AMERICAN UNIVERSITY OF BEIRUT

CHARACTERIZATION OF THE KALLIKREIN-KININ SYSTEM POST CHEMICAL AND TRAUMATIC BRAIN INJURY: AN *IN VIVO* AND *IN VITRO* BIOCHEMICAL APPROACH

by

AMALY MOHAMAD NOKKARI

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AN ABSTRACT OF THE THESIS OF

Amaly Mohamad Nokkari for Master of Science
Major: Biochemistry and Molecular Genetics

Title: Characterization Of The Kallikrein-Kinin System Post Chemical And Traumatic Brain Injury: An *In Vivo* And *In Vitro* Biochemical Approach.

Background: Traumatic Brain Injury (TBI) is the result of a mechanical impact on the brain that can provoke mild to moderate to severe symptoms. It is acknowledged that TBI leads to apoptotic and necrotic cell death; however, the exact mechanism by which brain trauma leads to neuronal injury is not fully elucidated yet. Noteworthy, the Kallikrein Kinin System (KKS) represents the first inflammatory pathway activated following tissue injury. Thus, the KKS's contributing role represents a good target to assess in the area of TBI.

Aims: The aim of this study is to investigate the expression and the role of the KKS's main players: Bradykinin and its receptors, in mediating neuronal injury. This will be evaluated under TBI (*in vivo*) and chemical (*in vitro*) neurotoxicity paradigms. The neuronal cell line PC12 will be treated with the apoptotic drug Staurosporine (STS) to simulate the cell activity after TBI to a certain degree.

Methods: The effect of STS on the viability and proliferation of pre-treated PC12 cells was investigated using MTT, ROS and LDH assays. DAPI nuclear staining was performed to assess for apoptotic bodies. MitoPT JC-1 and Annexin-V staining were presented as a proof of apoptosis. Intracellular calcium release was evaluated by Fluo 4-AM staining. Immunofluorescence (IF) was also performed on KKS markers, notably bradykinin 1 and 2 receptors (B1R and B2R). Western blotting (WB) was performed on both apoptotic and KKS markers. Real-time PCR, IF and WB were conducted to study the effect of STS on the transcriptional, translational and cellular localization of KKS markers. The same methods were employed on primary neurocortical cells and post-CCI ipsilateral cortical tissues. Finally, proteomics was used to find relevant proteins associated to STS and KKS in PC12 cells.

Results: STS inhibited the proliferation of pre-treated PC12 cells in a time-dependent manner. DAPI, WB and IF confirmed that apoptosis is the mechanism that STS is inducing and that it is mainly the B2R that is being altered after exposure to experimental brain injury insult and STS chemical neurotoxicity. Interestingly, inhibition of the B2R was shown to prevent calcium release following STS treatment and was showing the highest transcriptional level 3h and 12h post chemical injury. Proteomics results confirmed the emergence of a "survival" capacity developed by the cell when treated with B2R inhibitor and STS.

Conclusion: Our data suggests that the B2R is a main player in the inflammatory pathway following TBI and its inhibition represents a potential therapeutic tool**.**

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ABBREVIATIONS

ACE	Angiotensin I-Converting Enzyme
AIF	Apoptosis Inducing Factor
Akt or PKB	Protein Kinase B
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ap-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor 1
araC	Cytosine Arabinoside
B1R	Bradykinin 1 Receptor
B2R	Bradykinin 2 Receptor
BBB	Blood Brain Barrier
BCl-2	B cell CLL/lymphoma 2
Bdk	Bradykinin
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CCI	Controlled Cortical Impact
cdc 42	Cell division cycle 42
CMP	Carboxypeptidase M
CNS	Central Nervous System
CO_2	Carbon Dioxide
CSF	Cerebro-Spinal Fluid
Ctrl	Control
DAG	Diacyl Glycerol
DAPI	4-6-Diamidino-2-Phenylindole
ddH_2O	Double distilled water
DISC	Death-Inducing signaling complex
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
Elk-1	ETS domain-containing protein 1
ERK	Extracellular Signal-Regulated Kinases
FADD	Fas-Associated Death Domain
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
GPCR	G Protein Coupled Receptor
H/R	Hypoxia/Reoxygenation
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HMWK	High Molecular Weight Kininogen
HO-1	Heme Oxygenase
HS	Horse Serum
IAP	Inhibitor of Apoptosis Protein
ICP	Intracranial Pressure
IF	Immunofluorescence
IP_3	Inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
KDa	Kilo Dalton
KKS	Kallikrein-Kinin System
KO	Knockout
KOH	Potassium Hydroxide
LDH	Lactate Dehydrogenase
LMWK	Low Molecular Weight Kininogen
MAPK	Mitogen-Activated Protein Kinase
MAPK	Mitogen-Associated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Maitotoxin
NAD	Nicotinamide adenine dinucleotide
NBT	Nitro-Blue Tetrazolium
NEP	Neutral Endopeptidase
NeuN	Neuronal Nuclei

NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Neuron Growth Factor
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NOX	NADPH-Oxidase
P/S	Penicillin/ Streptomycin
PARP	Poly ADP ribose polymerase
PBS	Phosphate Buffered Saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
rt-PCR	Real-time Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STS	Staurosporine
TBI	Traumatic Brain Injury
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
WB	Western Blot

“Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith.'”

Max Planck, Nobel Prize in Physics (1918)

CHAPTER I

INTRODUCTION

Prologue

Traumatic Brain Injury (TBI) is an invisible wound of war commonly referred to as the "war signature injury". The current conflicts in the Middle East as well as the growing acts of terrorism all over the world have resulted in an increased number of patients (militaries and civilians) with TBI. According to the department of defense (DOD) and the U.S. Department of Veterans Affairs (VA) it is estimated that 2.4 million people sustain TBI in the United States each year; among which, 52,000 persons die from their injuries, 275,000 people are hospitalized and 1.4 million people are treated and released from emergency departments. Moreover, the Centers for Disease Control and Prevention in the U.S. estimate that the direct and indirect medical costs of TBI totaled $76.3 billion each year. Although the prevalence of brain trauma in warzone is the highest, TBI is also seen in car accidents, sport injuries and falls, especially among the elderly population.

TBI is the result of a high mechanical impact on the brain that leads to physical and cognitive disabilities. It is known that TBI provokes apoptotic and necrotic cell death through the activation of the protease system, namely the calpain/caspase system. However, the exact mechanism by which brain trauma can lead to neuronal injury has not been fully identified yet and is still being investigated on the molecular, genomic and proteomic levels.

Of interest, the Kallikrein-Kinin System (KKS) represents the first inflammatory pathway activated following tissue damage. Although the KKS's functions have been well characterized in the areas of diabetic nephropathy, diabetic

retinopathy and cardiovascular diseases, its role in brain injuries remains highly controversial. Indeed, many studies strongly believe that some of the KKS components represent good therapeutic targets in decreasing cellular injury following TBI, but the various experimental models used and the different time points examined lead to contradictory results. Therefore, this study aims at better understanding the mechanism of Bradykinin (Bdk)-mediated neuronal injury via an *in vitro* and an *in vivo* approach (Figure 1).

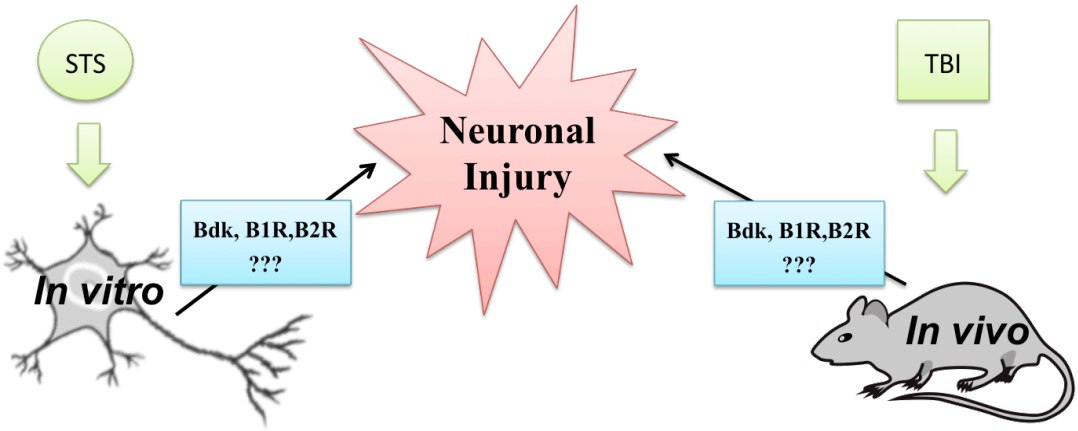

Figure 1: Identifying the KKS's putative role in the domain of neuronal injury. In the *in vitro* study neuronal cells will be subjected to the apoptotic drug STS, while in the *in vivo* study animals will be exposed to TBI. The role of the KKS players (Bdk, B1R, B2R) in inducing neuronal injury will then be assessed.

A. The Kallikrein-Kinin System

The KKS is a vasodilator system that plays essential roles in coagulation, vascular permeability, tissue inflammation and pain. Accordingly, disruption of the KKS was seen to be associated with diabetic nephropathy (Jaffa et al., 2012), diabetic retinopathy (Liu and Feener, 2013), cardiovascular diseases (Delemasure et al., 2013) and also neurological disorders (Guevara-Lora, 2012). In addition, KKS components present in the brain were shown to be up regulated upon brain injury (Leeb-Lundberg et

al., 2005). The major constituents of this system are the kallikrein enzymes which belong to the serine protease family and which role is to liberate kinins from the kininogens.

1. Kinins: Synthesis and Degradation

The KKS can be found under two forms in humans: the tissue KKS and the plasma KKS (Figure 2). The tissue KKS is mainly present in the kidney, the pancreas, the brain and the vascular system. Under normal conditions, the tissue kallikrein acts on the low molecular weight kininogen (LMWK) to liberate the decapeptide Kallidin (Abdallah et al., 2010)**.** In contrast, plasma kallikrein is synthesized by the liver and secreted into the blood as an inactive zymogen called prekallikrein. It is only upon injury and subsequent activation of the Hageman Factor (coagulation factor FXIIa) that the plasma prekallikrein is converted into plasma Kallikrein. The latter will then activate the high molecular weight kininogen (HMWK) that will release a nonapeptide called Bradykinin (Bdk) (Abdallah et al., 2010; Albert-Weissenberger et al., 2013). Bdk is a bioactive kinin peptide that is mainly found in blood and tissue. The sequence of this nine amino acids long peptide is as follows: Arg^{1}- Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9}. Bdk and Kallidin are made up of the same sequence of amino acids except that Kallidin has an additional Lysine amino acid at position 1: Lys^{1}-Arg^{2}- Pro^{3}-Pro^{4}-Gly^{5}-Phe^{6}-Ser^{7}-Pro^{8}-Phe^{9}-Arg^{10}.

Particularly, Bdk has a half-life that does not exceed thirty seconds (Kayashima et al., 2012). This is due to the fact that active kinins are rapidly broken down by kininases. Indeed, Angiotensin I-Converting Enzyme (ACE) inactivates kinins by removing the Phe^{8}-Arg^{9} dipeptide and then cleaving the Phe^{5}-Ser^{6} bond (Mark et

al.). Neutral endopeptidase (NEP) cleaves the Pro^{7}-Phe^{8} bond and can further hydrolyze the Gly^{4}-Phe^{5}, while carboxypeptidases M (membrane-associated) and N (tissue associated) remove the Arg^{9} from the C-terminus giving rise to an active product referred to as des-Arg^{9}-bradykinin or des-Arg^{10}-kallidin (Guevara-Lora, 2012).

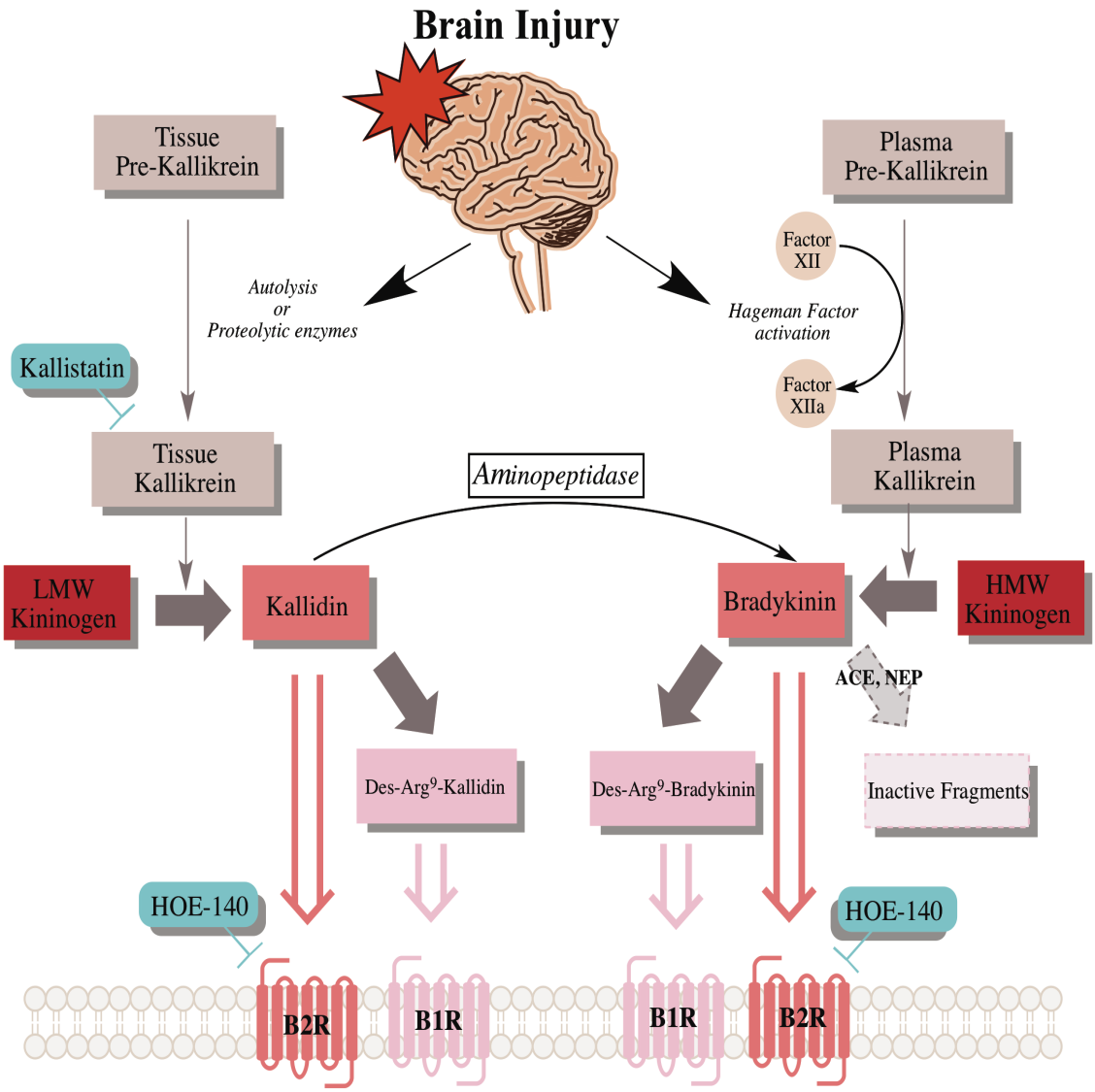

Figure 2: Tissue and plasma KKS activation pathway following brain injury. The tissue and the plasma kallikreins cleave the LMWK and HMWK, respectively, to give rise to the vasoactive kinins (kallidin and bradykinin), which are both agonists of the B2R. Kallidin and Bradykinin can be further broken down into B1R agonists.

2. Kininogens: Structure and Function

The LMWK and HMWK both belong to the cystatin superfamily and have identical N-terminus but differ at their C-terminus (Brown and Dziegielewska, 1997).

The mature HMWK is a single chain glycoprotein composed of 626 amino acids with a molecular weight of 120 KDa, and can be divided into 6 domains (Pathak et al., 2013). The first 3 domains compose the heavy chain of the HMWK while the last 2 domains make its light chain. Each domain has specific properties accounting for the versatile functions of the HMWK (Zhang et al., 2000):

- Domain D2: allows interaction with the endothelial cell receptor C1q
- Domain D3: allows interaction with platelets
- Domain D4: anchors Bdk
- Domain D5 (Histidine-rich domain): permits binding to anionic surfaces
- Domain D6: facilitates binding of the plasma kallikrein and Hageman factor

In contrast, the LMWK is a splice variant made of 409 amino acids with a molecular weight of 65 KDa. The heavy chain of the LMWK is composed of 3 domains identical to the ones composing the heavy chain of the HMWK, namely domains D1, D2 and D3. However, the light chain of the LMWK consists of only one domain, domain D5. Therefore, the main difference between the HMWK and LMWK is the lack of the D6 domain at the C-terminus of the LWMK (Colman and Schmaier, 1997).

The main role of kininogens is the production of the active kinins: Bdk and Kallidin (Figure 3). These oligopeptides have similar chemistry as mentioned earlier, and share physiological properties. The actions of kinins include vasodilatation, increased vascular permeability, increase in blood flow and plasma leakage, association with inflammatory and pain signaling (Walker et al., 1995).

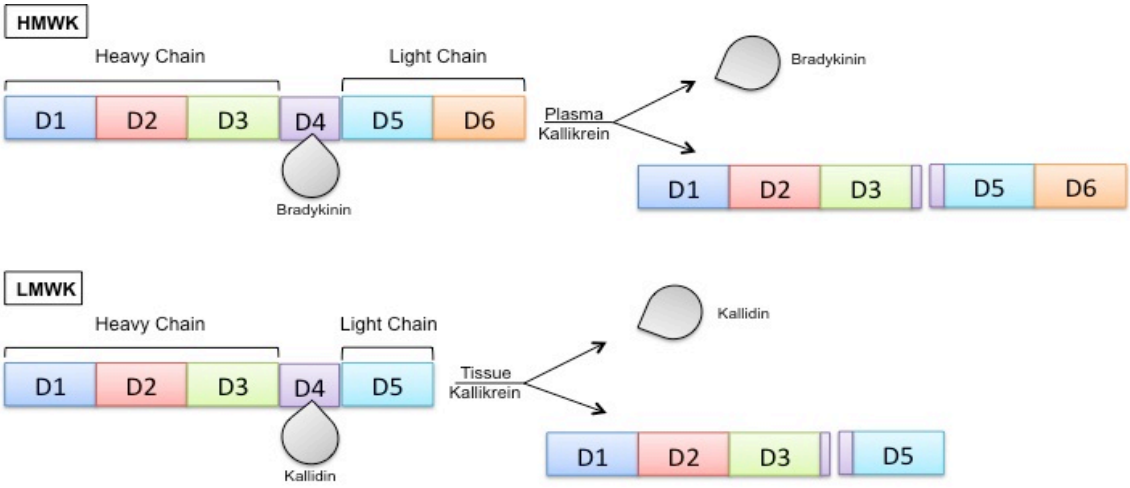

Figure 3: Schematic representation of the HMWK and LMWK domains.
The HMWK consists of 6 domains and releases Bdk from D4 upon plasma kallikrein cleavage. The LMWK consists of 5 domains and releases Kallidin from D4 upon tissue kallikrein cleavage.

3. Receptors of Kinins

There are two receptors through which kinins act: bradykinin 1 receptor (B1R) and bradykinin 2 receptor (B2R). B1R and B2R belong to the family of G protein coupled receptors (GPCRs). Activation of B2R by Bdk raises intracellular Ca^{2+} concentrations via activation of the phospholipase C that subsequently leads to the production of IP_3 (Pinheiro et al., 2013). This is followed by the elevation of inflammatory molecules such as prostaglandins and oxidative stress mediators including nitric oxide (NO) and reactive oxygen species (ROS) (Raslan et al., 2010).

These various pathophysiological functions account for the involvement of the B1R and B2R in vascular remodeling, edema formation and inflammation following injury. Of importance, Bdk and kallidin bind preferentially to the constitutive B2R, which is ubiquitously expressed in healthy tissues. On the other hand, des-Arg^9-bradykinin and des-Arg^{10}-kallidin (the degradation products) bind to the B1R that is expressed only under stress and inflammatory conditions (Trabold et al., 2010).

4. Signaling of Kinins

Taking into consideration that Bdk receptors belong to the Class A Rhodopsin-like family of seven transmembrane receptors, researchers focused on downstream proteins and pathways particular to this type of GPCRs.

Consequently, Bdk was shown to activate the ERK/ElK-1/Ap-1 pathway in mesangial cells and its signaling depended mainly on protein tyrosine phosphorylation (El-Dahr et al., 1998).

In another study, Blaukat and Dikic demonstrated the role of B2R in activating sphingosine kinase, leading to a dose and time-dependent increase of sphingosine 1 phosphate concentration (Blaukat and Dikic, 2001). This mechanism was inhibited in the presence of dihydrosphingosine, a sphingosine kinase inhibitor. Although the overexpression of sphingosine kinase did not increase the Bdk-induced ERK-MAPK activity, elevated doses of the sphingosine inhibitor disturbed the Bdk-induced ERK-MAPK activation and completely blocked the PKC-independent ERK/MAPK signaling cascade through B2R. Herein, sphingosine 1 phosphate plays a permissive rather than an active role in B2R-mediated mitogenic signaling (Blaukat and Dikic, 2001).

In addition, it was stated that B1R should heterodimerize with Carboxypeptidase M (CMP), an agonist-generating enzyme present on the same cell membrane, to efficiently generate B1R signals that play essential roles in inflammatory processes (Zhang et al., 2008). Besides, the B1R- and CMP- dependent calcium signal required the presence of the B2R agonist Bdk (Zhang et al., 2008).

A more recent study reported that tissue kallikrein diminished glutamate-induced apoptosis by activating B2R that phosphorylated ERK1/2 (mainly ERK1), and in turn activated the transcription factor NF-κB (Liu et al., 2009a) which controls the

expression of many inflammatory components and blocks cell apoptosis (Li and Stark, 2002).

Liu et al. have also shown that tissue kallikrein up-regulates the expression of the anti-apoptotic and survival genes: brain-derived neurotrophic factor (BDNF) and BCl-2 (Liu et al., 2009a). A parallel work emphasized the antioxidant features of tissue kallikrein, its ability to decrease ischemia-acidosis/reperfusion-induced injury, to inhibit apoptosis and to promote cell survival *in vitro* through the activation of the ERK1/2 signaling pathway (Liu et al., 2009b).

A study on the role of Bdk in rat brain astrocytes reported that activating the ERK/NF-κB and JNK/c-Jun cascades by a Nox/ROS-dependent event, which enhances c-Fos/AP-1 activity, is essential for Heme Oxygenase-1 (HO-1) upregulation and activation via Bdk (Hsieh et al., 2010). Indeed, HO-1 is a stress-inducible protein that acts downstream of interleukin-10 and represents a potential therapeutic target for treating inflammatory diseases (Lee and Chau, 2002). Also, ROS-dependent NF-E2-related factor 2's activation was shown to contribute to HO-1 induction by Bdk in astrocytes (Hsieh et al., 2010).

In another recent study, B2R was shown to play a critical role in promoting calmodulin kinase II-mediated neuronal differentiation and maturation in major b-series gangliosides GT1b, GD1b and GD3. Indeed, exogenous gangliosides do not only stimulate neuronal cells and induce calcium release from intracellular synaptic stores, they also activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cdc42, and thus promote reorganization of cytoskeletal actin and dendritic differentiation (Kanatsu et al., 2012).

B. The Kallikrein Kinin System and Neuronal Inflammation

Inflammation is the first line of defense of an organism following an injury or an infection. Inflammation results in local vasodilatation due to the release of mast cell and platelet substances including: histamine, bradykinin, leukotrienes, serotonin and prostaglandins. These mediators provoke blood-brain barrier (BBB) disruption, leading to edema, neuronal injury and finally cell death; thus underlining the significant role of kinins in brain inflammation (Raslan et al.; Su et al., 2009).

Kinins are well known inflammatory regulators outside the Central Nervous System (CNS). Although the kinin system is well distributed throughout the brain, the precise role of Bdk in the CNS is not clear yet (Sarit et al.). It has been proposed that, by acting on B1R and B2R, Bdk and substance P have a potent pro-inflammatory role and can initiate neurogenic inflammation resulting in vasodilation, plasma extravasation and subsequent development of edema, leading to neuronal degeneration (Raslan et al.; Thornton et al.).

C. Neuronal Cell Death Mechanisms

The mechanism through which the KKS responds to an injury is still under study; but has been linked to other cell death mechanisms such as apoptosis, necrosis and autophagy/excitotoxicity. Moreover, as mentioned earlier, TBI leads to apoptotic and necrotic cell death via the calpain/caspase system. Hence, this section provides a thorough review of these common cell death pathways, and focuses mainly on their occurrence and detrimental consequences in the brain.

1. Apoptosis

Apoptosis is also referred to as "programmed cell death". Apoptosis plays an essential role in normal development and homeostasis. The characteristic events of apoptosis include cell shrinkage and loss of normal contacts, dense chromatin condensation, cellular budding and fragmentation, as well as rapid phagocytosis by professional phagocytes or adjacent cells (Hockenbery, 1995).

Apoptosis is generally carried out by caspases. More importantly, apoptosis is activated through two principal signaling pathways: the intrinsic and extrinsic pathways (Elmore, 2007).

a. The Extrinsic Pathway

The extrinsic, caspase-dependent pathway is triggered in response to the activation of pro-apoptotic receptors, such as members of the tumor necrosis factor (TNF) receptor gene superfamily, death receptors DR4 and DR5, by specific pro-apoptotic ligands, such as TRAIL (TNF-related apoptosis-inducing ligand) and CD95. Ligand binding induces the rapid assembly of the DISC (death-inducing signaling complex) and the recruitment of initiator caspases 8 and 10 through the adaptor Fas-associated death domain (FADD) (Fulda and Debatin, 2006).

b. The Intrinsic Pathway

In contrast, the intrinsic, mitochondrial caspase-independent pathway is triggered in response to DNA damage and other types of severe cell stress, leading to transcriptional upregulation of pro-apoptotic members of the B cell lymphoma 2 (BCL2) family of proteins such as Bak and Bax (Portt et al., 2011). Bax causes the

release of cytochrome c from the mitochondria. The latter binds to the apoptotic protease-activating factor 1 (APAF1) leading to the formation of the apoptosome. The apoptosome will then recruit, dimerize and activate the initiator caspase 9 (Tait and Green, 2010). On the other side, Smac/DIABLO and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to the inhibitor of apoptosis proteins (IAPs) (Saelens et al., 2004).

c. The Caspase-Dependent Pathway

Eventually, both pathways merge into a caspase-dependent pathway where Caspases 8, 9 and 10 activate the effector caspases 3, 6 and 7, which are responsible for destroying critical components of the cell and inducing apoptosis (Agoston et al., 2009). Specifically, the role of caspases is to cleave the substrates in the cytoplasm or nucleus leading to many morphological features of apoptotic cell death. A worth mentioning target protein is PARP (poly-ADP-ribose polymerase). Once cleaved, PARP causes defective DNA repair mechanism. Similarly, lamins' cleavage results in disintegration of the nuclear envelop (Widmann et al., 1998), and cleavage of the 45 KDa DNA fragmentation factor DFF-45/40 leads to DNA degradation (Tang and Kidd, 1998). Finally, once actin is cleaved by caspases, the overall cell shape is lost (White et al., 2001).

d. The Caspase-Independent Pathway

Another apoptotic mechanism involves the mitochondrial release of the apoptosis inducing factor (AIF). AIF is anchored on the mitochondrial inner membrane and displays much slower release kinetics than cytochrome C. This is due to the fact

that AIF should be cleaved by proteases such as calpain I prior to its release from the permeabilized mitochondria (Tait and Green, 2010).

