The contribution of single Akt isoforms to neuronal survival: characterization of a new mechanim of Akt activation in the PDK1 K465E mice

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The contribution of single Akt isoforms to neuronal survival: characterization of a new mechanim of Akt activation in the PDK1 K465E mice

Memoria de tesis doctoral presentada por Xiangyu Zhou para optar al grado de Doctor en Neurociencias por la Universitat Autònoma de Barcelona.

Este trabajo ha sido realizado bajo la dirección del Doctor José Ramón Bayascas en la Unitat de Medicina del Departament de Bioquímica i Biología Molecular y en el Institut de Neurociències de la Universitat Autònoma de Barcelona.

El director

Dr. Jose Ramon Bayascas

El doctorando,

Xiangyu Zhou

Bellaterra, 2014

Nothing seek, nothing find!

无所求则无所获!

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SUMMARY

During development of the nervous system, neurons born from progenitors cells progress to their mature phenotype by means of intricate genetic programs which controls cell survival, growth, differentiation, migration and synapses formation. As development proceeds, neurons encounter a great diversity of growth factors and hormones that activate different signaling pathways. Combined pharmacological and overexpression studies carried out during the last two decades established the PI3K-Akt pathway as a central mediator required for neurotrophic factor-induced neuronal survival, and Akt is considered nowadays a drug target against neurodegenerative disorders and cancer.

However, mice lacking single Akt isoforms showed a mild nervous system phenotype only in the case of Akt3 that were far from confirming this notion. It is possible that different Akt isoforms could interchange and compensate each other deficiency. The implication of other substrates of PDK1 different from Akt in the regulation of the neuronal survival also cannot be neglected. Because specific Akt inhibitors have not been available until recently, genetic evidences demonstrating a role of Akt in regulating neuronal survival were needed.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a master kinase which mediates the action of growth factors by regulating a number of kinases of the AGC family including Akt, S6K, SGK, RSK and PKC isoforms. Akt regulation by PDK1 relies on the interaction of the PDK1 pleckstrin-homology domain (PH-domain) to PtdIns(3,4,5)P_3, the PI 3-kinase product. Following growth factor stimulation and PI 3-kinase activation, both PDK1 and Akt co-localize at the plasma membrane due to the ability of their PH-domains to bind the newly generated PtdIns(3,4,5)P_3 second messenger, where PDK1 can readily phosphorylate and activate Akt. Crystal structure high-resolution of the PDK1 PH-domain allowed the design of a specific point mutation, Lys465 to Glu, impairing the interaction of the PH-domain with PtdIns(3,4,5)P_3. This knock-in mice model was shown to be a good tool to analyze the contributory role of the Akt signaling pathway to glucose metabolism. The PDK1$^{K465E/K456E}$ mice were shown to be viable but smaller, with a modest reduction in

Akt activity compared with the wild type mice. By contrast, other PDK1 target such as RSK, S6K or SGK isoforms remained unaffected. This makes the PDK1$^{K465E/K465E}$ mutant mice model an excellent tool to explore the contribution of Akt to different human pathologies and to identify downstream substrates that could provide targets for therapeutic intervention. In particular, I aimed to use this genetic model to unravel the contribution of single Akt isoforms to neuronal survival and to understand the mechanism by which Akt activation can still take place in the absence of PDK1-PtdIns(3,4,5)P_3 binding.

Our former studies have indicated that neuronal survival was not compromised in the PDK1$^{K465E/K465E}$ mice, although activation of Akt was obviously reduced. Even after treated with the Akti-/1/2 inhibitor, which specifically inhibited Akt1 and Akt2 but not Akt3, cortical neurons were still surprisingly capable to survive. Regarding this, I proposed that Akt3 plays key role in regulating neuronal survival. In order to verify this hypothesis and re-define the contribution of single Akt isofroms to neuronal survival, optimized conditions for the specific detection of individual Akt isoforms were established. This allowed me to quantify the activation reduction of each Akt isoform in the PDK1$^{K465E/K465E}$ mice. I found that Akt activity levels were reduced by to 59% in the PDK1$^{K465E/K465E}$ neurons when compared to the controls, and that this reduction was contributed by all the three Akt isoforms, including the Akt3. Strikingly, when compared with Akt1 (55 %) and Akt2 (48 %), Akt3 activity was reduced only to 73%, which implies that the remained Akt3 activity can still play critical role to maintain the neuronal survival. I also found that Akt1 accounted for nearly half of Akt total activity (46.4%) and Akt3 accounted for 40.1% approximately, whereas Akt2 only accounted for 13.5% and was almost reduced to blank in the PDK1 K465E mice. These data imply the important roles that Akt1 and Akt3 may play in the nervous system. Consider that cell viability was not compromised and Akt2 was reduced to almost blank in the PDK1$^{K465E/K465E}$, I did not have much expectation on Akt2 role in regulating the neuronal survival.

In order to evaluate the contribution of single Akt isoforms to neuronal survival, I generated lentiviral particles containing corresponding Akt specific shRNAs to silence

the expression of each Akt isoform. After comparing the specificity and interference ability between different Akt shRNAs, appropriate shRNAs were selected which could reduce the expression of Akt1, Akt2 and Akt3 to 18%, 29% and 6%, respectively. Then I employed these lentiviral vectors to infect cortical neurons derived from the $PDK1^{K465E/K465E}$ and $PDK1^{+/+}$ mice and evaluated the survival responses of the infected cultures. and The hoechst analysis strongly suggested that neuronal survival was regulated by Akt in a quantitative manner, in which all the akt isoform-specific shRNAs could not increase the apoptotic percentages in PDK1 wild type cells, whereas Akt1 and Akt3 shRNAs significantly enhanced apoptosis in the $PDK1^{K465E/K465E}$ neurons. As predicted, , no significantly increase in apoptotic cell numbers was detected in neurons infected with the Akt2 shRNAs.

When the $PDK1^{K465E/K465E}$ mice physiologically expressing from the endogenous locus a mutant form of PDK1 incapable of phosphoinositide binding, were first generated, activation of Akt was not foreseen. We corroborated that the reduced activation of Akt that was still observed in the $PDK1^{K465E/K465E}$ cells was dependent upon the binding of PDK1 to the hydrophobic motif of Akt when phosphorylated at Ser473, and proposed that the PDK1 K465E mutation might reflect the ancient functions of this signaling branch, such as cell survival, whereas the mutual co-localization of PDK1 and Akt at the membrane might represent a functional innovation relying on the convergent acquisition of phosphoinositide binding domains by these two kinases, which in turn allowed faster and acute responses to particular second messengers. Indeed, inhibiting Akt Ser473 phosphorylation with the AZD8055 mTOR inhibitor totally inhibited the phosphorylation of Akt at Thr308 in the $PDK1^{K465E/K465E}$ mutant neurons at doses that did not affect Akt Thr308 phosphorylation in the control cells. As a consequence, the BDNF-elicited survival responses of the $PDK1^{K465E/K465E}$ cortical neurons were further decreased when compared to the control neurons.

In summary, my study provided new insights to understand the contribution of single Akt isoform to neuronal survival and also proposed a novel evolutionary mechanism of Akt activation.

ABBREVIATIONS

AGC	cAMP-dependent kinase, cGMP-dependent kinase and protein kinase C Family of kinases
Bad	Bcl-1/Bcl-XL-associated death domain protein
Bak	Bcl-2 homologous antagonist-killer protein
Bax	Bcl-2 associated X protein
Bcl-2	B-cell CLL/Lymphoma-2
Bcl-xl	Bcl-2 related gene, long isoform
Bim	Bcl-2 interacting mediator of cell death.
BDNF	Brain-derived neurotrophic factor
4EBP1	4E-binding protein 1
ERK	extracellular-signal-regulated kinase
IGF1	Insulin receptor substrate
IkB	IkB kinase
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukaemia 1
MDM2	Murine double minute 2
mTOR	mammalian target of rapamycin
NF-kB	Nuclear factor-k type B
NTs	Neurotrophins
NTD	Neural tube defects
PCD	Programmed cell death
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinase(also PI 3-kinase)
PIP2	Phosphatidylinositol-4,5-bisphophate
PIP3	Phosphatidylinositol-3,4,5-trisphophate

PKB/Akt	Protein kinase B
PKC	Protein kinase C
PRAS40	Proline-rich Akt Subtrate of 40 kD
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homologue enriched in brain
S6K	p-70 ribosomal S6 protein kinase
Trk	Tropomyosin-related kinase
TSC	Tuberous sclerosis complex

Less frequently used abbreviations are defined upon their first use in the text.

INTRODUCTION

Neurons are electrically excitable cells that are the basic building blocks of the nervous system, which includes the brain and spinal cord of the central nervous system (CNS), and the ganglia of the peripheral nervous system (PNS). There is one key difference between neurons and other cells in the animal body: neurons are specialized to process and transmit information through electrical and chemical signals which occur via synapses, specialized connections with other cells throughout the organism. A typical neuron possesses a cell body (soma), dendrites, and an axon (Fig. 1). In higher vertebrates, the survival of neurons is regulated by the programmed cell death, namely apoptosis, which results in the elimination of as much as half of the originally generated cells to determine the final number of neurons during the embryonic development (Oppenheim,1989). Neuronal survival is regulated by both cell-extrinsic and intrinsic programs. Any abnormality on neuronal survival during development can lead to various clinical manifestations. Mature neurons develop a specific mechanism to prevent the cell death by restraining apoptosis and therefore promoting survival. Neurodegenerative diseases are caused by abnormal neuronal death in the brain during adulthood, which indicates that completed development of neurons is crucial for the health of individuals. Although significant progress in the understanding of the signaling pathways supporting neuronal cell survival has been made, there still exist many unknown fields which need us to explore further this area of knowledge.

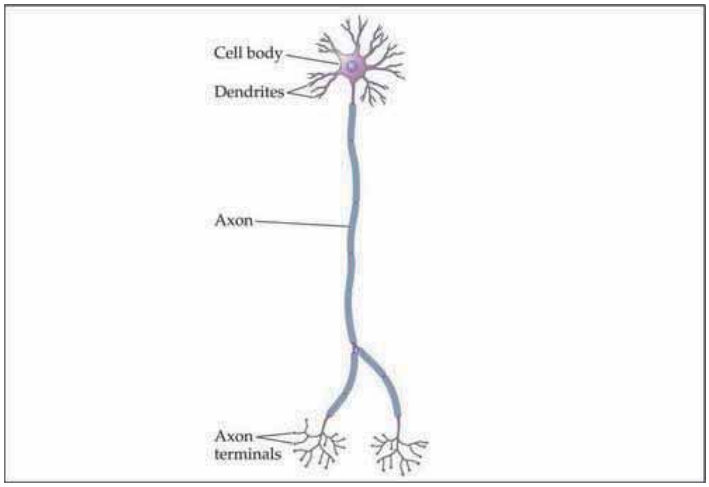

Figure 1. Structure of a typical neuron. Figure is adapted from internet (www.quizlet.com)

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1. NEURONAL CELL FATE DETERMINATION

Nervous system development is a intricate and fundamental process which starts with proneural genes expression and then neuronal fate determination, migration, axonal formation, and finally functional synapse generation followed by fine-tuning of connections (Kempermann, 2006). Neurons arise from regions of the brain that abound in high concentrations of neural stem cells. These cells own the potential ability to generate the variety of types of neurons which exist in the adult brain. Neural stem cells proliferate by splitting and producing either two new stem cells, or two early progenitor cells, or one of each. If a stem cell splits to produce two stem cells, it is called self-renew and these new cells have the potential to generate more stem cells. But if the stem cell produces an early progenitor cell, it is called differentiation. Differentiation implies that the progenitor cell is more specialized in form, shape and function. Once a neuron is produced, it has to migrate to the region of the brain where it performs the function. Not all the neurons can survive during this journey and only a part of them arrive their destination. Growth of axon and multiple dendrites is the first key step in neuronal polarization, an initial process of differentiation which is ended with synaptic specializations and establishment of functional networks (Craig *et al*., 1992). In the developing brain, shape and location of the neurons, and also the kind of neurotransmitter that neuron produces, are determined by molecular signals which are transmitted from other neurons. Although neurons have the longest lifetime in the body, a large number of neurons die by apoptosis during migration and differentiation. Numerous neurons which migrate to the cortex die at an early stage of development and subsequent many cells die when their task is done. This phenomenon is widely known as programmed cell death (PCD) which serves many significant functions in animal development and homeostasis. The pathogenesis of many diseases, including neurodegenerative disorders, is attributed to the malfunctioning of PCD.

1.1. NEURONAL BIRTH AND CELL CYCLE REGULATION

Neurogenesis (neuronal birth) is the process by which neurons are born from neural stem cells and progenitor cells. This process is most active during prenatal development and is responsible for populating the growing brain with neurons. Before the first evidence of adult neurogenesis was demonstrated by Joseph Altman, most scientists believed that the nervous system was a fixed system which was incapable of regeneration. Now many studies have indicated that neurogenesis could continue during the adulthood in two regions of the mammalian brain, the hippocampus and the subventricular zone (SVZ) (Altman *et al*.,1967 ; Peter S.Eriksson *et al*., 1998). The real role of adult neurogenesis has not yet been clearly determined. Some evidence indicates that this process is crucial to functions such as learning and memory (G.Neves *et al*., 2008; Becker S *et al*., 2005). These investigations have shown that new neurons increase memory capacity, reduce the overlap between different memories and also add information regarding time to memories.

In higher vertebrates, neurogenesis is initially controlled by neurogenic genes (Kim et al., 2008). Progenitor cells generated from neural stem cells (NSCs) need a specific command which regulates the cell size and identifies potential neuronal progeny, then the cell cycle progression is switched off to control and limit cell number (Maurange et al., 2008). Therefore, regulation of cell cycle entry and exit attracts major concerns for the neuronal development and should be considered parallel to other transcriptional pathways which prompt nervous system development (Ozlem et al., 2009). Among the numerous transcription factors, Pax (paired box) and Sox [SRY (sex determining region Y) box] family of proteins are particularly vital in the formation of neural tissues, and these factors are regulated by cell cycle regulatory proteins. Pax6 directly regulate the expression of the neurogenic factor Ngn2 and the cell cycle inhibitor p27 to increase the generation of cell adhesion molecules (Osumi

et al., 2008). Additionally, Sox proteins decrease neurogenesis and maintain neural cells in the undifferentiated condition (Sandberg et al.,2005). Collaboration of neurogenic transcriptional pathways with the cell cycle also occurs at the epigenetic level besides transcription factor level. DNA methylation is vital in the cell fate determination of adult neural stem cells within the subventricular zone for postnatal neurogenesis through the regulation of neurogenic genes such as Dlx2, Neurog2, and Sp8. Many microRNAs such as microRNA-124 and miR-9 have been shown to influence cortical size and layering during development (Xu,XL *et al*., 2012). Notch signaling also plays a key role in neurogenesis, restricting the neurogenic gene expression through lateral inhibition, thereby limiting the number of cells differentiating into neurons. In vertebrate neurogenesis, Notch and Delta signaling is limited to certain stages of the cell cycle (Cisneros et al., 2008). Timing of cell cycle exit does not always necessarily affect the differentiation profile: forced expression of cell cycle regulator cyclin D1 in motor neuron precursors (pMN) in the developing spinal cord of chick embryos does not yield to any significant change in either the differentiation program or timing, but simply leads to an increase in the pMN population (Lobjois et al., 2008). This implies that cell cycle exit, although important, is not the most crucial element in the differentiation program at least in the spinal cord, since the proliferating pMN express neuronal markers Ngn2 and HuC/D throughout their regular developmental program. Another cell cycle regulator, the cyclin dependent kinase (CDK) inhibitor (CDKI) p57Kip2 was shown to be expressed during cell cycle exit and in postmitotic cells (Ye et al., 2008). Similarly, prolonged p27Kip1 expression was shown to induce neuronal differentiation at the expense of astrocytic differentiation, which is mediated by TGF-β1 up-regulation and Smad activation (Misumi et al., 2008), thereby providing an autocrine regulation. Thus, cell cycle manipulation is an important aspect of neurogenesis as well as self-renewal of neural progenitor cells (Jablonska et al., 2007).

1.2. PROGRAMMED CELL DEATH IN THE NERVOUS SYSTEM

The programmed cell death (PCD) is considered an essential process that evolved to serve diverse roles, especially in the nervous system. During the development of the vertebrate nervous system, up to 50% of the developing neurons are naturally removed by programmed cell death which is a relevant process in the physiology and pathology of the nervous system. Genetically modified mouse model systems and complementary in vivo approaches in other vertebrates have provided valuable information to reveal the role of PCD in normal neural development, as well as the pathological consequences of its deregulation.

Apoptosis is the most important form of PCD, which was widely and intensively investigated by researchers in the past 20 years and provided valuable information regarding the regulatory mechanisms of PCD in the nervous system. Another form of PCD is autophagy, which consists on a basic catabolic mechanism that involves the cell degradation of redundant or dysfunctional cellular components, which are targeted and isolated within autophagosomes which in turn are then fused with lysosomes and then degraded or recycled (He and Klionsky, 2009). There is another process needed to be distinguished from PCD, necrosis, a form of cell injury which is caused by external factors from the cell or tissue, such as infection, toxins, or trauma, that result in the unregulated digestion of cell components (Proskuryakov SY *et al*., 2003). In contrast, apoptosis is a naturally occurring programmed and targeted process of cellular death. Apoptosis occurs during the normal development of the vertebrate nervous system and has been found in populations of proliferating neuronal precursors, differentiated postmitotic neurons and glial cells. Up to half or more of the nerve cells normally die soon after they are formed before completion of the sophisticated neural architecture. Currently, early neural cell death is accepted as a phase of physiological cell death during neural development and evidence obtained from genetically-modified animals shows that prevention of cell death results in expanded nervous tissue and embryonic lethality. However, the essential role of early neural cell death is uncertain, and a possible function in the adjustment of cell numbers is frequently reported (Davies, 2003). The neurotrophic theory suggests that

projecting neurons are generated in excess and have to compete for limiting amounts of survival factors produced by their innervating target cells. Neurons which receive enough neurotrophic factors can survive, while the rest need to undergo programmed cell death (Conlon and Raff, 1999; Davies, 2003). Thus, apoptosis adjusts the number of nerve cells to match the number of target cells that require innervation. Apoptosis of neuronal progenitor cells is crucial for normal brain formation. Some studies suggest that proliferation and cell death occur together in many systems. Recently, a compensatory mechanism related with neurogenesis has been evidenced in certain regions of the nervous system. The researchers observed that the mature neurons are likely to undergo programmed cell death that serves for the renewing of the neuronal circuits. Abnormality of this process can leads to the neurodegenerative diseases (Buss *et al*., 2006; Miguel-Aliaga *el a*l., 2009). A variety of neurodegenerative diseases including Alzheimer's disease, Parkinson disease and amyotropic lateral sclerosis have been related with abnormal death of neurons. Dysfunction of programmed cell death, which is required for normal morphogenesis of the neural tube, also occurs in conjunction with some congenital malformations, in particular neural tube defects (NTDs) (Greene and Copp, 2005). Thus, a detailed feature of the regulatory mechanisms and also functional roles of PCD , may promote the identification of new targets and drugs to diminish pathological cell death.

The apoptotic response is mediated via either the intrinsic or the extrinsic pathway, depending on the original stimuli (Putcha GV *et al*.,2002). The intrinsic pathway is mediated by the mitochondria, where the cytochrome c protein is released from the mitocondrial intermembrane space into the cytoplasm in response to the apoptotic stimuli. Cytochrome c protein activates the APAF1 (apoptotic protease-activating factor-1) in the cytoplasm, then APAF1 binds with ATP/dATP to form the apoptosome which leads to the activation of caspase-9, which then cleaves and activates caspase-3. Thus, a cascade of caspase activation is triggered (Fig. 2) (Riedl SJ *et al*., 2005). The extrinsic pathway is mediated by binding of an extracellular death ligand, such as FasL and TNF (tumor necrosis factor) to its cell-surface death receptor, such as FasR

and TNFR. These death receptors are activated by interaction with the corresponding death ligands (Fig. 3). Then, adaptor proteins transmit the activating signal to effector caspases (caspase-3, -8, and -10).

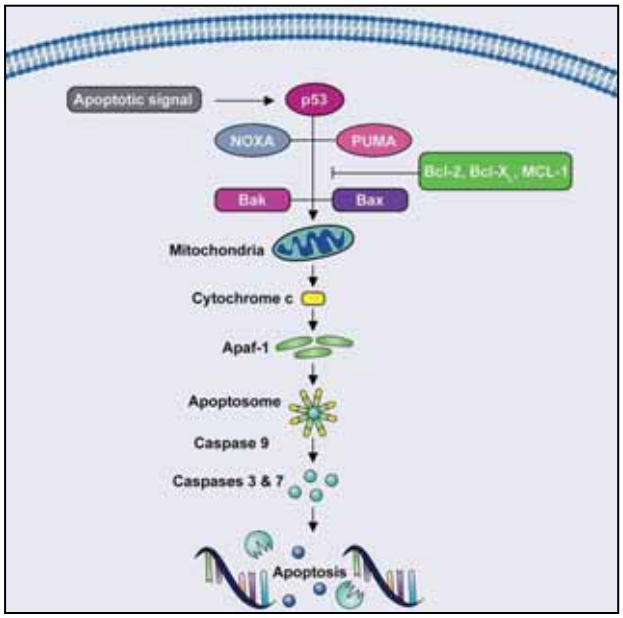

Figure 2. Elements of the mitochondrial pathway (intrinsic pathway). Mitochondria releases cytochrome *c*, which binds to and causes the aggregation of the adaptor protein Apaf-1. Then the Apoptosome is generated, which leads to the triggering of a caspase cascade. Figure is adapted from (Ashkenazi A, 2002)

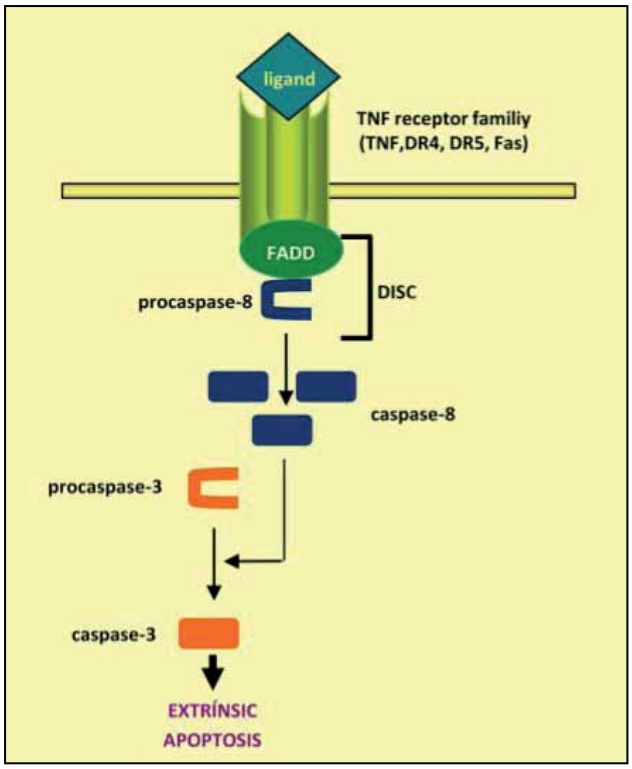

Figure 3. Schematic representation of extrinsic pathway. The extrinsic apoptosis pathway is triggered through activation of death receptors of the TNF family. Binding of the death ligand activates the TNF receptor on the surface of the target cell. FADD binds to the intracellular region of the aggregated receptor, causing the aggregation of procaspase-8 molecules. These then cleave each other to initiate the caspase cascade. The figure is adapted from (Raquel Gomez-Sintes., 2011)

Caspases are the major executors of apoptosis, the most frequent morphological manifestation of programmed cell death. The apoptotic caspases are generally classified into two groups: the initiator caspases, which include caspase-2, -8, -9 and -10 and the effector caspases, which include caspases-3, -6 and -7. All caspases are generated in cells as catalytically inactive zymogenes and have to undergo proteolytic activation during apoptosis (Riedl SJ *et al*., 2005). Effector caspases are activated by initiator caspases and then effector caspases are responsible for the proteolytic cleavage of a variety of cellular targets, which finally leads to apoptosis.

Activation of caspase-2 requires the adaptor protein RAIDD (RIP-associated ICH-1/CAD-3 homologous protein with a death domain). Knockdown of caspase-2 or RAIDD in neurons using shRNAs can reduce the rate of NGF withdrawal-induced cell death. Caspase-3 is very important because neurons don't express caspase-7 during the early stage of postnatal development. Caspase-3 and caspase-9 knockout mice show brain malformations, with an excess of cells in many regions of the cortex, hippocampus and cerebellum. Caspase-8 and -10 play key roles in initiating Fas associated death domain (FADD) induced apoptosis. Activated caspase-8 and -10 further activate caspase-3. Caspases-3 and -9 can be potently inhibited by a member of the IAP family, XIAP (X-linked inhibitor of apoptosis) (Tenev T *et al*.,2005). Caspase-3 and caspase-6 are necessary for the cleavage of a large number of nuclear proteins essential for apoptosis-associated chromatin margination, DNA fragmentation, and nuclear collapse (Slee EA *et al*., 2001). Recently, some studies suggested that caspase-2 may function upstream of the mitochondrial pathway by increasing the phosphorylation of c-Jun and the expression of the BH3-only protein Bim (Bcl-2-interacting mediator of cell death) (JeanYY *et al*.,2013).

Bcl-2 proteins are the main intracellular modulators of the cell death program (Garcia *et al*.,1992). All the members of the Bcl-2 family share the same four similar characteristic domains of homology named Bcl-2 homology (BH) domains BH1, BH2,

BH3 and BH4. The anti-apoptotic proteins always contain BH1 and BH2 domains, and some of them contain an additional N-terminal BH4 domain (Bcl-2, Bcl-X_L, Bcl-w). In addition, pro-apoptotic proteins contain a BH3 domain which is required for dimerization with other proteins of the Bcl-2 family. The BH3 domain is also present in some anti-apoptotic proteins. Bcl-2 proteins regulate mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic or anti-apoptotic (Fig. 4) (Lindsten *et al.*, 2005).

Pro-apoptotic	Anti-apoptotic
Bax	Bcl-2
Bak	Bcl-X_L
BOK	Bcl-W
BIM	MCL-1
BID	Bcl-B
BAD	+ viral homologs
NOXA	
PUMA	

Figure 4. Selected proteins in the Bcl-2 superfamily. Figure is adapted from (Lessene G *et al*., 2008)

Pro-apoptotic Bcl-2 proteins form mitochondrial apoptosis-induced channels (MAC) in the outer mitochondrial membrane thereby promoting MOMP, which enables the release of cytochrome c and other cytotoxic proteins that can enter into the cytosol via these channels. Pro-apoptotic Bax is expressed at high levels both in the developing and in the adult brain. Over-expression of Bax is sufficient to promote the cytochrome c release and apoptosis in the presence of NGF (Deckwerth *et al*., 1996). In agreement with that, in $Bax^{-/-}$ knock out mice the apoptotic cell death is remarkably reduced in a variety of regions of the central and peripheral nervous system in both developmental and postnatal brain, with 2.5 fold increased cell number compared with wild type mice. Bax and Bak are functionally important for MOMP. Bak mostly exists in the outer membrane of mitochondria. Like Bax, Bak promotes the cell death following

the corresponding stimuli (Lindsten et al.,2006). However, deletion of Bak in mice has no significant consequences on the neuronal cell death. Although $Bax^{-/-}$ deficient mice show only modest increases of cell numbers in particular areas of the brain, the double deletion of Bax and Bak leads to more severe phenotypes, with increased brain size and an increased number of myeloid and lymphoid cells (Lindsten et al .,2000).

Bid is a pro-apoptotic Bcl-2 protein containing only the BH3 domain. During death receptor apoptotic signaling, Bid is cleaved and activated by caspase-8 and the COOH-terminal part migrates to mitochondria where it interacts with Bax to trigger cytochrome c release (Luo *et al*., 1998). Bax is considered to switch on the mitochondrial voltage-dependent anion channel. Increasing evidence suggest that activated Bax and Bak form a mitochondrial apoptosis-induced channels (MAC) in the outer mitochondrial membrane. This leads to the release of cytochrome c and other pro-apoptotic factors from the mitochondria, leading to activation of caspases. Bcl-2-associated death promoter (BAD) protein, another member of the BH3-only family, is able to form a heterodimer with anti-apoptotic proteins, Bcl-2 and Bcl-xL, inactivating them and allowing Bax/Bak-triggered apoptosis (Yang *et al*., 2005). BAD is phosphorylated by Akt/protein kinase B and form the BAD-(14-3-3) protein heterodimer (Zha *et al*.,1996). This makes Bcl-2 free to inhibit the Bax/Bak triggered-apoptosis. So Bad releases Bax from Bcl-2 inhibition and promotes cell death (Yang E et al.,1995). Thus, BAD phosphorylation is anti-apoptotic and BAD dephosphorylation is pro-apoptotic.

The anti-apoptotic Bcl-2 proteins, including Bcl-2 itself, can also bind to BID and compete with Bax. As a result, the anti-apoptotic Bcl-2 proteins may inhibit apoptosis by sequestering BID, leading to reduced Bax activation. Over-expression of Bcl-2 prevent the NGF withdrawal-induced cell death and inhibits the mitochondrial cytochrome c release in sympathetic neurons (Putcha GV *et al*., 1999). In contrast, mice deficient in Bcl-2 complete embryonic development, but displayed growth retardation, early postnatal mortality and exhibited significant loss of sympathetic,

motor and sensory neurons (Michaelidis et al.,1996;Veis et al., 1993). Consistent with these findings, Bcl-2 expression determines whether a neuron dies or survives.

Besides Bcl-2 family proteins, another important intracellular apoptosis regulators are the IAP (inhibitor of apoptosis) family. The activity of activated caspases -3, -7 and -9 can be restrained by XIAP (X-linked inhibitor of apoptosis), which is a member of IAP family (Wu G et al., 2000). These proteins inhibit apoptosis in two ways: they bind to pro-caspases to block their activation, or bind to caspases to dysfunction their activity. IAP proteins were originally discovered as proteins produced by certain insect viruses, which use them to prevent the infected cell from killing itself before the virus has had time to replicate. Researchers also observed that mitochondria can release Smac, a novel protein that blocks IAPs, thereby greatly increasing the efficiency of the death activation process (Chunying Du *et al*., 2000) The intracellular cell death program is also regulated by extracellular signals, which can either activate apoptosis or inhibit it. These signal molecules mainly act by regulating the levels or activity of members of the Bcl-2 and IAP families.

1.3. NEURONAL SURVIVAL MECHANISMS

Since the first neurotrophin was discovered in 1979, the great curiosity of researchers has been motivated to figure out how these trophic factors maintain the neuronal survival. One pioneer investigation was done by EM Johnson's group who suggested that neurotrophins maintain the survival by preventing neurons from initiating programmed cell death (Martine *et al*.,1988 and 1992). Neurotrophins regulate the growth, survival and differentiation of neurons by binding to two kinds of cell receptor: the TrkA tyrosine kinase and the p75NTR receptors. Neurotrophin family have four structurally related factors: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). The balance between apoptosis and survival in developing neurons is determined by the competition for the limited amount of neurotrophic factors which are secreted by the

target tissue.

