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The Role of PKCδII in Insulin-Mediated Neurogenesis

Mishka Peart
Honors Thesis
Spring 2011

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ABSTRACT

Protein Kinase C delta (PKCδ) is a Serine/Threonine signaling kinase involved in a number of different cellular functions including cell differentiation, proliferation, and apoptosis. PKCδ improves learning and memory by preventing neuronal loss and maintaining the synapses. Data from our lab demonstrates that insulin regulates the alternative splicing of PKCδ pre-mRNA and promotes the expression of PKCδII, the splice variant that promotes neuronal survival. PKCδI, another splice variant of PKCδ, causes apoptosis of neurons. Our preliminary data and previous studies demonstrate that intranasal insulin improves cognitive ability in mice. The goal is now to determine the downstream components through which insulin is able to accomplish this task. Based on our data, it is hypothesized that insulin acts through PKCδII to increase the expression of other survival proteins in a cascade effect to improve cognition. What now needs to be determined is whether or not the increased levels of PKCδII will in fact increase the levels of the pro-survival genes Bcl2 and Bcl-xL, promoting neuronal survival and thereby increasing cognition. We expect that insulin treatments will increase the expression of PKCδII, Bcl2, and Bcl-xL. We propose that PKCδII is the crucial mediator of the insulin signaling pathway and directly increases the expression of Bcl2 and Bcl-xL.

INTRODUCTION

As the proportion of older people in the United States continues to rise, age-related cognitive decline among this population does as well. Currently, there are no therapies to slow or prevent cognitive decline, which can eventually lead to dementia and other neurodegenerative diseases. Through epidemiological studies and observing laboratory animals *in vivo*, strong links between insulin and cognitive function are supported, as have been proven by the Patel lab.

While it is true that the delivery of insulin to the central nervous system (CNS) improves memory, the exact mechanism remains unknown. Our data demonstrates that insulin increases PKCδII alternative splicing, and we would now like to discover the other downstream components of the mechanism. There are multiple pathways in the survival cascade that may be affected by the presence of insulin, and the purpose of this project is to investigate its target genes.

An Introduction to Protein Kinases

A kinase, sometimes referred to as a phosphotransferase, is an enzyme that transfers phosphate groups to specific substrates (Goodyear, 2010). This phosphorylation is responsible for directing most cellular activities, protein localization, and the overall function of many proteins. Kinase activity is also very prominent in signal transduction and other complex functions such as the cyclin-dependent kinases which regulate the cell cycle. Because kinases are responsible for mediating such essential functions, it is not surprising that over 50 distinct kinase families have been conserved between most eukaryotic organisms (Goodyear, 2010). It is this conservation that makes studying kinases among different species predictable in how they will react to certain conditions.

There are many different types of kinases, but protein kinases constitute one of the largest and most functionally diverse gene families that exist among them (Revil *et. al*, 2007). The protein kinase family can further be broken down into groups depending on the type of molecule that they phosphorylate. Tyrosine-specific protein kinases add a phosphoryl group to the amino acid Tyrosine, Histidine-specific kinases are found mainly in prokaryotes and transfer a phosphate group initially from ATP, then to Histidine, and finally to Aspartate or sometimes the

kinase itself. Serine/Threonine protein kinases phosphorylate the hydroxyl groups of the respective amino acids, and mixed kinases as the name suggests can have multiple specificity. Although protein kinases only constitute about 2% of the human genome, over 30% of all human proteins are modified by kinase activity (Goodyear, 2010).

The Protein Kinase C Family and Protein Kinase C Delta (PKCδ)

The Protein Kinase C (PKC) family consists of serine/threonine kinases (STK) that are activated by an increase in calcium ions and is involved in cell differentiation, cell growth, and apoptosis. There are currently eleven different known isoforms of PKC that exists. These eleven isoforms are then separated into three subfamilies: classical, novel, and atypical (Nishizuka, 2003). Classical, also known as conventional isoforms require calcium ions, diacylglycerol (DAG), and a phospholipid in order to be activated. This subfamily includes α, $β_I$, $β_{II}$, and γ isoforms (Newton, 2004). The novel isoform of the kinase requires DAG but no calcium ions for activation and include PKC- δ, ε, η, and θ. The atypical PKCs are the ζ, λ, and ι isoforms and require neither DAG nor calcium, and instead use phospholipids for activation and are usually on within the cell (Newton, 2004).

Protein Kinase C delta (PKCδ) belongs to the novel PKC subfamily and is a key mediator in apoptosis (Jiang *et. al*, 2008). Apoptosis can be defined as programmed cell death, and is a biochemical cascade that destroys molecules required for cell survival (Jiang *et. al*, 2008). It is necessary for cellular turnover and to restructure the neuronal network in a developing nervous system. PKCδ has a dual role in determining the fate of a cell: as a mediator of apoptosis as well as in survival mechanisms. A few contradictory roles that PKCδ has been implicated in include tumor suppression as well as the survival of several cancers; or as a

proapoptotic protein during DNA damage-induced apoptosis and as an antiapoptotic protein during receptor-initiated cell death (Basu and Pal, 2010). This is explained by the presence of alternatively spliced variants. Alternative splicing in PKCδ gives rise to PKCδI (constitutively expressed in all species), PKCδII (mouse), PKCδIII (rat), PKCδIV-VII (mouse), and PKCδVIII (human). The Patel lab has shown that PKCδI promotes apoptosis while PKCδII and PKCδVIII promote survival. The functions of PKCδIII-VII are unknown.

Mouse PKCδ has a hinge region between exons 9 and 10, which includes the caspase-3 recognition sequence. In the PKCδI pre-mRNA (~78 KDa), caspase-3 cleavage releases the C-terminal catalytic fragment which then triggers cell apoptosis. In PKCδII (~80 KDa), 78 base pairs are included after exon 9 due to utilization of an alternative 5' splice site, disrupting the binding site of caspase-3 and rendering caspase-3 protein unable to cleave the PKCδII pre-mRNA. Previous studies by Dr. Patel's lab have shown that PKCδII promotes survival of neuronal cells (Patel, Song, and Cooper, 2006). In humans, the hinge region is located between exons 10 and 11 and undergoes the same alternative splicing mechanism described above. PKCδVIII is the human homolog of the mouse PKCδII, and also exhibits anti-apoptotic properties.
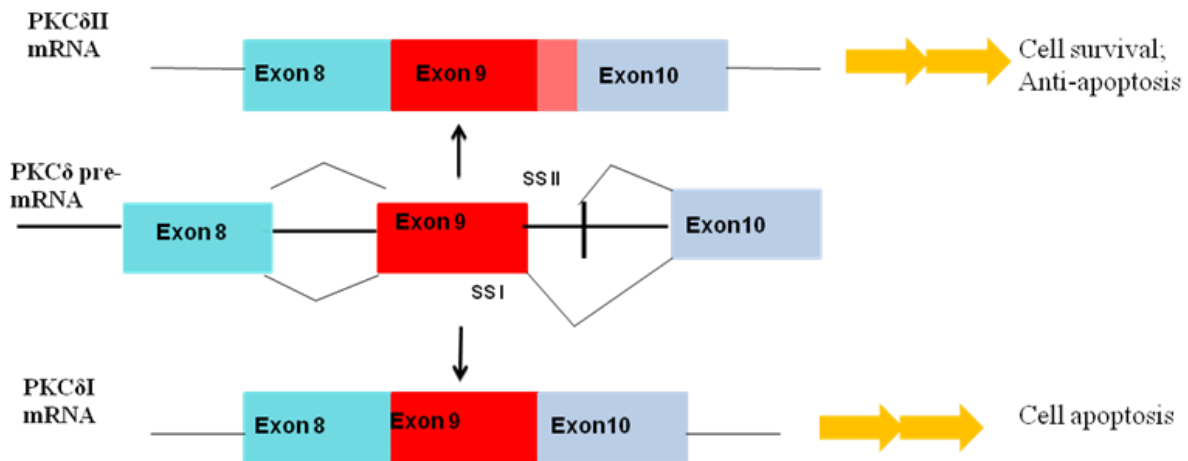
Figure 1. Schematic of mouse PKCδ pre-mRNA alternative splicing: Alternative 5' splice site selection in exon 9 of mouse PKCδ pre-mRNA creates PKCδI and PKCδII mRNAs which differ by 78 base pairs in the V3 hinge domain. Insulin promotes SSII selection. SSI: 5' splice site I; SSII: 5' splice site II. PKCδII promotes cell survival while PKCδI is apoptotic. Insulin increases PKCδII alternative splicing.

Survival Genes Bcl2 and Bcl-xL

Other genes that function in cell proliferation and apoptosis include those of the B-cell Lymphoma 2, or Bcl2 family. Bcl2 (26 KDa) is an apoptosis regulator protein that promotes cell survival by preventing apoptosis (Hetz, 2010). The Bcl2 gene has been implicated in a number of cancers and conditions such as schizophrenia and auto immunity. It is also thought to be resistant to most conventional forms of cancer treatment; its behavior in the pathogenesis of cancer and tumor formation supports its role in decreased apoptosis (Bronchud *et. al*, 2008). Bcl-x is a member of the Bcl2 family that undergoes alternative splicing to generate Bcl-xL (27 KDa), a pro-survival isoform, and Bcl-xS (17 KDa), a pro-apoptotic isoform (Wilhelm, Pellay, Benecke, and Bell, 2008). The larger Bcl-xL isoform (also known as Bcl-x Long form) is produced when splicing takes place at a specific 5' splice site located in exon 2. On the other hand, Bcl-xS (Bcl-x Short form) is derived from the use of a 5' splice site 189 nucleotides upstream from the Bcl-xL splice site (Exley *et. al*, 1999).
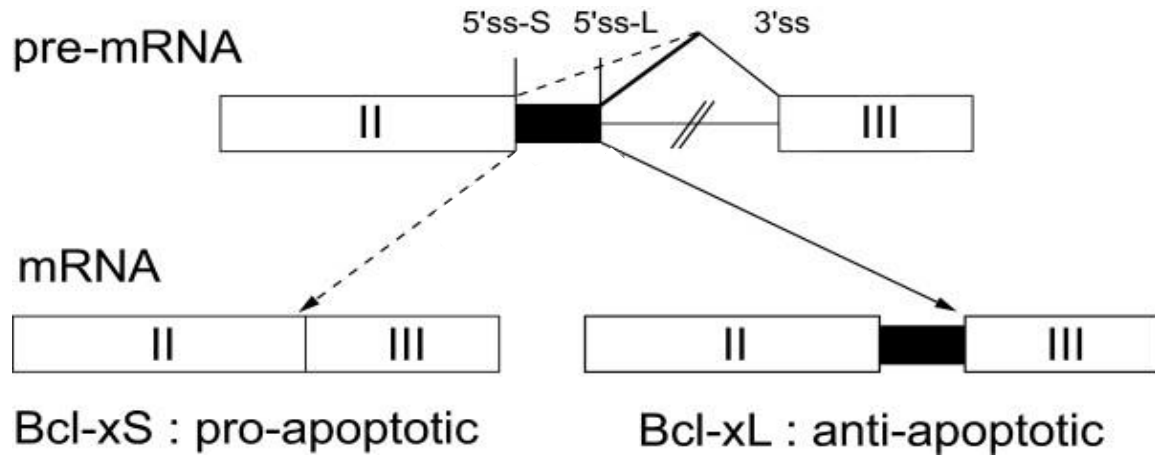
Figure 2. Alternative splicing of Bcl-x pre-mRNA: the 5' splice sites are located between exons 2 and 3, producing a difference of 189 base pairs between Bcl-xL and Bcl-xS.