2. Necrosis

Necrosis is defined as an acute, non-apoptotic form of cell death (Syntichaki and Tavernarakis, 2003). Necrosis is characterized by cell swelling, collapse of plasma membrane and rapid cell lysis. It is accompanied by a rapid efflux of cell constituents in the extracellular space (Proskuryakov et al., 2003).

Different agents such as viruses, bacteria, and protozoa can induce necrosis. Necrosis can also be induced by an improper secretion of cytokines, nitric oxide (NO), and reactive oxygen species (ROS), following pathological conditions. A typical example of necrosis is ischemia that leads to a severe depletion of oxygen and glucose, and causes extensive necrotic death of endothelial cells and nonproliferating cells of surrounding tissues (neurons, cardiomyocytes, renal cells, etc.) (Bramlett and Dietrich, 2004).

Other necrotic agents include members of the TNF receptor family (TNF, FAS, TRAIL) (Vandenabeele et al., 2010), purinergic receptors (P2Z) (Pizzo et al., 1992), lipids and some products of their peroxidation (Zoula et al., 2003), and under rare conditions survival factors such as insulin or NGF (Caucanas et al., 2011). Interestingly, activation of glucocorticoid receptors can be either antinecrotic (Arantes et al., 2000) or pronecrotic (Morita et al., 1999). In addition, Ca^{2+} is commonly removed from medium to protect cells from necrosis induced by starvation and anoxia (Kristian and Siesjo, 1998). Although hydrogen peroxide is a necrotic inducer, the antioxidants glutathione or N-acetylcystein (NAC) can prevent necrosis from occurring. Likewise, the pro-

necrotic effect of peroxinitrite in neuronal cells can be suppressed by NAC (Peristeris et al., 1992).

Regarding the mechanism through which necrosis operates, it is documented that activation of AKT kinase and MAP kinase ERK can protect against necrotic death (Lee et al., 2003). Similarly, proteins of the Bcl-2 family can suspend or prevent necrosis (Poliseno et al., 2004). Necrosis was also associated with activation of the cytosolic Ca^{2+}-dependent phospholipase A2 (Wissing et al., 1997).

Furthermore, necrotic cell death also occurs during normal processes such as tissue renewal, embryogenesis, and immune response (Zong and Thompson, 2006).

3. Excitotoxicity and Autophagy

Excitotoxicity and autophagy are two intertwined cell death mechanisms. Indeed, excitotoxicity results from excessive release of the neurotransmitter glutamate, elevation of intracellular calcium, accumulation of oxidizing free radicals, impairment of mitochondrial function and activation of apoptotic and autophagy programs (Dong et al., 2009).

a. Excitotoxicity

Excitotoxicity can be defined as an excessive activation of neuronal amino acid receptors (Mark et al., 2001). Excitotoxic neuronal cell death is a key feature of neurodegenerative CNS diseases such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, Huntington's disease, epilepsy, trauma, ischemia etc… (Beal, 1992).

The main mediator of excitotoxic damage is the excessive synaptic release of

glutamate that leads to the disregulation of Ca^{2+} homeostasis. Glutamate binds to and activates the postsynaptic receptors: N-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA), and kainate receptors. Upon extreme activation, these receptors lead to excessive influx of Ca^{2+}, which together with any Ca^{2+} release from intracellular compartments overwhelms Ca^{2+}-regulatory mechanisms and leads to cell death through necrosis or apoptosis (Arundine and Tymianski, 2003).

Remarkably, a novel study suggested that ramified microglia plays an active protective role in excitotoxicity-induced neurodegeneration in hippocampal slice cultures exposed to NMDA (Vinet et al., 2012).

b. Autophagy

Autophagy is an intracellular phenomenon that results from cell stress and is characterized by the engulfment of cytoplasmic organelles by double membrane vesicles called autophagosomes. The autophagosomes deliver the organelles to the lysosomes where the organelles are broken down by lysosomal proteases and the amino acids recycled back into the cell machinery to assist cell survival (Dunn, 1990).

Neurons can undergo either apoptotic (type I) or autophagic (type II) or oncotic/necrotic (type III) cell death depending on the nature of the insult (Liu et al., 2004). Of interest, acute excitotoxicity insults induce both oncotic and apoptotic cell deaths (Ankarcrona et al., 1995).

A recent study reported that NMDA-mediated excitotoxicity resulted in type II prolonged autophagy cell death, in cultured rat cerebellar granule neurons (Sadasivan et al., 2010).

Still in another study, it was revealed that Bdk is neuroprotective against NMDA-induced excitotoxicity, which is not the case in the presence of a B1R agonist that induces apoptosis via MEK/MAPK; thus highlighting the neuroprotective role of B2R in NMDA-induced excitotoxicity in rat hippocampal slices (Martins et al., 2012).

D. The Kallikrein Kinin System and Traumatic Brain Injury

The current surge in military and terrorist conflicts all over the world have resulted in a pronounced increase of patients (soldiers and civilians) with military and military-type injuries. However, TBI is only the tip of the iceberg; patients will also suffer from vasospasm, pseudoaneurysm formation, visual and/or auditory deficits and will have a higher risk of developing post-traumatic epilepsy, persistent post-concussive systems, post-traumatic stress disorder and chronic pain (Risdall and Menon, 2011).

Brain injury consists of two phases (Table 1). The initial primary mechanical damage occurs at the time of the traumatic incident, is irreversible and only amenable to preventative measures. The primary insult is followed by a secondary phase, which is a multifactorial process initiated at the time of impact and evolving over the subsequent hours to days (Vink and Nimmo, 2009). This secondary phase is made up of a variety of physiological, cellular and molecular responses aimed at restoring homeostasis. If not controlled, these processes will lead to secondary injury. Indeed, kininogen level increases immediately post brain trauma and remains as such for 15hrs; implying that the secondary post injury events are related to endothelial and vascular repair mechanisms (Ellis et al., 1989).

Primary Insult	Secondary Insult
-Occurs at the time of traumatic incident	-From time of impact to subsequent hours to days
-Mechanical damage	-Physiological, cellular and molecular responses
-Irreversible	-Restore homeostasis
-Necrotic cell death near impact site	-Apoptotic cell death in regions caudal to impact site
-Mediated by calpain	-Mediated by caspases

Table 1: Characteristics of primary and secondary insults following TBI.

Many studies have been conducted in order to tackle the role of Bdk following brain trauma. It was shown that plasma and cerebro-spinal fluid (CSF) levels of Bdk (1-5) were significantly and markedly increased after acute trauma patients (34700 fmol/ml vs. 34.9 fmol/ml in normal volunteers) (Marmarou et al., 2005).

Moreover, the KKS was shown to be an important mediator of secondary brain damage, notably vasogenic brain edema. Indeed, in vasogenic edema-induced mongrel cats by cold injury and following disruption of the BBB by focal trauma, plasma kininogens were demonstrated to penetrate into necrotic and edematous brain tissue where activation of the KKS provoked additional cerebral ischemia (Maier-Hauff et al., 1984). Also, amongst the pathological changes, disruption of the BBB has been demonstrated in the acute post-traumatic period, allowing not only an increase in intracranial pressure (ICP), but also the entry of circulating neutrophils, monocytes and lymphocytes to the injured site, directly impacting on neuronal survival and death (Thornton et al.).

Recent experimental work has provided evidence for involvement of the KKS in the progression of edema (Stover et al., 2000). In a study where aprotinin and

soybean trypsin inhibitor have been used as inhibitors of the KKS to treat brain swelling due to perifocal cold injury edema, a marked reduction of vasogenic edema as quantitatively assessed by the decrease in weight of the traumatized hemisphere was showed (Unterberg et al., 1986).

In addition, CP-0127, a specific Bdk antagonist, has been found to reduce cerebral edema in a cold lesion model in rats, as well as in a randomized, single blind pilot study, with a 7 day infusion of CP-0127 (3.0 ug/kg/min) in patients with focal cerebral contusions presenting within 24±96 hours of closed head injury with an initial GCS 9±14. CP-0127 seemed to block the secondary brain swelling by acting on the cerebral vasculature and limiting dys-autoregulation or by acting on the BBB to reduce cerebral edema. This Bdk antagonist proved highly beneficial in a way that the majority of the treated patients didn't need further surgical operation (Narotam et al., 1998).

Still another study underlined the neuroprotective effect of B2R antagonist HOE140 as compared to the B1R antagonist B 9858. Indeed, HOE140 was shown to reduce lesion volume and brain swelling induced by cold injury in the parietal cortex of rat and mouse, a therapeutic effect that was not reproduced while using B1R antagonist (Gorlach et al., 2001).

The therapeutic role of B2R antagonist was further demonstrated in a study conducted by Hellal et al. In fact, the B2R antagonist LF 16-0687 Ms given 30min post-injury reduced neurological deficit and cerebral edema when evaluated 4h after closed head trauma. Similarly neurological function was enhanced in $B2R^{-/-}$ mice when compared to $B2R^{+/+}$ mice; thus confirming the detrimental role of B2R in the development of the neurological deficit and of the inflammatory secondary damage resulting from diffuse TBI (Hellal et al., 2003). Comparable results where obtained with

the B2R antagonist LF 18-1505T. The latter reduced brain edema and improved neurological outcome after closed head trauma in rats (Ivashkova et al., 2006).

Other studies where conducted in a subtype of TBI known as controlled cortical impact (CCI). CCI represents an open wound injury model that mimics better what we observe in car accidents and war zone. The role of the KKS was also investigated in this type of injury and it was found that Bdk levels were significantly increased 2 hours following CCI before declining over subsequent hours (Trabold et al.). Moreover, in regards to the mRNA levels of B1R and B2R, expression of the B1R mRNA was increased four-fold at 6 hours after CCI. Further confirmation for the role of Bdk and its receptors in TBI has been obtained through the use of genetically engineered mice deficient for either B1R or B2R. $B2R^{-/-}$ animals had 50% less brain water content following injury when compared to wild-type animals and performed better on functional outcome tests (Trabold et al.).

E. Bradykinin Receptors as Potential Therapeutic Targets in Brain Injuries

Over the past years, numerous studies have focused on B1R and B2R as therapeutic targets in the treatment of different pathophysiological disorders. Nevertheless, the results obtained were controversial and misleading. Actually, some studies have highlighted the implication and protective role of the B1R in brain injury while others have underlined the critical role of the B2R. The disparity of the experimental results is summarized in Table 2.

Receptor	State	Effects	Benefit (+) Harm (-)	References
B1R	Activated	Less BBB leakage post cryogenic cortical injury	+	(Dutra et al., 2011)
	KO	Attenuated cortical damage in mice focal brain injury	+	(Austinat et al., 2009)
	Inhibited	Reduced brain edema and cerebral infarction in mice	+	(Austinat et al., 2009)
		No therapeutical benefit to attenuate 2^{ary} brain damage	-	(Thornton et al., 2010)
		Reduced inflammation and edema formation in a closed head model of focal TBI	+	(Raslan et al., 2010)
		Reduced axonal injury and astroglia activation post injury	+	(Albert-Weissenberger et al., 2013)
B2R	Activated	Prevention of H/R-induced astrocytic apoptosis	+	(Xia et al., 2004)
		Enhanced phosphorylation of ERK1/2 and Caspase-3 activity blockage	+	(Tang et al., 2009)
		Increased neuronal viability post H/R insult due to reduced LDH release	+	(Liu et al., 2011; Tang et al., 2009)
		Protection of pyramidal neurons against NMDA-mediated excitotoxicity	+	(Martins et al., 2012)
		Protection against ischemia and survival of CA1 neurons by blocking ROS release	+	(Danielisova et al., 2008)
		More 2^{ary} brain damage and edema formation, vascular permeability increase	-	(Trabold et al., 2010)
		IL-1β release contributing to BBB breakdown	-	(Woodfin et al., 2011)
	KO	Aggravated damage post MCAO	-	(Xia et al., 2006)
	Inhibited	Reduced brain edema and brain tissue loss by lowering intracranial pressure	+	(Zweckberger and Plesnila, 2009)
		Anatibant can treat 2^{ary} brain injured patients	+	(Marmarou et al., 2005)
		No protective effect after focal brain injury in mice	-	(Raslan et al., 2010)
		Therapeutical benefit to attenuate 2^{ary} brain damage	+	(Narotam et al., 1998)

Table 2: Controversial results regarding the protective effects of B1R versus B2R.

Although controversial, the results obtained offer encouraging therapeutic approaches for drug development (Heitsch, 2002; Rodell, 1996).

The protective role of Bdk originates from a study by Martins et al, where Bdk showed marked neuroprotection of pyramidal neurons against NMDA-mediated excitotoxicity. This vital protective role involved the activation of PI3 kinase, which then is responsible for Bad phosphorylation and subsequent anti-apoptotic activity. Bdk-mediated neuroprotection did not depend on the MEK/MAPK activation cascade. However, MEK/MAPK was involved in the induction of apoptosis mediated by the B1R (Martins et al.).

Due to their demonstrated role in brain injury, several studies have evaluated the role of Bdk antagonist as therapeutic targets after brain trauma. In one study by Zweckberger, it has been demonstrated that Anatibant, a selective and potent antagonist of the B2R, reduces the apparition of brain edema by lowering intracranial pressure, and diminishes the "secondary loss of viable brain tissue" (Zweckberger and Plesnila, 2009). Moreover, it has been demonstrated that a single subcutaneous dose of injected Anatibant can treat secondary brain injured patients with no adverse effects after experimental brain injury (Marmarou et al., 2005). Furthermore, Raslan et al. showed that $B1R^{-/-}$ mice or the use of acute B1R antagonist R-715 attenuates cortical damage after focal brain injury in mice whereas blockade of B2R has no significant protective effect. Mechanistically, the inhibition of B1R was associated with reduced CNS inflammation and BBB leakage after cryogenic cortical injury (Raslan et al.).

In one study by Waldner et al, it has been shown that following cerebral ischemia, Bdk promotes the secondary brain damage through an increase of vascular permeability and brain edema formation, again hallmarks of inflammation. Intracarotid

injection of Bdk resulted in increased numbers of rolling and adherent leukocytes as well as rolling platelets at the venular endothelium. This was reversed by administration of a B2R antagonist and was not observed upon administration of a B1R antagonist. Thus, it was proposed that to attenuate secondary brain damage, inhibition of B2 but not B1 receptors might be of therapeutical benefit (Waldner et al.). Similar result was shown in the experimental model of stroke (cerebral reperfusion injury) where permeability was prevented by applying the B2R antagonist HOE 140. In addition, it was shown that Bdk leads to IL-1β contributing to BBB breakdown. The acute permeability response to Bdk depends on arachidonic acid formation via phospholipase A2 (PLA2) activation and subsequent ROS generation via cyclooxygenase and lipoxygenase (Woodfin et al.). However, Albert-Weissenberger et al. demonstrated that blocking of B1R, but not B2R, protects from cortical cryolesion by reducing inflammation and edema formation in a closed head model of focal traumatic brain injury (TBI; weight drop). An increased expression of B1R and not B2R was observed in the injured hemispheres of wild-type mice after 7 days of injury. Blocking of B1R showed reduced axonal injury and astroglia activation after injury induction (Albert-Weissenberger et al.).

Since 1984, the KKS is known to be found in the CNS and to play an important role there (Scicli et al., 1984). In particular, the B2R is widely distributed in neuronal compartments suggesting its important role in brain pathological disorders such as TBI, ischemia, nerve regeneration and neurodegeneration (Chen et al., 2000). In addition to being present in the brain, the localization of B1R and B2R genes in human nervous tissue proved that the KKS is physiologically active in this body area (Mahabeer et al., 2000).

The next and most recent step in Bdk receptors' characterization is the study of their crystal structures. These templates will allow better understanding of the molecular basis of the KKS and will also allow the design and development of new medical inhibitors (Pathak et al., 2013).

F. Proteomics

Nowadays, proteomics is in the limelight. Indeed, this new approach gives researchers the ability to work on a new dimensional level with promising results in perspective. Moreover, the emergence of high-throughput techniques allows the processing of a large number of samples in a short time (Haynes and Yates, 2000) and permits the acquirement of more accurate results, leading to the discovery of so far unnoticed relevant details, which can be used to better understand and eventually treat diseases (Anderson, 2014).

1. Definition

"Proteome" is a term used to describe the entire set of proteins and modified proteins found in an organism or a cellular system, and was first coined by Marc Wilkins, a PhD student at Australia's Macquarie University in 1994 (Huber, 2003). Proteome also takes into consideration the various aspects a cell or an organism can pass through depending on its needs, and varying with time and external influences (Anderson and Anderson, 1998). "Proteomics" is the broad study of a single specific proteome that aims at understanding cellular processes by determining protein amounts, distinctions, alterations and connections with other proteins and networks (Blackstock and Weir, 1999).

2. Usefulness of Proteomics

Although the symptoms of TBI are known, their early detection and possible treatment requires a better understanding of the injury initiation and progression while looking at the molecular level (Berger, 2006). The real challenge though, is to assess what is happening in a patient in real time by finding telltale proteins and thus taking a closer look at the biological processes triggered by brain injury (Shen et al., 2014).

The discovery of a unique biomarker can also serve as an early specific sensor to prevent the emergence or propagation of the injury (Mondello et al., 2014). Indeed, biomarkers are traceable proteins found in tissues and body fluids such as plasma, serum, urine, CSF, and are particular to a type of disease (Biomarkers Definitions Working, 2001). Importantly, proteomics has the ability to accurately identify and quantify such proteins, in any bio specimen. Therefore, the quest for "biomarkers" is extremely promising and will allow scientists and clinicians to better understand the cellular processes involved in brain injury diffusion, with the hope to identify an effective therapeutic tool (Frantzi et al., 2014).

3. Proteomics versus Genomics

Although genomics have been more extensively elaborated and studied in the past few years, genes only hint to us what may occur as uttered by the genetic code (Weber, 2000). In other words, genes are only the "ingredients" of the cell, while the proteins encoded by the genes are the crucial functional players that determines both normal and disease phenotypes (Harry et al., 2000).

As opposed to genomics, the main challenge in proteomics is the assessment of the highly complex proteome (Tyers and Mann, 2003). Actually, the high complexity of

the proteome is due to the fact that:

- One gene can encode more than one protein. Although, the human genome contains between 20,000 and 25,000 protein-coding genes, the total number of proteins in human cells is estimated to be between 100,000 to ten million (Baak et al., 2003).
- In contrast to the genome, which is relatively static, proteins are dynamic**.** Proteins are continually undergoing spatiotemporal modifications (Henzler-Wildman and Kern, 2007). They can be synthesized, degraded, recycled, they can bind to each other to form complexes, they can anchor to the plasma membrane, enter and exit the nucleus or the cell, and move from one cytosolic organelle to the other (2009; Bahar et al., 2010; Bu and Callaway, 2011).
- Proteins are co- and post-translationally modified. They can undergo glycosylation, de-/phosphorylation, sulfation, ubiquitination, acetylation and nitrosylation (Karplus and Kuriyan, 2005; Pandey and Mann, 2000). Consequently, two individuals under different environmental factors will present different types of proteins. Different proteins can also be seen within the same person at different stages (age, health) of his life (Farley and Link, 2009; Ribet and Cossart, 2010).
- Proteins are found in a broad range of concentrations in an organism from pg/mL to mg/mL**.** This characteristic impedes the detection of low- and high- concentration proteins at the same time (VanMeter et al., 2012). Furthermore, body fluid protein concentrations with several orders of magnitude difference represent a big challenge for multiplexed detection of potential biomarkers. For example, the clinical biomarker Prostate Specific Antigen (PSA) is found in the serum of diseased patients at a concentration of 4-10 ng/mL and completely masks the presence of a second relevant biomarker interleukin 6 (Il-6), found in the serum at a concentration

of 20-1000 pg/mL (Rusling et al., 2010). To overcome this issue, one recent study revealed a novel technique called "broad-range biomarker assay" that makes use of "solid to liquid phase change nanoparticles". This method allows the reading of multiple biomarkers with concentration difference over 3 orders of magnitude (Wang et al., 2011).

4. Tools in Proteomics

Proteomics data depend heavily on high-throughput robotic systems capable of identifying and quantifying a myriad of proteins in a short lapse of time. The most common technologies used nowadays are presented below.

a. Mass Spectrometry

Mass spectrometry (MS) is a very clear-cut method that allows researchers to identify and quantify proteins in a given biological milieu. In addition, this highly precise machine can discriminate proteins differing by a single Hydrogen atom. However, separation of complex protein mixtures requires not only better sensitivity of instrumentation but also additional methods such as protein fractionation or affinity capture, which will enrich a group of proteins of interest, reducing the complexity of the protein mixture (Corthals et al., 2000).

Mass spectrometers consist of three essential parts: an ionization source, a mass analyzer and an ion detector. The ionization source converts molecules into gas-phase ions. The mass analyzer separates the ions according to their mass-to-charge ratios (m/z), which correlate with the time-of-flight (TOF) of each ion. Finally, the ion detector reads and plots the ions as per their m/z values (Sauer and Kliem, 2010).

Two important techniques are used to create ions from large molecules. The first technique is called Matrix-Assisted Laser Desorption Ionization (MALDI). This method consists in using a laser that will strike a crystalline matrix, causing the rapid excitation of certain molecules that get converted into ions and are ultimately ejected from the matrix to the gas-phase. The second technique is referred to as Electrospray Ionization (ESI). ESI works by applying a potential to a running liquid that gets charged and spray as small droplets retaining the analyte. Once in the mass spectrometer the droplets are subjected to gas collision-induced dissociation (CID) or heat, leading to the creation of many charged ions (Kast et al., 2003).

Importantly, MALDI-TOF is mostly used to obtain accurate measurement of molecular weight at low ppm level, while tandem mass spectrometers (MS/MS) is mainly used to diagnose amino acid sequence (Dutt and Lee, 2000). Tandem MS instruments include: the triple quadrupole, ion-trap, hybrid quadrupole-time-of-flight (Q-TOF) and are usually applied in liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments or with ESI (Gygi and Aebersold, 2000). MS/MS apparatus consist of an ion source, a first mass analyzer that will select a particular m/z value, a gas-phase collision chamber that will dissociate this m/z value by cleaving the amide bonds, a second mass analyzer that will analyze the fragmented ion products and an ion detector that will plot the fragmentation pattern of this particular peptide (Yates, 2000).

Furthermore, shotgun proteomics is a new tool for proteome analysis and is based on multidimensional LC-MS/MS (Sprung et al., 2009). The method consists in digesting tissue or biofluid specimens into tryptic peptides and fractionating them by basic reverse phase HPLC. The peptide fractions are then analyzed by reverse phase-LC coupled to LC-MS/MS (Yates, 2013). Since each MS/MS spectrum encodes the

sequence of a peptide, it is important to collect MS/MS spectra from as many of the peptides in the sample as possible. A database of sequences corresponding to all known human proteins is used to determine the peptide sequences found. The results are then gathered into an inventory of proteins that can account for the identified peptides. Ultimately, shotgun analysis serves to spot proteome differences between normal and diseased phenotypes, single gene mutation events and responses to drugs stimuli (Li et al., 2011).

Finally, multidimensional protein identification technology (MudPIT) is another proteomic strategy to identify proteins from a complex mixture (Kislinger et al., 2005). Once the proteins in the mixture are enzymatically or chemically cleaved, they are sorted by one or more chromatographic separation steps, such as strong cation exchange (SCX) coupled to reversed-phase chromatography (Washburn et al., 2001).

b. Protein Microarrays

Protein microarrays are highly powerful devices often referred to as multiplexed tools since they can identify many different biomarkers at the same time (Sutandy et al., 2013). Protein chips usually consist of a glass or plastic grid coated with various "capture agents"; each agent will grab a specific protein/biomarker. Then, the isolated biomarkers can be used for clinical diagnostics and for monitoring disease states (Hartmann et al., 2009).

Protein microarray technology can be used for many purposes such as identification of protein-protein and protein-phospholipid interactions, detection of small molecules and recognition of protein kinase substrates (Stoevesandt et al., 2009).

There are three kinds of protein microarrays: analytical, functional and reverse-phase microarrays. Analytical microarrays are also called antibody microarray since they make use of aptamers (stable, short single-stranded oligonucleotides capable to bind a broad range of target molecules with high specificity and affinity) or affibodies (small protein bundles generated by combinatorial protein engineering with specificity for a wide range of targets including human, bacterial and viral proteins) disposed on a glass microscope slide and is mostly used to measure protein expression level, binding affinities and specificities, from a complex mixture of proteins (Chandra et al., 2011). Functional microarrays consists of full-length functional proteins or protein domains in order to assess different types of protein-interactions: protein-protein, protein-DNA, protein-RNA, protein-phospholipid, and protein-small molecule interactions (Chen and Zhu, 2006). Finally reverse phase microarrays are made of lysed cells isolated from tissues of interest and placed on a nitrocellulose slide. Then, antibodies are used to bind proteins of interest and are detected by chemiluminescent, fluorescent or colorimetric assays (Mueller et al., 2010). Quantification of proteins can also be performed. Interestingly, reverse phase microarrays can detect post-translational modifications frequently seen in the altered diseased phenotype (Hall et al., 2007).

c. Nanotechnologies

Nanotechnology is an important tool that allows scientists to work at the nanolevel, and thus detect low abundance proteins *in vivo* (Chen et al., 2013). Nanotechnology devices are capable of crossing physical and biological barriers to detect a potential protein biomarker that can be used as a novel therapeutic tool in the treatment of a particular disease (Ding et al., 2013; Espina et al., 2003). In addition, it

was recently discovered that nanomaterials could serve as a scaffold for tissue regeneration, thus outwitting tissue engineering (Dvir et al., 2011).

Nowadays, there are many nano-devices available, comprising nanoparticles. Nanoparticles are microscopic molecules between 1 and 10 nm and are used as vehicles for targeted drug delivery i.e. delivery of the intact, fully effective drug at a specific site without dispersion of the latter in the body (Gu et al., 2013). Recently, gold nanoparticles have been used as tumor-specific nanoparticles in radiation therapies, where they interact with X-rays (Mesbahi, 2010). Nanoparticle-based contrast agents are valuable tools for *in vivo* long-term quantitative imaging, such as magnetic resonance imaging (MRI), computed tomography (CT) and ultrasound (US). Indeed, they are sensitive and selective for the target, can be used at low doses and are readily cleared from the body (Hahn et al., 2011). Other nano-devices include nanowires and nanocantilever arrays that can be used in biosensors to measure infinitesimal quantities of biomarkers in biological fluids (Biedermann et al., 2010).

d. Bioinformatics

Bioinformatics research is necessary to study how multiple proteins work together in a complex biological system, analyze data and compute the results in a clear, straightforward design. Controlled vocabularies, gene ontology and system biology are bioinformatics initiatives used to analyze gene and protein expression, construct pathways and find relationships among genes, proteins, cell processes, and diseases (Alawieh et al., 2012; Mayer et al., 2014).

Some new developed bioinformatics tools with biological perspectives include DAVID (Huang da et al., 2009), PANTHER (Mi et al., 2013), VENNY (Oliveros,

2007), MetaCore (http://www.genego.com/metacore.php), Pathway Studio (Nikitin et al., 2003) and are extremely valuable for integrative analysis of genomic and proteomic data, as well as computational interpretation of the results (Bruce et al., 2013).

In particular, Pathway Studio comes with a built-in resource named ResNet, which is a database of molecular interactions based on natural language processing of scientific abstracts in PubMed. With ResNet, a researcher can import his targeted gene product/protein list onto a new pathway diagram and build a pathway using well-known interactions discussed in existing literature.

