PKCε-Mediated Arc-Dependent Preconditioning and Aging

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PKCε-MEDIATED ARC-DEPENDENT PRECONDITIONING AND AGING

By
Charles H. Cohan

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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PKCε-MEDIATED ARC-DEPENDENT PRECONDITIONING AND AGING

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Cardiac arrest is a leading cause of death in the United States and results in a large population with cognitive deficits. These deficits are due to transient global ischemia and the resulting cell death within the CA1 region of the hippocampus and associated brain regions. The administration of the specific protein kinase C epsilon activator drug, ψε-Receptor for Activated C Kinase (ψεRACK), protects neurons within the CA1 region; however, the mechanism of action has not been fully elucidated. In this study, it was determined that ψεRACK treatment increased brain derived neurotrophic factor (BDNF) protein expression, tropomyosin related kinase B (TrkB) phosphorylation, and the expression of the protein activity-regulated cytoskeleton-associated protein (arc). The neuroprotective effect of ψεRACK is dependent upon TrkB phosphorylation and arc expression. ψεRACK treatment led to electrophysiological changes that were dependent upon arc expression, including decreased mEPSC amplitude and increased latency until anoxic depolarization. Additionally, it was determined that a middle-aged model of cardiac arrest resulted in cell death and behavioral deficits. Furthermore, it was determined that administration of ψεRACK at a higher dosage can overcome age-dependent reduction of arc protein expression and is a therapeutic candidate in the population most affected by ischemic injury, the elderly.
Dedication page:

I would like to dedicate this first and foremost to Holly Stradecki. She has been there for me every day throughout this long journey. She was essential for the completion of this document; she was a helping hand, a critical pair of eyes, and at times a voice of reason. I wouldn’t have made it through my time as a graduate student without her. Also, I would like to dedicate this to my family for their unconditional support and for feigning interest in my work long enough for me to attempt to explain it. Furthermore, I would like to thank my support group of friends who helped keep me sane and often served as a great sounding board. Additionally, I would like to thank Dr. Perez-Pinzon and Dr. Wright, for taking a chance on me and putting me into a great lab environment that allowed me to learn what it means to be a scientist. Finally, I would like to dedicate this to my dog Westen G. Barkingsworth Stradecki Cohan, who always seems excited about my ideas.
Acknowledgement page:

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Chapter 1. Introduction

1.1 Global Cerebral Ischemia and Excitotoxic Injury

Approximately 400,000 people every year suffer an out-of-hospital cardiac arrest (CA) in the United States (Go et al., 2014) with a survival rate of only 8% (McNally et al., 2011a). Aged individuals are the most at risk for CA, as over 80% of CAs occur in individuals over the age of 50 (McNally et al., 2011a). Survivors of CA often suffer from brain damage and irreversible learning and memory deficits (Lim et al., 2004) as a consequence of the global cerebral ischemia-induced neuronal damage (Richmond, 1997). The lack of oxygen and glucose during cerebral ischemia results in bioenergetics failure and leads to a phenomenon known as anoxic depolarization (AD), a sustained and potentially irreversible depolarization of neurons (Leao, 1947, Hansen et al., 1982, Czeh et al., 1993). Vulnerable regions of the brain, such as the Cornus ammonis 1 (CA1) region of the hippocampus, are particularly affected by this type of cerebral ischemia, (Petito et al., 1987), (Horstmann et al., 2010) and cell death can ensue days following this insult (Olsen et al., 1994, Hickey et al., 1996, Neigh et al., 2004).

The main consequence of CA is that it alters cerebral blood flow (CBF) resulting in low oxygen and glucose levels both during and following the insult. CBF alterations decreases mitochondrial adenosine triphosphate (ATP) production (Richmond, 1997) and results in the accumulation of metabolic by-products (Hossmann, 1997). The brain is a particularly susceptible organ to
sustain damage from global ischemia due to its high energy consumption and a need to maintain ion balance while sustaining functional electrical activity. Thus, in the brain, ischemia-related decrease in ATP production results in numerous consequences, including the loss of ion gradients (Hansen, 1985), release of excitatory neurotransmitters (i.e. glutamate), increase in intracellular calcium (Ca\(^{2+}\)) levels, and neuronal excitotoxicity (Choi, 1985).

1.1.1 Ion channel conductance changes

Global cerebral ischemia is a multifaceted disorder that affects the entire brain; however, the majority of research has focused on the hippocampus, as this region is one of the areas affected and is an important mediator in learning and memory (Petito et al., 1987, Calle et al., 1989). Each CA1 neuron of the hippocampus has an estimated 30,000 excitatory and 1,700 inhibitory synaptic inputs (Megias et al., 2001). This enormous amount of excitatory input is one factor that increases the sensitivity of the CA1 neurons to ischemia (Schmidt-Kastner and Freund, 1991). In general, CA1 neurons respond to ischemia in different phases, where initially the onset of oxygen/glucose deprivation (OGD) is associated with a small neuronal depolarization and increase in excitability (Hansen et al., 1982, Fujiwara et al., 1987, Leblond and Krnjevic, 1989). This phase is superseded by a reversible hyperpolarization phase from an increase in K\(^+\) conductance (Hansen et al., 1982, Fujiwara et al., 1987, Leblond and Krnjevic, 1989, Hyllienmark and Brismar, 1999). This hyperpolarization phase consists of different types of K\(^+\) channels, including the Ca\(^{2+}\)-activated K\(^+\) channels and ATP-sensitive K\(^+\) (K\(^+_\text{ATP}\)) channels (Leblond and Krnjevic, 1989).
This hyperpolarization phase is then proceeded by a large depolarization (i.e. AD) that spreads to the surrounding neurons (i.e. hypoxic or spreading depression) stimulating a massive glutamate release (Collewijn and Harreveld, 1966, Fujiwara et al., 1987).

1.1.2 Synaptic changes after global cerebral ischemia

After ischemia, neurons can undergo apoptosis/necrosis or survive by maintaining cellular/ionic homeostasis (Kass and Lipton, 1989, Kobayashi et al., 2003). For neurons that undergo apoptosis/necrosis, H2O and Ca2+ ions accumulate in the cytosol during the AD phase inducing neuronal swelling (Ekstrom von Lubitz and Diemer, 1982, Park et al., 1996, Werth et al., 1998, Jourdain et al., 2002, Kovalenko et al., 2006), mitochondrial swelling (Ekstrom von Lubitz and Diemer, 1982), and free radical damage (Braughler et al., 1985). Unlike other sub-regions of the hippocampus, CA1 neurons undergo delayed neuronal death, which can occur between 2-7 days after ischemia (Kirino, 1982, Petito et al., 1987, Kovalenko et al., 2006).

Non-apoptotic/necrotic neurons after ischemia undergo numerous modifications, including synaptic remodeling. Originally, pre-synaptic terminals were considered to be unaffected by ischemia (Kitagawa et al., 1992, Morioka et al., 1997); however, ischemia induces pre- and post-synaptic terminal alterations (Yokota et al., 2001, Bolay et al., 2002, Jung et al., 2004, Ito et al., 2006). After ischemia, synaptic remodeling occurs immediately through an increased growth of filopodia, synapse formation, projection of spines, and altered protein expression (Jourdain et al., 2002, Jung et al., 2004, Kovalenko et al., 2006).
Results from animals post middle cerebral artery occlusion (i.e. focal ischemia) have suggested that synapsin-I (a protein that dissociates synaptic vesicles from actin filaments) phosphorylation is decreased after ischemia (phosphorylation dissociates vesicles from actin), producing a decrease in neurotransmitter release (Bolay et al., 2002). Additionally, there is a pre-synaptic reduction of the synaptic vesicle associated proteins (synaptosomal associated protein 25 and synaptophysin) two days following ischemia, which would reduce neurotransmission (Ishimaru et al., 2001). In the subsequent days following ischemia, numerous spines swell, become concave, have post-synaptic density thickening, pre-synaptic depletion of synaptic vesicle pools, decreased extracellular space, and decreased synaptic spine density (Ekstrom von Lubitz and Diemer, 1982, Kovalenko et al., 2006). There are also decreases in the number of synapses and spines, total percent volume of axon terminals (Ito et al., 2006), and number of mitochondria per synapse in the CA1 region of the hippocampus (Radenovic et al., 2011).

Synaptic electrophysiological changes also occur after ischemia, which include increased N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor conductance’s (Andine et al., 1988, Hori et al., 1991, Andine et al., 1992, Hori and Carpenter, 1994b, Tsubokawa et al., 1995). This increased conductance has been described as a form of long-term potentiation (LTP) from the glutamate released during ischemia (ischemic LTP, iLTP) (Ai and Baker, 2006, Di Filippo et al., 2008). The molecular mechanisms involved in iLTP are similar to those of traditional LTP including the
activation of NMDA receptors, Ca\textsuperscript{2+} influx, calmodulin kinase 2 activation, and cAMP response element binding protein activation (Andine et al., 1988, Shackelford et al., 1995) reviewed in (Di Filippo et al., 2008)). iLTP is also dependent on the activation of protein kinase C (Hsu and Huang, 1998) and nitric oxide signaling (Huang and Hsu, 1997).

One common electrophysiological neurological problem after global cerebral ischemia is seizures (McNamara, 1979, Calle et al., 1989, Bladin et al., 2000, Kirkham, 2011, Rittenberger et al., 2012). Seizures have been attributed to an increase in hyperexcitability of CA3 neurons (Mittmann et al., 1998, Crepel et al., 2003), most likely from the resting membrane potential of CA3 neurons being permanently depolarized (Congar et al., 2000, Crepel et al., 2003). This depolarized state could originate from a loss of inhibitory neurons in the dentate gyrus or CA3 region and an increase in excitatory glutamate stimulation (Hsu and Buzsaki, 1993, Arabadzisz and Freund, 1999, Epsztein et al., 2006).

Overall, global cerebral ischemia results in a massive glutamate release, stimulating numerous neurons. These neurons process this glutamate signal as an increase in excitatory output (LTP) and respond through an increased growth of spines, synaptic protein levels, and conductance of receptors (Jourdain et al., 2002, Kovalenko et al., 2006).

1.2 Global Cerebral Ischemia and Cognitive Dysfunction

CA and the resulting prolonged transient global cerebral ischemia result in a multitude of cognitive dysfunctions in humans including deficits in memory (Roine et al., 1993, Lim et al., 2004, Horstmann et al., 2010, Alexander et al., 2011),
executive function (Torgersen et al., 2010, Alexander et al., 2011), motor function (Roine et al., 1993, Alexander et al., 2011), and anxiety (Roine et al., 1993, Drysdale et al., 2000, Wachelder et al., 2009, Alexander et al., 2011). In humans, the behavioral outcomes after CA vary depending on the type and length of ischemic injury varying within the individual; nevertheless, some aspects of cognitive dysfunction post-global cerebral ischemia have been characterized. The most susceptible regions of the brain to global cerebral ischemia are the CA1 region of the hippocampus (Petito et al., 1987, Lim et al., 2004, Horstmann et al., 2010), the cerebral cortex (Petito et al., 1987, Horstmann et al., 2010), the prefrontal cortex (PFC) (Horstmann et al., 2010), the thalamus (Horstmann et al., 2010), the cerebellum (Horstmann et al., 2010), and the putamen (Petito et al., 1987). The wide spread nature of global cerebral ischemia limits a researcher’s ability to dissect apart individual brain regions and determine which damaged region is responsible for specific cognitive deficits. Therefore, in order to separate which brain region is responsible for each cognitive deficit, studies using animals have been a necessity.

1.2.1 Spatial memory changes

One of the best defined systems of memory in both humans (Alexander et al., 2011) and animals (Davis et al., 1986, Roberge et al., 2008) is spatial memory. Spatial memory is the ability to learn a surrounding environment and orientation, where this ability is impaired with lesions of the hippocampus (Broadbent et al., 2004) or entorhinal-perirhinal cortex (hippocampus projection to the neocortex) (Nagahara et al., 1995). In animals, a common method for testing spatial memory
is the Morris water maze (Morris, 1984), which involves placing the animal (commonly rodents) in a pool of opaque water with a hidden underwater platform. The animal navigates the pool using spatial cues located outside the pool to find the correct location of the platform. The animal’s ability to learn the location of the platform is tested over a period of days, allowing for the animal to recall the platform location on subsequent trials. Deficits in spatial learning are determined by the time the animal takes to locate the platform and the overall distance traveled. For example, Morris water maze performance after global cerebral ischemia is impaired with an inability to locate the platform compared to sham controls (Olsen et al., 1994, Hickey et al., 1996). This deficit has been attributed to a decreased dendritic spine density and cell death of CA1 neurons (Olsen et al., 1994, Neigh et al., 2004, Hartman et al., 2005).

While the Morris water maze can be a useful technique to determine spatial memory deficits, this test requires animals to swim and could be an issue for animals that suffer from motor dysfunction after cerebral ischemia (Yamamoto et al., 1988, Wahl et al., 1992, Yonemori et al., 1998). Therefore, the Morris water maze may be an unsuitable model for testing animals with impaired motor function. Due to this disadvantage, alternative spatial memory tasks that are less physically demanding have been developed and include the Barnes circular platform maze (Barnes, 1979) and contextual fear conditioning (Wiltgen et al., 2006). In the Barnes circular maze, the animal is tasked with finding a dark box beneath a hole on the outside of a circular platform by using spatial cues (similar to the Morris water maze); deficits in spatial memory will cause and animal to
commit an increased number of incorrect hole entries (e.g. errors) and have longer overall distance traveled to locate the correct hole (Fig. 4A) (Barnes, 1979). Contextual fear conditioning measures freezing behavior of animals when returned to an environment where an electric shock was delivered; reduced freezing in upon return to the environment is indicative of reduced contextual memory (Sun et al., 2009). Overall, the Barnes circular maze and contextual fear conditioning spatial memory tasks may be more suitable in animal models with motor deficits due to the reduced motor requirements to complete the tasks.