Housekeeping Genes

GAPDH, or Glyceraldehyde 3-Phosphate Dehydrogenase (~37 KDa), is a housekeeping gene that is necessary for glycolysis (Pierce, 2005). The term 'housekeeping gene' refers to the fact that it is found in virtually all cells and is expressed at a relatively constant level regardless of experimental conditions. GAPDH is one of the most commonly used housekeeping genes used in comparison of gene expression data (Barber, Harmer, Coleman, and Clark, 2005). Another commonly used housekeeping gene is β-actin (~43 KDa). GAPDH and β-actin are used throughout this project as an internal control to ensure that the amount of mRNA and protein being analyzed among the different samples are constant and that any increase in gene expression is due to the experimental conditions, not the fact that different amounts of the gene is being analyzed. Further, their expression levels were not affected by our conditions and treatments.

Insulin

Insulin is a peptide hormone that is mainly responsible for glucose metabolism to keep sugar levels in the bloodstream low (Craft and Christen, 2010). When glucose is in abundance, such as shortly after a meal, insulin directs the body's cells to remove it from the blood for the production of energy or for storage in the liver in the form of glycogen. Other functions of insulin include increasing lipogenesis, decreasing lipolysis, and stimulating growth. There are also a few non-classical insulin target organs, such as the brain, where insulin modulates transcription and DNA synthesis, regulates the amount of food intake, and improve cognitive function (Craft and Christen, 2010). Because of the many functions and target organs of insulin, the route of administration is important to achieve a specific effect. For our purposes, administering the insulin to the mice intranasally was most plausible because it gives the insulin the opportunity to directly cross the blood-brain barrier for maximal absorption by insulin receptors in the brain (Strachan, 2005). As opposed to other routes, this yields minimal systemic absorption so the glucose levels in the bloodstream remain relatively constant before and after insulin treatment.

Techniques involved in the Project: RT, PCR, Western Blotting

This project focuses on evaluating the levels of gene expression in varying samples via RT-PCR and Western Blot analyses. The RT-PCR method analyzes gene expression levels based on the amount of mRNA present, and the Western Blot technique evaluates the level of protein in a sample. Transcription is the process of synthesizing RNA from a DNA template. The newly created RNA is then used by the cell to synthesize proteins and enzymes. In reverse transcription (RT), the process is turned around and RNA is used as a template to create complementary DNA,

or cDNA (Gilbert, 2010). This mechanism is characteristic of retroviruses, such as HIV, which transcribes its single-stranded RNA genome into double-stranded DNA, incorporating it into its host's genome (Coffin, Hughes, and Varmus, 1997).

In vitro, performing a reverse transcription requires the use of nucleotides, buffer, salts, and primers. The dNTPs (dATP, dCTP, dGTP, and dTTP) are incorporated into the newly synthesized DNA as Adenine, Cytosine, Guanine, and Thymine respectively. There are three common types of primers that are commonly used for RTs: random primers, oligo (dt) primers, and gene-specific primers. For this project oligo (dt) primers were used, which consists of short strands (~18 bases long) of Thymine nucleotides and target the poly-A tail (3' end) of mRNA. This choice of primers ensures a complete pool of all mRNA being reversed transcribed in our reaction. It also eliminates all other types of RNA, such as rRNA and tRNA.

There are three basic steps to carrying out an RT: denaturation, annealing and synthesis, and termination. During the denaturation step, the RNA sample is heated and it uncoils exposing its nucleotide bases. The sample is cooled during the annealing stages, and the primers adhere to the RNA and start synthesis of new DNA strands by incorporating the free nucleotides in solution through complementary base pairing. During termination, the temperature is again raised, inactivating the Reverse Transcriptase enzyme and ending the process (Walker and Rapley, 2008).
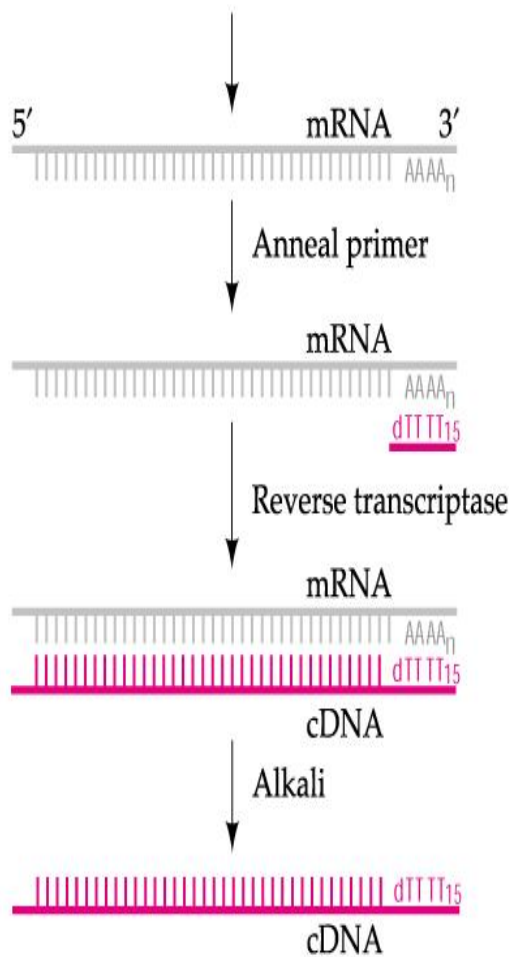
Figure 3. Method of preparing complementary DNA (cDNA) from the reverse transcription method. The cDNA can be isolated by raising the pH of the solution, thereby denaturing the double-stranded hybrid and cleaving the RNA. The double stranded-DNA can be isolated if the pH is kept in a neutral zone.

After the reverse transcription, the next step is the polymerase chain reaction (PCR) to amplify, or copy, the newly synthesized cDNA. Created in 1983 by Kary B Mullis, PCR is capable of synthesizing thousands to millions of copies of a specific DNA sequence (Micklos and Freyer, 2003). A significant amount of DNA is usually needed for molecular analysis, making PCR a necessary invention for studying DNA. PCR requires the use of a DNA template, forward and reverse primers, a DNA polymerase, dNTPs, and a buffer solution. The most commonly used DNA polymerase and the polymerase used in this lab is Taq Polymerase (National Human Genome Research Institute, 2010). Taq Polymerase, isolated from the

thermophile *Thermus aquaticus,* is stable at high temperatures and binds to the primer-template hybrid during the annealing stage of PCR in order to use the dNTPs to synthesize a new DNA strand.

PCR consists of 20-40 repeated temperature changes (cycles) with each cycle consisting of three discreet temperature steps. Similar to the RT, the PCR entails the denaturation step, annealing, and then elongation. In denaturation, the temperature is raised, the hydrogen bonds disrupt the complementary base pairing of the DNA molecule, and two single-stranded DNA molecules result. In the annealing step, the temperature is lowered and the primer and polymerase adhere to the DNA template. Finally during the elongation step, the temperature is raised slightly and the polymerase incorporates dNTPs in the new DNA strand complementary to the template. The cycle is repeated multiple times, resulting in thousands of new DNA fragments identical to the initial template (Walker and Rapley, 2008). The primers used in PCR reactions are specific for the target gene and optimized for linear amplification. In our experiments, we used PKCδ primers such that they amplify PKCδI mRNA and PKCδII mRNA simultaneously. This design also provides for a quantitative amplification.
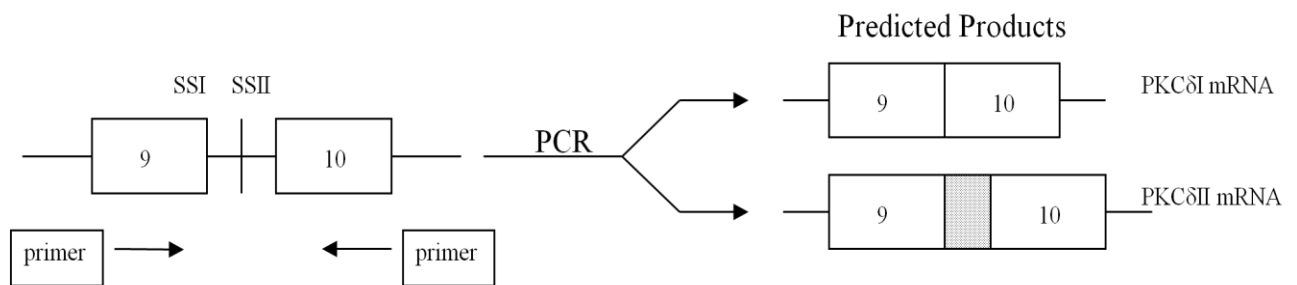
Figure 4. Simultaneous amplification of PKCδI and PKCδII mRNA. The arrows indicate the position of the primers which are designed to amplify PKCδI and PKCδII simultaneously.
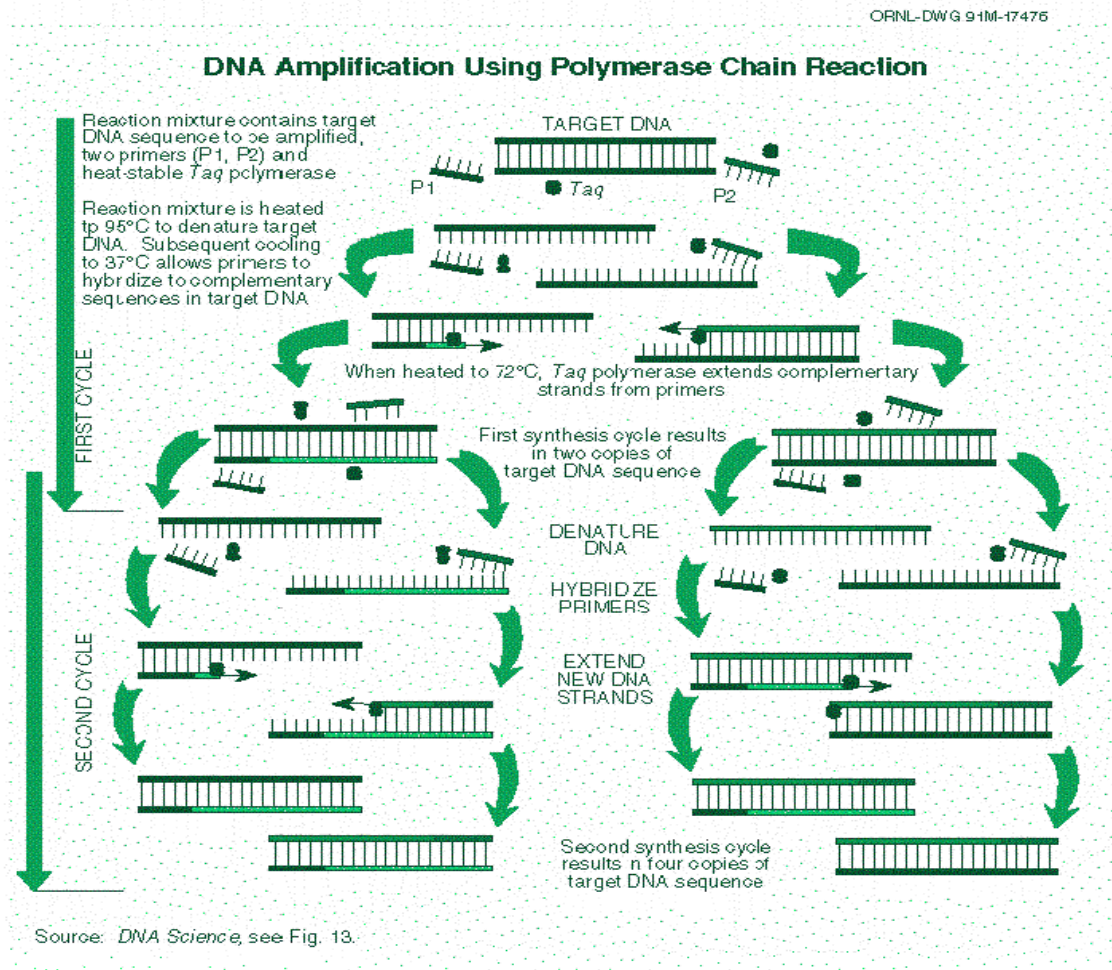
Figure 5. DNA Amplification using Polymerase Chain Reaction. The number of DNA strands is doubled with each cycle that the template undergoes. Each fragment contains the DNA region of interest.