When NGF binds to TrkA, the receptor transmits positive signals that enhance sympathetic neurons growth and survival. The binding of this neurotrophic factor to TrkA activates the small GTPase Ras which promotes neuronal survival by activating a variety of signaling cascades, including the PI3K-Akt and the Ras-MAPK pathways (Fig 5). Each of these pathways differentially contribute to cell viability depending on the neuronal cell type and the type of survival factors. Besides activating the above mentioned two main pathways, activated Ras can also inhibit the JNK pathway to prevent the cell death. In addition, the p75NTR receptor can transmit survival signals together with TrkA in response to neurotrophins, whereas it the absence of NGF, p75NTR turns to promote the cell death. This can be counteracted by anti-apoptotic proteins via TrkA, TrkB, or TrkC signaling in cells (M Kristiansen and J Ham, 2014).
Here, I firstly discuss the PI3K–Akt pathway in the first part as this pathway is particularly important in mediating neuronal survival, whereas the Ras/MAPK pathway is discussed in the second part.

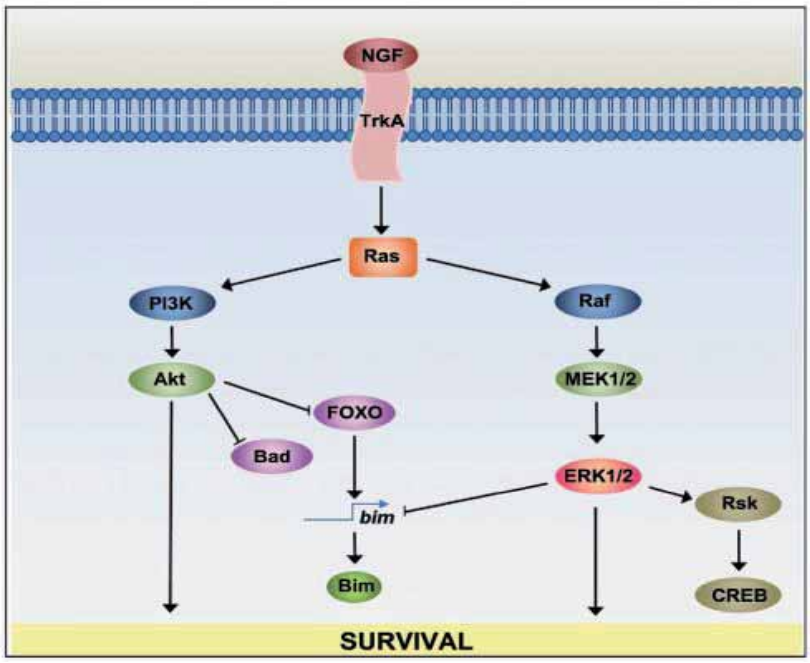

Figure 5. Schematic representation of survival pathways activated by the binding of NGF to TrkA. NGF binds to its receptor TrkA, which leads to the activation of the small GTP-binding protein Ras. Then activated Ras further activate the PI3K-Akt signaling pathway, which can inhibit apoptosis and promote cell survival by phosphorylating, and therefore inactivating, pro-apoptotic proteins such as the BH3-only protein Bad and the transcription factor FOXO. On the other hand, activated Ras can also activate the Raf-MEK-ERK signaling pathway, which promotes survival by inhibiting the expression of the pro-apoptotic Bim, as well as by activating RSK. RSK phosphorylates and activates the transcription factor CREB that promote the transcription of the anti-apoptotic Bcl-2 (M Kristiansen and J Ham, 2014).

1.3.1 Neuronal survival promoted by the PI3K–Akt signaling pathway

Upon binding of the survival factors to their corresponding tyrosine kinase receptors, PI3K is recruited to the plasma membrane. The catalytic subunit of PI3K generates the phosphoinositide phosphatydilinositiol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3, or PIP3) at the inner surface of the plasma membrane. PIP3 is a critical second messenger that promotes, among other effects, the phosphorylation and activation of several serine/threonine kinases, including Akt/protein kinase B, serum and glucocorticoid inducible kinase (SGK), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), and protein kinase C (PKC) isoforms (Vanhaesebroeck B *et al*.,2000). Akt is recruited to the plasma membrane through the interaction of its pleckstrin homology (PH) domain with the phospholipid products of PI3K. At the plasma membrane, the activation of Akt is dependent on phosphorylation at the Threonine 308 site within the activation loop of the kinase domain by the protein kinase PDK1 (phosphoinositide-dependent protein kinase-1). SGK, a protein kinase related to Akt and activated by PI3K, is also involved in mediating survival signals in several types of neurons (Vanhaesebroeck B *et al*.,2000). In the past twenty years, the PI3K–Akt pathway has been found to be sufficient and necessary for the trophic-factor-induced cell survival of many neuronal cell types.

A number of Akt substrates have been identified by the characterization of a consensus peptide phosphorylation motif (RXRXXpS/T) that is preferred by Akt (Alessi DR *et al*.,1996). Database screen indicate that this motif exist in a large number of proteins, including transcription factors Bcl-2 family members (Datta *et*

al.,1997), FOXO family members (Brunet *et al*.,1999), 4EBP1 (Eukaryotic translation initiation factor 4E-binding protein 1) (Fulton D *et a*l., 1999), the tumor suppressor BRCA1 (Altiok S et al., 1999), and glycogen synthase kinase-3 (GSK-3) (Cross DA *et al*., 1995). As the PI3K–Akt pathway regulates cell proliferation and metabolism as well as cell survival, it becomes crucial to distinguish which particular Akt targets mediate the neuronal survival effects of Akt.

In addition to its function as a suppressor of critical death genes, the activation of the PI3K–Akt survival pathway also triggers under some circumstances the expression of survival genes. Recent evidence suggests that the two transcription factors cAMP-responsive element binding protein (CREB) and nuclear factor κB (NF-κB), which induce the expression of survival genes, may be regulated by Akt (Ozes ON *et al*., 1999; Romashkova JA *et al*., 1999; Du k *et al*., 1998)

Several target genes of these transcription factors have been identified, which may account in part for the survival effect of the PI3K–Akt pathway. For example, the genes encoding the pro-survival Bcl-2 family members Bcl-xL (Chen c *et al*.,2000) and several IAPs (Chu zl *et al*., 1997) are upregulated by NF-κB, whereas the genes encoding Bcl-2 (Riccio A *et al.*, 1999) and the pro-survival neurotrophin BDNF (Tao X *et al*., 1998) are induced by CREB.

1.3.2 Cell Survival Promoted by the Ras-MAPK Signaling Pathway

Acting as serine and threonine protein kinases, mitogen-activated protein kinases (MAPK) are a family of protein kinases highly conserved from yeast to human. This classic MAPK family consists of three subfamilies: extracellular signal-regulated kinase (ERK1 and ERK2), c-Jun N-terminal kinase (JNK1, JNK2 and JNK3), and p38-MAP kinase (MPAK13, MAPK11, MAPAK, and MAPK 13) (Wada T and Penninger JM, 2004). MAP kinases are activated by dual phosphorylation on a conserved Thr-Xaa-Tyr motif in their activation loop by an upstream MAP2K. MAP2Ks are themselves activated by phosphorylation via an upstream MAP3K. Many studies suggested that ERKs are important for cell survival, whereas JNKs and

p38-MAPKs were deemed stress responsive to changes in osmolarity or metabolism, DNA damage, heat shock, ischemia, inflammatory cytokines, shear stress, UV irradiation, ceramide, or oxidative stress, and thus involved in apoptosis (Seger and Krebs, 1995; Tibbles and Woodgett, 1999; Widmann *et al.*, 1999; Davis, 2000; Chang and Karin, 2001; Johnson and Lapadat, 2002).

The mechanism by which the Ras-MAPK (mitogen-activated protein kinase) signaling pathway mediates neurotrophic factor-dependent cell survival has been extensively characterized. By phosphorylating specific serines and threonines of target protein substrates, MAP kinases regulate a wide range of processes: cell growth and differentiation, gene expression, mitosis, cell motility, metabolism, cell survival and apoptosis, and embryogenesis. The MAPK-activated kinase RSK catalyze the phosphorylation of the pro-apoptotic protein BAD at serine 112. BAD can also be phosphorylated by PKA and Akt (Lizcano JM et al.,2000). Phosphorylation of BAD suppressed BAD-mediated apoptosis in neurons. RSK is also known to phosphorylate the transcription factor CREB (cAMP response element-binding protein) (De Cesare D et al.,1998; Xing J et al.,1998). . Activated CREB promoted cell survival, and inhibition of CREB phosphorylation triggered apoptosis (Wada T and Penninger JM, 2004).These findings suggest that the MAPK signaling pathway promotes cell survival by a dual mechanism comprising the post-translational modification and inactivation of components of the cell death machinery, and the increased transcription of pro-survival genes. Therefore, like PI3K-Akt pathway, the Ras-MAPK pathway also controls cell survival or apoptosis by regulating the activity of anti- and pro-apoptotic transcription factors (Kaplan DR and Miller FD, 2000). In the next section, I will describe the detailed molecular mechanism of interaction between survival and apoptosis.

1.4. THE BALANCE BETWEEN SURVIVAL AND APOPTOSIS

The physiological interplay between neuronal apoptosis and survival that occurs

during the development of the nervous system has been confirmed in several types of primary neurons, including sympathetic, hippocampal, cortical, cerebellar granule and motor neurons. All of these neuronal cell types can be successfully cultured in the presence of defined neurotrophic stimuli, and die through an apoptotic process after withdrawal of trophic support. For example, brain-derived neurotrophic factor (BDNF) is a vigorous survival factor for cerebellar granule neurons. Mice which are genetically deficient for BDNF receptor genes display an excess of apoptotic cells in the cerebellum, indicating that the *in vitro* primary culture system may accurately recapitulate cellular events that occur during normal nervous system development *in vivo*.

The Bcl-2 family of proteins controls a critical step in the commitment to apoptosis by regulating permeabilization of the mitochondrial outer membrane (MOM). The Bcl-2 family is divided into three classes: multi-region pro-apoptotic proteins that directly permeabilize the MOM; BH3 proteins that directly or indirectly activate the pore-forming class members; and the anti-apoptotic proteins that inhibit this process at several steps (Shamas DA *et al*., 2013). Several models have been propossed to understand the consequences of the interaction among these three subgroups of Bcl-2 proteins. These interactions are regulated by the concentrations and activities of proteins and can occur both in membranes and cytoplasm.

The different mechanisms accounting for these models were summarized and reviewed by David W.Andrews group in 2013. In the first model, Bcl-2 multiregion pro-apoptosis proteins (Letai *et al.*, 2002) are activated by direct interaction with BH3 proteins such as Bax and Bak. BH3 proteins were divided into two groups: Activator (tBid, Bim, and Puma) that bind to both the anti or pro-apoptosis Bcl-2 proteins (Kim *et al*., 2006); Sensitizer (Bad, Noxa, Bik, Bmf, Hrk and Bnip3) that only bind to the anti-apoptosis proteins, thus leaving activator BH3 proteins free to promote the apoptosis. If the apoptosis need to be evaded in a cell, BH3 proteins must be isolated from anti-apoptosis to prevent Bax/Bak induced apoptosis (Kuwana et al.,2005; Certo et al.,2006).

In the second model, researchers proposed an indirectly binding mechanism. Normally, Bax and Bak proteins are inhibited by anti-apoptosis proteins for the neurons to survive. When apoptosis is initiated, BH3 proteins displace Bax and Bak from the anti-apoptosis proteins to promote the Bax/Bak mediated MOMP. The formation of heterodimers of Bak with the anti-apoptotic Mcl-1 and Bcl-XL in dividing cells provide potent evidence to support this model. Overexpression of Noxa can lead to the release of Bak from the complex with Mcl-1 and form Noxa-Mcl-1 heterodimers. Thus Noxa plays a important role in inducing apoptosis by neutralizing the effects of both Bcl-XL and Mcl-1 (Willis *et al*., 2005).

In the third model, multiregion Bcl-2 proteins adjust the affinity for binding to BH3 proteins by changing the conformations after interaction with MOM, which allow the insertion in the membrane (Leber *et al*., 2007,2010;Garcia-Saez *et al*., 2009). The sensitizer BH3 proteins neutralize the dual function of the anti-apoptosis proteins by displacing both the activator BH3 proteins and Bax/Bak from the membrane -embedded anti-apoptosis proteins (Billen *et al*., 2008). The last model, unified one, is based on the third model (Llambi *et al*., 2011), proposed that both models 1 and 2 take place simultaneously in cells. However, inhibition of apoptosis via model 1 is less efficient and is easier to overcome by BH3 sensitizers to promote than inhibition through model 2 (Fig 6).

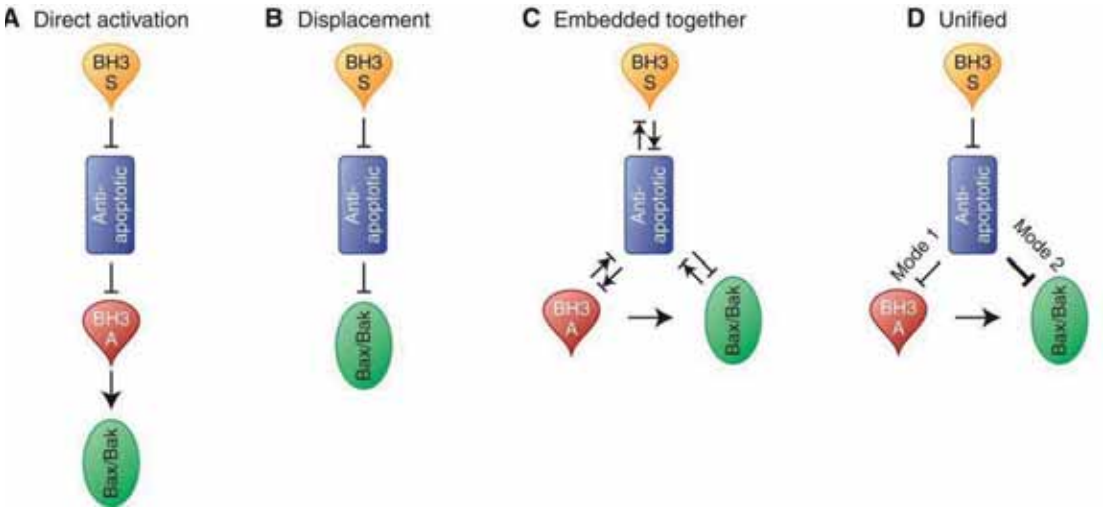

Figure 6. Schematics of the core mechanisms proposed by various models for the regulation of MOMP by Bcl-2 proteins. (↑) Activation; (⊥) inhibition; (⊥↑) mutual recruitment/sequestration. Paired forward and reverse symbols indicate the model makes explicit reference to equilibria. Figure is adapted from (Shamas DA *et al,* 2013)

In vertebrates, there are at least 4 anti-apoptosis Bcl-2 family members including Bcl-2, Bcl-XL, Bcl-w and MCL-1 containing the four BH domains BH1 to BH4. The Bcl-2 gene was originally identified as a translocated gene in follicular lymphoma (Tsujimoto *et al*., 1984) and its overexpression has been shown to prevent different types of cells from apoptosis (Ibrado *et al*., 1997;Shimizu *et al*., 1995). The Bcl-2 family members are engaged in cell death prevention mostly by interacting with pro-apoptosis Bax or Bak (Willis *et al*., 2007). Specific mutations in BH domains abrogated anti-apoptotic function and binding to Bax (Hirotani *et al*.,1999).

Mouse genetic models studies indicate that the survival of the cell requires at least one Bcl-2 homologue. Overexpression of Bcl-2 results in reduced naturally occurring cell death, which leads to hypertrophy of the nervous system. Furthermore, Bcl-2 overexpression can protect nerve growth factor-deprived neurons from apoptosis Martinou *et al*.,1994; Gagliardini *et al*.,1994). Bcl-2 knockout mice complete embryonic development, but displayed growth retardation and early postnatal mortality and exhibited obvious loss of sympathetic, motor and sensory neurons (Michaelidis *et al*., 1996; Veis *et al*.,1993). All these findings support the conception that Bcl-2 expression determines whether a neurons dies or survives.

The fact that Bcl-2 deficient mice finish the normal development and exhibit only mild defects within the nervous system might be explained by the functional redundancy and overlapping expression patterns with other Bcl-2 family members. Pro-survival Bcl-XL is specially expressed in the developing brain, and expression of Bcl-XL decreases after birth but is retained in the adult central nervous system. The Bcl-$XL^{-/-}$ mice are embryonic lethal at E13 displaying severe enhanced apoptosis of postmitotic differentiating neurons of the developing brain (Gonzalez-Garcis *et al*.,1995). In addition, Bcl-XL deficient neurons display increased susceptibility to

serum deprivation (Roth *et al*.,1996). These studies demonstrate that Bcl-XL is a critical anti-apoptosis factor in maintaining neuronal survival during brain development. Thus, Bcl-XL might provide the supplement to Bcl-2 in promoting cell survival within the developing nervous system.

When the neurons undergo cellular stress, synthesis of MCL-1 is blocked and the protein rapidly degraded (Nijhawan *et al*.,2003). MCL-1 deficient mice exhibit severe phenotype, with embryos dying at around embryonic day 3.5-4 and showing increased apoptotic activity (Rinkenberger *et al*.,2000). Loss of MCL-1 in the brain resulted in widespread apoptosis death of both neural progenitors and newly committed neurons during the development of the nervous system (Arbour *et al*.,2008). Moreover, both MCL-1 and Bcl-XL have been shown to block Bax and Bak-mediated apoptosis.

Another anti-apoptosis Bcl-2 family member, Bcl-w, was shown to be expressed in the axons of sensory neurons and its regulation by target-derived neurotrophin stimulation suggests that Bcl-w may be involved in the promotion of neuronal cell survival in the phase of establishing synaptic connections (Pazyra-Murphy *et al*.,2009). Furthermore, Bcl-w deletion caused abnormalities in size and function of axonal mitochondria of sensory neurons, manifested in progressive small fibber sensory degeneration and neuropathy, thereby suggesting that this protein may play a critical role in the viability of axonal processes (Courchesne *et al*.,2011).

The execution of apoptosis is mainly controlled by Bcl-2 family-members which locate in the outer mitochondrial membrane and control mitochondrial permeability. Once pro-apoptosis proteins-induced pathway is activated, the outer mitochondrial membrane becomes permisive to candidate proteins, including cytochrome *c*. As mentioned in previous sections, Cytochrome *c* released from the mitochondria to the cytoplasm participates in the formation of apoptosome, which consists of dATP, apoptosis protease activation factor-1 (APAF1), and the cysteine protease caspase 9. The direct consequence of formation of the apoptosome is the activation of caspase 9, which initiate in motion the activation of a cascade of effector caspases, such as caspase 3, which in turns induce the cell death by irreversible proteolysis of critical

cellular constituents.

Recent studies have suggested that apoptosis can still be inhibited even after cytochrome *c* has been released into the cytoplasm. Proteins of the inhibitor of apoptosis (IAP) family prevent apoptosis by specifically binding to and inhibiting the caspases. Researchers have recently shown that the anti-apoptosis role of the IAPs is antagonized by Smac/Diablo, which is a mitochondrial protein released into the cytoplasm during the apoptosis. The release of Diablo blocks the anti-apoptosis function of IAPs by inhibiting caspase activity, thereby inducing cell death. In sympathetic neurons, release of cytochrome *c* from the mitochondria is not sufficient to trigger apoptosis, and a second event is required for nerve growth factor (NGF) withdrawal to induce apoptosis, possibly the release of Diablo, or other closely related proteins, from the mitochondria. In addition to controlling Diablo release from the mitochondria, one can speculate that survival factors may also regulate the expression or activity of Diablo (Verhagen AM and Vaux DL, 2002; Wang K and Lin B, 2013).

PI3K/Akt promotes the cell survival by phosphorylating downstream target proteins such as FOXO, p53 and Bad. FOXO proteins are a group of class O subfamily of the forkhead family of transcription regulators (FKHR, FKHRL1 and AFX) which are characterized by a distinct fork head DNA-binding domain (Woods YL and Rena G, 2002). Akt directly phosphorylates FOXOs and inhibits their ability to induce the expression of death genes (Brunet *et al*.,1999). In the absence of survival factors, when Akt is inactive, FOXOs are localized in the nucleus and activate gene transcription to induce the apoptosis in a transcription-dependent manner. Recently, Bcl-2 family member Bim-1 and cell-cycle inhibitor p27KIP1 have been identified as FOXO target genes which may participate in the regulation of apoptosis (Stahl M *et al.,* 2002; Rathbone CR *et al*.,2008). In the presence of survival factors, Akt becomes activated, phosphorylates FOXOs at several regulatory sites, and block the re-localization of FOXOs from the nucleus to the cytoplasm, away from their target genes. Recent evidence shows that SGK also phosphorylates the FOXO family member FKHRL1. As the phosphorylation of each regulatory site of FKHRL1

appears to be critical for the efficient exclusion of FKHRL1 from the nucleus, it is likely that SGK and Akt cooperate to promote cell survival by coordinately regulating FOXO transcription factors (Brunet A *et a*l.,2001; Shin I *et a*l., 2001). In addition to regulating the FOXO-dependent transcription, some studies also indicate that Akt also promotes survival in hippocampal neurons by inhibiting the activity of the tumor suppressor p53. Active p53 is known to induce the expression of death genes, including the pro-apoptosis Bcl-2 family member BAX (Shen Y and White E, 2001; Blagosklonny MV, 2000).

Bad is another Bcl-2 family-member, which is directly phosphorylated by Akt in order to inhibit BAD pro-apoptosis functions. When neurons undergo trophic deprivation, BAD form the complex with pro-survival Bcl-2 family member, Bcl-XL, thereby preventing Bcl-XL from promoting survival. In the presence of survival factors, Akt is activated and phosphorylates BAD at serine. BAD is then released from the Bcl-XL and Bcl-2 complexes and the release of cytochrome c inhibited. The dissociated Bad binds to the adaptor protein 14-3-3 which firstly sequesters Bad from the mitochondria and also prevents the binding of Bad to Bcl-2 and Bcl-XL (Datta *et al*., 1997). Akt can also phosphorylate Bax on Ser184, which leads to the inhibition of the conformational change required for Bax translocation to mitochondria and thus blocks the pore formation and release of cytochrome c (Yamaguchi and Wang, 2001). Taken together, there is abundant evidence that Akt controls Bcl-2 family proteins which ensure that mitochondrial integrity is preserved during stress.

2. PI3K SIGNALING PATHWAY

PI3Ks are a family of intracellular lipid kinases that phosphorylate the 3'-OH group of phosphatidylinositols (PIs). They are classified based on their own lipid substrate specificity into class-I, class-II and class-III. Classical class-I PI3Ks are activated when the growth factors bind with their receptors, which leads to the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) at the D3 position of the

inositol ring to generate the phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3,or PIP3) second messenger, whereas class-II and class-III PI3Ks act on PIs to generate phosphatidylinositol-3-phosphate PI3P (Fig. 7). Class-I PI3Ks are divided into class-Ia and class-Ib PI3Ks. Class-Ia PI3K encompasses a p85 adapter/regulatory subunit and a p110 catalytic subunit and is activated by receptors with protein tyrosine kinase activity (RPTK), whereas class-Ib is activated by G-protein coupled receptors (GPCRs) (Vanhaesebroeck *et al*., 2001;Vanhaesebroeck *et al*., 1997). Class-Ia PI3K is activated following its recruitment to the corresponding receptor through the interaction of the p85 regulatory subunit with specific phospho-tyrosine residues presented by adaptor proteins such as IRS1 or GAB1 (Holgado-Madruga *et al*., 1997). PI3K activation results in production of phosphoinositides which take a role in propagation of the signal acting as the second messengers. This function of phosphoinositides is mediated by the specific interaction with lipid-binding domain-containing proteins, such as proteins containing pleckstrin-homology (PH) domains. Among them, the PDK1 and Akt kinases are key effectors of the PI3K signaling pathway.

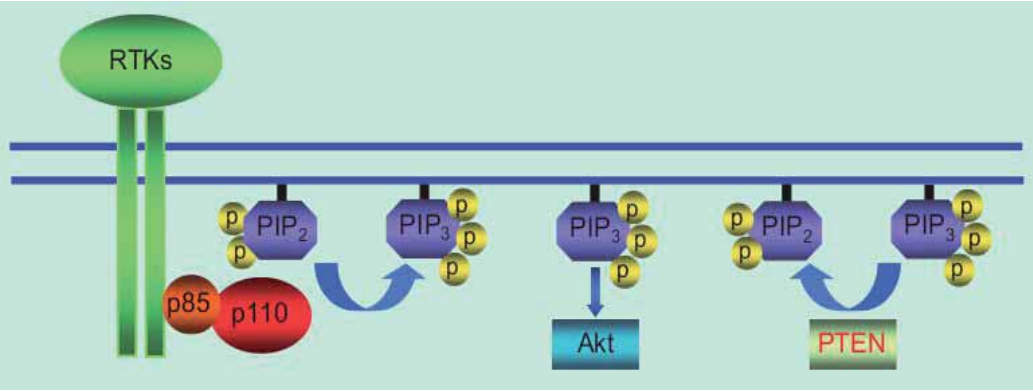

Figure 7. Class I PI3 kinase is activated by upstream signals from receptor tyrosine kinases (RTKs). Activated PI3 kinase catalyzes the production of the second messenger phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) by phosphorylating the phosphatidylinositol-(4,5)-bisphosphate (PIP2). PIP3 recruits the Akt protein kinase to the membrane. The phosphatase and tensin homolog deleted from chromosome 10 (PTEN) acts as a critical negative regulator of the PI3K signaling by removing the D3 phosphate from PIP3 to produce PIP2.The figure is adapted from (Cheng CK *et al* .,2009)

2.1. PKB/Akt, THE KEY MEDIATOR OF PIP3 SIGNALS.

PKB/Akt is a serine/threonine protein kinase of 60KD which attracts a major focus of attention because of its critical regulatory role in diverse cellular processes. Since its discovery as a proto-oncogene, Akt was widely studied and revealed as a key mediator of the PI3K pathway. In mammals, PKB/Akt comprises three highly conserved isoforms (termed as PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3) which are encoded by three different homologous genes and share more than 85 percent amino acid sequence identity (Fig 8). PKBα/Akt1 is ubiquitously and widely expressed at high levels in all tissues, whereas PKBβ/Akt2 is expressed in insulin-responsive tissues such as heart, adipose, liver and muscle. PKBγ/Akt3 expression is mainly restricted to cortex and testes. PKB/Akt isoforms possess an amino-terminal Pleckstrin Homology domain (PH-domain). Akt is activated upon dual phosphorylation by PDK1 at the T308 and by mTORC2 at the S473 activation sites (Vanhaesebroeck and Alessi, 2000).

PKB/Akt is involved in many cellular processes and pathologies, such as metabolism, proliferation, survival, growth, cancer and neurodegenerative disorders. Activated Akt phosphorylates serine/threonine residues on downstream target proteins at a recognition sequence Arg-X-Arg-X-X-Ser/Thr-Hyd, where X means any amino acid and Hyd means bulk hydrophobic residues (Alessi *et al*., 1996), and regulates numerous physiological processes through the phosphorylation of these downstream substrates. The requirement of Arg residues at the -3 and -5 terminal on peptides phosphorylated by Akt distinguishes it from other AGC kinases, which prefer the Lysine residue at the same sites.

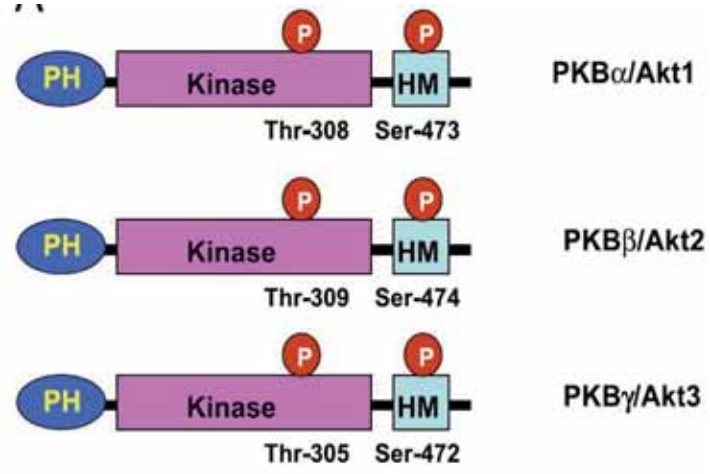

Figure 8. Domain structure of Akt isoforms. All the Akt isoforms possess the kinase domain in the central region of the molecule. The PH (pleckstrin homology) domain act as a phosphoinositide-binding module. The hydrophibic motif (HM) is located at the carboxyl-terminus adjacent to the kinase domain. Figure is adapted from (Hanada M *et al*. 2004)

PKB/Akt is a key signaling molecule highly conserved among many species, from primitive metazoans to humans, that belongs to the AGC group of protein kinases. Akt was originally cloned by different groups (Bellacosa *et al*, 1991; Coffer and Woodgett 1991; Jones *et al*.1991) twenty three years ago following the initial identification of its viral homolog, the v-Akt proto-oncogene, which is encoded by a transforming retrovirus (AKT-8) isolated from a spontaneous thymic lymphoma of an AKT mouse (Staal *et al.*,1977; Staal, 1987).

The three different Akt isoforms share an analogous structural organization which contains three domains: N-terminal Pleckstrin homology (PH) domain, the kinase domain of the AGC family and a carboxyl-terminal regulatory domain including the hydrophobic motif. This structure is conserved from fly, worm, mouse, to human. The C-terminal regulatory domain containing the hydrophobic motif is highly preserved among a subgroup of growth factor-stimulated AGC kinases including PKC, p70 S6 kinase (S6K), p90 ribosomal S6 kinase (RSK) and serum and glucocorticoid induced kinase (SGK) (Hanada M *et al*. 2004) (Fig. 9). In order to reach the full activation of Akt, both of the T308 site within the activation loop of the catalytic domain and the

S473 site in the hydrophobic motif of the regulatory domain need to be phosphorylated (Alessi *et al*. 1996a, 1997). The crystal structure resolution of the Akt kinase domain provided a chance to understand how these two phosphorylation sites contribute to the enzymatic activation of the kinase (Yang *et al*. 2002a, b). The catalytically active conformation is achieved upon phosphorylation of T308, whereas phosphorylation of S473 could enhance the stabilization of this active conformation. This stabilization is formed by intramolecular interactions between the hydrophobic motif and a corresponding acceptor structure within the catalytic domain, termed the hydrophobic groove. As mentioned before, activated Akt phosphorylates a number of substrates regulating diverse cellular and physiological processes, including cell cycle progression, cell growth, cell differentiation, cell survival and apoptosis, metabolism, angiogenesis and motility.