1.2.2 Executive function changes

Executive function is a set of neurological processes that control and regulate the ability to organize thoughts, perform tasks, and make decisions. One well defined type of memory required for executive function in humans (Spieker et al., 2012) and animals (Davis et al., 1986) is working memory, the ability to remember (for a brief period of time) items or facts that are distinguishable from previously learned information (Bizon et al., 2009). Working memory is dependent on the PFC in humans (Freedman and Oscar-Berman, 1986) and rodents (Seamans et al., 1998). PFC damage has been identified following global cerebral ischemia in humans (Horstmann et al., 2010) and rodents (Erbil et al., 2008, Garcia-Chavez et al., 2008). Damage to the PFC (Wikmark et al., 1973, Delatour and Gisquet-Verrier, 1996), hippocampus (Stevens and Cowey, 1973, Johnson et al., 1977, Aggleton et al., 1986, Mariano et al., 2009), entorhinal cortex (Ramirez and Stein, 1984), anterior thalamic nuclei (Aggleton et al., 1996), and septum (Fitz et al., 2008) have been suggested to impair executive function.
Working memory after global cerebral ischemia in humans has led to a mix of reports, as some investigators suggest a decrease in working memory performance (Torgersen et al., 2010, Alexander et al., 2011) and others found no working memory deficits (Grubb et al., 1996). Interestingly, minimal work has characterized executive function deficits after global cerebral ischemia in rodents, in comparison to other types of cognitive dysfunction (e.g. spatial memory or motor function). One study has indicated a deficit in T-maze alternation task, a task examining working memory where animals will alternate which arm of T-shaped maze they explore each trial due to a natural propensity to investigate novel areas, months after global cerebral ischemia. However, these results are difficult to interpret as working memory deficits and motivation of these animals were also affected (Kiryk et al., 2011). Although T-maze performance is altered with PFC damage (Delatour and Gisquet-Verrier, 1996), this task may be dependent on additional brain structures that are affected by global cerebral ischemia (i.e. hippocampus) (Johnson et al., 1977), further confounding the relationship between PFC damage and working memory performance. A potential method of studying executive function can employ the Barnes Maze, through determining the search strategy used in order to locate an escape tunnel (O'Leary and Brown, 2009, O'Leary et al., 2011). Overall, the assessment of working memory and other aspects of executive memory after global cerebral ischemia remains understudied and new techniques that accurately assess working memory in animals need to be developed.
1.3 Aging and Global Cerebral Ischemia

The incidence of sudden CA is less than 70 per 100,000 individuals aged 32 – 36 years but steadily increases to over 700 per 100,000 individuals over the age of 82 (Becker et al., 1993, McNally et al., 2011b), where this increased rate of CA is similar among races and sex (Deo and Albert, 2012, Roger et al., 2012).

Epidemiological studies have underscored the importance to study aged animals to better correlate research findings to the human population. A few studies have examined growth curves and survival characteristics of various rat strains, where the lifespan of the laboratory rat varies from strain to strain and between sex (Goodrick et al., 1983, Turturro et al., 1999). For example, Brown Norway/RijNia rats survive an average of 1200 days, while the lifespan of Fisher 344-NIH rats are reduced by 20% (Turturro et al., 1999), and Wistar rats have relatively short lifespan of 600-700 days (Goodrick et al., 1983). Turturro et al. (1999) also noticed that survival curves for gender were non-significant for Brown Norway/RijNia rats; however, female rats had increased lifespan in Fisher 344-NIH rats and decreased lifespan in a hybrid strain derived by crossing Brown Norway/RijNia and Fisher 344 rats as compared to male rats (Turturro et al., 1999). The dilemma in the aging field has been how to “compare the age of rats to humans”. Quinn (Quinn, 2005) indicates that a simple conversion of average human lifespan to rat lifespan may not be appropriate for all ages, because the rate of development in humans and rats contrast during different stages of life. For example, the period of birth to weaning is comparatively longer in rats than in humans, while the period of birth to musculoskeletal maturity is relatively shorter
in rats compared to humans (Quinn, 2005). Similarly, the post-senescence period in rats is relatively short compared to that in humans (Quinn, 2005). Investigators studying the process of aging in rodents consider 50% survival as the correlative age for old humans (i.e. approximately 65 years old) (Walford, 1976, Mos and Hollander, 1987), and as per this criterion, a 24 month-old Fisher 344 rat is considered as an aged rat.

The Stroke Treatment Academic Industry Roundtable (STAIR) preclinical recommendations have emphasized the importance of testing neuroprotective therapies in aged animal models (Fisher et al., 2009). However, most studies examining the effects of CA on the brain use 3-4 month-old rats and only a few studies have used aged rats (Xu et al., 2007, Xu et al., 2008, 2010). Poor post-CA survival is one of the major factors discouraging investigators from performing studies in aged rats. Xu et al. (2010) observed that 4 day post-CA survival in 24 month-old rats is less than 40% compared to approximately 70% survival in a 6 month-old rats (Xu et al., 2010). The presence of age-related diseases prior to the induction of CA and relatively poor recovery may account for poor survival in aged rats. Furthermore, another drawback to aged animals is that a 24 month-old Fisher 344 rat is about 5 times more costly than a 3 month-old one (2010). Another issue with using aged animals is that normal aging can result in cognitive deficits (for review see, (Rosenzweig and Barnes, 2003)), which must be taken into account when designing experiments for age appropriate models of global cerebral ischemia. For example, working memory deficits (see 1.2 Global Cerebral Ischemia and Cognitive Dysfunction, Page 5).
have been reported in aged animals on the delayed alternation task (Mizoguchi et al., 2009). These deficits in aged animals emphasize the importance of using aged matched controls and the potential for inherent variability when examining cognitive dysfunction after global cerebral ischemia. Overall, the epidemiological studies from humans suggests increased incidence of CA in the aged population, where characterizing/developing an appropriate animal model of CA with decent long-term survival remains a major challenge in the field.

1.4 Neuroprotective Strategies and Pharmacological Preconditioning

1.4.1 Post ischemia neuroprotective strategies

Currently, the most widespread use of a neuroprotective strategy following global cerebral ischemia is hypothermia. Hypothermia has been shown to be effective in animal models when administered after global cerebral ischemia. In animal models, acute hypothermia following global cerebral ischemia can result in reduced CA1 cell death and restore cognitive outcomes (Horn et al., 1991, Sterz et al., 1991, Hicks et al., 2000, D'Cruz et al., 2002). Interestingly, hypothermia administered following ischemic injury may delay cell death following ischemia but not prevent it (Dietrich et al., 1993). Despite this finding, in 2003, the advanced life support task force of the international liaison committee on resuscitation made a recommendation based upon two previous clinical trials (2002, Bernard et al., 2002) that mild hypothermia should be administered to adults for 12 to 24 hours following cardiac arrest (2005). Interestingly, a recent clinical trial that administered hypothermia at shorter time frames following cardiac arrest showed no additional benefit in reduction of mortality rate or
improvements in neurological status (Kim et al., 2014). Combining pharmacological treatments with hypothermia before, during, or after cardiac arrest may be an alternative method to further increase protective mechanisms provided by hypothermia.

Inhibition of glutamate receptors has been considered a potential neuroprotective pathway for over 30 years (Simon et al., 1984). When NMDA antagonists were administered immediately following a 2 vessel occlusion (model of global cerebral ischemia), it resulted in decreased cell death in the CA1 region (Simon et al., 1984, Church et al., 1988). Additionally, NMDA antagonism can result in increased cognitive performance and decreases latency until anoxic depolarization (Grotta et al., 1990, Tanaka et al., 1997). Interestingly other groups reported a neuroprotective effect against ischemic injury specifically using AMPAR receptor antagonists but not in NMDA antagonists (Sheardown et al., 1993, Pellegrini-Giampietro et al., 1994). Application of CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione, a competitive AMPA/kainite receptor antagonist) also decreases CA1 cell death and increases latency until anoxic depolarization (Pellegrini-Giampietro et al., 1994, Tanaka et al., 1997). Unfortunately, despite the promise observed in animal models, side effects have been reported in clinical trials against ischemic injury (Albers et al., 1999, Elting et al., 2002) limiting the clinical relevance of these antagonists. However, alternative regulation of glutamate receptors, such as regulating the number of AMPARs present at the synapse, may be a more subtle manipulation with fewer side effects but still provide neuroprotective effects.
1.4.2 Preconditioning strategies for neuroprotection

While the administration of therapeutic agents after global cerebral ischemia is a major target for neuroprotection, there is a large population who may be at risk for cerebral ischemia and would benefit from “preconditioning” (i.e., individuals susceptible to recurrent ischemic events (Mandic and Rancic, 2011, Roger et al., 2012) or high-risk individuals (Shpargel et al., 2008, Alkan, 2009). Preconditioning is the activation of protective cellular mechanisms, whereby the tissue is protected against future ischemic events (hours to days). The first mechanism of preconditioning described was ischemic preconditioning (IPC), where a sublethal ischemic insult protects neurons against a subsequent lethal insult (Murry et al., 1986, Schurr et al., 1986). Numerous compounds have the ability to precondition neurons by activating pathways similar to IPC (Table 1).
<table>
<thead>
<tr>
<th>Category</th>
<th>References</th>
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<tr>
<td>Adenosine agonists</td>
<td>(Heurteaux et al., 1995, Blondeau et al., 2000)</td>
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<td>Antibiotics</td>
<td>(Koerner et al., 2007); Reviewed in (Fagan et al., 2011)</td>
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<td>ATP-sensitive K⁺ (K⁺&lt;sub&gt;ATP&lt;/sub&gt;) channel agonist</td>
<td>(Perez-Pinzon et al., 1996, Reshef et al., 1998, Blondeau et al., 2000)</td>
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<tr>
<td>Ca²⁺-activated K⁺ channels</td>
<td>(Allen et al., 2011)</td>
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<td>Diazoxide</td>
<td>(Shake et al., 2001)</td>
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<tr>
<td>GABA receptor antagonist</td>
<td>(Tauskela et al., 2008)</td>
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<td>PKC epsilon (PKCε) agonist</td>
<td>(DeFazio et al., 2009)</td>
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<tr>
<td>Electroacupuncture</td>
<td>(Yang et al., 1999, Pang et al., 2003); Reviewed in (Li et al., 2012)</td>
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<td>Ethanol</td>
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<td>Ginkgo biloba</td>
<td>(Nada and Shah, 2012)</td>
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<td>Iron chelators</td>
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<td>K⁺ channel antagonist</td>
<td>(Tauskela et al., 2008)</td>
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<td>Magnesium</td>
<td>(Chan et al., 2005)</td>
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Table 1: Agents or methods used to induce preconditioning.

Numerous studies have indicated the involvement of NMDA receptor activation or modulation as a prerequisite for preconditioning (Kato et al., 1992, Bond et al., 1999, Raval et al., 2003, Yin et al., 2005, Goto et al., 2006, Zhang et al., 2009, Navon et al., 2012). Preconditioning-induced activation of synaptic NMDA receptors (not AMPA receptors) (Bond et al., 1999) activates pro-survival extracellular signal-regulated kinase (Navon et al., 2012), increases brain-derived neurotrophic factor release (Marini et al., 1998, Jiang et al., 2005), decreases
heat shock protein 70 (Saleh et al., 2009), and decreases glucose-regulated protein 94 for neuroprotection (Saleh et al., 2009). However, not all researchers have found this NMDA receptor dependence (Duszczyk et al., 2005) or have suggested that NMDA receptors activation is only partly responsible for IPC-induction (Prior et al., 2005). The differing results between these studies may be dependent on the type of preconditioning used; additionally, IPC may induce numerous modifications for preconditioning and may not be exclusively dependent on NMDA receptor activation.

Beyond NMDA receptor activation, numerous synaptic targets (direct or indirect) can induce neuroprotection (Fig. 3). For example, treatment with plasmalemma K$^{+}$ATP channel agonist (i.e., cromakalim) 3 days prior to IPC in vivo (global cerebral ischemia) induced neuroprotection in the CA1 and CA3 region of the hippocampus (Blondeau et al., 2000). Another study suggested that preconditioning with 3-nitropropionic acid (mitochondrial respiration chain inhibitor) modulates synaptic K$^{+}$ATP channels to delay the onset of hypoxic depolarization, decreasing the excitotoxic glutamate release for in vitro neuroprotection (Nakagawa et al., 2002). Adenosine A1 receptor agonists (e.g., N$^{6}$-cyclopentyladenosine) induce immediate neuroprotection through K$^{+}$ATP channel opening (Heurteaux et al., 1995, Perez-Pinzon et al., 1996, Reshef et al., 1998); additionally, in vivo administration of adenosine A1 receptor agonist 3 days prior to global cerebral ischemia induced neuroprotection through enhanced expression of heat shock protein 70 (Blondeau et al., 2000) suggesting differential preconditioning mechanisms of the adenosine A1 receptors. Also, the
Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel agonist, 1-ethyl-benzimidazolinone, administered 30 min before global cerebral ischemia induced neuroprotection by maintaining of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel expression and activity (Allen et al., 2011). Interestingly, the contribution of AMPARs to preconditioning mechanisms has been understudied. Research has shown that lethal ischemia can increase the calcium permeability of AMPARs in the CA1 region of the hippocampus due to a decrease in the subunit responsible for gating calcium, GluR2 (Pellegrini-Giampietro et al., 1992). Interestingly, IPC can reduce this increase in calcium permeability as observed after the injury (Tanaka et al., 2002). Despite this evidence and the evidence that AMPAR antagonism can inhibit cell death after a lethal ischemic event (Sheardown et al., 1993, Pellegrini-Giampietro et al., 1994), preconditioning mechanisms examining the regulation of AMPAR currents have not been elucidated.

1.5 The Role of PKC\textepsilon, BDNF and Arc in Preconditioning and Neuroprotection

Another preconditioning method is the activation of protein kinase C epsilon (PKC\textepsilon). PKC\textepsilon is a calcium-independent novel PKC (Ono et al., 1988), for review see (Mochly-Rosen et al., 2012) activated by IPC and is necessary for the neuroprotective effect of IPC (Raval et al., 2003, Dave et al., 2008). PKC\textepsilon preconditioning increases the average amplitude of GABA-related inhibitory post-synaptic events (DeFazio et al., 2009), maintains ion homeostasis during ischemia by inhibiting voltage gated Na\textsuperscript{+} channels and the expression of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (Dave et al., 2009), improves cerebral blood flow (Della-Morte et
al., 2011), and phosphorylates the mitochondrial $K^+_{ATP}$ channel (Raval et al., 2007). The plethora of responses that are produced from PKCε activation indicates the necessity for a preconditioning agent to have numerous targets for neuroprotection. As ischemia induces several pathological cascades, a pharmacological preconditioning agent that would allow for numerous systems to be modulated/activated could combat several of these dysfunctional cascades to induce neuroprotection.

