochemical cascade responsible for controlling low-oxygen tolerance in the fruit fly during both acute (Dawson-Scully et al., 2010) and prolonged (Wingrove and O'Farrell, 1999) hypoxia. PKG activity in the fruit fly is encoded by the *foraging (for)* gene, a homolog of the human *PRKG1* gene (Sokolowski, 1980). In nature,

the *for* gene is expressed as a polymorphism where 70% of flies have high PKG transcript and enzyme activity and 30% have low PKG (Osborne et al., 1997).

Modulating PKG levels affects behaviors, such as feeding strategies (Pereira and Sokolowski, 1993) and learning and memory (Mery et al., 2007), and modulates neural function in response to physiological stresses such as hyperthermia (Dawson-Scully et al., 2007), and hypoxia (Dawson-Scully et al., 2010). This biochemical signaling pathway is conserved across species and has also been shown to regulate neural function during acute hyperthermia and hypoxia in the locust (Dawson-Scully et al., 2007), tadpole (Robertson and Sillar, 2009), and neonatal mouse (Armstrong et al., 2010). A recent study demonstrated that PKG activation increases fruit fly survival in low oxygen environments and implicated potassium channels as the downstream effectors of PKG-mediated protection during hypoxia/anoxia (Dawson-Scully et al., 2010). However, little is known about how this signaling mechanism confers cellular protection and survival during hypoxia/anoxia and, more specifically, which K^+ channel(s) are involved remains a matter of debate.

Drosophila melanogaster Schneider 2 (S2) cells were derived from a primary culture of late stage (20-24 hrs old) embryos (Schneider, 1972). S2 cells are one of the most commonly used *Drosophila* cell lines. S2 cells have many advantages over other cell types such as its ease of maintenance, an optimal growth at 26-28°C without the need for CO_2, the ability to grow in suspension as a loose, semi-adherent monolayer, fast cell growth rates with the ability to reach high cell concentrations, and tolerance to

environmental stresses such as hypoxia (Moraes et al., 2012). Furthermore, full resolution of the *Drosophila* genome and ease of transfection make this cell line amenable for not only small interfering RNA-based studies but also for high level protein expression studies and have been used to better understand innate mammalian signaling and pathogenesis pathways (Luce-Fedow et al., 2008; Yang and Reth, 2012). Therefore, using this cell line enables the use of this stress tolerant organism to probe cellular mechanisms that confer protection against acute oxidative stress such as the PKG pathway.

To further explore the mechanism by which PKG activation leads to cellular protection and increases survival, the present study utilized a pharmacological assay to mimic acute oxidative injury in *Drosophila* S2 cells. Acute oxidative stress was induced by exposing cells to tert-butyl hydroperoxide (tbH_2O_2) or hydrogen peroxide (H_2O_2) to directly elevate intracellular reactive oxygen species (ROS). Increased ROS is known to occur in many stress events such as hyperthermia (Flanagan et al., 1998), hypoxia (Waypa et al., 2001), oxygen deprivation (Liu et al., 2005), and reperfusion following an ischemic event (Ferriero, 2001). Previous data indicates that the PKG pathway controls synaptic transmission tolerance to acute oxidative stress (Chapter 1); therefore, direct PKG manipulation was employed to determine if PKG activation could induce cytoprotection during acute oxidative stress. S2 cell viability was determined using the colorimetric [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] MTS assay and Trypan blue exclusion method.

Results

Acute oxidative stress decreases *Drosophila* S2 cell viability

To determine the effects of acute oxidative stress on *Drosophila* S2 cell viability, we designed an acute pharmacological assay where cells were exposed to tbH_2O_2 (11 mM) or H_2O_2 (24 mM) for one hour before a MTS assay was conducted. Most cell viability assays are performed over a 24 hour period; however, the goal of this experiment was to design a novel acute assay that could mimic acute disorders such as traumatic brain injury and stroke. Before addressing this goal, we needed to determine the concentration of S2 cells that would yield optimal results in the MTS cell viability assay. Various numbers of S2 cells were seeded in a 96-well plate in Schneider's media and were allowed to equilibrate for one hour before absorbance was recorded. The correlation coefficient of the line was 0.97, indicating a linear response between cell number and absorbance at 490 nm (Figure 32A). Therefore, wells were seeded at a concentration of 1*10^6 cells/well for all further MTS assays. Exposing cells to tbH_2O_2 or H_2O_2 for one hour significantly reduced S2 cell viability (Tukey Test, $p < 0.05$; Figure 32B).

Pharmacological PKG intervention does not affect reduced viability

To determine if *Drosophila* S2 cell viability can be altered by manipulating the PKG pathway and confer protection against or increase susceptibility to acute oxidative

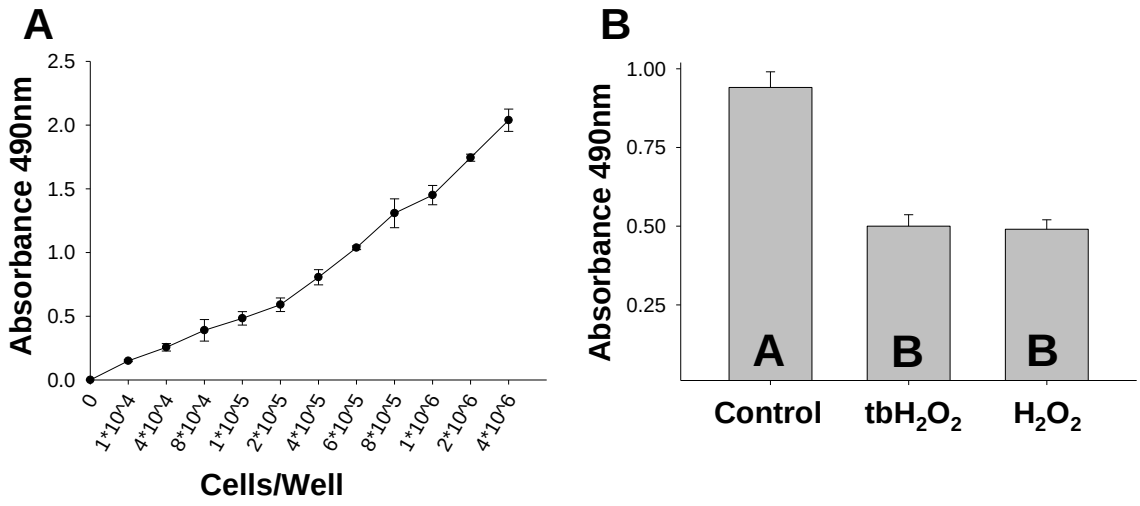

Figure 32. Acute oxidative stress decreases *Drosophila* S2 cell viability. A: the correlation coefficient demonstrates a linear response between the number of S2 cells/well and absorbance at 490nm. 96-well plates were seeded at a concentration of 1*10^6 cells/well for all MTS assays. B: S2 cells in Schneider's media exposed to tbH2O2 (11 mM) or H_2O_2 (24 mM) for one hour significantly reduced viability in a standard MTS assay (Tukey Test, $p < 0.05$; N = 16 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

injury, cells were exposed to tbH_2O_2 (11 mM) or H_2O_2 (24 mM) and pharmacological agents that modulate PKG activity for a one hour incubation period before conducting a MTS assay. PKG activity was directly activated (+PKG) or inhibited (-PKG) using 8-Bromo-cGMP (40 μM) or Rp-8Bromo-cGMP (50 μM), respectively. Decreased cell viability during acute oxidative stress induced by tbH_2O_2 or H_2O_2 was not significantly altered by PKG pathway manipulation (One-Way ANOVA, $F_{(8,63)} = 12.97$, $p < 0.001$; Figure 33). Therefore, additional work revising this acute stress protocol was required to determine if PKG modulation could alter cell viability during acute oxidative stress.

Revision of the acute stress protocol: incubation, media, and length of assay

To revise the acute cell viability assay, the next experiments took the experimental design into consideration and explored several protocol changes such as drug incubation and pre-incubation periods, type of media, drug concentrations, and the assay length of time. The first assay revision took into account that the pharmacological PKG modulators were added at the same time as the acute oxidative stress agents. In the next experiments, *Drosophila* S2 cells were given a pre-incubation period of one hour with PKG modulators before acute oxidative stress was induced by adding tbH_2O_2 (11 mM) or H_2O_2 (24 mM). A prior incubation with either PKG manipulator (8-Bromo-cGMP, +PKG, 40 μM or Rp-8Bromo-cGMP, -PKG, 50 μM) had no affect on S2 cell viability and the absorbance remained the same across treatment groups (One-Way ANOVA, $F_{(8,63)} = 12.97$, $p < 0.001$; Figure 34).

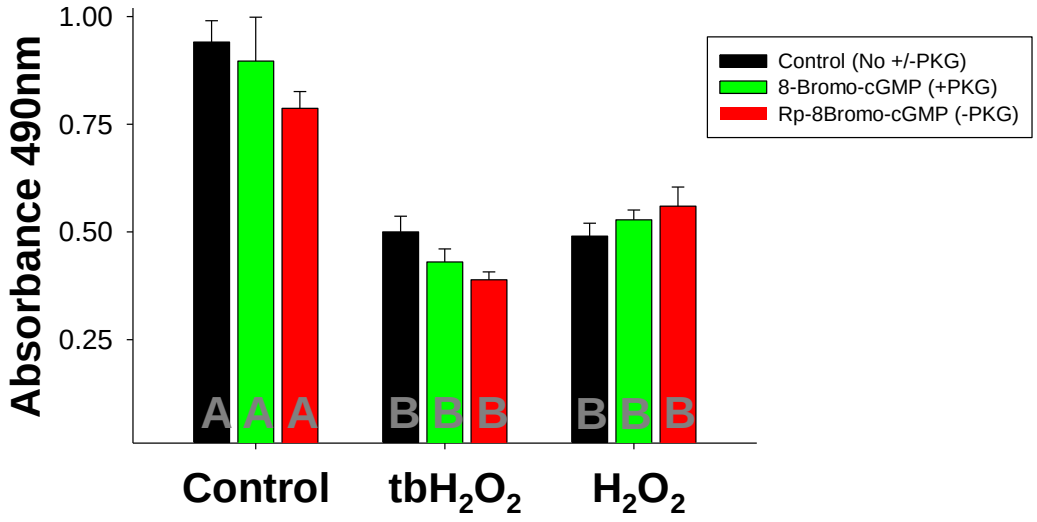

Figure 33. S2 cell viability during acute stress is not altered by PKG manipulation. *Drosophila* S2 cells were exposed to either tbH_2O_2 (11mM) or H_2O_2 (24mM) and PKG modulators (8-Bromo-cGMP, +PKG, 40 μM or Rp-8Bromo-cGMP, -PKG, 50 μM) simultaneously for one hour before absorbance readings were taken in Schneider's media. There was no significant difference between treatments (One-Way ANOVA on Ranks, $H_8 = 52.35$, $p < 0.001$; N = 8 for all groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