Currently, clinicians and researchers are using these methods and techniques in order to conduct translational bench-to-bedside studies.

e. Biospecimens

Measurement of genetic and protein expression depends heavily on the quality of biospecimens. Human biospecimens and biofluids are an important source of molecular data to identify molecular mechanisms, classify different types of injuries and discover potential therapeutic targets. The real challenge in TBI research is the collection, storage and processing of biospecimens with high consistency and perduring quality (Manley et al., 2010).

Nevertheless, to enhance the probability of a biomarker to reach the clinical stage, an appropriate number of biospecimens should be considered, and a statistical experiment design should be used to quantitatively assess the reliability and robustness of a biomarker candidate (Skates et al., 2013).

Moreover, in order to generate the best biospecimens possible, scientists require the help of big companies such as the Organization for Economic Co-operation

and Development (OECD), the European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI), the Cancer Genome Atlas, the Human Proteome Project, the Biospecimen Research Network (BRN), the Biospecimen Reporting for Improved Study Quality (BRISQ) and the Standard Preanalytical Coding for Biospecimens (SPREC). All these international efforts are crucial for global data access among the scientific community, large-scale biospecimens collection and financial support (Moore et al., 2011).

f. Reagents

The two principal aims of proteomic technologies are high specificity and reproducibility. This can be achieved by using well-defined standard reagents.

Antibodies are the most widely used reagents in the proteomic research. These best-characterized antigen binding ligands are used in proteomics applications to determine the features of a gene product, such as "tissue distribution, cellular localization, post-translational modifications, expression levels and complexes formed, using standard techniques such as immunofluorescence, immunohistochemistry, western blots and immunoprecipitation" (Bradbury et al., 2003).

Recently, a new affinity reagent called aptamer has shown great promise as an alternative to antibodies. These short single-stranded oligonucleotides are a class of nucleic acid-based molecules; they fold into diverse shapes and bind with high affinity and specificity to proteins, peptides and small molecules (Brody and Gold, 2000). The high potential of aptamers in the proteomics field lead to the creation of a new class of aptamer called the Slow Off-rate Modified Aptamer (SOMAmer). SOMAmers were demonstrated to have a high-affinity for nearly any protein target including complexes

and are used in multiplexed proteomic assays for biomarker discovery (Gold et al., 2010).

There are four proteomic quantitative methods that rely on standard proteomic reagents: 2D-DIGE, ICAT, iTRAQ and SILAC.

Two Dimensional Difference In-Gel Electrophoresis (2D-DIGE) uses fluorescent reagents to label proteins, which are then separated by 2D-PAGE according to isoelectric point (pI) and molecular weight (Unlu et al., 1997). Commonly, three different dyes are available Cy2, Cy3 and Cy5; thus, three samples can be run together on the same gel. After completion of electrophoresis each gel is scanned three times, at three different wavelengths (red, blue and green), the images are superimposed and analyzed using sophisticated computer software to detect subtle changes in protein expression (Tonge et al., 2001).

The Isotope-Coded Affinity Tags method (ICAT) is another strategy to quantify differential protein expression. ICAT reagents consist of three elements: an affinity tag (biotin) that isolates ICAT-labeled peptides, a linker, which can bind to stable isotopes, and a reactive group with specificity toward thiol groups. There are two forms of the reagent: heavy (containing eight deuteriums) and light (containing no deuteriums). The proteins from a whole lysate mixture are harvested, denatured, reduced, and labeled at cysteines with either the light or heavy ICAT reagents. The differently labeled samples are then combined and digested with trypsin. ICAT-labeled peptides are isolated by biotin-affinity chromatography and then analyzed by online HPLC coupled to a tandem mass spectrometer. Protein quantification is realized by measuring the peak ratios obtained by HPLC, while protein identification is provided by the tandem mass spectrum (Gygi et al., 1999; Gygi and Aebersold, 2000).

Still another proteomic quantitative method that relies on reagents is the Isobaric Tag for Relative and Absolute Quantitation (iTRAQ). iTRAQ reagents are non-polymeric, isobaric tagging reagents consisting of a reporter group, a balance group, and a peptide reactive group that will covalently bind to the lysine side chain and N-terminal group of a peptide via an amide bond. Since four different iTRAQ reagents are available, four samples can be analyzed and compared within a single MS run. As for ICAT, labeled proteins (primary amino groups in this case) are digested by proteolytic enzymes, quantified and analyzed by MS/MS (Wiese et al., 2007).

Last but not least, the Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) is a method that consists in adding isotopically labeled essential amino acids in an amino acid deficient cell culture media so that all synthesized proteins can readily incorporate them (Regnier et al., 2002). Proteins are then harvested, purified, combined, digested with trypsin and quantitated by MS. Importantly, the purified proteins will retain the exact ratio of the labeled to unlabeled protein since no more synthesis will be taking place (Ong et al., 2002).

To sum up, there are two main types of standard proteomic reagents. On one hand, reporter molecules reveal the presence of a target prior and after modifications, in a certain biological sample. On the other hand, capture molecules are used to purify the target from a complex biological sample in order to identify and quantify it.

G. Proteomics and Traumatic Brain Injury

Diagnosis and classification of patients with TBI depended on inadequate traditional approaches, namely clinical examination (Glasgow Coma Scale) and neuroimaging (Computed Tomography scan and Magnetic Resonance Imaging). The

complexity and heterogeneity of the brain stresses the need for sensitive and specific biomarkers. Specifically, in the case of TBI, cellular damage and disintegration results in the release of proteins into biofluids (CSF and blood). These proteins can be identified, measured and used as biomarkers to reveal the type and magnitude of the injury (Mondello et al., 2014).

The CSF represents a rich source of recognized biomarkers. Indeed, peptides, proteolytic fragments and antibodies are capable of crossing the BBB. However, macromolecules enter the CSF only in pathologic conditions when the BBB is disrupted (Datta et al., 2011). The goal of proteomics nowadays is to find early neurologic biomarkers in order to prevent the development of diseases. However, the main challenge of identifying the CSF proteome is the low abundance of proteins, peptides and proteolytic fragments as compared to serum. Among the commonly found components of human CSF figures: *tau* protein fragments, K6, Aβ fragments, apolipoproteins, etc… (Romeo et al., 2005).

Notably, serum biomarkers proved useful in predicting the outcome following TBI in adults and children. In particular, S100B, a major low-affinity calcium binding protein in astrocytes, is heavily released in the serum after astrocyte injury or death (Agoston et al., 2009; Kochanek et al., 2008), allowing clinicians to predict the severity of TBI and its outcome (Berger, 2006). Other TBI biomarkers include: myelin basic protein (MBP), neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), αII-spectrin breakdown products, microtubule-associated protein-tau (Kobeissy et al., 2008a) and ubiquitin carboxy-terminal hydrolase L1 protein (UCHL1) (Guingab-Cagmat et al., 2013).

The identification and discovery of new candidate biomarkers, in the TBI field, require the use of bioinformatics tools, including comparative 2D-DIGE and shotgun LC-MS/MS methods. Then, biological significance of the makers can be assessed by multiple reaction monitoring (MRM)-MS before being clinically validated (Shen et al., 2014).

H. Hypothesis and Aims of the Study

In this study we hypothesize that Bdk and its receptors are being altered after exposure to experimental brain injury insult and chemical neurotoxicity including Staurosporine (STS) exposure leading to apoptotic cell death phenotype. In this, work, we will utilize both an *in vitro* and *in vivo* approach in an attempt to decipher the mechanism of Bdk-mediated neuronal injury. This is fundamental to develop new intervention strategies that would benefit brain trauma worldwide.

The first aim of this study is to evaluate the KKS and Bdk receptor expression *in vitro* after exposure to the neurotoxic drug STS which display an apoptotic phenotype. Several parameters will be assessed such as cell death, ROS production, LDH release, immunofluorescence detection of apoptotic markers and KKS markers, translational and transcriptional level expression of KKS markers and apoptotic markers. The experiments will be conducted in the neuronal cell line PC12.

The second aim consists in evaluating the effects of Bdk spiking along with the B2R agonist ([Hyp^3]-Bradykinin), B2R antagonist (HOE-140), B1R agonist (Bradykinin Fragment 1-8 acetate salt hydrate) and B1R antagonist (R-715 TFA salt), following STS application in PC12 cells. The same parameters as in the first aim will be assessed.

Finally the third aim of this study is to evaluate the KKS and Bdk receptors expression after exposure to the neurotoxic drugs STS, NMDA and MTX that provoke apoptosis, excitotoxicity and necrosis, respectively. In this section, the experiments will be conducted in primary neurocortical cells extracted from mice pups and from traumatically injured adult mice *(in vivo).* Again, several parameters will be assessed; notably with translational expression of different KKS and inflammation markers.

MATERIALS AND METHODS

A. PC12 cell line

PC12 is a cell line derived from a pheochromocytoma, a catecholamine-secreting tumor of the rat adrenal medulla. PC12 cells stop dividing and terminally differentiate when treated with nerve growth factor. Thus, PC12 cells are a useful model system for neuronal differentiation.

B. PC12 cell culture

PC12 cells were cultured and maintained in Dulbecco's Modified Eagle Media DMEM (Lonza) supplemented with 10% of heat inactivated fetal bovine serum FBS (Sigma), 5% of heat inactivated horse serum HS (Sigma) and of 1% Penicillin/Streptomycin P/S (Sigma). Cells were incubated at 37°C in a humidified incubator (5% CO_2).

C. Brain dissection

1-day old Sprague-Dawley rat pups were wiped with 70% ethanol before being rapidly decapitated with a sharp pair of scissors. Then a small scissor was used to make a midline incision in the skin. The skin was then flipped over the eyes to free the skull. The optical nerve was cut and the eyes were removed. Afterward, a cut along the sagittal suture was performed and the parietal bone was tilted from one side and the other and broken down to reveal the brain. Then the brain was gently tilted upside down and freed from underneath cranial nerves. Once lifted out of the skull, the brain was transferred into a petri dish containing washing buffer (490mL sterile PBS, 2.25g

D-Glucose, 1.3g of 10mM HEPES, 1% P/S, 1% Sodium pyruvate, the whole mixture was filtered by suction filtration in sterile conditions) and placed on ice.

Animal handling and dissection was in compliance with guidelines set forth by the American University of Beirut Institutional Animal Care and Use Committee (IACUC).

D. Primary cerebrocortical cells harvesting

Following dissection, the brain was de-vascularized by removing the meninges under a stereoscope. The anterior olfactory bulbs and the cerebellum were removed. The cortex was collected and placed in a new petri dish containing the digestion solution (0.25% of 1X trypsin/EDTA, prepared in washing buffer). With forceps, the cortex was chopped and placed in a 50mL conical tube that was placed on a shaking water bath for 5min, at 37^{o}C. Then, for every 5ml of solution, 50uL of DNAse I (Roche) was added to get rid of glial cells, and the tube was placed again for 5min in the 37^{o}C shaking water bath. The cortical cells were re-suspended in primary media 1 (DMEM with 10% FBS, 1% P/S and 2% Sodium Pyruvate)**,** centrifuged at 900rpm for 5min and the pellet was re-suspended in this primary media I. Next, the content was filtered with a 70 microns filter and the cells were counted and plated on poly-L-lysine coated 6-well plates at a density of 1.5×10^{6} cells/mL. Cultures were maintained in primary media I in a humidified incubator of 37°C, 5% CO_2. After 3-4 days in culture, 1uM of cytosine arabinoside (araC) was added to prevent further glial growth. After another 2-3 days, the media was changed to primary media II (DMEM with 10% HS, 1% P/S and 2% Sodium Pyruvate). Subsequent media changes were performed three times a week. Treatments were performed on days 10 to 21 when the

neuronal cells were 60-70% confluent.

E. Pre-treatment

PC12 cells were pretreated for one hour with either:

- B2R agonist B2A (0.1uM): [Hyp^3]-Bradykinin (Sigma), reconstituted in ddH_2O
- B2R antagonist B2I (1uM): HOE-140 (Enzo Life Sciences), reconstituted in ddH_2O
- B1R agonist B1A (0.1uM): Bradykinin Fragment 1-8 acetate salt hydrate (Sigma), reconstituted in ddH_2O
- B1R antagonist B1I (1uM): R-715 TFA salt (Sigma), reconstituted in ddH_2O

F. Treatments

1. Neuron Growth Factor

In some experiments, after 48 h incubation, PC12 cells were washed and the modified DMEM was replaced with medium containing 10% FBS, 1% P/S and 50ng/ml of neuron growth factor NGF (Calbiochem) to induce sprouting. The cells were cultured for another 7 days with this medium and the media was changed every two days.

2. Staurosporine

Staurosporine (STS) is a natural product originally isolated in 1977 from the bacterium *Streptomyces staurosporeus*. The main biological activity of STS is the inhibition of protein kinases through the prevention of ATP binding to the kinase. In research, STS is used to induce apoptosis.

Following pre-treatment, PC12 cells were treated for 3h, 12h and 24h with STS 1uM (Calbiochem), reconstituted in DMSO.

3. Maitotoxin

Maitotoxin (MTX) is an extremely potent toxin produced by *Gambierdiscus toxicus*. Maitotoxin activates Ca^{2+} permeable, non-selective cation channels, leading to an increase in levels of cytosolic Ca^{2+} ions. In research, MTX is used to induce necrosis.

PC12 cells and primary neurocortical cells derived from few hours old rat pups were treated for 4h with MTX 2.25nM (University of Florida).

4. N-Methyl-D-Aspartate

N-Methyl-D-Aspartate (NMDA) is a specific NMDA receptor agonist that mimics the action of the neurotransmitter glutamate. Of interest, NMDA has no effect on the other glutamate receptors: AMPA and Kainate receptors. Moreover, NMDA receptors are ligand-gated cation channels with high permeability for Ca^{2+} and are implicated in many neurological diseases. In research, NMDA is used as an excitotoxin; it kills nerve cells by over-exciting them.

Primary neurocortical cells derived from few hours old rat pups were treated for 4h with NMDA 0.6mM (Sigma-Aldrich).

G. MTT / Cell viability assay

The antiproliferative effects of STS were measured *in vitro* by using MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay (Roche).

PC12 cells ($10x10^3$ cells/well) were cultured in three different 96-well plates - one plate per time point (3h, 12h, 24h) - and exposed to the different treatments: STS, B2A, B2A+STS, B2I, B2I+STS, B1A, B1A+STS, B1I, B1I+STS. Then the cells were incubated at 37°C, 5% CO_2, and when the time point was reached, 10ul of the MTT yellow dye was added in each well. After another 4h of incubation, the resultant formazan purple crystals were dissolved in 100ul/well of the solubilizing agent and incubated overnight at 37°C, 5% CO_2. Then, the absorbance intensity was measured by the microplate ELISA reader (Multiscan EX) at 595nm. All experiments were performed in triplicate and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

H. LDH release / Cytotoxicity assay

The cytotoxic effects of STS were measured *in vitro* by using LDH (lactate dehydrogenase) assay (Roche). Dead cells or cells with damaged plasma membrane will release the cytoplasmic enzyme lactate dehydrogenase that catalyzes the conversion of lactate to pyruvate via reduction of NAD^+ to NADH. Then, the diaphorase enzyme uses NADH to reduce the tetrazolium salt (INT dye) into a red formazan product. PC12 cells ($10x10^3$ cells/well) were cultured in 3 different 96-well plates - one plate per time point (3h, 12h, 24h) - and exposed to the different treatments: STS, B2A, B2A+STS, B2I, B2I+STS, B1A, B1A+STS, B1I, B1I+STS. The cells were incubated at 37°C, 5% CO_2. Then, all supernatants were collected and distributed in a new 96 well plate. The Reaction mixture consisting of the catalyst and the dye (ratio 11250:250) was added (50ul/well). The plate was left in the dark, at room temperature, for 20min and then 25ul of the stop solution was added to dissolve

the red formazan product. Finally, the absorbance intensity was measured by the microplate ELISA reader (Multiscan EX) at 492nm. All experiments were performed in triplicate and the relative cell cytotoxicity (%) was expressed as a percentage relative to the untreated control cells.

I. NBT reduction assay or Intracellular ROS production

NBT (Nitro Blue Tetrazolium) is a salt that gets reduced by ROS (reactive oxygen species) into a blue, insoluble form of NBT called formazan. Thus, NBT reduction is inversely proportional to intracellular ROS production. To perform this colorimetric assay, PC12 cells ($10x10^3$ cells/well) were cultured in 3 different 96-well plates - one plate per time point (3h, 12h, 24h) - and exposed to the different treatments: STS, B2A, B2A+STS, B2I, B2I+STS, B1A, B1A+STS, B1I, B1I+STS. The cells were incubated at 37°C, 5% CO_2. Then, at each time point 100ul/well of NBT (1mg/mL) was added and the plate was incubated for 1h, at 37°C, in the dark. After what, cells were fixed with methanol and left to air dry for 20min. The insoluble crystals formed were dissolved by adding 140ul/well DMSO and 120ul/well 2M KOH. Finally, the absorbance intensity was measured by the microplate ELISA reader (Multiscan EX) at 630nm. All experiments were performed in triplicate and the relative cell NBT reduction (%) was expressed as a percentage relative to the untreated control cells.

J. Detection of cell death (Apoptosis)

1. DAPI nuclear staining

DAPI (4-6 diamidino-2-phenylindole) staining was performed to assess the

integrity of the nucleus when cells are treated with STS. PC12 cells were seeded on glass cover slips in 12-well plates ($150x10^3$ cells). Once the cells were confluent and after 3h of STS treatment, the media was removed; cells were washed with PBS, fixed with 4% formaldehyde for 10min and washed again with PBS. Then the glass cover slip was fixed on a microscope slide covered with a drop of the mounting media (Santa-Cruz) containing DAPI. Slides were then visualized under a confocal microscope (Zeiss LSM 7-10).

2. Annexin V-Fluos staining

Annexin-V is an apoptotic marker. It binds with high affinity and in a calcium-dependent manner to phosphatidylserine, which is translocated from the interior side of the plasma membrane to the outer leaflet during the early stages of apoptosis. PC12 cells were seeded on glass cover slips in 12-well plates ($150x10^3$ cells). Once the cells were confluent and after 3h of STS treatment, the media was removed; cells were washed with PBS, fixed with 4% formaldehyde for 10min, washed again with PBS and incubated with 20ul/ml of Annexin-V-Fluos (Roche) for 15min in the dark. Then, cells were washed twice with PBS and the glass cover slip was fixed on a microscope slide covered with a drop of the mounting media (Santa-Cruz) containing DAPI. Slides were then visualized under a confocal microscope (Zeiss LSM 7-10).

3. MitoPT JC-1 assay

Mitochondrial Depolarization (MitoPT) is an apoptotic hallmark. JC-1 is a lipophilic dye that can easily penetrate cell and mitochondrial lipid bilayer membrane

barriers. MitoP JC-1 reagent is in the aggregated form and fluoresces orange in healthy negatively charged mitochondria. In contrast, the reagent is dispersed in a monomeric form and fluoresces green when the mitochondrial potential collapses in apoptotic cells. PC12 cells were seeded on glass cover slips in 12-well plates ($150x10^3$ cells). Once the cells were confluent and after 3h of STS treatment, the media was removed; cells were washed with PBS and incubated with 10ul/ml of MitoPT^{TM} JC-1 reagent (ImmunoChemistry Technologies) for 15min in the dark. Then, cells were washed with PBS and the glass cover slip was fixed on a microscope slide, visualized under Olympus BH2-RFCA fluorescence microscope at a magnification of 20x.

K. Immunofluorescence

PC12 cells were seeded on glass cover slips in 12-well plates ($150x10^3$ cells). Once the cells were confluent and after treatment, the media was removed; cells were washed with PBS, fixed with 4% formaldehyde for 10 min, washed again with PBS, permeabilized with 0.5% Triton X-100 for 5 minutes and blocked with 1X PBS containing 2% BSA for 1 hour at room temperature. Cells were probed with one of the following primary: B2R (1:500, Abcam), B1R (1:50, Santa Cruz), Vimentin (1:200, Santa Cruz), GFAP (1:100, BD Pharmingen), NeuN (1:100, Millipore), Beta III Tubulin (1:250, Abcam), Cd11b (1:50, BD Pharmingen), overnight at 4^{o}C in 1X PBS containing 2% BSA. The next day, cells were washed with 1X PBS-Tween, and incubated with a fluorophore-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody at a dilution of 1:200 for 1hr at room temperature. Nuclei were stained with DAPI. Images were taken using LSM 7-10 Confocal Laser microscope at 63X magnification.

L. Fluo-4 AM calcium staining

PC12 cells were seeded on glass cover slips in 12-well plates ($150x10^3$ cells). Once the cells were confluent and after treatment, the media was removed; cells were washed with PBS and incubated with 2uM Fluo-4 AM (Molecular Probes™ Life Technologies) for 15min in the dark. Then, the cells were washed with PBS and the glass cover slip was fixed on a microscope slide, visualized under a confocal microscope (Zeiss LSM 7-10).

M. Transcriptional expression of B1R and B2R genes upon treatment with STS using real-time Polymerase Chain Reaction (rt-PCR)

1. RNA extraction

PC12 cells were seeded in petri dishes (2.5×10^6 cells) and STS treated for 3h and 12h. At each time point, the media was removed and cells were washed with PBS and stored at -80°C. RNA extraction was performed using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Finally, RNA content was quantified with nanodrop (Spectrophotometer ND-1000, USAID) and stored at -80°C for subsequent cDNA synthesis.

2. cDNA synthesis and real-time PCR

Following RNA extraction, cDNA was synthesized from 1 μg of total cellular RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was then performed using the homemade 2x Real-time PCR Master Mix (gift from Dr. Daoud's Lab) in a CFX96 system (Bio-Rad). Amplification of Real-time PCR was done using primers (TIB Molbiol) listed in table 3. This reaction was performed as follows: precycle of 95°C for 10 minutes for an initial denaturation

followed by 40 cycles each consisting of an initial 95°C step for 10 seconds, then 58°C for 30 seconds, 72°C for 1 minute and finally a step of extension after the cycles consisting of 72°C for 10 minutes. The experiment was prepared in duplicates and the results were analyzed to obtain the fold change: each gene has a threshold cycle (Ct), which was normalized to GAPDH (gift from Dr. Jaffa's lab), the housekeeping gene, and the control was used as a reference for comparative analysis.

Gene of Interest	Primer sequence
B1R	F: GCGACGGCAAGCCCAAGCTA R: TGCCAAGCCTCGTGGGGGAA
B2R	F: GCTTGGCGTGCTGTCGGGAT R: TCGGAAGCGCTTGCCCACAA
GAPDH	F: TGGTGCTCAGTGTAGCCCAG R: GGACCTGACCTGCCGTCTAG

Table 3: Primer sequences for real time PCR.

N. Translational expression of apoptotic and KKS proteins upon treatment with STS using Western Blotting analysis

1. Primary neurocortical cell lysate collection and preparation

Primary neuronal cell cultures were harvested and lysed for 90 min at 4^{o}C with 50 mM Tris (pH 7.4), 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT and 1x protease inhibitor cocktail (Roche). The neuronal lysates were then centrifuged at 15000g for 5 min at 4^{o}C to clear and remove insoluble debris, snap-frozen, and stored at -80^{o}C until use.

2. PC12 cells protein extraction

PC12 cells (2.5 x 10^6 cells) were cultured in petri dishes and incubated at 37°C, 5% CO_2. Once the cells where confluent they were treated with: STS, B2A,

B2A+STS, B2I, B2I+STS, B1A, B1A+STS, B1I and B1I+STS. When time point was reached (3h, 12h or 24h), cells were collected by trypsinization and stored at -80°C, in PBS. Protein extraction was performed by adding Ripa lysis buffer (250mM Tris-HCl pH=8, 750mM sodium chloride, 5% NP-40, 5% sodium deoxycholate and 5% sodium dodecyl sulfate), along with protease and phosphatase inhibitors and dithiothreitol (DTT).

3. Protein quantification

Protein quantification was performed using the DC Protein Assay (Bio-Rad) as per manufacturer's recommendations employing bovine serum albumin (BSA) as a standard. Finally, equal amount of proteins (30ug) suspended in lysis buffer and Leammli buffer 2X (Biorad), were boiled for 5min and stored at -20°C.

4. Western Blotting

WB is performed to study protein expression levels. Samples were loaded into a 6%, 10% or 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), subjected to electrophoresis, and transferred onto a methanol activated polyvinylidene fluoride (PVDF) membrane overnight. The membranes were then blocked with 5% fat-free milk prepared in phosphate-buffered saline containing 0.05% Tween-20 for 1h at room temperature. Membranes were then incubated overnight at 4°C with the following primary antibodies: AIF (1:1000, Santa Cruz), BCl2 (1:1000, Cell Signaling), PARP (1:200, Santa Cruz), B2R (1:1000, Abcam), NOX-4 (1:1000, Abcam, gift from Dr. Jaffa's lab), Bax (1:150, BD Pharmingen) and anti-β Actin to verify equal loading. The next day, membranes were washed with TBST (Tris-

Buffered Saline and Tween 20), incubated with HRP-conjugated secondary antibodies (1:7000, Santa Cruz), washed again with TBST and then protein bands were visualized using electrochemiluminescence ECL (Roche).

5. Statistical Analysis

Protein levels were quantitated using the Image J software. Results of all measurements were expressed as mean +/- SE. Statistical significance was assessed by the Student's unpaired *t* test, and probability values (*p*) less than 0.05 were considered significant.

O. Proteomic analysis

Proteomic analysis was applied to evaluate the role of the KKS in PC12 cells post STS-induced apoptosis, mimicking brain protein dynamics following traumatic brain injury. Proteins were extracted from PC12 cells and subjected to LC-MS/MS analysis.

Table 4 shows the PC12 samples sent for proteomic analysis with the different treatments used: Ctrl, STS. B1A+STS, B2A+STS, B1I+STS and B2I+STS. PC12 cells were pre-treated for one hour before being treated with STS for 3 hours.

Tube number	1	2	3	4	5	6
Pre-treatment (1h)	Ctrl	-	BR1 agonist (0.1uM)	BR2 agonist (0.1uM)	BR1 inhibitor (1uM)	BR2 inhibitor (1uM)
Treatment (3hrs)		STS				

Table 4: PC12 samples sent for proteomic analysis.

LC-MS/MS was acquired using Dionex 3000 Ultimate nano-LC system (Dionex, Sunnyvale, CA), LTQ Orbitrap Velos and TSQ Vantage mass spectrometers (Thermo Scientific, San Jose, CA). The LC elution gradient of solvent B was: 5% over 10 min, 5%-20% over 55 min, 20-30% over 25 min, 30-50% over 20 min, 50%-80% over 1 min, 80% over 4 min, 80%-5% over 1 min and 5% over 4 min. Solvent B consisted of 98% ACN containing 0.1% formic acid (FA) while solvent A composed of 98% HPLC water containing 0.1% FA. The LTQ Orbitrap Velos mass spectrometer was operated in data-dependent acquisition mode comprised of two scan events. The first scan event was a full MS scan of 380-2000 *m/z* at a mass resolution of 15,000. The second scan event was CID MS/MS of parent ions selected from the first scan event with an isolation width of 3.0 m/z, at a normalized collision energy (CE) of 35%, and an activation Q value of 0.250. The CID MS/MS scans were performed on the 30 most intense ions observed in the MS scan event. The dynamic exclusion was set to have repeat count of 2, repeat duration of 30s, exclusion list size 200 and exclusion duration of 90s.