PKCε preconditioning and the neuroprotection it affords, has recently been shown to be dependent upon the brain derived neurotrophic factor (BDNF) signaling pathway (Neumann et al., 2015). BDNF, a member of the nerve growth factor family (Barde et al., 1982), is a 14 kDa protein that is cleaved from a 30 kDa pro-neurotrophin form (Seidah et al., 1996). BDNF is a ligand for the tropomyosin related kinase B (TrkB) and p75 neurotrophin (NTR) receptors (Klein et al., 1991, MacPhee and Barker, 1997). The uncleaved proform of BDNF, when bound to the p75 receptor, has previously been shown to trigger apoptosis (Teng et al., 2005) and may play a role in triggering long term depression (Woo et al., 2005). Within the field of ischemia, binding of BDNF to TrkB has been previously been shown to mediate its neuroprotective effects in the disease models of both focal (i.e. stroke) and global ischemia (Kiprianova et al., 1999a, Saarelainen et al., 2000). The targets modified by BDNF signaling are diverse as it is known to promote changes at the synapse, in nuclear transcription, and in mitochondrial function (Tyler and Pozzo-Miller, 2001, Groth and Mermelstein, 2003, Markham et al., 2004). Increases in BDNF expression
increase AMPAR miniature excitatory post synaptic current (mEPSC) frequency and also promote an increase in synapse number in CA1 neurons when continuously administered for 24-72 hours (Tyler and Pozzo-Miller, 2001, Tyler and Pozzo-Miller, 2003). As previously mentioned, it was recently determined that activation of PKCε can lead to increased BDNF expression (Neumann et al., 2015). Additionally, IPC can lead to an increase in BDNF mRNA levels indicating that BDNF is an essential component to preconditioning paradigms (Truettner et al., 2002). However, the exact mechanism by which BDNF confers neuroprotection during preconditioning has not previously been elucidated.

BDNF expression is capable of increasing the expression of an important immediate early gene (IEG) activity-regulated cytoskeleton-associated protein (arc). Arc, which was discovered in 1995, was originally shown to be activated by cellular activity (Lyford et al., 1995). It was discovered that both short and prolonged bouts of BDNF expression led to the increased expression of arc protein (Messaoudi et al., 2002, Ji et al., 2010). Interestingly, the duration of BDNF signaling activation was found to result in very different levels of arc expression (Ji et al., 2010). Increases in arc expression have been speculated to be neuroprotective (Otsuka et al., 2009). When arc is expressed in COS-7 cells, it sequesters the apoptosis inducing protein AMIDA in the nucleus, preventing it from triggering cell death (Irie et al., 2000). The best characterized actions of arc deal with its role at the synapse. Increased expression of arc has previously been found to increase AMPAR glutamate receptor endocytosis (Chowdhury et al., 2006) and decrease AMPAR expression (Okuno et al., 2012). Recently, this
mechanism of action has been linked with the ability of arc to bind to TARPy2, a protein known for its ability to regulate the length of time AMPARs remain in the synapse (Kaibara and Koga, 1989). As previously mentioned, AMPA antagonism can delay latency until anoxic depolarization (Tanaka et al., 1997). Thus, the ability of arc to regulate AMPARs may have neuroprotective benefits against ischemic injury.

1.6 Concluding Remarks and Hypothesis

Our central hypothesis that increased BDNF levels through PKCε activation enhances an age-dependent arc expression, thereby delaying the latency until AD through an AMPAR-dependent mechanism conferring protection against a lethal ischemic injury. This project will uncover a novel neuroprotective mechanism in preconditioning that may help develop new treatments for ischemic injury, while explaining the reduced effectiveness of preconditioning caused by aging. Furthermore, it will focus on optimizing the effects of PKCε preconditioning for the age group most at risk for CA, the elderly.
Chapter 2. BDNF and arc are necessary for PKCε-dependent neuroprotection

2.1 Summary

PKCε activation has previously been found to be neuroprotective in susceptible brain regions against a lethal OGD; however, the mechanism behind its neuroprotection has not been completely elucidated. In this chapter, the role of BDNF and arc are examined in PKCε-mediated neuroprotection against OGD in the hippocampus. It was determined that BDNF protein expression was increased 6 hours following administration of the PKCε activator ψε-Receptor for Activated C Kinase (ψεRACK). Additionally, the downstream signaling receptor TrkB showed increased phosphorylation. Furthermore, in an organotypic hippocampal cultured slice model, when TrkB receptor signaling was inhibited using the specific TrkB inhibitor ANA-12, PKCε-mediated neuroprotection was blocked. In addition to increases in BDNF, increases in arc protein expression were observed in the hippocampus 48 hours following administration of ψεRACK by both immunofluorescence and western blotting experiments. Furthermore, when arc protein expression was inhibited using arc antisense oligodeoxynucleotides (arc AS ODNs), PKCε-mediated neuroprotection was also impaired. This is the first report of arc AS ODN inhibition impairing a neuroprotective effect provided by a pharmacological agent.
2.2 Introductory Remarks

PKCε activation has previously been found to be neuroprotective against ischemic injury (Lange-Asschenfeldt et al., 2004, Neumann et al., 2015) and has also previously been found to be necessary in several forms of preconditioning including ischemic preconditioning and NMDA preconditioning (Raval et al., 2003). Previous research has found increased BDNF mRNA (Truettn er et al., 2002) and protein expression (Neumann et al., 2013) following administration of the specific PKCε activator, ψεRACK. Additionally, MEK/ERK activation has been implicated as a necessary step in PKCε-mediated neuroprotection against ischemic injury (Lange-Asschenfeldt et al., 2004, Kim et al., 2010). Interestingly, a BDNF/TrkB/MEK/ERK signaling pathway is involved in the expression of another potentially neuroprotective protein, arc (Yin et al., 2002). Arc decreases AMPA receptor (AMPARs) number and currents at excitatory synapses (Rial Verde et al., 2006) and protects neurons against the expression of an apoptosis inducing protein, AMIDA, in COS-7 cells (Irie et al., 2000). The role of TrkB signaling and arc protein expression in preconditioning-mediated neuroprotection has not previously been investigated. In this chapter, we investigated if TrkB phosphorylation and arc expression were increased in the hippocampus following administration of ψεRACK in vivo, and determined if either were necessary for neuroprotection against OGD in organotypic slices in vitro.
2.3 Materials and Methods

In vivo injections

For in vivo injections, Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were injected intraperitoneal (i.p.) with 0.2 mg/kg (25 day old rats) or 0.5 mg/kg (3 month old rats) of the PKCε-specific activator, ψεRACK (TAT-conjugated; KAI Pharmaceuticals, San Francisco, CA, USA) or with TAT peptide control (KAI Pharmaceuticals). Brains were used for western blotting and immunofluorescence experiments. Unless specified, all chemicals were purchased from Sigma-Aldrich.

Western blotting

Tissue was homogenized in RIPA buffer solution (pH 8.0) containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, supplemented with 1% protease (Sigma) and 1% phosphatase inhibitor (Roche Molecular Systems Inc., Branchburg, NJ, USA) cocktails. Lysates were centrifuged at 16,000G for 15 minutes. Supernatant was collected and the protein concentration determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein samples (30 or 40 µg) were separated on a 10 or 12% SDS-PAGE gel. Proteins were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad). Blots were then blocked in 5% milk in tris-buffered saline with 1% tween then incubated overnight in primary antibody in 5% milk. Blots were washed then incubated for 1 hour in horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary (Sigma). Antibodies used include: actin
(Sigma, 1:5000), BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200), TrkB (Cell Signaling Technologies, Boston, MA, USA, 1:1000), phosphorylated TrkB (pTrkB) (Millipore, 1:1000), and arc (Santa Cruz, 1:200).

**Immunofluorescence**

Rats underwent transcardial perfusion under anesthesia. Rats were perfused first with saline followed by 4% paraformaldehyde and post-fixed overnight. Brains were removed, cryoprotected, frozen on dry ice, then cryosectioned (30 µm). Coronal hippocampal sections were taken beginning at -3.8 mm bregma. Sections were washed in PBS with 0.8% triton X-100 (PBST) three times and then were blocked overnight at 4°C using a 10% goat serum PBST solution. Following blocking, sections were washed 3 times in PBST then incubated with 1:500 NeuN (Millipore) and 1:50 Arc (H-300 Santa Cruz) in PBS for 72 hours. Slices were then washed in PBS 3 times and secondary anti-mouse alexa fluor 568 (Life Technologies, Grand Island, NY, USA, 1:500) and anti-rabbit hilyte fluor 488 (AnaSpec, 1:500) were applied for 2 hours at room temperature in the dark. Slides were mounted using Prolong Antifade reagent (Molecular Probes, Carlsbad, CA, USA) and visualized using confocal microscopy at 150X magnification. Images were acquired with FLUOVIEW FV1000 (Olympus).

**Organotypic hippocampal cultured slice preparation**

Hippocampal cultured slices were prepared as previously described (Raval et al., 2003). Briefly, P9-13 pups were anesthetized using 1 mg/kg ketamine and decapitated, then hippocampi were isolated and placed on a tissue
chopper. Transverse slices (400 µm) were obtained and placed into 4°C Gey’s Balance salt solution (Sigma). Slices with distinct and intact hippocampal morphology containing the dentate gyrus, CA3, and CA1 region were then plated onto 0.4 µm Millicell well inserts (Millipore) in 1 mL of media containing 25% horse serum, 25% Hanks balanced salt solution (HBSS), 50% minimal essential media , supplemented with 1 mM glutamine and 30 mM glucose. Media was changed one day following plating and subsequently twice a week. Experiments began 14 days after culturing. Slices were used for electrophysiology, western blotting experiments, or neuronal death experiments after OGD.

**Drug administration for hippocampal cultured slices**

For PKCε-activation, 200 nM of ψεRACK (Raval et al., 2003) was added to each well for 60 minutes then removed and fresh media added. Slices were kept at 36°C throughout the experiment. For experiments blocking tropomyosin-related kinase B (TrkB), the specific TrkB inhibitor, ANA-12 (Cazorla et al., 2011) (10 µM), was administered 24 hours prior to ψεRACK treatment. To prevent the increase of arc expression following PKCε-activation, 1 nM arc antisense oligodeoxynucleotide (arc AS ODN) or 1 nM scrambled control ODN (SC ODN) was added to each well 24 hours prior to administration of ψεRACK or TAT treatment, The sequence 5’-GTCCA GCTCCATCTGCTCGC-3′ (arc AS ODN) or 5’-CGTGCACCTCTCGCAGCTTC-3′ (SC ODN) was used, where the three outer most linkages contain phosphorothioate linkages (Midland Certified Reagent Company, Midland, TX, USA) (Guzowski et al., 2000, McIntyre et al., 2005,
Ploski et al., 2008). ODNs were prepared in phosphate-buffered-saline (PBS) (pH 7.4).

**Oxygen and glucose deprivation**

Slices were washed three times in glucose-free HBSS containing 1.26 mM CaCl$_2$ · 2H$_2$O, 5.37 mM KCl, 0.44 mM KH$_2$PO$_3$, 0.49 mM MgCl$_2$, 0.41 mM MgSO$_4$ · 7H$_2$O, 136.9 mM NaCl, 4.17 mM NaHCO$_3$, 0.34 mM Na$_2$HPO$_4$ · 7 H$_2$O, 30 mM sucrose. Slices were then placed in fresh glucose-free media and moved to a sealed chamber. Oxygen was removed by flushing the chamber with 90% N$_2$/5%CO$_2$ /5%H$_2$ gas at 36°C for 5 minutes. The slices were then moved into a second sealed incubator containing the same air gas mixture where the temperature was 36°C for 35 minutes. Following oxygen and glucose deprivation (OGD), slices were removed and returned to fresh culturing media containing glucose as described above (Raval et al., 2003), (DeFazio et al., 2009).

**Propidium iodide measurements of cell death**

For each treatment, slices were incubated in culture medium supplemented with 2 µg/mL propidium iodide (PI). PI fluorescence was measured at 4 time points: (1) prior to ODN administration, (2) prior to OGD, (3) 24 hours after OGD, and (4) 24 hours following a 1 hour application of 500 µM NMDA application (causing complete cell death of neurons). Images of the CA1 region were captured with an Olympus IX50 fluorescent microscope using SPOT CCD camera and software and quantified with ImageJ. Cell death was quantified as %
PI fluorescence expressed as \( \frac{\text{post OGD} - \text{prior to OGD}}{\text{post NMDA} - \text{prior to OGD}} \times 100 \) (Lange-Asschenfeldt et al., 2004).

2.4 Results

2.4.1 \( \psi \varepsilon \text{RACK} \) increases hippocampal BDNF expression and TrkB phosphorylation in vivo.

Recently, our laboratory reported that BDNF protein expression increases following PKC\( \varepsilon \)-activation \textit{in vitro} (Neumann et al., 2015). To test the hypothesis that PKC\( \varepsilon \) increases BDNF expression \textit{in vivo}, 25 day old Sprague Dawley rats were administered \( \psi \varepsilon \text{RACK} \) (0.2 mg/kg; i.p.) and hippocampal expression of BDNF and tropomyosin related kinase B phosphorylation (pTrkB) were measured 6, 24, and 48 hours following administration via western blot (Fig. 1 A). Following PKC\( \varepsilon \)-activation of \( \psi \varepsilon \text{RACK} \), BDNF expression was significantly increased 2.11 +/- 0.35 fold at 6 hours and 5.96 +/- 0.11 fold at 24 hours compared to a TAT peptide \( (n = 4, p<0.05, p< 0.005 \text{ Student’s t-test}) \) (Fig. 1 B). 48 hours following administration of \( \psi \varepsilon \text{RACK} \), there was no observed change in BDNF protein levels compared to a TAT peptide administered control \( (n = 5, p>0.05, \text{ Student’s t-test}) \) (Fig. 1 B). TrkB phosphorylation (pTrkB) was increased 2.94 +/- 0.32 fold compared to a TAT peptide at 24 hours following \( \psi \varepsilon \text{RACK} \) administration \( (n=4, p<0.01, \text{ Student’s t-test}) \) (Fig. 1 C). There were no observed changes in TrkB phosphorylation at 6 or 48 hours following \( \psi \varepsilon \text{RACK} \) treatment \( (n=5, p>0.05, p>0.05 \text{ Student’s t-test}) \).
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Figure 1. ΨεRACK administration increases BDNF protein levels and TrkB phosphorylation in the hippocampus in vivo. 25 Day old Sprague Dawley rats were treated with 0.2 mg/kg of the Protein kinase C ε specific activator, ΨεRACK, or TAT peptide control. (A) Whole cell homogenates were obtained 6, 24, or 48 hours following treatment and BDNF, pTrkB, and TrkB levels were quantified via western blot. (B) BDNF levels were increased compared to a TAT peptide at 6 and 24 hours respectively following ΨεRACK injection compared to TAT (n = 4, p<0.05, p<0.005 Student’s t-test). (C) TrkB phosphorylation was increased compared to a TAT peptide at 24 hours following ΨεRACK administration (n=4, p<0.05, Student’s t-test). There were no observed changes in BDNF or TrkB phosphorylation observed at 48 hours.

2.4.2 ΨεRACK increases hippocampal BDNF expression and TrkB phosphorylation in vivo and is necessary for PKCε-mediated neuroprotection.