A Western Blot, more formally called a protein immunoblot, is used to measure the relative amounts of protein present in different samples (Walker and Rapley, 2008). This determines whether the mRNA levels detected by PCR truly reflect the protein levels observed in a biological system. Since there is a possibility that mRNA can be degraded and destabilized prior to translation into a functional protein, it is necessary to determine the protein levels in response to treatments. Once the proteins are separated on a PAGE gel, they are transferred to a sheet of nitrocellulose blotting membrane. The blot is incubated with a generic protein (such as

milk protein) to bind the remaining open places of the membrane. The primary antibody, or the antibody that will bind to the protein of interest directly, is added to the blot. After the blot is washed with buffer, the primary antibody will only be attached to its target protein. The blot is then incubated with the secondary antibody, or the antibody that binds to the primary antibody. The secondary antibody is usually labeled with an enzyme or probe that makes detection relatively easy. Fluorescent detection is one of the most sensitive types of detection used for blotting analysis, and it is what is used for this project. The enzyme on the secondary antibody emits light when the secondary antibody finds and binds to the primary antibody, which is in turn attached to the protein of interest. A photograph can then be taken of the developed membrane (Walker and Rapley, 2008).
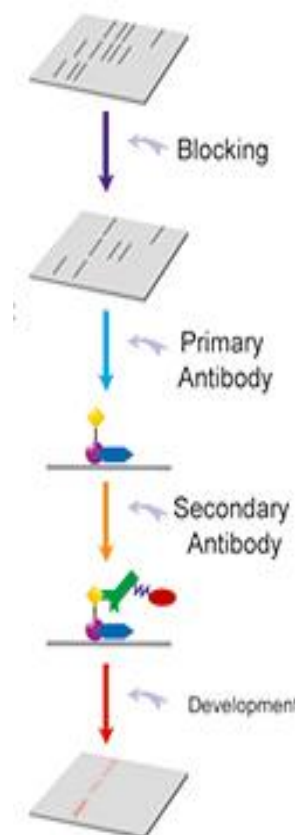
Figure 6. Schematic of protein analysis by Western Blotting. The membrane is blocked with a general protein, and then incubated in primary antibody specific to the protein of interest. The membrane is then incubated with secondary antibody, which binds to the primary antibody. An enzyme conjugated to the secondary antibody gives a visual cue that the secondary antibody has bound the first.

Summary of the Project

In this project we examined the levels of expression of PKCδI, PKCδII, Bcl2, and Bcl-xL present in the primary neuronal cell line established from the hippocampus of adult mice that were treated with varying doses of insulin. All analyses were done via RT-PCR and Western Blot analysis. Levels of GAPDH were also analyzed as an internal control as well as a loading control between samples.

BACKGROUND

Treating Mice with Insulin and the Radial Arm Water Maze (RAWM) Test

During previous *in vivo* studies in Dr. Niketa Patel's lab, mice were aged to 10 months before being assessed by the Radial Arm Water Maze (RAWM) test. The RAWM test, which was initially constructed for rats, is now also adapted for mice to test their hippocampal-dependent spatial learning, memory, and overall cognitive abilities (Salazar, 2007). The test is conducted with a large pool of water and a transparent platform, located in one of the six arms, submerged 1-2 centimeters beneath the water. External cues are strategically placed around the pool to help the mice remember where the platform is located. The mouse's task is to use the spatial configuration of the room and the cues to find the hidden platform (Shukitt-Hale, McEwen, Szprengiel, and Joseph, 2004). This was done for twelve trials and the mice were evaluated on 1) their ability to find the platform, 2) the number of mistakes they made when trying to find the platform and 3) the time it took to achieve the results.

Figure 7. Radial Arm Water Maze Test: Note the transparent platform located in the spoke in the upper left hand corner of the picture.

To further test their learning ability, once the mouse has mastered the task of finding the platform, it is moved to a different location, where they are again evaluated in twelve different trials. To test their short-term memory, the mouse is taken out of the pool and returned to the cage for an hour before they are placed back in the pool and evaluated in twelve different trials. When testing the long-term memory, the mouse is removed from the pool and returned the next morning for evaluation.

RAWM was administered to the mice prior to beginning treatments to establish a baseline. The mice were then treated with insulin intranasally for four weeks. Simultaneously, a control group was given saline for four weeks instead of insulin. On completion of their treatment regime, the mice were assessed by the Radial Arm Water Maze where their short- and long-term memories and their cognitive performances were compared. Results show that the administration of insulin to aging mice improves their memory and learning abilities. These results are shown on the following page.

(a)

(b)
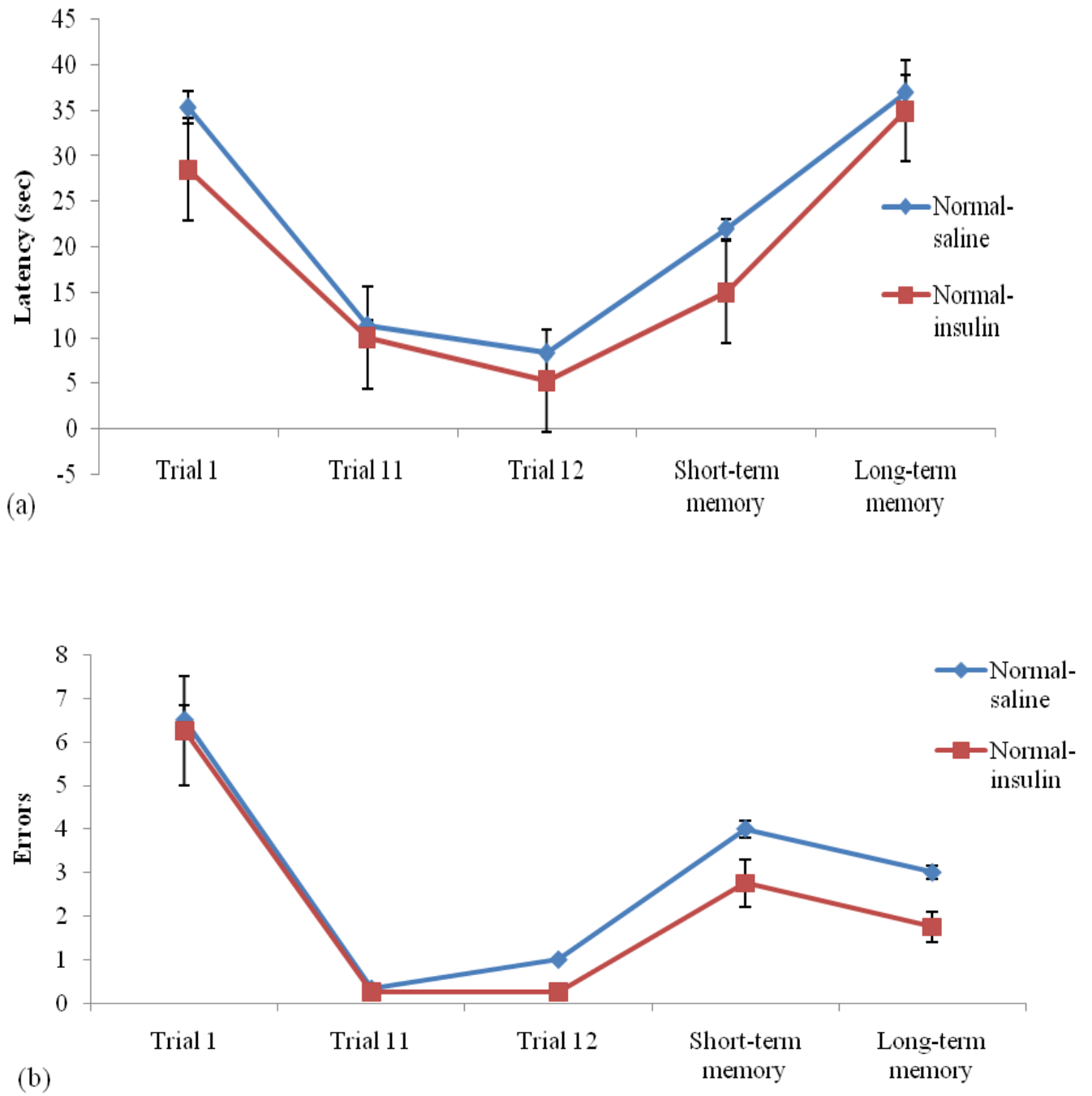
Figure 8. **Behavior tests after intranasal insulin treatment:** Performance during the last block of pre-testing in the RAWM task during working memory trials T11 and/or T12; retention trial (short term memory) and long term memory retention for both latency (a) and error (b) measurements. Results revealed insulin treatment improved performance in memory tests in mice.

