NOVEL EFFECTS OF SEROTONIN 1A RECEPTOR-MEDIATED SIGNALING THROUGH MAP KINASE

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

The City University of New York

2004

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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An ischemic stroke is associated with a massive depolarization of the brain tissue, which is followed by an onset of apoptotic changes as a result of excitotoxicity and/or due to reoxygenation of the tissue. The hippocampal regions have been repeatedly reported to be a common site of the neuronal loss independent of the exact location of the ischemic core. Serotonin 1A receptors (5-HT_{1A}-R), which are particularly abundant in hippocampal regions, exhibit some neuroprotective properties when activated by agonists. Until recently, the possibility of using the 5-HT_{1A}-R agonists as alternative treatment of ischemic stroke patients was overlooked. Overall neuroprotection offered by the agonists of the 5-HT_{1A}-R is a collective effect of receptor-mediated signaling through multiple pathways. In this study, we chose as a model a mouse hippocampus-derived cell line HN2-5, which expresses 5-HT_{1A}-R, and is devoid of expression of voltage-gated Calcium ion channels. Equipped with this model, we studied the role of 5-HT_{1A}-R in stimulating Ca-channel-independent pathways, which block apoptosis. Prolonged agonist stimulation of the 5-HT_{1A}-R results in a phosphoinositide *tris*-phosphate (PI-3K) independent, slow and persistent MAPK activation that has been shown to be a part of a neuroprotective pathway

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leading to inhibition caspase-3 processing. This neuroprotection is equally effective against anoxia- and H_2O_2-induced apoptosis, suggesting that a general anti-apoptotic mechanism is mediated by the 5-HT_{1A}-R agonists in suppressing the apoptotic machinery downstream of the mitochondria. This neuroprotection is dependent on PL-Cβ, ERK 1/2 and PKCα. The ERK 1/2 enzymes play a key role in mediating anti-apoptotic signaling via regulation of PKCα activity. Detergent soluble cellular membrane fractions of agonist-stimulated HN2-5 cells showed ERK 1/2 dependent increase in phospho-PKCα (pSer^{657}, and pThr^{638}), which is indicative of the activation of PKCα. Membrane associated phospho-PKCα profile closely followed the phospho-ERK 1/2 profile and co-immunoprecipitated with ERK-2 from membrane-bound but not cytosolic fractions of the cell. This study presents a novel mode of regulation of caspase-3 by MAPK through its association with PKCα. It is the first documented report on co-immunoprecipitation of ERK and PKCα kinases, suggestive of direct phosphorylation of PKCα turn motif (Thr^{638}) by the ERKs.

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This thesis work is dedicated to my loving family: my parents, my wonderful boys Daniel and Denis and my husband Gene, who made it all possible.

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ACKNOWLEDGMENTS

This work could not be brought to completion without the dedicated efforts of numerous wonderful people. The author wishes to express deep appreciation to all members of the Biology/Neuroscience Doctoral Program at the Graduate School and University Center and IBR/CSI Center for Developmental Neuroscience for moral and financial support. This project was supported by the OMRDD Fellowship from IBR/CSI Center for Developmental Neuroscience. A special note of gratitude goes to the mentor of this project Dr. Probal Banerjee for his scientific and editorial wisdom, as well as to members of supervisory committee for guidance and helpful discussions. Also at IBR/CSI CDN and at the research lab, I would like to thank my fellow graduate students for their enthusiastic support.

Finally, I thank once again the members of my immediate family, who were patient and understanding enough to see me accomplish this work and share the happy moments with me.

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TABLE OF CONTENTS

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Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES AND FIGURES

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ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
5-HT_{1A}-R	$5\text{-hydroxytryptamine}_{1A}$ receptor, serotonin 1A receptor
AGC	denoting protein kinases A, G and C
Akt	protein kinase B, PKB
Apaf1	apoptosis activated factor 1
BSA	bovine serum albumin
CPP-32	Caspase-3, casp-3, Yama
Ca	calcium
Ca/CaM	calcium/calmodulin
Cyto C	cytochrome c
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EGFR	epidermal growth factor receptor
ERK 1/2	extracellular signal regulated kinase 1/2, MAPK
FBS	fetal bovine serum
GFX	Bisindolylmaleimide I
GFP	green fluorescent protein
GPCR	G protein coupled receptor
H_2O_2	hydrogen peroxide
HRP	horse-redish peroxydase
IAP	inhibitor of apoptosis protein

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IP	immunoprecipitation
KRB	Krebs-Ringer's buffer
MAPK	mitogen activated protein kinase, ERK 1/2
MBP	myelin basic protein
MEK	MAPK kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDK-1	phosphoinositide-dependent kinase-1 (PDK-1)
PKB	protein kinase B, Akt
PI-3K	phosphoinositide *tris*-phosphate kinase
PIP2	phosphoinositide-*bis*-phospate
PKC	protein kinase C
PL-Cβ	phospholypase Cβ
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
RIPA	radioimmune precipitation buffer
ROS	reactive oxygen species
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBST	Tris-buffered saline + Tween-20

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Chapter 1

Introduction

SEROTONIN

Structure and Synthesis

Serotonin (5-hydroxytryptamine, 5-HT) is a unique monoamine product of tryptophan (Trp) that belongs to a group of aromatic compounds called indoles, with a pyrolle ring fused to a benzene ring. It is produced and utilized by both neuronal as well as non-neural cells. Despite the relative simplicity of its structure and two-step synthetic pathway (Figure 1.1), this molecule plays an important role in regulating numerous biological processes in the central and peripheral nervous system as well as in the cardiovascular and gastrointestinal systems (Peroutka, 1988).