Due to our findings that BDNF and TrkB phosphorylation were increased in vivo following administration ΨεRACK, we hypothesized that PKCε-dependent activation of TrkB was necessary for conferring neuroprotection against a lethal
OGD. In our previous study, we found that PKCε-mediated neuroprotection was dependent upon Trk receptor activation by using a non-specific Trk inhibitor K252a (Neumann et al., 2015). To test our hypothesis that specifically TrkB receptor activation is necessary in conferring neuroprotection against OGD following administration of ψεRACK, we used an in vitro model of the hippocampus, the organotypic hippocampal cultured slice (Raval et al., 2003), and used a specific TrkB receptor inhibitor, ANA-12 (Cazorla et al., 2011). Organotypic slices were supplemented with ANA-12 (10 µM) or DMSO control (0.01%) 24 hours prior to administration of ψεRACK (200 nM) or TAT control peptide (200 nM) in order to inhibit TrkB receptor due to early changes in BDNF expression seen at 6 hours. Slices were subject to a lethal OGD 48 hours after ψεRACK administration and cell death was expressed as a percentage of maximum cell death using PI (2ug/mL). Inhibiting TrkB with ANA-12 impaired the protective effect of ψεRACK. With ψεRACK + ANA-12 treatment there was 60.67 +/- 12.11 % CA1 cell death as compared to 20.80 +/- 6.20 cell death with ψεRACK + a DMSO control treatment (n = 7, p<0.005 Student’s t-test) (Fig. 2 A,B).
Figure 2. TrkB phosphorylation is necessary for ψεRACK dependent neuroprotection. In organotypic hippocampal cultured slices, the TrkB pathway was inhibited using the specific inhibitor ANA-12 during and following a one hour 200 nM administration of ψεRACK 48 hours prior to a lethal 35 minute oxygen and glucose deprivation (OGD). Example images of cell death observed via PI staining for (A) TAT, (B) ψεRACK (C) ψεRACK + DMSO, and (D) ψεRACK + ANA-12 groups after being subjected to a lethal OGD. Example images of the same slices following a 500 µM NMDA treatment for (E) TAT, (F) ψεRACK (G) ψεRACK + DMSO, and (D) ψεRACK + ANA-12 groups. (I) Neuroprotection was impaired in organotypic hippocampal cultured slices when ψεRACK + ANA-12 was administered compared to its ψεRACK + DMSO control (n = 7-8, p<0.01, ANOVA, Bonferroni).

2.4.3 PKCε increases hippocampal arc expression in vivo.

BDNF expression has previously been shown to increase the expression of the potentially neuroprotective protein arc. (Irie et al., 2000, Ying et al., 2002). We hypothesized that administration of ψεRACK increases hippocampal arc
expression *in vivo*. To test this hypothesis, we injected 0.2 mg/kg of ψεRACK (the specific PKCε activator). ΨεRACK administration increased arc expression 2.6 +/- 0.46 fold compared to a TAT peptide treated control in 25 day old Sprague Dawley rats (n = 4, p<0.05, Student’s t-test) (*Fig. 3 A,B*). To determine if this PKCε-mediated increase in arc expression occurred in the region of the hippocampus that is most susceptible to ischemia (i.e., CA1 pyramidal cell layer), we carried out immunohistochemistry. CA1 pyramidal cells were probed for arc and the neuronal marker, NeuN (*Fig. 3 C,F*). To determine if PKCε-activation increases arc expression in adult rats, we administered 0.5 mg/kg of ψεRACK in 3 month old Sprague Dawley rats and found that PKCε-activation increased the intensity of arc expression in the cell body of CA1 pyramidal neurons (*Fig. 3 D-H*)
Figure 3. \(\psi\)εRACK administration increase of arc protein levels 48 hours in CA1 neurons in vivo. 25 Day old Sprague Dawley rats were treated with 0.2 mg/kg tat peptide, The Protein kinase C \(\varepsilon\) (PKC\(\varepsilon\)), activator \(\psi\)εRACK, or TAT peptide control. (A) Whole cell homogenates were obtained 48 hours following and activity-regulated cytoskeleton-associated protein (arc) levels were quantified via western blot. (B) Arc proteins levels were elevated fold compared to a TAT peptide treated control in 25 day old Sprague Dawley rats (\(n = 4\), \(p<0.05\), Student’s t-test). (C-H) To determine that this pathway remained active in adult rats, 3 month old Sprague Dawley rats were injected with 0.5 mg/kg \(\psi\)εRACK i.p. Immunofluorescence Images were obtained 48 hours following administration of \(\psi\)εRACK. Sections were stained for NeuN and arc protein to verify CA1 pyramidal neurons (\(n = 3\)). A visual increase in somatic arc protein was observed.

2.4.4 PKC\(\varepsilon\)-dependent arc expression is necessary for PKC\(\varepsilon\)-mediated neuroprotection against oxygen and glucose deprivation in organotypic hippocampal cultured slices.

In order to determine the importance of arc in PKC\(\varepsilon\)-mediated neuroprotection, arc protein expression was inhibited in organotypic slices prior to PKC\(\varepsilon\)-activation by administering an arc AS ODN peptide (a well characterized technique to inhibit arc expression, (Guzowski et al., 2000). Arc AS ODN decreased protein expression of arc detected via western blot 56.84 +/- 24.08% of SC ODN (\(n = 3\), \(p<0.05\), paired t-test) (Fig. 4 A,B). \(\psi\)εRACK administration protected slices from lethal OGD, reducing cell death from 71.89 +/- 5.33% in the Tat control to 29.91 +/- 10.92% in the \(\psi\)εRACK treated slices (\(n = 6\), \(p<0.005\) ANOVA, Bonferroni) (Fig. 5 A,B,E). The \(\psi\)εRACK + SC ODN group was also protected against a lethal OGD, 35.91 +/- 5.97% cell death within the CA1 region was observed. In comparison, the \(\psi\)εRACK + Arc AS ODN group showed an abolishment of this protective effect with 74.93 +/- 4.24% cell death in the CA1 region (\(n = 6\), \(p<0.005\) ANOVA, Bonferroni) (Fig. 5 C,D,E).
Figure 4. Arc AS ODNs decrease arc expression in organotypic hippocampal cultured slices. (A) Organotypic hippocampal cultured slices were treated with arc antisense oligodeoxynucleotide (Arc AS ODN) (1 nM) or a scrambled control oligodeoxynucleotide (SC ODN) (1 nM) for 24 hours. (B) Arc protein expression was quantified via western blot and a decrease in arc protein expression was observed following 24 hours of Arc AS ODN treatment compared to control scrambled control (n = 3, p<0.05, paired t-test).
Figure 5. Inhibition of Arc blocks PKCε-dependent neuroprotection in organotypic hippocampal cultured slices. Example images of cell death observed via PI staining for (A) TAT, (B) ψεRACK (C) ψεRACK + SC ODN, and (D) ψεRACK + Arc AS ODN groups after being subjected to a lethal OGD. The same slices following administration of a lethal dose of NMDA (500 µM), (E) TAT, (F) ψεRACK, (G) SC ODN + ψεRACK, (H) Arc AS ODN + ψεRACK following a lethal NMDA application Organotypic hippocampal cultured slices were treated with TAT peptide (200 nM), ψεRACK (200 nM), Arc AS ODN + ψεRACK or a SC ODN + ψεRACK. Cell death in the CA1 region was observed 24 hours following the lethal OGD using a 2ug/mL concentration of propidium iodide (PI). (I) ψεRACK administration protected slices from a lethal oxygen and glucose deprivation (OGD), reducing PI fluorescence (expressed as a % of maximal CA1 cell death 24 hours following NMDA administration) (n = 6 p<0.005 ANOVA, Bonferroni). Inhibition of arc with an arc AS ODN impaired ψεRACK-dependent neuroprotection against a lethal OGD reducing its protective effect within the CA1 region of the hippocampus (n = 6, p<0.05 ANOVA, Bonferroni).
Chapter 3. PKCε activation regulates AMPAR currents and latency until anoxic depolarization through an arc-dependent mechanism

3.1 Summary

In order to determine the mechanism by which arc mediated the neuroprotective effect triggered by PKCε activation, a number of electrophysiology outcomes were examined due to arc's known role as a mediator of synaptic strength. Using organotypic hippocampal cultured slices, it was determined that ψεRACK administration decreased the size of AMPAR mEPSC after 48 hours. Additionally, if arc expression was impaired using arc AS ODNs, this reduction in AMPAR mEPSC current was lost. Similarly, following administration of ψεRACK, there was an arc-dependent delay in latency until anoxic depolarization in organotypic hippocampal slices when exposed to a permanently anoxic and aglycemic condition. Although a change in latency until anoxic depolarization was observed, it was not correlated to action potential threshold, amplitude, or maximum sodium current, all of which have been suggested to play a role in latency until anoxic depolarization. Furthermore, administration of arc AS ODNs did not cause any significant changes in these observed parameters. Additionally, there were no observed changes in calcium permeability indicating that there was no ψεRACK-dependent change in AMPAR subunit receptor surface expression.
3.2 Introductory Remarks

Previous research in our laboratory has determined that activation of the epsilon isoform of protein kinase C (PKCε) is necessary for the neuroprotective effect of IPC and that application of a specific activator of PKCε, ψε-Réceptor of Activated C Kinase (ψεRACK), can be used as a neuroprotective, pharmacological preconditioning agent (Raval et al., 2003) (Sun et al., 2013). Activation of PKCε can also modulate the synapse by increasing the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) (DeFazio et al., 2009). The combination of PKCε and PKCα activation with the drug byrostatin has previously been shown to increase spontaneous IPSCs in CA1 hippocampal neurons (Xu et al., 2014), synaptogenesis following stroke in neonatal rats (Sun et al., 2008), and survival in aged rats following stroke (Tan et al., 2013). The mechanism through which PKCε-specific activation confers neuroprotection by modulation of excitatory synapses has not previously been investigated despite its known roles in regulation of synaptic activity.

Previous research in our lab found that two days following an application of ψεRACK brain derived neurotrophic factor (BDNF) protein expression increased, CA1 neuronal action potential firing rates decreased, and latency until AD increased (Neumann et al., 2015). BDNF protects neurons against hypoxia through a mitogen activated protein kinase (ERK)/mitogen activated protein kinase kinase (MEK)-dependent pathway (Han and Holtzman, 2000). In Chapter 2 (see Page 22), it was uncovered that PKCε activation with ψεRACK was capable of driving the expression of the protein arc, a known regulator of AMPAR
currents. Previous research has determined that inhibiting AMPAR currents through AMPA antagonism is capable of conferring neuroprotection (Sheardown et al., 1993, Kawasaki-Yatsugi et al., 1997) and additionally delaying latency until anoxic depolarization (Tanaka et al., 1997). Thus, it was investigated if PKCε-dependent changes in arc expression were responsible for regulation of AMPAR mEPSCs and/or played a role in increasing latency until anoxic depolarization. Furthermore, the ability of arc to cause the internalization of specific GluR subunits was examined (Chowdhury et al., 2006, Rial Verde et al., 2006, Shepherd et al., 2006). Lastly, a relationship between action potential properties and latency until anoxic depolarization was also examined as inhibition of sodium current by tetrodotoxin can delay latency until anoxic depolarization (Raley-Susman et al., 2001).

3.3 Materials and Methods

**AMPA miniature excitatory postsynaptic AMPAR current, AMPAR glutamate triggered current, and calcium rectification measurements**

Whole-cell voltage clamp was used to record and observe miniature excitatory postsynaptic (mEPSC) AMPAR currents. Organotypic hippocampal cultured slices were submerged in bath perfusing external solution containing 150 mM NaCl, 3.0 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.3 mM NaPO₄, 25 mM NaHCO₃, 10 mM glucose, 1 µM tetrodotoxin, 10µM bicuculine, 20µM APV, and bubbled with 95% O₂/5% CO₂. The internal solution of the pipette contained 122.5 mM CsMSO₄, 10 mM CsCl, 10 mM HEPES, 10 mM EGTA, 10 mM
glucose, 8 mM NaCl, 2 mM MgATP, 1 mM CaCl₂, and 0.3 mM NaGTP at pH 7.4 (with NaOH) and 317 mOsm. Bicuculine, (D-(-)-2-amino-5-phosphonopentanoic acid (APV), and tetrodotoxin (TTX) were added to the external solution to inhibit GABA receptor currents, NMDA receptor currents and spontaneous EPSCs respectively. Pipettes (2-5 MΩ) made from borosilicate glass capillaries (Word Precision Instruments) were used. CA1 hippocampal pyramidal neurons were exclusively targeted and visualized using Alexa 488 dye. One cell was patched per slice equating to an n of 1. A GΩ seal was obtained followed by a whole cell configuration in voltage clamp mode. Access resistance was maintained under 20 MΩ and recordings that drifted more than 4 MΩ were discarded. Two minute gap free recordings were obtained in current clamp mode using the program pCLAMP 9.0 (Molecular Devices, USA). The first 100 miniature events over each gap free recording for each cell were used for analysis of maximum amplitude, miniature maximum amplitude distribution, and event frequency. If less than 100 events occurred over the course of a 2 minute recording, the total number of events in the recording was used. mEPSCs were selected using Clampfit event detection software using a custom made filter. For glutamate triggered current recordings, a second pipette filled with 10µM glutamate was placed 100 µm from the cell body of the cell being recorded from near with the dendrites of the cell (visualized with Alexa 488 dye in the internal solution). Glutamate was administered onto the cell with a 10 second pulse of glutamate in order to maximally measure glutamate receptor current. For calcium rectification measurements the second pipette filled with 10µM glutamate placed in the same
location as previously described. Then a short 20 ms application of glutamate was applied to the cell voltage clamped at -60 and +40. The +40 / -60 ratio was used as the calcium rectification index as previously described (Noh et al., 2005).

**Anoxic depolarization**

Organotypic slices were used to obtain whole cell recordings 48 hours following treatment with TAT or \( \psi \)RACK in the presence or absence of SC or arc AS ODNs. Slices were in the same external solution described above. The internal solution of the pipette contained 140 mM K\(^+\)-gluconate; 5 mM KCl; 10 mM HEPES; 10 mM EGTA; 2 mM MgCl\(_2\); 2 mM MgATP; and 0.5 mM NaGTP, adjusted to a pH of 7.33. CA1 hippocampal pyramidal neurons were again exclusively targeted and visualized using Alexa 488 dye. One cell was patched per slice equating to an \( n \) of 1. Whole cell configuration was obtained as indicated above. Access resistance was maintained as indicated above. To initialize the OGD, the external perfusate was switched over to a glucose free media, where sucrose replaces glucose, in a solution that was vigorously bubbled with a 95% N\(_2\) and 5% CO\(_2\) gas mixture. From the onset of the OGD, the membrane potential was continuously recorded for the duration of the OGD. AD was marked as a rapid change from resting membrane potential (Tanaka et al., 1997).
3.4 Results

3.4.1 PKCε-mediated arc expression decreases AMPAR miniature excitatory postsynaptic current amplitudes in organotypic hippocampal cultured slices.