LC-ESI-MS/MS data was searched using SwissProt database (Rattus) in MASCOT version 2.4 (Matrix Science Inc., Boston, MA). Scaffold Q+ (Proteome Software, Portland, OR) was employed for spectral counts quantitation.

Finally, a systems biology analysis was performed on the proteins exhibiting significant up- or down- regulations, using Protein Analysis Through Evolutionary Relationships PANTHER system (http://www.pantherdb.org/genes/batchIdSearch.jsp) and Pathway Studio 8.

Although, the number of proteins per cell compartment is further defined with PANTHER, a protein may be represented in more than one category so that the total

number of proteins per pie chart does not equal the total number of proteins detected per analysis. Therefore, Venn diagrams were used to show the number of overlapping proteins when comparing the different conditions/treatments used on PC12 cells, using VENNY software.

RESULTS

A. Neurite outgrowth in differentiated PC12 cells

Typical PC12 cells are spherical in shape and do not produce neurites. It is only after exposure to NGF that they fully differentiate, stop proliferating and extensions start sprouting in the periphery. Phase-contrast images of NGF-treated PC12 cells clearly show neurite outgrowth and intercellular contacts (Figure 4). A yellow arrow is used to show the location of the neurites and a line delineates the length of an extended neurite. Formation of intercellular contacts i.e. physical interaction of one neurite with another is considered an essential factor of PC12 cells in neuronal morphology study.

Maturity of the neuron and neurite sprouting ability were further investigated using immunofluorescence for NeuN and beta III tubulin, respectively. Figure 5 depicts the maturity of PC12 cells reached after NGF treatment post 7 days. Intercellular contacts and neurite outgrowth are highlighted by the high fluorescent intensity of NeuN (green) and beta III tubulin (red) staining markers. Figure 6 shows immunofluorescent staining of undifferentiated cells (Figure 6A) and NGF-induced differentiated PC12 cells (Figure 6B and 6C), with apparent well-formed microtubules extending in the periphery.

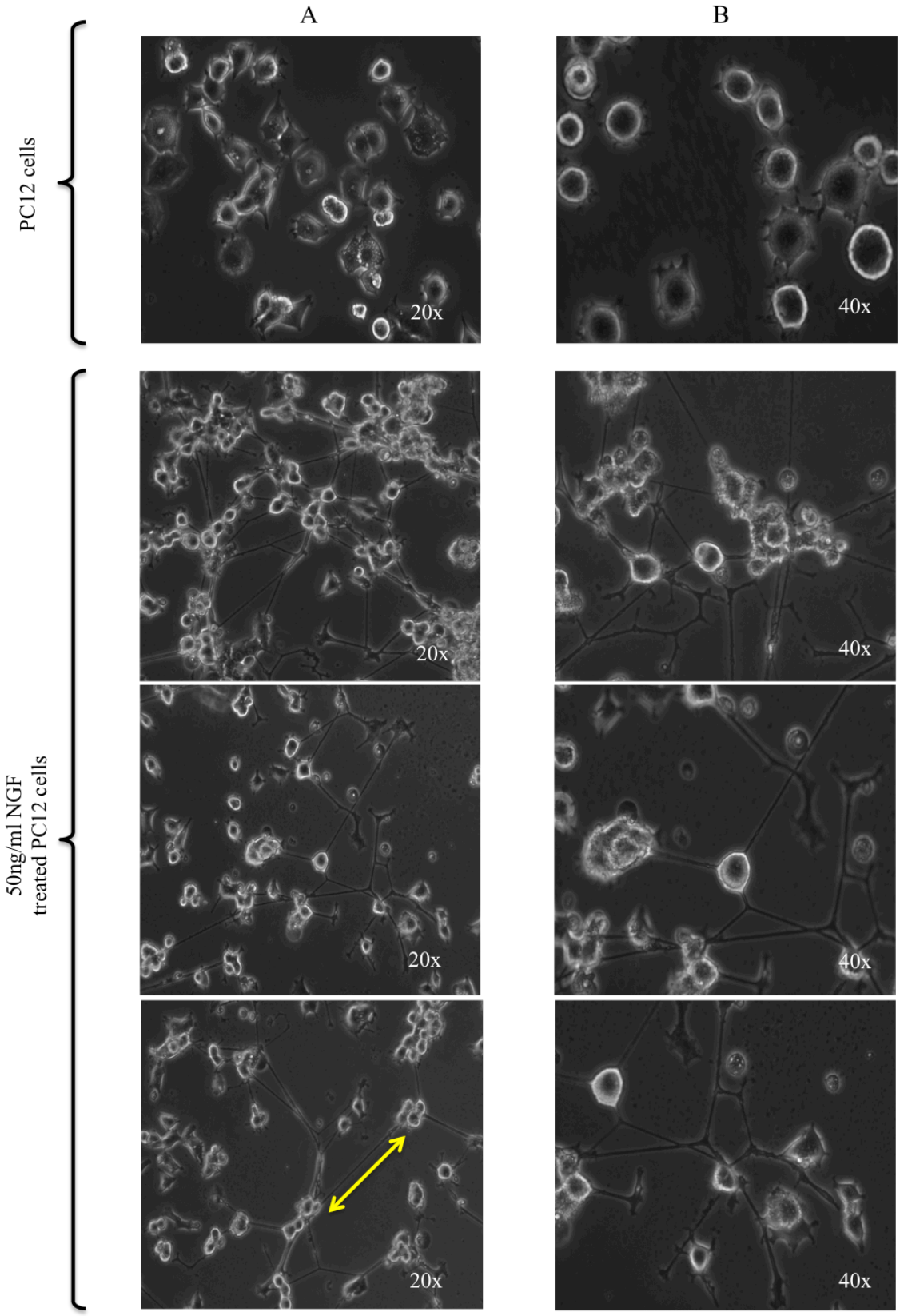

Figure 4: Phase contrast images of PC12 cells following 50ng/ml NGF treatment post 7 days. Images were taken using Zeiss Microscope at 20X (A) and 40X (B).

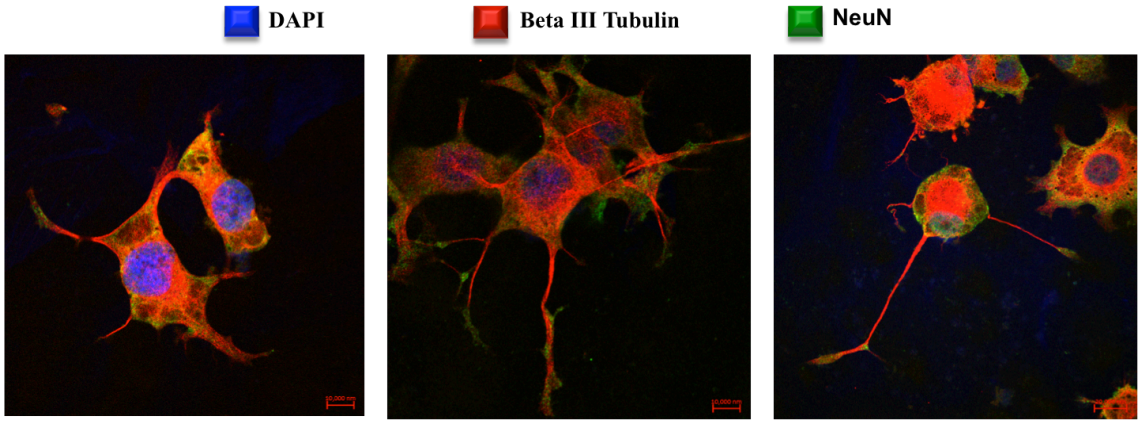

Figure 5: Neuronal maturity and sprouting ability investigated by NeuN (green) and beta-tubulin III (red) staining of PC12 cells exposed to 50ng/ml NGF for 7 days, using oil lens LSM 7-10 Confocal Laser microscope at 63x magnification.

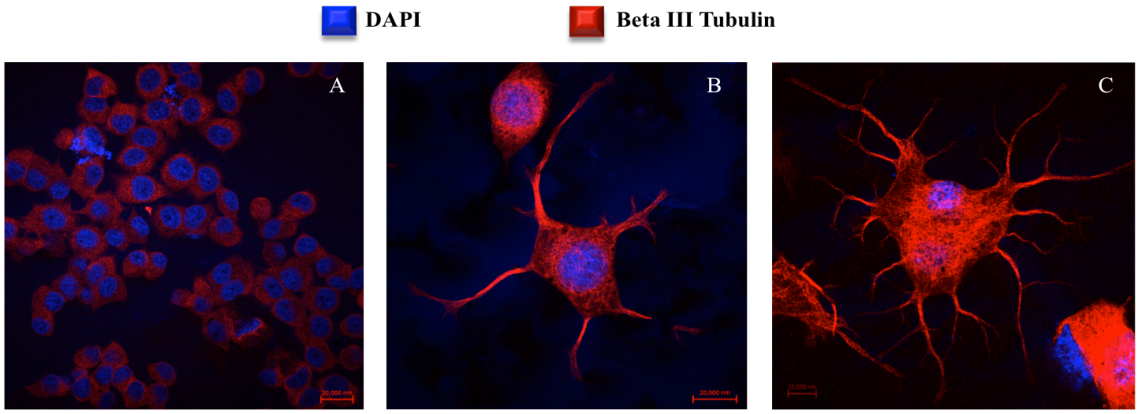

Figure 6: Sprouting analyzed by beta III tubulin (red) staining of PC12 cells after incubation with 50ng/ml NGF for 7 days, using oil lens LSM 7-10 Confocal Laser microscope at 63x magnification. (A) Undifferentiated PC12 cells, (B and C) NGF treated PC12 cells.

B. Apoptotic effect of STS on PC12 cells: qualitative interpretation

1. DAPI nuclear staining

DAPI is a fluorescent nuclear stain used to visualize neurons undergoing DNA fragmentation and nuclear condensation. DAPI preferentially binds to double stranded DNA and particularly associates with AT clusters in the minor groove. Assessment of apoptotic cell death using DAPI staining confirmed the harmful effect of STS on PC12

cells after only 3 hours of treatment. Indeed, STS appeared to disturb the integrity of the nucleus, causing nucleus shrinkage, fragmentation and the formation of apoptotic bodies, as shown in figure 7.

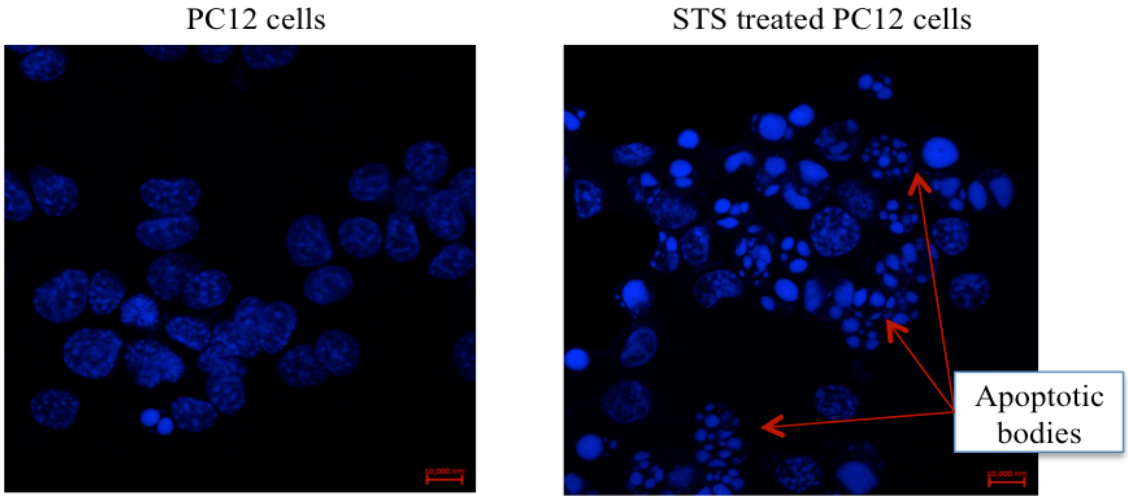

Figure 7: DAPI nuclear staining showing apoptotic bodies upon 3h STS treatment of PC12 cells.
Images were taken using 7-10 LSM Confocal Laser Microscope at 63X magnification.

2. Annexin-V Fluos staining

Annexin-V assay is an effective method to detect one of the earliest event in apoptosis: translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer membrane. Annexin-V is a phospholipid binding protein that is labeled with a fluorophore and can identify apoptotic cells by binding to the externalized exposed phosphatidylserine with strong affinity. The harmful apoptotic effect of STS is once more confirmed by the apparent Annexin-V fluorescent apoptotic marker, in STS treated PC12 cells after 3h of treatment (Figure 8).

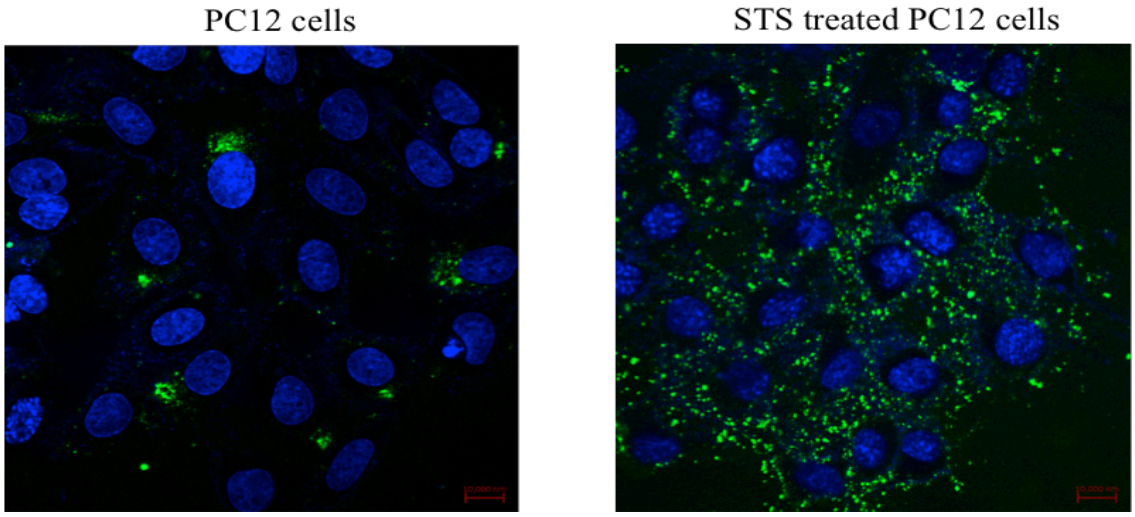

Figure 8: Annexin-V Fluos staining showing membrane flipping as a result of apoptosis induced by 3h STS treatment of PC12 cells.
Images were taken using 7-10 LSM Confocal Laser Microscope at 63X magnification.

3. MitoPT JC-1 assay

Mitochondrial permeability transition was visualized using the fluorescent JC-1 reagent. Orange fluorescence implies intact mitochondria whereas green fluorescence denotes a collapse in mitochondrial potential. Figure 9 shows that STS induces a mitochondrial depolarization in PC12 cells after 3 hours of treatment.

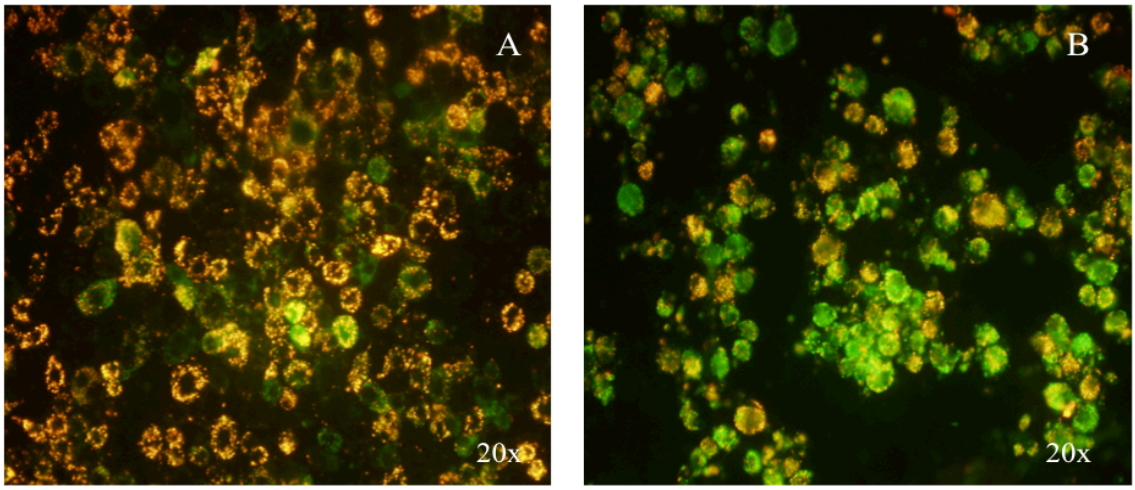

Figure 9: MitoPT JC-1 staining showing mitochondrial depolarization upon 3h STS treatment of PC12 cells. (A) PC12 cells, (B) STS treated PC12 cells.
Images were taken using Fluorescent Microscope at 20X magnification.

C. Visual confirmation of cell death and morphological changes

PC12 cells were pre-treated with the respective agonists and antagonists of B1R and B2R for 1h before being treated with 1uM STS for 3h and 12h (Figure 10). The aim was to determine an eventual protective role of the KKS players when the cell was subjected to the neurotoxic compound STS.

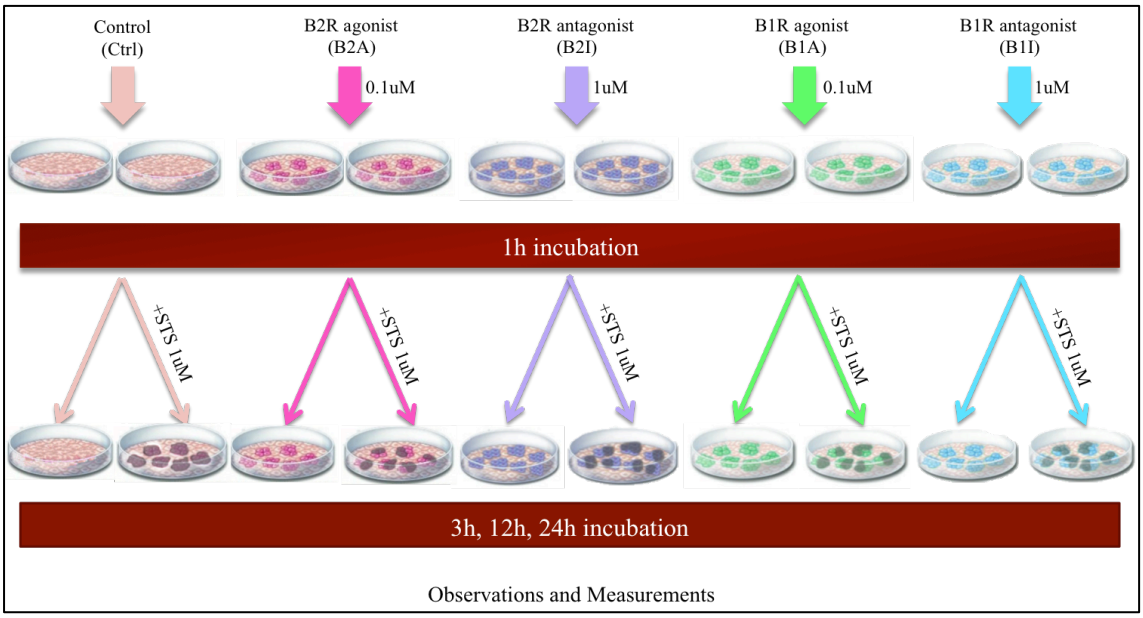

Figure 10: Schematic representation of PC12 cells in cell culture. PC12 cells were pre-treated with B1A (0.1uM), B2A (0.1uM), B1I (1uM) or B2I (1uM) for 1h before being treated with 1uM of STS. The cells were incubated for another 3h, 12h or 24h before being measured and compared to the control and to STS-non-treated cells.

Nevertheless, it was visually observed that the KKS components were solely capable of slightly postponing apoptotic cell death triggered by STS. Indeed, at 3h post STS-treatment B1A, B1I, B2A, B2I pre-treated cells show less nuclear disintegrity than STS treated cells only (Figure 10A). However, this was not the case at 12h post-treatment were PC12 cells shrinkage and nuclear collapse were clearly observed in all conditions (Figure 10B). Importantly, there are no morphological distinction following activation or inhibition of B1R and B2R.

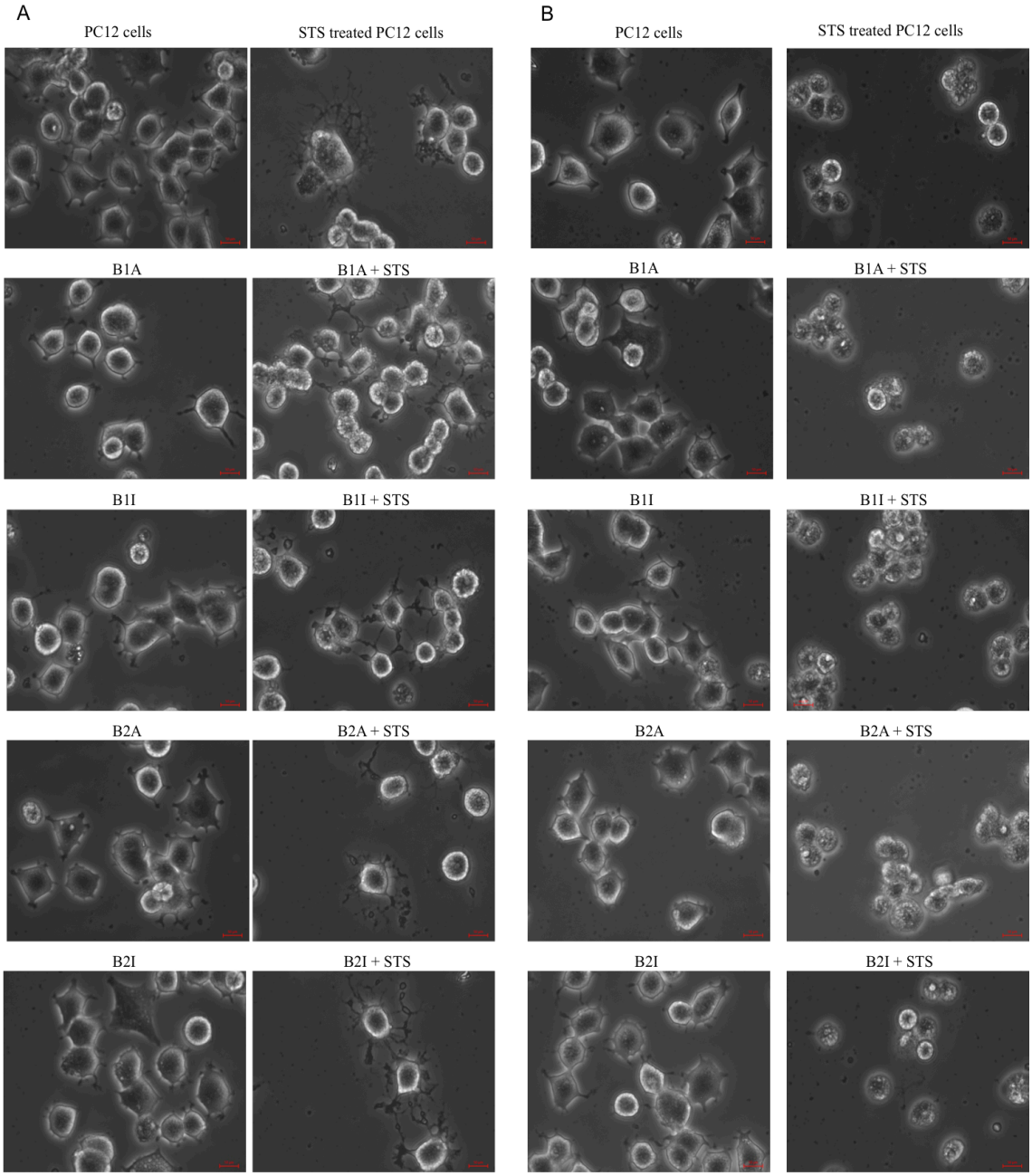

Figure 11: Cell death and morphological changes of pre-treated PC12 cells following STS treatment for (A) 3h and (B) 12h.
Cells were visualized by Carl Zeiss microscope at 40x magnification.

D. Effect of STS on the proliferation of PC12 cells: MTT assay results

MTT assay was performed to reflect the effect of STS on the mitochondrial enzymatic activity of PC12 cells and to confirm quantitatively the non-protective role of KKS players, observed visually. The results obtained by MTT assay are shown in figure

11. It is observed that after 3h of treatment, STS is killing approximately 60% of PC12 cells and on average 50% of pre-treated PC12 cells. This minor protective role of KKS players was denoted previously in figure 10A. Nevertheless, the potent role of STS is highlighted by the fact that at this same time point, B1R and B2R agonists and antagonists are showing an increase in the proliferative activity of PC12 cells but fail to protect PC12 cells from the harmful effect of STS. Although B1A, B1I, B2A and B2I still increase the mitochondrial enzymatic activity of PC12 cells at 12h and 24h post-treatment, they do not exhibit any protection against STS treatment that kills 90% of PC12 cells. It is also important to note that activation or inhibition of B1R and B2R are showing similar results, at all time points: increase in proliferative activity of PC12 cells before STS treatment, and decrease in proliferative activity of PC12 cells following STS treatment.

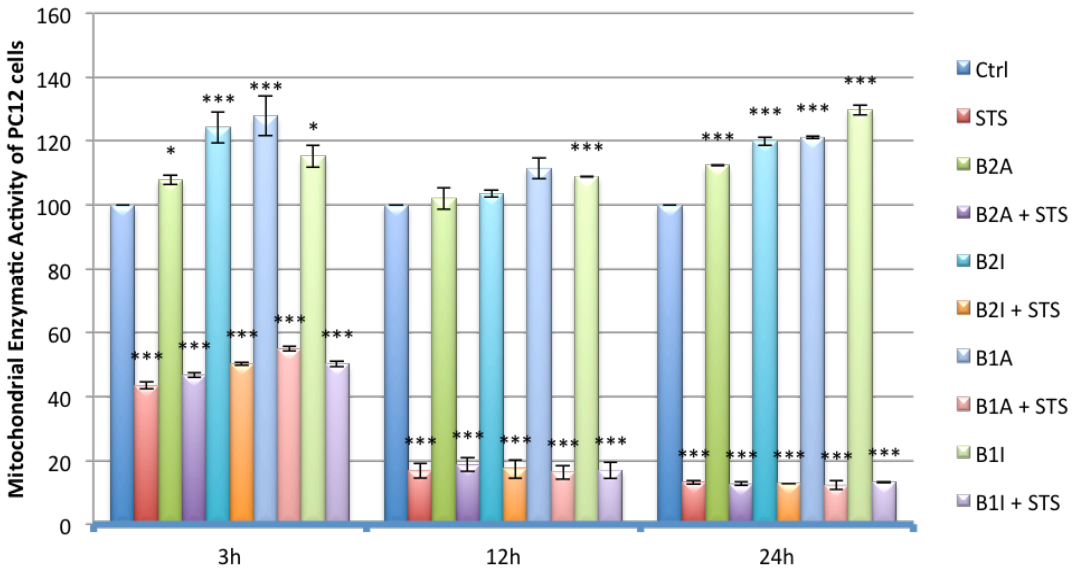

Figure 12: Inhibition in the proliferative activity of PC12 cells in response to STS treatments after 3h, 12h and 24h, using MTT assay (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

E. Cytotoxic effect of STS on PC12 cells: LDH assay results

LDH is a cytosolic enzyme released in the media when cells are damaged and serves therefore as a marker for cellular cytotoxicity and cytolysis. The results obtained by LDH assay are presented in figure 12. We can observe that at 3h post-STS treatment, LDH release is minimal in pre-treated PC12 cells and STS treated cells. However, at 12h and 24h post-STS treatment there is a huge increase in LDH release in STS treated PC12 cells as compared to untreated and solely pre-treated cells. The difference is the highest at 12h. These results correlate with the increase cell death observed in PC12 cells at 12h and 24h post-STS treatment. In other words, STS is reducing the proliferative activity of PC12 cells (MTT results) by increasing cell damage (LDH results) whether KKS players are present or not and whether they are activated or inhibited.