Analysis of Hippocampal Tissue via RT-PCR and Western Blot Analysis

From the *in vivo* experiments, we have found that insulin improves cognitive function and now need to determine the mechanism by which this occurs. It is suspected that PKCδII plays a role in the cognitive function signaling pathway, so its levels of gene of expression were tested both in the presence and absence of insulin. Tissues from the hippocampus from the above group of mice were processed for RT-PCR and Western Blot analysis. In both the Western Blot and RT-PCR analyses, the levels of PKCδII visibly increased from the control samples (saline), to the samples that were treated with intranasal insulin. The RT-PCR shows that the expression levels of PKCδI remained constant between the insulin-treated and untreated hippocampal samples. The studies done by Patel lab demonstrated that insulin does not affect the levels of the pro-apoptotic PKCδI, but only increases the pro-survival PKCδII gene. The membrane and PAGE gel from the Western Blot and RT-PCR respectively can be seen below.

(3a) Western Blot Analysis

WT

C   Ins

PKCδII

GAPDH

(3b) RT-PCR

WT

C   Ins

PKCδII

PKCδI

GAPDH
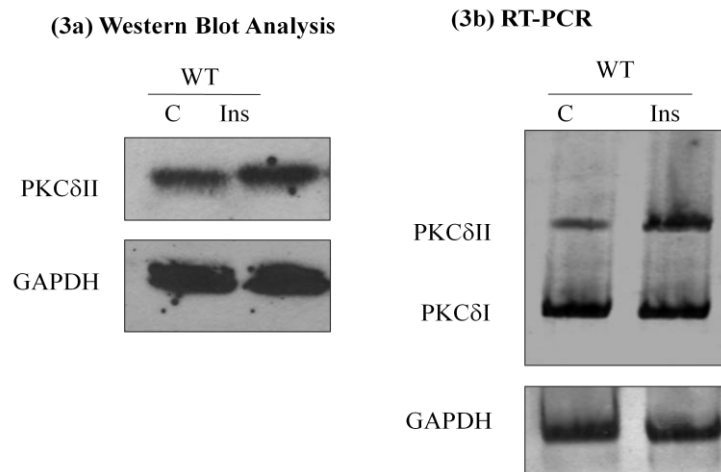
Figure 9. Insulin increased PKCδII expression in vivo: Hippocampus from aged mice treated with or without intranasal insulin were processed for Western Blot analysis (a) and RT-PCR (b) as indicated above. In both methods, it is evident that the levels of PKCδII increased visibly in the hippocampuses of mice that were treated with insulin.

*Immunohistochemical Analysis of Mouse Hippocampal Tissue*

In order to increase cognitive function, two components have to be satisfied: neurogenesis has to be promoted and the survival of these newly formed cells has to be maintained. In previous studies carried out by the Patel lab, Bromodeoxyuridine (BrdU) is used to detect neurogenesis and proliferating cells in living tissues (Bd Pharmingen, 2007). In actively replicating cells, BrdU can be incorporated into the DNA as an analog of thymidine. Antibodies specific for BrdU can detect the presence of the chemical, and an enzyme conjugated to the antibody produces a visual cue (usually fluorescence) that indicates new cell synthesis. The cells are co-stained with NeuN, a marker for neurons. This analysis was done on both hippocampal tissues from mice that were untreated and those that were treated with insulin for four weeks. The amount of fluorescence (correlated to the amount of neurogenesis taking place) was compared to determine whether or not insulin was able to promote neurogenesis. Results (data not shown) showed that insulin promotes the formation of new neurons in the hippocampus of mice and is again capable of improving cognitive function.

Microarray for Apoptotic Genes

In order to determine which genes of the apoptosis pathway were affected by insulin, an apoptosis microarray analysis was performed by the Patel lab. Results demonstrated the expression levels of 84 apoptosis genes in response to insulin treatment. and hence the genes we would like to study throughout the project. Control and insulin samples (10 nM) were tested in triplicate and the expression level of each gene in the control sample versus each test sample (those with insulin) were calculated. The pro-survival genes that yielded the largest response became our candidate genes to study. We chose to study the pro-survival genes because PKCδII

is pro-survival and previous studies have shown that PKCδII expression levels increase when insulin is administered.

The results suggested that amongst the pro-survival genes, Bcl2 and Bcl-xL gave the largest response to insulin and were thus selected to be studied for the project. There were several other pro-apoptotic genes whose expression was decreased by insulin, but we focused our study to the pro-survival genes that would explain insulin-mediated increases in neuronal survival. The microarray profile shown below compares control samples to those treated with 10 nM of insulin.
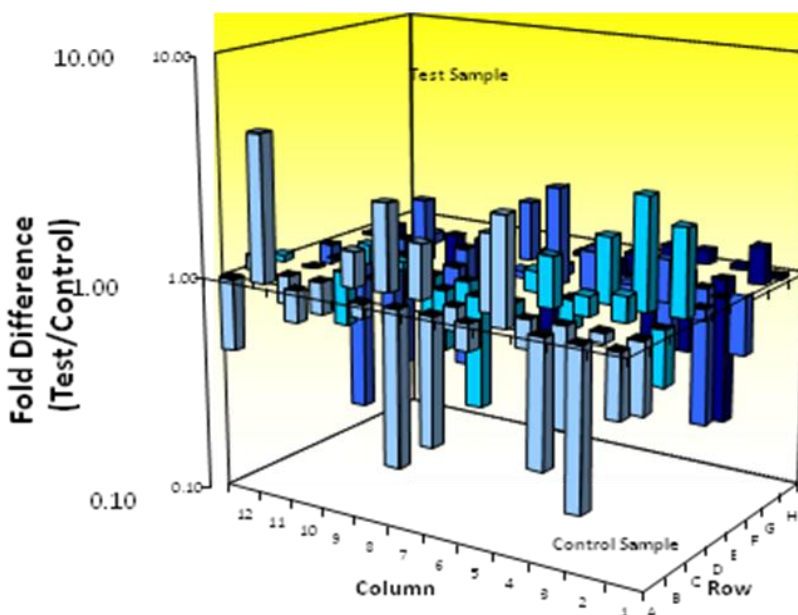
Figure 10. 3D profile of results from apoptosis microarray: The graph represents an average of control and insulin samples carried out in triplicate. The expression level ($2^{-\Delta Ct}$) of each gene in the control sample versus the test (insulin) sample is calculated. Results showed that the expression levels of Bcl2 and Bcl-xL increased the most amongst the pro-survival genes in the presence of insulin.

In the normal signaling cascade, the expression levels of Bcl2 and Bcl-xL are not enough to suppress the effects of the pro-apoptotic proteins and enzymes and thus cell death is inevitable. When the levels of Bcl2 and Bcl-xL in the cell are high, the apoptotic machinery such as Bax, Bak, and Caspase-3 cannot function and cell survival and neurogenesis are increased. A broad overview of the roles of Bcl2 and Bcl-xL involved in the neuronal survival signaling pathway can be viewed on the following page.
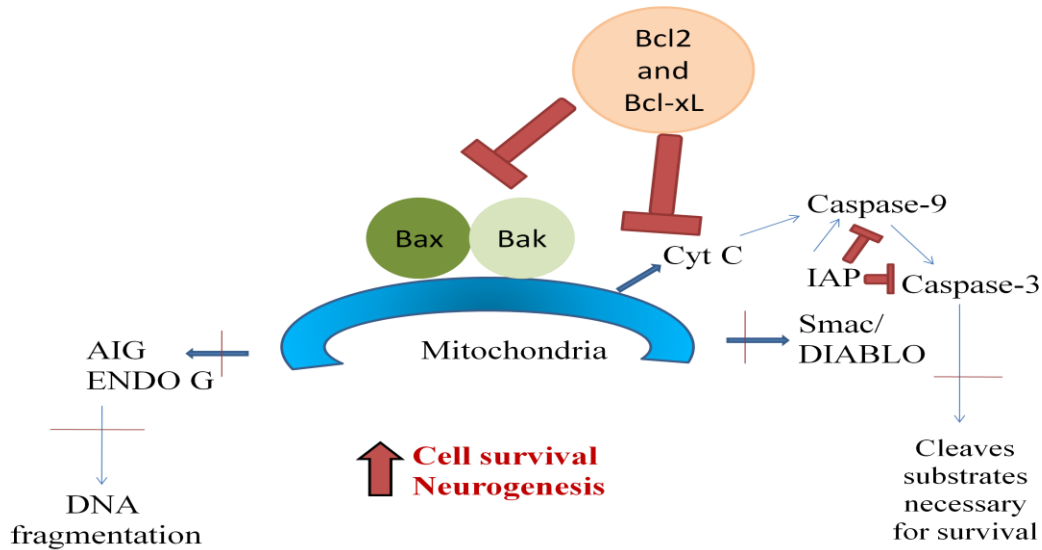
Figure 11. Broad schamatic of signaling components involved in neuronal survival. Bcl2 and Bcl-xL inhibits the apoptotic cascade, thus increasing cell survival and neurogenesis.

MATERIALS AND METHODS

Primary Neuronal Cell Culture

A primary neuronal cell line derived from the hippocampus of normal adult mice was previously established by the Patel lab for use in our *in vitro* experiments. The cells were plated at a density of 10^6 in a six-welled plate, maintained in a total of 6 ml of 0% Dulbecco's modified Eagle's medium (DMEM) and 10% Fetal Bovine Serum (FBS), and were then incubated at 37 ºC in a 95% air and 5% CO_2 cell incubator. The medium was changed and the cells were replated every three days.