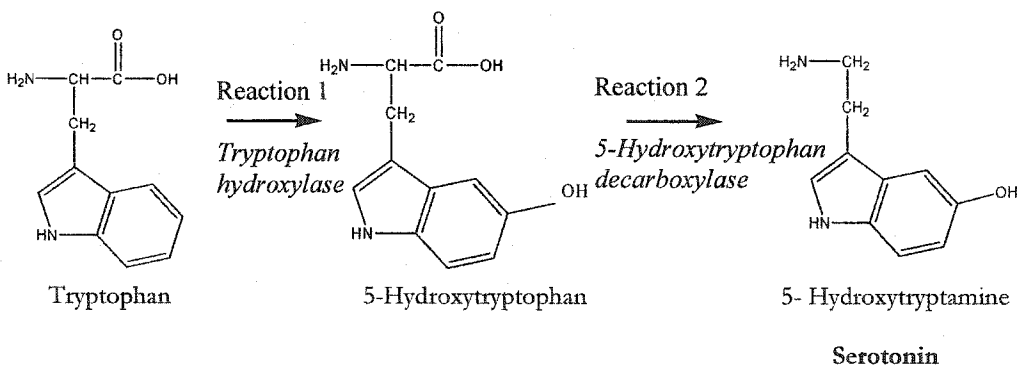

Figure 1.1. Serotonin biosynthesis. Tryptophan (Trp) hydroxylase catalyses the first, rate controlling reaction converting Trp to 5-Hydroxytryptophan (5-HTP). The second enzyme in the synthetic pathway is 5-HTP decarboxylase, that catalyses the final conversion of 5-hydroxytryptophan into 5-hydroxytryptamine.

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Neuronal Serotonin

Neuronal serotonin has been implicated in more behaviors, physiological and disease processes than any other neurotransmitter of the nervous system. Besides its role in synaptic transmission, serotonin is known to act as a regulatory factor during neural development (Azmitia et al., 1992). Extensive, 5-HT- directed research efforts for over 60 years have unraveled numerous functional details that aid in our understanding of the diverse range of effects produced in the brain by this single chemical. Developmentally, serotonergic neurons are formed as a group of relatively large multipolar neurons, collectively known as raphé nuclei, located on the midline of the brainstem. Raphé neurons appear early during embryonic development (in humans, they emerge by six weeks of gestation) and begin synthesis of serotonin soon after their last cell division. They are among the first neurons to differentiate and have been implicated in governing neurogenesis, differentiation and maturation. The raphé neurons reach practically all structures through axonal projections far in advance of final development and maturation of those structures and in so doing exert their effect on target areas. Such structures include serotonergic projections that reach the primordial cortical plate during mitosis and peak of maturation of this region (Dori et al., 1996). Thus serotonin plays a pivotal role in brain maturation, development and plasticity through interactions with post synaptic neurons and adjacent glia, promoting release of trophic factors and glucose necessary for neuronal survival.

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Serotonin receptors (5-HT_{1A} and others)

The nature of the effect produced by serotonin on a target region is encoded by the target itself, namely by the type of serotonin receptor present at the synapse. Serotonin exerts its function by interaction with fourteen receptors that are currently reported in the literature. These receptors have been grouped into seven classes (5-HT_{1-7}, see Table 1) generally on the basis of their operational, structural and functional characteristics (review Humphrey et al. 1993; Hoyer and Martin 1997). Thus, the effects are mediated by, as many as, thirteen heptahelical G protein-coupled receptors (GPCRs), and one ligand-gated ion channel (5-HT_3 receptor). These receptor subtypes share amino acid homology with one another and with other members of the GPCR superfamily (Shih and Chen 1990). The diversity of the effects produced by serotonin is further expanded by selective coupling of the receptor sub-class to distinct types of the G-proteins (*i, o, q, s,* etc.).

Out of the whole family of serotonin receptors, the 5-HT_{1A} receptor seems to be of most interest, not only for being first on the list (see Table 1), but mainly due to its contribution to the serotonergic system on the whole and its functional role in normal physiology of the brain and in the pathogenesis of numerous disorders. This mainly CNS receptor is encoded by an intronless gene, mapped to chromosome 5 in humans. In the CNS its expression begins early during development and has region specificity timed to differentiation and maturation. (Daval et al., 1987; Dyck et al., 1993; Hillion et al., 1994) Trophic role of serotonin during brain development is proposed to be mediated by the 5-HT_{1A} receptor since fetal expression of receptor mRNA in the brain stem shows a pattern of a high rate for a limited time. In rat brain stem 5-HT_{1A} receptor transcripts were first detected at

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Table 1. Serotonin receptors, distribution and implications