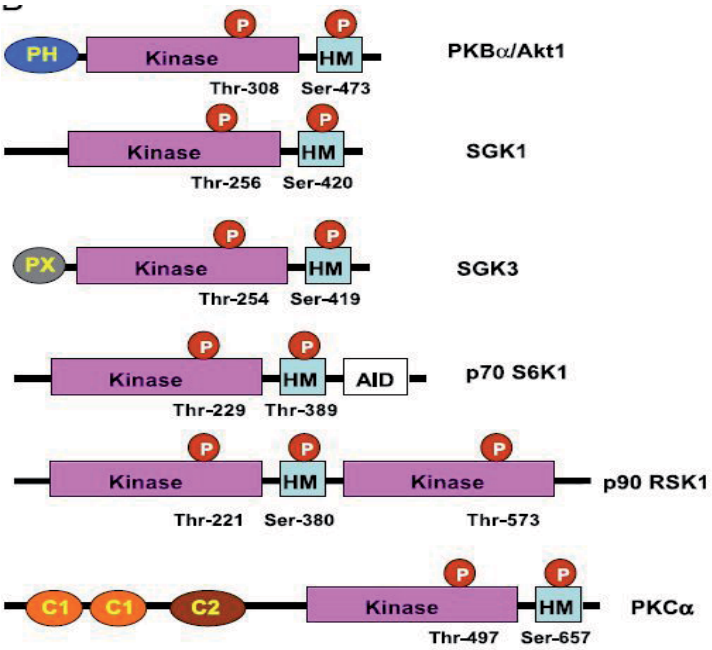

Figure 9. Domain structure of AGC kinases. All the AGC kinases possess the kinase domain in the central region of the molecule. The hydrophobic motif (HM) is located at the carboxy-terminus adjacent to the kinase domain. Phosphorylation sites in the activation loop in the kinase domain and the hydrophobic motif are indicated. SGK, serum and glucocorticoid induced protein kinase; AID, autoinhibitory domain; C1, phorbol esters/diacylglycerol binding domain; C2, calcium-dependent membrane targeting domain, PX, Phox domain. Figure is adapted from (Hanada M *et al*. 2004)

2.1.1. PKB/Akt IN NEURONAL SURVIVAL

The PI3K/Akt signaling pathway has been proposed to contribute to diverse processes in the cell among which the prominent function in cell survival (Fig. 7). Akt as a major downstream target of growth factors enhances cell survival by different mechanisms including inhibition of apoptosis, as already discussed. Glycogen synthase kinase-3 (GSK3) is a bona fide, first characterised physiological substrate of Akt which activity is negatively regulated downstream of the PI3K pathway. GSK3 play essential roles in regulating neurogenesis, neuronal survival and neuronal plasticity and it is believed to be deregulated in a number of psychiatric disorders (Beaulieu *et al*., 2009). Initially, GSK3 was found to be an important component of the apoptotic response occurring after growth factor withdrawal in cultured cortical neurons (Hetman *et al*., 2000). Indeed, Akt inactivates GSK3 isoforms by phosphorylating them on an inhibitory site, namely Ser21 in GSK3α and Ser9 in GSK3β (Cross *et al*., 1995). Inhibition of GSK3 represent another mechanism by which the Akt kinase protects cells from programmed cell death. GSK3 promote cell death through the mitochondrial intrinsic apoptotic pathway by targeting several key proteins promoting the disruption of the mitochondria membrane. For example, GSK3 can directly phosphorylate the pro-apoptotic Bcl-2 family member Bax on Ser-163, which results in its activation (Linseman *et al*., 2004). Also, stress-induced expression of pro-apoptotic Bim requires GSK3 activity, which can be counteracted by lithium (Hongisto *et al*., 2003). Additionally, it has been reported that GSK3 exerts its pro-apoptotic function by phosphorylation and degradation of the anti-apoptotic Bcl-2 family member MCL-1 (Maurer *et al*., 2006). Apart from the direct regulation of proteins implicated in mitochondrial function during apoptosis, GSK3 also regulates the expression of other components of the apoptotic signaling. The important tumour suppressor transcription factor p53 is regulated by GSK3, which binds directly to p53 and promotes p53-mediated transcription of specific genes (Watcharasit *et al*., 2003). In addition to regulation of gene expression, GSK3 also regulates translation by

phosphorylating and inhibiting elF2B, which appears to contribute to the control of cell survival by the Akt signaling pathway (Pap and Cooper, 2002). Akt was also meant to inactivate human caspase-9 by phosphorylating Ser196, thereby providing a new mechanism for its anti-apoptotic role (Cardone *et al*., 1998). However, the fact that the anti-apoptotic properties of Akt activation downstream of cytochrome c release were also observed in species such as mouse and monkey, in spite that the Ser196 phosphorylation site of caspase-9 is not conserved in these species (Fujita *et al*., 1999), strongly argue against the notion of Akt directly regulating caspase-9 inactivation. By contrast, a prominent role of ERK1/2 in promoting caspase-9 inactivation by phosphorylating Thr125 (Allan *et al*., 2003), a well conserved site which become an integration point for multiple signaling pathways, is nowadays widely accepted (Allan and Clarke, 2009). Akt directly regulates the cytoplasmic apoptotic machinery by regulating Bcl-2 family members activity. Upon growth factor stimulation, Akt enhances the cell survival by different mechanisms, one of which is blocking the function of pro-apoptotic proteins (Dudek *et al*.,1997). Akt phosphorylates the Bcl-2 homology domain 3 (BH3)-only protein Bad at Ser136 thereby inhibiting its pro-apoptotic effect (Datta *et al*., 1997). As described before, Bad pro-apoptotic function involves the binding with pro-survival Bcl-2 family members such as Bcl-XL resulting in their inactivation (Yang *et al*., 1995). Akt-mediated phosphorylation of Bad at Ser136 is essential for the accessibility of other survival-promoting kinases at the Bad Ser155 phospho-site within the BH3 domain, which results in the binding of 14-3-3 proteins and disruption of Bad/Bcl-XL complex (Datta *et al*., 2000). The survival effect of Akt among different cell types was also validated in primary neurons (Datta *et al*., 2002).

Akt contributes to cell survival not only by directly inhibiting the pro-apoptotic BH3-only proteins, but also by regulating different transcription factors such are Forkhead, NF-kB and CREB that control the expression of survival or apoptotic genes. Akt phosphorylates the Forkhead transcription factor FOXO1 on Thr24 and Ser256, as well as FOXO3 and FOXO4 on their equivalent sites. Akt phosphorylation of

FOXOs occurs in the nucleus and once phosphorylated they are sequester by 14-3-3 proteins, which triggers the release of FOXO transcription factors from the promoter of target pro-apoptotic genes, after which FOXO is exported from the nucleus. Among the gene promoters regulated by FOXO transcription factors, FasL and its receptor Fas, TRAIL (TNF-related apoptosis inducing ligand), TNFα and its receptor (Birkenkamp and Coffer, 2003) as well as Bim-1 (Dijkers *et al*., 2000) represent relevant transcriptional targets that accounts for Akt-induced cell survival. In addition to Akt function as a suppressor of critical death genes, it also triggers the expression of survival genes. Akt has the capacity to phosphorylate and activate IKKα and IKKβ, which are the upstream kinases that regulate the activation of the NF-κB family of transcription factors. IKKs directly phosphorylate IκB on a serine residue thereby targeting it for proteosomal degradation. As a result, NF-κB translocates into the nucleus where it activates transcription of pro-survival genes such as cIAP-1 and cIAP-2 (Dan *et al*., 2008).

The Akt signaling pathway also promotes survival by inhibiting the activity of the tumour suppressor protein p53. Akt activation initiated by survival factors promotes nuclear entry of the oncoprotein MDM-2 by phosphorylating it on Ser-166 and Ser-186. Once in the nucleus, MDM-2 promotes the ubiquitinization and subsequent inactivation of p53 (Mayo and Donner, 2001), which is a major regulator of apoptosis. This function of p53 was mostly notable when chromosomal aberrations were detected due to DNA damage induced by environmental stresses. MDM-2 binds to the transactivation domain of p53, thereby blocking the recruitment of factors necessary for the induction of gene expression. MDM-2, which forms a tight complex with p53, controls its function not only through inhibition of p53-mediated transcriptional activity, but also by promoting p53 degradation acting as an E3 ligase to maintain low protein levels of p53 under non-stressed conditions. Conversely, MDM-2 is positively regulated by p53 in an autoregulatory feedback loop in which p53 positively regulates MDM-2 expression and MDM-2 negatively regulates p53 (Momand *et al*., 1992). MDM-2 has been also shown to be associated with MDMX, which is considered to be

a direct substrate of Akt. Akt phosphorylates MDMX on Ser 367 generating a 14-3-3 binding site and the resultant association with 14-3-3 stabilizes MDM-2. The molecular details may need to be worked out but it is clear that Akt activity is important in keeping MDM-2 active and thus suppressing p53 activity (Lopez-Pajares *et al*.,2008).

The other transcription factors which may account in part for the survival effects of the Akt signaling are CREB/CBP and E2F. In response to growth factor stimulation, Akt promotes the phosphorylation of CREB at Ser133, which stimulates the recruitment of CBP (CREB binding protein) to the promoter thereby activating cellular gene expression of pro-survival genes via the CRE-dependent mechanism. Several studies indicate that CREB and its paralog CREM are indeed important in cell survival. Overexpression of a dominant-negative CREB transgene enhances apoptosis (Du and Montminy, 1998) whereas targeted disruption of CREM gene causes a defect in germ cell apoptosis (Blendy *et al*., 1996). The transcription factor E2F is known to have an important role in the regulation of apoptosis (Field *et al*., 1996). It was shown that active Akt induces E2F inactivation, thereby promoting survival (Brennan *et al*., 1997).

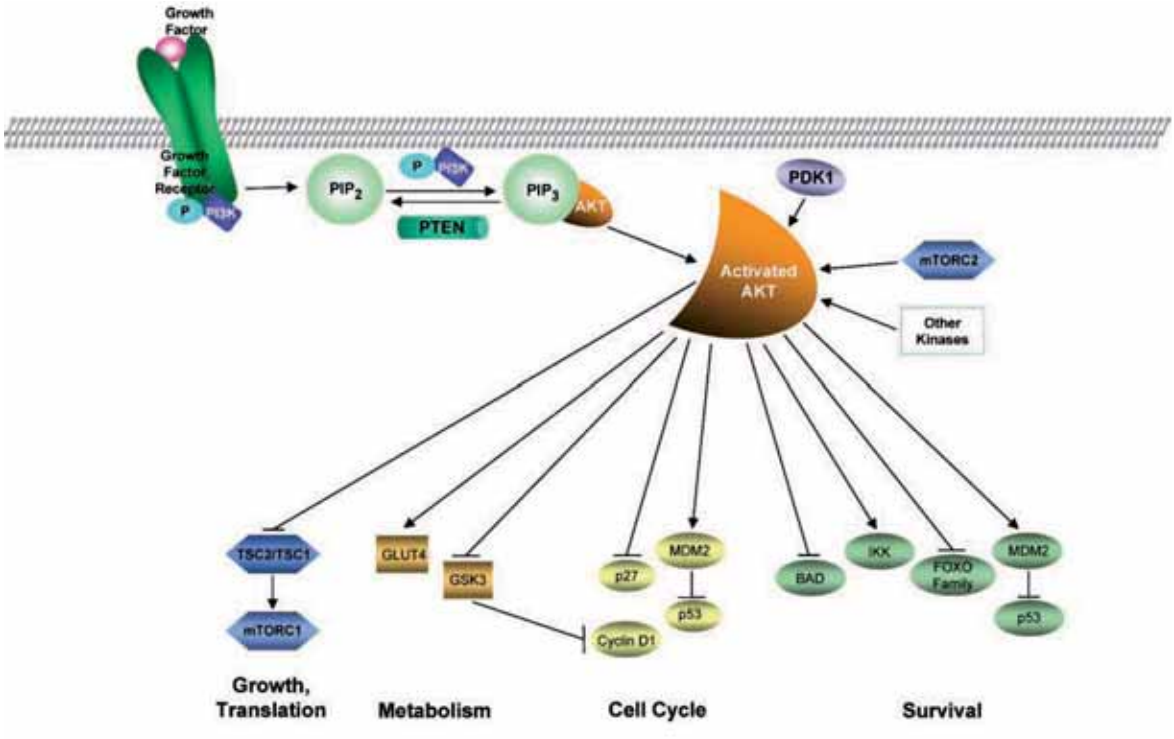

Figure 10. Akt substrates controlling cell survival (marked in green). Through the phosphorylation of a diverse set of substrates, Akt mediate some of these biological processes, which are indicated on the figure (Crowell *et al*., 2007).

2.1.2. PKB/Akt IN CELL GROWTH

One of the most conserved functions of Akt is promoting cell growth, which is a fundamental process of eukaryotic cell life. Nutrients and growth factors control cell growth by activating sensory signaling pathways. Akt contributes to this process by activating mTORC1 downstream of PI3K through the phosphorylation of two mTORC1 inhibitory proteins, namely PRAS40 and TSC2. In response to growth factors TSC2, also called tuberin, is phosphorylated and functionally inactivated by active Akt (Inoki *et al*., 2002). TSC2 is a negative regulator of the GTP binding protein Rheb (Ras homolog enriched in brain) which turns out as a main regulator of mTORC1 activation. In the GTP-bound state, Rheb binds directly to the kinase domain within the mTOR complex-1 and activates somehow its kinase activity. TSC2 contains a GTPase activating protein (GAP) domain at its C-terminus and in complex with TSC1, also named hamartin, is characterized with enhanced GAP activity towards Rheb, leading to its inactivation. In fact, TSC1 does not have any recognizable functional domain, but in complex with TCS2 prevents its degradation. Once TSC2 is phosphorylated by Akt it does not have the functional GAP domain and can no longer convert GTP-bound active Rheb into GDP-bound inactive form, whereas functionally active TSC1/2 complex maintains Rheb in an inactive state (Castro *et al*., 2003). In cells lacking either TSC1 or TSC2, the functional TSC1/2 complex is disrupted and consequently the levels of Rheb-GTP are elevated. This leads to deregulated mTORC1 activity and disorganized overgrowth within many organs that are seen in tuberous sclerosis hamartomas (Kwiatkowski and Manning, 2005). Akt also phosphorylates and inhibits PRAS40, a mTORC1 binding protein that inhibits mTOR kinase activity, thereby allowing mTORC1 activation (Sancak *et al*.,

2007;Vander *et al*., 2007).

2.2. THE AGC FAMILY

Both PDK1 and Akt belong to the AGC family of protein kinases, which encompasses about 60 members out of the 518 kinases of the human kinome and are highly conserved throughout eukaryotic evolution (Manning *et al*., 2002b). This AGC family was originally named after the cAMP-dependent kinase (PKA), cGMP-dependent kinase (PKG) and protein kinase C (PKC) family members. Protein kinases of the AGC family share the structural similarity and the most remarkable feature relying on the mechanism controlling their activity. For many AGC kinases, this activation mechanism involves the dual phosphorylation at two regulatory motifs which are highly conserved: the activation segment also termed activation loop or T-loop located in the catalytic domain, and the hydrophobic motif found within the non-catalytic region following the kinase domain (Pearce *et al*., 2010). AGC kinases are activated downstream of a wide range of extracellular stimuli by different mechanisms. The 3-phosphoinositide-dependent protein kinase 1 (PDK1) acts as the upstream activator phosphorylating a specific Thr or Ser residue located at the T-loop of the kinase domain of at least 23 members of the AGC family (Bayascas, 2010). This subgroup includes all the three Akt/Akt isoforms (Alessi *et al*., 1997b;Walker *et al*., 1998), the p70 ribosomal S6 protein kinases (S6K) -1 and -2 (Alessi *et al*., 1998; Lee-Fruman e*t al*., 1999; Pullen *et al*., 1998), the three serum and glucocorticoid inducible kinase (SGK) isoforms (Kobayashi and Cohen, 1999;Kobayashi *et al*., 1999;Park *et al*., 1999), p90 ribosomal S6 protein kinases (RSK) -1,-2 and -3 (Jensen *et al*., 1999;Richards et al., 1999), nine protein kinase C (PKC) isoforms (Chou *et al*., 1998;Dutil *et al*., 1998;Le Good *et al*., 1998) and the three PKC related kinase (PRK) isoforms (Dettori *et al*., 2009;Flynn *et al*., 2000;Leenders *et al*., 2004).

PDK1 was originally identified as the kinase capable of phosphorylating Akt at Thr308 in a phosphoinositide-dependent manner. PDK1 is one of the few kinases present in the vertebrate genome as a single copy gene which is expressed

ubiquitously. PDK1 is synthesized as a constitutively active enzyme which is activated following the trans-autophosphorylation of its own activation segment residue Ser241 (Casamayor *et al*., 1999), but it is still able to coordinately regulate the activation of the different downstream substrates in response to different stimuli.

In contrast to Akt, the rest of PDK1 substrates lack PH domains to directly sense PtdIns(3,4,5)P_3 levels. Instead, these AGC kinases are phosphorylated at the hydrophobic motif that is not sufficient for their activation but creates a docking site that can be recognized by a groove within the PDK1 catalytic domain, termed PIF pocket, which functions as a phospho-docking site recognition motif. Once bound to the substrate, PDK1 then is able to phosphorylate the T-loop and activate targets such as S6K, RSK, PKC and SGK isoforms (Collins *et al*., 2003). Phosphorylation of the S6K hydrophobic motif residue (Thr389) is mediated by mammalian target of rapamycin complex 1 (mTORC1) (Fig. 6B) (Hara *et al*., 2002; Kim *et al*., 2002). RSK isoforms are characterized by the presence of an N-terminus kinase domain (NTKD) of the AGC family and a C-terminus kinase domain (CTKD) of the CAMK family which is activated downstream of ERK1/2. The CTKD autophosphorylates then the hydrophobic motif, which is located in the linker region (Dalby *et al*., 1998). mTORC2 regulates the hydrophobic motif phosphorylation and activity of SGK1 (Garcia-Martinez and Alessi, 2008). Deregulation of the activity of these enzymes and mutations in the enzyme encoded genes contribute to pathogenesis of many human disorders including neurodegenerative diseases. Much effort is being applied in the development of drugs that target AGC kinases to treat these conditions. Despite intensive studies, still many basic questions remain.

2.3. mTORC1 AND ITS DOWNSTREAM EFFECTORS

mTOR (mammalian target of rapamycin) is a serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K) related kinase (PIKK) family. mTOR plays a vital role in cell growth signaling pathways. mTOR exist in two huge functionally distinct complexes: mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2)

(Sarbassov *et al.*, 2005a). The unique proteins that distinguish these complexes are RAPTOR (regulatory-associated protein mTOR) within mTOR complex-1 that may have a role in substrate binding and in rapamycin-FKBP12 binding and sensitivity, and RICTOR (rapamycin-insensitive companion of mTOR) within mTOR complex-2 (Sarbassov *et al.*, 2005a). Another distinguishable set of components are PRAS40 (proline-rich Akt substrate 40kD) for mTORC1 which is a negative regulator of the whole complex (Sancak *et al*., 2007) and PROTOR for mTORC2 which is likely to help in assembly the other mTORC2 subunits inside the complex (Pearce *et al*., 2007). Besides that, mTORC2 contains mSIN1 (mammalian stress-activated protein kinase interacting protein 1) which is absent in the complex 1. mLST8 (the mammalian lethal with Sec13 protein8), also known as GβL, and DEPTOR (DEP domain-containing mTOR interacting protein) are shared components for both mTOR complexes.

The proline-rich Akt substrate of 40-kDa (PRAS40) is a component of mTORC1, which has a regulatory function at the intersection of the PKB/Akt and mTORC1 signalling pathway. Phosphorylation of PRAS40-Thr246 by PKB/Akt, and PRAS40-Ser183 and PRAS40-Ser221 by mTORC1 but not mTORC 2 results in dissociation from mTORC1, and its binding to 14-3-3 proteins (Nascimento EB et al., 2010).Akt also phosphorylates the two tumor suppressor genes TSC1 and TSC2, which are negative regulators of the activity of mTOR. (Martin DE *et al*.,2005; Tee AR *et al.*,2005).Phosphorylation of TSC1 and TSC2 results in suppression of their inhibitory activity. Epistatic analysis and biochemical studies indicate that AKT-dependent phosphorylation of TSC2 prevents the TSC2-dependent inhibition of mTOR signaling. Whereas PI3K/AKT appears to be the major growth factor-mediated signal that controls TSC2 phosphorylation and mTOR activation, TSC2 is also phosphorylated on other residues by the activation of PKC, MEK1, ERK, and p90 RSK1, which apparently contributes to AKT-independent mTOR activation (Fingar DC *et al*.,2004;Naegele S *et al*.,2004).

mTORC1 is a major driver of cell growth in response to both growth factors and nutrients by regulating its downstream effectors S6 kinase-1 (S6K1) and 4EBP1.

mTORC1 controls mRNA translation through activation of S6K1 and inactivation of eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1), which is a translation suppressor that associates with and inhibits eIF4E. mTORC1 phosphorylates 4EBP1 at specific serine and threonine residues, which leads to release of eIF4E from 4EBP1 and permits its participation in translation initiation complex (Hara *et al*., 1997). mTORC1 phosphorylates S6K at the hydrophobic motif (Thr389) allowing the phosphorylation by PDK1 in the S6K activation loop (Thr229). When phosphorylated by mTORC1, S6K promotes mRNA translation initiation and progression by phosphorylating multiple proteins. Several S6K targets have been described, including the S6 ribosomal protein (Kozma *et al.*, 1989), eEF2K (eukaryotic elongation factor 2 kinase) (Wang et al., 2001) and eIF4B (eukaryotic translation initiation factor 4B) (Holz *et al*., 2005). It is worth to mention that aside from the ability of mTORC1 to enhance protein synthesis through its downstream targets, active mTORC1 phosphorylates the Ulk1 kinase at Ser757 thereby inhibiting its autophagy-inducing activity (Kim *et al.*, 2011).

Unlike mTORC1, mTORC2 is the hydrophobic kinase for Akt, which is widely implicated in cell survival and differentiation (Sarbassov *et al*., 2005b); mTORC2 is also the hydrophobic motif regulator for serum-and glucocorticoid-regulated kinase (SGK) (Garcia-Martinez *et al*., 2008) and also, mTORC2 promotes the regulation of PKC isoforms which regulate cell cycle progression (Ikenoue *et al*., 2008). All these findings collectively put mTORC2 upstream of important cellular processes such as survival or cell cycle progression.

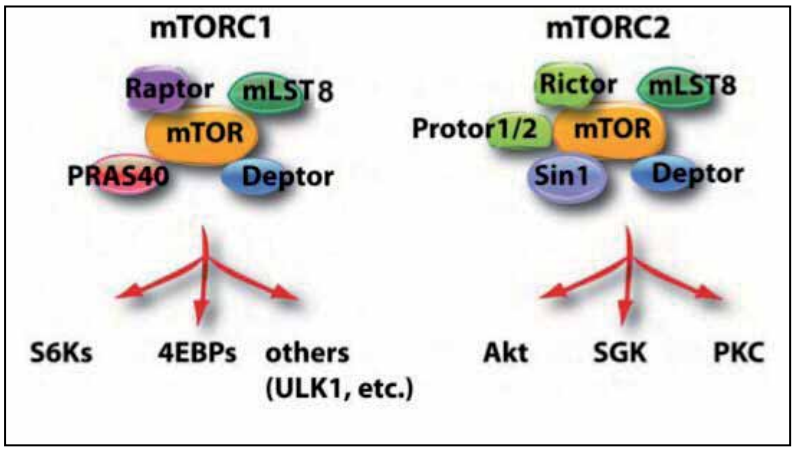

FIGURE 11. mTOR complexes. The diagram shows the components of mTORC1 and mTORC2 and their well-known substrates. Figure is adapted from (Limon and Fruman, 2012)

3. PDK1/Akt GENETIC MODELS

In order to learn more about the functions of PDK1 in mammals, different genetic mice models were generated and analyzed. Studies have indicated that the deletion of the PDK1 gene resulted in an embryonic lethal phenotype and that PDK1 is indispensable during embryonic development (Lawlor et al 2002). However, this essentiality of PDK1 during embryonic development precluded the analysis of the PDK1 function in the adult tissues and organs. To solve this problem, many tissue-specific conditional knockout mice were generated. For example, conditional deletion of PDK1 on T lineage leads to impaired T cell differentiation (Hinton *et al*., 2004). Muscle-specific knockout mice displayed dilated cardiomyopathy and died within 5-12 weeks of age (Mora et al 2004). Liver specific PDK1 knockout mice lacking PDK1 in hepatocytes displayed post-pandrial glucose intolerance and died between 4-16 weeks of age due to liver failure (Mora et al., 2005), whereas deletion of the PDK1 gene specifically in pancreatic cells developed progressive hyperglycemia and reduction in islet density due to both reduced cell number and size (Hashimoto *et al*., 2006).

However, in the tissue-specific PDK1 knockout models, it was impossible to find which of the downstream AGC kinase signal transduction pathways was responsible for the resultant phenotype. Crystal structure high-resolution of the PDK1 PH-domain

(Komander *et al*., 2004) and the PDK1 kinase domain (Biondi *et al*., 2002) lead to the rational design of two specific PDK1 point mutations which impair the function of either the PH-domain or the PIF-pocket domain. Jose R Bayascas exploited this exclusive mechanism of activation of Akt by generating two mice strains expressing the PDK1 Lys465Glu (K465E) knock-in allele disrupting the PH domain, and the Leu155Glu (L155E) mutation within the PIF pocket. These mutant enzymes retain catalytic activity but are incapable of either phosphoinositide or docking site binding. As a consequence, the activation of Akt, or substrates others than Akt (S6K, SGK, RSK, PKC), was correspondingly affected (Collins *et al*.,2003; McManus *et al*., 2004). These new genetic tools were called to become instrumental in defining the physiological roles that the PDK1 PIF pocket and the PDK1 PH domain play in the activation of diverse PDK1 substrates and in indentifying the contribution of the different PDK1-regulated kinases to the observed PDK1 mutant mice phenotypes.

3.1. PDK1 PH DOMAIN KNOCK-IN GENETIC MODELS

PDK1 contains a PH domain that binds PtdIns(3,4,5,)P_3 with high affinity and PtdIns(4,5)P_2 and PtdIns(3,4)P_2 with less affinity. This interaction does not affect PDK1 catalytic activity, but enhances its ability to activate Akt by co-localizing both PDK1 and Akt to the plasma membrane (Currie *et al*., 1999; Stokoe *et al*., 1997). In order to abolish PDK1-PtdIns(3,4,5,)P_3 binding and Akt activation, PDK1 (RRR472-474LLL) knock-in mice, which expressed a mutant form of PDK1 with a triple mutation of three consecutive Arg residues to Leu, were generated. In response to insulin-like growth factor (IGF1), $PDK1^{R472,473,474L/R472,473,474L}$ knock-in ES cells showed normal activation of RSK, whereas Akt activation and phosphorylation at Thr308 was totally abolished. However, in homozygous knock-in ES cells as well as in mouse embryos, the PDK1 (RRR472-474LLL) mutant protein was only expressed at 20% of the normal levels, which resulted in embryonic lethality. At day E8, knock-in embryos were of similar size than their heterozygous or wild type littermates and the only phenotypic difference that became apparent between

PDK1$^{R472,473,474L/R472,473,474L}$ and PDK1$^{+/+}$ mice embryos was the presence of head blood vessel and placental development defects (McManus *et al*., 2004). Reduced expression of the mutant PDK1 protein could have contributed to the effects seen on Akt activation in ES cells as well as to the the embryonic lethality. Since the homozygous PDK1$^{R472,473,474L/ R472,473,474L}$ have never ever developed to term, no much could be learned about the role of the PH domain of PDK1 from this animal model. Lately, structural studies of the PDK1 PH domain-PIP3 complex suggested that the mutation of Arg473 to Leu could destabilize the PDK1 PH domain fold, which might explain the decreased expression or stability of the mutant PDK1 (RRR472-474LLL) protein (Komander et al., 2004). Thus, it was impossible to determine whether the resultant phenotype was caused by the mutation or changes in protein expression or by the combination of both of these. Subsequent to this study, a knock-in point mutation (K465E) was designed that abrogated phosphoinositide binding without affecting the stability of the PDK1 PH domain (Fig. 9). Based on this, the PDK1$^{K465E/K465E}$ mice were generated. Homozygous PDK1$^{K465E/K465E}$ mice were found to be viable, born at the expected Mendelian frequency and fertile.

The significance that the interaction of the PDK1 PH domain with phosphoinositides plays in the activation of Akt has been evaluated in vivo in the PDK1$^{K465E/K465E}$ knock-in mouse model (Bayascas *et al*., 2008), which expresses this rationally-designed point mutant form of PDK1 that retains catalytic activity but is incapable of phosphoinositide binding (Komander *et al*., 2004). The inability of the mutant form of PDK1 to bind phosphoinositides was further confirmed by the experiment in which the PDK1$^{K465E/K465E}$ protein did not interact with PIP3-coated beads, whereas wild type PDK1 interacted with this resin. Furthermore, in lysates derived from six different tissues from wild type and PDK1$^{K465E/K465E}$ mice, PDK1 was immunoprecipitated and assayed employing the T308tide peptide, encompassing the PDK1 phosphorylation site on the Akt T-loop as a substrate, and the extracts were immunoblotted with a specific PDK1 antibody. The results drawn from this experiment provided a clear evidence that the PDK1 protein was expressed in the

PDK1$^{K465E/K465E}$ knock-in mice at normal levels and moreover the catalytic activity of PDK1 was unaffected by the K465E mutation, as judged by the ability of PDK1 to phosphorylate the T308tide peptide (Bayascas *et al*., 2008). In tissues derived from these mice, activation of Akt by growth factors was significantly reduced, whereas activation of the rest of PDK1 substrates proceeds normally. These mice were smaller and prone to diabetes. Deficient activation of Akt resulted in incomplete phosphorylation of some of its substrates, for example PRAS40 and TSC2 proteins. As a consequence, the growth factor-induced release of mTORC1 activation was significantly damaged, leading to reduced mTORC1 and S6K activities, which most likely explains the small size phenotype of the PDK1$^{K465E/K465E}$ mice (Bayascas *et al*., 2008;Waugh *et al*., 2009; Wullschleger *et al*., 2011). Moreover, the PDK1$^{K465E/K465E}$ mice were protected from PTEN-induced tumourogenesis (Wullschleger *et al*., 2011). The PDK1$^{K465E/K465E}$ mouse is a genuine model in which activation of Akt is only moderately reduced but not ablated, that has been proved instrumental in dissecting the PDK1 signaling (Bayascas, 2008). This has permitted to establish that in T-cells, an Akt signaling threshold depending on PDK1-phosphoinositide interaction dictates specific cellular responses, such as cell migration, but not cell proliferation (Waugh *et al*., 2009).

Stereological analysis of embryonic brain sections showed that the PDK1$^{K465E/K465E}$ mice displayed reduced brain size due to a reduction in neuronal cell size rather than cell number, since the number of cortical and hippocampal neuronal populations between PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ mice was not significantly different, whereas the volume of the mutant neuronal soma was approximately 80% of the volume of the wild type neuronal soma (Zurashvili et al 2013).