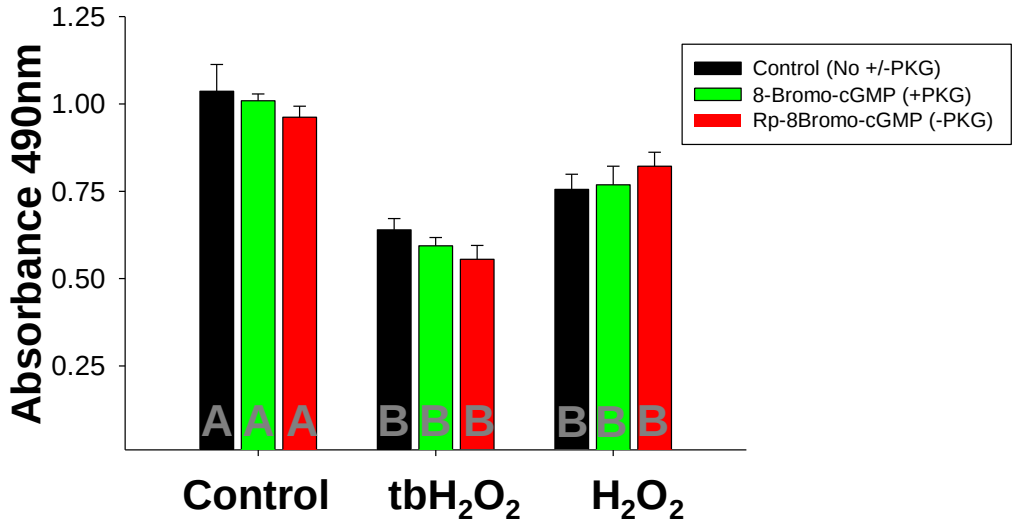

Figure 34. Incubation with PKG modulators does not affect S2 cell viability. *Drosophila* S2 cells were exposed to PKG drugs (8-Bromo-cGMP, +PKG, 40 μM or Rp-8Bromo-cGMP, -PKG, 50 μM) for one hour before tbH_2O_2 (11 mM) or H_2O_2 (24 mM) exposure. Pre-incubation with PKG agents did not significantly alter S2 cell viability during acute oxidative stress (One-Way ANOVA, $F_{(8,63)} = 12.97$, $p < 0.001$; N = 8 for all groups). Absorbance readings were taken in Schneider's media. Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

The next factor taken into consideration was the type of media present during the experiment. Schneider's media was replaced with HL-3 saline, which was used previously for all electrophysiology experiments at the *Drosophila* larval NMJ (for complete protocol see Chapter 2). Replacing the media with HL-3 saline lowered the absorbance readings and overall cell viability of all treatment groups (control, +PKG, -PKG, H_2O_2, H_2O_2/+PKG, H_2O_2/-PKG) and pharmacological PKG manipulation still had no effect (Figure 35). The final parameter changed the assay length of time and concentrations of chemically-induced stress agents. *Drosophila* S2 cells were exposed to acute oxidative stress conditions (2.25 - 10 mM) for four hours rather than the one hour time frame used previously. S2 cells exposed to saline/H_2O_2 solutions (6 -10 mM) during this longer period of time showed significant differences in cell viability among treatment groups (One-Way ANOVA, $F_{(3,44)} = 6.01$, $p = 0.002$; Figure 36).

PKG modulation alters S2 cell viability using Trypan blue exclusion

With the experimental design revised, the next experiments determined whether pharmacological PKG manipulation could alter *Drosophila* S2 cell survival following a four hour exposure to acute oxidative stress induced by H_2O_2 (6 mM). These experiments analyzed cell viability using the Trypan blue exclusion method. Under normal conditions, S2 cell toxicity was similar between the PKG drug treatment groups and controls (Figure 37). Controls displayed an average of 20% cell death and cells exposed to PKG activation (8-Bromo-cGMP, +PKG, 40 μM) or inhibition (Rp-8Bromo-cGMP, -PKG, 50 μM) exhibited 25% and 22% cell death, respectively. Since the PKG drugs were dissolved in

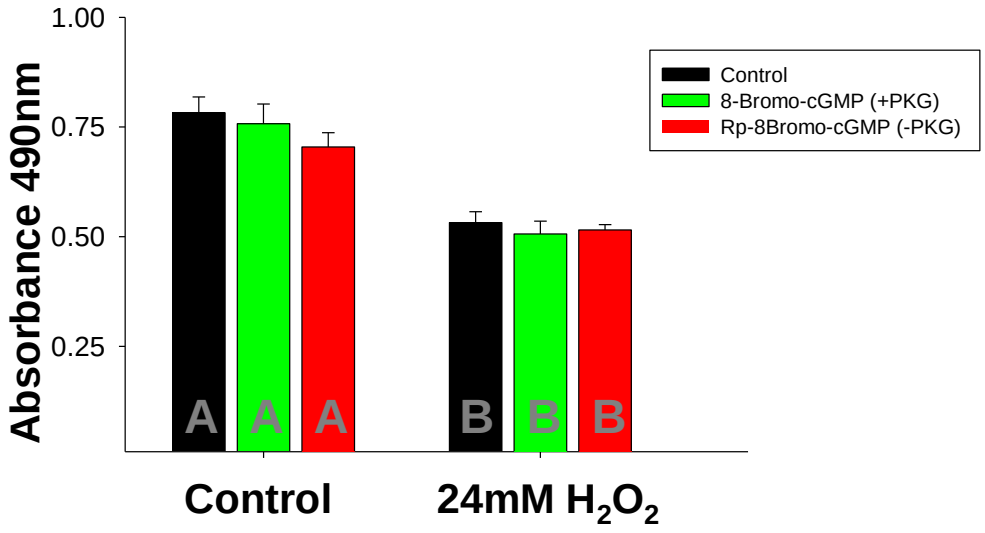

Figure 35. HL-3 saline does not affect S2 cell viability. Schneider's media was replaced with saline before the addition of H_2O_2 (24 mM) and pharmacological PKG modulators (8-Bromo-cGMP, +PKG, 40 μM or Rp-8Bromo-cGMP, -PKG, 50 μM). No significant difference was observed between treatment groups (Tukey Test, $p < 0.05$; N = 8 for all groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

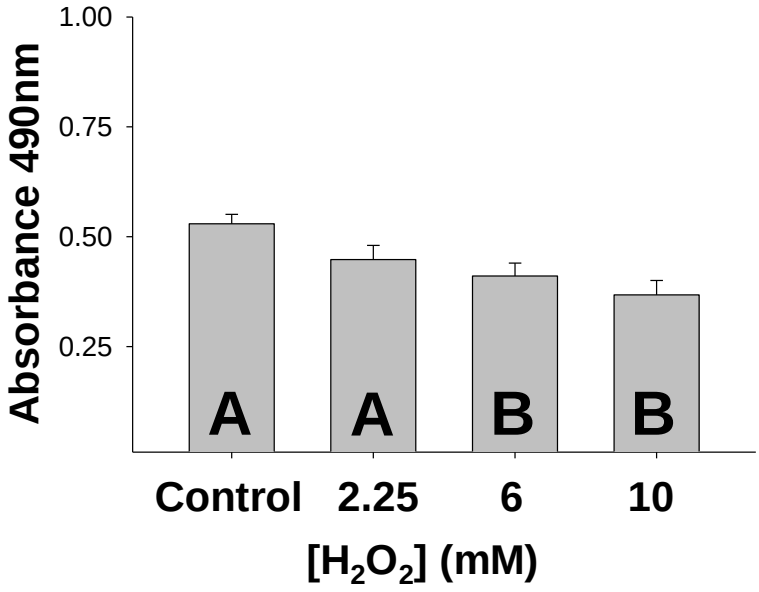

Figure 36. A four hour acute oxidative stress period decreases S2 cell viability. *Drosophila* S2 cells were exposed to different concentrations of H_2O_2 (2.25 mM, N = 8; 6 mM, N = 16; 10 mM, N = 8) for four hours before conducting an MTS assay. There was a significant difference in cell viability between controls (N = 16) and acute oxidative stress treatment groups at higher H_2O_2 concentrations (One-Way ANOVA, $F_{(3,44)} = 6.01$, $p = 0.002$). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

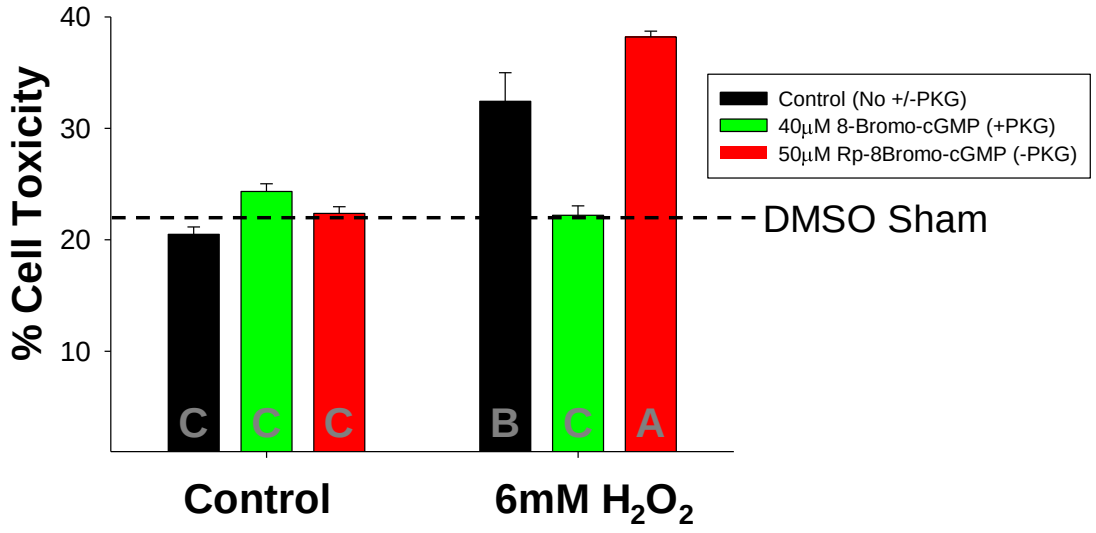

Figure 37. PKG manipulation during acute oxidative stress alters S2 cell viability. *Drosophila* S2 cells were exposed to H_2O_2 (6 mM) and pharmacological PKG agents (8-Bromo-cGMP, +PKG, 40 μM or Rp-8Bromo-cGMP, -PKG, 50 μM) for four hours. PKG activation significantly protected cell viability during H_2O_2 treatment (One-Way ANOVA, $F_{(6,14)} = 32.95$, $p < 0.001$; N = 3 for all treatment groups). Letters in histogram bars represent statistical rankings, where different letters are statistically significant. All vertical bar charts are shown as means ± SEM.

DMSO, we conducted a sham control and found no difference in the amount of cell death at 22%. During acute oxidative stress, direct PKG modulation significantly altered S2 cell death (One-Way ANOVA, $F_{(6,14)} = 32.95$, $p < 0.001$). PKG activation during H_2O_2 exposure protected S2 cells by lowering cell death from 35% to 23%, while inhibition increased cellular toxicity to 38%. Further work is required to determine if PKG activation also protects against lower amounts of H_2O_2. These data are opposite to PKG-induced synaptic effects during acute oxidative injury where inhibition yields functional protection (Chapter 3), suggesting that PKG signaling has an inverse protective effect during acute oxidative stress.

Discussion

The overall goal of the present study was to design a novel pharmacological acute injury assay to mimic the effects of acute disorders, such as traumatic brain injury and stroke, and demonstrate that PKG modulation can alter these effects depending on whether the pathway is activated or inhibited. These experiments designed a novel oxidative injury assay to analyze cell viability from acute oxidative trauma within a short time period of one to four hours. We took many factors into account when designing this experimental protocol such as drug pre-incubation and incubation periods, the type of media, drug concentrations, and the assay length of time. There were many experiments that did were not successful, but ultimately the experimental protocol was revised and PKG pathway manipulation was found to alter *Drosophila* S2 cell survival during acute H_2O_2 exposure. As opposed to what was observed for prolonging synaptic function, PKG

activation was found to protect S2 cells while inhibition increased cell toxicity during acute oxidative exposure.

Recently, it was demonstrated that natural variation in the *foraging* gene, which encodes PKG activity, differentially affects the tolerance of behavior and survival of the fruit fly during acute hypoxia (Dawson-Scully et al., 2010). This study led to the hypothesis that the PKG pathway controls anoxia tolerance in an inverse manner whereby inhibition protects neural function at the expense of survival, while activation increases cell survival but reduces the tolerance of neural function to acute hypoxia. To further explore this hypothesis, we developed a pharmacological assay that can be expanded to analyze multiple types of chemical stress agents, multiple cellular mechanisms, and adjusted for any desired time period over multiple hours.