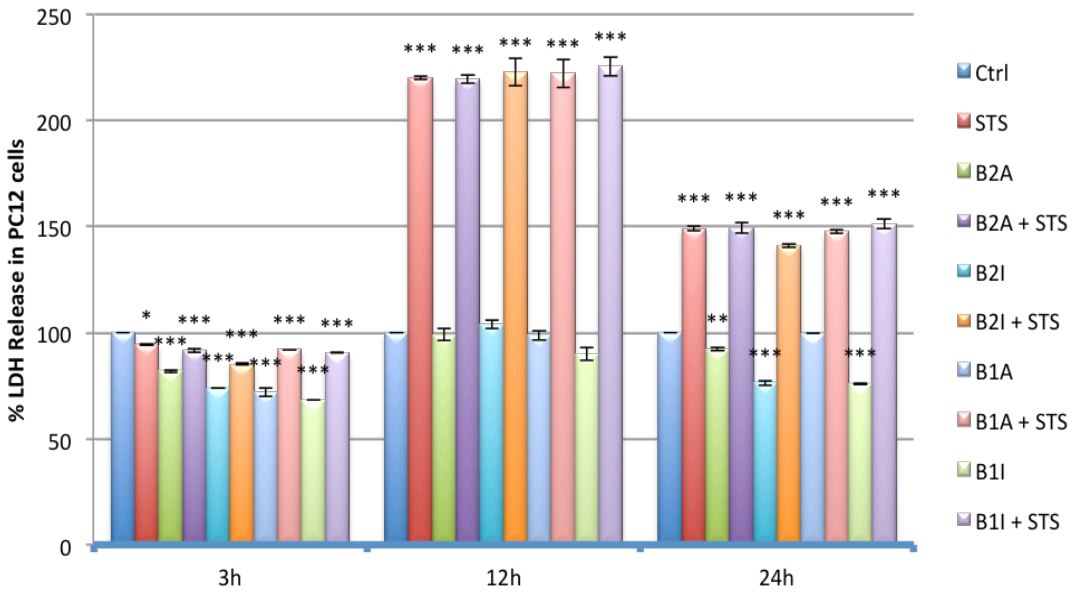

Figure 13: PC12 cell membrane damage in response to STS treatments after 3h, 12h and 24h, using LDH assay (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

F. Effect of STS on intracellular ROS production: NBT reduction assay

NBT is a salt that gets reduced when oxygen free radicals are oxidized. Thus, NBT reduction inversely represents the intracellular presence of ROS such as superoxide anion, peroxide and hydroxyl radical. According to the results obtained and listed in figure 13, STS has only a slight oxidizing effect on PC12 cells that does not vary much at the different time points. Indeed, at 3h, 12h and 24h, intracellular ROS production of STS treated PC12 cells does not exceed 50%. Once again, KKS components did not interfere with STS effect on intracellular ROS production.

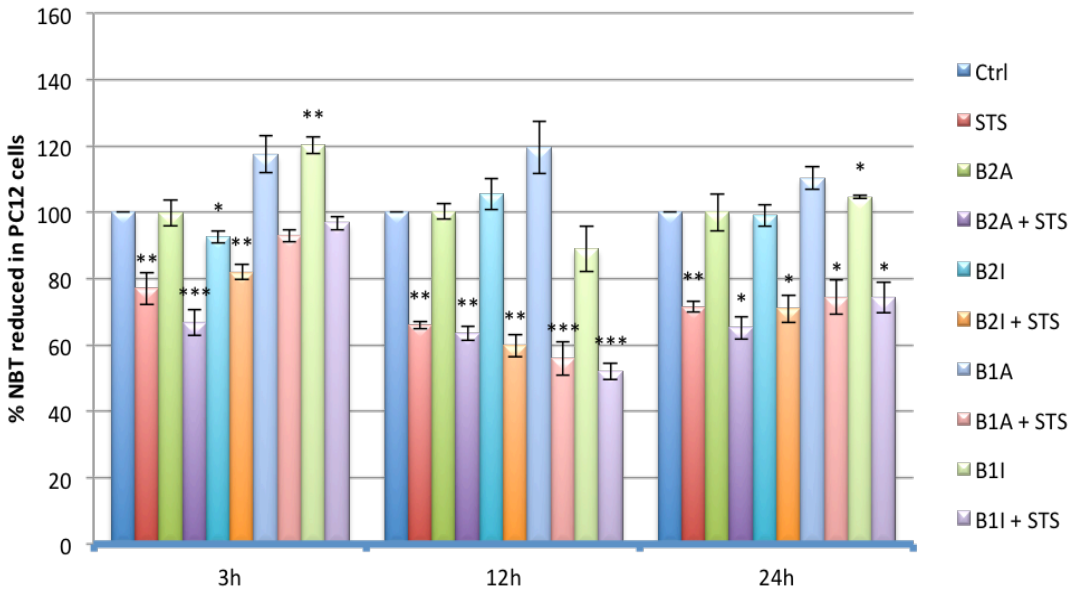

Figure 14: Intracellular ROS production in PC12 cells in response to STS treatment after 3h, 12h and 24h, using NBT assay (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$)**.**

G. Immunostaining analysis of B1R and B2R in PC12 cells

B1R is localized in the endoplasmic reticulum, and exists with a very low expression on the plasma membrane. In contrast, B2R is capable of efficiently exiting the endoplasmic reticulum, targeting the plasma membrane. Moreover B2R is a

constitutive receptor commonly found in healthy tissues, while B1R gets induced upon tissue injury and inflammation. Although previous experiments disproved the protective role of the KKS receptors following apoptotic cell death induced by STS, immunostaining of these receptors confirmed their involvement in the cell death mechanism. Interestingly, it is not until 12h post-STS treatment that B1R will be induced (Figure 14A). Inversely, B2R is expressed before STS treatment and gets highly expressed 3h post-STS treatment (Figure 14B). Consequently, the next experiments will mainly focus on the effect of the B2R 3h post-STS treatment.

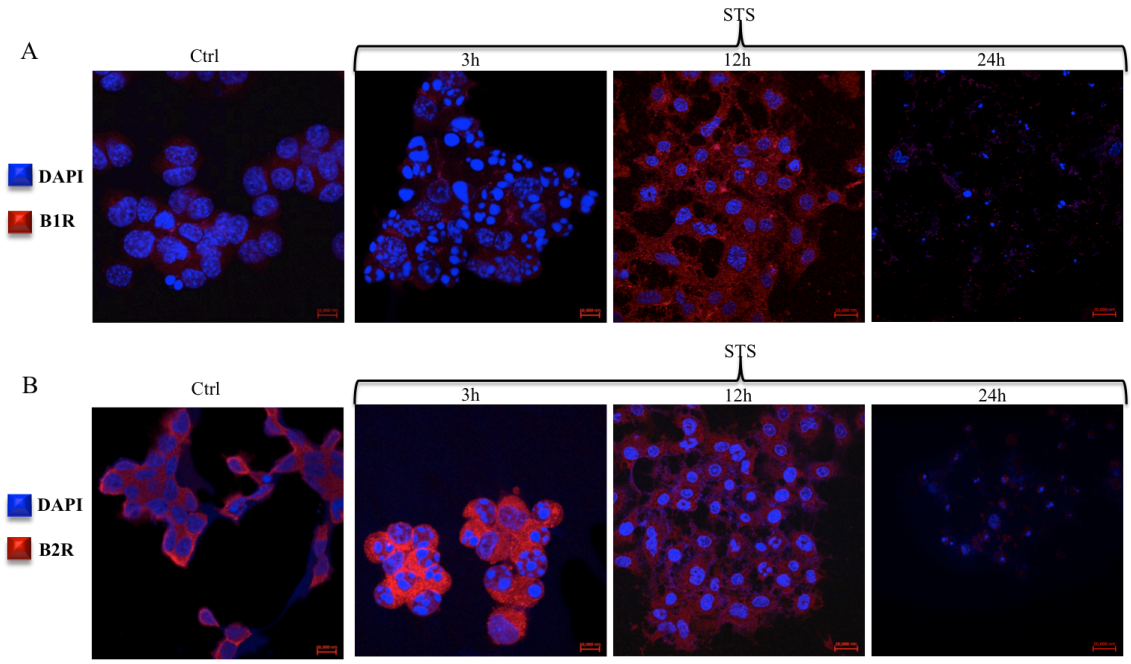

Figure 15: Immunostaining of (A) B1R and (B) B2R in PC12 cells in response to 3h, 12h and 24h of STS treatment. Images were taken using oil lens LSM 7-10 Confocal Laser microscope at 63x magnification.

H. Calcium detection in PC12 cells following STS treatment

It is classically admitted that severe calcium dysregulation promotes necrotic cell death. Nevertheless, new studies suggest a link between apoptosis and calcium

(Hajnoczky et al., 2003; Mattson and Chan, 2003; Orrenius et al., 2003). It is stated that controlled intracellular calcium increase induced by mild insult encourages cell death through apoptosis (Pinton et al., 2008). In compliance with this statement STS did not fail to show high intracellular calcium level 3h post-treatment as depicted in figure 15B. Likewise, calcium release was observed in B2A pre-treated PC12 cells and the effect was exacerbated following 3h STS treatment (Figure 15C and D). However, calcium release was completely inhibited in B2I pre-treated PC12 cells and even following 3h STS treatment (Figure 15E and F). B2R inhibition was unsuccessful protecting PC12 cells from apoptotic cell death following STS treatment, yet it appears to play a significant role in shutting down intracellular calcium release.

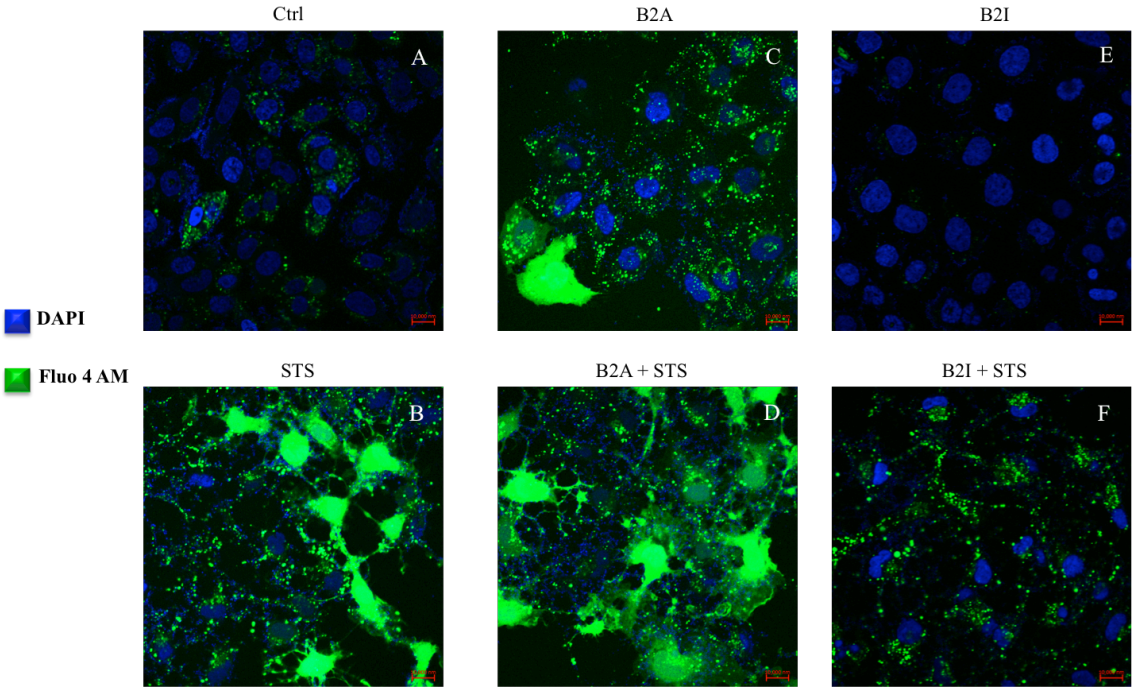

Figure 16: Fluo-4 AM staining of PC12 cells 3h post-STS treatment depicting intracellular calcium release. (A) PC12 cells control, (B) STS treated, (C) B2A pre-treated, (C) B2A pre-treated and STS treated, (D) B2I pre-treated and (E) B2I pre-treated and STS treated. Images were taken using oil lens LSM 7-10 Confocal Laser microscope at 63x magnification.

I. STS effect on the transcription of B1R and B2R genes: rt-PCR results

Since B1R and B2R were shown to be involved in STS mediated apoptotic cell death of PC12 cells but each one at a different time point, it was interesting to study the expression level of these receptors at the molecular level. rt-PCR was performed on PC12 cells 3h and 12h post STS treatment to assess the level of B1R and B2R gene expression. In figure 16, we can observe that at 3h post-STS treatment, B2R gene expression is higher than B1R gene expression in all conditions. Noteworthy, B2R gene expression is the highest following STS treatment and upon inactivation of B1R followed by STS treatment, suggesting a compensatory role of B2R when B1R is inactivated.

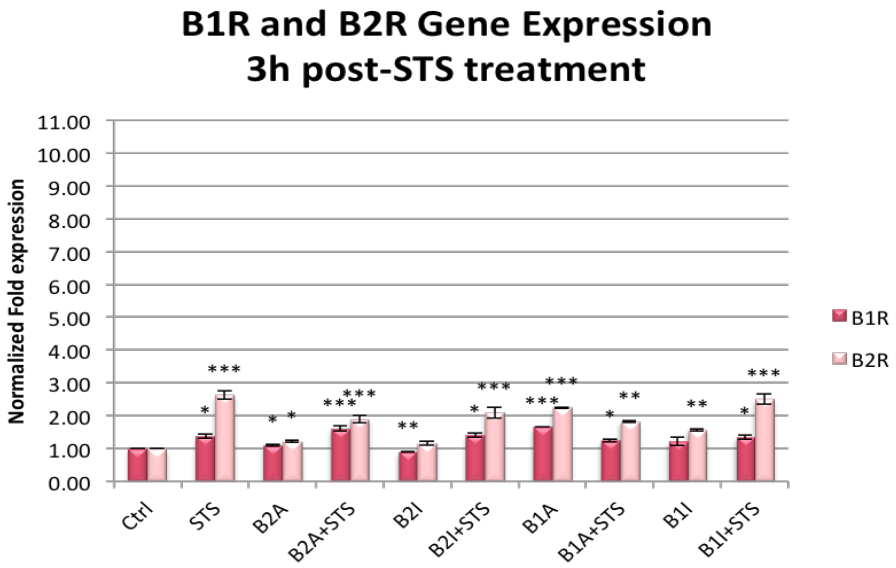

Figure 17: Expression of the B1R and B2R genes in PC12 following 3h treatment with STS, using rt-PCR assay (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

In addition, in figure 17, we can observe a tremendous increase in B1R and B2R gene expression 12h post-STS treatment. However, this time B1R gene expression is higher than B2R gene receptor in all conditions except STS, B2A+STS and B1I+STS

conditions. These 3 conditions show the highest gene expression of B1R and B2R with an obvious advantage for B2R, suggesting that B2R is the main player in STS induced apoptotic cell death in PC12 cells and is still compensating for B1R inhibition. Moreover, it is now observed that B1R can also compensate for B2R inhibition.

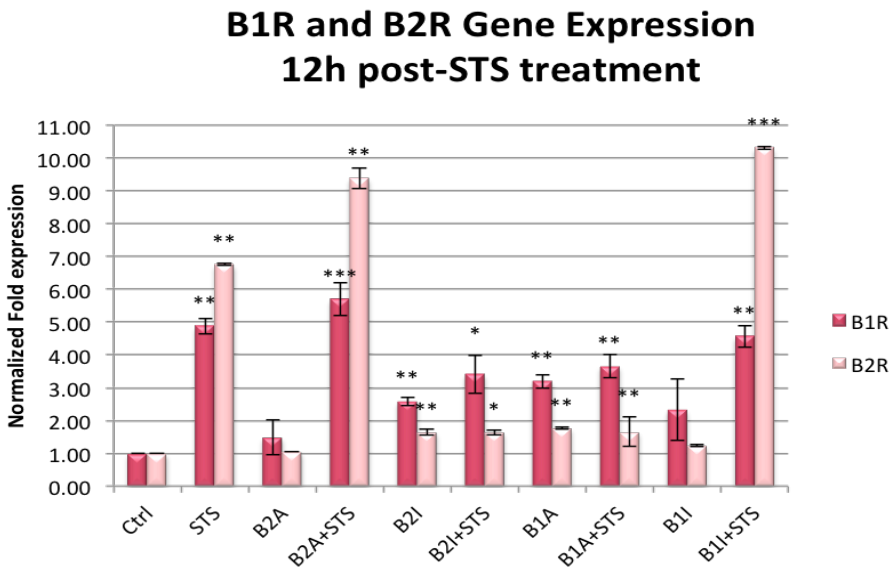

Figure 18: Expression of the B1R and B2R genes in PC12 following 12h treatment with STS, using qPCR assay (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

J. STS effect on the translational level of B1R and B2R in PC12 cells: WB results

1. Quantitative analysis of apoptotic markers following STS treatment

To further investigate the effect of Bdk receptors on STS-induced apoptosis, Western Blotting was performed and the protein expression of the apoptotic markers PARP, BCl2 and AIF was assessed. The experiment was carried out at 3h after STS treatment and the results are shown in Figure 18. It is observed that PARP, an enzyme playing a role in DNA repair mechanism and programmed cell death, is inhibited by cleavage of its full-length form (116 KDa peptide) into its 89 KDa polypeptide form

(Figure 18A). Moreover, AIF, a protein involved in initiating a caspase-independent pathway of apoptosis by causing DNA fragmentation and chromatin condensation, is increased following STS treatment (Figure 18B). Finally, BCl2, an anti-apoptotic regulator protein, is significantly inhibited with STS treatment (Figure 18C). Remarkably, the increase of AIF and the decrease of BCl2 were independent of Bdk receptors activation or inhibition, underlining once more their non-protective roles during STS mediated apoptosis in PC12 cells. In addition, these results establish an evidence for STS induction of caspase-dependent and caspase-independent apoptosis.

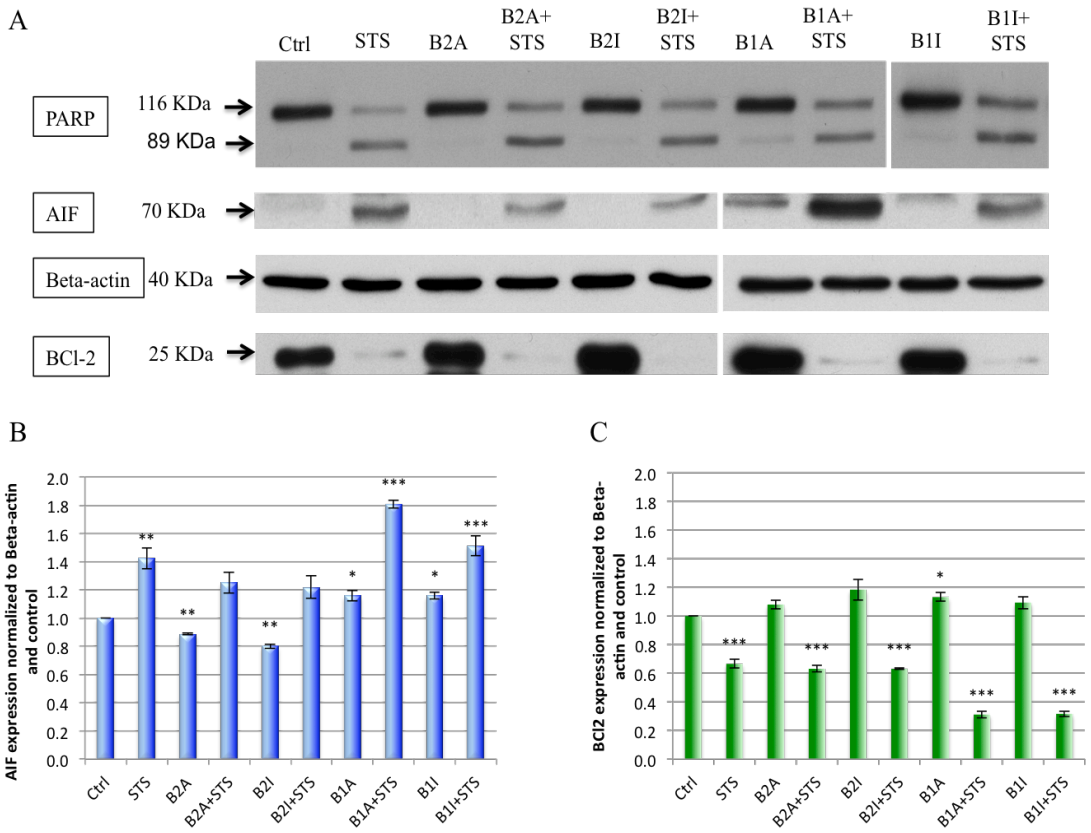

Figure 19: AIF protein expression increase, BCl2 protein expression decrease and PARP cleavage upon 3h STS treatment of PC12 cells.

(A) Western Blot of protein extracts from pre-treated and treated PC12 cells.
(B) Histogram representing densitometry analysis of AIF expression using Image J software (n=3)
(C) Histogram representing densitometry analysis of BCl2 expression using Image J software (n=3)

2. *Quantitative analysis of B2R following STS treatment*

Since it was previously demonstrated that B2R is the KKS player that is mainly involved in STS mediated apoptosis, B2R protein expression was studied in PC12 cells 3h post-STS treatment. In Figure 19, we can clearly see that B2R protein expression is high in STS treated PC12 cells and in B1A, B1A+STS, B1I, B1I+STS conditions. Hence, the involvement of B2R in STS mediated apoptosis is undeniable. It can also be stated that B2R is acting to help B1R when the latter is activated, and to compensate for it once it is inhibited.

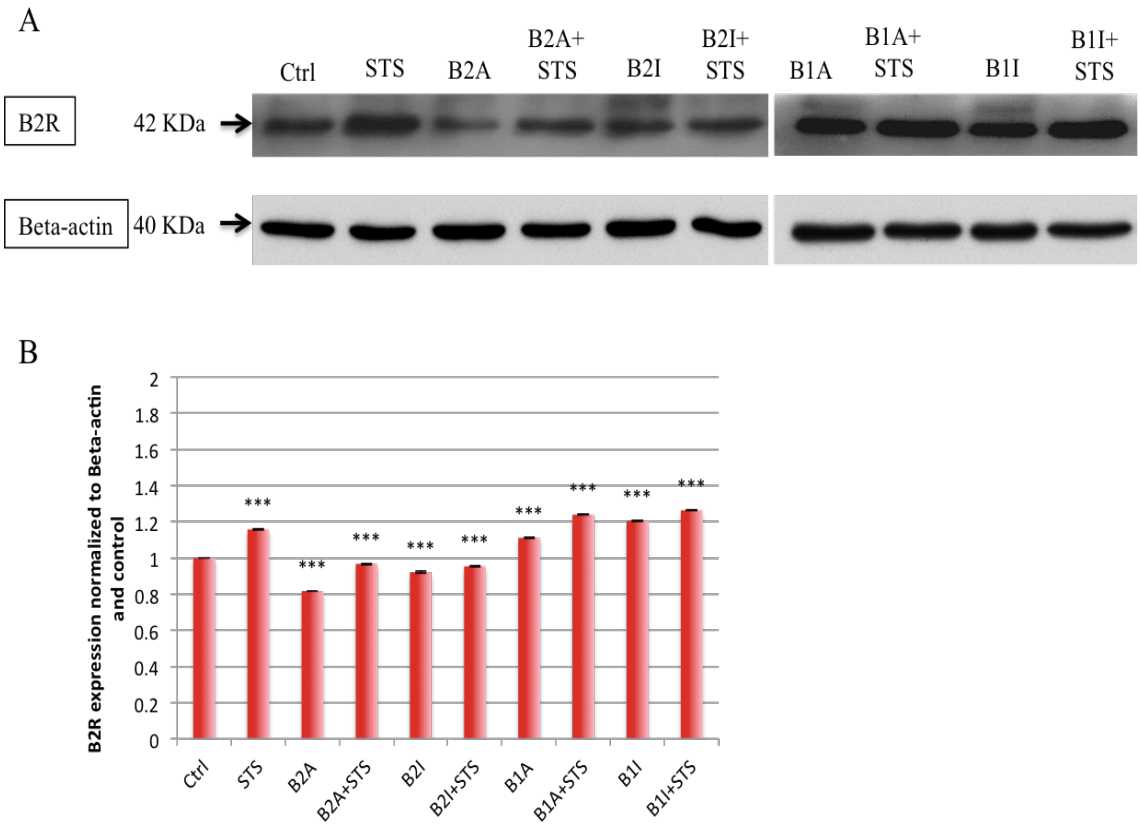

Figure 20: B2R protein expression upon 3h STS treatment of PC12 cells.
(A) Western Blot of protein extracts from pre-treated and treated PC12 cells.
(B) Histogram representing densitometry analysis of B2R expression using Image J software (n=3)

K. Proteomics data

1. Overall Proteome

LC-MS/MS (2hrs) analysis of proteome extracted from PC12 cells permitted the total identification of 440 proteins.

By comparing the STS treated group with the control group, 62 proteins were identified; among which no proteins were unique to the STS treated group while only 1 protein was unique to the control group.

Likewise, a comparison between the B1A+STS treated group and the control group revealed 261 proteins. 30 proteins were found unique to the treated group and 8 proteins were unique to the control group.

Similarly, 264 proteins were identified in B2A+STS treated group and control group, with 32 proteins unique to the treated group and 4 proteins found only in the control group.

Also, a comparison between B1I+STS treated group and control group show 269 proteins with 35 proteins unique to the treated group and 7 proteins solely found in the control group.

Finally the highest number of proteins, 301 proteins, was found while comparing the B2I+STS treated group with the control group. Among these proteins, 43 proteins were unique to the treated group and just 3 proteins were exclusively found in the control group.

The results are summarized in Table 5 and Venn Diagrams (Venny software) are used to better visualize proteins comparison between the control group and each treated group (Figure 21).

Groups Compared	Total number of proteins	Proteins unique to treated group	Proteins unique to control group
Ctrl vs. STS	62	0	1
Ctrl vs. B1A+STS	261	30	8
Ctrl vs. B2A+STS	264	32	4
Ctrl vs. B1I+STS	269	35	7
Ctrl vs. B2I+STS	301	43	3

Table 5: Number of proteins identified while comparing treated and control groups.