Stimulation of cortical neurons with BDNF induced a robust phosphorylation of Trk receptors followed by the phosphorylation of Akt at Thr308 in the PDK1$^{+/+}$ cells, which was blunted in the PDK1$^{K465E/K465E}$ neurons, whereas Akt phosphorylation at the mTORC2 site (Ser473) proceeded normally in both type of cells. The moderate reduction of Akt activation was not rate-limiting for the phosphorylation of those Akt

substrates governing neuronal survival and apoptosis such as FOXO and GSK3. Then, it was questioned whether such mutation could affect survival responses in primary neuronal cultures. The findings from this study illustrate that the integrity of the PDK1 PH domain is not essential to support the survival of different embryonic neuronal populations analyzed. Cell viability was compromised after trophic factor deprivation, whilst BDNF treatment rescues cells from death to the same extent in both $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ neurons (Zurashvili et al 2013).

In contrast, the moderate reduction of Akt activity in the $PDK1^{K465E/K465E}$ neurons markedly reduced phosphorylation of the PRAS40 and TSC2 substrates, leading to decreased mTORC1/S6K activation and also reduced BRSK protein synthesis. The $PDK1^{K465E/K465E}$ neurons in culture showed reduced neurite outgrowth, delayed polarization and deficient axonogenesis. To establish the possible causal relationship between the Akt pathway defects and axon formation, the impact of specific pharmacological treatments with Akt and mTORC1 inhibitors on neuronal differentiation were assessed, which provided strong evidence that the differentiation defects were due to reduced Akt activity and inefficient activation of the mTORC1 signaling. Moreover, the overexpression of BRSK isoforms rescued the axonogenesis defects of the $PDK1^{K465E/K465E}$ hippocampal cells (Zurashvili et al 2013). Altogether, these findings illustrated how the binding of PDK1 to PIP3 creates a Akt signaling threshold which was sufficient to support survival, but not differentiation of neuronal cells. In this regard, there is increasing evidence that PI3K/PDK1 dependent, Akt independent pathways might be responsible for the control of essential cellular processes, for example cell survival, which rely on other members of the AGC family activated by PDK1.

These other PDK1-regulated members of the AGC family include SGK1, PKC, S6K and RSK. The activation of these kinases is not dependent on PDK1 binding to PIP3 and therefore they should be normally phosphorylated in the $PDK1^{K465E/K465E}$ knock-in mice neurons. However, we observed decreased phosphorylation of the SGK substrate NDRG1. This study clearly stated for the first time, that NDRG1 is regulated by Akt,

at least in neurons. Activation of S6K was found also incomplete in the PDK1$^{K465E/K465E}$ neurons due to reduced mTORC1 Akt-dependent activation, which could be overcome by nutrients. In fact, the only PDK1 substrate analyzed that appeared to not to be affected by the PDK1 K465E mutation was RSK, which served as a control of the specificity of this knock-in mutation (Zurashvili et al 2013).

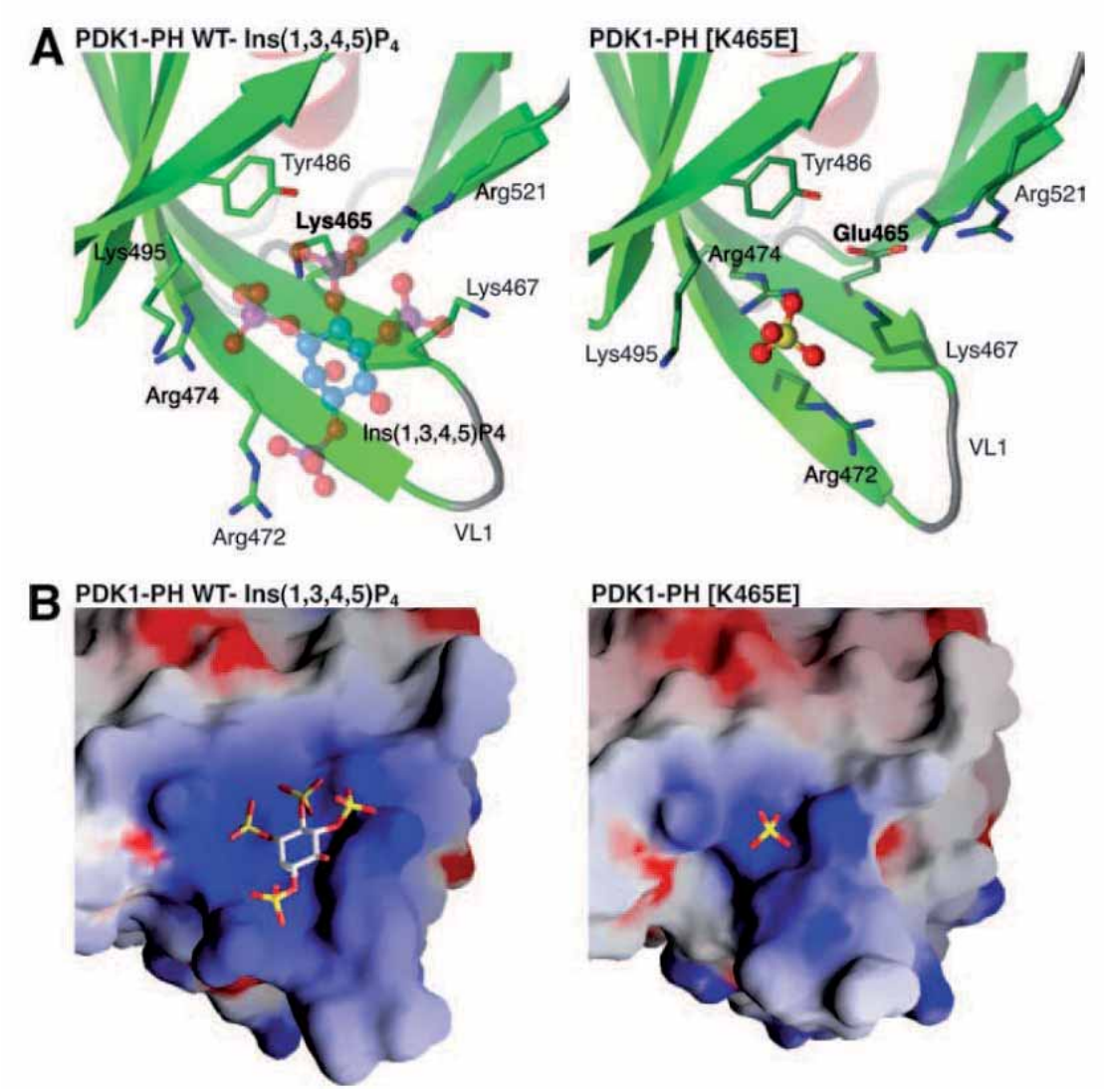

Figure 12. Crystal structure of the isolated PDK1[K465E] PH domain. (A) Comparison of the phosphoinositide-binding site of the wild-type PDK1 (left) with that of the PDK1 K465E mutant (right). A stick representation of the interactions of Ins(1,3,4,5)P4 (blue,

inositol ring; purple/red, phosphate groups) with protein residues (green) in the phosphoinositide binding site of the PDK1-PH domain is shown. Lys465 is the central residue in the back of the pocket, and its mutation to Glu does not affect the overall structure of the PH domain but leads to reorganization of the phosphoinositide-binding site by attracting surrounding positively charged residues. B) An electrostatic surface potential of the phosphoinositide binding site. Mutation of Lys465 to Glu alters the surface charge destroying the shape of the binding pocket (Bayascas *et al.*, 2008).

3.2. PDK1 PIF POCKET KNOCK-IN GENETIC MODELS

In order to assess the role of the PDK1 PIF-binding pocket in regulating the specificity of activation of AGC kinases in vivo, knock-in embryonic stem (ES) cells were generated in which Leucine 155 was changed to Glutamic acid in both copies of the endogenous PDK1 gene. The PDK1 PIF-pocket was functionally disrupted by this approach. Expression of PDK1 was maintained at similar levels in both $PDK1^{L155E/L155E}$ and $PDK1^{+/+}$ ES cells. Growth factor activated only Akt, which is a PDK1 substrate that was not meant to require hydrophobic motif phosphorylation to be phosphorylated by PDK1. In contrast, growth factor failed to activate S6K, RSK, and SGK in the knock-in ES cells, demonstrating that the PDK1 PIF pocket plays a crucial role in enabling PDK1 to activate those substrates lacking PH domains (Collins *et al*., 2003)

The $PDK1^{L155E/L155E}$ mice failed to develop to term. No $PDK1^{L155E/L155E}$ mice were ever isolated at E12,5 or later, indicating that the mutant embryos died and reabsorbed after this prenatal age. $PDK1^{L155E/L155E}$ embryos were retarded compared to control littermates of the same age. The mutant embryos displayed multiple abnormalities, including reduction in forebrain size and body axis defects.

To circumvent this prenatal lethality and to further dissect the contribution of the different PDK1 substrates to the regulation of cellular responses in vivo in tissues, a new strategy of transgenesis named tissue-specific conditional knock-in mice was engineered in which the expression of the mutant form of PDK1 was targeted to tissues expressing the Cre-recombinase under the control of a tissue-specific promoter. Thus, conditional knock-in mice expressing the PDK1 L155E mutation specifically in

muscle were generated. The tissue-specific knock-in strategy worked successfully and, as it was expected, insulin induced the phosphorylation and activation of Akt, but not S6K, in Cre-targeted tissues, whereas the activation of both Akt and S6K proceeded normally in non Cre-expressing tissues. The muscle specific $PDK1^{L155E/L155E}$ conditional knock-in mice were viable and of normal size and displayed no abnormality in glucose homeostasis (Bayascas *et al*., 2006). Recently, $PDK1^{L155E/L155E}$ neuronal-specific conditional knock-in mice have been generated, which are born alive and exhibit no gross abnormailities (Lluis Cordon Barris, personal communication, ongoing project in JR Bayascas`lab).

3.3. INSIGHTS FROM Akt ISOFORM KNOCKOUT MICE MODELS.

In order to understand the specific physiological functions of the individual isoforms, animal models deficient in PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 were generated. Mice lacking Akt1, the most ubiquitously expressed member of the Akt family, demonstrated defects in fetal and postnatal growth and increased apoptosis compared with the control littermates (Chen *et al*., 2001;Cho *et al*., 2001b). In addition, $Akt1^{-/-}$ mice displayed placental insufficiency and as a consequence fetal growth impairment and neonatal mortality (Yang *et al*., 2003). Also, Akt1 was specifically required for thymus normal development (Fayard *et al*., 2007). In contrast, Akt2 deficient mice displayed impaired ability of insulin to lower blood glucose levels. These mice were insulin resistant, with mild growth retardation and exhibited age-dependent loss of adipose tissue (Cho *et al*., 2001a; Garofalo *et al*., 2003). These observations establish Akt2 as the main regulator in maintaining normal glucose homeostasis. However, in contrast to diabetic $Akt2^{-/-}$ mouse phenotype, $Akt3^{-/-}$ mice display normal glucose metabolism, but reduced brain size by significant reduction in both cell volume and number. Akt3 deficiency in mouse brain was accompanied by a considerable reduction in total phosphorylated/activated Akt without any compensatory increase of other Akt isoforms, suggesting that Akt total activity failure was due to the loss of Akt3 isoform and further demonstrating that Akt3 is highly expressed in the nervous

system (Easton *et al*., 2005;Tschopp *et al*., 2005). According to these genetic models, all three Akt isoforms appear to have some differential, non-redundant physiological functions. Further studies were required to complete the understanding of the spectrum of common and isoform-specific roles of the Akt kinases. To address this, mice with combined Akt deficiencies were generated. Phenotypic analysis of double Akt isoform knockout mice revealed some overlap among the function of individual isoforms..Double Akt1 and Akt2 knockout mice develop to term but die shortly after birth by respiratory failure. These mice exhibit severe growth retardation with a very significant reduction in body weight, approximately 50% less than normal. They also showed skeletal muscle atrophy, impaired skin development and adipogenesis (Peng *et al*., 2003). By contrast, double Akt1 and Akt3 knockout mice were embryonic lethal. They died around embryonic day 12 with severe impairments of growth and cardiovascular development. However, $Akt1^{-/-}Akt3^{+/-}$ mice were born alive, but died several days after birth. Anatomic organ analysis of viable $Akt1^{-/-}Akt3^{+/-}$ mice at day 3 of age revealed hypotrophic thymus, heart and skin, which was much thinner than in the wild type (Yang *et al*., 2005).

On the other hand, the Akt2 and Akt3 double knockout phenotype resulted in particular organ size reduction, 35 % in brain and 40% in testis weights compared to wild types, whereas the organ/body weight ratio was not changed in all of the rest tissues analyzed (heart, lung, thymus, liver, spleen, and kidney) which were proportionally decreased in size (Dummler *et al*., 2006). Mice with a single functional allele of Akt1 ($Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$) were born alive despite the severe reduction of body weight and insulin intolerance (Dummler *et al*., 2006). Single knockout Akt isoform mice models have no survival disadvantages in the adulthood; however Akt1 knockout mice demonstrated increased neonatal mortality shortly after birth and displayed multiple developmental abnormalities. Furthermore, the pancreas-specific constitutively active Akt1 mouse model showed an increase in pancreas cell and organ size accompanied by improved glucose tolerance (Bernal-Mizrachi *et al*., 2001; Tuttle *et al*., 2001).

All this together is raising the intriguing possibility that the Akt1 is the most critical isoform for fetal development and postnatal survival in mammals. This notion was further corroborated by the double $Akt2^{-/-}Akt3^{-/-}$ knockout mice phenotype, which was fully viable with no growth abnormalities, as well as the $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ which were born alive in spite of having only a single Akt1 isoform, in contrast to the $Akt1^{-/-}Akt2^{-/-}Akt3^{-/-}$ triple knockout mice which were embryonic lethal (Dummler *et al*., 2006)

The Akt2 knockout mouse model was shown to be diabetic, whereas nor Akt1 neither Akt3 seem to contribute to glucose homeostasis. As far as combined deficiency of Akt2 and Akt3 isoforms manifested glucose and insulin intolerance, this strongly suggested the enrolment of Akt2 isoform in glucose homeostasis.

However, it was reported that the double knockout $Akt1^{-/-}Akt2^{-/-}$ mice with only Akt3 isoform retained, developed to term but died after birth with different severe abnormalities. The analogous phenotypical behaviour was found in $Akt1^{-/-}Akt3^{+/-}$ mice, whereas mice which were lacking both Akt1 and Akt2 died approximately at day E11 displaying multiple embryonic developmental defects. These observations provide the strong genetic evidence about Akt3 isoform playing more critical role in early development and survival than Akt2. Reduction in brain size was characteristic for Akt3 knockout mouse model, similar to this phenotype the simultaneous deletion of Akt2 and Akt3 isoforms led to brain size reduction. This argues for the Akt3 isoform preference to regulate the brain size.

Akt isoform single and double knockout mice genetic studies demonstrated different phenotypes, indicating that Akt isoforms play over-lapping but also differential roles in development and physiology (Fig. 10). Since Akt isoforms are structurally highly similar, the critical question raises how Akt activity is specified to discrete cellular functions in response to extracellular stimuli. It is considered that substrate specificity of Akt, abundance of each Akt isoform in a given type of tissue and specific cellular localizations may also in part account for Akt isoform specificity and the resultant phenotypes observed in the Akt isoform mutant mice. The understanding of Akt

isoform individual or combinatorial contribution to distinct physiological functions is further required to facilitate the development of therapeutic approaches directed to interfere with the Akt kinase functions. However, the PDK1$^{K465E/K465E}$ mutation, which specifically and partially impairs the activation of all the three Akt isoforms by PDK1, could be also a good alternative to define the physiological roles that the Akt kinases might play in adult tissues and in particular in the central nervous system, when compared to other PDK1 substrates.

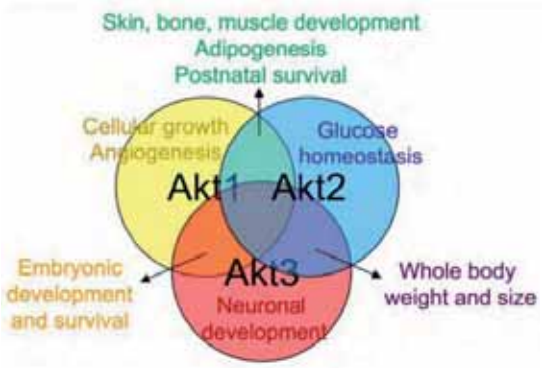

Figure 13. Overlapping and specific functions of PKB/Akt family members. Akt isoform-regulated common and distinct physiological functions, summarized from the phenotypic analysis of single and double Akt isoform knockout mice models. Figure adapted from (Gonzalez and McGraw, 2009)

We recently published the impact that the PDK1 K465E mutation had on the development of the central nervous system. Disrupting the interaction of PDK1 with PIP3 resulted in mice with reduced Akt activity due to reduced phosphorylation of Akt at Thr308, the PDK1 site, which is however not rate limiting for the phosphorylation of those Akt substrates promoting cell survival, such as FOXO1 or GSK3. Accordingly, the integrity of the PDK1 PH domain was not essential to support the survival of neuronal cells.

This observation is particularly important, since Akt has been widely accepted for years as the most critical factor promoting neuronal survival, and therefore challenges the notion of Akt being a good pharmacological target to treat neurodegeneration. We hypothesize than the importance of Akt was overestimated due to the lack of good genetic tools, and that other PDK1-regulated kinases might contribute to the neuroprotective functions of the pathway. It might as well be possible that the marginal activation of Akt reached in the PDK1$^{K465E/K465E}$ mice was sufficient to

support the survival of neurons. In this regard, inhibition of Akt1and Akt2 with the Akt-1/2i inhibitor did not compromise neuronal survival neither. Therefore, it become crucial to define whether the third isoform, Akt3, which is particularly enriched in neurons, is escaping the impact of the PDK1 K465E mutation in an analogous manner that it escapes the pharmacological inhibition, and whether Akt3 is responsible for neuronal survival in the PDK1$^{K465E/K465E}$ mice.

Genotype	Targeted Tissues	Viability	General findings and reference(s)
PDK1$^{-/-}$	All	Embryonic lethal E9,5	Lack of somites, forebrain and neural crest-derived tissue (Lawlor *et al*., 2002)
PDK1$^{flox/flox}$	All	Viable	Reduced size (Lawlor *et al*., 2002)
PDK1$^{-/-}$	Muscle	Lethality 5-11 weeks	Dilated cardiomyopathy (Mora *et al*., 2003)
PDK1$^{-/-}$	Liver	Lethality 4-16 weeks	Postpandrial glucose intolerance and liver failure (Mora *et al*., 2005)
PDK1$^{-/-}$	Pancreas	Viable	Hyperglycemia, islet mass loss (Hashimoto *et al*., 2006)
PDK1$^{-/-}$	T-cells	Viable	Impaired T-cell differentiation (Hinton *et al.*, 2004)
PDK1 (L155E)	All	Embryonic lethal E12	Developmental defects, forebrain reduction (Collins *et al*., 2003;McManus *et al*., 2004)
PDK1 (R131E)	All	Embryonic lethal E19,5	General growth retardation (Collins *et al*., 2005)
PDK1 (RRR/LLL)	All	Embryonic lethal E10,5	Head blood vessel defects, lack of placental development (McManus *et al*., 2004)
PDK1 (L155E)	Muscle	Viable	Normal glucose homeostasis (Bayascas *et al*., 2006)
PDK1 (K465E)	All	Viable	Small, insulin-resistant, hyperinsulinemic (Bayascas *et al*., 2008)
Akt1$^{-/-}$	All	Partial neonatal mortality	Small body size, increased apoptosis, placental defects, thymus developmental defects (Chen *et al*., 2001;Cho *et al*.,2001b;Fayard *et al.*, 2007;Yang *et al.*, 2003)
Akt2$^{-/-}$	All	Viable	Insulin resistance, hyperglycemia, age-dependent lipoatrophy (Cho *et al*., 2001a;Garofalo *et al*., 2003)
Akt3$^{-/-}$	All	Viable	Reduced brain weight (Easton *et al.*, 2005;Tschopp *et al*.,2005)

$Akt1^{-/-}Akt2^{-/-}$	All	Neonatal lethality	Small size, impaired skin, skeletal muscle, bone development and adipogenesis (Peng *et al*., 2003)
$Akt1^{-/-}Akt3^{-/-}$	All	Embryonic lethal E11	Severe impairments in growth, cardiovascular and nervous system development (Yang *et al*., 2005)
$Akt2^{-/-}Akt3^{-/-}$	All	Viable	Impaired glucose homeostasis, reduced body size, reduced brain and testis weights (Dummler *et al*., 2006)

TABLE 1. Genetically modified PDK1 and Akt mouse models. The table includes the information about phenotypes of genetically modified mouse models.

AIMS

AIM 1.- To evaluate the impact of the PDK1 K465E mutation in the activation of the individual PKB/Akt isoforms.

AIM 2.- To elucidate the relative contribution of each PKB/Akt isoform to the total Akt activity in cortical neurons.

AIM 3.- To evaluate neuronal viability in cells infected with lentiviral vectors producing isoforms specific PKB/Akt shRNAs

AIM 4.- To understand the mechanism of activation of PKB/Akt in the PDK1 K465E mice.

METHODS

Methods & Materials

1. Plasmid Construction

1.1. Bacterial Competent Cells Preparation

Inoculate DH5α cells in 250 of ml Luria Broth (LB) medium and grow overnight at 18°C with shaking at 180 rpm. In the morning after, the cell density should reach a OD 600 of 0.6. Place the bottle on ice for 10 min and then harvest at 4000 rpm for 5 min at 4ºC. Discard the supernatant carefully and resuspend the pellets in the correct volume of Transforming Buffer (80 ml TB buffer/pellet from 250 ml of OD 600=0.6 cells) (Table 2). Incubate on ice for 10 min and spin at 4000 rpm for 5 min at 4ºC. Then carefully resuspend the cell pellet in 64 ml of TB + 7% DMSO. Incubate for 5 minutes on ice and then aliquot directly into cold tubes (-20°C) quickly. Test an aliquot for competency after overnight storage at -80. The transformation is performed as detailed below.

TB buffer Reagents	Quantity	Final Concentration
Pipes	1.512 g	10 mM
$CaCl_2$	1.10 g	15 mM
KCl	9.30 g	250 mM
$MnCl_2$	5.44 g	55 mM
H_20	To total of 500 ml	-

Table 2. TB solution used for the competent cells preparation. A list of composition for and the final concentration are provided

1.2. Akt Isform-specific shRNAs from SIGMA Mission.

PLKO.1-puro retroviral plasmids containing different mouse Akt isoform-specific shRNAs were purchased from SIGMA Mission shRNA collection (Table 3). Four specific shRNA plasmids for each Akt isoform were prepared to proceed in the next step with the plasmid construction.

Target Protein	Reference	Sequences of ShRNAs
PKBα/Akt1	TRCN0000054703	CCGGGCAGAACTCTAGGCATCCCTTCTCGAGAAGGGATGCCTAGAGTTCTGCTTTTTG
	TRCN0000054704	CCGGGCCTGATCAAGATGACAGCATCTCGAGATGCTGTCATCTTGATCAGGCTTTTTG
	TRCN0000054705	CCGGGTGGCAGGATGTGTATGAGAACTCGAGTTCTCATACACATCCTGCCACTTTTTG
	TRCN0000054706	CCGGCCACAGTCATTGAGCGCACCTCTCGAGAGGTGCGCTCAATGACTGTGGTTTTTG
PKBβ/Akt2	TRCN0000055258	CCGGGCCACGGTACTTCCTTCTGAACTCGAGTTCAGAAGGAAGTACCGTGGCTTTTTG
	TRCN0000055259	CCGGCGCCTCTTTGAGCTCATTCTTCTCGAGAAGAATGAGCTCAAAGAGGCGTTTTTG
	TRCN0000055260	CCGGTGACCATGAATGACTTCGATTCTCGAGAATCGAAGTCATTCATGGTCATTTTTG
	TRCN0000055261	CCGGTCACTTCAGAAGTGGACACAACTCGAGTTGTGTCCACTTCTGAAGTGATTTTTG
PKBγ/Akt3	TRCN0000054724	CCGGCTATGCTATGAAGATTCTGAACTCGAGTTCAGAATCTTCATAGCATAGTTTTTG
	TRCN0000054723	CCGGCATCTGAAACAGACACCCGATCTCGAGATCGGGTGTCTGTTTCAGATGTTTTTG
	TRCN0000054725	CCGGCCGTGATCTCAAGTTGGAGAACTCGAGTTCTCCAACTTGAGATCACGGTTTTTG
	TRCN0000054727	CCGGCAGCTCAGACTATTACAATAACTCGAGTTATTGTAATAGTCTGAGCTGTTTTTG

Table 3. Nucleotide sequence of the Akt shRNAs purchased from Sigma Mission shRNA collection.

1.3. Transformation

Transformation is the process by which foreign DNA is introduced into a cell. Briefly, competent cells are taken out of -80 °C and thaw on ice (approximately 5 min). Then agar plates (containing the appropriate antibiotic, usually Ampicillin 100 μg/ml) are taken out from the fridge to warm up to room temperature or place in 37°C incubator. Mix 1 μl of DNA (usually 1 ng is enough) into 20-50 μl of competent cells in 1.5 ml Eppendorf tube. Gently mix by flicking the bottom of the tube with the finger a few times. Place the competent cell/DNA mixture on ice for 20-25 min. Heat shock each transformation tube by placing the tube into a 42 °C water bath for 30-60 seconds (45 sec is usually ideal, but this varies depending on the competent cells you are using). Put the tubes back on ice for 2 min. Add 500 μl LB (without antibiotic) and grow in a 37 °C shaking incubator for 45 min. Plate some or all of the transformation mixture onto a 10 cm LB agar plate containing the appropriate antibiotic. Incubate plates at 37 °C overnight.

1.3. Isolation of Plasmid DNA

Pick up a single colony of bacteria and grow on bacterial culture LB medium

supplemented with antibiotic (Ampicillin 100 µg/ml) overnight (O/N) at 37 °C with shaking at 200 rpm. Pour the O/N culture in a 2.0 ml Eppendorf tube and centrifuge at 13000 rpm for 1 min. Miniprep Kit (Campus Lab, Ref #0703) was used to isolate the plasmids. Briefly, discard the supernatant and add resuspension buffer (stored at 4°C) into the tube and pipette up and down to mix properly until the pellet dissolves. Add 100 µl of lysis buffer and mix by gently inverting the tube 10 times until the mixture becomes transparent. Then add 150 µl of neutralizing buffer into each tube and mix by inverting tube several times. At this point bacterial chromosomal DNA is usually seen as a white precipitate complexed with denatured proteins. Incubate it on ice for 10 minutes. Centrifuge the sample at 14000 rpm for 10 minutes and filter the supernatant into a fresh Eppendorf tube. Fill the tube with one volume of ice-cold isopropyl alcohol (stored at -20°C) and incubate in cold for 2 minutes. Centrifuge the sample at 14000 rpm for 10 min. Discard the supernatant and fill the tube with 500 µl 70% cold ethanol, then centrifuge again, discard the supernatant and let the pellets air dry. Finally, resuspend the DNA pellet with 50 µl TE and completely dissolve the pellet by pipetting the solution several times.

1.4. Plasmid Quantification

The Qubit® (previously known as Quant-iT™) dsDNA HS (High Sensitivity) Assay Kit (Life Technologies #Q33120) is used in this assay and the concentration of plasmid can be read using the Qubit® 2.0 Fluorimeter. The assay is performed at room temperature, and the signal is stable for 3 hours. Firstly, prepare dye working solution in a plastic tube by mixing 199 µl of buffer and 1 µl of dye for each sample, then aliquot 198 µl of working solution into two assay tubes for standards, add 2 µl of each standard to an assay tube and mix by vortexing. Aliquot 198 µl of working solution into assay tubes for samples. Add 2 µl of each sample and vortex. Incubate for 2 min and read the results in the Qubit® 2.0 Fluorometer.

DNA Gel electrophoresis experiment was performed to verify the concentration obtained from the Qubit kit and verify the integrity and size of the DNA. Mix 1g of

agarose in 100 ml of 1X TAE, which was diluted from 50X TAE (Tris-Base 121 g, Acetic Acid 28.55 ml, EDTA 0.5 M 50 ml, then H_20 to 500 ml), to prepare standard 1% agarose gel. Microwave for 3 min until the agarose is completely dissolved and there is a nice rolling boil. Let agarose solution cool down for 5 min and add the 5µl DNA dye to a final concentration of 1 µg/ml. Then pour the agarose into a gel tray with the well comb in place. Let sit at room temperature for 20 min until it has completely solidified. Add loading buffer into each sample tubes and move the gel into the tank which is filled with 1X TAE. Load a molecular weight ladder into the first lane of the gel and load the samples into additional wells of the gel. Run the get at 110 V until the dye line is approximately 70% of the way down the gel. Finally visualize the DNA fragments under the UV light and take the images.

1.5. Restriction Digest of Plasmid DNA and Gel Purification

Before proceeding with this step, correct restriction enzymes were chosen to digest the plasmid and an appropriate reaction buffer was determined by reading the instructions of the manufacturers. In a 1.5 ml tube combine the following:

Reagents	Quantity
DNA	10 µg
EcoRI	2 µl
SpeI	2 µl
100x BSA	0.5 µl
10x Buffer	5 µl
dH20	Total volume to 50 µl

Table 4. Table of solutions used in the preparation of Double Enzyme Digestion. A list of composition for reaction is provided.

Mix gently by pipetting. Incubate tube at appropriate temperature (usually 37 °C) for 2 hours. Prepare appropriate percentage of agarose gel (normally 0.7 %) to isolate the plasmid fragments of interest. Load all the volume of each digested sample into the gel and run the gel as usual. Once running is finished, move the gel to an open UV box and slice out the desired DNA fragment from the gel. Place the chosen band into

a labeled Eppendorf tube. Using a scale, weigh the tube with the gel fragment after zeroing the scale with an empty tube. Alternatively, you can just subtract the weight of the empty tube from the weight of the tube with the gel fragment. The weight of the gel is directly proportional to its liquid volume and this is used to determine how much of each buffer to add during the DNA isolation step. Finally, isolate the DNA from the gel. This was done with a commercial gel purification kit (QIAquick Gel Extraction Kit #28704) by following the manufacturer's instructions. At the last step, recover the DNA in TE buffer and keep it in -20 °C

1.6. DNA Ligation

The final step in the construction of a recombinant plasmid is connecting the insert DNA fragment into a compatibly digested vector backbone. Before setting up the ligation reaction itself, it is important to determine the amount of insert and vector to use for the ligation reaction. In my experiments, 3 insert : 1 vector ratio was used. Combine the following in a PCR tube as stated in Table 5. Incubate at at 16°C overnight (following the manufacturer's instructions). Then proceed with bacterial transformation, analyze single colonies and verify the cloning by digestion and sequencing. All sequencing was performed at the corresponding authomated DNA sequencing service of the Servei de Genomica of the UAB.