5-HT Receptors	Type	Effect on AC	Distribution	Comments
5-HT_1	**GPCR**	**Inhibit**		Family of intronless genes
5-HT_{1A}			Raphe nuclei (somatodentritic autoreceptors), septum, hippocampus, hypothalamus, amygdala, cortex, spinal cord (postsynaptic, heteroreceptors) (Gerard et. al., 1994)	Gene mapped to ch. 5 in humans; signaling through G_i/G_o coupling; implicated in anxiety, depression, mood disoders, eating disorders; involved in sexual behavior, appetite control, thermoregulation, and cardiovascular function; developmental diseases (DS, Rett's syndrome, etc)
5-HT_{1B}		**Inhibit**	Presynaptic, and postsynaptic in substantia nigra, globus pallidus, and dorsal subiculum of rodents (Zifa and Fillion, 1992)	Presynaptic receptors synthesized locally at the serotonergic terminal (Doucet et al., 1995); correspond to human 5-HT_{1D}
5-HT_{1D}		**Inhibit**	First identified in bovine caudate. In human brain found in the CA3 field of hippocampal Hammond's horn; trigeminal ganglia; human cerebral blood vessels	In humans potential involvement in Huntington's disease (Pasqualetti et al., 1996)
5-HT_{1E}		**Inhibit** or **Stimulate**	Human: Cortical areas, caudate, putamen and amygdala, in rodent brain: cortical areas, the hippocampal formation and claustrum (Bruinvels et al., 1994)	May couple to adenylate cyclase via two distinct pathways: Gi-mediated inhibition cAMP levels at low agonist concentrations or Gs - potentiated cAMP accumulation at higher agonist concentrations (Adham et al., 1994) encoded by human 6q14-q15.
5-HT_{1F}		**Inhibit**	Non-neural: uterus and mesentery; CNS: Cerebrovascular and neural tissue (Bouchelet et al ., 1996)	Plays role in vascular contraction Potential involvement in cerebrovascular functions and dural inflammation, migraines.

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Table 1. Serotonin receptors, distribution and implications (*Continued*)

5-HT$_2$ 5-HT$_{2A}$	**GPCR**		Frontal cortex, nucleus accumbens, tuberculum olfactorium, cell body and dendrites of cortical pyramidal neurons (Wu et al, 1998), layer IV of stiatum and pyramidal cells of hippocampal CA1 region (Pasqualetti et al., 1996); DRG; blood vessels.	Share the same intron/exon organization, signal through PLC to produce IP_3 and DAG second messagers. Play a role in appetite control, thermoregulation, sleep, potentate hallucinogenic behavior; involved in cardiovascular function and muscle contraction
5-HT$_{2B}$			High levels in human liver and kidneys; low levels in pancreas and spleen; vascular endothelium. In CNS low levels in cerebral cortex (Bonhaus et al., 1995); DRG.	Involved in embryonic development and postnatal maturation of the enteric nervous system; regulates cardiac embryonic development, implicated in vascular growth in hypertension.
5-HT$_{2C}$			CNS at axon terminals: choroids plexus, cerebral cortex, septum, hippocampus, amygdale, some of basal ganglia, the substantia nigra, the substantia innominata, ventro-medial hypothalamus (Pasqualetti et al., 1999). Epithelial cells	Regulates septo-hippocampal activity
5-HT$_3$ 5-HT$_3$	Ion channel		Found in periphery as well as in CNS: area postrema, entorhinal cortex, frontal cortex, and hippocampus	nonselective Na+/K+ ion channel receptors
5-HT$_4$ 5-HT$_4$	**GPCR**	**Stimulate**	Broad tissue distribution, in the brain in nucleus accumbens, collicular and hippocampal neurons	In CNS mediate slow excitatory responses to 5-HT may be involved in memory and learning; in periphery involved in peristalsis

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Table 1. Serotonin receptors, distribution and implications (*Continued*)

5-HT$_5$	**GPCR**			Sequence similarity (encoded by two exons separated by one large intron)
5-HT$_{5A}$		**Inhibit**	Primary sites of expression are considered to be non-neuronal. In human CNS: cerebral cortex (layer II-III, V- VI), hippocampus (DG, pyramidal cell of CA1, CA3), cerebellum (high levels in Purkinje cells and dentate nucleus, lower at granule cells) (Pasqualetti et al., 1998).	Mediates effect of 5-HT on cerebella function
5-HT$_{5B}$		?	DRG, embryonic sensory neurons (Chen et al., 1998a); in rodent brain, 5-HT5B gene expression occurs predominantly in the medial habenulae and hippocampal CA1 cells of the adult. (Wisden et al., 1993)	Does not appear to alter either cAMP accumulation or phosphoinositide turnover.
5-HT$_6$	**GPCR**	**Stimulate**	Almost exclusively in the CNS: striatum, olfactory tubercle, nucleus accumbens and hippocampus (Ruat et al., 1993); in human brain, highest levels in the caudate nucleus.	Possibly controls cholinergic neurotransmission
5-HT$_6$				
5-HT$_7$	**GPCR**	**Stimulate**		Mapped to human chromosome 10.
5-HT$_7$			High levels in CNS (SCN, hypothalamus); Low level in periphery	Implicated in serotonergic regulation of circadian rhythm (Lovenberg et al., 1993)

CNS = central nervous system, GPCR = G protein coupled receptor, AC = adenylate cyclase, PLC = Phospholipase C, IP_3 = inositol triphosphate, DAG = diacylglycerol, DG = dentate gyrus, DS-Down Syndrome, DRG = dorsal root ganglia, SCN = suprachiasmatic nuclei

embryonic day (ED) 12, peaking at ED15 and then decreasing progressively to relatively low levels by birth (Hillion et al., 1993). Serotonin binding to 5-HT_{1A}-R exerts a trophic effect on maturing neurons and augmentation of the receptor expression may prevent neuronal loss (Singh et al., 1996*a*). Besides its role in brain development and neuronal plasticity, 5-HT_{1A}-R has been implicated in the pathogenesis of anxiety, depression, mood and eating disorders, hallucinations, motion sickness etc. Serotonin 1A receptor knockout mice have been studied as an animal model of anxiety-related disorder (Ramboz et al., 1998).