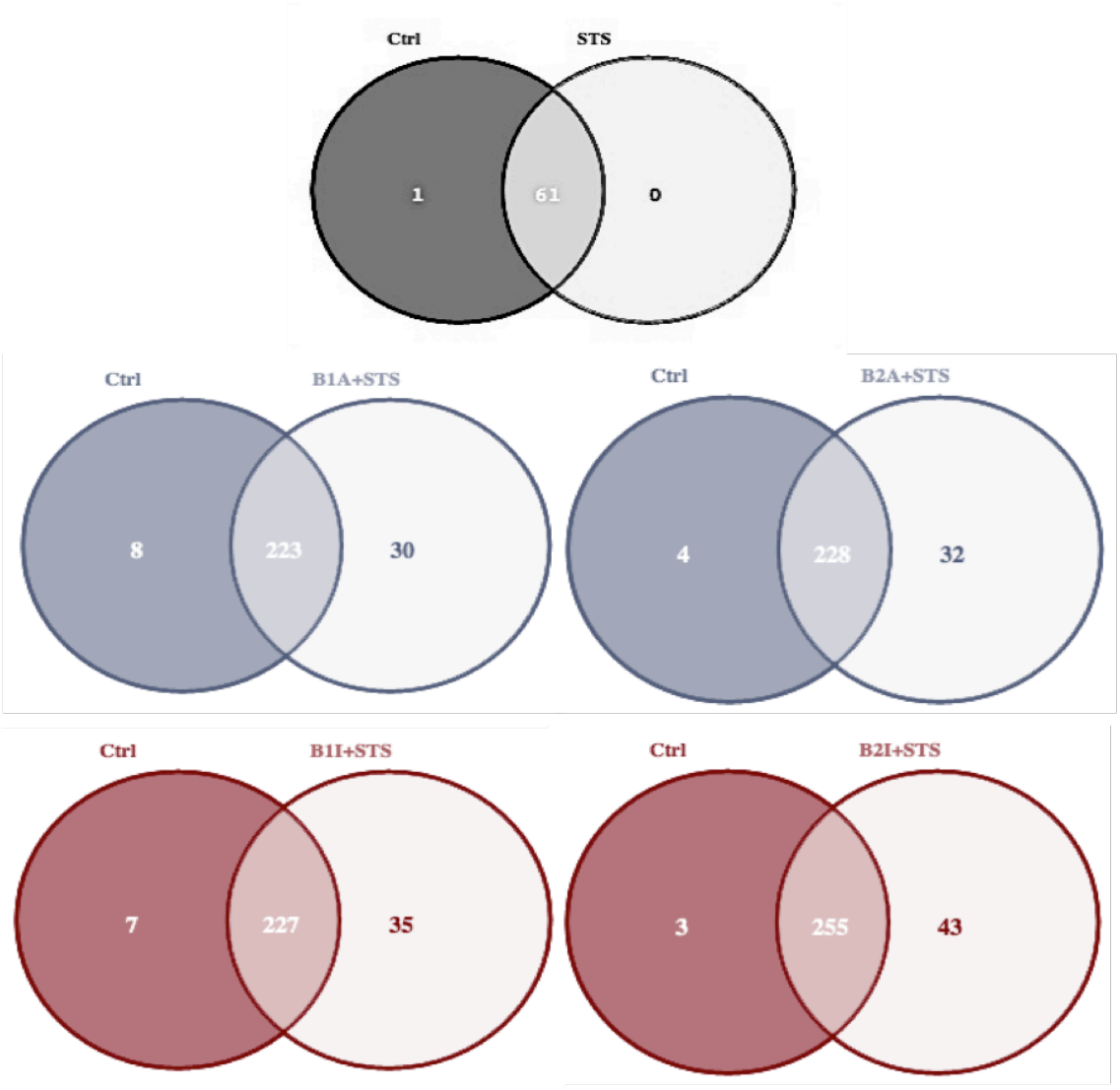

Figure 21: Venn Diagrams depicting protein list comparison between treated and control groups, using Venny Software.

2. Spectral Count Quantitation

Spectral counts of identified proteins were used to perform a preliminary label free quantitation.

Among the total 62 proteins identified in the Ctrl vs. STS group, 61 proteins were thought to be significantly different based on an independent t-test ($p< 0.05$). According to the spectral count data, 29 proteins were down-regulated while 32 proteins were up-regulated after STS treatment.

Similarly, for the 261 proteins identified in Ctrl vs. B1A+STS group, 223 proteins were thought to be significantly different. 125 proteins were down-regulated while 98 proteins were up-regulated.

Likewise, in the Ctrl vs. B2A+STS group, 228 out of 264 proteins were significantly different; 130 proteins were down-regulated and 98 proteins were up-regulated.

Equally, among the 269 proteins detected in the Ctrl vs. B1I+STS group, 227 proteins were significantly different. 124 proteins were shown to be down-regulated while 103 proteins were up-regulated.

Lastly, in the Ctrl vs. B2I+STS group, 255 out of 301 proteins were identified as significantly different. 161 proteins were down-regulated while 94 proteins were up-regulated.

Results are summarized in Table 6 and Venn Diagrams (Venny Software) are used to compare the amount of up-regulated (figure 22A) and down-regulated (figure 22B) proteins in the different groups. We can observe that among the different groups, 18 proteins are commonly up-regulated while 39 proteins are down-regulated.

Importantly, the highest number of unique up- and down-regulated proteins is

seen in the Ctrl vs. B1A+STS group when compared to the Ctrl vs. B2A/B1I/B2I groups.

Groups Compared	Total number of proteins	Significant proteins	Down-regulated proteins	Up-regulated proteins
Ctrl vs. STS	62	61	29	32
Ctrl vs. B1A+STS	261	223	125	98
Ctrl vs. B2A+STS	264	228	130	98
Ctrl vs. B1I+STS	269	227	124	103
Ctrl vs. B2I+STS	301	255	161	94

Table 6: Number of up- and down- regulated proteins identified while comparing treated and control groups.

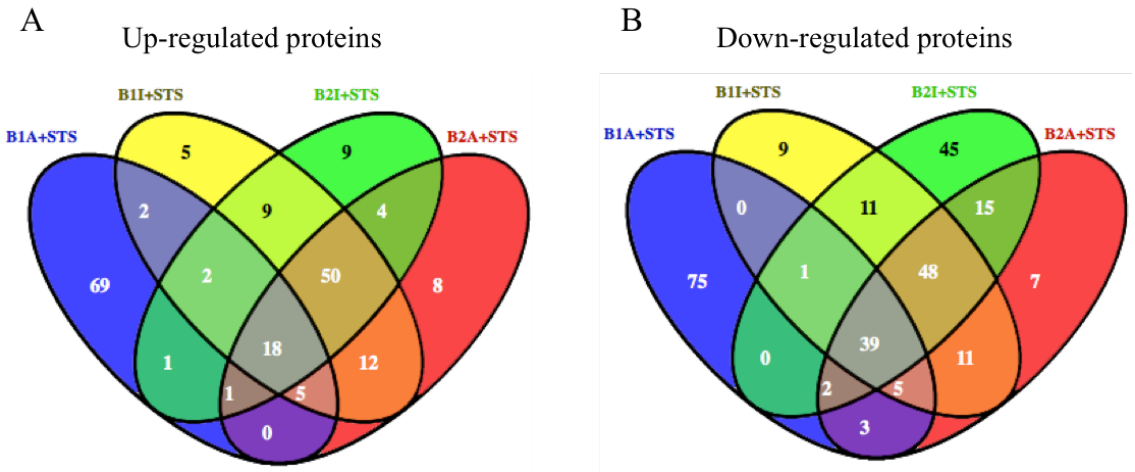

Figure 22: Venn Diagrams for comparison of up-regulated (A) and down-regulated (B) proteins in the different PC12 samples.

Also, a principal component analysis (PCA) was performed on the spectral count data to investigate internal variation between the different treated groups and the control group. Figure 23 depicts the PCA scoring plots of PC12 samples proteome. According

to PCA, the different experimental and control groups of PC12 proteome were clearly parted from each other. Indeed, the data points of STS, B1A+STS, B1I+STS, B2A+STS and B2I+STS treated groups appeared to be far-apart from the control group suggesting that these treatments have a clear influence on PC12 cells. Of importance, the data points of B2A+STS and B2I+STS groups, although remote from the data points of the control group, appeared close to each other. This aspect enforces the fact, previously observed, that activation or inhibition of B2R following STS treatment does not differ in effect.

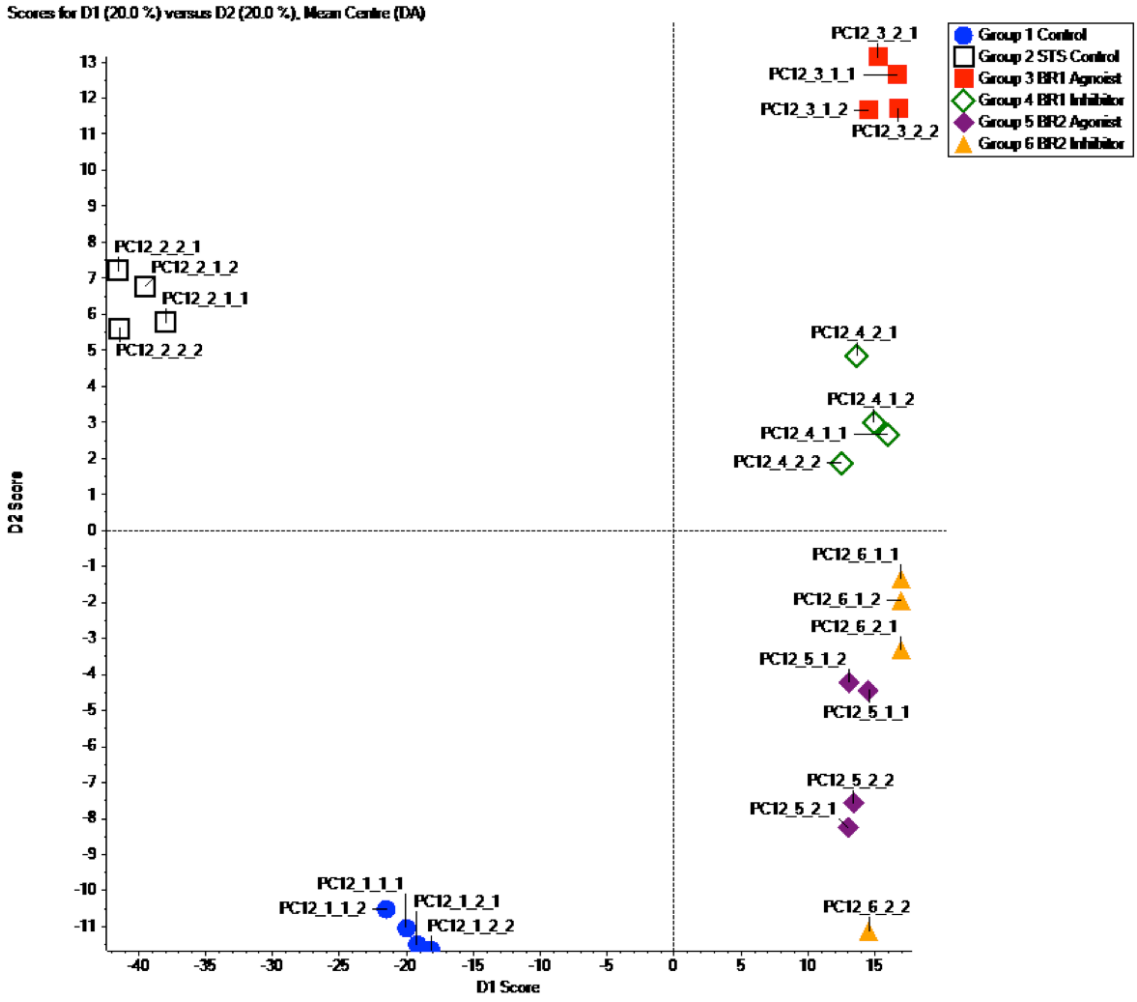

Figure 23: Principal Component Analysis (PCA) of spectra count data of PC12 treated proteome. Data points of Ctrl (blue circles), STS (black squares), B1A (red squares), B1I (green diamonds), B2A (purple diamonds) and B2I (yellow triangles).

3. Systems Biology Study

For better understanding of the proteins found in PC12 cells following STS, B1A+STS, B2A+STS, B1I+STS and B2I+STS–induced altered proteome, PANTHER analysis was used to classify proteins, utilizing rat protein gene ontology database, into distinct categories of molecular function (figure 24), biological process (figure 25) and protein class (figure 26).

The most common molecular function of STS–induced altered proteins was demonstrated to be binding, 32.4% of the proteins were shown to have this function, while metabolic process (29.9%) was the prominent biological process related to STS–induced altered proteins. In addition, most of the identified proteins appeared to belong to the cytoskeletal protein class (21.3%).

In contrast, in B1A+STS-, B2A+STS-, B1I+STS-, B2I+STS- induced altered proteins, catalytic activity and metabolic process were shown to be the most predominant molecular function and biological process, respectively, with almost the same percentage. Nonetheless, B1A+STS-, B1I+STS- and B2I+STS- induced altered proteins were demonstrated to belong to the oxidoreductase protein class, while B2A+STS-induced altered proteins were mostly chaperones.

Table 7 summarizes the major molecular function, biological process and protein class found in PC12 cells STS, B1A+STS, B2A+STS, B1I+STS and B2I+STS–induced altered proteome, with their relative percentage taken from PANTHER pie charts.

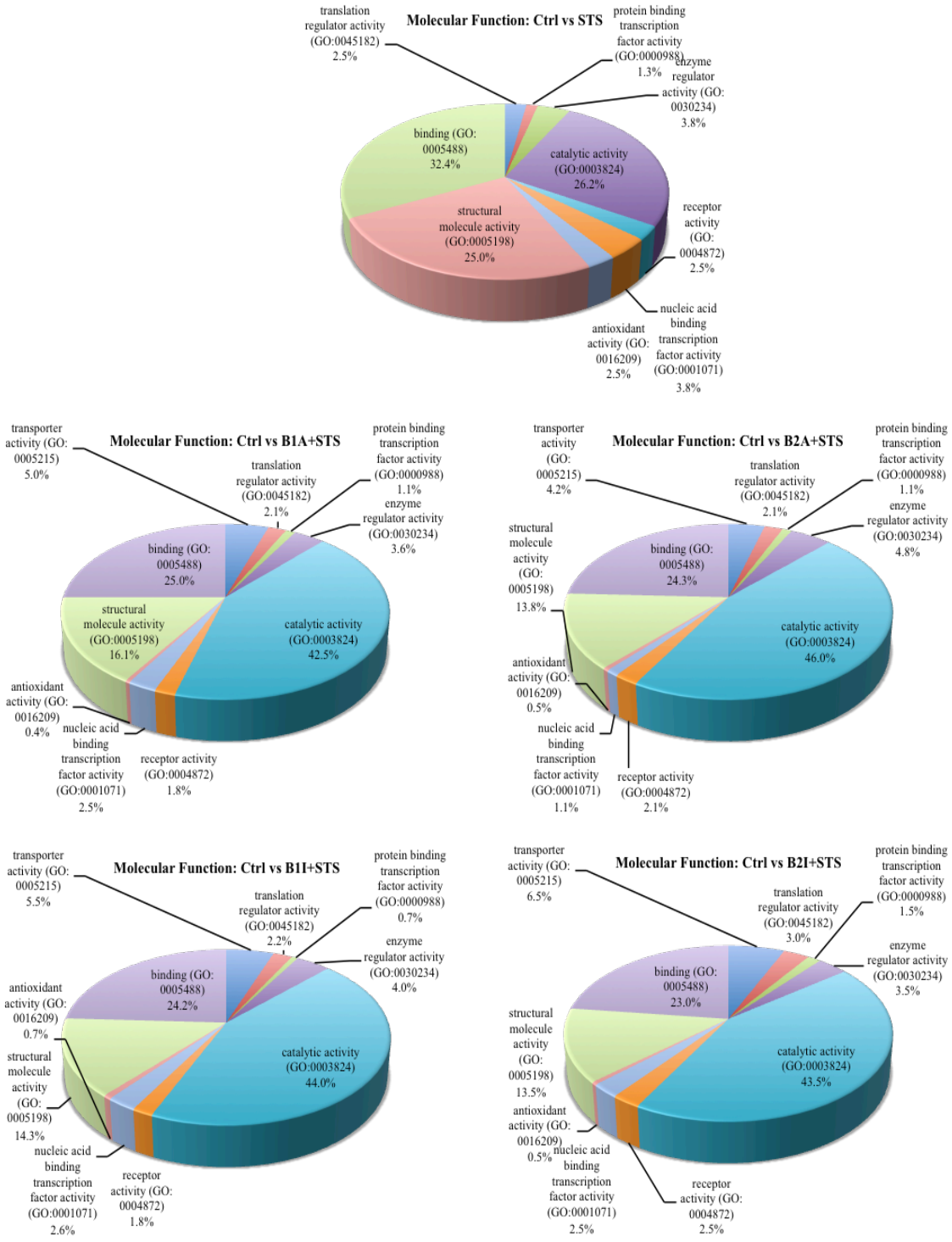

Figure 24: Molecular function of PC12 cells following STS, B1A+STS, B2A+STS, B1I+STS and B2I+STS–induced altered proteome.

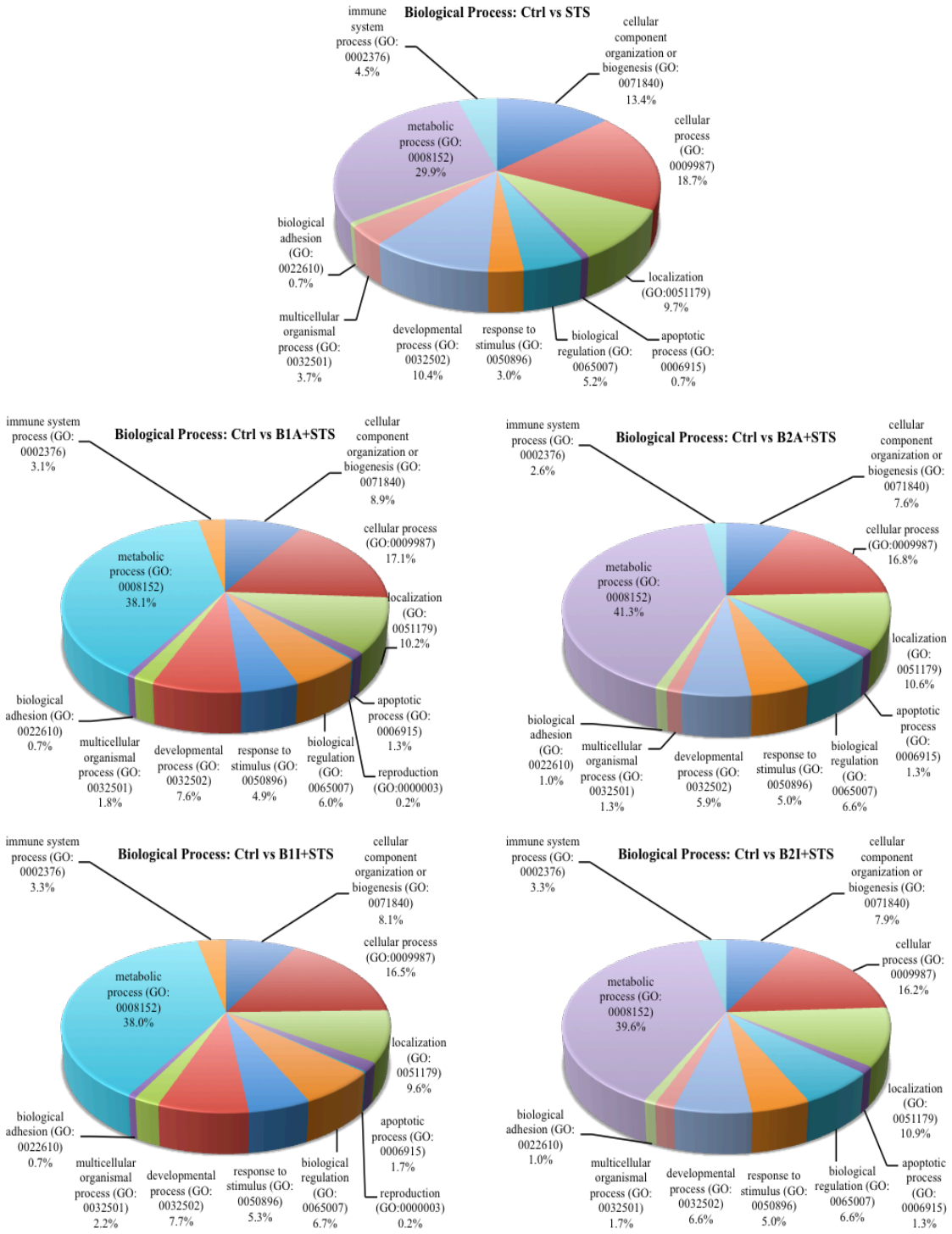

Figure 25: Biological process of PC12 cells following STS, B1A+STS, B2A+STS, B1I+STS and B2I+STS–induced altered proteome.

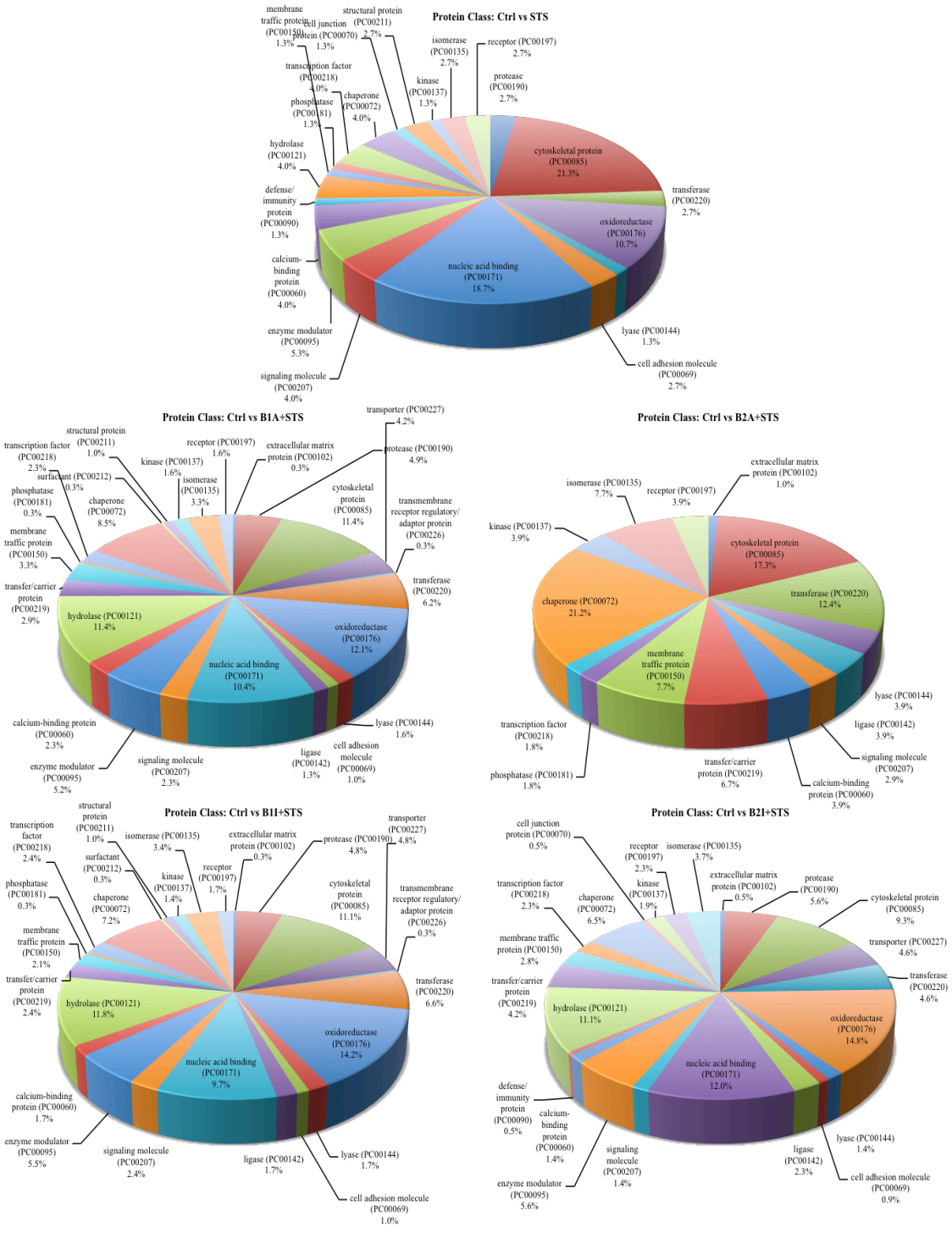

Figure 26: Protein class of PC12 cells following STS, B1A+STS, B2A+STS, B1I+STS and B2I+STS–induced altered proteome.

Groups Compared	Molecular Function	Biological Process	Protein Class
Ctrl vs. STS	Binding 32.4%	Metabolic 29.9%	Cytoskeletal 21.3%
Ctrl vs. B1A+STS	Catalytic activity 42.5%	Metabolic 38.1%	Oxidoreductase 12.1%
Ctrl vs. B2A+STS	Catalytic activity 46.0%	Metabolic 41.3%	Chaperone 21.2%
Ctrl vs. B1I+STS	Catalytic activity 44.0%	Metabolic 38.0%	Oxidoreductase 14.2%
Ctrl vs. B2I+STS	Catalytic activity 43.5%	Metabolic 39.6%	Oxidoreductase 14.8%

Table 7: Major molecular function, biological process and protein class in the different PC12 cells groups. Percentages were taken from PANTHER pie charts.

The software Pathway Studio 8 (2011) was used to search possible protein-protein interactions, common regulators, cell processes, and related pathways for associations with STS–induced altered proteins before and after treatment with B2I. The network was generated using Shortest Path algorithm to map interaction between altered proteins and only the identified proteins with 2 or more references were kept. A simplified picture of their interactions is shown in figure 27. Figure 27A depicts the global interaction proteome of the STS group while figure 27B reflects the global interaction proteome of the B2I+STS group. By this approach, we found that proteins belonging to different structural and functional families were involved in processes such as mitochondrial damage, cell death, inflammation, oxidation, and apoptosis (highlighted in red in STS group). Nevertheless, the most important discovery was that, in the B2I+STS cohort, fewer proteins were involved in these detrimental processes (highlighted in cyan in B2I+STS group) and new proteins appeared to be involved in cell survival (highlighted in red in B2I+STS group). The results are summed up in Table 8.