These data laid the foundation for another set of experiments performed in collaboration with Olena Makhnyeva that fine-tuned this method to mimic ischemia-reperfusion injury in *Drosophila* S2 cells and probe the mechanism by which PKG activation leads to cellular protection and increases survival (data not shown). Chemical hypoxia was induced using sodium azide (NaN_3) or cobalt chloride ($CoCl_2$) to inhibit mitochondrial ATP production and increase intracellular ROS (Clyne et al., 2001; Stannard and Horecker, 1948; Zou et al., 2001). In addition to modeling ischemic stress, this work mimicked reperfusion injury using H_2O_2 to directly elevate intracellular ROS. To elucidate the cellular mechanisms involved in PKG-mediated protection against cellular damage during these insults, upstream components and downstream effectors of

the PKG pathway were analyzed. Since mitoK_{ATP} channels have been implicated as targets of PKG signaling (Chai and Lin, 2010) and opening of these channels is critical for protection against ischemia-reperfusion injury (Garlid et al., 1997; Ardehali and O'Rourke, 2005) these channels were investigated as potential mediators of PKG-induced cytoprotection during acute hypoxia and oxidative stress in *Drosophila* S2 cells.

CHAPTER 6. DISCOVERY OF NEUROPROTECTIVE MARINE NATURAL PRODUCTS

Introduction

Throughout human history, natural remedies have been used to treat disease and improve health and shaped traditional medicinal practices. Modern scientific advances have led to the discovery and isolation of pure bioactive compounds derived from plants and animals or micro-organisms, commonly referred to as natural products. Natural products have played an invaluable role within the drug discovery and development process and provided an overwhelming contribution to the current pharmacopeia (Newman and Cragg, 2009; 2012). Half of the top 20 best-selling pharmaceuticals are related to natural products (Harvey, 2000) and several new drugs have been developed based on natural product structures (Newman and Cragg, 2014). Until relatively recently, the ocean was largely unexplored as a diverse and unique source of bioactive compounds for drug discovery. The two major problems with using marine natural products in therapeutics are supply issues and scaffold complexity; however, these issues are progressively being resolved as evidenced by recent approvals for clinical use: five of seven marine-derived/inspired agents received FDA approval status since 2007, thirteen are in different phases of clinical trials, and fourteen are in preclinical evaluation (Gerwick and Moore, 2012).

Spanning two-thirds of the earth's surface, marine ecosystems are an exceptional reservoir of biodiversity that could contain as much as 80% of the world's plant and animal species (McCarthy and Pomponi, 2004). This untapped biological and chemical diversity is an enormous resource for the discovery of marine natural products that could eventually be developed into therapeutics to treat human diseases (Newman and Cragg, 2012). Evolutionary progress has equipped many marine organisms with complex biochemical mechanisms including the production of bioactive compounds that allow them to thrive in the expansive, ever-changing ocean environment. Marine organisms produce a plethora of natural products with unique structural features and potent biological activities that bolster their biomedical potential (Kijjoa and Sawangwong, 2004). Since these organisms are exposed to high levels of oxidative stress on a daily basis due to photochemical interactions and thermal variations (Wong et al., 2003), their structurally-unique bioactive metabolites are gaining interest as lead compounds in the creation of novel drugs (Ji et al., 2009).

Oxidative stress, the overproduction of reactive oxygen species (ROS), is inherent in the pathophysiology of an array of devastating human ailments including ischemic vascular diseases, heart failure, myocardial infarction, stroke, cancer, and numerous neuropsychiatric and neurodegenerative disorders (Siti et al., 2015; Reuter et al., 2010; Réus et al., 2015; Federico et al., 2012). Natural products have played an important role in the treatment of a variety of human diseases in traditional medicine and continue to drive the modern drug discovery and development process (Cragg et al., 2014). For many

conditions, few treatment options exist and more effective strategies could be developed by identifying natural products as novel drug targets and/or therapeutic agents.

Pseudopterosins are a collective group of marine diterpene glycosides isolated from the gorgonian soft coral, *Pseudopterogorgia elisabethae* (Look et al., 1986a and b; Fenical, 1987; Roussis et al., 1990). Thirty-one structurally unique pseudopterosin derivatives have been identified based upon three different isomeric aglycone skeletons (Berrue et al., 2011; Figure 38A). Structural differences among pseudopterosins, such as the position of glycosylation on the terpene skeleton and the type of sugar moiety, affect their biological and cytotoxic activities (Rodriguez et al., 2004; Correa et al., 2011).

These compounds possess an array of potent biological activities including anti-inflammatory and analgesic (Look et al., 1986a and b; Mayer et al., 1998; Ata et al., 2003; Correa et al., 2009; Dayan et al., 2009), wound-healing (Montesinos et al., 1997; Mayer et al., 2010), antibacterial (Ata et al., 2004; Correa et al., 2011), anticancer, antiviral, anti-malarial, and anti-tuberculosis (Rodriguez et al., 2004) in both *in vitro* and *in vivo* assays with a novel mechanism of action (Look et al., 1986a and b; Fenical, 1987; Ettouati and Jacobs, 1987; Roussis et al., 1990; Mydlarz and Jacobs, 2004; Moya and Jacobs, 2006; Mayer et al., 2010). They are in Phase II clinical trials as an anti-inflammatory and wound healing agent (Ata et al., 2004) and are the first commercially licensed natural product for use as an additive in Estée Lauder skin care and cosmetics products (Newman and Cragg, 2004), which are commercially harvested from a natural, renewable source.

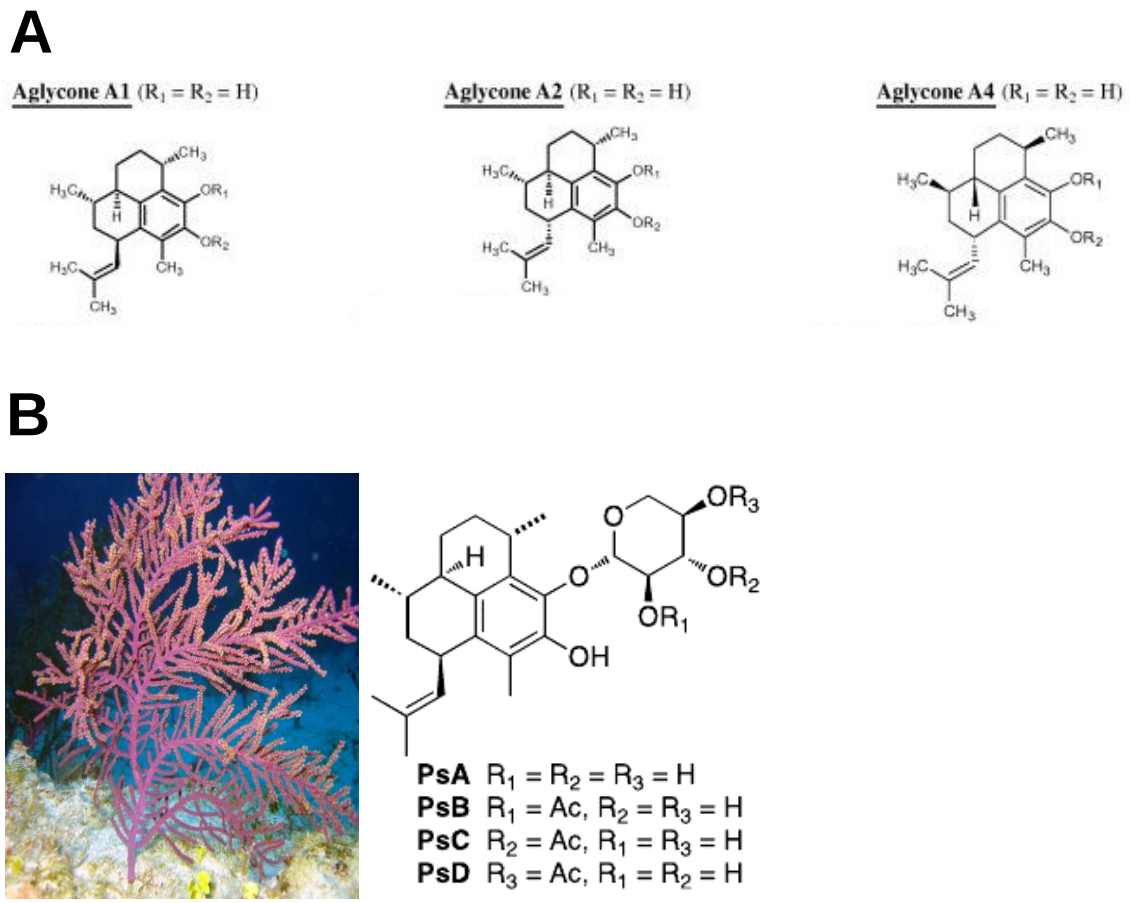

Figure 38. Pseudopterosins isolated from *Pseudopterogorgia elisabethae*. A: the general structure of pseudopterosins is based upon three different isomeric aglycone skeletons (A1,A2, and A4). Other structural differences include the position of glycosylation on the terpene skeleton and the type of sugar moiety. B: picture of the gorgonian sea whip *Pseudopterogorgia elisabethae*. Pseudopterosins A-D were first isolated and reported by Fenical and co-workers in 1986 (Look et al., 1986a and b; Fenical, 1987). These compounds have the same terpene skeleton but differ in the degree of acetylation of the sugar subunit.

Pseudopterosin A (PsA), which contains a non-acetylated xylose sugar subunit (Figure 38B), is one of the most extensively studied pseudopterosins and exhibits cell membrane stabilization properties with a novel mechanism of action (Ettouati and Jacobs, 1987). PsA has also been shown to alter intracellular calcium and inhibit phagocytosis in free living ciliates and reduce oxidative bursts during cellular stress in unicellular protists with a unique mode of action (Mydlarz and Jacobs, 2004; Moya and Jacobs, 2006). Few studies have examined PsA's effects during cellular stress and, to our knowledge, no studies have explored its potential as a novel neuromodulatory agent.

Recently, the West laboratory here at Florida Atlantic University, Department of Chemistry and Biochemistry, was supported with a NIH grant (5P41GM079597) to generate a chemically diverse pilot-scale marine natural product library for evaluation by the Molecular Library Probe Production Centers Network (MLPCN) to identify lead compounds for probe development. This project resulted in the creation of a pseudopterosin library consisting of twelve known pseudopterosins, two novel pseudopterosin K analogues differing in the position of acetylation, and a new series of pseudopterosins isomeric with that of pseudopterosin A, C and D, in which the β-D-xylose was linked to the C-10 position of the terpene skeleton instead of C-9, and therefore named iso-PsA, iso-PsC, and iso-PsD. In collaboration with the West laboratory, we designed a project to investigate the potential neuromodulatory properties of pseudopterosins using *Drosophila melanogaster*.

To determine whether pseudopterosin analogues have neuromodulatory effects during oxidative stress, the present study analyzed their ability to alter synaptic transmission at the larval neuromuscular junction (NMJ) in the fruit fly, *Drosophila melanogaster*. Invertebrate model systems have helped pave the way to understanding key aspects of the mammalian central nervous system including synapse structure, function, and regulation (Anderson et al., 1976; Patlak et al., 1979). The *Drosophila* larval NMJ is a well characterized model for studying the cellular mechanisms of synaptic development and neurotransmission (Jan and Jan, 1976a and b; Gramates and Budnik, 1999; Collins and DiAntonio, 2007).

Oxidative stress was mimicked pharmacologically using two paradigms that generate physiologically relevant oxidant species: mitochondrial superoxide production induced by sodium azide (NaN_3) and hydroxyl radical formation via hydrogen peroxide (H_2O_2). NaN_3 induces ischemic stress by inhibiting cytochrome c oxidase (Bennett et al., 1996), ATP production (Noumi et al., 1987), superoxide dismutase (Misra and Fridovich, 1978), DNA synthesis and cell division (Ciesla et al., 1974). H_2O_2 propagates hydroxyl radicals by reacting with transition metals (Halliwell, 2001). Furthermore, these experiments will lay the foundation to expand this assay for use with *Drosophila* primary neural cultures that will enable genetic targeting of specific neuronal populations to identify pseudopterosin *in vivo* targets and determine their mechanism using a novel bioimaging/optogenetics approach that combines the use of genetically-encoded calcium indicator (GECI) and the light activated ion channel, channelrhodopsin-2 (ChR2), to visualize calcium dynamics in single cells.