Vector DNA	25 ng
Insert DNA	75 ng
Ligase Buffer 10X	1µl
T4 DNA Ligase	1µl
H_2O	To total of 10 µl

Table 5. Table of solutions used in the preparation of ligation experiment. A list of composition for reaction is provided

2. Experimental Animal Model

In order to explore the objectives of this project, I used the PDK1$^{K465E/K465E}$ knock-in mice, which express a rationally-designed point-mutant form of the PDK1 protein kinase which blocks the phosphoinositide binding pocket, whilst the catalytic activity

is unaffected. The generation and genotyping of the PDK1$^{K465E/K465E}$knock-in mice expressing the single-amino acid substitution of Lysine 465 for Glutamic acid in the PDK1 PH domain have been described previously (Bayascas et al., 2008). Mice were maintained in the Animal House faculity of the Universitat de Lleida under standard husbandry conditions: animals were housed with access to food and water ad libitum in a colony room kept at a constant temperature between 19-22 ºC and at 40-60 % humidity, under a 12/12 hours light/dark cycle.

Mice homozygous for the knock-in allele expressing the PDK1 mutation in the whole body including brain have always been obtained from heterozygous breeding. To obtain embryonic primary cultures, experimental matings were set in which the day of pregnancy was determined after the appearance of the vaginal plug. At the appropriate day, pregnant mice were euthanized by cervical dislocation and embryos dissected out from the uterus. In this project I have used the homozygous PDK1$^{K465E/K465E}$ knock-in mice and cells for all the experiments, and the results were compared to control PDK1$^{+/+}$ littermates.

All animals studies and breeding performed in the study were approved by the Comité de Bioseguridad de la UAB, the Comité Ético y de Experimentacion Animal y Humana de la UAB (CEEAH, project 2291) and the Departament de Medi Ambient i Habitatge de la Generalitat de Catalunya (DMAH, project 7493).

3. Primary Cell Cultures

Primary neuronal cultures of embryonic cerebral cortex were obtained from gestational stage E15.5. Pregnant female were sacrificed by cervical dislocation and embryos extirpated from uterus by caesarean section. Cerebral cortical tissues were dissected and the cells were enzymatically dissociated for 10 min at 37 ºC in Solution 2 which consist on Krebs Ringer Buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.3 mM glucose) containing 0.3% BSA, 0.03% $MgSO_4$ and 0.25 mg/ml of trypsin.

The enzymatic digestion was stopped by adding Solution 4 (see Table 3) and the digested tissue was then pelleted by centrifugation for 30 seconds at 1500 rpm. The

supernatant was discarded and the pellets were resuspended in Solution 3 consisting on Krebs Ringer Buffer containing 0.3% BSA, 0.03% $MgSO_4$, 0.08 mg/ml DNase and 0.52 mg/ml trypsin inhibitor by gentle pipetting up and down 10 times by using a fire-polished Pasteur pipette to produce a single cell suspension. Cells were collected and added into Solution 5 consisting on Krebs Ringer Buffer which contains 0.3% BSA, 0.03% $MgSO_4$ and 0.0014% $CaCl_2$. Cells were centrifuged for 5 min at 1000 rpm and resuspended, then counted with Scepter™ 2.0 Handheld Automated Cell Counter (Millipore) and finally diluted in DMEM (Dulbecco´s Modified Eagle Medium, Sigma Aldrich #D5796) complemented with 30 mM glucose, 2 mM L-Glutamine, 250 U/ml penicillin-0.25 mg/ml streptomycin and 10% FBS (Fetal Bovine Serum, GIBCO #10270). The cortical cells were plated onto poly-D-Lysine (50 μg/ml) coated 24-well plates for cell viability study, or 6-well plates for Western blot analysis, at a density of 2 x 10^5 cells/ml. The cells were allowed to attach to the plate for two hours and then the medium replaced by Neurobasal™ medium (GIBCO #21103) complemented with 30 mM glucose, 2 mM L-Glutamine, 250 U/ml penicillin-0.25mg/ml streptomycin and 2% B27 supplement (GIBCO #17504). Cells were maintained at 37 ^oC in a humidified incubator containing 5% CO_2 in normoxia conditions.

SOLUTION	REAGENTS	QUATITY
PBS(1X)	H_20	430 ml
	PBS 10X	50 ml
	Glucose 30%	10 ml
	Penicillin 10000U/ml-Streptomycin 10mg/ml	12.5 ml
Krebs-Ringer Buffer(KRB) 10X	NaCl	70.70 g
	KCl	3.60 g
	KH_2PO_4	1.66 g
	$NaHCO_3$	21.40 g
	Glucose	25.70 g
	H_2O	till 1000 ml
Magnesium Stock 3.8%	$MgSO4.7H_2O$	19 g
	H_20	to 50 ml
Calcium Stock 1.2%	$CaCl_2.2H_2O$	0.12 g
	H_2O	to 10 ml

Solutions For Cell Culture, Prepared Freshly		
Solution 1	KRB 1X	50 ml
	BSA Fraction V	0.15 g
	Magnesium Stock 3.8%	0.4 ml
Solution 2	Solution 1	10 ml
	Trypsin	2.5 mg
Solution 3	Solution 1	10 ml
	DNase	0.8 mg
	Trypsin Inhibitor	5.2 mg
	Magnesium Stock 3.8%	0.1 ml
Solution 4	Solution 1	8.4 ml
	Solution 3	1.6 ml
Solution 5	Solution 1	5 ml
	Magnesium Stock 3.8%	40 µl
	Calcium Stock 1.2%	6 µl

Table 6. Solutions and media used for primary culture preparation and maintenance. A list of recipes for the preparation of solutions and media used in the primary culture is provided.

4. Trophic Deprivation, Stimulation and Drug Treatment

Experiments for cell viability and biochemistry were always performed at day 6 *in vitro* (DIV 6). Medium was aspirated and cells were washed twice with serum-free DMEM and then incubated for 24 h in serum-free Neurobasal supplemented with 30 mM glucose, 2 Mm L-Glutamine, and 0.25 mg/ml of penicillin/streptomycin. The experimental controls included sham treatments consisting of two washes with serum-free media and incubation with same original conditioned media. BDNF (human Brain-Derived Neurotrophic Factor, Alomone #B-250) was diluted in DMEM without any supplement. The inhibitors Akti-1/2 (Calbiochem #124018), AZD8055 (AxonMedChem #1561) and GSK650394 (AxonMedChem #1570) were dissolved in dimethi1 sulfoxide (DMSO, Sigma #D5879). For viability and apoptosis analysis, growth factors and inhibitors were added at the onset of trophic deprivation. For biochemistry analysis, cells were pre-treated for 30 min with inhibitors and the stimulated with BDNF for 5 min as indicated.

5. Lentiviral Production

Plko.1-puro-GFP retroviral plasmids containing different mouse Akt isoform-specific shRNAs were propagated in HEK 293T cells to produce the corresponding lentiviral particles following standard procedures.

5.1. Transfection of HEK 293T cells.

Frozen HEK 293T Cells were recovered and maintained in DMEM medium (Dulbecco´s Modified Eagle Medium, Sigma Aldrich #D5796) complemented with 50 ml Fetal Bovine Serum (FBS) (GIBCO #10270) and 250 U/ml penicillin-0.25 mg/ml streptomycin. Then cells were cultured in 15-cm tissue culture flasks by spliting them twice a week at a ratio of 1/10 using trypsin/EDTA. Cells were seed 3 days before the transfection by plating 10^6 cells/10 cm dish. This allows using only few amounts of culture and results in tightly adherent cells. Ideal cells should reach 80-90 percent confluence on the day of transfection. Two hours before transfection, the media was replaced with 5 ml of fresh preheated DMEM medium, then the concentration of the different plasmids was adjusted to 1 mg/ml in TE buffer pH 8.0 and mixed as follows: 15.9 μg of PMD2G and 29.1 μg of PPAX2 packaging plasmids, as well as 45 μg each of the plasmids contain Akt-isoform-specific shRNAs in 1,3 ml TE and 727 μl of buffered water. After this step, 223 μl of $CaCl_2$ 2.5 M were added into this mixture and pipetted strongly (see Table 4). Finally, the mix was added to 2.25 ml of 2X HBS dropwise under agitation by vortexing during 2-3 seconds and the precipitate left at room temperature at least 20 minutes, then added to the cells at 1.5 ml/dish dropwise and incubated overnight. Next day morning, the medium was aspirated and 5 ml of fresh new DMEM medium added. 10-12 hours later, the culture medium from each plate was harvested into a 50 ml centrifuge tube and the collection repeated every 10-12 hours. The pooled supernatant was filtered by using a 0.45-um filter unit, then the supernatant was pipetted into 38.5 ml Beckman ultraclear tubes (put at least 26 ml). Ultracentrifugation for 120 min at 50.000 g and 16 ^oC was performed and then the supernatant gently discarded by inversion. Finally, the pellet was resuspended with 150

µl of PBS 1X, aliquoted and stored at -80ºC.

5.2. Titration of Lentiviral vectors

The first day, HEK 293T cells were seeded on 24 well plates in 0.5 ml medium/well. After 24 hours, the volume of the well was reduced by half and the cells transduced with 6-fold serial dilution: 2 ul, 1 ul, 0.5 ul, 0.25 ul, 0.125 ul, 0.0625 ul of the lentiviral stock. The third day, 1 ml of fresh DMEM medium was added. The fifth day, the medium was removed and the cells washed twice with PBS 1X. Next 200 µl of tripsin-EDTA/well were added for 5 min at 37 ºC. Then 600 µl of medium with serum were added, the cells collected and centrifuged for 30 seconds at 1000 rpm. The pellet was washed with 500 µl PBS and then centrifuged again. Finally, 500 µl PBS 1X were added and the fluorescence analyzed by FACS (Cytomics FC 500, Beckman Coulter) by reading the percentage from linear values.

Solution	**Reagents**	**Quantity**	**Sterilize & Storage**
2.5 M $CaCl_2$	$CaCl_2.2H_2O$ H_2O	9.18 g 25 ml	0.22-µm nitrocellulose filter +4^{0}C
2 x HBS (HEPES-Buffered Saline) pH 7.00	NaCl HEPES $NaHPO_4$ NaOH H2O	8.18 g 5.95 g 0.201 g 5 M Till 500 ml	0.22-µm nitrocellulose filter -70 ^{0}C For 2 Years
TE Buffer 1X pH 8.0	Tris HCl EDTA	10 mM 1 mM	0.22-µm nitrocellulose filter +4^{0}C
TE Buffer 0.1X pH8.0	TE Buffer 1X PH 8.0 H_2O	10 ml 90 ml	0.22-µm nitrocellulose filter +4^{0}C
2.5 mM Buffered Water	1 M Hepes H_2O MiliQ	125 µl Till 50 ml	0.22-µm nitrocellulose filter +4^{0}C

Table 7. Table of solutions used in the preparation of lentiviral production.

6. Lentivirus infection

Cortical cells were plated into 24-well plates at a density of 2 x10^5 cells/ml in a final volume of 500 µl. After 3 days (DIV3), 250 µl of conditioned medium was taken out from each well prior the infection and kept in the bath at 37 ºC. Then lentiviruses were added on top of the cells directly and swirl gently to mix well. Cells were incubated with viruses at 37 ºC in the incubator. After 6 hours, medium was aspirated and replaced with the kept 250 µl conditioned medium supplemented with 250 µl of complete fresh Neurobasal™ medium (GIBCO #21103) topping up to 500 µl/well. Infection efficiency was evaluated after 48 hours by means of the fluorescent marker GFP.

7. Western Blot Analysis

7.1. Generation of Protein Extracts from Cortical Cells

After the corresponding different treatments medium was aspirated and cells were rapidly lysed in ice cold Lysis Buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1mM sodium orthovanadate, 50mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium beta glicerophosphate, 0.27 M sucrose, 1% (by mass) Triton-X 100, 0.1% (v/v) 2-mercaptothanol, 1:100 Protease Inhibitor Cocktail) using the scraper for cell harvesting. The collected lysates were left on ice for 30 min and then centrifuged at 4º C for 12 min at 12000 rpm. The supernatants were transferred into fresh Eppendorf tubes and finally kept at -20 ºC. Protein concentrations were determined by the Bradford method (Coomassie protein assay reagent, Pierce #23200) using bovine serum albumin (BSA) as a standard.

7.2. Protein Immunoprecipitation Experiment

For this experiment, 1 µg of the corresponding antibody was conjugated with 10 ul of beads (Streptavidin Sepharose, GE Healthcare, #71-5004-70). Sepharose beads and antibody were incubated at 4º C for 60 min on a tube rotator mixer. Beads were washes

three times with washing buffer (Lysis buffer plus 0.5 M NaCl). Then, the beads incubated with the corresponding antibody were added to lysates containing 100 μg of protein and the immune complexes were allow to form overnight at 4º C on a tube rotator mixer. Beads were collected by centrifugation and washed three times with washing buffer, while the supernatants were recovered and kept after the first centrifugation. Finally, the immune complexes were denatured by boiling 10 min at 95º C in a Laemmli-SDS sample buffer and resolved on a SDS-PAGE. Western blot analyses were carried out as below. The supernatants were kept as a control of the specificity of the procedure.

Lysis Buffer	**Reference**	**grams/L**	**Volume from stock**
50 mM Tris-HCl, pH 7.5	Amresco 0497		50 ml Tris-HCl 1M pH7.5
1 mM EGTA	Sigma E0396		4 ml EGTA 250 mM pH7.5
1 mM EDTA	Sigma 443885		2 ml EDTA 500 Mm pH8.0
1 mM Na-orthovanadate	Sigma S6508		
50 mM Na-fluoride	Sigma 7920	2.100	
5 mM Na-pyrophosphate	Sigma 221369	2.230	
10 mM Na-B-glycerol-P	Sigma G6501	2.161	
0.27M sucrose	Sigma S9378	92.421	
1%(by mass) Triton X-100	Sigma X100	n.a	0.1 ml Triton X-100/10ml buffer
0.1% 2-mercaptoethanol	Sigma M7154	n.a	0.01 ml 2-mercapto/10ml buffer
Proteinase inhibitor	Sigma P8340	n.a	0.1 ml Prot.Inhibit/10ml buffer

Stock	**Preparation**
Tris-HCl 1 M, pH 7.5	60.7g TRIS in 500 ml water, pH to 7.5 with HCl
EGTA 250 mM, pH 7.5	19.02g EGTA in 200 ml water, pH to 7.5 with HCl
EDTA 500 mM, pH 8.0	93.06g EDTA in 500 ml water, pH to 8.0 with HCl
Na-OTV 100 mM	3.68g Na-OTV in 200 ml water, pH to 7.0 with HCl and microwaves to achieve yellow colour

Table 8. Lysis buffer. The composition and procedure to prepare lysis buffer are provided

Solutions (ml)	**Running Gel**						**Stacking Gel**	
Percentage (%)	5%	6%	7.5%	10%	12%	15%	3.6%	5%
d H2O	11.6	10.4	10	8.3	7	5	5.8	3.8
30% acryl	3.4	4	5	6.7	8	10	1.3	0.85
1.5M Tris pH8.8	5	5	5	5	5	5	-	-
0.5M Tris pH6.8	-	-	-	-	-	-	2.5	0.325
20%SDS	0.25	0.25	0.25	0.25	0.25	0.25	0.05	0.025
10%APS	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.05
TEMED	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.005

Table 9. Table of solutions used in the preparation of SDS-PAGE running and stacking BIO-RAD gels. SDS: sodium dodecy1 sulphate; TEMED: N, N, N', N'-tetramethylethylenediamine; APS: ammonium persulfate.

7.3 SDS Gel Electrophoresis

Polyacrylamide gels were prepared in BIO-RAD mini PAGE (polyacrylamide gel electrophoresis) system (10 well, thickness 1 mm) using solutions listed in table 9. 5X Laemmli sample buffer (Table 10) was added to normalized lysate samples, which were then boiled for 5 min at 95^{o} C before loaded on the SDS-PAGE gel alongside prestained protein markers (250-10kDa) from Bio-Rad (#161-0373). Gels were run on 1X Tris-Glycine Buffer 0.1% (w/v) SDS (see Table 10) at 160 V for 60-100 min depending on the nature of the gel and the protein of interest.

Laemmli-SDS Sample Buffer

Concentration	**5X**	**1X**	**For 100ml of 5X**
Tris-HCl p H6.8	125 mM	25 mM	25 ml Tris-HCl 0.5 M 6.8
Glycerol	50 %	10 %	50 ml
SDS	10 %	2 %	10 g
Bromophenol Blue	0.01 %	0.0025 %	12.5 mg
ß-Mercaptoethanol		1 %	Freshly added

Tris-Glycine SDS Electrophoresis Buffer (10X)

Concentration	1X	10X	For 1 litter of 10X
Tris base	25 mM	250 mM	30.3 g
Glycine	192 mM	1.92 mM	144.1 g
SDS	0.1% (w/v)	1% (w/v)	10 g

Tris-Glycine Transfer buffer(10X)

Concentration	1X	10X	For 1 litter of 10X
Tris base	25 mM	250 mM	30.3 g
Glycine	192 mM	1.92 mM	144.1 g

TBS Buffer (10X)

Concentration	1X	10X	For 1 litter of 10X
NaCl	150 mM	1.5 M	87.6 g
Tris Base	25 mM	250 mM	30.3 g

Table 10. Western Blot Buffers. Recipes of western blot buffers are provided as well as the concentration of their components.

7.4 Transfer of Proteins to Nitrocellulose Membranes

After the gel has finished running, remove the stacking gel. Wet the nitrocellulose membrane (Whatman®GmbH) with transfer buffer and carefully lay it across the exposed running gel, making sure that air bubbles between the gel and membrane are excluded. In the same fashion, add two layers of pre-wetted filter paper to the membrane. Pre-wet one of the cassette sponges and add to the stack. Top with the outer half of the cassette itself and carefully turn the entire stack of glass plate, gel, membrane, filter paper, sponge and cassette over. Place the stack in the container with transfer buffer. The transfer was then performed at 100 V for 90 min. Then membranes were blocked in 10 % non-fat dry milk/TBS/0,2 %-Tween at room temperature for 1 hr. Primary antibody were diluted in 5 % milk/TBS/0,2 %-Tween (total protein antibodies) or in 0.5 % BSA/TBS/0,2 %-Tween (phospho-protein antibodies) as indicated in Table 12 and incubated for 16 hours at 4 ºC. Antibodies were diluted by following the manufacturer's instructions. After 16 hour incubation, the membrane was washed three

times for 5 min in TBS/0,2 %-Tween and then incubated with the corresponding secondary antibodies for 1 hour at room temperature, secondary antibodies were always diluted 1:5000 in 5% milk/TBS 0,2%-/Tween. Then the membrane was washes three times for 10 min with TBS/0,2 %-Tween. Detection of proteins was performed by the enhanced chemiluminescence (ECL) system. Membranes were treated with the ECL reagent mixture for 1 min before exposed to Super RX Fujifilm (MTB) in the dark room. The film was developed to produce the images based on interest.

Solution	Reagents	For 50 ml
Ecl 1	Tris-HCl 1 M pH 8.5	5 ml
	Luminol 0.5 M in DMSO	0.25 ml
	P-Coumaric Acid 79.2 mM in DMSO	0.25 ml
		H_20 till 50 ml
Ecl 2	Tris-HCl 1 M pH 8.5	5 ml
	Hydrogen Peroxide 8.8 M	0.032 ml
		H_20 till 50 ml

Table 11. Recipe of enhance chemiluminescence (ECL) reagents

7.5. Primary and Secondary Antibodies for Detection

The following antibodies were kindly provided by Prof. Dario Alessi from the University of Dundee. All were raised in sheep and affinity purified on the appropriate antigen. The Akt1 total antibody was raised against the sequence RPHFPQFSYSASGTA corresponding to residues 466-480 of rat Akt1 while the Akt2 total antibody was raised against the sequence RYDSLDPLELDQRTH corresponding to residues 455-469 of the mouse Akt2; The total TSC2 antibody was raised against a sequence encompassing residues 1719-1814 of mouse TCS2; The total PRAS40 antibody was raised against the peptide DLPRPRLNTSDFQKLKRKY corresponding to residues 238-256 of human PRAS40; The rest of the primary antibodies used for western blot and immunofluorescence analysis were purchased from commercial company and listed below.

Primary Antibodies	Host	Supplier	Reference	Dilution
Phospho-Akt Thr308	Rabbit	CST	#9275	1:1000
Phospho-Akt Ser473	Rabbit	CST	#9271	1:1000

Phospho-S6K Thr389	Rabbit	CST	#9205	1:1000
Phospho-SK6 Thr229	Rabbit	CST	#9379	1:1000
Total S6K	Rabbit	CST	#9202	1:1000
Phospho-S6 Ser235/236	Rabbit	CST	#2211	1:1000
Total S6	Rabbit	CST	#2217	1:1000
Phospho-FOXO-1 Thr24	Rabbit	CST	#9464	1:1000
Total FOXO1	Rabbit	CST	#2880	1:1000
Phospho-GSK3α/β Ser21/9	Rabbit	CST	#9331	1:1000
Total GSK3α/β	Mouse	SCB	#sc-7291	1:1000
Phospho-RSK Ser380	Rabbit	CST	#9335	1:1000
Total RSK 1/2/3	Rabbit	CST	#9355	1:1000
Phospho-TSC2 Thr1462	Rabbit	CST	#3611	1:1000
Phospho-PRAS40 Thr246	Rabbit	CST	#2997	1:1000
PKBγ/AKT3	Mouse	Millipore	# 05-780	1:1000

Secondary antibody	**Host**	**Supplier**	**Reference**	**Dilution**
Anti-Rabbit HRP-conjugated	Goat	Thermo	#31460	1:5000
Anti-Mouse HRP-conjugated	Goat	Thermo	#31430	1:5000
Anti-Mouse HRP-conjugated	Rabbit	Thermo	#31480	1:5000

Table 12. Primary and secondary antibodies for western blot and immunofluorescence analysis. A list of primary and second antibodies is provided, as well as their source and the dilution that was used for western blot analysis. CST: Cell Signaling Technology, SCB, Santa Cruz Biotechnology.

8. Evaluation of Cell Viability

8.1. Quantification of Apoptosis

After 24 hours treatment, cells were stained with 1 μg/ml of the DNA dye Hoechst 33342. Hoechst 33342 is a membrane-permeable nuclear stain and labels the nuclei of all cells in the sample. Apoptotic nuclei are characterized by a higher density of the

chromatin and clear fragmentation pattern. Then cells were fixed in 2% paraformaldehyde and visualized under the fluorescence microscope. Apoptosis was quantified at each condition point by scoring the percentage of apoptosis cells in the adherent cell population. Cells exhibiting fragmented or condensed nuclei were scored as apoptotic whilst cells showing uniformly stained nuclei were scored as viable. At least 200 cells from 5-6 randomly selected fields per well were counted.

8.2. MTT Reduction Assay

The MTT assay is a colorimetric assay for assessing cell viability. The yellow tetrazolium MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide salt) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Briefly, MTT (Sigma #M2128) was added to the medium directly at a final concentration of 0.5 mg/ml. Plates were then returned to the incubator for 60 min. After incubation, the medium was aspirated and the resulting formazan crystals were dissolved by mixing with 300 µl of DMSO. Absorbance intensity was measured at 570 nm, with background measured at 690 nm, using a spectrophotometer running the Labsystem Multiskan^{R} Software.

9. Statistical Analysis

Statistical significance was determined using the Student´s t test or One Way Anova analysis. *$p<0.05$, **$P<0.01$ between categories or conditions as indicated in the figure legends.

RESULTS

1. Establishment of the conditions for the specific detection of individual PKB/Akt isoforms.

As it was mentioned before, Akt comprises three mammalian isoforms: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. Although PKB isoforms are encoded by separate genes, each of these isoforms shares more than 80 % amino acid sequence identity and a similar structure organization. In order to elucidate the relative contribution of the different Akt isoforms to neuronal survival and differentiation, it was firstly needed to establish precise and sensitive conditions for the specific detection of each individual Akt isoform. To achieve that, I characterized the specificity of antibodies raised against the different Akt isoforms. As described in the methods section, I employed the Akt1 total antibody raised in sheep against the sequence RPHFPQFSYSASGTA corresponding to residues 466-480 of mouse Akt1/PKBα and the Akt2 total antibody raised against the sequence RYDSLDPLELDQRTH corresponding to residues 455-469 of the mouse Akt2/PKBβ, which were kindly shared by Professor Dario R Alessi; the Akt3 total antibody purchased from Millipore was raised in mouse against the sequence CSPTSQIDNIGEE corresponding to residues 119-131 of human Akt3/PKBγ; HEK 293T cell lysates overexpressing GST-tagged versions of the different Akt isoforms were analyzed by western blot and immunoprecipitation assays (Figure 14). The selected antibodies were validated and will be next optimized in cortical neuronal extracts for the detection of the different endogenously expressed Akt isoforms.

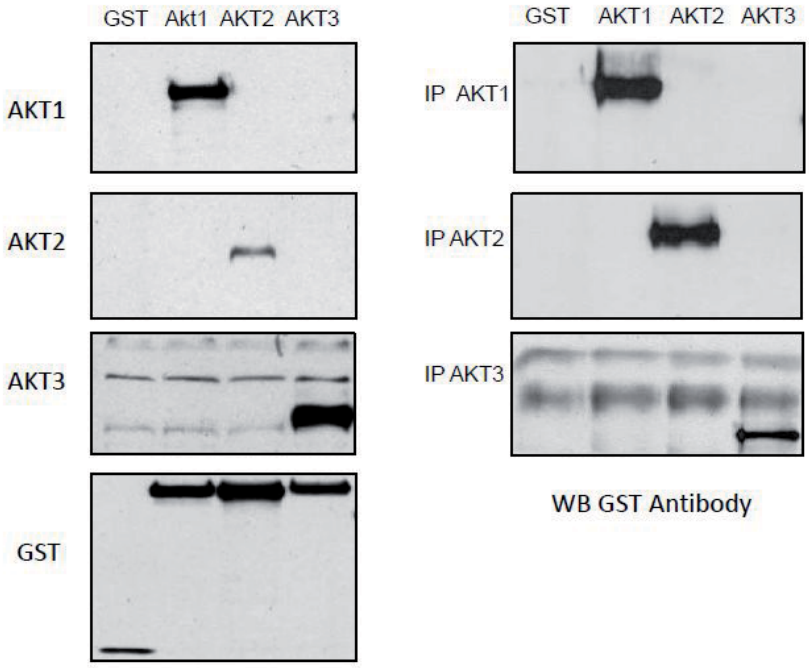

Figure 14. The specificity of Akt antibodies. Different Akt/PKB antibodies raised against isoform-specific peptides were tested on total protein extracts from HEK 293T cells over-expressing GST-fusion proteins for akt1, akt2 or akt3, as indicated, either by western blot with the designated Akt antibody (left) or after immunoprecipitation (IP) with the appropriate Akt antibody and western blot with the anti-GST antibody (right). As a control, lysates from cells expressing the GST empty vector were also included.

2. Generation of pLKO-GFP vectors.

pLKO.1-puro plasmids purchased from Sigma company were firstly amplified by transforming into DH5α cells and a MIDI Prep Kit was used to isolate the plasmids released from the *E.coli* cells. Then the quality of plasmids was confirmed by running DNA Gel electrophoresis (Figure 15A). After quantification of the plasmids by the Qubit® dsDNA Assay Kit, 10-15 ug PLKO.1-puro plasmids containing different shRNAs were double-digested with EcoRI and SpeI restriction enzymes by following the standard condition detailed in Methods part to separate the indicated 480bp fragment from the vector. Meanwhile, PLKO.1-puro-CMV-TurboGFP™ was double-digested with the same restriction enzymes to release the 1801bp fragment containing the TurboGFP™ tag (Figure 16), which was then retrieved by Gel purification Kit to perform the ligation reaction with the purified vectors. Before the

DNA ligation reactions, the quality and quantity of insert fragments and vectors need to be determined by DNA gel electrophoresis experiment (Figure 15B).

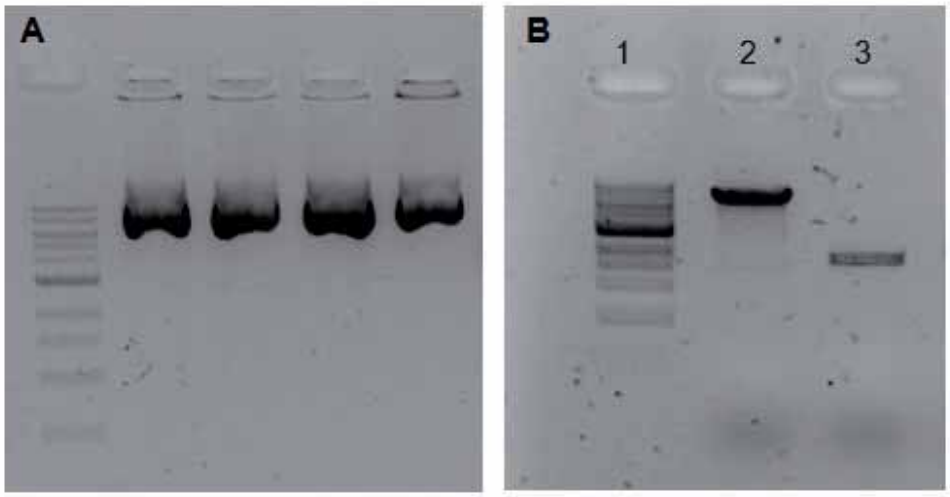

Figure 15. DNA preparation for cloning. Agarose gel electrophoresis of plamids and DNA fragments from the MIDI Prep Kit (A) and Gel Purification Kit (B), respectively. Lane 1: marker; Lane 2: vector; Lane 3: Insert fragment.

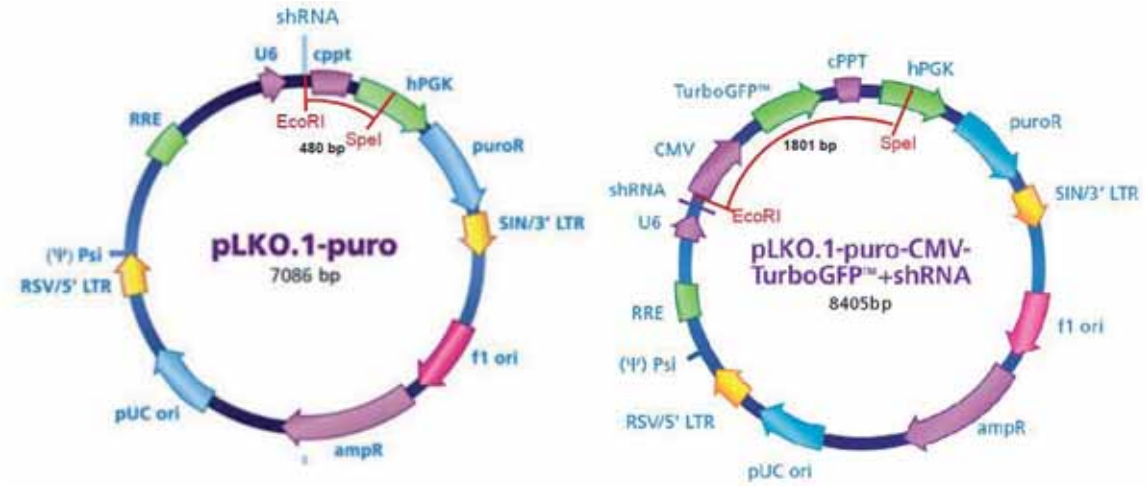

Figure 16. The diagram of pLKO,1-puro and pLKO,1-puro-TurboGFP™ structure and the strategy of plasmid construction. Upon EcoRI and SpeI double-digestion, the 1.8kb fragment from the second vector containing the GFP cassette plus cPPT sequence and half of the the hPGK prooiter is cloned onto the first vector lacking the 0.5Kb fragment containing the same cPPT sequence and half of the hPGK promoter.