Cell Harvesting and RNA Isolation for mRNA Analysis

To harvest the cells, they were removed from the incubator after treatment and the media was eliminated by suctioning with an unfiltered pipette tip. They were washed with 1M Phosphate Buffered Saline (PBS) to remove excess media in the wells. RNA was isolated from

each sample by first using 500 µl of RNA Bee™ to lyse the cells. The cells were scraped from the walls of the six-welled plate, collected in a microcentrifuge tube, and left at room temperature for 5 minutes. 100 µl of chloroform were added, the tubes were shaken (not vortexed) for 30 seconds, and the samples were again kept at room temperature for 5 minutes. The samples were centrifuged at 4 ºC for 15 minutes, or until the formation of two distinct layers were visible. 250 µl of the colorless upper aqueous layer was transferred into a new microcentrifuge tube; an equal amount of chilled isopropanol was added to the nucleic acids, and stored at room temperature for 10 minutes.
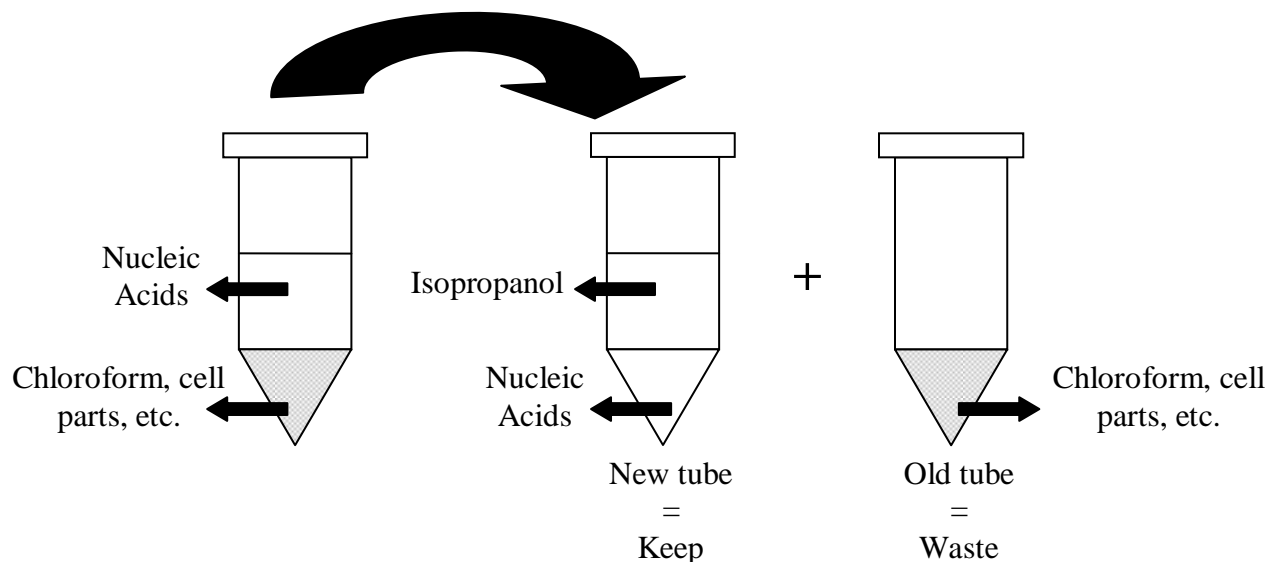
Figure 12. Layer Components after centrifugation for RNA isolation. The nucleic acid is transferred to a fresh tube and is mixed with isopropanol. The organic layer is discarded as waste.

The samples were centrifuged again for 15 minutes at 4 ºC, or until the RNA begins to precipitate as a translucent pellet in the bottom of the tube. The supernatant was removed and the pellet was washed with 500 µl of 75% ethanol. The samples were centrifuged for 5 minutes at 4 ºC, and the supernatant was removed by pipetting. The pellet of RNA was air-dried and dissolved in nuclease-free water. The RNA concentration in each sample was found using a gene quant.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) Analysis

A reverse transcription (RT) was done where 2 μg of RNA was used to synthesize complementary DNA (cDNA) using Oligo (dT) primers, dNTPs, RNAse inhibitor, 10X RT buffer, and RNA Omniscript. Once the cDNA was synthesized, 1-2 μl was used to perform a Polymerase Chain Reaction (PCR). Taq polymerase, 5% DMSO, dNTPs, sense, and antisense primers were also included in the reaction. The primers used for the PCR included:

1. Mouse PKCδ
   - sense 5'-CACCATCTTCCAGAAAGAACG-3'
   - antisense 5'-CAACAACGGGACCTATGGCAAG-3'

2. Mouse Bcl-xL
   - sense 5'-CTCTCCTACAAGCTTTCCCAG-3'
   - antisense 5'-CCAGCGGTTGAAGCGCTCC-3'

3. Mouse Bcl2
   - sense 5'-ATCTTCTCCTTCCAGCCTGA-3'
   - antisense 5'-TCAGTCATCCACAGGGCGAT-3'

4. Mouse GAPDH
   - sense 5'-GCACAGTCAAGGCCGAGAAT-3'
   - Antisense 5'-GCCTTCTCCATGGTGGTGAA-3'

Following 30 cycles of amplification in a Biometra T3000 thermocycler (PKCδ: 94 °C, 30 s; 54 °C, 30 s; and 72 °C, 1 min. Bcl-xL: 94 °C, 1 min; 59 °C, 1 min; and 72 °C, 20 s. Bcl2: 95 °C, 45 s; 65 °C, 45 s; and 72 °C, 2 min. GAPDH: 94 °C, 30s; 60 °C, 30 s; and 72 °C, 45 s), the products were resolved on 6% PAGE gels and detected by silver nitrate staining. The gels were soaks briefly in 10% ethanol for 3 minutes, followed by soaking in 1% nitric acid for 3 minutes. The gel was then incubated in 0.1% $AgNO_3$ for 10 minutes. The developing solution (37% formaldehyde, 10 mg/ml sodium thiosulfate, and 6% Na_2CO_3) was then added and the gel was rocked until bands appeared. The reaction was stopped by the addition of 10% glacial acetic

acid. The gel was then dried between cellophane sheets. Analysis via RT-PCR was done at least 3 times per experiment.

Protein Lysis and Western Blot Analysis

To harvest the cells, they were removed from the incubator after treatment and the media was eliminated by suctioning with an unfiltered pipette tip. They were washed with 1M Phosphate Buffered Saline (PBS) to remove excess media in the wells, and 250 μl of protein lysis buffer were added to each sample. The samples were then mixed with Laemmlli loading buffer to a final concentration of 1 M and placed in a heating block at 90 ºC for 6 minutes. The samples were cooled and then loaded onto a 10% polyacrylamide gel for electrophoretic separation at 60 V through the stacking layer and 150 V through the resolving layer for 2 hours. The samples were run in 1 M running buffer against a known molecular marker.

Once the gels were run, the protein had to be transferred to a nitrocellulose membrane. For each gel, 2 Scotch-Brite pads, 2 pieces of filter paper, and 1 piece of membrane are needed. The pads, filer paper, and membrane are to be soaked in 1 M transfer buffer for 15 minutes. A medium-sized magnetic stir bar and ice box were placed in the loading tank for the transfer. The gels were removed from the first apparatus and the stacking layers were cut off. The pads, filter paper, gel, and membrane were stacked in the ribbed screens, removing all bubbles, in the following manner: dark bubble screen, pad, filter paper, gel, membrane, filer paper, pad, and the clear bubble screen. The bubble tray was closed, slid into the tray holder, and the entire cassette was loaded into the loading tank. The tank was filled with transfer buffer and placed on a stir plate, and the anode and cathode were connected. The stir plate was turned on, and the membranes were run at ~100 V for 1.5 hours.
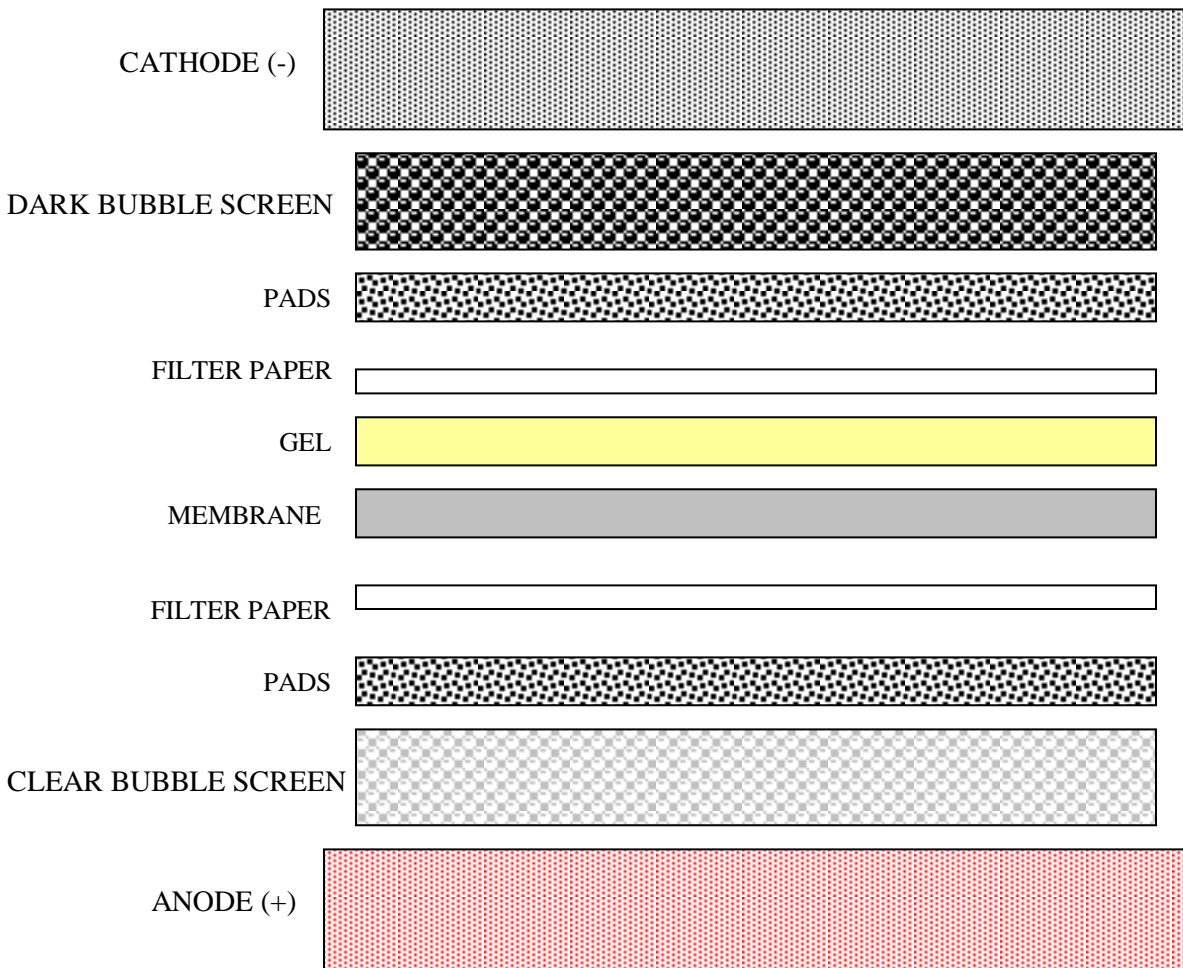
Figure 13. Constituents of the cassettes and orientation in the loading tank. The overall process of the transfer runs from "dark" to "light", with the gel closer to the cathode and the membrane closer to the anode.