Selective immunolabeling of the 5-HT_{1A} receptor in postnatal brains in rodents and humans shows similarity of distribution, with strong immunoreactivity in the median raphé nucleus and structures of the limbic system such as dentate gyrus, CA1 region of the hippocampus, lateral septum, and entorhinal cortex (Gerard et al., 1994; Kia et al., 1996).

5-HT_{1A}-R subtypes

Serotonin$_{1A}$ receptors are sub classified further into two distinct categories based on their cellular localization: somatodentritic autoreceptors and postsynaptic heteroreceptors. Somatodendritic 5-HT_{1A} receptors are found on serotonergic neurons of raphé nuclei, where they regulate a feed back inhibition, controlling global serotonergic outflow to the target areas. Activation of those receptors lead to the opening of K^+ channels that hyperpolarize neurons, offsetting the membrane potential and inhibiting the firing-rate of the serotonergic neurons, and decreasing the amount of serotonin released from the neuron (Hutson et al., 1989; Penington et al., 1993). Postsynaptic 5-HT_{1A} receptors are found outside of the raphé region on

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the target neurons and the function of those receptors depends entirely on the neuronal function of the target. The pattern of expression of postsynaptic receptors in forebrain regions in rodents is very low during embryonic stages and first detected at ED17. Subsequently, the expression of this receptor gradually increases to reach nearly adult levels by postnatal day 21 (PD 21). According to a recent 5-HT_{1A}-R knockout study, selective expression of forebrain 5-HT_{1A} heteroreceptors is required during early postnatal period for normal anxiety-like behavior in adult mice (Gross et al., 2002).

G protein-coupled receptors

As a typical member of GPCR superfamily, 5-HT_{1A} receptor is composed of seven transmembrane domains and hydropathy analysis predicts that the amino-terminus of the protein is exposed to extracellular surface while the carboxy-terminus remains in the cytosol. A G protein-coupled receptor signaling mechanism have been described by the ternary complex model, according to which the receptor exists in a ground state (R) and also in a partially activated state (R*) that is able to couple to G protein. Agonist (A) then stabilizes R* and R*G to form the active state AR*G, the so-called ternary complex. In the ternary complex, GDP bound to the G protein is exchanged for GTP and the complex then dissociates, releasing α and $\beta\gamma$ subunits of the G protein that can then alter the activity of the effector molecules such as adenylyl cyclase. Seven transmembrane α-helical regions are linked by loops inside and outside the membrane. The three extracellular loops in addition to the amino-terminus form ligand binding site, while intracellular loops are responsible for the coupling of the receptor to a second messenger pathway. The 5-HT_{1A} receptor

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binds serotonin (its natural agonist) with high affinity (1 X 10^{-9} M) (Albert et al., 1990). An aminotetraline derivative 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH DPAT) shows 5-HT_{1A} receptor specificity and is widely used for biochemical and functional studies of this receptor. Specificity of coupling to different Guanyl nucleotide binding proteins (G-proteins) is assigned to the intracellular loops. The third intracellular loop (i3) of the receptor is thought to participate in signaling through G protein that couple the receptor to a variety of effector-molecules. Mutations in the regions of i3 could affect the ability of the receptor to couple to one or the other pathway (Lembo & Albert, 1995). Different subtypes of G-protein that couple to the 5-HT_{1A} receptor can connect it to various signaling pathways through either Gα or Gβγ (Martin et al., 1998).

5-HT_{1A}-R signaling

The serotonin$_{1A}$ receptor is coupled to pertussis toxin-sensitive G-proteins, such as G_i and G_O (Fargin et al., 1989), and has a characteristic inhibitory signaling to adenylate cyclase through Gα (De Vivo & Maayani, 1990, Banerjee et al., 1993). It also increases conductance of potassium ions through neuronal membrane causing a decrease in excitability of the neurons, leading to a decrease in frequency of action potential and reduces opening of voltage sensitive calcium channels (Penington et al., 1990; 1993). In addition, agonist binding to this receptor causes inhibition of N-type Ca-channels, probably *via* G_O, and activation of phospholipase C, through G_O or G_q (also termed GPLC)(Hamon et al., 1990). Serotonin 1A-receptor coupling to such a variety of G-proteins, however, has a common denominator - in all these receptor-G

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protein interactions, the Gßγ complex is always released, which can then activate multiple effector molecules or pathways (Lopez-Ilasaka et al., 1998), such as the phospholipase Cß➔ERK1/2 pathway or the PI-3 kinase (PI-3K) pathway (Stoyanov et al., 1995; Lopez-Ilasaka et al., 1997; Zwick et al., 1999; Della Rocca et al., 1999). Mutational studies showed that the second intracellular loop (i2) of the 5-HT_{1A} receptor has a conserved threonine residue, Thr149, which is particularly responsible for Gβγ-mediated signaling (Albert et al., 1998).

5-HT_{1A}-R in cell lines

There are a few 5-HT_{1A}-R cell lines that have been recently studied. Heterologous expression of the receptor in non-neural cells, the Chinese hamster ovary and human embryonic kidney 293 cells, unraveled some of the signaling properties (Garanovskaya et al., 1996, 1998, Cowen et al., 1996, Della Rocca et al., 1999). HN2-5 cells is a neuronal cell line with engineered expression of the 5-HT_{1A} receptor. It is derived from mouse hippocampal cell line HN2 (mouse neuroblastoma x hippocampal cells, Lee et al.,1990) via stable transfection with a DNA construct encoding the human 5-HT_{1A}-R (Banerjee et al., 1993, Singh et al., 1996*c*). The HN2-5 cells undergo differentiation in the presence of Retinoic acid or in phosphate-free Kreb's Ringer Buffer within 16-24 hours, exhibit neuronal morphology and strongly positive staining for neuronal markers (Banerjee et al., 1996; Das et al., 2003). Both differentiated and non-differentiated cells express the functional 5-HT_{1A}-R. The HN2-5 cells do not express any voltage-gated Ca^{2+} currents, although some Na^{+} currents are noticed (Adayev et al., 1999, El-Sherif et al., 2001).