A well characterized function of arc is to alter the number of cell surface AMPARs either through an increase in endocytosis (Rial Verde et al., 2006) or through regulation of AMPAR transcription (Korb et al., 2013) leading to a decrease in AMPAR mEPSCs. In order to determine if ψεRACK administration alters AMPAR currents, analysis of AMPAR mEPSCs was performed. 48 hours following ψεRACK treatment (200 nM) in organotypic hippocampal cultured slices, AMPAR mEPSCs were recorded (Fig. 6 A). Electrophysiological properties capacitance (pF), membrane resistance (MΩ), access resistance (MΩ), tau (ms), and holding current (pA) were measured in order to determine similar cells in terms of size, health, and quality of the patch (Table 2). Treatment of organotypic slices with ψεRACK decreased the average maximum AMPAR mEPSCs amplitude to 12.75 +/- 0.35 pA as compared to 14.80 +/- 0.39 pA in TAT control treated slices (n = 20, p<0.05, ANOVA, Bonferroni) (Fig. 6 B,D). The ψεRACK + SC ODN treated group showed a similar decrease in average AMPAR mEPSC maximum amplitude, 12.47 +/- 0.49 pA, as compared to the TAT control (n = 20, p=0.01, ANOVA, Bonferroni-post hoc). However, this change in mEPSC amplitude was lost in the ψεRACK + Arc AS ODN treated group as there was no statistically significant change in mEPSC amplitude when compared to the TAT peptide (n = 20, p>0.05, ANOVA, Bonferroni-post hoc) (Fig. 6 C,D). There were no observed changes in event frequency between any
of the groups measured (n = 20, p>0.05, ANOVA, Bonferroni) (Fig. 6 E). In addition to decreasing the average maximum mEPSC amplitude, the distribution of the maximum amplitudes for AMPAR mEPSCs was shifted towards smaller amplitude responses for the ψεRACK group compared to TAT peptide controls (n = 1853 and n = 1905, respectively, p<0.005, Kolmogorov-Smirnov test) (Fig. 7 A). There was no observed difference in the distribution of the maximum amplitude for the total events measured between the ψεRACK and ψεRACK + SC ODN groups (n = 1853 and n = 1964, respectively, p>0.05, Kolmogorov-Smirnov test) (Fig. 7 B). The administration of the ψεRACK + Arc AS ODN shifted the distribution of the maximum amplitude for total events measured towards larger responses when compared to the ψεRACK + SC ODN group (n = 1977 and n = 1964, respectively, p=0.034, Kolmogorov-Smirnov test) (Fig. 7 C). The distribution of the maximum amplitude for the ψεRACK + Arc AS ODN was still shifted smaller when compared to the TAT control treated group (n = 1977 and n = 1905, respectively, p<0.005, Kolmogorov-Smirnov test) (Fig. 7 D).

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Table 2. Electrophysiological membrane properties of CA1 pyramidal neurons patched for mEPSC recordings. The capacitance, series resistance, access resistance, tau, and holding current (pA) were measured for each group. There were no
significant differences observed between any of the groups (n = 20, one way ANOVA, Bonferroni post-hoc).

**Figure 6. Average amplitude of AMPAR mEPSCs are decreased following PKCε-dependent arc expression.** Organotypic hippocampal cultured slices were treated for one hour with TAT (200nM), ψεRACK (200nM), Arc AS ODN (1 nM) + ψεRACK or SC ODN (1 nM) + ψεRACK. 48 hours following administration of TAT or ψεRACK AMPAR miniature excitatory postsynaptic currents (mEPSCs) were measured in the presence tetrodotoxin (TTX) (1 µM nM) bicuculine (10µM), and APV (20 µM). (A) Typical mEPSC tracings observed. (B) An average tracing of every mEPSC recorded for each group. (C) Following administration of ψεRACK the maximum amplitude was decreased as compared to a TAT control group (n = 20 slices, p<0.05 ANOVA, Bonferroni). ΨεRACK + SC ODN also decreased the maximum mESPC amplitude as compared to a TAT control group (n = 20 slices, p<0.01 ANOVA, Bonferroni). The ΨεRACK + Arc AS ODN showed no decrease in maximum amplitude observed as compared to the TAT group (n = 20 slices p>0.05 ANOVA, Bonferroni). (E) There were no significant changes in event frequency between any of the groups (n = 20, p>0.05, ANOVA, Bonferroni).
Figure 7. The distribution of AMPAR mEPSCs are decreased following PKCε-dependent arc expression. Cumulative fraction plots examining the changes of the distribution for all of the events for the Tat (n = 1905), ψεRACK (n = 1853), ψεRACK + SC ODN (n = 1964) & ψεRACK + Arc AS ODN (n = 1977) groups. (A) The distribution of amplitude of total mEPSCs was shifted towards smaller maximum amplitudes in slices treated with ψεRACK compared to a Tat control (p<0.001, Kolmogorov-Smirnov Test). (B) There was no change in the distribution of the maximum amplitude of total events for the ψεRACK group and the ψεRACK + SC ODN (p>0.05, Kolmogorov-Smirnov Test). (C) The presence of the arc AS ODN shifted the distribution of the maximum amplitude larger in slices treated with ψεRACK + SC ODN (p<0.05, Kolmogorov-Smirnov Test). (D) The ψεRACK + arc AS ODN still displayed a shifted distribution from the TAT control (p=0.005, Kolmogorov-Smirnov Test).
3.4.2 PKCε-activation does not modify glutamate triggered responses or calcium rectification

Measurements of glutamate triggered responses and changes in the GluR1 to GluR2 ratio were measured following treatments with TAT control or ψεRACK with SC ODNs or arc AS ODNs as described above. (Fig 8 A-D). No observed changes in maximal glutamate triggered responses or GluR1 to GluR2 ratio were observed (n=3-7, p>0.05, ANOVA, Bonferroni).

Figure 8. No observed changed in AMPAR glutamate triggered currents or calcium rectification. (A) An example glutamate triggered AMPAR response following application of 10 µM glutamate through a picospitzer 100 µm away from the cell body of a CA1 neuron in an organotypic hippocampal cultured slice. (B) No significant differences were observed in maximum glutamate triggered AMPAR response between any of the groups (n =3-7, p> 0.05, ANOVA, Bonferroni). (C) To measure changes
between the GluR1 and GluR2 subunit, the +40 to -60 ratio of AMPAR current during the application of glutamate (calcium rectification index) was compared for each group. There was no significant difference in calcium rectification between any of the groups (n = 3-7, p > 0.05, ANOVA, Bonferroni).

3.4.3 PKCε-mediated arc expression delays the latency until anoxic depolarization in organotypic hippocampal cultured slices.

A previous study showed that inhibition of AMPARs can increase the latency until AD in acute hippocampal slices (Tanaka et al., 1997). Therefore, decreasing postsynaptic AMPARs through an increased arc expression may decrease excitability during OGD, leading to a similar delay in AD. To test the hypothesis that administration of ψεRACK would cause an arc-dependent increase in latency until AD, we measured latency until AD in organotypic hippocampal cultured slices. Electrophysiological properties capacitance (pF), membrane resistance (MΩ), access resistance (MΩ), tau (ms), and holding current (pA) were measured in order to determine similar cells in terms of size, health and quality of the patch as above (Table 3). Slices were treated with TAT, ψεRACK, ψεRACK + SC ODN, or ψεRACK + Arc AS ODN (as described above). 48 hours following TAT or ψεRACK treatment, recordings were obtained from CA1 neurons and membrane potential was measured following an irreversible OGD (Fig. 9 A,B). Latency until AD was increased from 29.27 +/- 3.6 minutes in the TAT group to 50.77 +/- 5.08 minutes and 52.73 +/- 6.78 in the ψεRACK and ψεRACK + SC ODN groups respectively (n = 15-13, p < 0.05 and p < 0.05 respectively, ANOVA, Bonferroni) (Fig. 9 C). However, latency until AD was 39.5 +/- 5.63 minutes for the ψεRACK + Arc AS ODN group, not significantly different from the TAT controls (n = 14, p > 0.05, ANOVA, Bonferroni).
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Table 3. Electrophysiological membrane properties of cells patched for anoxic depolarization data. The capacitance, series resistance, access resistance, tau, and holding current (pA) were measured for each group. There were no significant differences observed between any of the groups (n = 15-13, one way ANOVA, Bonferroni post-hoc).
Figure 9. PKCε-mediated increase in latency until anoxic depolarization is partially dependent upon expression of arc. (A) Example images of TAT treated slices that have undergone 0 and 40 minutes of anoxic depolarization (AD). Two cells that have undergone significant swelling during the AD are circled. (B) Example membrane potential changes over time for TAT and ψεRACK groups during OGD experiment. (C) Latency until AD was increased in the ψεRACK group and the ψεRACK + SC ODN groups compared to TAT (n =15-13, p<0.05, p<0.05, ANOVA, Bonferroni). However, latency until AD in the ψεRACK + Arc AS ODN group was not significantly different from the TAT controls (n = 14, p>1.000, ANOVA, Bonferroni).
3.4.4 Action potential properties are not correlated with latency until anoxic depolarization.

A recent publication in our lab determined that application of ψεRACK modified action potential threshold and maximum amplitude after 48 hours (Neumann et al., 2015). The triggering of action potentials has previously been implicated in determining latency until AD due to sodium influx (Ye et al., 2010). Action potential threshold, maximum amplitude, and sodium current influx were measured between each group to determine if these parameters were modified by ψεRACK or arc-dependent ψεRACK expression. There were no observed differences in any of these parameters (n = 8-11, p >0.05, ANOVA, Bonferroni) (Fig 10, A,B,C). In order to determine if action potential threshold, maximum amplitude, or sodium influx were related to latency until AD, a correlation analysis between each parameter and latency until anoxic depolarization was performed (Fig 10 D,E,F). No relationship between any of these three parameters and latency until anoxic depolarization was observed (n = 8-11, r² = 0.025 (threshold), r² = 0.0152 (amplitude), r² = 0.0057 (sodium current)).
Figure 10. No relationship was observed between ψεRACK, arc, anoxic depolarization, and action potential properties. 48 hours following administration of ψεRACK, action potential (A) threshold, (B) amplitude, and (C) sodium current were measured for all four groups. There were no significant differences found for any of these measurements (n = 8-11, p>0.05, ANOVA, Bonferroni). Additionally, correlation analysis for action potential (D) threshold, (E) amplitude, (F) sodium current showed no correlation between each of these parameters and latency until anoxic depolarization (n = 8-11, $r^2 = 0.025$ (threshold), $r^2 = 0.0152$ (amplitude), $r^2 = 0.0057$ (sodium current).
Chapter 4. An age-dependent decrease in PKCε-triggered arc expression may be overcome by an increased dosage of the PKCε activator, ψεRACK

4.1 Summary

In this chapter, the effects of cardiac arrest on aging and potential neuroprotective treatments are examined. Previous research in middle aged and aged rats has indicated that global cerebral ischemia can trigger an age-dependent worsening cell death within the hippocampus (Soli and Soonpaa, 1979). However, a robust characterization of the electrophysiological, cell death and behavioral changes in a cardiac arrest global cerebral ischemia model has not previously been conducted. In this chapter, we detail a middle aged Fischer 344 rat model of cardiac arrest and then determined cell death, electrophysiological, and behavioral changes that occurred. We found that 9 month Fischer 344 rats that have undergone a mild cardiac arrest, show cell death within the CA1 region and subiculum, however, do not show cell death within the CA3 region or the entorhinal cortex, the input and output of the hippocampus respectively. Additionally, we observed no changes in LTP 7 days post injury, however, we did see an increase in paired pulse response. Additionally, we observed spatial memory deficits between groups on the Barnes Maze and contextual fear conditioning tasks, and observed an increase in random search strategy used, indicating a potential deficit in executive function.

After characterizing the ACA model in middle aged rats, we examined the potential treatment of administering the PKCε activator ψεRACK in aged animals. We determined that there was no change in PKCε or RACKII protein levels. Then we determined whether PKCε activation with the same dose of ψεRACK resulted...
in different levels in arc expression as arc is known to be decreased with aging. We determined that 48 hours following administration of ψεRACK in 9 or 24 month old Fischer 344 rats there was no increase in arc expression. We then determined if this inhibition of arc expression could be overcome by increasing the dosage of ψεRACK. We found that increasing the concentration of each dose 5 fold (from 0.2 mg/kg to 1.0 mg/kg) in another subset of aged animals, 18 month old Fischer 344 rats, we could restore a PKCε-dependent increase in arc expression.

4.2 Introductory Remarks

Over 80% of ischemic injuries in this country occur in individuals over the age of 50 (Go et al., 2014). Often, most studies using animal models of ischemic injury employ young animals due accessibility and mortality issues with aged animals. Thus, many neuroprotective treatments are often developed and titrated in young animal models. A previous group found that the efficacy of IPC may be decreased against ischemic injury in aged rats (He et al., 2005). This is consistent with what previous literature has found with the potential neuroprotective mechanism detailed in Chapters 2 (Page 22) and 3 (Page 36). Neuronal activity dependent arc mRNA expression has previously been found to be down regulated in aged animals (Penner et al., 2011). Specifically, increases in arc expression occur in the CA1 region of aged animals, but at lower levels due to enhanced arc promoter methylation (Penner et al., 2011). This is consistent with the idea that aging may impair activation of the neuroprotective mechanisms triggered by IPC, of which PKCε activation is a key mediator.
(Lange-Asschenfeldt et al., 2004). In this chapter, we characterize a middle aged model of cardiac arrest in Fischer 344 rats. Then we examined the PKCε-activated neuroprotective pathway detailed in Chapters 2 (Page 22) and 3 (Page 36), the PKCε-mediated increase in arc protein expression, and determined whether this pathway was still activated in aged animal models. We then investigated the machinery of the PKCε pathway, namely expression of PKCε and its downstream receptor of activated C kinase II (RACKII) protein expression in aging. Finally we determined if an increased dosage of ψεRACK could overcome the inhibition of PKCε-triggered arc expression that was observed due to aging.

4.3 Materials and Methods

Animals

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Animal Care and Use Committee of the University of Miami. Animals were separated into either sham (n = 22) or asphyxial cardiac arrest (ACA; n = 45) groups for each experiment. Only sham (n = 21) or CA (n = 17) animals that survived 7 days post-surgery were used for data analysis. Animals that underwent histological analysis (11 total, 6 sham, 5 ACA) did not undergo Barnes maze or fear conditioning testing due to the ability of enriched environment to increase cell survival post-ischemic injury (Wadowska et al., 2014). Animals that underwent long-term potentiation (LTP; 12 total, 6 sham, 6 CA) testing were trained on a Barnes maze in order to reduce any variability.
caused by exposure to the Barnes maze and did not undergo fear conditioning as fear conditioning can impair LTP induction (Sacchetti et al., 2002). Remaining animals were used for Barnes maze testing and fear conditioning.