Results

Pseudopterosins extend neuronal function during acute oxidative injury

We know from our previous work that exposure to the strong oxidizing agent H_2O_2 significantly disrupts synaptic function at the *Drosophila* larval NMJ and that this disruption can be protected by pharmacological or genetic manipulations (Chapter 3). To determine if pseudopterosins have neuromodulatory effects during acute oxidative stress, we analyzed their ability to alter synaptic function at the *Drosophila* larval NMJ during oxidative overload induced via H_2O_2 exposure (2.25 mM) under two stimulation protocols, single and dual axon fiber stimulation used previously (Chapters 3 and 4). Before addressing whether pseudopterosins can protect synaptic activity, we performed dose-response curves to determine the effective concentrations of pseudopterosin analogues that could extend synaptic function at the *Drosophila* larval NMJ during H_2O_2-induced stress (Figure 39).

The ability of pseudopterosins to mitigate oxidative stress-induced synaptic failure was compared to that of the well-known antioxidant Trolox. During single axon fiber stimulation, all drug treatments significantly prolonged synaptic function during acute oxidative stress (One-Way ANOVA, $F_{(6,34)} = 87.131$, $p < 0.001$; Figure 40A). H_2O_2 treatment reduced the time until synaptic failure occurred from 53 ± 2 minutes for control preparations to 20 ± 1 minute. IsoD, PsD, and Trolox showed the greatest levels of protection, which were all comparable to controls not exposed to the acute stress or any

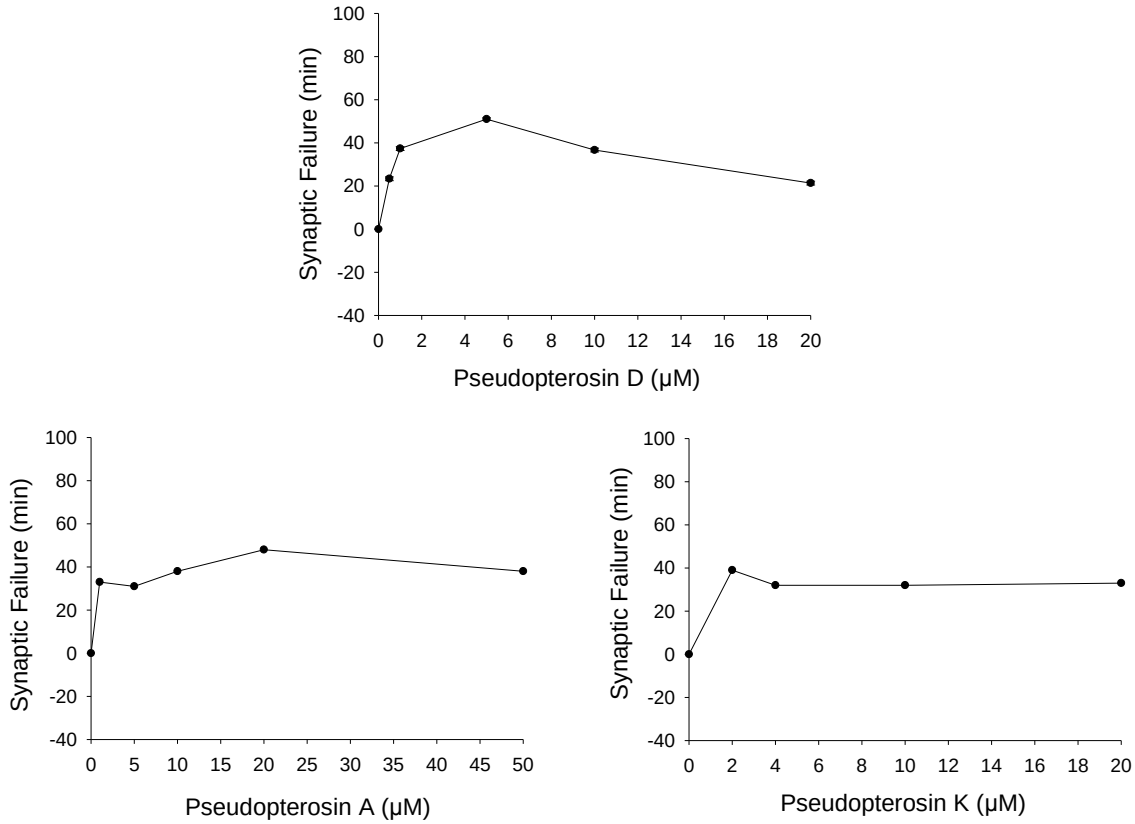

Figure 39. Pseudopterosin dose response curve in *yw Drosophila*. Representative dose response curves for pseudopterosin effects on synaptic function during simultaneous H_2O_2 (2.25 mM) and pseudopterosin exposure (μM). Data for pseudopterosins A, D, and K (PsA, PsD, and PsK) are shown.

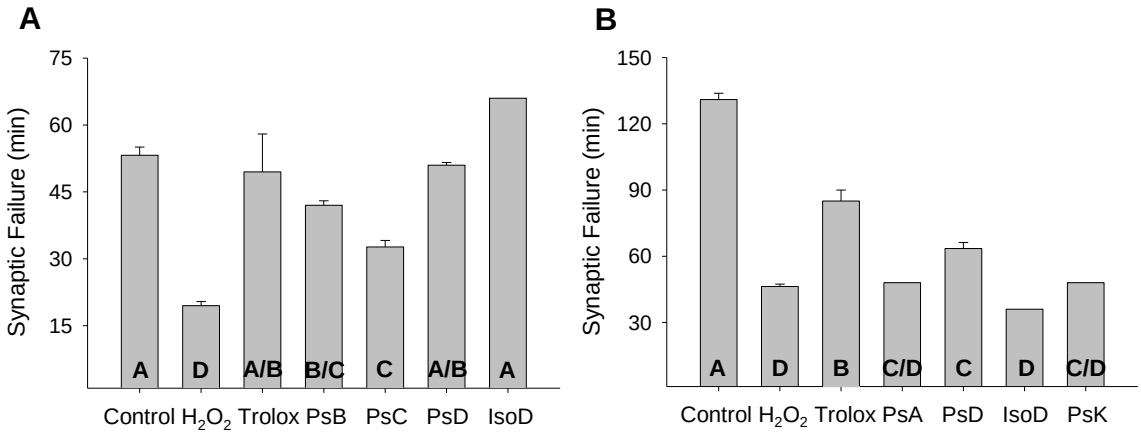

Figure 40. Pseudopterosin-induced synaptic effects during acute oxidative stress. A: average NMJ synaptic failure times during single axon fiber stimulation of muscle 6 at the NMJ of *yw Drosophila* larvae. Animals were exposed to the following conditions: control (N = 9), H_2O_2 (2.25 mM, N = 18), H_2O_2/Trolox (N = 3), H_2O_2/PsB (N = 3), H_2O_2/PsC (N = 3), H_2O_2/PsD (N = 3), or H_2O_2/IsoD (N = 1). Trolox (5 μM) and pseudopterosins (5 μM for all trials) significantly extended the time until synaptic failure during acute oxidative stress (One-Way ANOVA, $F_{(6,34)} = 87.131$, $p < 0.001$). B: average NMJ synaptic failure times during dual axon fiber stimulation of muscle 6 at the NMJ of *yw Drosophila* larvae. Animals were exposed to the following conditions: control (N = 6), H_2O_2 (2.25mM, N = 12), H_2O_2/Trolox (N = 3), H_2O_2/PsA (N = 1), H_2O_2/PsD (N = 4), H_2O_2/IsoD (N = 1), or H_2O_2/PsK (N = 1). Trolox (5 μM) and PsD (5 μM) significantly extended the time until synaptic failure during acute oxidative stress, while PsA (20 μM), IsoD (2.5 μM), and PsK (5 μM) were less effective (One-Way ANOVA, $F_{(3,20)} = 400.10$, $p < 0.001$).

drug treatments. PsB and PsC were also effective at extending neural function during H_2O_2 treatment showing average failure times of 42 ± 1 minute and 33 ± 1 minute, respectively. During dual axon fiber stimulation, control preparations displayed synaptic failure for an average of 131 ± 1 minute. Trolox still demonstrated the highest level of protection by prolonging synaptic function from 46 ± 1 minute during H_2O_2 treatment to 85 ± 5 minutes (Figure 40B). Of the pseudopterosins, PsD showed the greatest ability to protect NMJ synaptic activity by extending function for an average of 64 ± 5 minutes during induced acute oxidative stress.

Pseudopterosins protect synaptic function of wild-type Canton-S *Drosophila*

The next experiments explored pseudopterosin effects on synaptic function during acute oxidative stress using wild-type Canton-S *Drosophila melanogaster*. This fly line was chosen because they are a naturally occurring strain of fruit flies that are not inherently protected against stress such as the *yw* fly line, which is genetically predisposed to better cope with stress. Acute oxidative stress was induced using two different oxidative stress paradigms: oxidative overload via H_2O_2 exposure and mitochondrial inhibition with NaN_3. Once again, we performed dose response curves on pseudopterosin analogues and determined the effective concentrations to extend synaptic function at the *Drosophila* larval NMJ during acute oxidative injury (Figure 41).

Using the same protocol as with the *yw* flies, Canton-S larval preparations were exposed to H_2O_2 (2.25 mM) and one pseudopterosin analogue (PsA, IsoA) or Trolox.

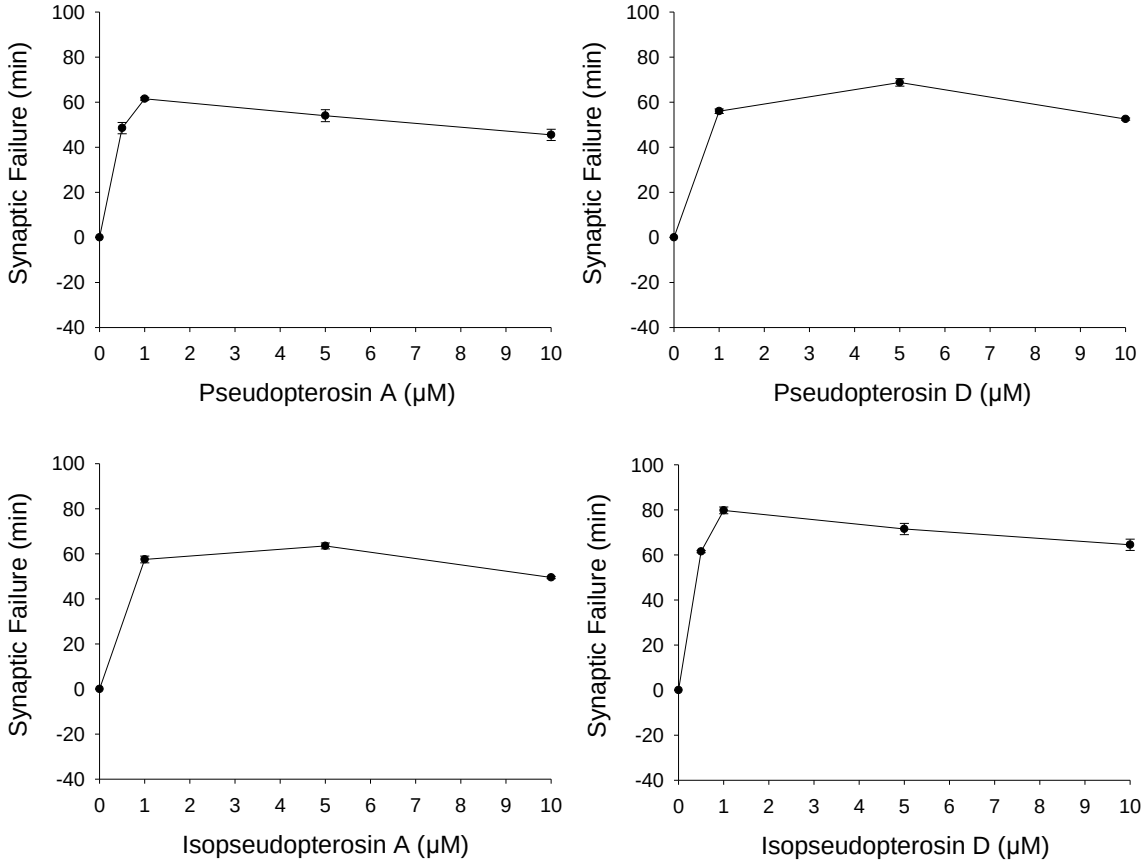

Figure 41. Pseudopterosin dose response curve in wild-type Canton-S *Drosophila*. Dose response curves for pseudopterosin effects on synaptic function during simultaneous H_2O_2 (2.25 mM) and pseudopterosin exposure.