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The cell lines with endogenous expression of the receptor: RN46A, derived from transformation of embryonic day-13 (E13) rat raphe cells using the temperature-sensitive SV-40 T antigen (White et al., 1994), and the SN48 cell line – a hybrid of septal neurons from postnatal day-21 (P21) mice and N18TG2 neuroblastoma cells (Lee et al., 1990). Both cell lines share the property of expressing a low level of 5-HT_{1A}-R, which potentially could be increased upon differentiation of the cells.

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MAPK – Mitogen-Activated Protein Kinase

In various cell-types, agonist-stimulated release of the Gβγ complex results in the activation of Extracellular signal Regulated Kinases (ERK1/2), which are members of the mitogen-activated protein kinase (MAPK) family. MAPK are a family of serine/threonine protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as proliferation, differentiation, motility and death. MAPK signaling cascades are organized hierarchically into three-tiered modules (Figure 1.2).

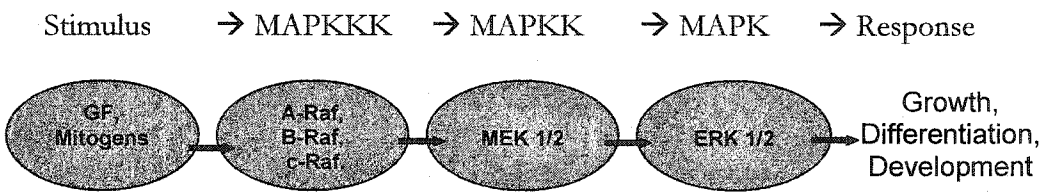

Figure 1.2. Schematic representation of stimulus signal transduction through MAPK pathway. ERK 1/2 mediated growth factor (GF) signaling is given as an example. Growth factor acting through specific receptor tyrosine kinase leads to activation of Raf kinases (MAP-kinase-kinase-kinase, MAPKKK) that phosphorylate MEK (MAP-kinase-kinase, MAPKK), that in turn phosphorylates ERK 1/2 (MAP kinase, MAPK), that further acts on its down stream targets to result in stimulus-specific effects.

MAPKs (ERK1/2) are regulated by tyrosine and threonine phosphorylation via phosphorylation and activation by MAPK-kinases (MAPKKs also termed as MEK), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs).

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The MAPKKK is in turn activated by interaction with a family of small GTPases and/or other protein kinases connecting the MAPK module to the cell surface receptor or external stimuli. Thus, activation of multiple signaling proteins could precede the stimulation of the key enzymes ERK1/2, which finally regulates many important cellular events, such as mitosis or survival. Such preceding, signaling molecules are the non-receptor tyrosine kinases Pyk2 and Src, the calcium binding protein Ca/Calmodulin (CaM), the low molecular weight G-protein Ras and the kinases Raf-1 and MEK (Della Rocca et al., 1997, 1999). As reviewed in the literature, divergent mitogen-activated protein kinase cascades can connect G protein-coupled receptors to the nucleus (Gutkind, 1998). Earlier studies in a neuron-derived cell line have demonstrated that agonist stimulation of the 5-HT_{1A} receptor causes upregulation of the MAP kinase pathway, which results in an inhibition of the pro-apoptotic, cysteinyl aspartate-specific protease, caspase-3 (Adayev et al., 1999).

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Apoptosis and ischemia

The neuronal injury is an umbrella term that combines different causes of neuronal loss such as traumatic brain injury, neurodegenerative diseases, ischemia/anoxia etc. When interference with the cause of the injury is not possible, one of the therapeutic strategies is to mitigate the after effect in the attempt to salvage the neuronal tissue. In acute ischemic stroke, for instance, neuroprotective agents are designed to work either during acute ischemia or during the reperfusion (reoxygenation) period, when additional brain injury occurs. Although, causes of neuronal injuries could be different, consequences are often associated with apoptotic cell death. The hippocampus (especially CA1 region) is crucial in many physiologic brain functions, including memory formation, and reported to be the area of most insult during ischemic-anoxic stress. Ongoing research efforts are focused on reducing apoptosis-associated cellular processes to prevent neuronal loss.

While, extensive data have been collected demonstrating the involvement of cell cycle machinery in the onset of apoptosis, some research also shows the lack of involvement of the cell nucleus in the apoptotic signaling cascades. Many cytosolic and mitochondrial proteins are involved in the process of apoptosis. Mitochondrial impairment caused by diverse types of tissue insults (Figure 1.3) leads to apoptosis through a generally accepted pathway. This cascade involves the release of cytochrome c from impaired mitochondria, its association with the apoptosis activating factor Apaf-1 and caspase-9, and subsequent partial activation of caspase-9 (Bossy-Witzel, Green, 1999; Datta, R. et al., 1997; Yoshida et al., 1998). The partially activated caspase-9 undergoes autocatalytic cleavage to yield fully active caspase-9. Procaspase-3 is direct a downstream target of the caspase-9.