After picking up the candidate clones from the bacterial transformation, the next step

was enzyme digestion and DNA sequencing to verify that constructed vector contains the TurboGFP insert and the corresponding shRNA. Then sequences alignment were performed and analyzed to distinguish the false positives. For example, here I constructed one mouse shRNA-Turbo-GFP plasmids for Akt1 isoform. Two candidate clones for shRNA-Turbo-GFP plasmids were chosen to sequencing. Then sequences of different candidate clones were analyzed by Chromos software (Figure 17).

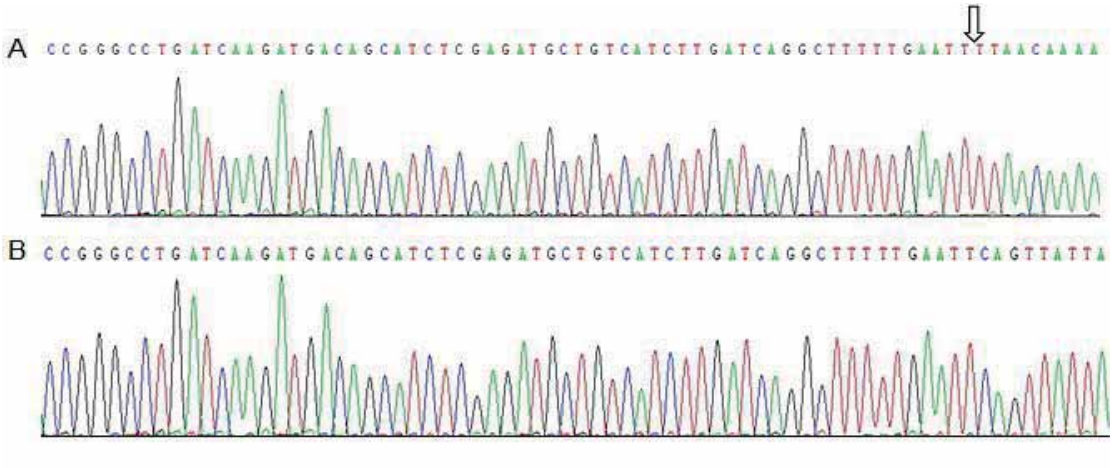

Figure 17. Sequence diagram of shRNA1 Akt1 candidate clones. Arrowhead indicates the nucleotide from which the two sequences diverge.

Next, sequences were aligned by online software (www.ebi.ac.uk/Tools) and compared with empty pLKO.1-turbo-GFP vector sequences to decide which one was the right clone (Figure 18). In the upper panel, sequence of clone A, which contains the shRNA1, is consistent with pLKO.1-turbo-GFP sequence. In the bottom panel, although clone B contains the correct shRNA, its sequence does not match with pLKO.1-turbo GFP sequence. From here, one can decide that clone A is the correct one. The enzyme digestion experiment was also performed to further confirm this conclusion.

Figure 18. Sequence alignment between pLKO.1-turbo-GFP plasmid (C) and candidate clones (A and B). The gap in the upper panel contains the sequence of Akt1 shRNA (CCGGGCCTGATCA AGATGACAGCATCTCGAGATGCTGTCATCTTGATCAGGCTTTTTG).

Finally 11 pLKO.1-puro-GFP plasmids containing different mouse Akt isoform-specific shRNAs were constructed. Among them, 4 plasmids contained mouse Akt1 -specific and Akt3-specific shRNAs, respectively. The last 3 plasmids contained Akt2-specific shRNAs.

3. Production of lentiviral particles and optimization of the infection conditions for cortical cells.

After plasmid construction, the next procedure was to test whether the turbo-GFP vectors generated express the green fluorescent protein (GFP) in HEK-293 cells. PLKO.1-puro-GFP plasmids containing different isoform-specific Akt shRNA were transfected into the HEK-293T cells following the standard protocol. Fluorescence intensity was observed under the microscope (Figure 19) and all the constructed

plasmids were confirmed to express intensively the GFP protein in HEK 293T cells. Then these plasmids were subsequently propagated in HEK 293T cells to produce the corresponding lentiviral particles as depicted in the method section.

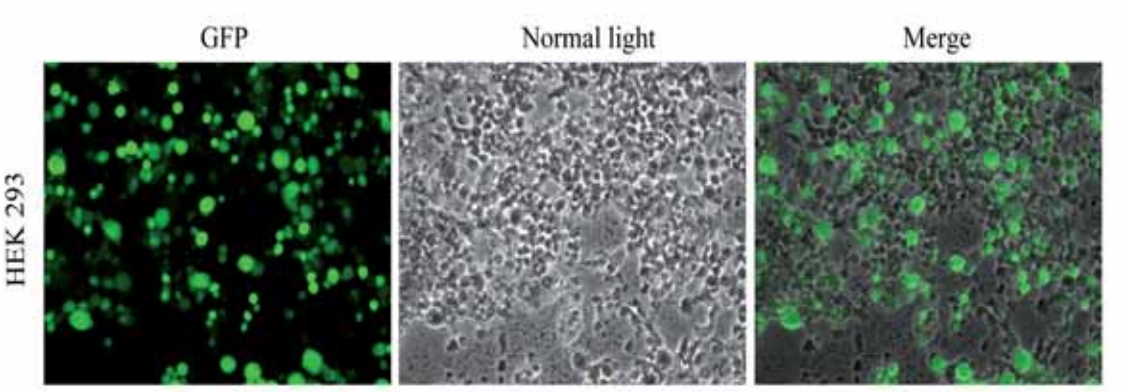

Figure 19. Representative micrographs of HEK-293 cells transfected with a pLKO.1-puro-GFP plasmids containing Akt isoform-specific shRNA.

Before using lentiviral particles to infect the cortical cells, the ability of the different lentiviral stocks to specifically interfere with the expression of the corresponding Akt isoform was assessed on cell lines by western blot with the specific Akt antibodies which were previously optimized. In order to achieve that, mice neuronal cell lines were needed, since the shRNAs were designed to target the different mouse Akt messengers, and the neurons one of the few cell types expressing all the three different Akt isoforms. The Neuro-2A mouse neuroblastoma (N2A) cell line was chosen to perform this experiment, and the expression of the different Akt isoforms corroborated. N2A cells were then infected with the lentiviral particles containing different Akt shRNAs, where the infection efficiency almost reached 80%. The interference ability and specificity of corresponding lentiviruses were evaluated and compared among different shRNA plasmids by western blot (Figure 20). In the upper panel (Figure 20A), Akt1 #2 and #3 specific shRNAs had better ability than #1 and #4 shRNA to interfere and silence the Akt1 protein expression in N2A cells, whereas Akt3 expression remained unaffected. Meanwhile, Akt3 #3 and #4 specific shRNA had higher ability than #1 and #2 to interfere with the Akt3, but not Akt1, protein expression. In the bottom panel (Figure 20B), Akt2 #2 and #3 shRNAs had higher

ability than #1 to interfere the Akt2 expression in N2A cells, but they did not compromised the expression of Akt1. After comparing the interference ability among different Akt isoform-specific shRNAs, the #2 and #3 shRNAs for Akt1 isoform, #2 and #3 shRNAs for Akt2 isoform, #3 and #4 shRNAs for Akt3 isoform were preliminary selected to further evaluate their efficiency.

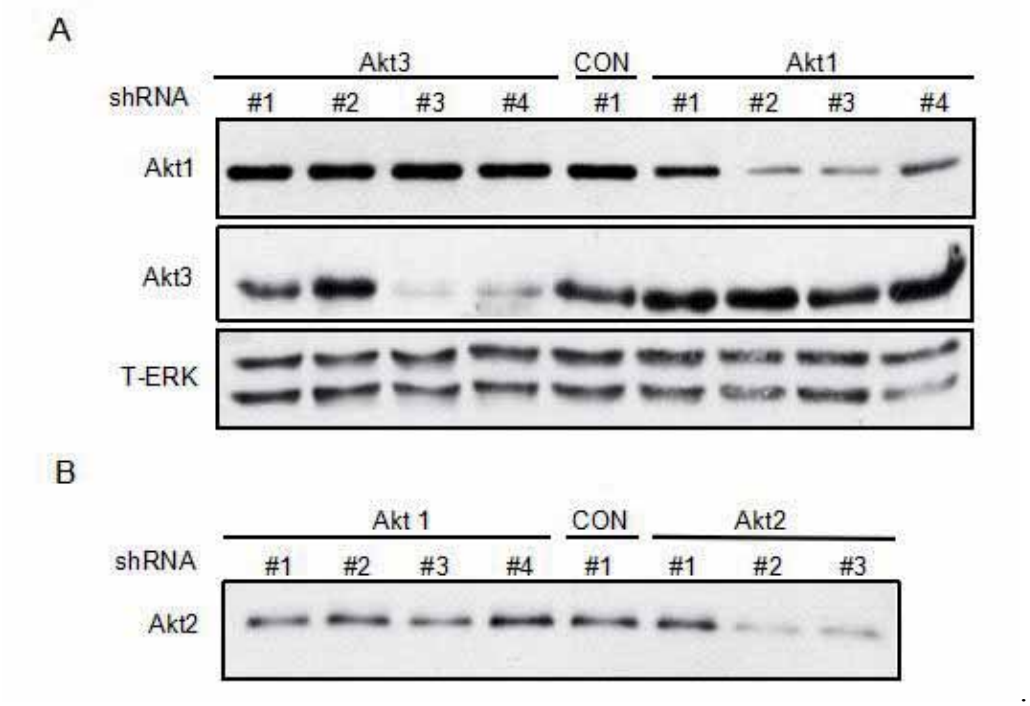

Figure 20. The ability and efficiency of the different lentiviral vectors were tested by western blot with isoform-specific Akt antibodies on protein extracts of Neuro-2A mouse neuroblastoma cells infected with the indicated lentiviral constructs. A representative Western blot out of three independent experiment is shown.

To further confirm the efficiency of the Akt shRNAs chosen, the concentration of lentiviruses was diluted gradiently to search for the optimized conditions of infection. N2A cells were thus infected with those optimized doses of lentiviruses, which yielded as much as 90% efficiency. The interference ability and specificity of the corresponding lentiviruses were evaluated by western blot as described before (Figure 21). I found that after optimization the selected Akt3 shRNAs almost totally silenced

the Akt3 protein expression levels and have full specificity to target the Akt3, but not Akt1 and Akt2 messengers. The interference efficiency of the selected Akt1 shRNAs can reach 80% efficacy to down-regulated the expression of Akt1, especially #2 shRNAs almost reach 82% reduction in Akt1 protein levels. Meanwhile the expression of Akt2 and Akt3 was not affected by Akt1 shRNA. Compare to Akt1 and Akt3 shRNAs, the interference efficiency of Akt2 shRNAs was relative lower but still reached approximately 71% reduction. The specificity of Akt2 shRNAs was also high against Akt1 and Akt3 shRNAs. After quantification and analysis of the relative intensity of the different detected western blot signals (Figure 22), I finally chose #2 shRNA for Akt1, #2 shRNA for Akt2, and #3 shRNA for Akt3 to evaluate the roles of the different Akt isoforms on neuronal survival in cortical cells.

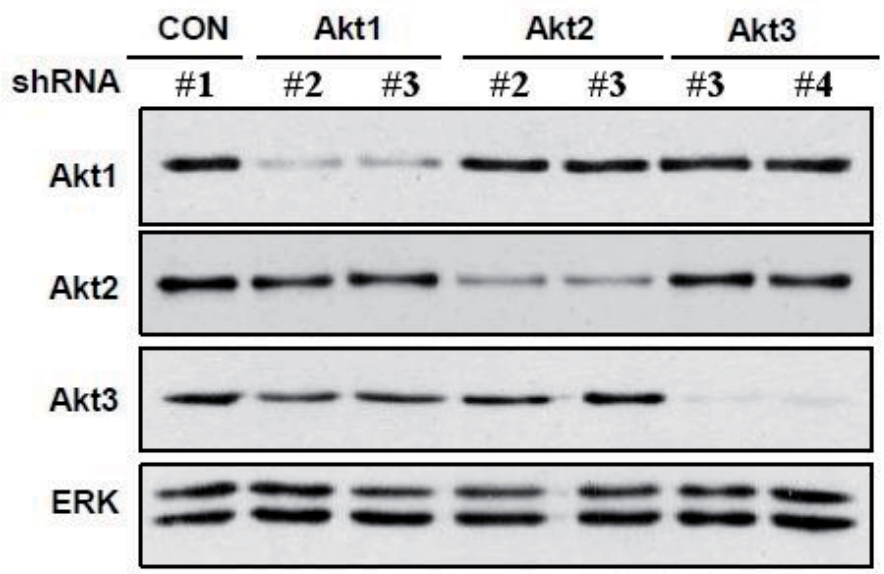

Figure 21. Protein extracts from Neuro-2A mouse neuroblastoma cells infected with optimized doses of the selected lentiviral vectors were immunobloted with the indicated isoform-specific antibodies. A representative Western blot out of three independent experiment is shown.

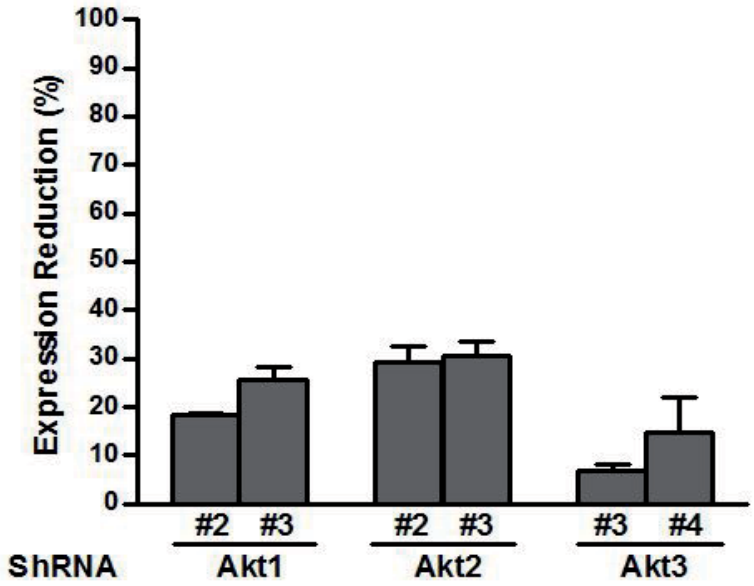

Figure 22. Expression reduction of each Akt isoform induced by different shRNAs was determined by quantifying the signal intensity of the corresponding bands. Values are expressed as as a percentage of control cells, and are presented as the means ± standard errors of the means for protein extracts derived from three independent experiment.

Since I established the optimal conditions to specifically detect the protein expression of each individual Akt isoform, as well as interfere with their expression by selected shRNAs, I took advantage of these excellent tools to investigate the contribution of single-specific Akt isoforms to neuronal survival in cortical cells. Before carrying out this experiment, suitable conditions for infecting the cortical cells were needed to be tested. Cortical cells were thereby infected with the corresponding concentration of pLKO.1-puro-GFP lentiviral particles at 1 DIV and 3 DIV. After three days of infection, the efficiency of infection was estimated by measuring the intensity of the fluorescenct GFP reporter signal under the microscope. The infection efficiency was found higher at 3 DIV compared with 1 DIV, which can reach as much as 35%, whereas the status of the cortical neurons infected at 3 DIV was more differentiated and healthier in morphology than that of the neurons infected at 1 DIV. Considering all these results together, I decided to infect the cortical cells at 3 DIV in the next step (Figure 23).

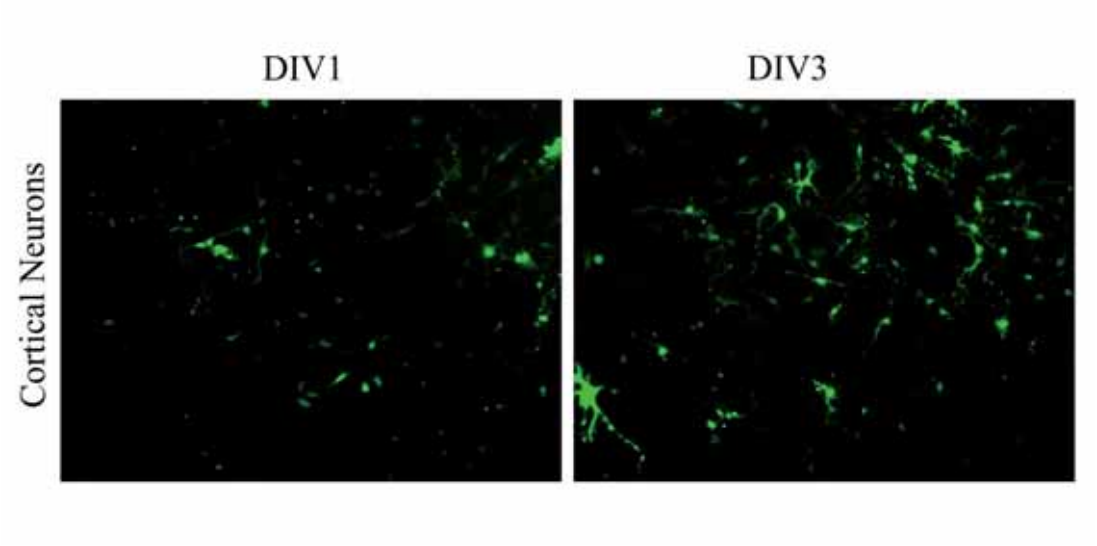

Figure 23. Representative photographs of cortical cells infected with lentiviral particles containing the pLKO.1-puro-GFP vector at 1 DIV and 3 DIV. Images were take at 4DIV and 6 DIV, respectively.

In order to quantify the apoptosis of cortical cells, the Hoechst 33342 nuclear die was used to stain the cells. Apoptosis could be then quantified at each condition by scoring the percentage of apoptotic cells in the adherent cell population which can express the green fluorescent protein. The conditions of staining were achieved after following the standard protocol (Figure 24).

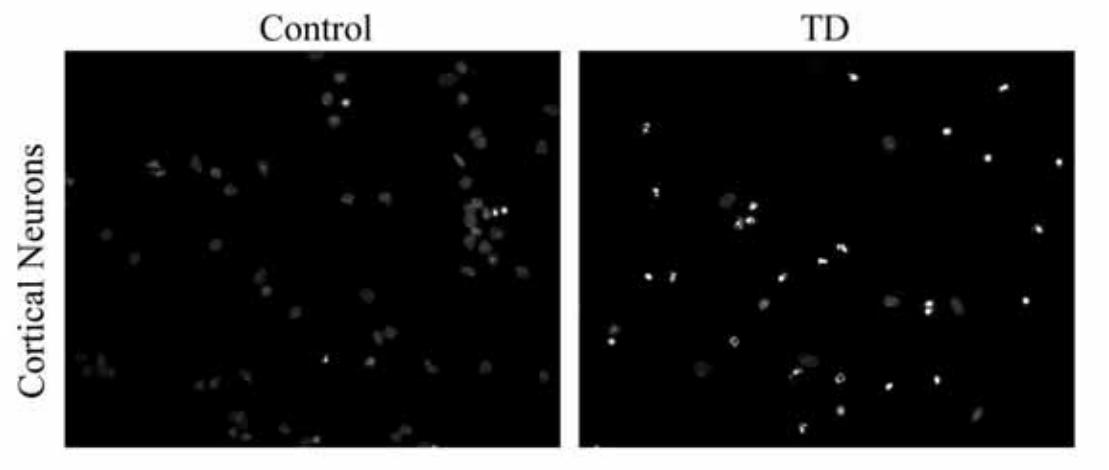

Figure 24. Representative photographs of cortical neurons stained with the Hoechst 33342 nuclear die after there days of lentiviral infection. Before staining, cortical cells were cultured with completely medium (Control) or upon Trophic Deprivation (TD) for 24 hrs.

4. Activation of Akt3 by phosphorylation at the T305 site was reduced by the PDK1 K464E mutation.

We recently published the impact that the PDK1 K465E mutation had on the development of the central nervous system (Zurashvili et al., 2013). Disrupting the interaction of PDK1 with PIP3 results in mice with reduced Akt activity due to reduced phosphorylation of Akt1 at Thr308, the PDK1 site, which was however not rate limiting for the phosphorylation of those Akt substrates promoting cell survival, such as FOXO1 and GSK3. Accordingly, the integrity of the PDK1 domain was not essential to support the survival of neuronal cells.

This observation was particular important, since Akt has been widely accepted for many years as the most critical factor promoting neuronal survival, and therefore challenges the notion of Akt being a good pharmacological target to treat neurodegeneration. We hypothesize that the importance of Akt was overestimated due to lack of good genetic tools and other PDK1-regulated kinases might also contribute to the neuroprotective functions of this pathway. However, it might as well be possible that the marginal activation of Akt reached in the PDK1 K465E mice was sufficient to support the survival of neurons. In the regard, inhibition of Akt1 and Akt2 with the Akt1/2i inhibitor did not compromise neuronal survival. Since in the past decade many studies have indicated that Akt1 plays a key role in the regulation of cell survival, this result was really surprising to us. Considering that Akt3 isoform successfully escapes the inhibition of the Akt1/2i compund, although it shares highly similar amino acid sequence and structure with Akt1 and Akt2, and that Akt1 and Akt2 activities were shown to be equally affected by the PDK1 K465E mutation, it became crucial to define whether the third isoform, Akt3, which is particularly enriched in neurons, was escaping the impact of the PDK1 K465E mutation in the similar manner that it escapes the pharmacological inhibition, and whether Akt3 is responsible for neuronal survival in the PDK1 K465E mice. In order to study whether Akt3 activity was also reduced, like Akt1 and Akt2, in the PDK1 K465E mice, I carried out immunoprecipitation experiments to pull down the Ak1, Akt2 and Akt3

isoforms one by one with corresponding antibodies, then proteins released from beads were immunobloted with the indicated antibodies (Figure 25).

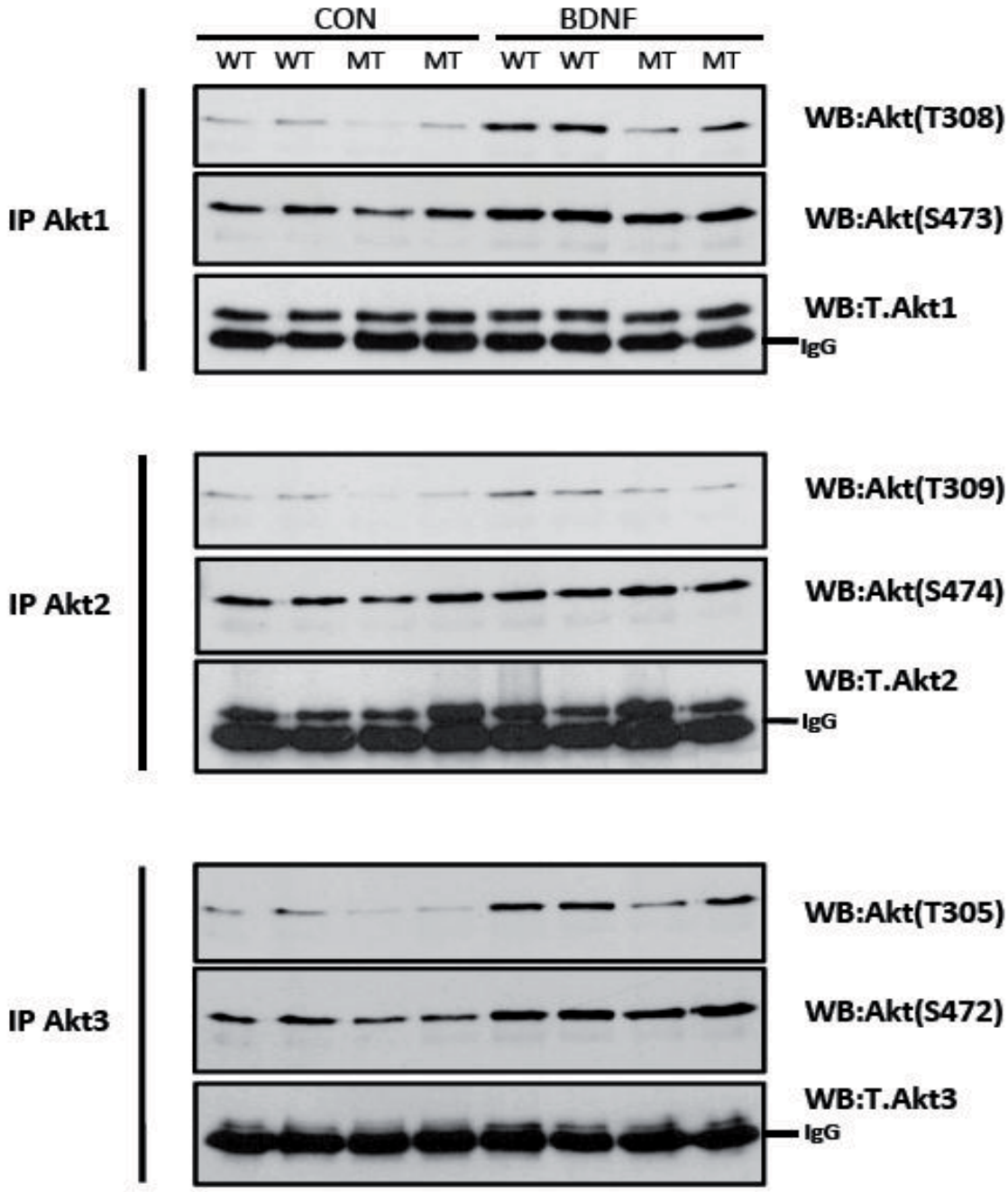

Figure 25. Deficient Akt3 activation in the PDK1$^{K465E/K465E}$ cortical neurons. PDK1$^{+/+}$ (WT) and PDK1$^{K465E/K465E}$ (MT) cortical neurons were cultured for 6 DIV, then serum starved for 4 hours and then either left unstimulated or stimulated with 50 ng/ml BDNF for 5 min. Immunoprecipitation experiment was performed to pull down Akt1, Akt2 and Akt3 isoform with appropriate antibodies one by one. Then the beads containing the targeted Akt isoforms were eluted and the released proteins immunoblotted with the indicated phospho and total antibodies. A representative Western blot out of three independent experiment is shown, where each lane represents a sample derived from a different embryo.

The results of the immunoprecipitation experiment show that the phosphorylation of all three Akt isoforms including the Akt3 one was also reduced at this site in the PDKI$^{K465E/K465E}$ mice. The phosphorylation of Akt2 (51.14 %) at Thr309 site was reduced more than that of Akt1 at T308 (45.98%) and Akt3 at T305 (26.96%) in the PDK1$^{K465E/K465E}$mice (Fig 26C). Furthermore, the proportion of each Akt isoform was also analyzed by quantifying the signal intensity of the Ser472/3/4 site. The data shows that Akt1 is the highest one (46.4%) and Akt2 is the lowest one (13.45 %), the proportion of Akt3 is 40.15 % but still less than Akt1 (Fig 26B). Here we also calculated the total activity reduction(39.1 %) of three Akt isoforms (Akt 1/2/3) at the PDK1 site by comparing the signal intensity in wild type and mutant mice, which is coincidence with the data from our former studies(Fig 26A).

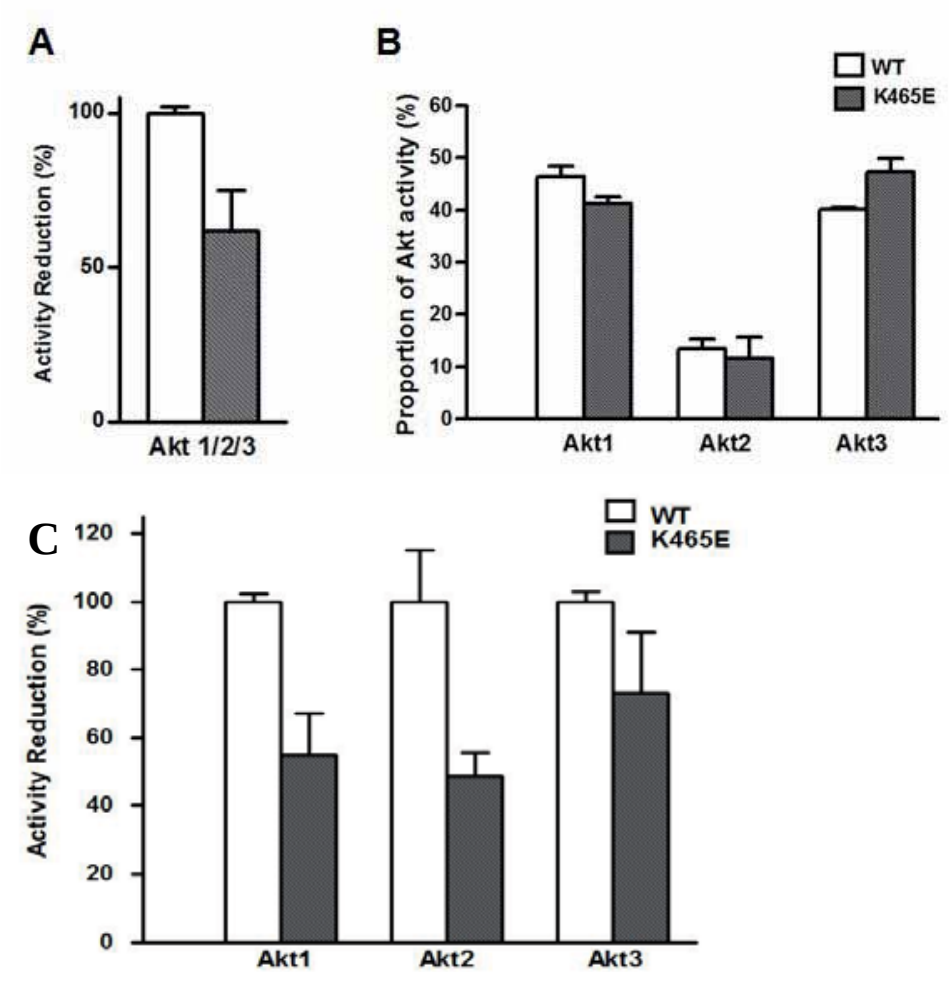

Figure 26. The effect of the PDK1 K465E mutation on the phosphorylation of Akt at the PDK1 site. (A) The activity reduction of total Akt phosphorylation at the PDK1 site was determined by quantifying the signal intensity of corresponding bands on total lysates. (B) The proportion of each Akt isoform and (C) the activity reduction of each Akt isoform at the T305/8/9 site were determined by quantifying the signal intensity of the corresponding bands after immunoprecipitation. Values are expressed as as a percentage of control cells (A and C), or as a percentage of total activity (B) and are presented as the means ± Standard errors of the means for protein extracts derived from at least three independent experiments.