Interestingly, we found that while H_2O_2 treatment had a similar effect on these flies with an average synaptic failure time of 35 ± 2 minutes, however, only Trolox showed significant protection of synaptic function (One-Way ANOVA, $F_{(4,17)} = 370.15$, $p < 0.001$; Figure 42). Pseudopterosins did not extend the time until synaptic transmission failure. Since there was a limited response in the number of drug treatments that were able to attenuate synaptic failure during H_2O_2 treatment (2.25 mM), the concentration of H_2O_2 (1 mM) was lowered to determine if these compounds could protect synaptic activity in other *Drosophila* fly lines.

Using this revised protocol for Canton-S flies, we found that all treatments (Trolox, PsD, IsoD, PsA, IsoA) significantly extended the time until neurotransmission failure during acute oxidative injury (One-Way ANOVA, $F_{(6,21)} = 256.36$, $p < 0.001$; Figure 43). Acute H_2O_2 exposure reduced synaptic function from 132 ± 3 minutes in controls to 50 ± 2 minutes. IsoD demonstrated a strong protection by prolonging average synaptic activity to 80 ± 2 minutes, which was equivalent to that of Trolox during H_2O_2 treatment. PsD, PsA, and IsoA were also protective during the acute oxidative injury and extended the time until synaptic failure to 69 ± 2 minutes , 62 ± 1 minute, and 64 ± 1 minute, respectively. These data demonstrate that pseudopterosins can protect synaptic function during acute oxidative stress and this protection occurs at levels equivalent to that of antioxidants for some analogues.

When the EJP amplitude is plotted as a function time, the differential drug treatments separated into clear patterns of varying synaptic transmission tolerance during

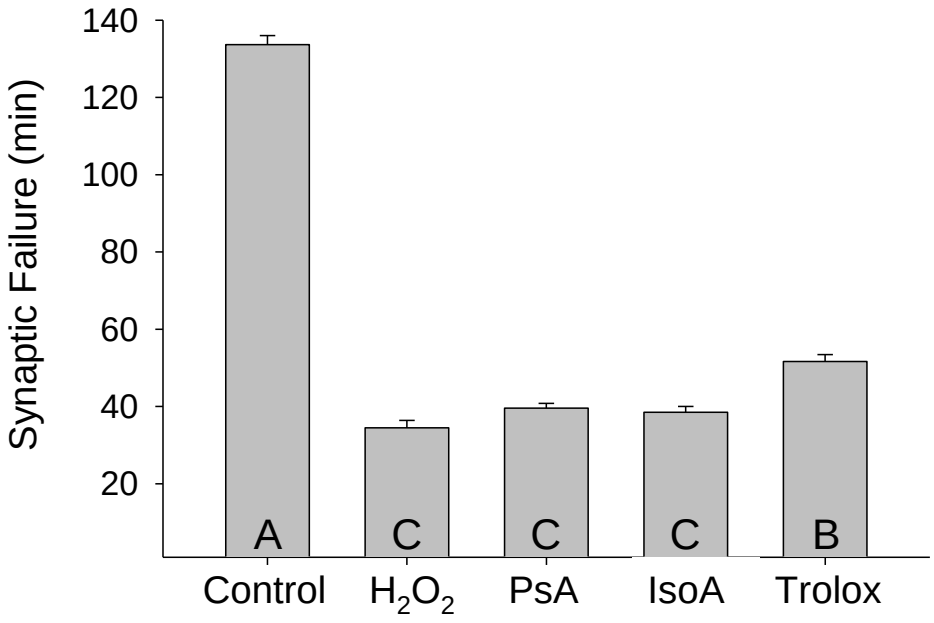

Figure 42. Pseudopterosins are not protective during H_2O_2 exposure (2.25 mM). Wild-type Canton-S larval preparations were exposed to control (N = 4), H_2O_2 (N = 8), or H_2O_2 and simultaneous treatment with one of the following agents: PsA (N = 5), IsoA (N = 2), or Trolox (N = 3). Trolox significantly extended neurotransmission during the H_2O_2 treatment (Holm-Sidak, $p < 0.05$).

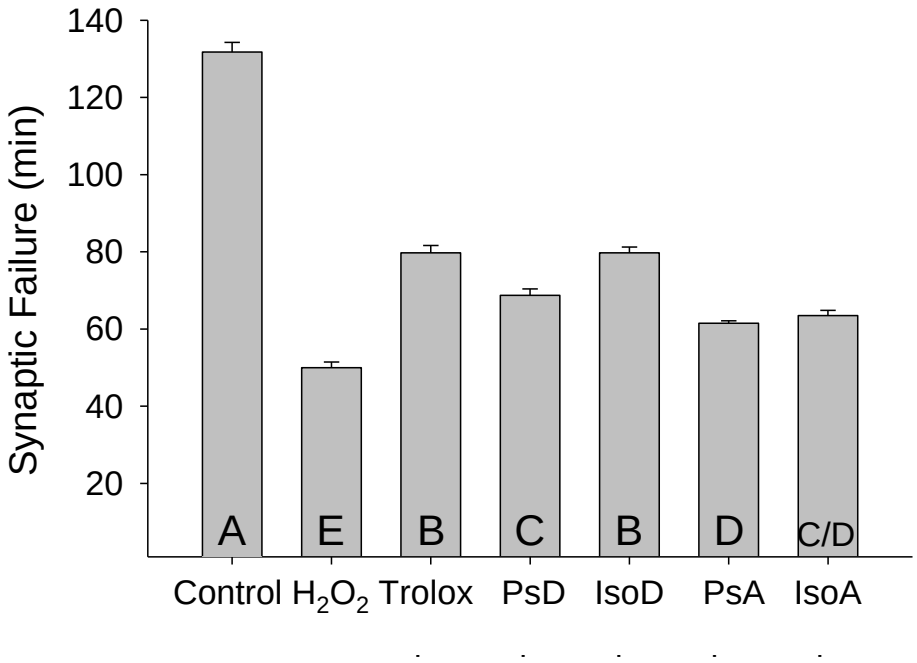

Figure 43. Pseudopterosins extend neurotransmission during H_2O_2 exposure (1 mM). Wild-type Canton-S larval preparations were exposed to control (N = 5), H_2O_2 (1 mM, N = 4), or H_2O_2 and simultaneous treatment with one of the following agents: Trolox (5 μM, N = 4), PsD (5 μM, N = 4), IsoD (1 μM, N = 4), PsA (1 μM, N = 5) or IsoA (5 μM, N = 4). Trolox and all pseudopterosin analogues significantly prolonged NMJ synaptic function during the H_2O_2 treatment (One-Way ANOVA, $F_{(6,21)} = 256.36$, $p < 0.001$).

the acute stress condition (Figure 44). Controls displayed synaptic function for a maximum of 138 minutes and NaN_3 exposure (1 mM) reduced the ultimate time until NMJ synaptic failure to 55 minutes. Pseudopterosins (PsA and IsoA) and Trolox reduced the rapid decay caused by H_2O_2-induced oxidative stress and extended neuronal function to 85 minutes (PsA), 70 minutes (IsoA), and 71 minutes (Trolox).

To determine if pseudopterosins could protect synaptic function during exposure other physiologically relevant oxidant species, the next experiments exposed Canton-S flies to NaN_3 (75 µM). Pseudopterosins significantly extended neurotransmission during NaN_3-induced oxidative stress at higher levels (73 - 156%) than that afforded by Trolox, which increased synaptic function by 24% above NaN_3 treatment (One-Way ANOVA, $F_{(6,22)} = 110.5$, $p < 0.001$; Figure 45). Control preparations not exposed to any drug treatment displayed synaptic function for an average of 131 ± 3 minutes and NaN_3 reduced this time frame to 46 ± 3 minutes. Trolox protected synaptic activity and extended the time until synaptic failure occurred to 55 ± 3 minutes. However, pseudopterosins prolonged neurotransmission more effectively than Trolox with average synaptic failure times between 61 - 114 ± 3 minutes during the acute stress, with PsA demonstrating the greatest amount of protection, which extended synaptic function for an average of 87 ± 5 minutes in nanomolar concentrations.

These data demonstrate a difference between pseudopterosins and Trolox during acute oxidative stress and suggest that pseudopterosin-induced synaptic effects act in a mechanism distinct from scavenging activity. Further, the difference between

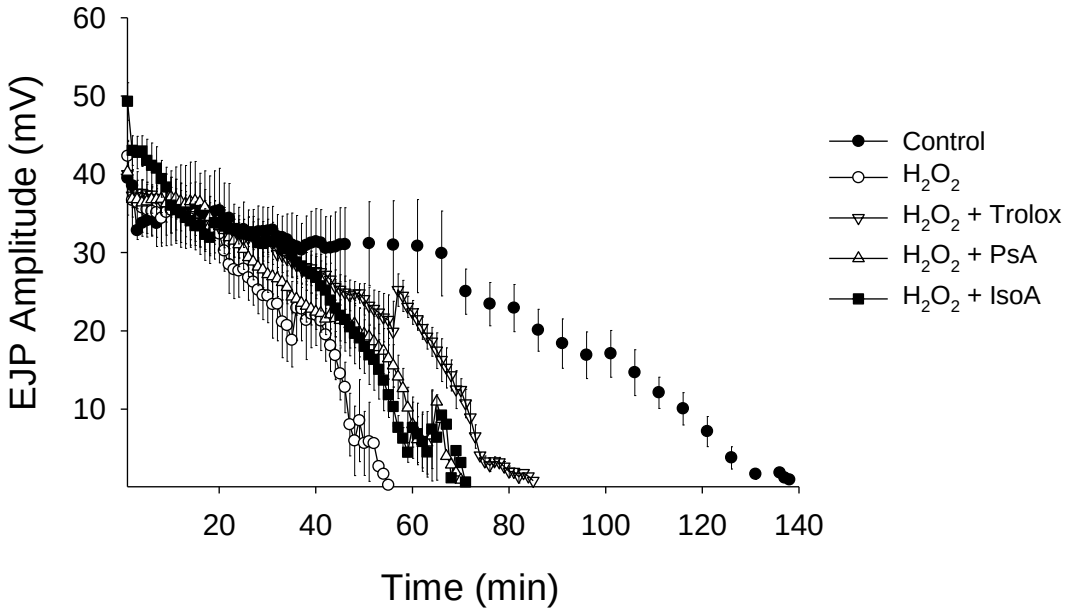

Figure 44. EJP amplitude decline during pseudopterosin and H_2O_2 exposure. The evoked EJP response in Canton-S *Drosophila* larvae reduce over time at different rates in control preparations (saline only, N = 5) and those exposed to H_2O_2 (1 mM, N = 4). Larvae with simultaneous exposure to Trolox (5 μM, N = 4) or a pseudopterosin analogue (PsA, 1 μM, N = 5 or IsoA, 5 μM, N = 4) during the acute stress maintained synaptic function for a longer period of time.