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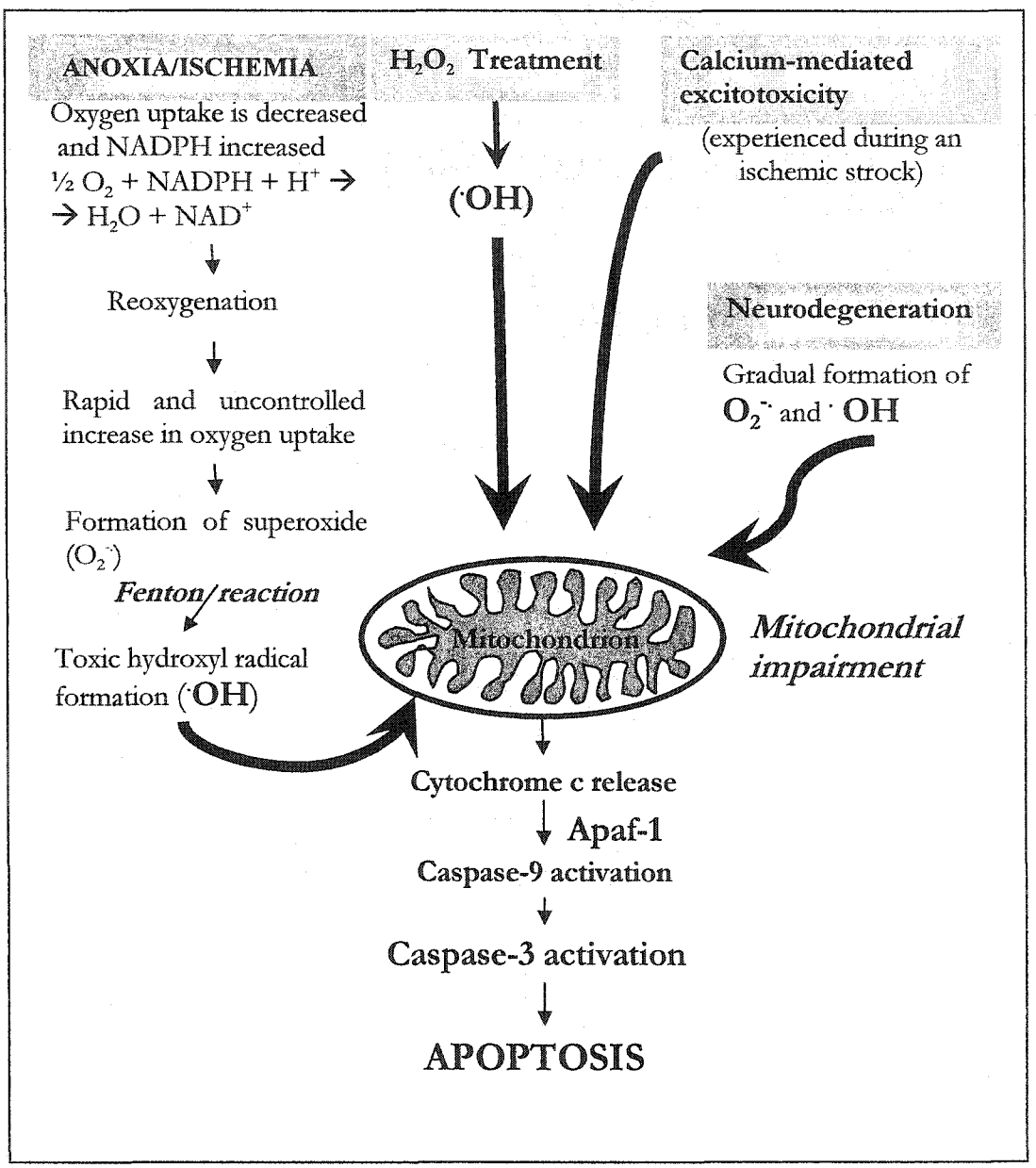

Figure 1.3 Possible mechanisms of mitochondrial impairment and the subsequent pathway to caspase activation and apoptosis

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Another pathway to apoptosis initially bypasses the mitochondria. It is set off by ligated death receptors (Fas), which, once activated through aggregation with adapter molecule Fas Adhering Death Domains (FADD), causes proteolytic activation of upstream protease caspase-8. Later it was found that caspase-8, which initiates the cascade of apoptotic events channels its proteolytic activity in two directions: (1) activation of other caspases (casp-3,7), (2) targeting cytosolic factors (Sun et al., 1999). Cytosolic BID was found to be a specific, proximal substrate of Caspase-8. It is a BH3 domain-containing pro-apoptotic Bcl-2 family member, which promotes apoptosis by inhibiting the death-antagonist members of the same family. Once truncated, tBID translocates to mitochondria and causes loss of mitochondial membrane potential, followed by cytochrome c release (Li et al., 1998). Cytochrome c release mediates mitochondrial amplification loop of caspase activation (as just described above). Thus, inhibition of the post-mitochondrial pathway is expected to attenuate apoptosis caused by diverse conditions, such as anoxia/ reoxygenation, neurodegenerative diseases, or excitotoxicity triggered by various conditions of neuronal injury.