The membranes were blocked with PBS/0.2% Tween 20, and 4% nonfat dried milk for one hour at room temperature. They were then washed and incubated for 24 hours at 4 ℃ with a primary monoclonal antibody against anti-PKCδII, anti-Bcl2, anti-Bcl-xl, anti-PKCδ C-terminal antibody, or anti-GAPDH. The membranes were washed again and incubated with secondary anti-rabbit or anti-mouse antibody for either 2 hours at room temperature or 24 hours at 4 ℃. The membranes were washed again and were ready to be developed.

To develop, the membranes were incubated in equal amounts of solution 1 and 2 from the electrochemiluminescence (ECL) for five minutes before being placed a plastic-lined cassette. In

a dark room, the film was placed in the cassette and photographs of the blots were developed at different exposures. Analysis via Western Blotting was done at least three times per experiment.
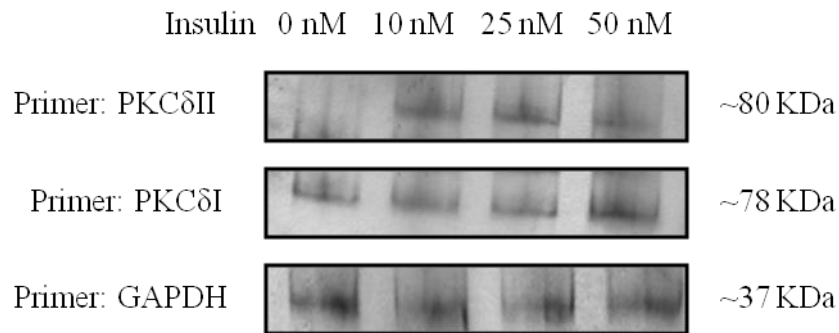
Densitometric and Statistical Analysis

Photographs of the blots and gels were scanned into a computer and analyzed via Un-Scan-It® software program. A densitometric analysis was done to compare the percent of densitometric units in each sample for each gene tested for. The densitometric units of each sample were also standardized against β-actin, our housekeeping gene. Finally a statistical analysis was done to find the five percent error in each analysis.

RESULTS

Insulin Dose Curve Experiments

The cells were grown to 80% confluency and treated with increasing doses of insulin (10 nM to 200 nM). The cells were incubated again at 37 ºC in a 95% air and 5% CO_2 environment for 24 hours and harvested for RT-PCR and Western Blot analysis. Our results demonstrate that PKCδII levels increase in an insulin dose-dependent manner: the higher the concentration of insulin in each sample, the stronger the band that corresponds to PKCδII in both RT-PCR and Western Blot analyses. Our results also show that the expression levels of the survival genes Bcl2 and Bcl-xL increase in response to insulin. Note: Because kinases are capable of becoming phosphorylated and having their molecular weights affected, the weight of each mRNA/protein is estimated using molecular markers.

RT-PCR Analysis

Insulin   0 nM   10 nM   25 nM   50 nM

Primer: PKCδII   ~80 KDa

Primer: PKCδI   ~78 KDa

Primer: GAPDH   ~37 KDa

Figure 14. The effects of insulin on PKCδI and PKCδII expression levels. As the concentration of insulin increased, the levels of PKCδII increased as well. The expression levels of PKCδI remain unaffected by insulin. GAPDH is used as an internal control to normalize the genes and compare equal amounts of protein. NOTE: This data was generated by the Patel lab.
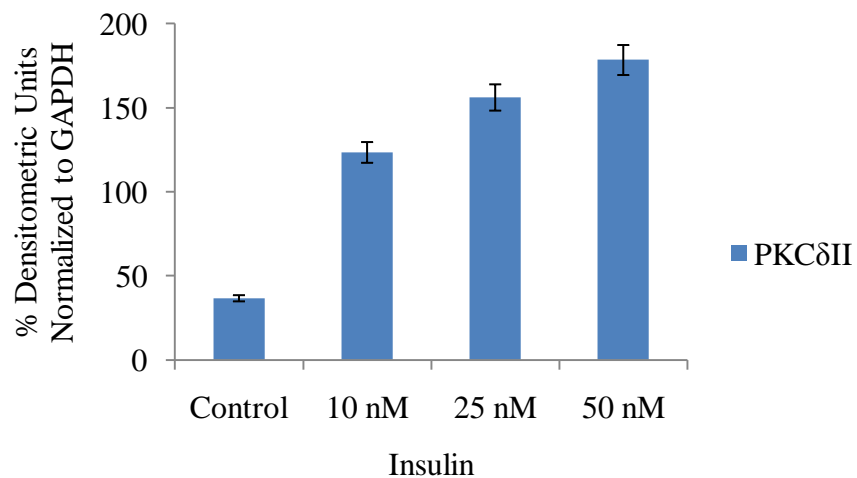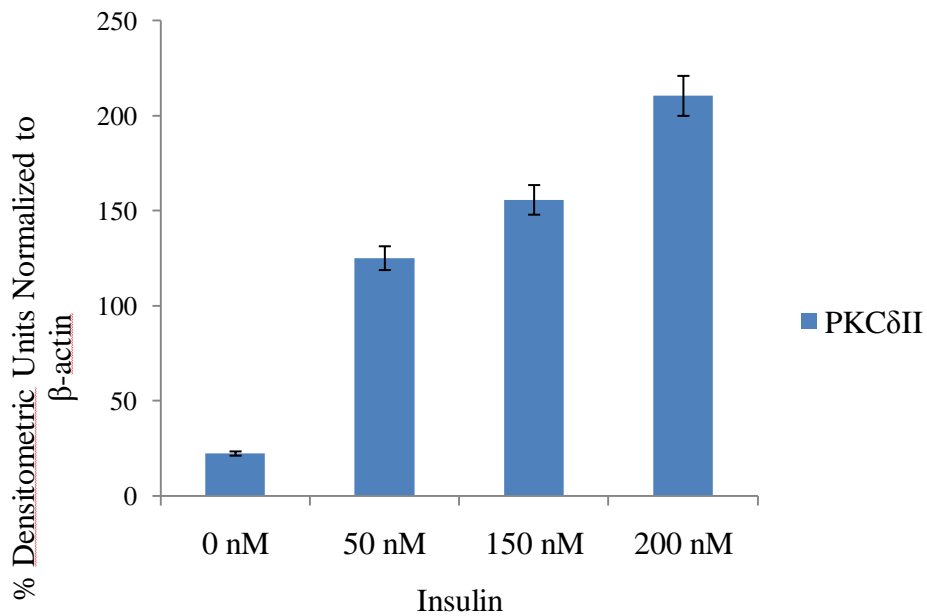
Graph 1. Densitometric analysis of PKCδII after being normalized against GAPDH. The densitometric units of PKCδII increase with increasing concentrations of insulin as demonstrated by RT-PCR analysis. NOTE: This Data was generated by the Patel lab.

Western Blot Analysis
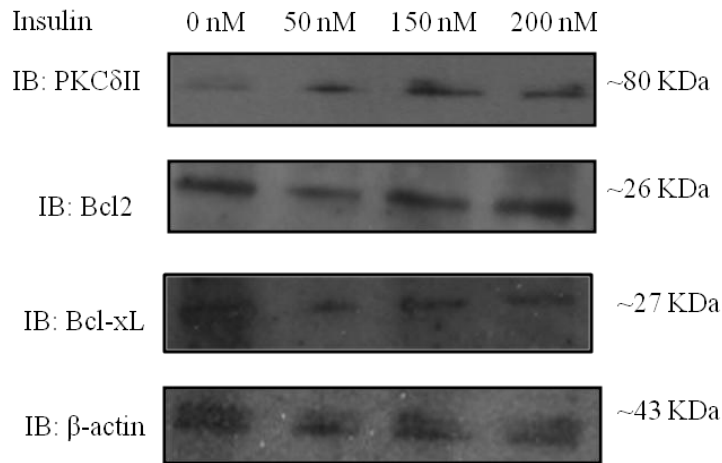
Figure 15. The effects of insulin on PKCδII, Bcl2, and Bcl-xL expression levels. As the concentration of insulin increased, the levels of PKCδII, Bcl2, and Bcl-xL proteins increased as well. β-actin is used as an internal control to normalize the genes and compare equal amounts of protein.
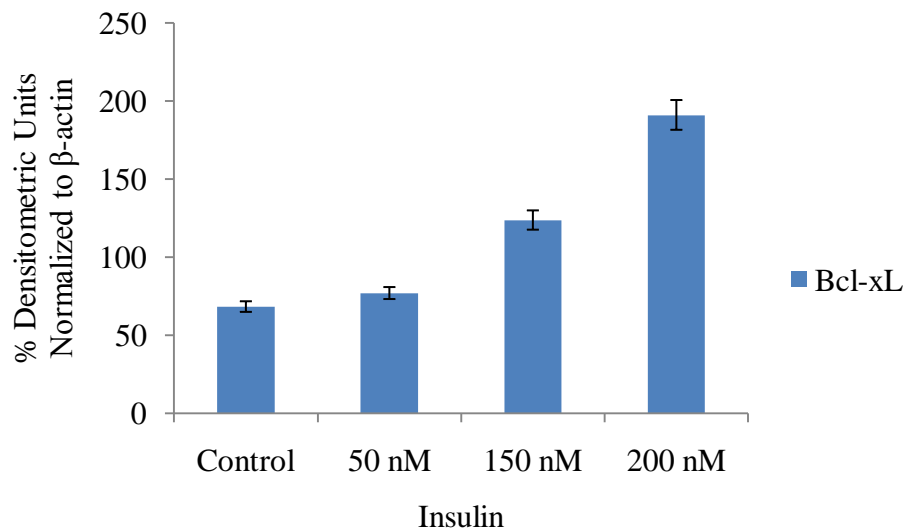