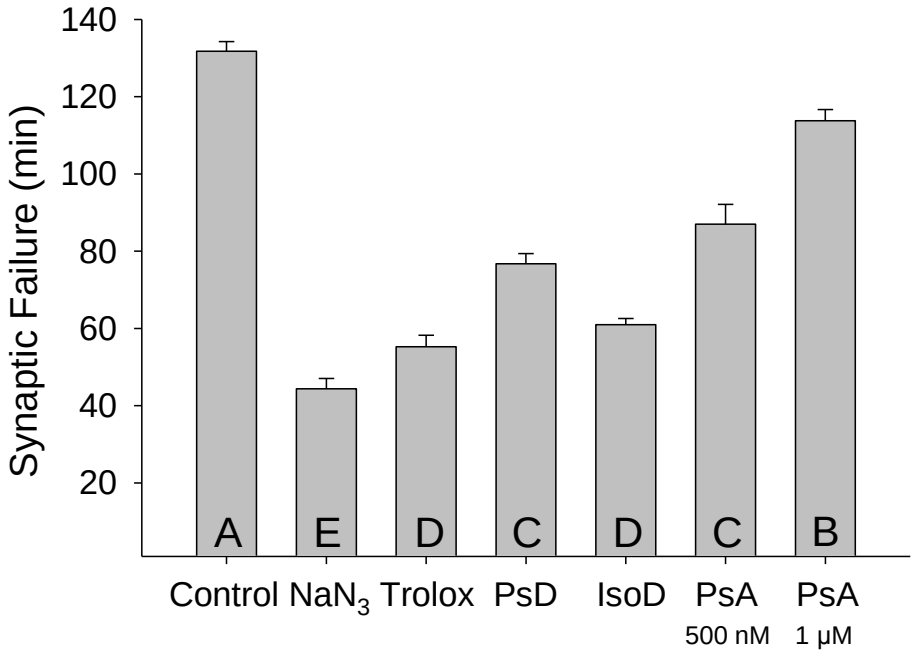

Figure 45. Pseudopterosins extend neurotransmission during ischemic stress. Wild-type Canton-S larval preparations were exposed to control (N = 4), NaN_3 (N = 4), or NaN_3 and simultaneous treatment with one of the following agents (N = 4 for all treatment groups): Trolox (5 μM), PsD (5 μM), IsoD (1 μM) or PsA (500 nm and 1 μM). Trolox and all pseudopterosin analogues significantly prolonged NMJ synaptic function during NaN_3-induced stress with PsA demonstrating the greatest level of protection (Holm-Sidak, $p < 0.05$).

pseudopterosin and Trolox activities is evidenced by the EJP amplitude decline over time (Figure 46). During NaN_3-induced stress, Trolox exposure declines in a similar fashion as it did during H_2O_2 treatment albeit at a slightly faster decay. However, PsA acts in a different manner causing a synaptic functional decline mirroring that of controls not exposed to stress. Future work will investigate if other pseudopterosin analogues can modulate synaptic function during oxidative stress and if this ability acts differentially depending on the type of induced stress.

Combined expression of GECI/ChR2 in *Drosophila*

The following experiments describe the development of a novel neuroactivity assay to determine the *in vivo* target(s)/mechanism of action of pseudopterosins during acute oxidative stress using an optogenetic approach that utilizes advanced *Drosophila* genetic techniques. This unique strategy will enable the study of pseudopterosin effects on neuronal structure, function, and survival at specific synapses, within a neuronal population, or in subsets of neurons within a population. Using the *Drosophila* GAL4/UAS expression system, we genetically expressed a genetically-encoded calcium indicator (GECI) and the light-activated ion channel channelrhodopsin-2 (ChR2) in motor neurons at the larval NMJ (Figure 47).

This transgenic fly line combines expression of ChR2 and a red-shifted calcium indicator (RGECO), which has an excitation wavelength of 561 nm and will not overlap with that of ChR2 at 488 nm. Using a red-shifted GECI in concert with blue light-

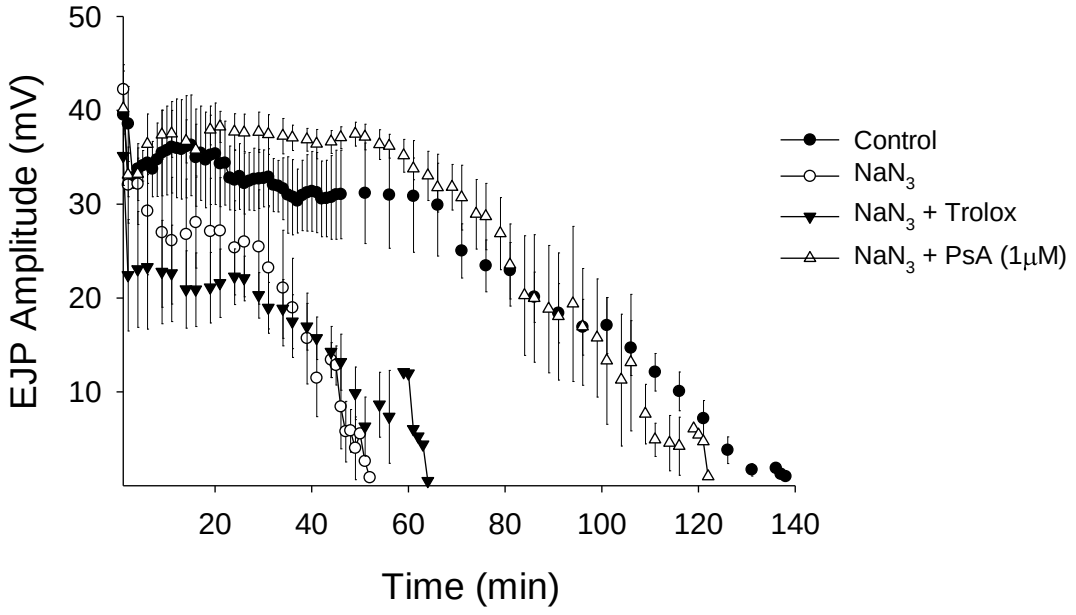

Figure 46. EJP amplitude decline during NaN_3 and pseudopterosin exposure. The evoked EJP response in Canton-S *Drosophila* larvae reduce over time at different rates in control preparations (saline only, N = 4) and those exposed to NaN_3 (75 μM, N = 4). Larvae with simultaneous exposure to Trolox (5 μM, N = 4) or PsA (1 μM, N = 4) during the acute stress maintained synaptic function for a longer period of time. However, the rate of EJP amplitude decline during PsA exposure mirrored that of the controls while Trolox did not.

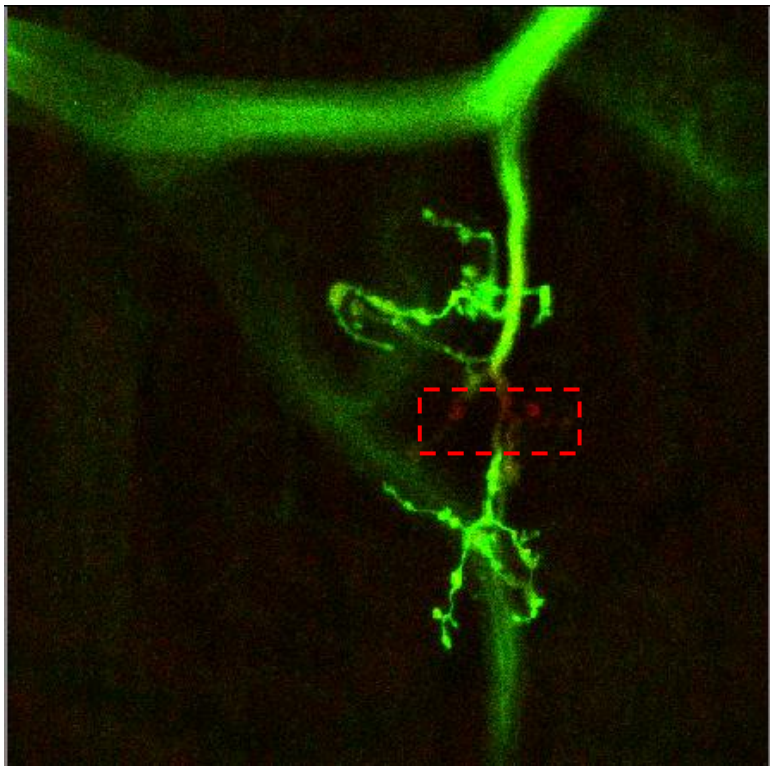

Figure 47. Synaptic boutons at the larval NMJ of RGECO/ChR2 *Drosophila*. RGECO and ChR2 were expressed in *Drosophila* motor neurons at the larval NMJ allowing for visualization of internal calcium changes following neuronal stimulation via blue light. The red box indicates where line scans were performed across synaptic boutons.

activated ChR2 permits independent manipulation of the light-gated channels during calcium imaging. This specific RGECO/ChR2 combination allows us to simultaneously control cellular activity via light activation (optogenetics) and view resultant functional changes that indicate neuronal activity (fluorescent imaging of calcium influx) at specific synapses (NMJ) or in selective neuronal populations (neurons expressing RGECO and ChR2).

Calcium imaging of RGECO/ChR2 transgenic *Drosophila*

Using calcium imaging to monitor neuronal activity triggered by ChR2 activation makes it possible to analyze natural products, such as pseudopterosins, for potential neuroactive and/or neuromodulatory properties. Like many biological processes, the influx of calcium into the cell after photo- activation of ChR2 occurs rapidly. Calcium transients are normally recorded using line scan mode because analyzing changes in intracellular calcium dynamics requires the fastest scanning option available. Using RGECO/ChR2 transgenic *Drosophila*, we can measure the ability of pseudopterosins to mitigate intracellular calcium influx caused by oxidative stress and determine potential *in vivo* targets by comparison to compounds with known targets/cellular membrane effects such as ion channel agonists and antagonists.

Performing line scans across synaptic boutons at the NMJ, we measured intensity changes due to NaN_3-induced oxidative stress and found that PsA lowered the intracellular calcium increase (Figure 48), which is in agreement with previous findings

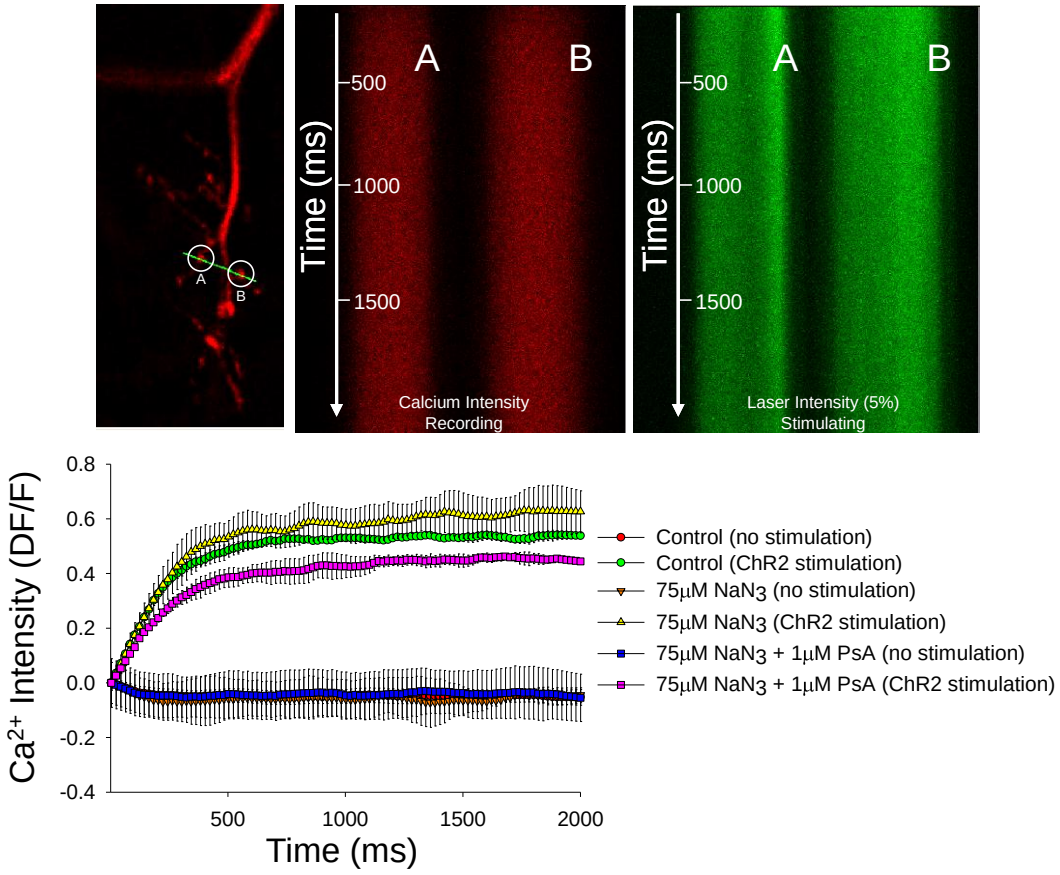

Figure 48. Calcium imaging of RGECO/ChR2 transgenic *Drosophila*. *Top:* Image of synaptic boutons at the NMJ. The green line (20 μm) indicates where 2 s line scans were performed. Line scan images of an RGECO/ChR2 transgenic fly where the RGECO calcium indicator (red) and ChR2 activation (green) is shown for a control (ChR2 stimulation) preparation. *Bottom:* Example of intracellular calcium changes for control, NaN_3, and NaN_3 + PsA combination (n = 2 - 3 boutons per group). Error bars = SEM.

that PsA blocks extracellular calcium induced phagosome formation in *T. thermophila* (Moya and Jacobs, 2006). These results together with the synaptic function experiments and other findings that pseudopterosins inhibit ROS production (Mydlarz and Jacobs, 2006), alter intracellular calcium concentration (Moya and Jacobs, 2006), and stabilize cell membranes (Ettouati and Jacobs, 1987) during cellular stress demonstrate that these compounds have a neuromodulatory effect during oxidative stress.