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Anti-apoptotic role of 5-HT_{1A}-R

The hippocampal formation is the site of projection of serotonergic neurons and is therefore, a region rich in postsynaptic serotonin receptors. Preferential stimulation of certain subtypes of serotonin receptors might either promote apoptosis or offer neuroprotection. Such selective neuroprotection is a candidate for the therapeutic strategies used to block an ischemic insult to hippocampal neurons. Some studies have demonstrated the neuroprotective effect of stimulation of 5-HT_{1A}-R by the agonist 8-OH-DPAT (Singh et al., 1996a). Recently, the novel aminomethyl chroman derivative, repinotan BAY × 3702 was described as a high affinity 5-HT_{1A} receptor agonist with pronounced neuroprotective properties (De Vry et al., 1997a; 1997b; 1998; Horváth et al., 1997).

This neuroprotection may occur via multiple mechanisms; firstly, activation of the 5-HT_{1A}-R mediates a neuronal hyperpolarization which presumably offsets the ischemia-induced depolarization (Clarke et al., 1987) via opening of K^+ channels (through Gi) or closing of the Ca^{2+} channels (through Go). This would reduce excitotoxicity that is usually associated with ischemic conditions. Additionally, the 5-HT_{1A}-R agonists have been shown to prevent apoptosis and attenuate anoxia-induced impairment in protein synthesis (Suzuki et al., 1995).

Earlier studies have shown that suppression of apoptosis can occur via signaling from growth factor receptors through PI-3 kinase-mediated activation of Akt, which exerts its kinase activity to inhibit either the proapoptotic mitochondrial protein Bad (Datta et al., 1997) or the proapoptotic protease caspase-9 (Cardone et al., 1998). By contrast, previous studies in mouse hippocampus-derived HN2-5 cells have shown that a G protein-coupled receptor, the 5-HT_{1A} receptor, causes

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inhibition of caspase-3 and apoptosis by stimulating a novel, PI-3K-independent pathway that involves MAPK (Adayev et al., 1999). A mouse hippocampal neuron-derived cell line, engineered to express 5-HT_{1A} receptor (HN2-5), lacking the expression of N- or L-type Ca channels made it an ideal model for investigation and dissection of the 5-HT_{1A} receptor mediated neuroprotective pathway. The 5-HT_{1A} receptor-mediated neuroprotective pathway is possibly linked to the etiology of numerous neurodegenerative conditions. Therefore, a careful analysis of signaling molecules involved in this cascade is of utmost importance.

Generally the MAPK pathway, which is believed to block apoptosis by interfering with signals that leads to loss of mitochondrial membrane potential (i.e. acting upstream of mitochondria) results in an inhibition of the pro-apoptotic enzyme caspase-3. Ischemia, stress, and aging are usually associated with the generation of highly toxic ·OH (hydroxyl) radicals that affect the mitochondria directly to result in caspase-3 activation followed by apoptosis. Hydrogen peroxide (H_2O_2) treatment also follows the same pathway in causing impairment of the mitochondria. Given that 5-HT_{1A}-R-mediated MAPK signaling attenuates anoxia-induced apoptosis through the reduction of the caspase-3 activity, the point of interception of the apoptotic pathway (up- or downstream of mitochondria) is yet to be found. Since many conditions of neural insult and degeneration culminate in the activation of caspase-3, and the inhibition of its activity is essential for neuronal survival, the 5-HT_{1A}-R-mediated inhibition of caspase-3 could be used as a general tool to prevent neuronal apoptosis.

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Protein Kinase C

In the field of oncology research, the AGC family of serine/threonine kinases - protein kinase C (PKC) - has long been known to mediate the resistance of a cancerous cell to undergo apoptosis and, at times, is responsible for aggressive tumor invasion. Pharmaceutical companies are currently testing therapeutic strategies including development of antisense oligonucleotide PKCα expression inhibitors for the potential treatment of refractory solid tumors (Roychowdhury and Lahn, 2003). A calcium-dependent PKC isozyme mediates caspase-3 inhibition and proliferation of human adenoma cells, where the proliferation/survival could be effectively blocked by inhibitors of PKC-alpha (Go6976) and MEK 1 (PD98059) (McMillan et al., 2003). In a generally accepted pathway of PKC signaling, initiated by G protein-coupled receptors, PKC is reported to be active upstream of MAPK (Figure 1.4), regulating cell division, proliferation and tumor growth. Activation of PKC signaling pathway could occur through either Gβγ or Gα subunit mediated coupling to phospholipase C_β(PLC_β) with or without involvement of PI-3K. Generation of diacylglycerol (DAG) and intracellular Ca increase, second messagers of PLCβ and PI-3K respectively, provide necessary cofactors for PKC activation, which act through recruitment of small adaptor molecules SOS, Ras GRP etc. to cause activation of Ras and the MAPK pathway.