**Induction of Cardiac Arrest**

ACA was induced as previously described (Dave et al., 2004, Della-Morte et al., 2009). Male Fischer 344 rats (nine month old) weighing 430.2 ± 3.5 g were fasted overnight and then anesthetized with 4% isoflurane and 30:70 mixture of O₂ and N₂O by inhalation. The femoral artery was cannulated for blood pressure measurements and arterial sampling of blood gases. Arterial blood gases (Radiometer, Copenhagen, Denmark) and plasma glucose levels (One Touch glucose monitor; LifeScan, Milpitas, CA) were measured throughout the experiment. Head and body temperature were maintained at 37°C throughout the experiment. Rats were immobilized throughout the procedure with vecuronium bromide (2.0 mg/kg, intravenous [IV], administered every 10 minutes). ACA was induced through apnea by disconnecting the ventilator from the endotracheal tube. Six minutes after asphyxia, resuscitation was initiated by administering a bolus injection of epinephrine (0.005 mg/kg, IV) and sodium bicarbonate (1 meq/kg, IV) followed by mechanical ventilation of 100% O₂. Sham animals were subjected to similar surgical procedures without the induction of asphyxia, resuscitation drugs, and mechanical ventilation of 100% O₂. Following ACA or sham procedures, animals were monitored daily for body weight, rectal temperature, hydration, and blood glucose. If needed, animals were gavage-fed
with liquefied animal chow, injected with saline, and/or maintained in a humidified warm incubator.

**Histology**

Seven days after sham or ACA procedures, rats were anesthetized with isoflurane, perfused with physiologic saline for one minute, and then perfused for 19 minutes with FAM (a mixture of 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume). The perfusate solution was delivered into the root of the ascending aorta at a constant pressure of 110–120 mmHg, as previously described (Perez-Pinzon et al., 1997). The head was removed and immersed in FAM for one day at 4 °C before the brains were removed from the skull and gross sectioned using a rat brain matrix (ASI instruments, MI, USA). Coronal brain blocks were processed in a tissue processor (Leica TP1050, Germany) and embedded in paraffin using a Histo-Center-II embedding station (Fisher Scientific, PA, USA). Coronal brain sections (10 μm) were cut using a high profile Teflon coated disposable blade (Ted Pella, CA, USA) on a rotatory microtome (Leica RM2135). Brain sections were then placed on a glass slide and paraffin was removed by incubating overnight in an oven at 54 ± 1°C. The sections were then stained with hematoxylin (Gill’s Formulation #2, Fisher Scientific, PA, USA) and eosin (0.5% eosin solution in 80% ethanol and 0.5% glacial acetic acid). Neuronal counts (“non-compromised” neurons) were made by an investigator blinded to the experimental conditions within the CA1 and subiculum regions.
using three coronal brain sections 200 µm apart starting at the level of -3.8 mm from bregma. Cell counts were average per hemisphere and field of view.

**Acute Slice Preparation**

Hippocampal slices were prepared from rats seven days following ACA (Moyer and Brown, 2007). Animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneal and placed on ice. Animals were then transcardially perfused with a sucrose artificial cerebrospinal fluid (aCSF) (in mM: sucrose, 206; KCl, 2.8; CaCl₂, 1.0; MgCl₂, 1.0; MgSO₄, 2.0; NaHCO₃, 26; Na₂HPO₄, 1.25; ascorbic acid, 0.4; glucose, 10; and oxygenated with 95% O₂ – 5% CO₂) at 4°C that contained 20 µM 6-cyanono-7-nitroquinoxaline-2,3-dione and 20 µM R-2-amino-5-phosphonopentanoate. The animals were decapitated, the brain was rapidly removed. Coronal slices of 400 µm in thickness were sectioned using a Leica VT1000S microtome in sucrose aCSF at 4°C and transferred into a solution containing 50% sucrose aCSF/50% aCSF at room temperature for 30 min before being transferred/stored in aCSF (in mM: NaCl, 126; KCl, 3.5; CaCl₂, 2.0; MgSO₄, 2.0; NaHCO₃, 26; Na₂HPO₄, 1.25; sodium ascorbate, 0.4; glucose, 10; and oxygenated with 95% O₂–5% CO₂) at room temperature. Individual slices were then transferred to an interface recording chamber (BSC-BU chamber, Warner Instruments, Hamden, CT, USA), superfused with warmed (33 ± 1°C) aCSF (TC-344C, Warner Instruments) at a rate of 1 ml/min, and oxygenated with humidified 95% O₂ – 5% CO₂ (Dave et al., 2004).
**Long term potentiation and paired pulse recordings**

General population measurements of field excitatory postsynaptic potentials (fEPSP) were recorded with NaCl-filled (150 mM) glass micropipettes inserted into the stratum radiatum of the CA1 hippocampal subfield using a SUPER-Z head-stage attached to a BMA-931 AC/DC Bioamplifier (CWE-inc, Ardmore, PA, USA), Digidata1200 (Molecular Devices, Sunnyvale, CA, USA) and pClamp 9.0 software (Molecular Devices). Schaffer collaterals were electrically stimulated (0.3 ms constant current pulses) with bipolar tungsten electrodes using a S48 square pulse stimulator (GRASS technologies, Warwick, RI, USA). After slice recovery in the recording chamber (approx. 30 min), input–output curves relating stimulus current intensity to fEPSP slope and amplitude were generated. Stimulus intensity required for half-maximal fEPSP slope was selected. Paired-pulse experiments were performed using an intra-pulse duration of 50 ms. Stimulation for fEPSP was performed at a rate of 1 stimuli every 30 sec for baseline recordings. Long-term potentiation (LTP) was then induced using tetanic stimulation (100 Hz) for 1 sec. One minute following LTP, test stimulation (1/30 sec) resumed for a period of 1 h, and afterwards input–output curves were repeated. Slices from each animal were averaged together to give a n-value of one.

**Barnes Circular Platform Maze**

The Barnes circular platform maze is a less physically demanding alternative to the Morris water maze (Harrison et al., 2009), which is used to evaluate hippocampal dependent spatial memory deficits (Barnes, 1979).
Animals were tested three days after ACA for four consecutive days; however, to acclimate the animals to human touch, each rat was handled for three days (10 min session) prior to ACA induction. Testing was performed just after onset of the dark cycle for the rats. The Barnes circular platform is a 122 cm diameter circular platform on a 1.4 m stand with 18 evenly spaced 9.5 cm diameter holes around the circumference, where a black box (escape tunnel) was placed underneath one of the holes (Med-Associates Inc., St. Albans, VT). Four bright lights were positioned above the platform as an aversive stimulus and cause the rat to seek out the escape tunnel using spatial cues. Prior to the first test trial, animals were subjected to a habituation trial by placing the rat next to and allowing the rat to enter the escape tunnel for one minute before being returned to its home cage. On all subsequent trials, the animals were placed into the center of the apparatus under a dark container for 30 seconds before the container was lifted and the rat was allowed to navigate the maze using spatial cues surrounding the apparatus (images). Each rat was allowed 180 seconds to locate and enter the escape tunnel per trial. If the rat was unable to locate the escape hole after 180 seconds, the rat was gently guided to the correct hole location and allowed to enter the escape tunnel. Once the rat entered the escape tunnel (either guided or on their own), they remained in the tunnel for two minutes before returning to their home cage. Each day, the respective animal would attempt two test trials spaced 15 minutes apart for a total of eight trials. In between each trial, the platform and escape tunnel were cleaned with 70% ethanol and water. Video recordings were made using an EQ610 Polestar II Everfocus camera. The latency to entry into the
escape tunnel and distance traveled, were quantified using EthoVision 8.5 video tracking software (Noldus, Leesburg, VA).

**Search Strategy**

The search strategy for each animal was analyzed in a manner adapted from the previously published methods (Bach et al., 1995, O'Leary and Brown, 2009). Briefly, each Barnes maze trial was classified into one of three search strategies: random, serial, or spatial. Use of a spatial strategy indicates use of cues in a spatial manner in order to locate the target quadrant. The strategy was classified as “spatial” if from the start of the trial the animal entered the correct quadrant and made two or less errors before entering the escape tunnel. Use of serial strategy indicates a systematic, non-random method for locating the target hole independent of spatial cues. The strategy was classified as “serial” if the rat circled around the outside of the maze in a systematic fashion for the length of the trial. The use of a random search strategy indicates a non-systematic, non-spatial method for locating escape tunnel, indicating an inability to develop a cognitive strategy to locate the hole. The strategy was classified as “random” if the rat looked in one or more incorrect hole(s) then crossed the midline of the maze more than once. After classification of search strategies, two separate analyses were conducted to compare search strategy utilization differences between sham and ACA animals. The first analysis examined the ability of the rats to use spatial cues. The number of trials where animals used a spatial strategy versus a non-spatial strategy (i.e. a systematic or random strategy) was counted; differences between sham and cardiac arrest animals were compared.
The second analysis examined use of non-random search strategy. The number of trials where systematic non-random search strategies (i.e. spatial or serial) versus non-systematic, random search strategy (i.e. random strategy) was quantified; again, differences in utilization between sham and cardiac arrest animals were compared. Comparisons were made using chi square analyses. Null hypothesis testing was conducted comparing the expected results if ACA had no effect on search strategies (i.e. the observed sham values), compared to the observed ACA search strategy values. Significance was determined at p < 0.05.

**Contextual Fear Conditioning**

To determine if spatial memory deficits persisted 6-7 days after ACA, contextual fear conditioning was performed. Fear conditioning occurred in a conditioning apparatus (12" W x 10" D x 12" H) placed inside of an isolation cubicle (30" W x 17.75" D x 18.5" H) (Coulbourn instruments, Whitehall, PA). The isolation cubicle contained an overhead stimulus light and a 28 V exhaust fan that were left on during the trials. A drop pan was placed underneath an electrically active floor grid that was connected to a precision animal shocker (Coulbourn instruments, Whitehall, PA). Six days following ACA, after completing the Barnes circular platform maze, fear conditioning was induced. For this process, rats were placed into the testing room containing the conditioning apparatus for 30 minutes prior to testing. The conditioning apparatus was cleaned with 70% ethanol immediately prior to each trial. For the induction of fear, rats were placed into the conditioning apparatus for 340 seconds, at which
time the animals receive a two second shock (1.5 mA). The rats would then spend an additional 28 seconds in the apparatus before completing the trial and returning to their home cage. On the second day, the animals were again returned to the room containing the conditioning apparatus again for 30 minutes prior to the trial. The rats were then placed into the apparatus for eight minutes with no shock to measure freezing behavior. Freezing behavior for both trials was quantified as the percent of time spent frozen using the visual tracking software FREEZEFRA (Coulbourn instruments, Whitehall, PA).

**In vivo injections**

For *in vivo* injections, Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were injected intraperitoneal (i.p.) with 0.2 mg/kg (25 day old rats) or 0.5 mg/kg (3 month old rats) of the PKCε-specific activator, ψεRACK (TAT-conjugated; KAI Pharmaceuticals, San Francisco, CA, USA) or with TAT peptide control (KAI Pharmaceuticals). Brains were used for western blotting and immunofluorescence experiments. Unless specified, all chemicals were purchased from Sigma-Aldrich.

**Western blotting**

Tissue was homogenized in RIPA buffer solution (pH 8.0) containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, supplemented with 1% protease (Sigma) and 1% phosphatase inhibitor cocktails (Roche Molecular Systems Inc., Branchburg, NJ, USA). Lysates were centrifuged at 16,000G for 15 minutes. Supernatant was collected and the protein
concentration determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein samples (30 or 40 µg) were separated on a 10 or 12% SDS-PAGE gel. Proteins were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad). Blots were then blocked in 5% milk in tris-buffered saline with 1% tween then incubated overnight in primary antibody in 5% milk. Blots were washed then incubated for 1 hour in horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary (Sigma). Antibodies used include: actin (Sigma, 1:5000), BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200), TrkB (Cell Signaling Technologies, Boston, MA, USA, 1:1000), phosphorylated TrkB (pTrkB) (Millipore, 1:1000), and arc (Santa Cruz, 1:200).

**Statistics**

All data are expressed as mean ± S.E.M. Statistical evaluation of the data was performed using one-way ANOVA, two way ANOVA, general estimating equation model or Student's t-test when appropriate. For the search strategy measurements a chi square analysis was used to compare between spatial and non-spatial search strategies and the random and systematic search strategies.

**4.4 Results**

4.4.1 Cardiac arrest decreases survival and increases hippocampal cell death in middle aged rats.

In order to determine that the animal model of cardiac arrest led to hippocampal cell death and behavioral deficits in middle aged rats, as has previously been observed in young animals, asphyxia cardiac arrest and sham surgeries were performed on 9 month old Fischer 344 rats. Before the induction
of sham or ACA procedures, the physiological parameters were measured and no statistical differences were observed between groups (Table 4). While a majority of animals survived four days following ACA (83%), the overall seven-day survival in animals subjected to ACA was significantly reduced to 38% (n = 22 sham, n = 45 ACA, p<0.05, Log-Rank test) (Fig. 11 A). Most of the animals subjected to ACA died of unknown complications; however, some deaths ensued from hypothermia, kidney failure, and/or lung edema. Rats subjected to sham or ACA surgeries were sacrificed seven days after ACA for histopathology. Neurons with “non-compromised” characteristics are cells that do not exhibit ischemic cell change such as eosinophilic cytoplasm, dark-staining triangular-shaped nuclei, and eosinophilic-staining nucleolus were counted in the CA1 region of the hippocampus and subiculum (Fig. 11 B). The number of non-compromised CA1 neurons in the hippocampus from the ACA group was significantly lower in both the left and right hemispheres compared to sham animals by 26%; p<0.05; Sham n=6 and ACA n=5) (Fig. 11 B,C). Similarly, ACA-treated animals resulted in a 26% reduction of non-compromised neurons in the right hemisphere of the subiculum compared to sham-operated rats (p<0.05; Sham n=6 and ACA n=5) (Fig. 11 B,C). Conversely, the left hemisphere of the subiculum had a 17% reduction in non-compromised neurons, however this reduction was non-significant (Sham n=6 and ACA n=5) (Fig. 11 B,C).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Variable</th>
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<tr>
<td></td>
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<td>Before</td>
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<tr>
<td>Sham</td>
<td>Body weight (grams)</td>
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<td></td>
<td>pH</td>
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<td></td>
<td>pCO₂ (mm Hg)</td>
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<td></td>
<td>pO₂ (mm Hg)</td>
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<td>Plasma glucose (mg/dl)</td>
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<td>Asphyxial Cardiac arrest</td>
<td>Body weight (grams)</td>
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<tr>
<td></td>
<td>pH</td>
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</tr>
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<td></td>
<td>pCO₂ (mm Hg)</td>
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<tr>
<td></td>
<td>pO₂ (mm Hg)</td>
<td>130 ± 6</td>
</tr>
<tr>
<td></td>
<td>Plasma glucose (mg/dl)</td>
<td>138 ± 4</td>
</tr>
</tbody>
</table>

Table 4. Physiological parameters prior to and following asphyxial cardiac arrest or sham surgery in 9 month Fischer 344 rats. (**p<0.01, ***p<0.001)
Figure 11. Survival rate and hippocampal damage following Sham or ACA in 9 month old Fischer 344 rats. (A) Kaplan-Meier curve of animal survival for seven days following Sham or ACA procedures. At day seven, the survival of sham animals was 95% and 38% for ACA rats (Sham n=22 and ACA n=45, p<0.05, Log-Rank test). (B) Representative histological images of hippocampal coronal slices of sham and ACA animals with 40X images of the i) CA1 and ii) subiculum region. There was a visible increase in small pyknotic and eosinophilic cells (arrows) following ACA in the CA1 and subiculum regions. (C) Bar graph depicting the number of non-compromised neurons per field to left (LH) or right (RH) hemisphere to their respective groups, where there was a significant decrease in the number non-compromised neurons following ACA in the CA1 region and RH of the subiculum (p<0.05; Sham n=6 and ACA n=5).