Discussion

Marine organisms possess an array of structurally-unique bioactive metabolites that have been an invaluable source of inspiration in the drug discovery pipeline (Cragg et al., 2014). Evaluating the biological properties of marine natural products has not only led to the creation of novel therapeutic agents but also bridged natural products chemistry with modern molecular biology (Gerwick and Moore, 2012). Many marine natural products have been evaluated for their drug-like potential. Though their mechanism of action remains unknown (Look et al., 1986a and b; Fenical, 1987), pseudopterosins have been analyzed as novel anti-inflammatory, analgesic, wound-healing, antimicrobial, and anticancer agents (Mayer et al., 2010). Since few studies have investigated pseudopterosin effects during cellular stress, the present study examined the ability of pseudopterosins to modulate synaptic function at the *Drosophila* larval NMJ during acute oxidative stress. Acute oxidative stress was induced pharmacologically using two paradigms: mitochondrial inhibition with NaN_3 (75 µM) and oxidative overload via H_2O_2 (1 mM).

Organisms respond differently to stress and the cellular basis for stress resistance is still poorly understood. The mammalian brain is especially sensitive to ROS-induced oxidative stress and irreversible damage and cell death can occur within minutes of uncontrolled oxygen fluctuations (Haddad and Jiang, 1993). Unlike mammals, insects can survive in a critically low oxygen environment for hours without pathology (Wingrove and OFarrell, 1999; Haddad, 2006). Invertebrate stress resistance mechanisms are believed to have evolved very early and be highly conserved (O'Farrell, 2001). Previously, we demonstrated that oxidative stress rapidly reduces neural function at the *Drosophila* NMJ and that this disruption can be protected by pharmacological manipulations (Chapter 3). To determine if pseudopterosins have neuromodulatory effects during acute oxidative stress, we analyzed their ability to alter synaptic function at the *Drosophila* larval NMJ. Synaptic transmission rapidly declined during both oxidative stress models (NaN_3 or H_2O_2) and simultaneous exposure to Trolox or a pseudopterosin analogue extended neurotransmission during both insults (Figures 43 and 45). Trolox, a water-soluble analog of vitamin E, was selected as a positive control due to its ability to protect against oxidative stress via biological oxidation-reduction reactions.

In comparison to the well-known antioxidant Trolox (Re et al., 1999), pseudopterosins demonstrated differential effects depending on the method of oxidative stress induction. During NaN_3-induced ischemic stress, pseudopterosin analogues prolonged synaptic transmission at higher levels than Trolox, with PsA showing the greatest level of protection. Interestingly, PsA promoted synaptic function less effectively than Trolox during H_2O_2-induced oxidative overload (Figure 43). Mechanistically,

Trolox has been shown to protect cells from oxidative damage through scavenging activity (Hamad et al., 2010; Nguyen et al., 2010) and the data presented here also confirm the ability of Trolox to attenuate H_2O_2-induced synaptic failure. Additionally, these data suggest that pseudopterosins act in a mechanism that is distinct from typical antioxidant activity and agrees with previous findings that direct scavenging is not the primary mechanism of action of pseudopterosins (Mydlarz and Jacobs, 2004). It has been suggested that the pseudopterosin site of action may occur at cellular membrane receptors, such as G protein coupled receptors (Moya and Jacobs, 2006). Elucidating the pseudopterosin mechanism of action could be possible using the expansive *Drosophila* toolkit to target specific genes, pathways, and cell surface receptors in many cell types using commercially available fly stocks (i.e. genetic mutants and RNA interference RNAi lines).

Similar to what was observed in the average synaptic failure rates, when the EJP amplitude decline was plotted as a function of time it further demonstrates the difference between Trolox and pseudopterosin-induced extension of neurotransmission during oxidative stress (Figures 44 and 46). During both oxidative stress paradigms, the rate of EJP amplitude decline was similar during simultaneous Trolox but not pseudopterosin exposure demonstrating a difference between the mechanistic activities of these compounds. Furthermore, PsA's ability to slow the EJP amplitude decay rate during induced stress in a similar fashion to controls is suggestive of a mechanism that has the potential to not only stabilize cellular membranes, as previously suggested (Ettouati et al., 1987; Mayer et al., 2010), but also promote synaptic activity. These results demonstrate

that pseudopterosins are able to modulate neuronal activity during oxidative stress and mediate synaptic transmission tolerance by extending the time until neurotransmission breakdown. Since the structure-activity relationships of pseudopterosins are not fully understood, future work is needed to investigate if other pseudopterosin analogues can modulate synaptic function during acute oxidative stress and if this ability acts differentially depending on the type of induced stress.

The rapidly growing new field of optogenetics has produced breakthrough technologies that make it possible to regulate the activity of specific cell types within complex neural circuits of the nervous system with spatial and temporal resolution (Packer et al., 2013). Optogenetic techniques rely upon cellular properties, such as intracellular calcium concentration, to serve as functional indicators of activity. Calcium plays crucial roles in regulating a variety of cellular functions such as muscle contraction, neurotransmitter release, neuronal excitability, neurite outgrowth, synaptic plasticity, gene transcription, and cell survival (Wang and Michaelis, 2010). Intracellular calcium acts as a signaling molecule that not only plays an essential role in signal transduction but also represents a powerful tool for examining neuronal activity. Action potential number, timing, frequency, as well as levels of synaptic input, can all be quantified by measuring changes in intracellular free calcium (Yasuda et al., 2004). Therefore, calcium-sensitive probes are a popular choice for optically monitoring neural activity.

New optogenetic methods for examining and manipulating neural function *in vivo*, *in vitro*, and high-throughput *ex vivo* studies in a number of model systems has

expanded the study of a variety of topics from neural architecture and function to therapeutics in disease model systems (Smedemark-Margulies, 2013). Optogenetics uses light to control cellular excitability and calcium influx via expression of microbial-type rhodopsins such as ChR2. ChR2 is a light-gated, non-selective cation channel from the green alga *Chlamydomonas reinhardtii* that upon activation causes membrane depolarization and a rapid rise of intracellular calcium due to its partial permeability to calcium (Nagel et al., 2003). The combination of a genetically-encoded photosensor (GECI) and photoactuator (ChR2) is a powerful tool that allows for simultaneous visualization and control of neural activity in defined populations of neurons (Reiner and Isacoff, 2013). Using this concept, we genetically expressed RGECO/ChR2 in motor neurons at the *Drosophil*a larval NMJ and used calcium imaging to detect pseudopterosin-induced changes in intracellular calcium concentration (Figures 47 and 48). These preliminary data demonstrate that we can use this optogenetic protocol to visualize ionic current changes, such as calcium influx, that can impart major changes in neuronal activity and use this assay to examine pseudopterosins for neuromodulatory properties.

Additionally, we could potentially determine their mechanism of action using advanced *Drosophila* genetic techniques that allow us to target specific genes, pathways, and cell surface receptors using commercially available transgenic fly stocks [i.e. genetic mutants and RNA interference (RNAi) lines]. This technique can also be applied for use in different *Drosophila* models, such as primary neural cultures, that will enable us to genetically target specific neuronal populations (serotonergic, dopaminergic, etc) within a

group and analyze potential cell specificity of pseudopterosin compounds. More specifically, we can directly analyze synaptic function at specific synapses (such as the larval NMJ) or we can analyze effects on neural activity either pan-neuronally or in specific subsets of neurons using genetically-targeted expression of RGECO and ChR2 via the *Drosophila* GAL4/UAS system.

To examine pseudopterosin distribution profiles and ability to access *in vivo* neuronal targets, in collaboration together with Dr. Catherine White, University of Georgia, we developed a bioanalytical method for determining pseudopterosin concentration in mouse plasma, liver, brain and kidney following intravenous injection (data not shown). This method was used to determine PsA distribution within mouse tissues and concentration-time profiles revealed that PsA levels in the plasma, liver and brain declined in a bi-exponential fashion after reaching peak concentration, which indicates a rapid equilibrium was achieved between plasma and liver or brain (data not shown). In contrast, the concentration-time profile of PsA in kidney indicated that it does not achieve a rapid equilibrium with plasma, which suggests the possible existence of an intrinsic clearance mechanism for PsA in the kidney. Among these tissues, brain had the largest PsA concentration, indicating that it distributes within the brain.

These data demonstrate that pseudopterosins alter synaptic activity by promoting function during acute oxidative stress. PsA was shown to readily cross the BBB, indicating its potential as a novel neuromodulatory agent (data not shown). Furthermore, its physiochemical properties allow PsA to be administered intravenously, which is the

preferred route of administration for the treatment acute neurological conditions such as traumatic brain injury and stroke. Given that many pharmaceuticals have been inspired from natural products, and derivatives thereof (Cragg et al., 2014; Newman and Cragg, 2009; 2010), and pseudopterosins, in general, have an expansive list of biological activities, these compounds warrant further investigations into their neuroactive properties that may shed light on their elusive mechanism of action and harness their therapeutic potential.

CHAPTER 7. CONCLUSIONS

Endogenous protection mechanisms, such as the HS and PKG pathways, were investigated to determine their ability for regulating synaptic tolerance of the *Drosophila* larval NMJ during acute oxidative stress. A combination of genetics and pharmacological approaches were used to explore the hypothesis that these mechanisms are involved in controlling cellular responses to acute physiological stress by modulating neurotransmission and ion channel conductance. Potential interactions between the HS and PKG pathways were investigated to determine if the overall level of functional protection could be additively increased by combining these mechanisms. Differences between stimulating a single axon versus both axonal fibers in muscle 6 during acute oxidative stress were analyzed. Furthermore, this work applied the pharmacological oxidative stress assay that was developed to screen marine natural products that can alter synaptic function and potentially identify novel neuromodulatory agents. This chapter summarizes and discusses the main findings and significance of this work and makes additional suggestions for future research that will extend these findings.

Protection of synaptic function during acute oxidative stress

It is well known that a prior HS protects organisms from a variety of stresses and this mechanism protected synaptic function at the *Drosophila* larval NMJ during acute

oxidative stress by prolonging the time until synaptic failure. These results are similar to other findings that demonstrate a cross tolerance between HS and anoxic shock, where both are able to protect synaptic function against the other insult (Klose and Robertson, 2004). Furthermore, this study investigated if a potential interaction existed since both of these mechanisms confer protection against a variety of cellular stressors and affect physiological neuronal properties (Renger et al., 1999; Ramirez et al., 1999). Combining these protective treatments did not additively increase synaptic function during acute oxidative stress. Though it is possible that there is a maximal level of protection that can be achieved, these data suggest that these protection mechanisms are distinct. Though both of these pathways are known to have similar effects, such as modulating neuronal K^+ efflux, the HS pathway is believed to confer cellular protection against stress via the upregulation of heat shock proteins that act as molecular chaperones and aid in the refolding of denatured proteins (Feder and Hofmann, 1999).