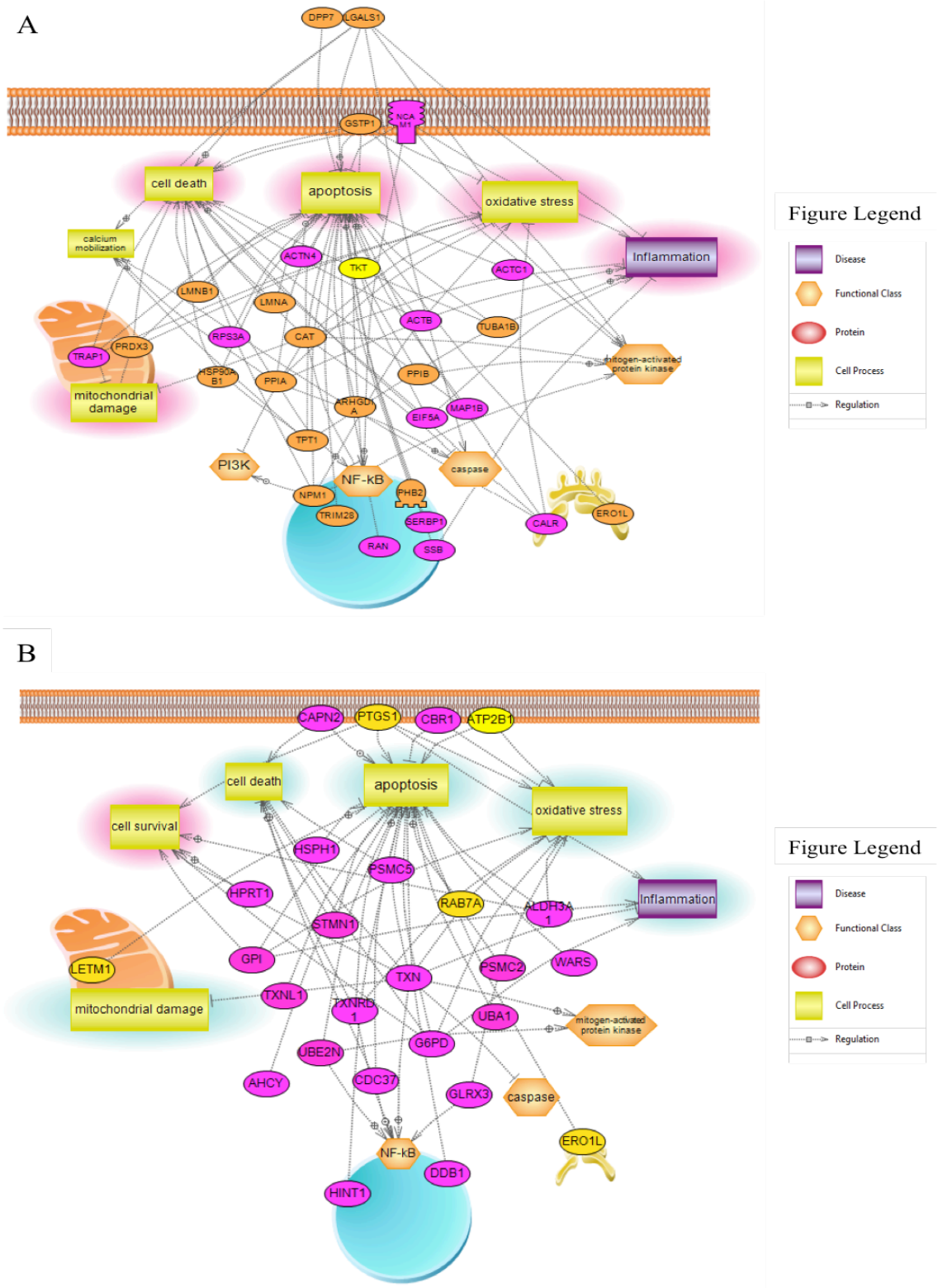

Figure 27: Pathways study of STS-induced altered proteins. Global Interaction Proteome of (A) STS treated cells and (B) B2I+STS treated cells. The yellow rectangles, violet rectangles and orange hexagons are reflective of biological processes, disease processes and functional classes, respectively, as shown in the legend. Also, the different colors of proteins reflect their alteration: up-regulated (pink), down-regulated (orange), down-regulated and unique to the treated group (yellow).

	Number of Hits (proteins)	
	STS group	B2I+STS group
Apoptosis	36	32
Oxidative stress	14	13
Cell death	27	17
Mitochondrial damage	6	3
Inflammation	11	5
Caspase	6	3
MAPK	11	7
NF-κB	9	11

Table 8: Comparison of the number of proteins identified in the STS and B2I+STS groups and involved in different processes (biological or disease) or belonging to different functional classes.

From the above table it can be observed that the number of proteins related to the biological processes: apoptosis, oxidative stress, cell death and mitochondrial damage, as well as the disease process: inflammation, and the caspase and MAPK functional classes, are all diminished in the B2I+STS group when compared to the STS group. However, the NF-κB is the only functional class that contains more hits in the B2I+STS group than in the STS group. Therefore, it can be proposed that the inhibition of the B2R and the presence of the transcription factor NF-κB are essential to reduce the harmful effects of STS in PC12 cells.

In general, although the inhibition of the B2R following STS treatment is not totally impeding apoptosis, it is activating some downstream proteins necessary for the survival of the cell. By providing the cell with a "survival" capacity, B2I is a legitimate therapeutic tool candidate, especially to treat diseases where apoptosis and excessive intracellular calcium release are the main players, such as Alzheimer's disease (Stutzmann, 2005) and Huntington' disease (Bezprozvanny and Hayden, 2004).

L. Tissue panel analysis for B2R

Before starting the *in vivo* work, it was interesting to detect the presence of the B2R in brain tissues and to compare its protein expression with different tissues. Figure 28 shows that the highest B2R protein expression is seen in muscle, striatum, heart left ventricle and kidney. Importantly, B2R is present in all brain tissues, namely: hippocampus, cortex, cerebellum and striatum.

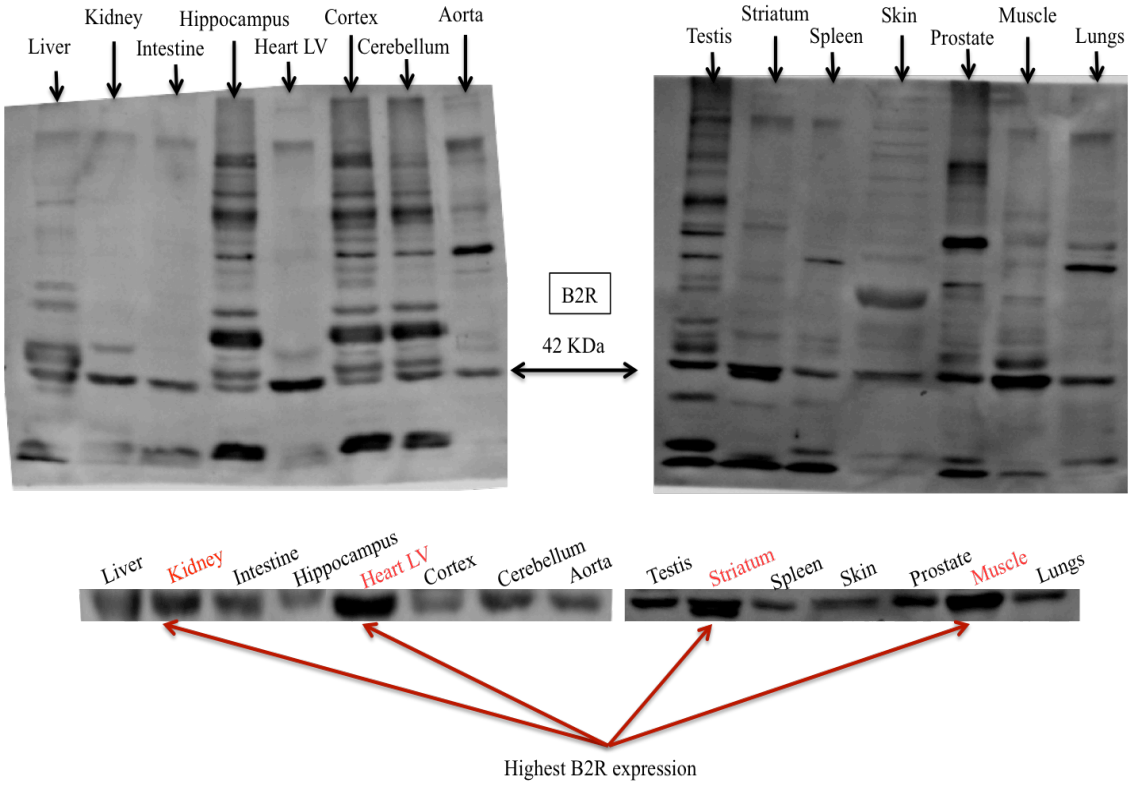

Figure 28: Tissue panel analysis of B2R, showing highest B2R protein expression in muscle, striatum, heart left ventricle and kidney.

M. Video sequencing of neonatal rat cortices dissection

The brain of few hours old rat pups was dissected and cortical cells were extracted and harvested for culture experiments, as illustrated in Figure 29 (the procedure is detailed in the materials and methods section).

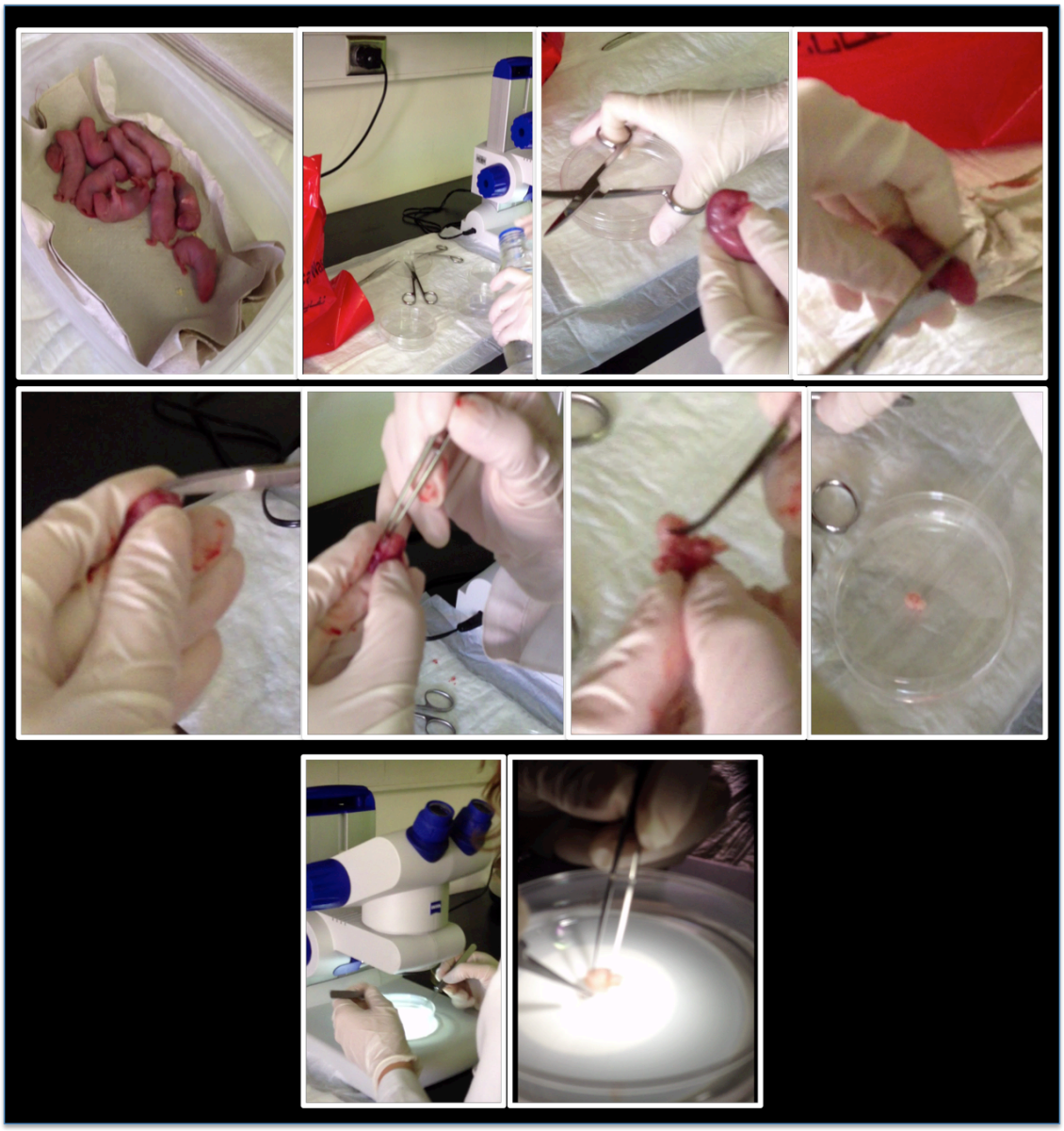

Figure 29: Video sequencing of rat pups' brain dissection. After quick decapitation and removal of the brain, the brain is freed from meninges and the cortices are collected under a stereoscope.

N. Primary neurocortical cells

Primary cortical cells were maintained in culture for up to 21 days in an araC containing media to reduce the amount of glial cells (Figure 30). At this time point, neuronal cells were treated with different neurotoxic drugs along with B1R and B2R agonists and antagonists. Then, different parameters were assessed, such as neuronal

markers staining, mitochondrial enzymatic activity, cytotoxicity, intracellular ROS production and translational expression of apoptotic and KKS markers.

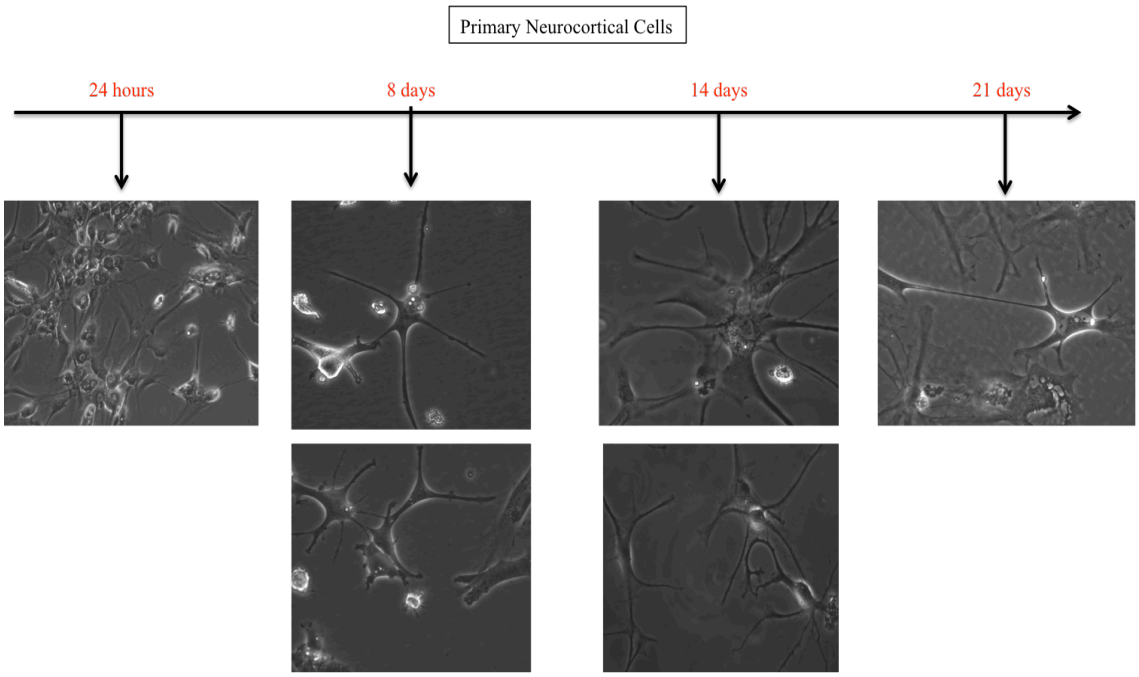

Figure 30: Timeline of primary cortical cells maintained in culture for up to 21days.

1. Neuronal markers staining

After 24h of incubation, the primary neurocotical cells were stained for different neuronal markers, namely: Cd11b: a microglial marker, GFAP: an astrocyte marker, Vimentin: a neural stem cell marker and NeuN: a neuronal maturation marker. As it is shown in Figure 31, the primary neurocortical cells are not completely free of glial cells since Cd11b and GFAP are still expressed. In addition, they are not fully mature and demonstrate early differentiation activity as attested by the presence of the NeuN and Vimentin markers, respectively.

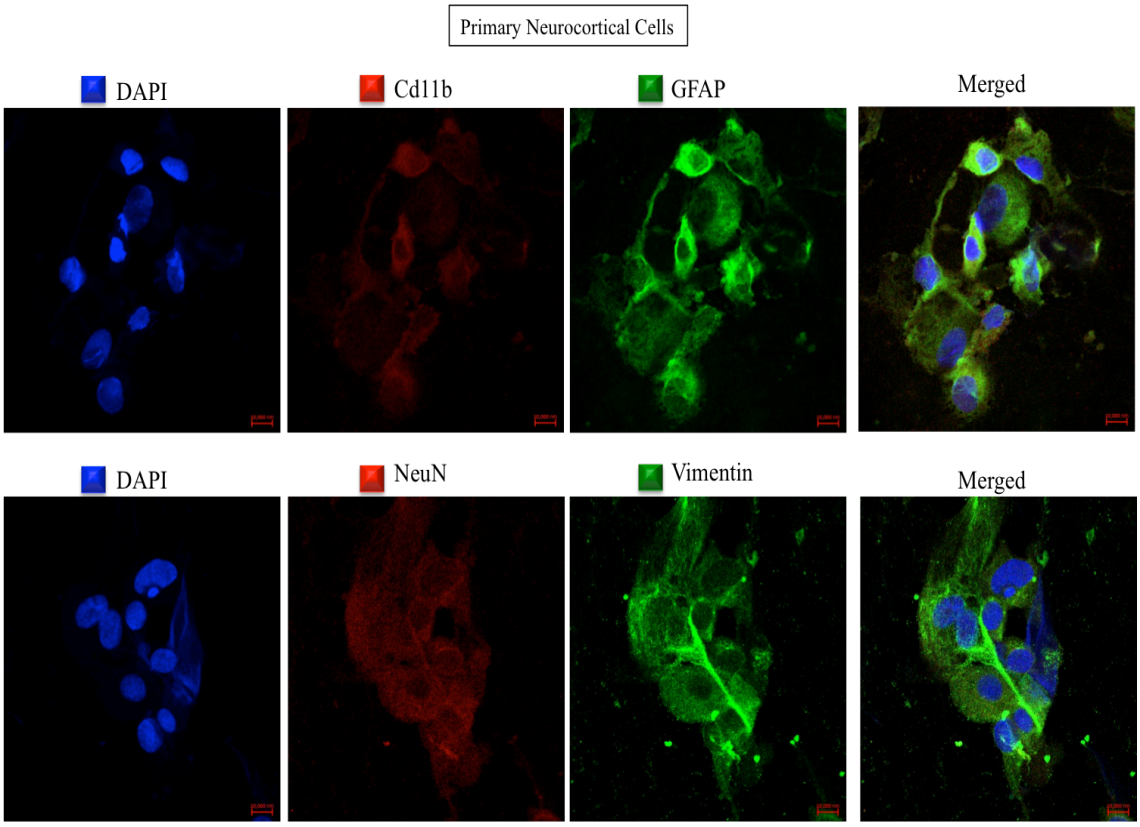

Figure 31: Cd11b, GFAP, NeuN and Vimentin staining of 24h old primary neurocortical cells. Images were taken using oil lens LSM 7-10 Confocal Laser microscope at 63x magnification.

2. Visual effect of STS, MTX and NMDA treatment

STS, MTX and NMDA are toxic agents that affect cells through different cell death mechanisms. As mentioned earlier, STS, MTX and NMDA induce apoptosis, necrosis and excitotoxic cell death, respectively. Morphological changes in primary cerebrocortical cells following 24h STS (0.5uM), MTX (0.5nM) and NMDA (5mM) treatments, is shown in Figure 32. Cell shrinkage and nuclear collapse are clearly observed in STS-mediated apoptotic cell death, while cell swelling is noticed in MTX-mediated necrosis. Interestingly, hair-like extensions start appearing following NMDA treatment. These extensions are nothing else than filopodia, extensions from which dendritic spines arise. As synapses form, the number of filopodia declines and more

dentritic spines become established at the postsynaptic sites of most excitatory synapses in the CNS (Fiala et al., 1998; Harris, 1999; Hering and Sheng, 2001; Ziv and Smith, 1996). Nevertheless, dendritic spines represent a major target of excitatory synapses; therefore, a continuous NMDA application can either desensitize NMDA receptors or become toxic to the neurons (Rocha and Sur, 1995).

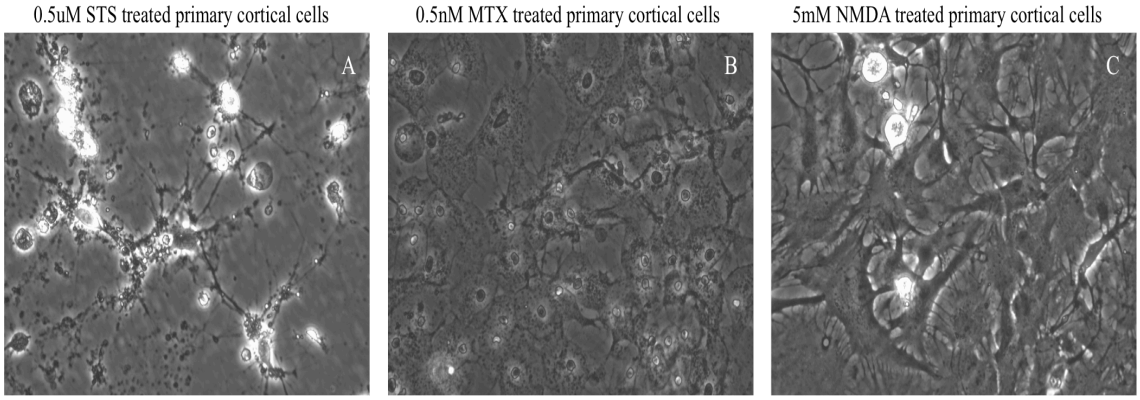

Figure 32: Morphological changes of primary neurocortical cells following 24h (A) STS, (B) MTX and (C) NMDA treatment.

3. Effect of STS and Bdk receptors on proliferation, cytotoxicity and intracellular ROS production

The role of Bdk receptors following 24h STS-mediated apoptosis was investigated in primary neurocortical cells. Figure 33 sums up the results of the MTT, LDH and NBT reduction assays. STS is observed to kill 80% of the neuronal cells by decreasing mitochondrial enzymatic activity (MTT results) and damaging cell membrane (LDH results), but STS provokes only 20% of intracellular ROS production. In addition, activation or inhibition of B1R and B2R did not help prevent apoptotic cell death. These results are similar to the ones observed in PC12 cells.

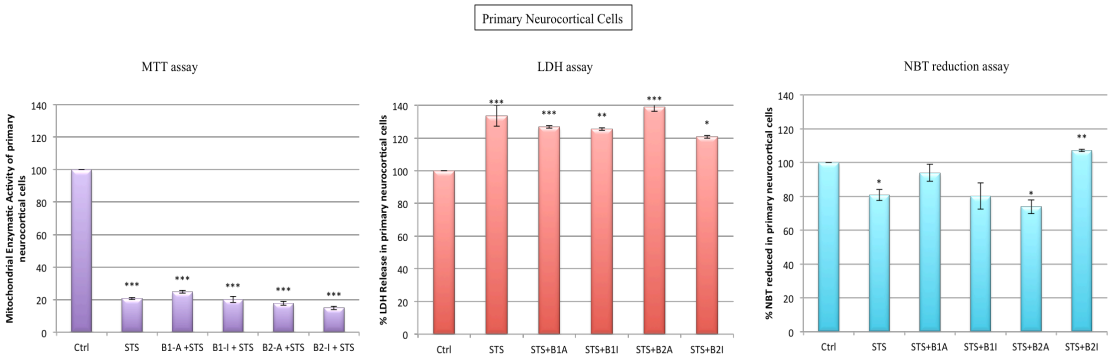

Figure 33: Effect of STS on cell viability, cytotoxicity and intracellular ROS production in primary neurocortical cells post 24h, using MTT, LDH and NBT reduction assays, respectively. (n=3, triplicate, *P<0.05, **P<0.01, ***P<0.001).

4. Western blotting

The translational expression of the pro-apoptotic Bax protein and the apoptosis-associated NOX4 protein, along with the B2R was investigated in primary neurocortical cells post 24h of STS (0.5uM) treatment. Figure 34 shows an increase in protein expression of B2R, Bax and NOX4. This result indicates the involvement of the B2R in STS-mediated apoptosis in primary neurocortical cells.

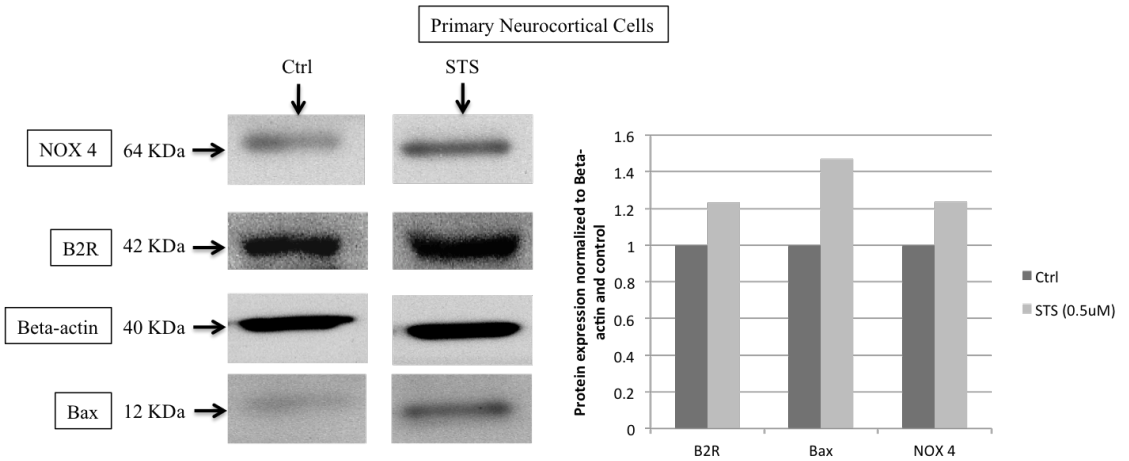

Figure 34: B2R, Bax and NOX4 protein expression upon 3h STS treatment in primary neurocortical cells.
(A) Western Blot of protein extracts from untreated and treated cells.
(B) Histogram representing densitometry analysis of B2R, Bax and NOX4 expression using Image J software (n=1)

DISCUSSION

The recent conflicts in Iraq and Afghanistan as well as the growing acts of violence in various regions of the world lead researchers and clinicians to focus on the development of possible body protections, elucidate TBI mechanism and search for eventual biomarkers and therapeutic tools. In fact, TBI is a "war signature" that makes a lot of victims, not only soldiers but also civilians. The Brain Injury Awareness Day held on March 12, 2014 by the Brain Injury Association of America, indicated that 5.3 million individuals live with life-long disability as a result of TBI. Of higher importance, TBI can also result from car accidents, sport injuries, domestic violence and falls mainly seen in the juvenile and elderly population.

TBI induces inflammation in the brain; therefore, several studies aimed at targeting apoptotic and necrotic pathways post-brain injuries. Interestingly, recent studies have focused on an inflammatory pathway that showed promising results in diabetic nephropathy and retinopathy and in cardiovascular diseases, namely the KKS. Indeed, while performing a tissue panel analysis of B2R (one of the main KKS component), it was observed that the translational expression of B2R was the highest in the kidney, the left ventricle of the heart, the striatum and in muscles. Nevertheless, the results obtained regarding the beneficial effects of the KKS components in TBI, were controversial and required more investigation. Thus, our study aimed at analyzing the mechanism followed by the KKS in PC12 cells and primary neurocortical cells post STS-induced apoptotic cell death, and compare the results with ipsilateral cortices of CCI subjected mice.