Graph 2. Densitometric analysis of PKCδII after being normalized against β-actin. The densitometric units of PKCδII increase with increasing concentrations of insulin.
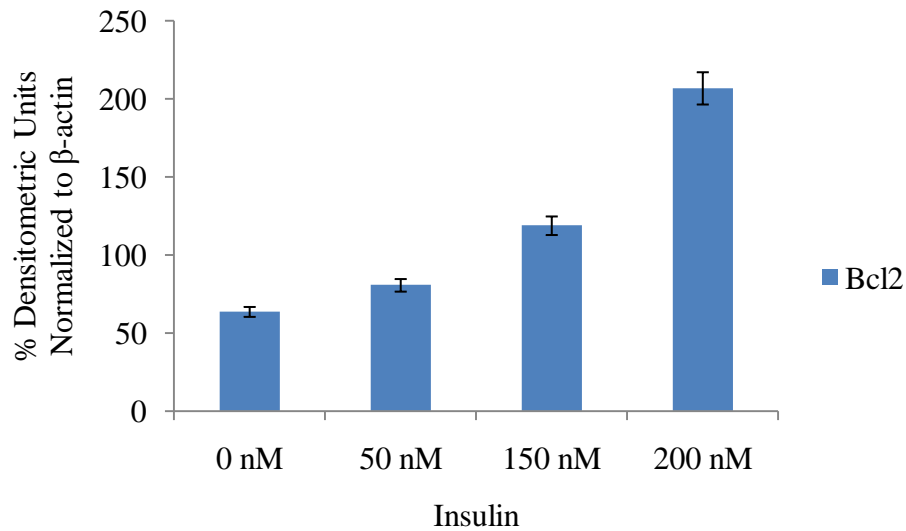
Graph 3. Densitometric analysis of Bcl2 after being normalized against β-actin. The densitometric units of Bcl2 increase with increasing concentrations of insulin.


Graph 4. Densitometric analysis of Bcl-xL after being normalized against β-actin. The densitometric units of Bcl-xL increase with increasing concentrations of insulin.

PCKδII Overxpression Experiments

The cells were plated, maintained, and incubated in the manner described above. When the cells were grown to 50% confluency, they were transfected with the PKCδII/pTracer plasmid

vectors. To do this, the TransIT®-LT1 reagent (8 µl per 1 g of DNA used) was diluted in 250 µl of 0% DMEM and incubated at room temperature for 20 minutes. The plasmid DNA was added, at a concentration of 3 µg per well, to the diluted solution, mixed by pipetting, and incubated again at room temperature for 30 minutes. The reagent/DNA complex mixture was added to the cells and once again incubated for 24 hours at 37 °C. The samples were harvested the next morning for RT-PCR and Western Blot analysis.
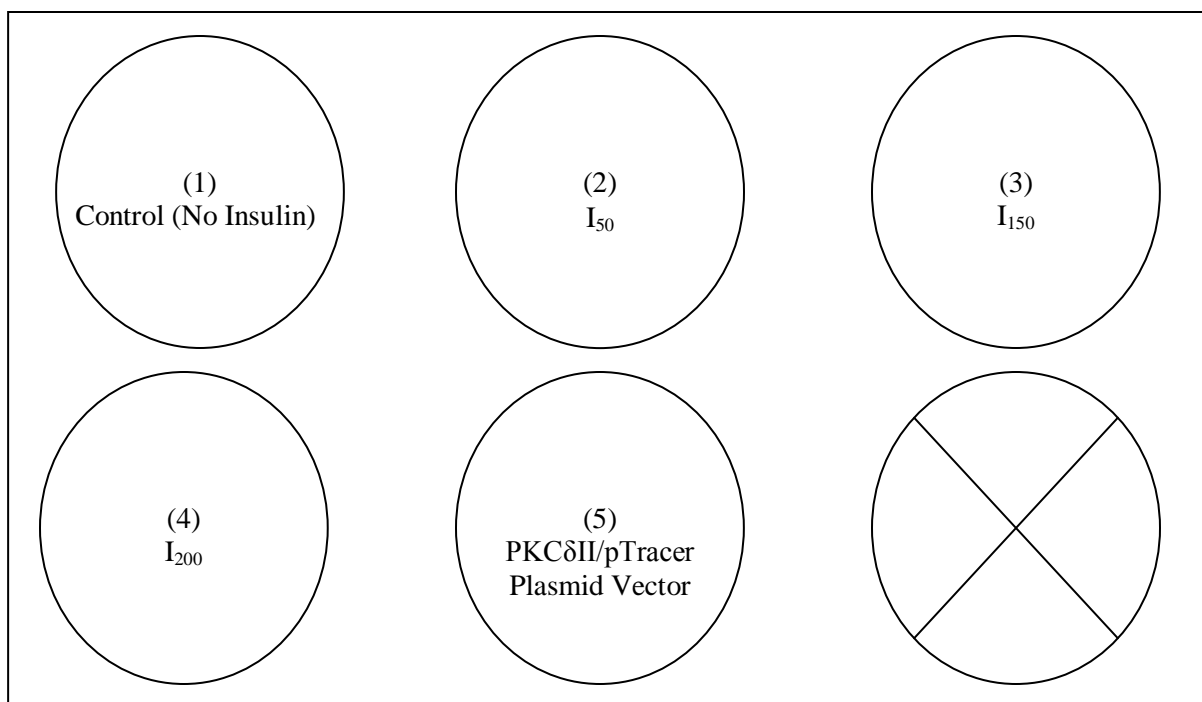
Figure 16. Treatment of the Primary neuronal cells. Sample 1) no treatment, 2) 50 nM insulin, 3) 150 nM insulin, 4) 200 nM insulin, and 5) overexpression of PKCδII using pTracer plasmid vector.

Our results demonstrate that the overexpression of PKCδII in a sample mimics the response of the cells to insulin: the expression levels of PKCδII, Bcl2, and Bcl-xL all show a visible increase in comparison to the control sample. Further, overexpression studies indicate that PKCδII is upstream in the Bcl2-mediated survival pathway.

Western Blot Analysis
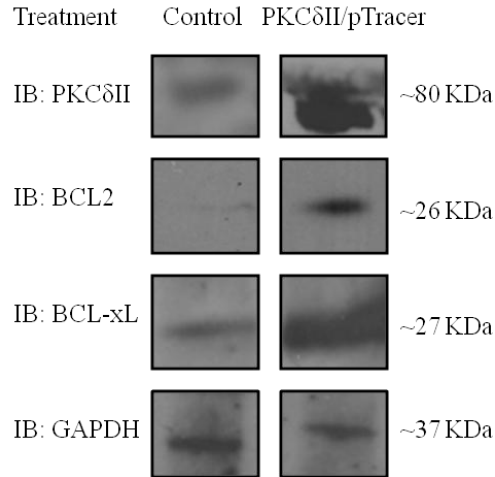
Figure 17. The effects of insulin on PKCδII, Bcl2, and Bcl-xL expression levels. As the concentration of insulin increased, the levels of PKCδII, Bcl2, and Bcl-xL proteins increased as well. GAPDH is used as an internal control to normalize the genes and compare equal amounts of protein.
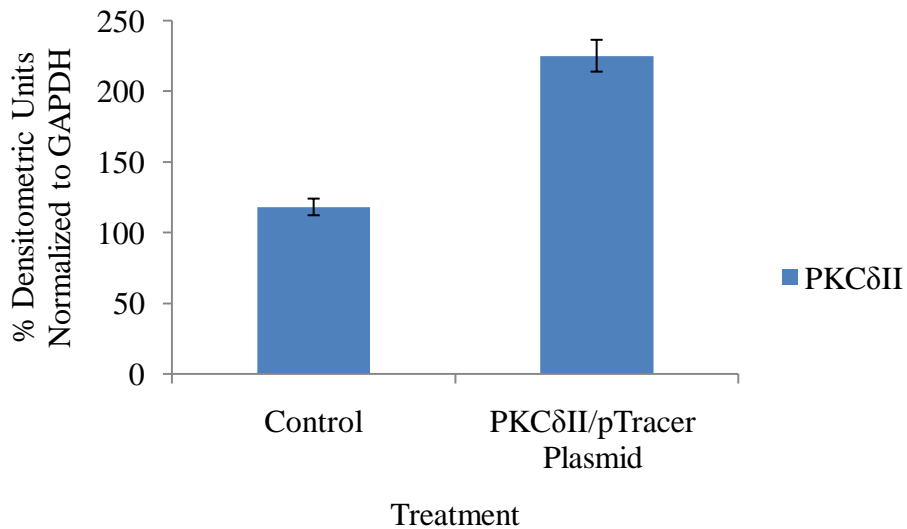
Graph 5. Densitometric analysis of PKCδII after being normalized against GAPDH. The densitometric units of PKCδII increase with increasing concentrations of insulin.
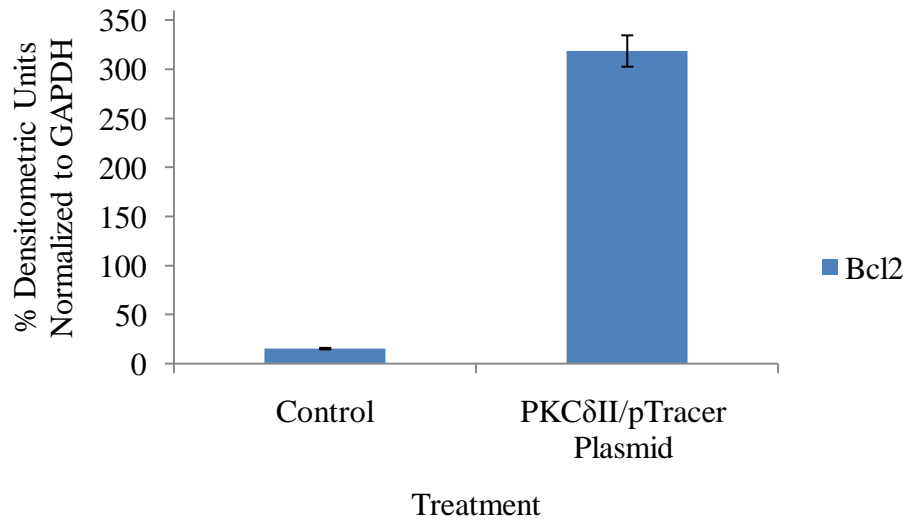
Graph 6. Densitometric analysis of Bcl2 after being normalized against GAPDH. The densitometric units of Bcl2 increase with increasing concentrations of insulin.
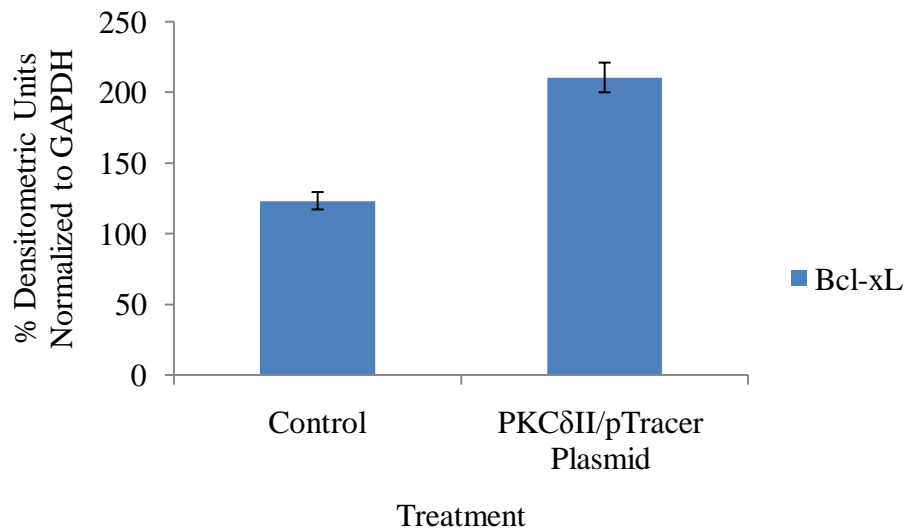
Graph 7. Densitometric analysis of Bcl-xL after being normalized against GAPDH. The densitometric units of Bcl-xL increase with increasing concentrations of insulin.