In order to get more convinced evidence regarding this conclusion, the specificity of the immunoprecipitation was also evaluated by Western blot. The results showed that the Akt1 protein was only detected after immunoprecipitation with Akt1 antibody. In the pull-downs obtained with Akt2 and Akt3 antibodies, Akt1 was not detected by western blot. Meanwhile, Akt1 was not detected in the supernatants in which Akt1 has already been depleted by immunoprecipitation with the Akt1 antibody. All this data means that the phosphorylation signal of Akt1 detected from the immunoprecipitation experiment was highly specific and correspond to the totality of the Akt1 protein in the extract. The specificity of Akt2 and Akt3 was also evaluated by following the above described procedures and the results further confirm that the immunoprecipitation was specific and fully effective (Figure 27 and 28).

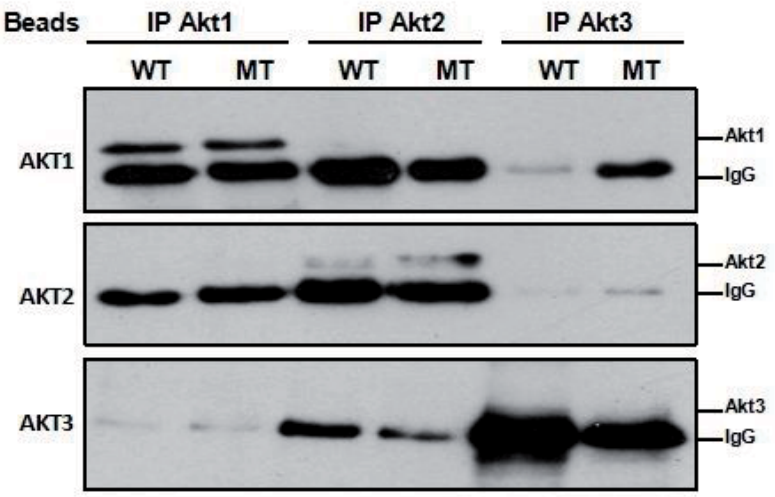

Figure 27. Evaluation of the specificity of the Pull Down assay. The purified beads were eluted to retrieve immunoprecipitated proteins. After centrifugation, supernatant were collected and analyzed by Western blot with the indicated antibodies. IP Akt1, beads incubated with the Akt1 antibody; IP Akt2, beads incubated with the Akt2 antibody; IP Akt3, beads incubated with the Akt3 antibody.

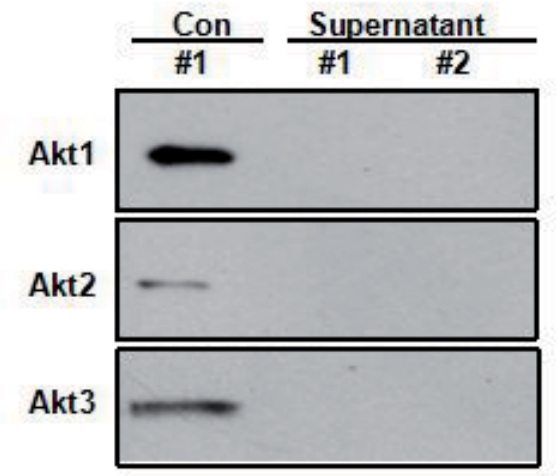

Figure 28. The indicated Akt antibody completely pulled down the corresponding Akt isoforms. After the three-step pull down assay, supernatants were kept and loaded to check whether any residual Akt protein still remained in the supernatants. Con, lysates without pull-down assay; Supernatant, lysates with IP treatment (Incubated with Akt1, Akt2 and Akt3 antibodies sequentially).

5. Akt1 and Akt3 play crucial roles in regulating the neuronal survival

The results showed that activation of Akt1 and Akt3, as judged by the phosphorylation at the Thr308/305 site, was almost four-fold higher than phosphorylation of Akt2 at the Thr309 site both in PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice. Considering that cell viability was not compromised by the PDK1 K465E mutation, it is reasonable to propose that the remained 59 % Akt activity measured in the PDK1$^{K465E/K465E}$ mice was enough to maintain the neuronal survival, and that Akt1 and Akt3 are most likely playing crucial roles in neuronal viability in the PDK1 K465E mice. In order to proof this hypothesis and to dissect the contribution of single-specific Akt isoforms to neuronal survival, primary cultures of cortical neurons derived from the PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice embryos at E 15.5 were infected with the lentiviral particles producing Akt isoform-specific shRNAs at 3 DIV. After 72 hours, infected cortical

neurons were then cultured in serum-free medium in the absence or presence of BDNF for 24 h, or left untreated. Neuronal survival was determined by scoring the percentage of apoptotic nuclei after Hoechst staining. Apoptotic morphological characteristics such as chromatin condensation and nuclear fragmentation were increased in cortical neurons deprived from serum, which were partially reverted by BDNF treatment. We first analyzed the survival of wild type cortical neurons infected with lentiviral particles without shRNA. Results shows that 18.38 % of apoptotic cells were observed in controls in which neurons were cultured with complete media and 49.65% of cell death was induced by serum deprivation in the absence of BDNF. The percentage of apoptotis decreased to 29.62 % when cortical neurons were serum deprived in the presence of 50 ng/ml BDNF. No different ratios were observed in the $PDK1^{K465E/K465E}$ mice when neurons were infected by lentiviral particles with GFP (Figure 30). This finding further confirm the conclusion from Tina Zurashvili's thesis that the PDK1 interaction to phosphoinositides is likely not essential for the neuroprotective actions of BDNF.

Next the percentage of dead cells was measured in cortical neurons infected with lentiviruses expressing different Akt1, Akt2 or Akt3 shRNA. In PDK1 wild type cortical cells, no significant difference was detected in the survival responses of cultures infected with different Akt shRNA, with the exception of Akt1 infection, which can slightly increase the percentage of apoptotic cells but no significant different in statistics (Figure 29A, C and E). In the $PDK1^{K465E/K465E}$ cells, both Akt1 (Figure 31) and Akt3 (Figure 32) significantly reduced the neuronal survival. When neurons from $PDK1^{K465E/K465E}$ mice were cultured in complete medium, Akt1 and Akt3 down-regulation increased the percentage of apoptotic cells to 27.76 % and 30.76 % (Figure 29B), respectively whereas Akt2 did not significantly increase the incidence of apoptotic cell dead. When neurons were deprived from serum in the presence of BDNF, both Akt1 and Akt3 down-regulation consistently leaded to an increase in the percentage of apoptosis, in which Akt1 had more profound effects on cell viability than Akt3 (Figure 29 B and F).

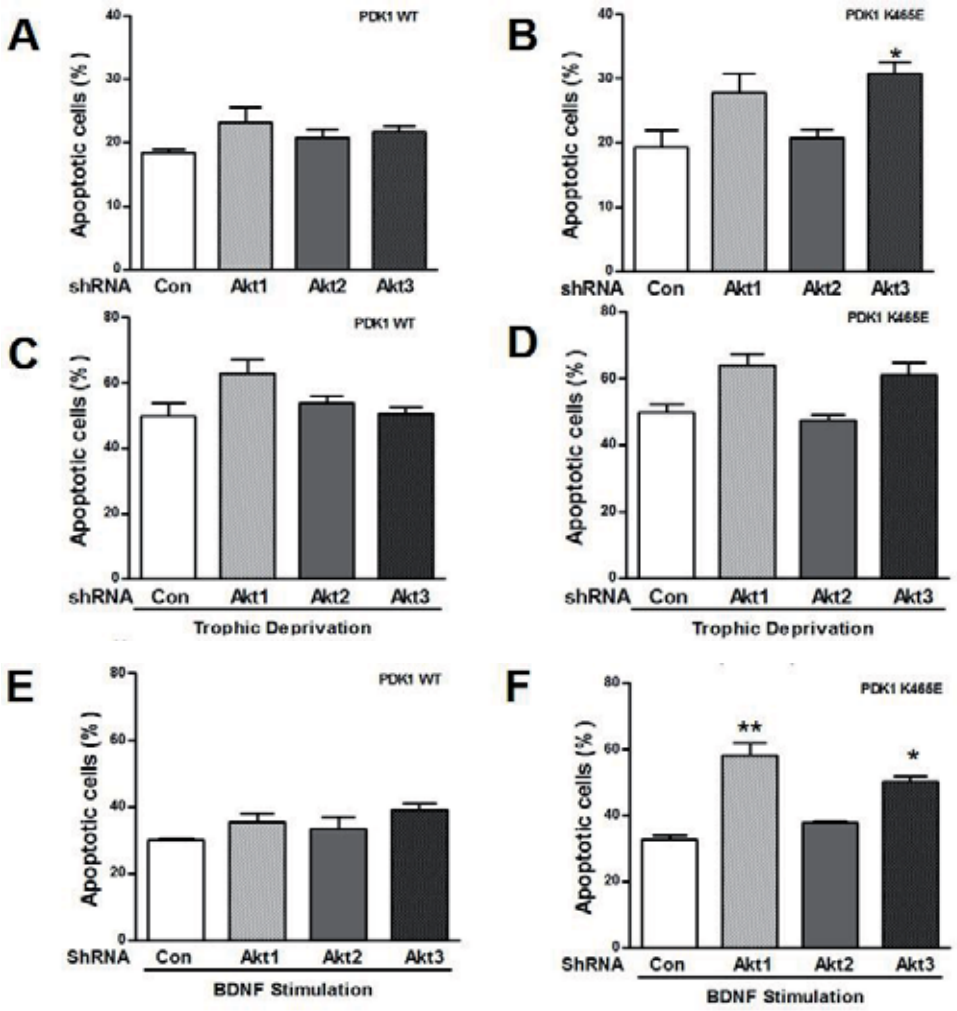

Figure 29. PDK1 wild type and K465E cortical cells were culture for three days in vitro (DIV) and then transduced with the indicated shRNAs. At DIV6, cortical cells were either sham treated (CONTROL) or trophic deprived for 24 hours in the absence (TD) or presence (TD+BDNF) of 50 ng/ml BDNF, and finally fixed and stained with Hoescht. The percentage of apoptotic cells on the GFP-expressing cell population was obtained by scoring the number of nuclei exhibiting chromatin fragmentation divided by the total. Values are expressed as percentage of apoptosis and are presented as the means ± standard errors of the means for 250-300 neurons from at least five independent mouse embryos per genotype from three independent litters for each conditon potint. *,$P< 0.05$ ** $P<0.01$ as determined by Student's t test.

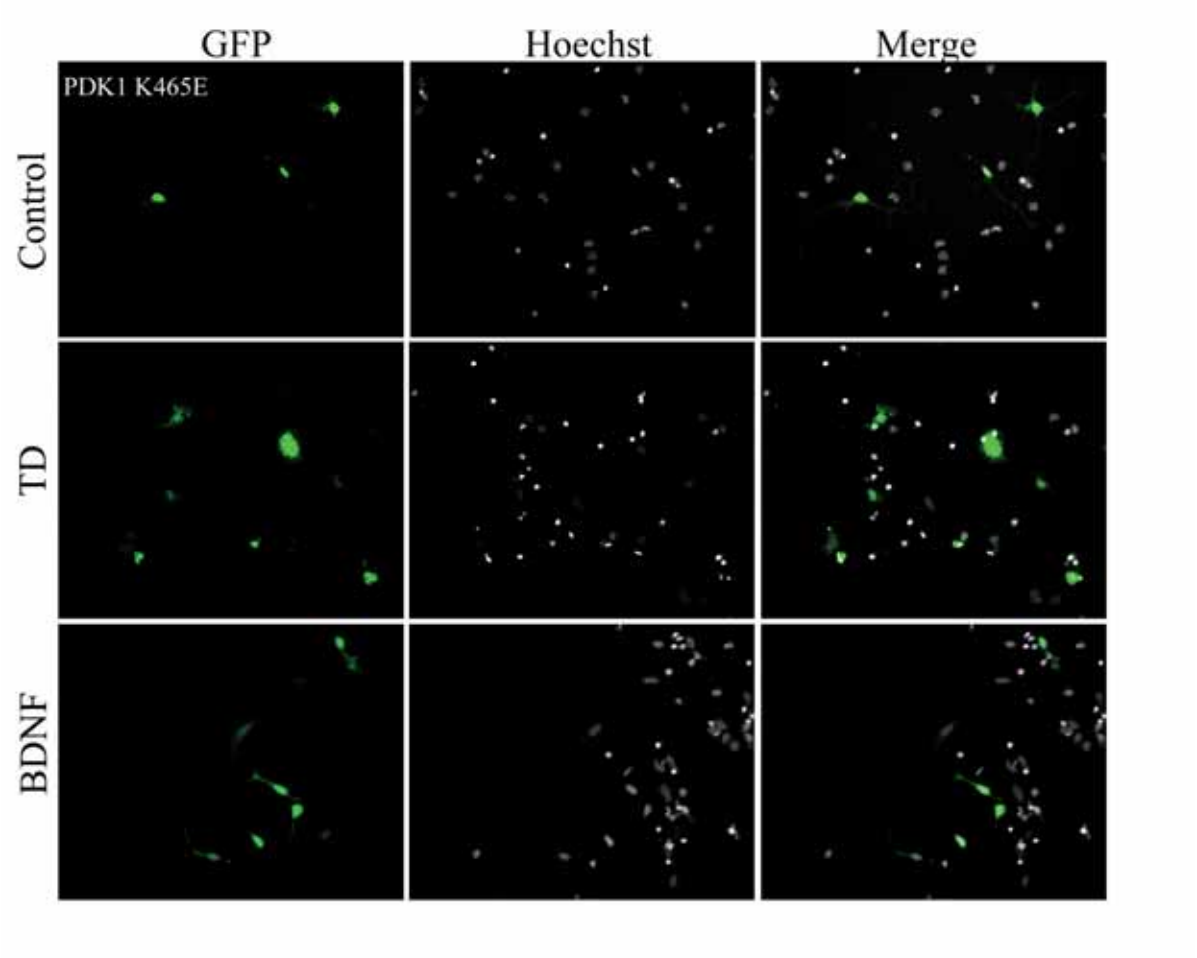

Figure 30. Representative photographs of cortical neurons infected at DIV3 with the shGFP lentiviruses and then cultured at DIV6 in complete medium (Control) or deprived of serum for 24 h in the absence (TD) or presence (BDNF) of 50 ng/ml of BNDF. Cells were then stained with Hoechst for 10 min. The GFP fluorescence, Hoechst staining and merged signals are shown as indicated.

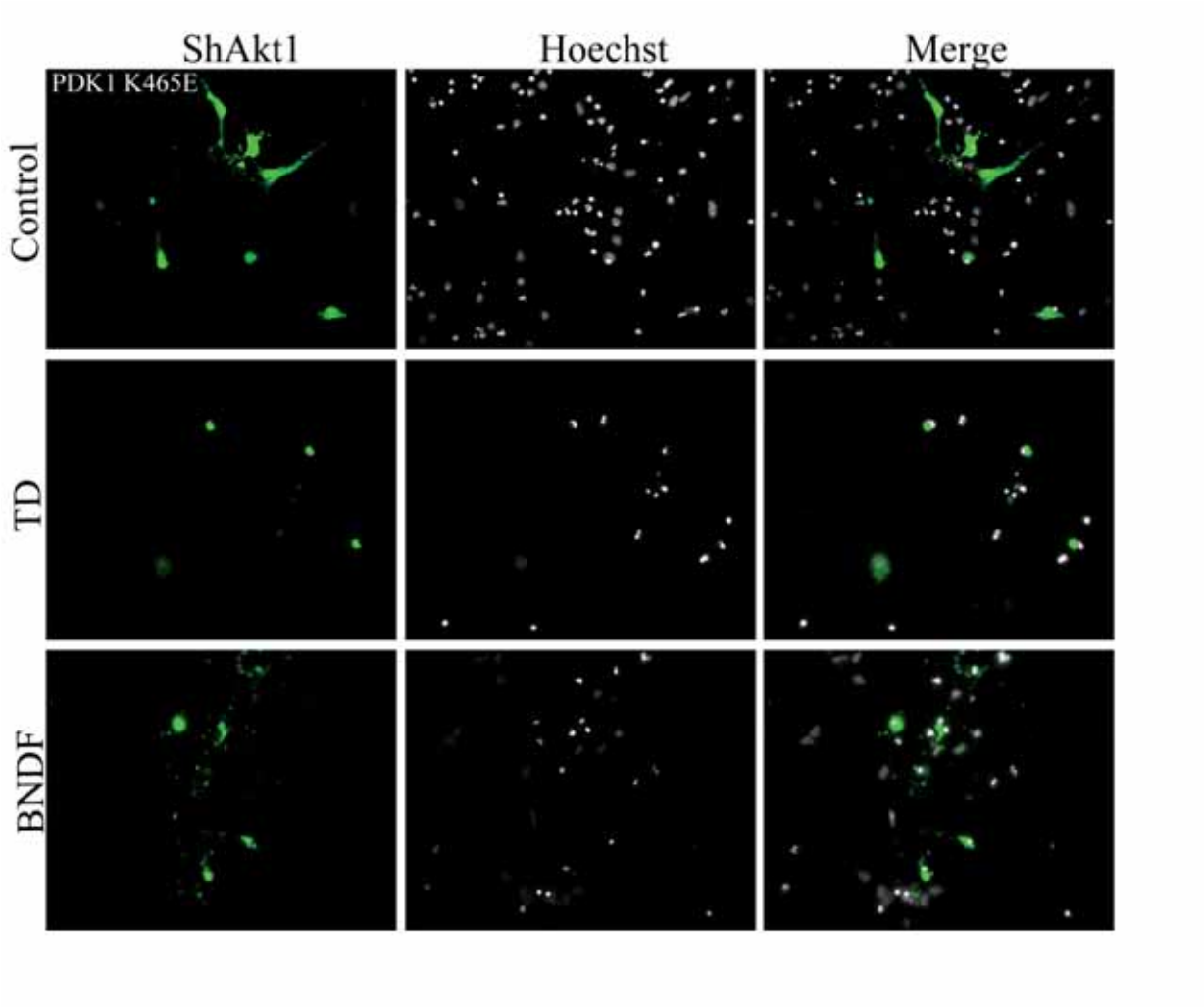

Figure 31. Representative photographs of cortical neurons infected at DIV3 with the shAKT1 lentiviruses. and then cultured at DIV 6 in complete medium (Control) or deprived of serum for 24 h in the absence (TD) or presence (BDNF) of 50 ng/ml of BNDF. Cells were then stained with Hoechst for 10 min. The GFP fluorescence, Hoechst staining and merged signals are shown as indicated.

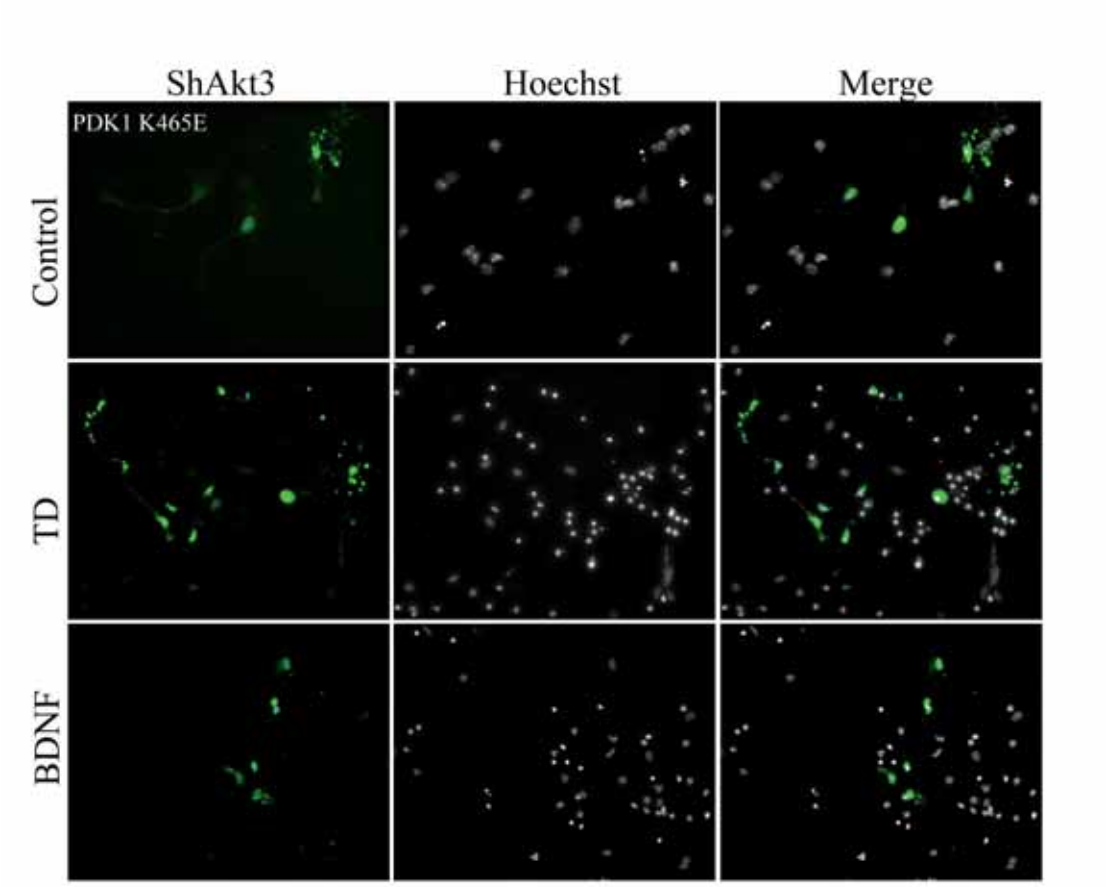

Figure 32. Representative photographs of cortical neurons infected at DIV3 with the shAKT3 and then cultured at 6DIV in complete medium (Control) or deprived of serum for 24 h in the absence (TD) or presence (BDNF) of 50 ng/ml of BNDF F. Cells were then stained with Hoechst for 10 min. The GFP fluorescence, Hoechst staining and merged signals are shown as indicated.

6. An evolutionary innovation in the mechanism of Akt activation that enabled diverse physiological responses.

In the PDK1$^{K465E/K465E}$ mice, PDK1 cannot interact with phosphoinositides, but we found that Akt could surprisingly be activated, albeit to a lesser extent when compared to control mice. Najafov and co-workers recently proposed that the reduced activation of Akt that was still observed in the PDK1$^{K465E/K465E}$ cells was dependent upon the

binding of PDK1 to the hydrophobic motif of Akt when phosphorylated at Ser473 (Najagov et al 2012). Therefore, in the absence of PtdIns(3,4,5)P_3 binding, PDK1 may still take advantage of the docking site mechanism to activate Akt, albeit less efficiently.

It is widely believed that the PI3K/Akt signaling axis plays essential roles in regulating neuronal survival and differentiation. Unexpectedly, we found that the low levels of Akt activation achieved in the PDK1$^{K465E/K465E}$ neurons were sufficient to support cell survival. By contrast, the differentiation capacity of primary cortical and hippocampal neuronal cultures was markedly affected by the PDK1 K465E mutation.

Since the activation of Akt in the PDK1$^{K465E/K465E}$ tissues most likely arises from the ability of the PDK1 PIF pocket to recognize and bind to the Akt hydrophobic motif when phosphorylated at Ser473, it is proposed that the cellular responses elicited by the low levels of Akt activity uncovered by the PDK1 K465E mutation might reflect the ancient functions of this signaling branch (Fig. 33). In basal conditions, or under stimuli that does not induce strong activation of the PI 3-kinase signaling pathway, Akt can still become phosphorylated to some extent at Ser473 within the hydrophobic motif, creating a substrate binding site for PDK1 that will gain access in this manner for phosphorylation of the Thr308 residue within the Akt activation loop. This might represent the ancestral mechanism by which the first Akt-like orthologues lacking PH domains were activated. In the PDK1$^{K465E/K465E}$ mice, the PIF pocket dependent activation of Akt was proved to be sufficient to guarantee essential functions such as cell survival and proliferation thought the phosphorylation of certain cellular substrates such as GSK3 or FOXO1. Mutual binding of both PDK1 and PKB to PtdIns(3,4,5)P_3 dramatically increases the rate of Akt activation, exceeding in this way a threshold of signal that enables efficient phosphorylation of other cellular substrates, namely PRAS40 or TSC2, controlling particular physiological responses such as neuronal morphogenesis (Zurashvili et al 2013) or T-cell migration (Waught et al 2009). Therefore, the phosphoinositide-dependent activation should be considered an evolutionary innovation that enabled the acquisition of novel functions.

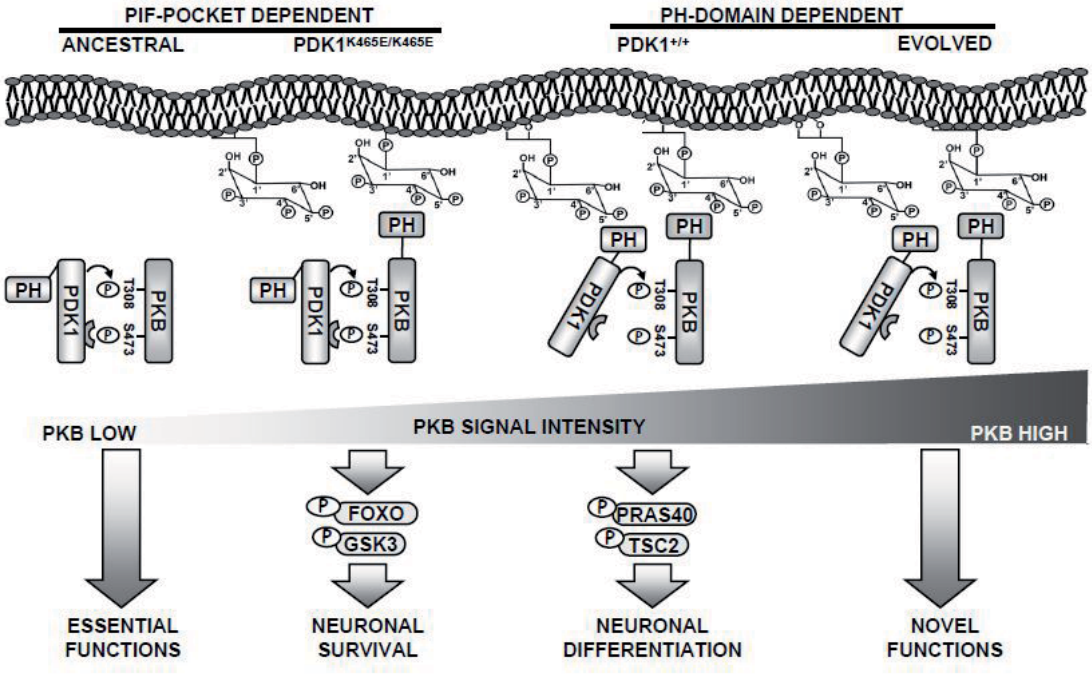

Figure 33. A model for the evolution of the PKB/Akt activating mechanisms. In ancestral eukaryotes lacking PH domain-containing PKB/Akt-like orthologues, interaction of PDK1 with PKB/Akt relied on the PIF-pocket mechanism. This promoted limited PKB/Akt activation that was responsible for essential functions such as supporting cell survival, a situation that is reproduced in the PDK1$^{K465E/K465E}$ knock-in mice neurons. At the amoebozoans-metazoans origin, the acquisition of a PH domain allowed PKB/Akt to co-localize with PDK1 in response to PtdIns(3,4,5)P3 raises. This enabled activating PKB/Akt with high efficiency, which allowed the recruitment of additional cellular substrates regulating novel functions, such as complex neuronal morphogenesis.

In order to test this model, I explored whether pharmacological inhibition of Akt Ser 473 phosphorylation affected the biochemical and cellular responses of the PDK1 K465E knock-in mice neurons. Treatment of cortical primary cultures with the AZD8055 mTORC2 inhibitor reduced the BDNF-induced phosphorylation of Akt at Ser473 to basal levels in both PDK1 wild type and PDK1$^{K465E/K465E}$ cortical neurons in a dose-dependent manner, while totally inhibited the phosphorylation of Akt at Thr308 in the PDK1$^{K465E/K465E}$ mutant neurons at doses that did not affect Akt Thr308 phosphorylation in the control cells. The differential inhibition of Akt resulted in impaired phosphorylation of the Akt substrates PRAS40 and TSC2, as well as GSK3 and FOXO, in the mutant cells (Fig 34B). As a consequence, the BDNF-elicited

survival responses of the PDK1$^{K465E/K465E}$ cortical neurons were further decreased when compared to the control neurons (Fig. 34A).