4.4.2 LTP is unaffected but paired pulse response changes occur following cardiac arrest in middle age rats.

To investigate the synaptic activity in the stratum radiatum of CA1 hippocampal neurons following sham or ACA, we harvested hippocampal slices seven days following surgery and tested the induction of LTP in CA1 neurons. There was no statistical difference in the input/output curve between sham and ACA slices (Fig. 12 A) and both the stimulation intensity and half-max response
were not significantly different between groups (Table 5). Using 50% maximum stimulation, baseline recordings were made every 30 sec for 30 min before and 60 min following tetanus stimulation (example sham LTP induction tracings, Fig. 12 B). The average slope calculated from each stimulus, normalized to the 30 min baseline recording, is shown in Fig. 12 C. Hippocampal slices from both sham and ACA-treated groups induced LTP following tetanic stimulation (Fig. 12 C; n=6). Paired-pulse facilitation was investigated seven days following sham or ACA procedures (example of sham paired-pulse facilitation tracing, Fig. 12 E). There was a significant increase in the maximum amplitude of the paired-pulse response following ACA at the stimulation interval of 50 ms, where sham animals had a 1.42 ± 0.05 fold increase in response compared to 1.62 ± 0.04 fold increase following ACA.

<table>
<thead>
<tr>
<th></th>
<th>Stimulation intensity (V)</th>
<th>50% fEPSP amplitude (mV)</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>8.54 ± 0.24</td>
<td>1.78 ± 0.20</td>
</tr>
<tr>
<td>ACA</td>
<td>8.55 ± 0.23</td>
<td>1.51 ± 0.13</td>
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</table>

Table 5. Average stimulation intensity (V) required to induce 50% fEPSP amplitude (mV).
Figure 12. Hippocampal acute slice electrophysiology seven days following Sham or ACA procedure. A) Mean (±S.E.M.) input/output curve (I/O) of fEPSP amplitudes across stimulation intensities (4-15 V) in control and ACA animals (not normalized). B) Example tracings of sham fEPSP recorded in the CA1 stratum radiatum region of hippocampal slices, before (black) and after (gray) LTP-induction. C) Average slope of fEPSP (averaged to the 30 min pre-tetanus values) before and after the induction of LTP. LTP was induced by 100-Hz tetanic stimulation for 1 s and is indicated by the arrow. D) Example tracings of sham paired-pulse response. E) Average fold increase of paired-pulse response; Stimulation 2 (S2)/Stimulation 1 (S1) amplitude) following sham and ACA procedures (*p<0.05, n=6).

4.4.3 Spatial memory deficits occur 7 days following ACA in 9 month old Fischer 344 rats.

To correlate a decrease in normal neurons (Fig. 11) and paired-pulse facilitation dysfunction (Fig. 12) with cognitive deficits, rats were tested using the Barnes circular platform maze to measure spatial memory deficits. The distance
traveled and latency (time to enter the escape tunnel) were measured and averaged for each day as an indication of spatial memory deficits (Fig. 13; n=10 sham and n=8 ACA). Both groups significantly improved in performance for both the distance traveled (Fig 13 A) [F (1, 3) = 8.30, *p<0.05] and latency (Fig 13 C) [F (1, 3) = 6.68, *p<0.05] measured over four days. Additionally, there was a difference between the two groups observed in both distance traveled (represented in Fig 13 B) [F (1, 3) = 6.07, **p<0.01), and latency [F (1, 3) = 35.69, ***p<0.005] (represented in Fig 13 D) averaged over all days. However, there was no significant interaction between the sham and ACA groups across the four day period for either distance [F (3, 14) = 0.70] or latency [F (3, 14) = 1.62] measures.

To further evaluate changes in memory and cognition, the search strategy used by each animal was evaluated for each trial on the Barnes maze (Fig. 13 E,F, and Table 6). The percentage of sham rats using a spatial search strategy (42.5%) was elevated compared to ACA animals (21.9%) (Chi-Square, *p<0.05) (Fig. 13 E). Additionally, sham animals used a systematic non-random strategy (either serial or spatial) 78.1% of the time, compared to the ACA animals that used a systematic non-random search strategy 57.5% of the time (Chi-Square, **p<0.005) (Fig. 13 F). To determine if changes in locomotor activity influenced spatial memory or search strategy changes observed on the Barnes maze, an open field activity test was carried out. There was no observed change in locomotor activity following cardiac arrest as compared to a sham animal (Fig.
Figure 13. Barnes maze cognitive measurements following Sham or ACA procedure. Average distance traveled A) per day \( F (1, 3) = 6.68, *p<0.05 \) (group effect) or B) across all trials \( **p<0.01 \) on Barnes circular platform maze (testing occurred 3, 4, 5, or 6 days following sham or ACA procedures). The average latency to escape tunnel C) per day \( *p<0.05 \), group effect) or D) across all trials \( ***p<0.005 \) (group effect) on Barnes circular platform maze. The percentage of each animal using a search strategy (spatial, serial, or random) was also quantified on the Barnes maze. E) Percentage of trials where an animal used a spatial versus a non-spatial (serial + random) search strategy \( *p<0.05 \). F) Percentage of trials where an animal used a systematic (spatial + serial) versus a random search strategy \( ***p<0.005 \) (Sham n=10 and ACA n=8).
Figure 14. Locomotor activity following Sham or ACA procedure. Average distance traveled per animal in one 30 minute trial in an open field chamber. Sham animals traveled an average distance of 1943 ± 100.2 cm compared to ACA animals which traveled 1644 ± 212.4 cm (n = 6, Student’s t-test, p >0.05)
Table 6. Search strategy analysis of Barnes maze spatial memory task.
Two separate analyses were run. The first analysis divided search strategies into spatial (Sp), or non-spatial (Non) strategies. ACA rats used a spatial less often than sham rats (Chi-Square, *p<0.05). The second analysis divided search strategies into a non-random systematic (sys) (serial + spatial), or random (ran) strategies. ACA rats used a non-random systematic strategy less frequently than sham rats (Chi-Square, ***p<0.005). Sp = spatial, non = non-spatial (serial + random), Sys = systematic (serial + spatial), ran = random. * = p<.05, ** = p<0.01

In addition to the Barnes circular platform maze, contextual fear conditioning was completed to confirm spatial memory deficits seven days following ACA. Contextual fear conditioning was induced six days after sham or ACA procedures. Upon returning to the conditioning apparatus the following day, the percent increase in freezing behavior from baseline in sham animals was
significantly higher (51.44 ± 5.21%) compared to the ACA animals (33.97 ± 2.90%, *p<0.05) (Fig. 15; n=9 sham and n=6 ACA), suggesting that ACA rats were unable to recall the context as compared to sham rats.

**Figure 15. Fear conditioning spatial memory deficits following Sham or ACA procedure.** Average increase in percent time frozen from baseline following fear conditioning was significantly higher in sham animals (51.44 ± 5.21%) compared to the ACA animals (33.97 ± 2.9%, *p<0.05) (Sham n=9 and ACA n=6).

4.4.4 Aging impairs ζεRACK induced hippocampal arc expression but does not affect hippocampal PKCε or RACKII expression.

To determine if PKCε activation would be an effective treatment in aging, overall levels of PKCε and RACKII and arc were examined in 4, 9, and 24 month old Fischer 344 rats. 0.2 mg/kg ζεRACK or TAT peptide was injected i.p. and samples were collected 48 hours later for western blot analysis. There was no significant difference in expression of PKCε (Fig 16 A,C) or RACKII (Fig 16 B,D) (n = 4, p > 0.05, ANOVA, Bonferroni).
Figure 16. PKCε and RACKII protein expression does not change with age. Example blots for protein expression fold change from 4 month old animals for 4, 9, & 24 month old animals for (A) PKCε and (B) RACKII. No significant changes in fold protein expression were observed for (C) PKCε and (D) RACKII (n = 4 p>0.05, ANOVA, Bonferroni).

Interestingly, ψεRACK administration triggered a 2.64 +/-0.60 fold increase in arc expression in 4 month old animals as compared to a tat control, however did not trigger an increase in arc expression in either 9 or 24 month old animals (n = 4-6, p < 0.05, Student’s t-test) (Fig. 17 A,B) despite having normal levels of PKCε and RACK II (Fig. 16).
Figure 17. Aging decreases ψεRACK driven arc expression. (A) Example western blot images, and (B) Western blot quantification of 4, 9, and 24 month old Fischer 344 rats treated with 0.2 mg/kg TAT or ψεRACK i.p. after 48 hours. ΨεRACK increased arc expression compared to TAT in 4 month old rats (n = 4 p<0.05, Student’s t-test). However, there was no significant increase in arc expression compared to TAT in both 9 month and 24 month old rats (n = 5-6, p>0.05, Student’s t-test).

4.4.5 A high dose of ψεRACK increases arc expression in aged animals

Additionally, we determined that increasing the dosage of ψεRACK in aged animals from 0.2 mg/kg to 1.0 mg/kg restored a PKCε-triggered increase in arc expression in the hippocampus after 48 hours from a 1.02 +/- .11 fold TAT with 0.2 mg/kg injection to a 1.94 fold TAT with 1.0 mg/kg injection (n =3, p< 0.05, ANOVA, Bonferroni) (Fig. 18 A, B).
Figure 18. Increased dosage of ψεRACK can increase arc expression in aged animals. 18 month Fischer 344 rats were injected with 1.0 mg/kg TAT, 0.2 mg/kg ψεRACK or 1.0 mg/kg ψεRACK i.p. (A) 48 hours following injection, the hippocampal arc expression was measured via western blot. (B) Quantification western blot indicating that 1.0 mg/kg ψεRACK has a significant increase in arc expression compared to a TAT control (n = 3, p<0.05, ANOVA, Bonferroni).
Chapter 5. Discussion

5.1 The roles of BDNF and arc in ψεRACK pharmacological preconditioning

PKCε is a novel PKC, which is calcium-independent and is activated by the phospholipase C signaling pathway through the binding of diacylglycerol (Mochly-Rosen et al., 2012). The signaling cascades activated by PKCε are multifaceted and may confer neuroprotection by modifications in mitochondria and at the synapse (Dasgupta and Milbrandt, 2007, Gutsaeva et al., 2008, Dave et al., 2009, DeFazio et al., 2009). Activation of PKCε has previously been implicated in modulating GABAergic activity at the synapse. For example, one hour administration of ψεRACK decreased the amplitude of inhibitory postsynaptic currents that are dependent upon the presence of the PKCε (Chou et al., 2010). However, two days following a 1 hour treatment with ψεRACK, there was an observed increase in GABA miniature inhibitory postsynaptic currents (mIPSC) (DeFazio et al., 2009). Together, these results indicate that the effects of PKCε-activation at the synapse may be time dependent and may initially enhance excitation, followed by a second phase of inhibition.

It has been suggested that the repeated activation of PKCε and PKCα using the drug byrostatin may increase BDNF expression in the CA1 region of the hippocampus (Sun et al., 2008). Additionally, we recently established a direct connection between PKCε-activation and expression of BDNF (Neumann et al., 2015) in in vitro models. Previously, Ying et al., (2002) demonstrated that BDNF triggers arc expression through MEK/ERK activation (Ying et al., 2002). We
previously showed that PKCε-induced neuroprotection requires the MEK/ERK pathway (Lange-Asschenfeldt et al., 2004). Although previous work has determined that activation of multiple PKC isozymes using the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) triggered mRNA expression of arc, no specific PKC isozyme was defined as responsible for triggering arc expression (Teber et al., 2004). ψεRACK is a selective activator of PKCε, therefore, our results suggest PKCε as the PKC isozyme involved in regulating arc expression. However, we cannot rule out the contribution of other PKC isozymes in regulating arc expression in different settings.

5.2 Arc-dependent modifications in AMPAR currents and anoxic depolarization

PKCs can modulate excitatory neurotransmission through the direct phosphorylation of AMPARs. The role of PKCs in regulating synaptic currents and receptor trafficking is more ambiguous due to the complex interplay between phosphorylation sites on the AMPARs themselves as well as surrounding machinery (for review see (Henley et al., 2011)). Phosphorylation of serine 816 and serine 818 on the GluA1 subunit following pan PKC activation by phorbol-esters can lead to an immediate insertion of AMPARs into a synapse and also play a critical role in the generation of long term potentiation (LTP) (Boehm et al., 2006, Lin et al., 2009a). Both PKCα and PKCγ have been implicated as kinases involved in the phosphorylation of these sites (Boehm et al., 2006). Interestingly, the role of AMPAR phosphorylation as an overall contribution to AMPAR trafficking is somewhat controversial, as a recent publication has implicated that
the percentage of phosphorylation occurring at an individual synapse is around 1% (Hosokawa et al., 2015).

Arc is a well-characterized protein that is most commonly used as a marker of neuronal activity as its mRNA transcription is tightly coupled to neuronal activity and excitation (Lyford et al., 1995, Guzowski et al., 1999). A prior publication determined that following a global ischemic event, the production of arc is impaired in a region susceptible to ischemic injury, the CA1, while its expression in a region that show less sensitivity to ischemic injury, the dentate gyrus, was preserved (Otsuka et al., 2009). Arc is known to increase AMPAR endocytosis (Shepherd et al., 2006). Another way to decrease AMPAR signaling is by inhibition. Inhibiting AMPARs reduces hippocampal damage in models of global ischemic injury (Sheardown et al., 1993, Kawasaki-Yatsugi et al., 1997). Decreasing AMPAR currents through an arc-dependent mechanism (as observed in our study) may be a different way to regulate the same neuroprotective mechanism. As both AMPAR antagonism (Tanaka et al., 1997) and PKCε-activation delay the latency until AD in CA1 neurons indicate it is likely that these two differential ways to decrease AMPAR signaling are leading to the same delay in AD, triggering neuroprotection through decreased excitotoxicity.