Manipulation of the PKG pathway, whether genetically or pharmacologically induced, modified synaptic tolerance of the *Drosophila* larval NMJ during acute oxidative stress induced by H_2O_2 exposure, resulting in protection against or vulnerability to neuromuscular communication breakdown. The time until synaptic transmission failure was prolonged by inhibition of this signaling cascade, while failure occurred more rapidly during PKG pathway activation. These data suggest that PKG activity controls synaptic tolerance to acute oxidative stress in a manner similar to previous findings investigating thermotolerance and anoxic tolerance (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). Furthermore, shunting K^+ channel kinetics during acute

oxidative stress extended neurotransmission and this protection continued in the simultaneous presence of a PKG agonist.

These data suggest the potential for PKG signaling as part of an endogenous neuroprotective scheme that regulates neurological function in the presence of acute trauma. In favor of this hypothesis, PKG activity has been implicated in regulating the function of neural circuits, behavior, and susceptibility to stress in insect and vertebrate model systems (Armstrong et al., 2010; Dawson-Scully et al., 2010; Robertson and Sillar, 2009), highlighting its potential as a relevant neuroprotective mechanism to combat a number of physiological stressors and potentially neurological disorders.

Larval motor axons of ventral longitudinal muscles

The *Drosophila* larval NMJ has been used as a model system to study the molecular mechanisms of synapse development, structure, regulation and function. Many basic neuroscience questions concerning ion channel function, neurotransmitter release, vesicle recycling, axonal transport, and functional and structural synaptic plasticity have been teased apart using *Drosophila* larval body wall muscles and its very accessible synapses. Combined with the vast *Drosophila* genetic toolkit and its amenability towards manipulation, the stereotypical innervation pattern of is efferent system has been well-characterized with almost all of its motor neurons being morphologically and physiologically described and target muscles and developmental origins assigned. The ventral longitudinal muscles, muscle 6 and 7, commonly used in physiological

examinations are innervated by two motor axons, Type Ib and Type Is, that both contribute to the postsynaptic response in the muscle.

These motor axons display differences in many morphological features where Type Ib have a larger subsynaptic reticulum and larger boutons with more mitochondria compared to Type Is (Atwood et al., 1993). Apart from their morphological differences, these motor neurons also display distinct physiological disparities. Though Type Ib motor neurons occupy a larger muscle surface area due to their bulky boutons, they induce smaller EJPs compared to those evoked from Type Is terminals in the same muscle (Atwood et al., 1993; Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). While this seems unlikely, many observations can account for this paradox. Type Is boutons have a higher release probability (Atwood et al., 1997), larger synaptic vesicles (Karunanithi et al., 2002), and different composition of glutamate receptors at its terminals (Marrus et al., 2004) compared to Type Ib motor neurons.

Furthermore, these motor neurons display differences in synaptic plasticity and excitability where short trains of pulses induces facilitation and spiking can be evoked with less current input in Type Ib, whereas Type Is display depression and require more current input to induce spiking (Kurdyak et al., 1994; Lnenicka and Keshishian, 2000; Choi et al., 2004). Such cellular refinement of the development, organization, function, and plasticity of the *Drosophila* neuromuscular system combined with its accessibility and array of powerful genetic tools make it an attractive preparation that has been well utilized to better understand neuronal networks.

Motor axon contributions during acute oxidative stress

Recruiting a desired evoked EJP response at the *Drosophila* larval NMJ can be achieved using a lower intensity stimulus, where suprathreshold stimulation of both motor axons is initiated and then the intensity is turned down until the Type 1b axon fails to spike (Kurdyak et al., 1994). The EJP amplitude decline of PKG manipulated animals, whether genetically or pharmacologically induced, during single and dual axon fiber stimulation revealed that there is an abrupt exponential decay during single fiber stimulation. These data suggest that the motor neurons innervating muscle 6 exhibit failure at different times, represented by an immediate drop in the amplitude of the evoked response. Sudden changes like these are believed to be a "dropping out" of one of the axons since the evoked EJP response is the combined output of both motor axons that innervate muscle 6/7 (Zhong and Wu, 1991). The observable differences in the EJP amplitude declines between single and dual axon fiber stimulation can likely be attributed to sudden axonal failure and the evoked EJP reduces at a more gradual rate during dual axon fiber stimulation since it recruits both axons.

Comparison of the latency durations of the evoked EJP responses during single and dual axon fiber stimulation demonstrated a difference between PKG genetic variants during single fiber stimulation, where for^{R} and for^{s2} animals had considerably lower latency values than for^{s} animals at synaptic failure which was not observed during dual axon fiber stimulation. During dual axon fiber stimulation of PKG allelic variants, the latency values at synaptic failure were similar, however, for^{s} and for^{s2} animals displayed

a gradual increase in the latency duration while the rate of increase was accelerated in *for*^{R} animals. These data are not surprising since the *foraging* gene, which encodes PKG activity, has been suggested to regulate neuronal excitability, synaptic transmission and nerve connectivity (Renger et al., 1999). Furthermore, *for*^{s} and *for*^{s2} animals have been shown to elicit spontaneous and supernumerary evoked excitatiory junctional currents (EJCs) with greater amplitudes following stimulation compared to *for*^{R} animals (Renger et al., 1999). Therefore, these data mirror this difference in motor axon excitability where animals with low PKG activity (*for*^{s}, *for*^{s2}) have a more gradual increase in the latency duration compared to those with higher activity (*for*^{R}).

Comparison of the latency durations of the evoked EJP responses of animals with chemically modulated PKG activity during single and dual axon fiber stimulation demonstrated a difference during dual fiber stimulation, where animals with low PKG activity (-PKG) had shorter latency values at synaptic failure than animals with high PKG activity (+PKG). Since the PKG pathway affects K^{+} channel conductance, which ultimately affects a cell's ability to reach threshold (the critical level of depolarization needed to elicit an action potential), these data suggest that manipulating PKG activity alters membrane excitability. Similar to what was observed in *for*^{s} and *for*^{s2} larvae, animals with PKG inhibition had a gradual increase in the latency duration compared to pathway activation. These data demonstrate that reducing K^{+} conductance slows the EJP amplitude decay and latency duration increase, which mediates neuronal excitability and enables synaptic transmission to continue for a longer period of time.

Promoting cell survival during acute oxidative insults

Natural variation in the *foraging* gene, which encodes PKG activity, differentially affects the tolerance of behavior and survival of the fruit fly during acute hypoxia, where inhibition protects neural function at the expense of survival and vice versa. (Dawson-Scully et al., 2010). To further explore this hypothesis, we designed an oxidative injury assay using *Drosophila* S2 cells to demonstrate that PKG modulation can alter cellular survival during acute stress induced by H_2O_2. The overall goal was to design an assay that could be expanded to analyze multiple types of chemical stress agents, multiple cellular mechanisms, and adjusted for any desired time period over multiple hours. These data laid the foundation for another set of experiments performed in collaboration with Olena Makhnyeva that fine-tuned this method to mimic ischemia-reperfusion injury in *Drosophila* S2 cells and probe the mechanism by which PKG activation leads to cellular protection and increases survival. Based off finding from this collaborative project, this assay could be used to analyze the potential role that cell-surface K_{ATP} channels play in PKG-mediated cell protection during hypoxia. Additionally, this pharmacological acute oxidative stress assay can be used to probe the mechanistic differences between inducing stress with different agents such as NaN_3 and H_2O_2.

Identifying novel compounds that protect against oxidant stress and injury

The fruit fly, *Drosophila melanogaster*, is an anoxia-tolerant organism that can withstand drastic oxygen fluctuations and survive in hypoxia (low oxygen) and anoxia

(no oxygen) for hours without pathology (Haddad et al., 1997). Fruit flies can tolerate drastic fluctuations in oxygen levels that cause irreversible injury and cell death in mammals and, therefore, is routinely used for investigations of the molecular mechanisms that underlie hypoxic and oxidative stress resistance (Zhao and Haddad, 2011). Oxidant stress and injury is inherent in many human diseases such as ischemic vascular and respiratory diseases, heart failure, myocardial infarction, stroke, perinatal and placental insufficiencies, diabetes, cancer, and numerous neuropsychiatric and neurodegenerative disorders. Finding novel therapeutics to combat the deleterious effects of oxidative stress is critical to create better therapeutic strategies for many conditions that have few treatment options. Natural products have played an invaluable role within the drug discovery pipeline and aided in the creation of many pharmaceuticals (Cragg et al., 2014). The overall goal of this project was to identify natural products capable of modulating neurotransmission during oxidative stress using *Drosophila melanogaster*.

For the first time, this study demonstrated that a group of marine natural products, pseudopterosins, are able to protect synaptic function at the *Drosophila* larval NMJ. This ability was rivaled by that of the well-known antioxidant, Trolox. These data suggest that pseudopterosins have potential as novel neuromodulatory agents that can protect neurological function during oxidative stress. To explore this hypothesis further, we analyzed pseudopterosin biological distribution within mammalian tissues and found that PsA is rapidly and efficiently distributed into the brain. These data demonstrate that PsA can readily cross the BBB and its physiochemical properties allow it to be administered intravenously, which is the preferred route of administration for the treatment acute

neurological conditions such as traumatic brain injury and stroke. While pseudopterosins have a plethora of biological activities, this is the first demonstration of their ability to modulate synaptic function.

Though these data demonstrate pseudopterosin-induced protection of synaptic function during oxidative stress, the mechanism of action behind these effects is still not clear. Therefore, it is of interest to develop novel bioassays to test for potential neuromodulatory and/or neuroprotective properties of not only these compounds but also natural products in general. The identification of novel drug targets using the fruit fly, *Drosophila melanogaster*, has provided enormous advancements in elucidating the molecular mechanisms behind many human diseases (Bier, 2005; Segelat, 2007; Stilwell and Westlund, 2008). Therefore, the next experiments designed a novel bioimaging/optogenetic approach to identify the mechanism of action of pseudopterosins on synaptic function and neuronal survival during oxidative stress at the well-characterized *Drosophila* larval neuromuscular junction. Using a *Drosophila* based bioimaging/optogenetic approach to determine the mechanism of action of pseudopterosins is an economical, fast and convenient solution in comparison to laborious, time-consuming and expensive mammalian transgenic model systems.

We genetically expressed RGECO/ChR2 in motor neurons at the *Drosophil*a larval NMJ and used calcium imaging to detect pseudopterosin-induced changes in intracellular calcium concentration, which is indicative of synaptic activity. PsA was found to reduce the intracellular calcium increase caused by NaN_3 exposure, indicating

that this optogenetic protocol can be used to examine pseudopterosins for neuromodulatory properties. Additionally, we could potentially determine their mechanism of action using advanced *Drosophila* genetic techniques that allow specific targeting of genes, pathways, and cell surface receptors.

This technique can also be applied for use in different *Drosophila* models, such as primary neural cultures, that will enable us to genetically target specific neuronal populations (serotonergic, dopaminergic, etc) within a group and analyze potential cell specificity and site of action of pseudopterosins. Additionally, using a cell culture system also enables the ability to find novel neuroprotective agents. Furthermore, this approach provides the framework for identifying *in vivo* target(s)/mechanism(s) of action of other natural products. Using *Drosophila* and its advanced genetic and optogenetic techniques to potentially identify novel neuromodulatory and neuroprotective compounds is a novel strategy that could lead to a new areas of research that will further unite natural products chemistry and modern molecular biology.

Taken together, the present study analyzed neuroprotective mechanisms, their potential interactions, and specific motor axon contributions during acute oxidative stress at the *Drosophila* larval NMJ. Two different pharmacological oxidative stress paradigms that induce the production of physiologically relevant oxidant species were utilized. Furthermore, these pharmacological oxidative stress paradigms were used to identify novel neuromodulatory agents.

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