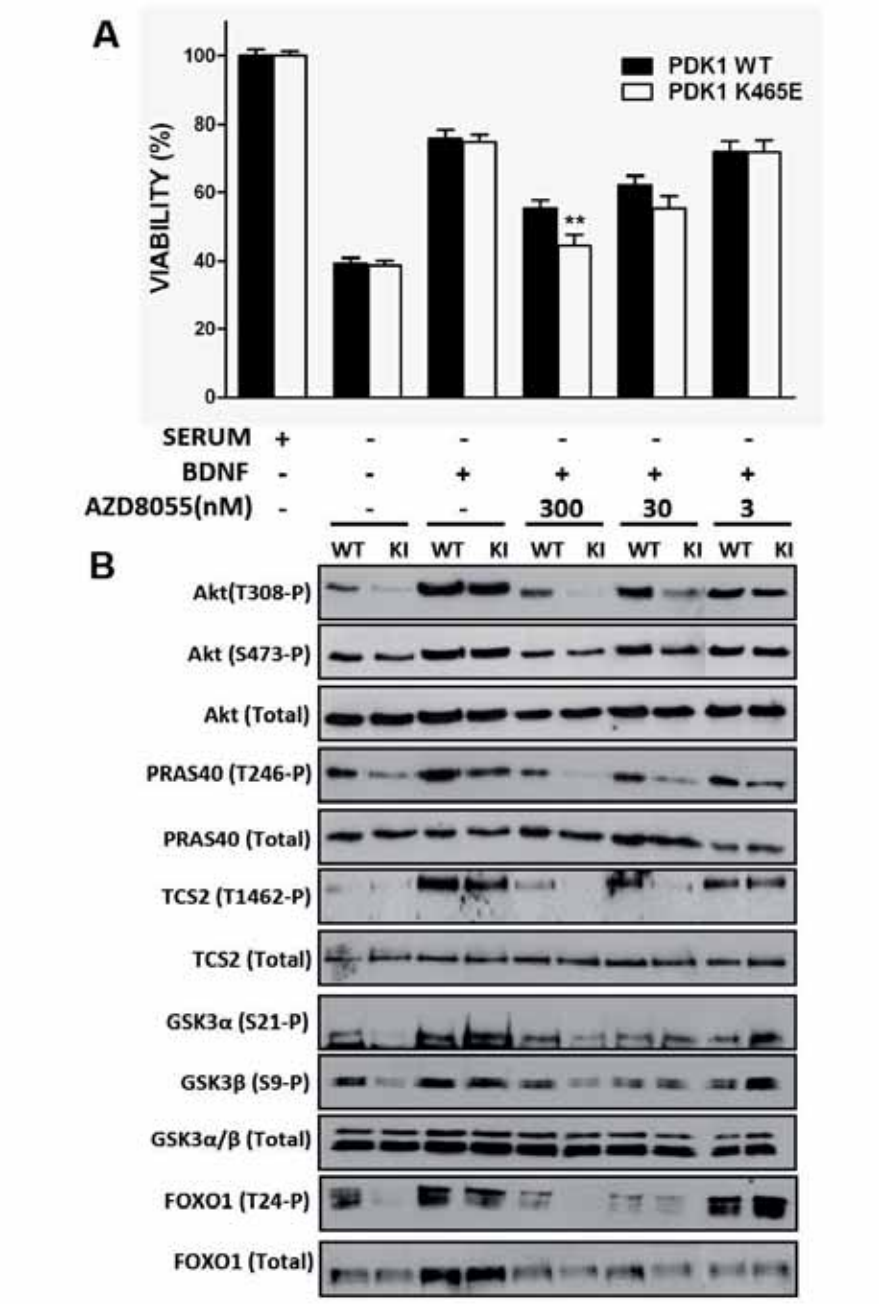

Figure 34. Primary cultures of cortical neurons derived from PDK1 wild type and PDK1 K465E E 15,5 embryo littermates were generated as described. After six days in culture, cells were incubated in complete medium or deprived of serum for 24 h in the absence or presence of 50 ng/ml of BDNF and the indicated concentrations of the AZD8055 inhibitor. Cell viability was determined by the MTT reduction assay and is represented as the mean ± standard errors of the mean from five independent embryos per genotype, with each sample assayed in quadruplicate. As a control for the different treatments, cell lysates from matched PDK1 wild type and PDK1 mutant littermate mice were immunoblotted with the indicated antibodies. **, $P < 0.01$ between genotypes as determined by the Student's t test.

DISCUSSION

The present study has dissected the contribution of single-specific Akt isoforms to neuronal survival by utilizing primary cultures of cortical neurons derived from the $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ mice embryos. I also analyzed the mechanisms leading to the reduced phosphorylation of each Akt isoform at the activation loop site observed in the $PDK1^{K465E/K465E}$ mice. By taking advantage of high quality research tools and optimized conditions that were established at the beginning, the results strongly suggest that both Akt1 and Akt3, but not Ak2, play crucial roles in regulating the survival of cortical neurons. In the past decade, although many studies have pointed out to the specific functions of each Akt isoform, my study demonstrated that the three Akt isoforms regulate the neuronal survival in a quantitative-dependent manner. Whatever Ak1, Akt2 or Akt3, once the reduction in total Akt activity meet the threshold for apoptosis, pro-apoptotic cascade reaction was trigered and cell viability was compromised. Here I didn't deny the specific functions that each Akt isoform may perform in other cellular processes such as neuronal cell cycle, neuronal differentiation, neuronal proliferation and migration, although the relative abundance of each particular Akt isoform in a particular cell type or tissue may also dictate its contribution to these specific functions. But regarding neuronal survival, the data presented strongly suggest that all the three Akt isoforms possess common functions and can compensate each other deficiency. Although silencing the expression of Akt2 didn't affect the neuronal survival in both PDK1 wild type and mutant mice, I propose that Akt2 might still be a regulator in cell viability and that the apparent lack of function in neuronal cells was just because Akt2 only accounted for marginal total Akt activity. Hence, when neuronal survival was threatened, all Akt isoforms were forced to maintain cell viability in a quantitative manner and exhibit no functional specificity. Once survival requirement was guaranteed, Akt isoforms were free to perform specific functions in regulating other cellular processes such as proliferation, differentiation, migration and cell cycle (Cheng YC *et al*., 2013; Yoo JK *et al*., 2014; Rahmani A *et al*., 2013).

1. Establishment of conditions for the specific detection of individual Akt isoforms.

In the first part of my project, I established the conditions for the specific detection of individual Akt isoforms. It was very important that the Akt isoform-specific antibodies were able to detect and immunoprecipitate only the isoform they should, and not the other two Akt isoforms. After this step, I established conditions for the detection and purification of individual Akt isoforms from cortical neurons protein extracts, and measured the contribution of each Akt isoform to the reduction in total Akt activity observed in the $PDK1^{K465E/K465E}$ mice. This experimental part also provided a useful tool for other members in our lab to further study the numerous functions of Akt isoforms, some of which we may still don't know yet.

2. Plasmids construction of pLKO.1-puro-GFP

In the second part, lots of time was devoted to the plasmid construction by inserting the GFP open reading frame fragment into the vectors containing different shRNAs. 12 PLKO.1-puro plasmids containing the corresponding isoform-specific shRNAs, but lacking the GFP marker were purchased. Finally, I successfully constructed 11 pLKO.1-puro-GFP plasmids which expressed the right shRNAs together with the GFP protein from the same biscistronic messenger, which provided me with very powerful tools to investigate the impact of individual Akt isoform expression silencing on cell viability. Meanwhile, performing this step rather than purchasing customized pLKO.1-puro-GFP plasmids to the supplier really saved lots of research funds.

3. Application of lentiviral particles to block the expression of target genes.

Lentivirus is primarily a research tool used to introduce a gene product into in vitro systems or animal models. Large-scale collaborative efforts are underway to use lentiviruses to block the expression of a specific gene using RNA interference technology in high-throughput formats. The expression of short-hairpin RNA (shRNA)

reduces the expression of a specific gene, thus allowing researchers to examine the necessity and effects of a given gene in a model system. These studies can be a precursor to the development of novel drugs which aim to block a gene-product to treat a particular disease.

Historically, cultured and primary neuronal cells have been extremely difficult to maintain and transfect. Media and serum sensitivity and cytotoxicity frequently result in low efficiency, reduced viability, and neurodegeneration in cultured primary neurons. In order to avoid and solve this problem, I used in my project lentiviruses to infect the cultured cortical neurons, which are very difficult to transfect by normal methods. For the production of lentiviral particles, three components need to be prepared, which included a lentiviral vector pLKO.1 containing the shRNA, a packaging vector psPAX2 and an envelope vector pMD2G. Compared with other transfection techniques, virus-based infection methods have many advantages which have been applied both in vitro and vivo. Lentiviral vectors have become a promising new tool for the establishment of transgenic animals and the manipulation of the mammalian genome. The relatively low transgenic efficiency of conventional microinjection-based methods has opened the door for alternative approaches, including lentiviral vectors. Lentiviral vectors are an appealing tool for transgenesis in part because of their ability to integrate into the genomic DNA with high efficiency, especially in cells that are not actively dividing. Lentiviral vector-mediated transgene expression can also be maintained for long periods of time. Recent studies have documented high efficiencies for lentiviral transgenesis, even in animal species and strains, such as NOD/scid and C57Bl/6 mouse strains, that are very difficult to manipulate using the standard transgenic techniques (Woods N. B. *et al*., 2003; K. P. Greenberg *et al*., 2003). These advantages of the lentiviral vector system have broadened its use as a gene therapy vector to additional applications that include transgenesis and knockdown functional genetics (Park F *et al*., 2007).

4. **Specificity and ability of lentiviral particles to interfere the expression of the corresponding Akt isoform**

The aim of the project was to evaluate the contribution of individual Akt isoforms to neuronal survival. In order to successfully and unequivocally reach this purpose, I must guarantee that the shRNAs used in the study were highly specific, which means that each Akt shRNAs can only target the corresponding Akt isoform, neither other Akt isoforms nor related proteins, especially the ones involved in cell viability. To reach this purpose, each shRNAs and other components were transfected into HEK-293T cells to produce the lentiviral particles, then their specificity was evaluated by Western blot. After analyzing the results, it was satisfying that the selected shRNA could reduce the expression of Akt1, Akt2 and Akt3 in Neuro-2A cells to 15%, 25% and 5%, respectively, and each selected shRNA possessed highly specificity to reach the above mentioned requirements.

Recently Francisco Wandosell laboratory has evidenced how the shRNA-mediated disruption of Akt2 and Akt3 protein levels significantly reduced cortical neurons viability (Diez et al., 2012). Although these researchers used the same tool, lentiviral particles, to interfere the expression of Akt isoforms, the specificity of Akt3 shRNA (TRCN0000054726) can be questioned and arguable, at least in the cell viability assay. From the figure they provided, it can be seen how Akt3 shRNA not only targets the Akt3 but also Akt2 isoforms. This means that when they evaluate the viability reduction induced by the Akt3 shRNAs, this is contributed by both Akt2 and Akt3 isoforms disruption. Besides the specificity, the interference ability of shRNAs is also very important. If the interference efficiency of shRNA is lower that 60%, the residual activity of the corresponding Akt isoform in neurons would be enough to maintain the survival. Thus, we will get the false negative results. Furthermore, lentiviral particles were also used to overexpress the active Akt isofoms. After lentiviral vectors were injected into the ischemic cortex, researchers found lentiviral-mediated overexpression of cAkt1 or cAkt3 can reduce neuronal death after in vitro and in vivo ischemia (Rong Xie *et al*., 2013).

5. Activation of Akt3 was preserved compared with Akt1 or Akt2 in the PDK1$^{K465E/K465E}$ mice.

Before I evaluated the activation of each Akt isoform by measuring the phosphorylation of the T-loop site, our former study indicated that neuronal survival was not compromised in the PDK1$^{K465E/K465E}$ mice, even after treatment with the Akti-1/2 inhibitor. Considering that the Akti-1/2 inhibitor can only target Akt1 and Akt2, but not Akt3 isoform, we had reason to believe that the Akt3 isoform can escape the impact of PDK1 K465E mutation in the same manner it escapes the pharmacological inhibition and therefore Akt3 might be responsible for the survival in the PDK1$^{K465E/K465E}$ mice. Our data indicate that the total activation of Akt, as judged by the phosphorylation at the activation loop site, was reduced by 41%, in which Akt2 and Akt1 were reduced by 52% and 45%, respectively. But surprisingly, activation of Akt3 was only 27% reduced, which is much less than Akt1 and Akt2 reduction. Because Akt3 activity was not reduced in the predicted way, and Akt3 is especially enriched in neurons, it can be concluded that most of the total Akt activity is contributed by Akt3 in the PDK1$^{K465E/K465E}$ mice, and that this remained Akt activity was enough to maintain the survival of cortical neurons. Besides Akt3, it was found that Akt1 is also expressed at high levels in cortical neurons, which implies that Akt1 might still play critical roles in neurons as it plays in other tissues. Since the activation of Akt2 was almost reduced to blank in the PDK1$^{K465E/K465E}$ mice, I don´t have much expect about the role it may play in regulating the neuronal survival. Phosphorylation of Akt1 and Akt3 at the Thr308 and Thr305 sites was almost 4-fold higher than that of Akt2 at Thr309 site. Actually, other members in the group previously had tried to measure the phosphorylation of Akt2 at the Thr309 site in the PDK1$^{K465E/K465E}$ mice and even no signal was detected by Western blot (Tinatin Zurashvili, personal communication). Many previous reports have shown that Akt3 has a more restricted distribution with high levels in the adult brain (Yang *et al*., 2003; Oliver Tschopp *et al*., 2004) and Akt1 is widely and highly expressed in most of tissues, whereas Akt2 is only expressed at higher levels in liver, muscle, fat and heart (Yang *et al*., 2005). The distribution and levels of individual Akt isoforms within the brain were also

investigated. Akt1 have higher level in the spinal cord, Akt2 is expressed at comparative moderate levels in cerebellum and hippocampus, whilst Akt3 is expressed in all examined regions but at higher levels in the cortex and the hippocampus. In the postnatal day 1 brains, Akt1 was accounting for 47% of the total protein, whereas the relative levels of Akt2 and Akt3 were 14 and 39%, respectively. But in the adult brains, Akt3 was the predominant isoform, representing about one-half of the total Akt protein, whereas Akt1 accounted for approximately 30% of the total and Akt2 made up the rest. (Rachael M.Easton *et al*.,2005).

In 2008, Jose Ramon Bayascas characterized the activation of Akt in the insulin-responsive tissues of the $PDK1^{K465E/K465E}$ mice. As expected, in control $PDK1^{+/+}$ mice, insulin induced a marked activation of Akt1 in skeletal muscle, heart, liver and adipose tissue. In the $PDK1^{K465E/K465E}$ mice, Akt1 was activated to a three to five fold lower level than in control mice at all time points analyzed. Akt1 activation was also more transient in the tissues of the $PDK1^{K465E/K465E}$ mice. The activity of the Akt2 isoform was also measured in liver and adipose tissue, which was found reduced to an extent similar to that of Akt1 in the $PDK1^{K465E/K465E}$ mice. By contrast, activation of Akt3 was only reduced by 20% in adipocytes, which express low levels of Akt3 (Bayascas, personal communication). At all time points, phosphorylation of Akt at the activating T loop residue phosphorylated by PDK1 was markedly reduced in the $PDK1^{K465E/K465E}$ tissues. Quantitative analysis of the immunoblots in skeletal muscle indicated that phosphorylation of PKB at Thr308 was reduced by 65% in $PDK1^{K465E/K465E}$ animals (Bayascas JR *et al.*, 2008). In my study, I characterized the activation of Akt in the cortex region of the brain in the $PDK1^{K465E/K465E}$ mice. As described in the insulin-responsive tissues, activation of Akt3 was reduced by only 27%. The $Akt3^{-/-}$ mice displayed normal glucose metabolism, but reduced brain size due to a significant reduction in both cell volume and number Easton *el al.*, 2005; Tschopp *et al*., 2005). Although brain size was also reduced in the $PDK1^{K465E/K465E}$ mice, only cell volume but not cell number was affected, which suggested that Akt3 activity plays important role in neuronal survival in the $PDK1^{K465E/K465E}$ mice.

6. The role of individual Akt isoforms in regulating the survival of cortical neurons.

Although activation of Akt was reduced to 59% in the PDK1$^{K465E/K465E}$ mice, cell number and cell viability were not affected by the PDK1 K465E mutation. These results really challenge the notion that Akt plays a key role in regulating the neuronal survival. Furthermore, even neuronal survival was not compromised after treatment with the Akti-1/2 inhibitor (Zurashvili et al 2013). Before evaluating the contribution of each Akt isoform to the total levels of Akt activation, as judged by phosphorylation at the activation loop, PDK1 site, some controversial was seed about this conclusion. Since activation of Akt3, which is richly expressed in brain, at Thr305 site was only 27% reduced in the PDK1$^{K465E/K465E}$ neurons, it seems explainable that cell viability was not affected and still Akt would play prominent roles in controlling such process. But this was only a proposal and it was not known whether Akt3 was responsible for neuronal survival in the PDK1$^{K465E/K465E}$ mice or whether other Akt isoforms, which were missed in previous investigation, also played important roles in the cell viability.

Then it was evaluated the contribution of each Akt isoform to neuronal survival both in the PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice. Compared with control shRNAs, apoptosis was not induced in wild type cortical neurons infected with the Akt2 or Akt3 shRNAs. Only Akt1 shRNA infection could slightly increase the number of apoptotic cells grown in complete media or deprived from serum in the absence or presence of BDNF, but the differences were not statistically significant when compared with controls. These findings were conflicted with conclusions from Francisco Wandosell laboratory, in which they claimed that Akt2 and Akt3 contributed to the neuronal survival, even Akt2 knockdown having more effects in cell viability (Diez *et al*.,2012). Considering that phosphorylation of Akt2 at Thr309 in the PDK1$^{K465E/K465E}$ cortical neurons was almost reduced to blank but neuronal viability was not compromised in the PDK1$^{K465E/K465E}$ mice, and that the number of apoptotic cells were not increased upon infection with highly specific Akt2 shRNAs, the contribution of single-specific Akt isoforms to cell viability in cortical neurons was in

the need of being re-evaluated. In parallel, I evaluated the neuronal survival in cortical cultures derived from the $PDK1^{K465E/K465E}$ mice. The results of Hoechst assay strongly suggested that both Akt1 and Akt3 isoforms play crucial roles in regulating the cell viability, since the infection with the Akt3 shRNA increased the percentage of apoptotic cells in all the different conditions tested, which was further increased upon Akt3 shRNA infection, whereas Akt2 viruses had no consequences on the survival of the mutant cells. The differential impact that the Akt1 and Akt3, the two more abundant isoforms in neuronal tissues, down-regulation had in the $PDK1^{+/+}$ and $PDK1^{K465E/KK465E}$ neurons strongly support a quantitative model of Akt function to explain the mechanism of neuronal survival regulation. In 2013, researchers from Stanford infected the ischemic cortex with lentiviral vectors expressing constitutively active Akt1 and Akt3 proteins, and the infarct sizes were evaluated. Akt1 and Akt3 proteins were degraded as early as 1 hour after stroke, whereas Akt2 proteins remained unchanged until 24 hours after treatment. Lentiviral-mediated over-expression of Akt1 and Akt3 reduced neuronal death after in vitro and in vivo ischemia. In this study the researchers claimed that Akt3 offers stronger protection than Akt1 by maintaining Akt levels and promoting mTOR activity (Xie R *et al*., 2013).

Actually in the last decade, the specific physiological functions of individual Akt isoforms have been intensively investigated. Mice lacking Akt1 have been characterized many years ago, which exhibited defects in fetal and postnatal growth and increased apoptosis. (Chen *et al*.,2001; Cho *et al*.,2001b). Surprisingly, $Akt2^{-/-}Akt3^{-/-}$ mice and even $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice developed normally and survived with minimal dysfunctions, despite a dramatic reduction of total Akt levels in all tissues. A single functional allele of Akt1 appears to be sufficient for successful embryonic development and postnatal survival. This is in sharp contrast to the previous described lethal phenotype of $Akt1^{-/-}Akt2^{-/-}$ mice and $Akt1^{-/-}$ $Akt3^{-/-}$mice (Bettina D *et al*., 2006). In 2013, Ekert PG group generated IL-3-dependent myeloid cell lines from mice lacing Akt1, Akt2 or Akt3. Akt1 deletion resulted in accelerated

apoptosis at low concentrations of IL-3. Expression of constitutively active Akt1 was sufficient to delay apoptosis in response to IL-3 withdrawal. Akt1 prolonged survival of Bim- or Bad-deficient cells, but not cells lacking Puma. This study showed a key role of Akt1 during IL-3 signaling in repressing p53-dependent apoptosis pathways (Green BD *et al* ., 2013). Furthermore, the apoptosis percentage in the heart of Akt $1^{+/-}$ $3^{+/-}$ mice was less than 5 %, whereas this number increased to 50% in the Akt$1^{-/-}3^{+/-}$ mice (Figure 35), which indicated that Akt1 gene is more essential than Akt3 for embryonic development and survival (Yang ZZ *et al*., 2005). All these data indicated that Akt1 is still the principal isoform regulating apoptosis.

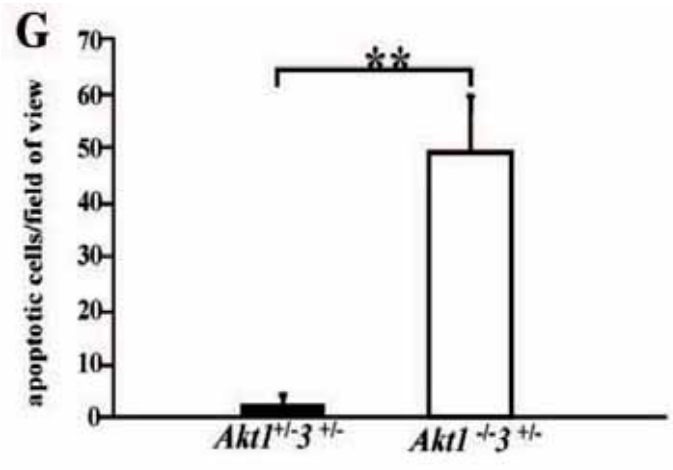

Figure 35. Quantification of apoptosis in the heart from Akt$1^{-/-}$Akt$3^{+/-}$ and Akt$1^{+/-}$Akt$3^{+/-}$ mice. Error bars indicate standard deviation. **. P<0.01. (Yang ZZ *et al*., 2005)

Unlike Akt1, which is widely expressed in all tissues, Akt3 is a tissue-specific expressed isoform. So investigation for specific functions of Akt3 was normally focused on the regions where it's rich in, such as brain and testis. In 2005, Akt$3^{-/-}$ mice was first characterized by reduced brain size which was caused by both cell volume and cell number reduction. Although Akt$1^{-/-}$ and Akt$3^{-/-}$ deficient brains were reduced in size to approximately the same degree, the absence of Akt1 leads to a reduction in cell number, whereas the lack of Akt3 results in smaller and fewer cells (Oliver T *et al*.,2005; Rachael ME *et al*., 2005). In Akt2 $^{-/-}$ mice, no reduction on brain weight and size was detected (Cho H *et al*., 2001a). This indicated that Akt3 plays a crucial role in the brain development. In this regard, induction of Akt3 is probably a key step in the activation of pathways for survival in the attempt to counteract motor neuronal

degeneration in amyotrophic lateral sclerosis (Peviani M *et al*., 2014).

Many examples are found in the literature supporting the notion of Akt1 and Akt3 controlling cell viability in cancer cells. In the neuroendocrine tumors (NETs) cells, knockdown of Akt isoform 1 and 3 suppressed NET cell viability and colony-forming capacity, in contrast to Akt2 knockdown. The inhibitory effect of simultaneous downregulation of Akt1 and Akt3 on tumor cell viability was significantly stronger than that caused by downregulation of all Akt isoforms, suggesting a particular role for Akt1 and Akt3 in NET tumourogenesis (Zitzmann K *et al*., 2012). In the single disseminated tumor cells (DTC), proliferation and survival of DTC was reduced by depletion of Akt3 and to a lesser extend by Akt1, but not after depletion of Akt2 (Grabinski N *et al*., 2011). Blocking Akt1 or Akt3, but not Akt2 expression, prohibits somatic cell proliferation and reprogramming (Tang Y *et al*.,2014).. Knockdown of Akt3 suppresses cell viability and induces the apoptosis of glioblastoma multiform T98G cells (Paul-Samojedny M *et al*.,2014).

As it was mentioned before, $Akt2^{-/-}$ mice displayed impaired ability of insulin to lower blood glucose levels, which was caused by defects in the action of the hormone on liver and skeletal muscle (Cho H *et al*., 2001a). Besides this, another study observed mild growth deficiency in the Akt2-/- mice and also exhibit age-dependent loss of adipose tissue (Garofalo RS *et al* ., 2003). These observations suggest that Akt2 is a main regulator in the maintenance of normal glucose homeostasis. Actually it's very difficult to define the specific functions of Akt2 isoforms, especially in the oncology. I have to say that there are so many studies which keep producing different conclusions at the time. Several studies indicate that Akt2 activation plays a critical role in cell migration (Zhang B *et al*., 2009; Bulj Z *et al*., 2013; Qiao J *et al* ., 2013; Cariaga-Martinez AE *et al*., 2013; Grabinski N *et al*., 2011), whilst other studies propose that Akt2 might play more important roles in regulating the survival of tumors than in neurons (Girouard J *et al*., 2013; Lee MW *et al*.,2011; Chin YR *et al*.,2014; Mure H *et al*., 2010) Even there is a study which claims Akt1 ablation significantly delays initiation of lung tumor growth, whereas Akt2 deficiency

dramatically accelerates tumourogenesis in this mouse model (Linnerth-Petrik NM *et al*., 2014). Ablation of Akt1 inhibits and ablation of Akt2 accelerates mammary tumor development, in which ablation of Akt1 promotes apoptosis, whereas the ablation of Akt2 inhibits apoptosis (Maroulakou IG *et al.*, 2008). Akt2, but not Akt1, prevents UV-induced cell death by inhibiting activation of JNK and p38 (Kim MA *et al.*, 2009). Akt2 deficient preadipocytes were significantly more sensitive to apoptosis induction by death receptor stimulation compared to Akt1 deficient cells (Fischer-Posovszky P *et al*.,2012). Akt1 and Akt2 are required for alpha/beta thymocyte survival and differentiation (Juntilla MM *et al*., 2007). Considering all these investigations, although each Akt isoform displayed numerous specific functions in different tissues and in different types of tumor cells or non-tumor cells, my results strongly suggested that Akt1 and Akt3 play critical role in regulating the neuronal survival, which also was supported by numerous previous investigations in the nervous system, and that the contribution of single specific Akt isoforms to cell viability might ultimately rely on the cell and tissue relative abundance of these proteins.

7. A novel mechanism in the activation of Akt was uncovered in the PDK1$^{K465E/K465E}$ mice

A former study in the lab indicated that binding of PDK1 to PtdIns(3,4,5)P_3 was not essential to support neuronal survival. At the time, it was really surprising that Akt could still be activated in the PDK1$^{K465E/K465E}$ mice. Recently, Najafov and co-workers proposed that the reduced activation of Akt that was still observed in the PDK1$^{K465E/K465E}$ cells was dependent upon the binding of PDK1 to the hydrophobic motif of Akt when phosphorylated at Ser473 (Najafov A *et al*.,2012). While characterizing the GSK2334470 PDK1 inhibitor, the authors found this compound more efficient at inhibiting S6K or SGK than at inhibiting Akt. Only under conditions that moderately activated the PI 3-kinase signaling, or in cells expressing mutant forms of either PDK1 or Akt incapable of PtdIns(3,4,5)P_3 binding, the inhibitor blocked Akt activation with the same efficiency than that observed for S6K or SGK

(Najafov A *et al*.,2011). Moreover, pharmacological inhibition of Akt Ser473 phosphorylation in several cancer cell lines, or genetic inactivation of different mTORC2 components in MEF cells, sensitized Akt to the PDK1 inhibitor. Also, the reduced activation levels of Akt observed in the PDK1$^{K465E/K465E}$ cells were totally blocked by mTOR inhibitors (Najafov A *et al.*,2012).

Therefore, in the absence of PtdIns(3,4,5)P_3 binding, PDK1 can still take advantage of the docking site mechanism to activate Akt, albeit less efficiently. This new uncovered ability of PDK1 to interact also with the phosphorylated Akt Ser473 hydrophobic motif indicates that the substrate docking site activation mechanism is shared by all the PDK1-regulated kinases and therefore should be considered as an ancestral mechanism of PDK1 regulation, which was conserved during eukaryotic evolution (Silber J *et al*.,2004). In contrast, the mutual co-localization of PDK1 and Akt at PtdIns(3,4,5)P3-rich surfaces might represent a functional innovation relying on the convergent acquisition of phosphoinositide binding domains by these two kinases, which in turn allowed faster and acute responses to particular second messengers.

In order to test this model, I explored whether pharmacological inhibition of Akt Ser 473 phosphorylation affected the biochemical and cellular responses of the PDK1$^{K465E/K465E}$ knock-in mice neurons. Treatment of cortical primary cultures with the AZD8055 mTOR inhibitor reduced the BDNF-induced phosphorylation of Akt at Ser473 to basal levels in both PDK1 wild type and PDK1$^{K465E/K465E}$ cortical neurons in a dose-dependent manner, while totally inhibited the phosphorylation of Akt at Thr308 in the PDK1$^{K465E/K465E}$ mutant neurons at doses that did not affect Akt Thr308 phosphorylation in the control cells. As a consequence, the BDNF-elicited survival responses of the PDK1$^{K465E/K465E}$ cortical neurons were further decreased when compared to the control neurons. We also observed that the AZD8055 inhibitor modestly compromised the viability of the PDK1 wild type neurons at doses that did not affected Akt activation. Since the mTORC1 specific inhibitor rapamycin has not effect on neuronal viability, and the contribution of other PI3K classes poorly targeted by the inhibitor to neuronal viability is highly unlikely, whilst the mTORC2 complex

acts also as the hydrophobic motif kinase for the SGK isoforms, these genotype-independent effects of the AZD8055 compound on cell viability point out to a role of SGK in promoting neuronal survival. Indeed, me and other members of the group have observed that in the PDK1 L155E mice expressing a mutant form of PDK1 incapable of activating SGK, a synergistic role of both Akt and SGK in controlling neuronal survival can be envisaged (Lluis Cordon-Barris and Jose R Bayascas, unpublished data). Altogether, these results further support the notion of low levels of Akt activity attained by means of the PIF-pocket mechanism being sufficient to guarantee essential functions such as neuronal viability, whereas more specialized functions necessitate the acute and rapid activation of Akt that is achieved upon phosphoinositide dependent co-localization with PDK1 in response to variations in the levels of the PtdIns(3,4,5)P3 second messenger.

CONCLUSIONS

V-CONCLUSION

1. The PKB/Akt activation in cortex was less reduced (39%) than in insulin responsive tissues (65%) in the PDK1$^{K465E/K465E}$ mice.

2. In the PDK1 wild type mice, the PKBα/Akt1 isoform accounted for 46.4% of the total Akt activity in cortical neurons, whereas the proportion of PKBγ/Akt3 is 40.1%. But in the PDK1$^{K465E/K465E}$ mice, PKBγ/Akt3 is the highest isoform, almost accounting for half of the total PKB/Akt activity.

3. The PKBβ/Akt2 only accounted for 13.4% activity of PKB/Akt in cortical neurons, which implies unimportant role in regulating the neuronal survival.

4. The PKBγ/Akt3 activity was preserved compared with PKBα/Akt1 or PKBβ/Akt2 in the PDK1$^{K465E/K465E}$ mice, only 27% activity reduction was detected fot Akt3 whereas Akt1 and Akt2 activities were reduced by 45% and 52%, respectively.

5. The number of apoptotic cells was not significantly increased by different PKB/Akt isoform shRNA in cortical neurons of the PDK1 wild type mice.

6. In the PDK1$^{K465E/K465E}$ mice, the percentage of apoptotic cells was increased by Akt1 and Akt3 shRNAs infection in cortical neurons, whereas Akt2 down-regulation had no significant effects on neuronal apoptosis.

7. I proposed a model that the neuronal survival was regulated by PKB/Akt isoforms in a quantitative manner.

8. All three PKB/Akt isoforms have the common functions in regulating the neuronal survival. Because of the high proportion of Akt phosphorylation at Thr 8/9 sites, Akt1 and Akt3 might play more important roles than Akt2 in neuronal survival.

9. The reason for neuronal survival being not compromised by the PDK1 K465E mutation or Akti 1/2 inhibitor is because residual PKB/Akt activity is enough to maintain the neuronal survival.

10. A novel mechanism in the activation of PKB/Akt was uncovered in the PDK1 $^{K465E/K465E}$ mice. In spite of the absence of PtdIns(3,4,5)P_3 binding, PDK1 can take advantage of the docking site mechanism to activate Akt.

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