DISCUSSION AND ANALYSIS

When insulin is administered intranasally, it functions in the CNS to improve cognition rather than in the systemic regulation of glucose uptake. The purpose of this project was to find a way to prevent dementia and cognitive decline before neurodegenerative disease becomes severe. Before a therapeutic drug can be proposed, its target genes and signaling pathways must be known. Because it has been demonstrated that PKCδII, Bcl2, and Bcl-xL are all crucial mediators in the insulin pathway that improves cognitive function, it is possible to synthesize therapeutic agents that will specifically target the increase of these pro-survival genes.

For the insulin dose curve RT-PCR analysis, the increase in PKCδII is visible. The 50 nM sample had a slightly higher amount of GAPDH present, leading us to conclude that there is somewhat higher concentration of protein in this sample that the other samples. This may explain why it may seem that the expression levels of PCKδI might have increased from the 25 nM to the 50 nM sample. When the densitometric analysis was done and each sample was normalized to GAPDH, we found that the levels of PKCδI remained relatively constant from between all four samples (between 84-100% densitometric units). This data was generated by Andre Apostolatos of the Patel lab.

For the insulin dose curve western blot analyses, the housekeeping gene β-actin showed an unequal distribution among our four samples. The control sample had a significantly larger amount of β-actin present than the samples for I_{50}, I_{150}, and I_{200}. This allows us to conclude that there is more protein present in the control sample. To account for the difference in β-actin levels between each sample, the genes were all normalized against the housekeeping gene β-actin. In the blots for Bcl2 and Bcl-xL, the band corresponding to the control seems to have a higher concentration of each gene than the I_{50} sample. After the normalization, it can be shown by the

percent densitometric units of each sample that the expression levels of PKCδII, Bcl2, and Bcl-xL all increase with increasing doses of insulin. The error bars represent 5% random error, which our collected data falls outside the range of. The data collected can be assumed to vary based on factors other than random error and our results are statistically significant.

In the western blot analysis for the overexpression experiment, the increase of PKCδII, Bcl2, and Bcl-xL can all be visibly seen from the control sample to the sample in which PKCδII was overexpressed. This was confirmed by densitometric analysis after being normalized against GAPDH.

Insulin Increases PKCδII Levels in a Dose-Dependent Manner

Hypothesis 1: The expression level of PKCδII is dependent on the concentration of insulin administered to each sample.

As the concentration of insulin administered increased, so did the expression levels of PKCδII. Since the levels of PKCδII in the sample is increased, it is assumed that there is less apoptosis occurring and more cells are surviving contributing to the increase in cognitive function that was seen in the mice experiments.

Insulin Increases the Expression of the Pro-Survival Genes Bcl2 and Bcl-xL

Hypothesis 2: The levels of the pro-survival genes Bcl2 and Bcl-xL increase with increasing doses of insulin.

When insulin was added to a sample, the levels of Bcl2 and Bcl-xL increased visibly from the control samples, as demonstrated by RT-PCR and Western Blot analysis. This supports the results from the microarray analysis previously performed by the Patel lab. The higher the

concentration of insulin administered, the higher the gene expression levels of Bcl2 and Bcl-xL. Since there is more Bcl2 and Bcl-xL in the cell, the apoptosis pathway is blocked, the cell survives, and neurogenesis occurs causing an increase in cognitive function.

Insulin Promotes the Expression of Pro-Survival Proteins via PKCδII

Hypothesis 3: Insulin acts through PKCδII to increase the expression of other survival proteins in a cascade effect to improve cognition.

In the overexpression experiment, the overexpression of PKCδII mimicked the same effect that insulin had on the samples. The levels of PKCδII, Bcl2, and Bcl-xL increased from the control samples. This signals that PKCδII is a crucial mediator in the cognitive function pathway, increasing the expression of the pro-survival genes Bcl2 and Bcl-xL, and in turn increasing cell survival and neurogenesis.

Future Work

Future studies include how the absence of PKCδII will affect the insulin-mediated increases in cognitive function. A siRNA knockdown experiment, in which the PKCδII gene is removed, will analyze how the expression levels of Bcl2 and Bcl-xL are affected by insulin treatments. If the expression levels of the pro-survival genes are diminished or totally absent, it would be feasible to assume that the PKCδII-mediated pathway is the only mechanism which acts to increase the expression levels of these genes. On the other hand, if the expression levels of Bcl2 and Bcl-xL are not completely inhibited by the PKCδII siRNA, then there is another mechanism other than the one mediated by PKCδII that affects the expression of these survival genes in the cell.

Conclusion

The findings from our studies can be summarized in the following statements:

- Insulin administered intranasally increases cognitive function

- Insulin promotes cognitive function via alternative splicing of PKCδII

- Increased levels of PKCδII in the cell results in an increase in expression levels of the pro-survival genes Bcl2 and Bcl-xL

- Insulin promotes alternative splicing of PKCδII which increases the expression of Bcl2 and Bcl-xL. This ultimately leads to increased neuronal survival and neurogenesis, which are key to increases in cognitive function.

Our hypothesis, that insulin acts through PKCδII to increase the expression of other survival proteins in a cascade effect to improve cognition, was supported by the findings of this project. The model on the following page visually represents our conclusions.
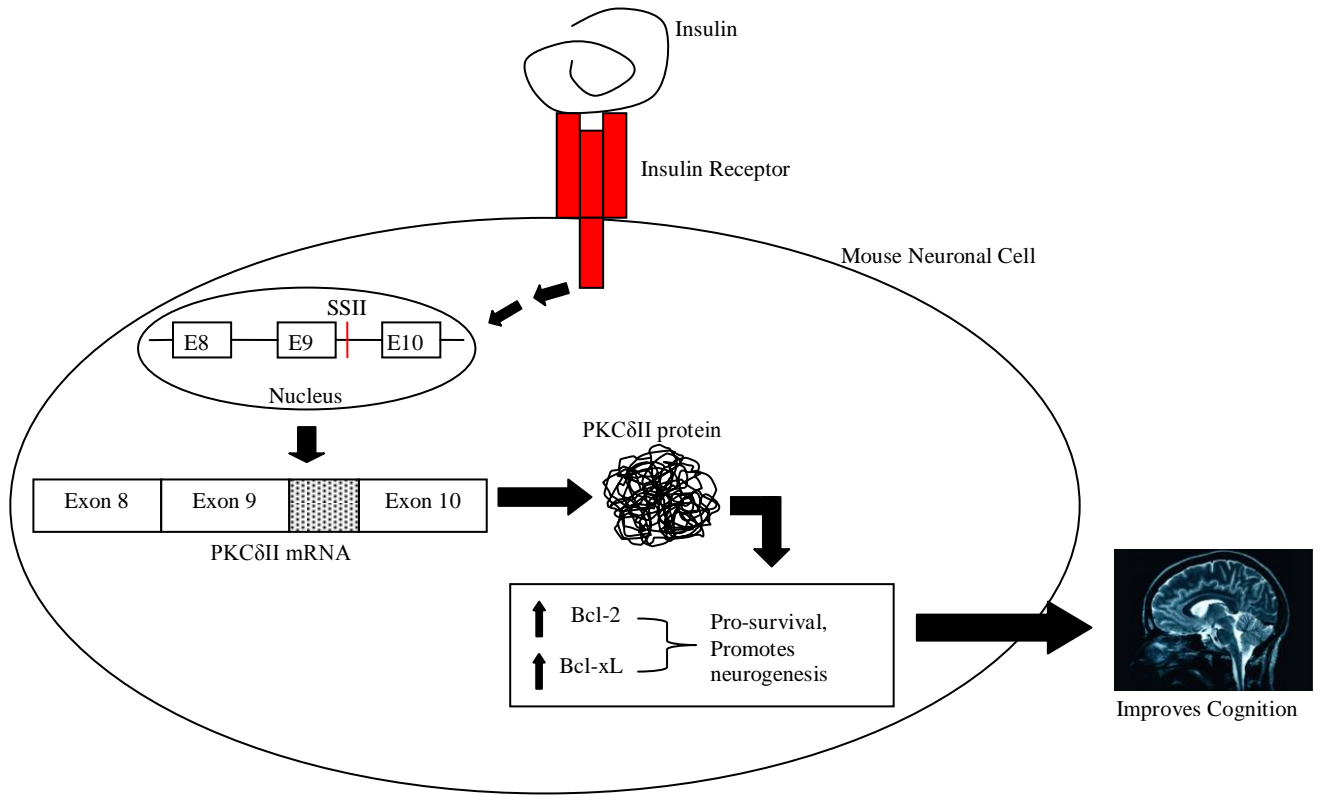
Figure 18. Summary of how the administration of insulin increases cognitive function through the use of PKCδII and the pro-survival genes Bcl2 and Bcl-xL.

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