On one hand, the results obtained confirmed the detrimental apoptotic effect of STS on the neuronal PC12 cell line. Indeed, apoptotic bodies, translocation of

phosphatidylserine from inner to outer plasma membrane and mitochondrial depolarization are all hallmarks of apoptosis and were clearly observed in STS treated cells when compared to the untreated control. It was also noticed that STS decreased mitochondrial enzymatic activity by approximately 60% post-3h treatment, 85% post-12h treatment, and 90% post-24h treatment. Similarly, LDH release from STS damaged cell membranes was observed in 90% of cells post-3h, 220% of cells post-12h, and 150% of cells post-24h. Finally, STS treated cells exhibited an average of 30% intracellular ROS production, suggesting the oxidizing effect of STS. Thus, STS is causing apoptotic cell death and its harmful effects are enhanced in a time-dependent manner. Moreover, 3h of STS treatment was shown to increase translational expression of the caspase-independent apoptotic AIF protein and to decrease the translational expression of the anti-apoptotic BCl2 protein. In addition, caspase-dependent PARP cleavage was observed in STS treated cells. Thus, STS is capable of inducing apoptosis through a caspase-dependent and caspases-independent way in PC12 cells.

On the other hand, the results revealed the involvement of the KKS components, mainly of the B2R in STS treated PC12 cells. Although, the agonist and antagonist of B1R and B2R increased the mitochondrial enzymatic activity of PC12 cells, they failed to protect the cells against STS-induced apoptosis. Similarly, they didn't reduce LDH release and ROS production triggered by STS. Importantly, activation and inhibition of any of the two receptors indicated similar results, suggesting a compensating mechanism between the B1R and B2R. Nevertheless, immunostaining of B1R and B2R in PC12 cells obviously revealed the presence of the constitutive B2R in control cells and its induction in STS treated cells post-3h. As predicted, the inducible B1R was not expressed before 12h of STS treatment but its expression was

still less than the expression of the B2R post-3h. In addition, 3h of STS treatment augmented transcriptional expression of the B2R by 2.5 fold and the expression of B1R by 1.3 fold when compared to the control, while 12h of STS treatment lead to a 6.8 and 4.9 fold increase of B2R and B1R transcriptional expression, respectively. Also, at 3h post STS treatment, the transcriptional expression of the B2R was slightly higher than the one of the B1R in all conditions. However, at 12h post STS treatment, B1R expression was higher than B2R expression in almost all conditions except B2A+STS and B1I+STS, where the B2R expression was 9.3 and 10.2 fold higher than the control. Likewise, translational expression of B2R in STS treated cells was the highest in STS, B1A+STS, and B1I+STS conditions. These results suggest again a crosstalk between the B1R and B2R and the main participation of the B2R. Nevertheless, it is important to highlight the fact that inhibition and activation of B1R or B2R, following STS treatment, did not prevent PARP cleavage, increased AIF and decreased BCl2 translational expression. Therefore, although KKS components are playing a certain role in STS-mediated apoptosis, they do not prevent its occurrence.

The work was repeated in primary neurocortical cells of neonatal rat pups for further validation. Here again, STS was shown to kill 80% of the cells post-24h and to extensively damage plasma membrane as implied by the approximately 135% LDH release. However, although the oxidizing effect of STS was prevalent in all conditions, it was completely inhibited in the B2I+STS condition; thus B2I is capable of reducing oxidative stress in primary neurocortical cells. Moreover, Western Blot was used to demonstrate the increased translational expression of the B2R, NOX4 and Bax proteins post 24h of STS treatment. Thus, it is once again suggested that inhibition of the B2R represents an interesting opportunity to control STS-induced apoptosis but is not

sufficient to completely avert its manifestation in primary neurocortical cells extracted from few hours old rat pups.

An interesting striking discovery though, was the ability of the B2R to prevent intracellular calcium release once inhibited, and even following STS treatment. Figure 35 sums up the effects of STS and B2I on calcium release and apoptosis, according to the results obtained in this study. Under normal circumstances and following agonist activation of the B2R, the alpha subunit of the G protein detaches from the $G_{\beta\text{-}\gamma}$ dimer and from the receptor, via GDP to GTP conversion (Smrcka, 2008). G_{α}-GTP then activates PLC_{β} that cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Liu and Wu, 2004). IP_3 is a soluble molecule capable of diffusing through the cytoplasm to the endoplasmic reticulum (ER). Once at the ER, IP_3 is able to bind to the IP_3 receptor on a ligand-gated Ca^{2+} channel that is found on the surface of the smooth ER. The binding of ligand IP_3 triggers the opening of the Ca^{2+} channel, and thus release of Ca^{2+} into the cytoplasm (Nakayama et al., 2004). In contrast, STS leads to the cleavage of the cytosolic pro-caspase 3 into caspase 3 (Assefa et al., 2004), through a yet to be determined mechanism. Caspase 3 binds and induces a conformational change of IP_3 receptor, triggering an increased intracellular Ca^{2+} release (Hanson et al., 2004). This high calcium concentration will be sequestered by the mitochondria (Tinel et al., 1999) (Rizzuto et al., 2004), but eventually, the mitochondrial calcium overload will induce opening of permeability transition pore, mitochondrial dysfunction and apoptosis (Szalai et al., 1999). Furthermore, it was demonstrated that inhibition of B2R by antagonist binding impeded intracellular calcium release since IP_3 is no more produced. Interestingly, the same result was observed when blocking the B2R and treating the

cells with the apoptotic inducer STS. Thus, it can be suggested that exacerbated calcium release provoked by STS is due to a third component, associated with the B2R and necessary for either the activation of pro-caspase 3 by STS or binding of caspase 3 to IP_3 receptor. In other words, inhibition of B2R will prevent the formation or activation of this third component that will in turn prevent the formation or action of caspase 3 and will not lead to increased calcium release. Nonetheless, apoptosis is still observed in all STS conditions. Thus, controlling increased intracellular calcium release by inhibiting B2R is not sufficient to prevent STS-induced apoptosis.

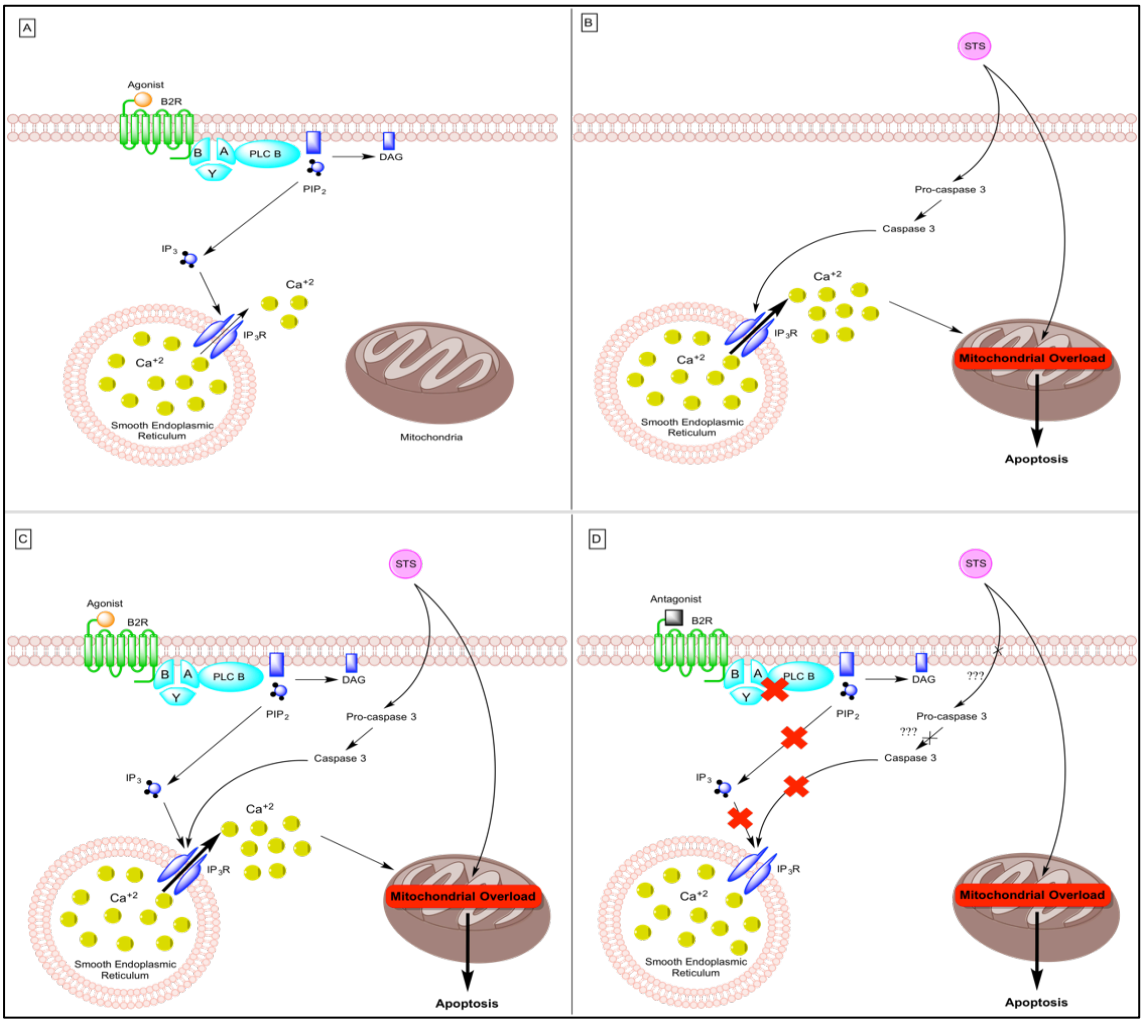

Figure 35: Effects of STS and B2R antagonist on calcium release and apoptosis. (A) B2R activated, (B) STS treatment, (C) B2R activated and STS treatment, (D) B2R inactivated and STS treatment.

Finally, according to the proteomics results of this study, it could be demonstrated that inhibition of the B2R and activation of the transcription factor NF-κB are essential for the survival of the cell but not sufficient to completely prevent STS-mediated apoptosis in PC12 cells. Therefore, we must focus our attention on the proteins promoting cell survival in the B2I+STS group, such as: TXN, CBR1, ALDH3A1, HINT1, MAPRE1 etc…. These proteins are shown in figure 36 and are pink colored, which means that they are up-regulated in the B2I+STS group.

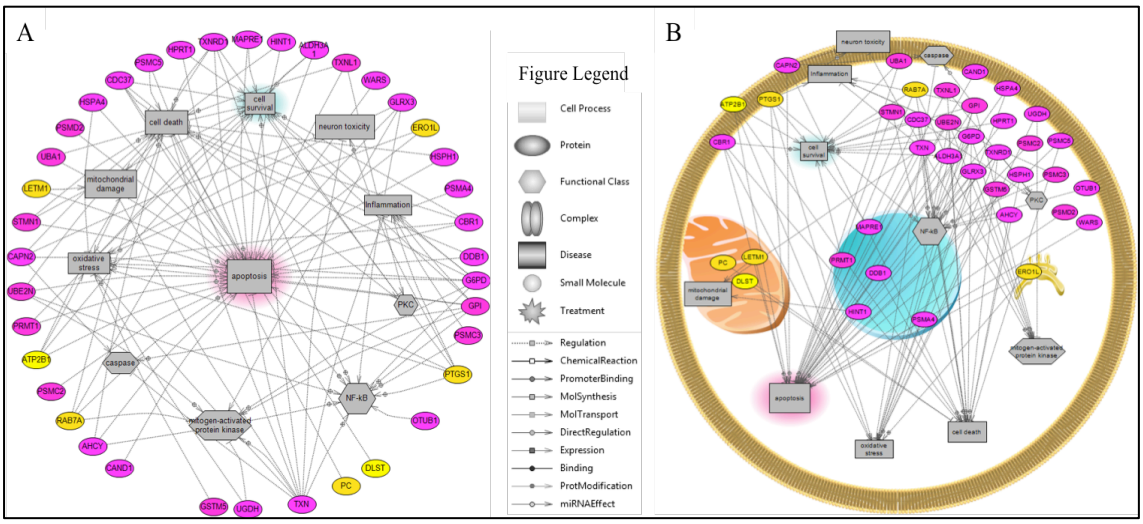

Figure 36: Enriched Pathways and networks (A) and global interaction proteome (B) of B2I+STS-induced altered proteins, showing the different proteins involved in the cell survival biological process.

LIMITATIONS AND FUTURE PERSPECTIVES

Some limitations have been encountered while working on this study and impeded the obtention of certain key information. Indeed, some of the elaborated plans in the grant couldn't be conducted for several reasons. These limitations constitute attractive future perspectives.

- Limitation 1: One main disadvantage was the absence of functional NMDA receptors in PC12 cells. This statement was advanced by Edwards et al. who demonstrated by immunohistochemical techniques and electrophysiological analysis that PC12 cells contain NMDA NR1, NR2C and NR2D subunits of the receptor but lack the NMDA NR2A and 2B subunits, rendering NMDA receptors non-functional in PC12 cells (Edwards et al., 2007). This significant discovery prevented the use of NMDA/glutamate as excitotoxic factors on PC12 cells since the latter are NMDA receptor specific agonists, i.e. they can't bind to other glutamate receptors such as AMPA and Kainate receptors. Therefore, NMDA was only employed in primary neurocortical cells, but not in PC12 cells.
- Future work 1: Animal TBI models are costly and lengthy to work with; therefore, a "TBI cell line" should be created to facilitate the *in vitro* work, which is more practical and less time-consuming. However, to achieve this goal, a mixture of toxic reagents such as STS (apoptotic), MTX (necrotic) and NMDA (excitotoxic) should be used to mimic the cell dynamic after TBI. Although PC12 cells are the preferred neuronal cells to study the effects of STS and MTX, they are not good candidates to study NMDA effects. Consequently, in order to test the effects of the excitotoxic NMDA drug, *in*

vitro studies should be performed on another neuronal cell line such as Neuro 2A (N2a). N2a is a mouse neural crest-derived cell line that can be used to study neuronal differentiation, axonal growth and signaling pathways. In particular, differentiation of N2a cells into dopamine neurons can be performed by treating the cells with dibutyryl cyclic adenosine monophosphate (dbcAMP) (Coleman et al., 2013; Tremblay et al., 2010).

- Limitation 2: Another inconvenience was the low signal and high background of the B1R antibody in WB experiments. Several measures have been taken to fix this issue: using B1R from different companies, trying different dilutions of primary and secondary antibodies, changing the length of time and temperature of incubation, preparing the antibody and blocking the membrane in 5% milk with tween 20 or BSA, contacting the companies. Unfortunately, the problem couldn't be solved till now.
- Future work 2: After ordering a monoclonal B1R antibody or requesting the antibody from a lab that developed its own B1R antibody and have tried it on PC12 cells, B1R protein expression should determined by Western Blot. Although the B2R was shown to be the main player in STS-induced apoptosis in PC12 cells, the B1R might play a major role in primary neuronal cells post chemically-induced neurotoxicity or in post-injury brain tissues. It is important to recall that the B1R is expressed only under stress and inflammatory conditions.

- Limitation 3: Still another limitation included the delivery of some compounds to Lebanon and to AUB. In particular, Methamphetamine (METH) is an illegal drug that is neurotoxic to the dopaminergic and serotonergic systems in the brain and that may

involve apoptotic, necrotic and autophagy cell death mechanisms (Kobeissy et al., 2008b). Interestingly, in a study conducted by Warren et al., it has been demonstrated that a dose of 40 mg/kg METH was not significantly different in effect as TBI (Warren et al., 2005). Unfortunately, research conducted on METH requires approval from the Health Ministry of Lebanon. Likewise, the recent tensions and conflicts in the Middle East, raised concern among the selling companies who are refusing to deliver the necrosis-inducing Maitotoxin (MTX) drug to Lebanon.

- Future work 3: An interesting experiment to perform is to intraperitoneally inject adult rats with 10 mg/kg doses of METH four times every 2 hours to achieve a desired dosage of 40 mg/kg in a bolus of 0.3 mL. Then, the rats will be sacrificed at 24 h post-intraperitoneal injection. This protocol was performed by Kobeissy et al. to study the psychoproteomic of rat cortex following acute METH exposure. (Kobeissy et al., 2008b).

- Limitation 4: Finally, the main lengthy issue was the arrival of the CCI machine. The shipment of this machine did not only take time to be delivered to Lebanon and to AUB but also encountered some political issues at its arrival at the airport of Beirut. Furthermore, a place was to be assigned for the machine and the machine had to be mounted on a flat solid inflexible surface capable of holding the high impact frequencies engendered by the trauma device. Most importantly, a student training session for the CCI machine is still awaited.
- Future work 4: CCI represents an open wound injury model and mimics better what we observe in car accident and war zone. For an *in vivo* model of experimental TBI, adult rats should be anaesthetized and placed in a stereotactic device in a prone position.

Then, a midline cranial incision is made to reveal the soft tissues, and a unilateral (ipsilateral to the site of impact) craniotomy (7 mm diameter) is performed adjacent to the central suture, midway between bregma and lambda. Brain trauma is produced by impacting the right cortex (ipsilateral cortex) with a 5 mm diameter aluminum impactor tip at a velocity of 3.5 m/s with a 1.6 mm (severe) compression and 150 ms dwell-time (compression duration). Sham-injured control animals will undergo identical surgical procedures but will not receive an impact injury (Brody et al., 2007).

Additional future works:

➢ Future work 5: Another attractive experiment to achieve is to explore and evaluate the expression of B1R and B2R in an animal model post-CCI injury and compare the results with STS- and METH- administrated animals. Thus, the study will comprise 7 groups of rats with 10 animals per group, as summarized in table 9.

Group Category	Group Description	Reference
1. Control	Saline group	
2. Sham-injured control	Surgical procedure	(Brody et al., 2007)
3. CCI	Surgical procedure and impact injury	
4. METH	Intraperitoneal injection of 10x 4mg/kg	(Thomas et al., 2004)
5. STS	CA_1 topical injection of 10ng/kg	(Hara et al., 1990)
6. B1I (*R-715*)+CCI	Intravenous administration of 1mg/kg	(Raslan et al., 2010)
7. B2I (*Anatibant*)+CCI	Subcutaneous administration of 3.0mg/kg	(Ongali et al., 2006)

Table 9: Groups for *in vivo* model of experimental TBI. (CA_1: pyramidal cells in the hippocampus).

These cohorts can be tested at 6h, 24h and 6days post-CCI injury and 24h post METH and STS exposure. Each group should be compared to control sham animals. Then several parameters can be assessed such as: bleeding, brain edema, inflammation, diffuse axonal damage and brain water content.

➢ Future work 6: The use of B1R and B2R KO models can also be beneficial to confirm the results obtained while using B1R and B2R inhibitors. As mentioned earlier, such experiments have already been conducted but the results obtained were conflicting and contradictory. This was in part due to the various experimental models used, the diverse brain areas targeted and the different time points examined. Hence, the elucidation of the KKS mechanism and its potential protective role in TBI require the use of *in vivo* and *in vitro* models treated with neurotoxic drugs such as NMDA, STS, MTX and METH, in the fundamental paradigm of CCI injury.

CONCLUSION

In conclusion, our study demonstrated that the main player of the KKS during STS-induced apoptosis in PC12 cells is the B2R. Moreover, the fact that B2I is capable of inhibiting calcium release following STS treatment suggests a potential therapeutic role of the B2R.

In addition, proteomic analysis revealed that inhibition of the B2R along with activation of the transcription factor NF-κB are inducing cell survival in PC12 cells following STS treatment. An interesting future approach would be to work with the "survival"-promoting proteins identified in the downstream mechanism of B2R post STS-induced apoptosis.

PRELIMINARY DATA

Western Blot was performed on post-CCI ipsilateral cortical tissues derived from adult mice, decapitated 2h, 6h, 24h, 2d and 5d post-injury (a gift from the University of Florida). B2R expression was shown to be significantly elevated directly post-CCI but the B2R expression decreased with time (figure 37).

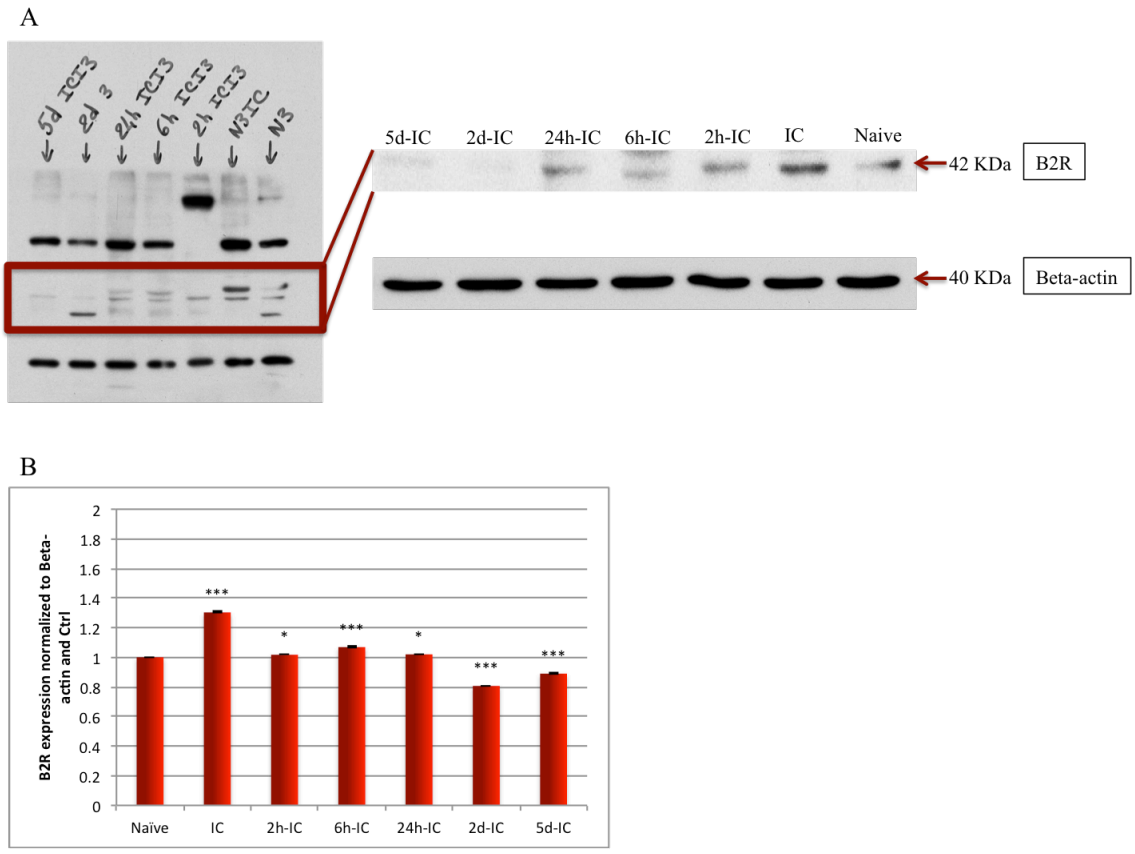

Figure 37: B2R protein expression in post-CCI mice cortical tissues.
(A) Western Blot of protein extracts
(B) Histogram representing densitometry analysis of B2R expression using Image J software (n=1)

In addition, the effects of MTX (2.25nM) were studied in PC12 cells post 4h and were compared to STS effects. As depicted in figure 38, MTX is a necrotic drug

that induced PC12 cell swelling. In contrast, treatment of PC12 cells with the apoptotic drug STS provoked cell shrinkage and nuclear collapse.

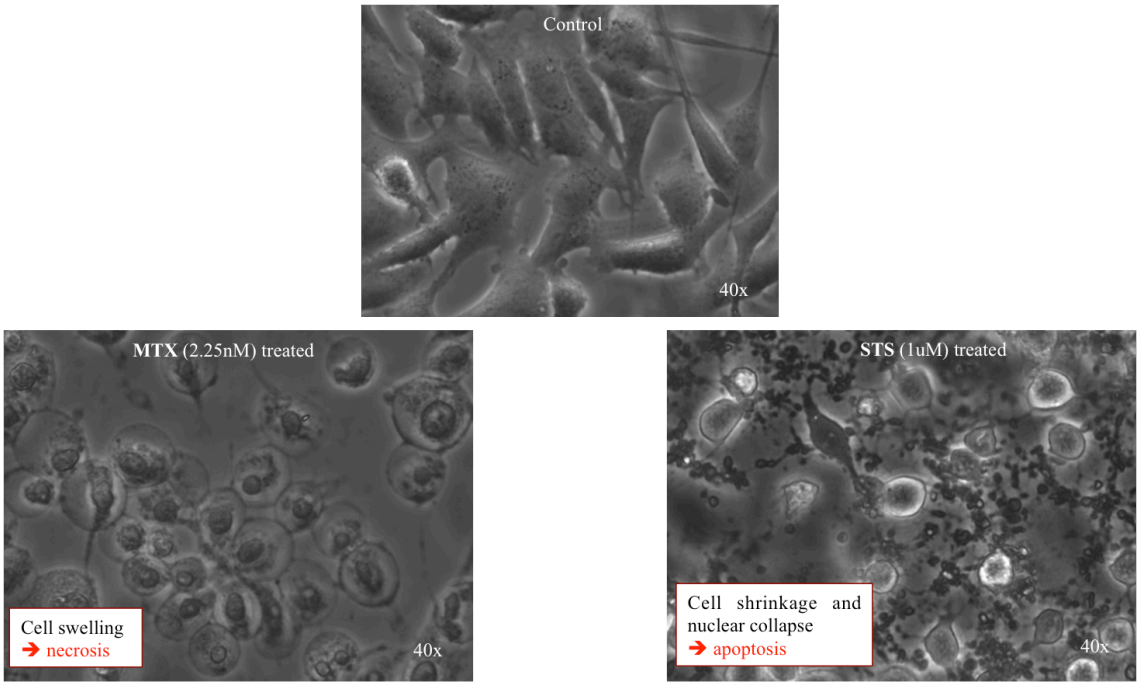

Figure 38: Morphological changes of PC12 cells, following 4h MTX and STS treatments.

In addition, MTT, LDH and ROS assays were performed on PC12 cells following 4h MTX treatment and were compared to 24h STS treatment. The potent effect of MTX on PC12 cells is clearly observed in figure 39. While STS (1uM) is inducing approximately 40% cell death post 24h, MTX (2.25nM) is killing almost 90% of the cells post 4h, according to the MTT results. In addition, cell membrane damage is seen in 40% of STS-treated cells after 24h and 98% of MTX-treated cells after 4h. However, intracellular ROS production does not exceed 50% in STS and MTX treated PC12 cells.

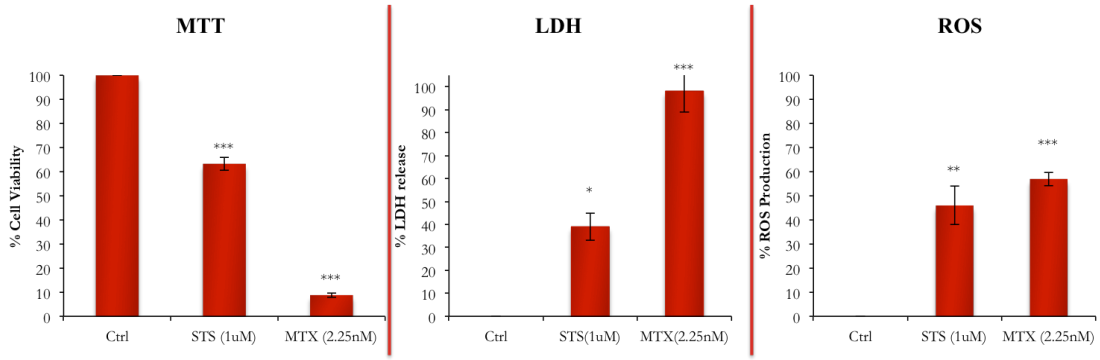

Figure 39: Effect of STS (1uM, 24h) and MTX (2.25nM, 4h) on cell viability, cytotoxicity and intracellular ROS production in PC12 cells, using MTT, LDH and NBT reduction assays, respectively. (n=3, triplicate, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

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