The organotypic hippocampal cultured slice model we used to examine AD has a longer latency until AD as compared to the acute slice model (Neumann et al., 2015, Tanaka et al., 1997). Latency until AD is model specific as there are differences in the extracellular space, which is determines the latency until glutamate builds up enough to trigger excitation (Arnth-Jensen et al.,
This is also reflected in the time of OGD required to promote CA1 cell death in organotypic slices (Xu et al., 2002) as compared to acute slice preparations (Neumann et al., 2015). Despite these model differences, CA1 neurons in both acute slices and in vitro models are excited from glutamatergic release from the CA3 pyramidal cells (Ye et al., 2010) suggesting that this mechanism of injury should be conserved between the two models.

Our result that inhibition of arc expression completely abolished the neuroprotective effect of PKCε-activation was initially unexpected as there are many neuroprotective mechanisms triggered by PKCε-activation (Dasgupta and Milbrandt, 2007, Gutsaeva et al., 2008, Dave et al., 2009, DeFazio et al., 2009). However, our findings that PKCε-activation triggered an arc-dependent delay in latency until AD, suggest that a delay in latency until AD may be necessary for PKCε-induced ischemic neuroprotection. Results from our current study taken together with our previous findings (DeFazio et al., 2009, Neumann et al., 2015), suggest that activation of PKCε can both increase mIPSC GABA currents and decrease mEPSC AMPA currents. These effects, in turn, can delay the latency until AD. Together, the tight regulation of synaptic transmission across both inhibitory and excitatory processes may be necessary for conferring neuroprotection against OGD. To our knowledge, ours is the first study to report that PKCε mediates arc expression, and that arc expression is necessary for PKCε-dependent neuroprotection.
5.3 Age-Dependent Changes in PKCε Pharmacological Preconditioning

Global cerebral ischemia induces a variety of alterations in the brain including neuronal damage and cognitive impairments (Petito et al., 1987, Calle et al., 1989, Neumann et al., 2013). Numerous investigators in the field of cerebral ischemia have used 2-vessel occlusion (VO) (Smith et al., 1984, Globus et al., 1992) or 4-VO models (Ordy et al., 1988, Volpe et al., 1989, Block and Schwarz, 1997) which have the primary drawback of only affecting the brain and not the entire body, as what would happen during a cardiac arrest. Additionally most studies have primarily used young animals (2-4 months) to investigate the resultant cognitive impairments and neuronal alterations as appropriately aged models have lower experimental feasibility and high attrition after CA (Katz et al., 1995, Dave et al., 2004, Della-Morte et al., 2009, Kiryk et al., 2011). Several pharmacological agents have been suggested as putative therapeutics through the use of young animals (Neumann et al., 2013); however, the translational feasibility to the clinic is still very limited (Ginsberg, 2008, 2009). Therefore, we sought to establish a model of CA and characterize potential memory deficits in middle-aged rats, as a putative model for testing potential therapeutics against mild ischemia.

Our findings indicate a significant decrease in the number of non-compromised hippocampal CA1 and subiculum neurons seven days following ACA in nine month old male Fischer 344 rats as compared to age-matched sham surgery controls. Similarly, a previous report has described an observed increase in hippocampal cell death following cardiac arrest in 12 month old Fischer 344
rats as compared to 6 month old rats (Xu et al., 2010); together with our results, it suggests that age may play a role in worse outcomes following cardiac arrest. In our study, the seven day survival following ACA in nine month old Fischer 344 rats was significantly reduced from 95% in the sham group to 38% in the ACA group (Fig. 11). This decline in survival is greater compared to our previous studies in 3 month old Sprague Dawley rats, where the seven day survival rates were around 80-90% at seven days (Lin et al., 2014a, Lin et al., 2014b). Therefore, this suggests that ischemic events may be increasingly detrimental as an individual ages as compared to their younger counterparts, a trend that has been previously suggested (Jin et al., 2004, Xu et al., 2007). In our study, ACA-treated rats also exhibited impairment in spatial memory formation when measured using the Barnes circular platform and contextual fear conditioning. Furthermore, potential executive function impairments were observed through the increased use random search strategies in ACA rats.

Dysfunction in synaptic transmission occurs following cerebral ischemia (Hori and Carpenter, 1994a, Kiprianova et al., 1999b, Calabresi et al., 2003, Dave et al., 2004, Wang et al., 2005, Dai et al., 2007). In this study, paired-pulse facilitation was measured seven days following ACA, where there was a significant increase in the maximal amplitude of the second stimulation fEPSP response (50 ms) compared to sham animals (Fig. 12 E). Paired-pulse facilitation is dependent on presynaptic sequestration of Ca$^{2+}$ to limit the amount of glutamate release during repetitive stimulation (Wu and Saggau, 1994). This pre-synaptic dysfunction may be due to a deficit in Ca$^{2+}$ sequestration or removal
from the cytoplasm of pre-synaptic neurons, a problem that is associated with aging (Verkhratsky and Toescu, 1998, Brewer et al., 2006, Sama et al., 2012). Other studies have shown that, deletion of either pre-synaptic Ca\(^{2+}\) regulatory protein synaptotagmin IV or scaffolding protein RIM1α leads to increased paired-pulse facilitation and exhibit deficits in contextual fear conditioning (Schoch et al., 2002, Ferguson et al., 2004, Powell et al., 2004). Overall, while the mechanisms underlying this pre-synaptic alterations in ischemic animals remains undetermined, the data presented are consistent with the hypothesis that pre-synaptic Ca\(^{2+}\) dysregulation may develop following CA, where increased synaptic Ca\(^{2+}\) overtime may lead to increased synaptic dysfunction; future studies should investigate this connection.

Previous studies have suggested that various degrees of synaptic transmission dysfunction (primarily deficits in LTP) are evident 7 to 14 days following 10-12 min of global cerebral ischemia in young rats (2-3 month old) (Kiprianova et al., 1999b, Wang et al., 2005, Dai et al., 2007); however, no discernable LTP deficit was observed in our study. Differences in the model used (ACA versus 2-VO or 4-VO), ischemia duration (6 versus 10-12 min), or the age of the animals (9 months versus 3-4) may explain the lack of an impairment of LTP. Overall, these results may suggest that CA3 to CA1 synaptic transmission is dysfunctional beyond the initial mild ischemic insult (lasting at least seven days). However, additional experiments are still needed to fully investigate this synaptic transmission dysfunction following global cerebral ischemia.
Various studies have indicated that following cerebral ischemia, animals display numerous cognitive deficits (Hickey et al., 1996, Neigh et al., 2004). Here, our data on nine month old Fischer 344 rats suggests that there is a significant impairment or delay in memory formation as there was an effect due to surgery (ACA vs. sham) observed on the Barnes maze in both latency and distance measurements, as well as differences in spatial memory performance on the fear conditioning task. These results are in agreement with reports indicating spatial memory deficits are apparent following CA in both rats and mice (Neigh et al., 2004, de la Tremblaye and Plamondon, 2011, Kiryk et al., 2011). Furthermore, we observed a difference between ACA and sham groups in the use of a spatial search strategy, as well as the use of a systematic non-random search strategy (either serial or spatial). Although, there is some controversy as to the role of the hippocampus in the resulting spatial memory deficits (Grubb et al., 2000, Roberge et al., 2008), our findings indicated extensive CA1 damage and spatial memory deficits, in agreement with a wide body of the literature (Auer et al., 1989, Volpe et al., 1992, Neigh et al., 2004, Wang et al., 2013, Kurinami et al., 2014). A potential explanation of the observed spatial memory deficits may be due to damage to the network connecting the medial prefrontal cortex and hippocampus (Bizon et al., 2012). It should be noted that this impairment was not due to a decrease in animal locomotor activity, as there was no significant difference in activity found in an open field test between sham and ACA animals (Fig. 14). While locomotor activity following CA has been controversial (Hicks et al., 2000, Kiryk et al., 2011), these differences in activity
could be dependent on the duration or model of global ischemia and the strain of animal used.

The cognitive dysfunction observed 7 days following mild CA in the Barnes circular platform maze and fear conditioning is suggestive of the synaptic transmission dysfunction and decreased number of non-compromised neurons observed in our electrophysiological and histological experiments. The data are in agreement with de la Tremblaye and Plamondon, 2011, who used 10-12 month old Wistar rats and 10 minutes of global cerebral ischemia (4-VO) to observe spatial memory deficits using the Barnes circular platform maze one week and contextual fear conditioning three weeks following ischemia (de la Tremblaye and Plamondon, 2011). Additionally, the spatial memory deficits may be long lasting, as Kiryk et al. (2011) found that spatial memory deficits were sustained for up to six months following cardiac arrest (Kiryk et al., 2011). It should be noted that hippocampal-dependent memory formation was only impaired and not completely inhibited, which is commonly observed in cardiac arrest survivors (Roine et al., 1993, Lim et al., 2004, Horstmann et al., 2010). This impairment, but not complete abolishment, was indicative of the animal's ability to encode and retrieve information about the spatial orientation within their environment over time during their Barnes circular platform maze trials. Our electrophysiology data was correlative with this observation as LTP was induced following tetanus, indicating that the molecular underpinning for memory formation is present at seven days.
Finally, due to the low survival rate of 38%, a possible limitation of this study may be that the animals that survived seven days were those with minimal ischemic damage, potentially minimizing the spatial memory and synaptic deficits observed. Interestingly, this outcome may closely resemble clinical paradigms, as the out-of-hospital CA survival rate is only 8-10% (McNally et al., 2011b, Roger et al., 2012).

In addition to establishing an aged cardiac arrest model of injury, this project sought to define a potential treatment that would prove to be effective in aged animals. Previous research has determined that the efficacy of IPC may be blunted by the aging process (He et al., 2005). Additionally, previous research has determined that the ability of neuronal activity to drive the expression of arc may be impaired in aged animals, particularly within the CA1 region of the hippocampus as a result of enhanced promoter methylation (Penner et al., 2011).

In order to investigate if a PKCε-mediated arc-dependent pathway would be an effective neuroprotective therapeutic in aged animals, we chose to investigate whether this pathway was fundamentally changed. Previous reports have indicated decreases in PKCα and β isoform signaling as a result of decreased RACKI receptor abundance (downstream receptor of PKCα and β). PKCε works through binding with its downstream receptor RACKII, (Battaini and Pascale, 2005). Consistent with previous literature (Battaini and Pascale, 2005), we observed no changes in PKCε protein levels or expression of its downstream receptor RACKII (Fig. 16). Our observation that administration of PKCε agonist, ψεRACK led to decreased activation of arc expression in aged animals is
consistent with known changes in arc protein expression in aging (Fig. 17), (Penner et al., 2011). Interestingly, we found that this decreased ability of ψεRACK to enhance arc expression in aged animals could be overcome by increasing the dosage of ψεRACK (Fig. 18). This finding has wide reaching implications for treatments within the aged population. Due to changes in methylation status of promoters or down regulation of the protein machinery involved in conferring neuroprotection, it is possible that other neuroprotective /preconditioning treatments (e.g. resveratrol, IPC, etc.) that have yet to be validated in aged populations, may need to be titrated specifically in these populations to overcome changes in underlying molecular mediators.

In summary, our results indicate that following ACA in nine month old Fischer 344 rats, there is a significant reduction in the number of non-compromised hippocampal CA1 and subiculum neurons, dysfunction in paired-pulse facilitation, and impairment in spatial memory encoding or learning. Despite these impairments in memory, animals displayed the ability to form spatial memories over-time. Additional studies are still needed to identify these pre-synaptic alterations following ACA and to further identify long-term cognitive deficits that could ensue from prolonged synaptic transmission dysfunction. Furthermore, we uncovered a potential treatment for the deficits and cell death observed in this middle aged model, through an increased dosage of the PKCε activator, ψεRACK.
5.4 Conclusion and Future Directions

CA and the resulting global cerebral ischemia is a major cause of neurological dysfunction that normally occurs in the aged population. Numerous CA models have been developed to mimic this type of ischemia (vessel occlusion to cessation of the heart/blood flow) that has allowed researchers to investigate this multi-faceted disease; however, most of these studies have been limited to younger animals. Synapses endure a variety of alterations after global cerebral ischemia from the resulting excitatory glutamate stimulus and have been a major target for neuroprotection. However, numerous neuroprotective agents have been advanced into clinical trials targeting glutamate excitotoxicity or other synaptic components without significant improvements (Ginsberg, 2008). While most of these neuroprotective agents have had very specific neuronal targets, the future of neuroprotective therapies could depend on therapeutics that are pleiotropic in nature. One such example is the PKCε activator, ψεRACK, which has multiple targets including those in the mitochondria and at the synapse (Raval et al., 2007, DeFazio et al., 2009, Morris-Blanco et al., 2014, Neumann et al., 2015) and as detailed in this project since it targets multiple sites for neuroprotection. This would allow for numerous targets and pathways to be activated and limit the immediate neuronal damage or protect CA1 hippocampal neurons from delayed neuronal cell death.

Furthermore, a shift in current models used to assess therapeutic neuroprotective treatments may be necessary. Currently, increasing the number of healthy neurons has become the primary endpoint for studies investigating the
efficacy of neuroprotectants; however, the future of these agents will also be dependent on their ability for clinically relevant behavior improvements. Therefore, future ischemia research should focus on characterizing cognitive deficits and how neuroprotectants reverse these behavioral deficits in addition to preserving healthy neurons. The probability of global cerebral ischemia increases with age suggesting the necessity for an aged-related clinically relevant model of ischemia (Goldberger et al., 2008, Reinier et al., 2011). Although testing on aged animals may be difficult and costly, determining how aging effects global cerebral ischemia will be critical for designing treatments for aged populations. Future studies continuing this project should include testing the efficacy of high dosages of PKCε to limit neuronal cell death and improve cognitive outcomes in an aged model of cardiac arrest.

Neuroprotection conferred by arc expression has implications beyond just PKCε-activated arc expression. If arc expression is neuroprotective and is tightly coupled to activity, this indicates that cellular activity itself may have neuroprotective properties. Previous studies show that an NMDA concentration that triggers depolarization of neurons can also be used as a preconditioning agent (Soriano et al., 2006). Currently, stroke and cardiac arrest have very few approved treatments to protect the brain against cell death following the ischemic event. Understanding the mechanisms by which PKCε-activation dependent preconditioning is protective against ischemic injury may help to develop future treatments against ischemic events such as stroke and cardiac arrest. The finding that a protein important for conferring neuroprotection is ubiquitous in the
processes of learning, memory, and cellular activation also may help shift the search for neuroprotective paradigms in a new direction.
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