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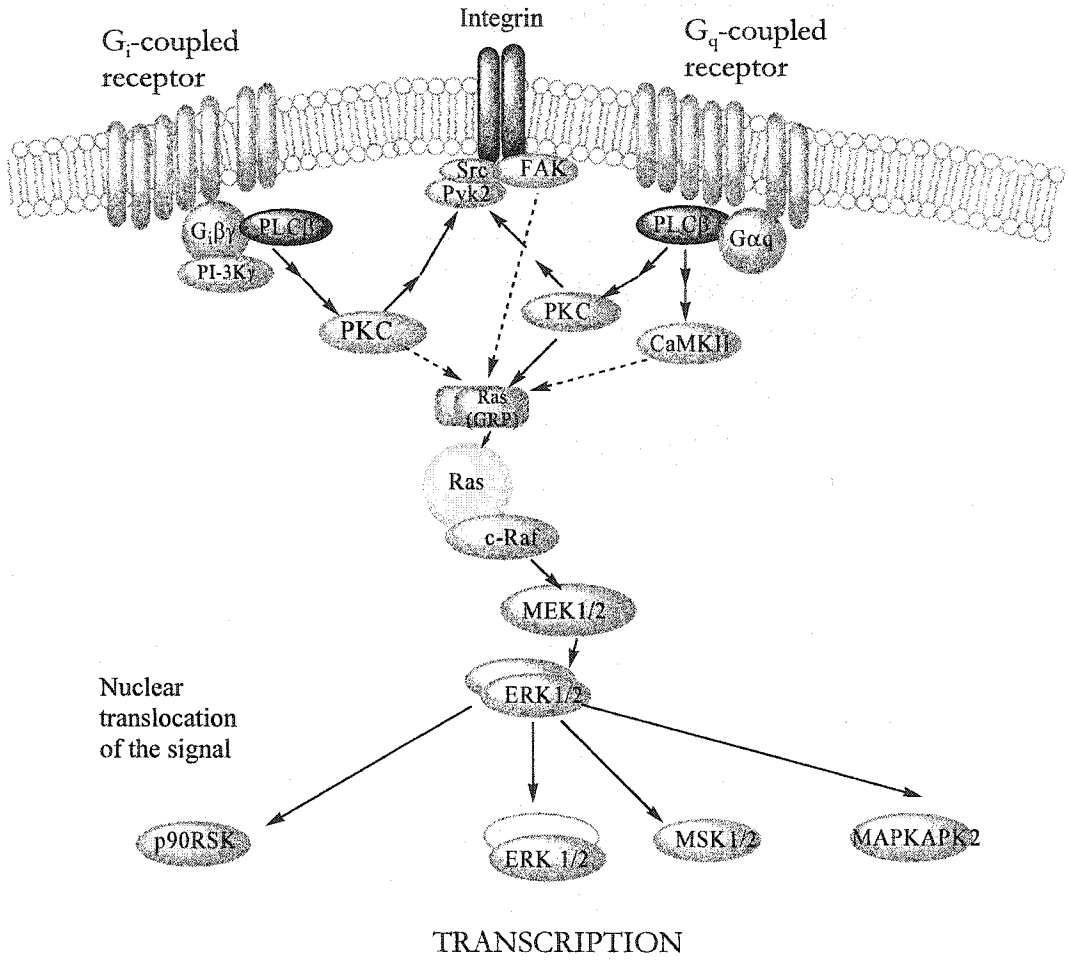

Figure 1.4 GPCR mediated PKC signaling

PKC regulation

PKC is highly regulated by three distinct mechanisms: phosphorylation, cofactor binding, and interaction with targeting proteins. PKC sequesters near its regulators and substrates that determines subcellular localization of PKC for its physiologic function (Newton review, 2001). Generally, newly synthesized PKC is attached to cellular scaffolds and has to undergo a series of three phosphorylations within the catalytic domain of the protein (Thr^{497}, Thr^{638}, and Ser^{657} for PKCα) one

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after the other (Figure 1.5). Those sites are conserved in all species of PKC as well as in its close relatives PKA and PKB/Akt. The fully phosphorylated species of PKC is released into the cytosol, now 'primed' for activation. In the presence of cofactors, Ca and DAG, PKC translocates to the membrane where it becomes active by releasing an autoinhibitory pseudosubstrate from its catalytic domain active site. The fully active PKC then carries out serine/threonine phosphorylation of its substrates.

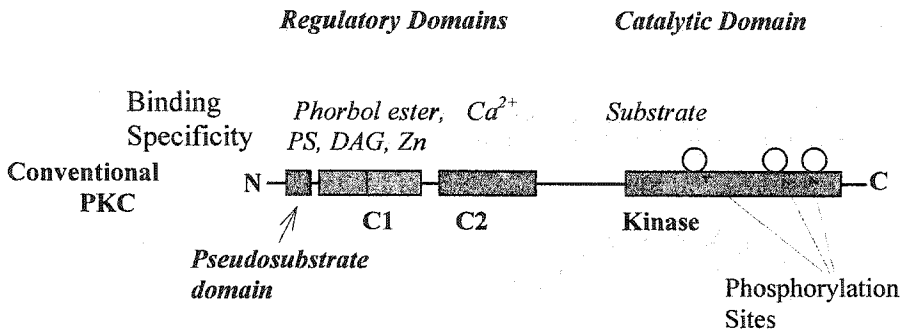

Figure 1.5. Schematic protein structure of conventional Protein Kinase C (PKC). PKC consist of regulatory and catalytic domain each with its own binding specificity: C1 domain responsible for binding of phorbol esters, diacylglycerol (DAG), phosphatidylserine (PS), and Zink (Zn); C2 domain – calcium binding (Ca^{2+}); kinase domain - responsible for substrate binding and its phosphorylation on serine/threonine residues. In addition aminoterminus of PKC has autoinhibitory pseudosubtrate domain that in inactive kinase remain bound to the active site of the kinase domain.

Phosphorylations within the catalytic domain are required for proper PKC processing and gain of catalytic competence. For instance, threonine[497] of PKCα is the first site to be phosphorylated and corresponds to the activation loop of the

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PKC molecule. The phosphoinositide-dependent kinase, PDK-1, is the necessary upstream kinase absolutely essential for the first and rate-limiting step of this activation loop phosphorylation. Phosphoinositide 3-kinase (PI-3K) activation is traditionally reported to be an apical event for PDK-1 activation; however, PDK-1 is also reported to phosphorylate conventional PKC isozymes by a mechanism that is independent of PI-3K (Sonnenburg et al., 2001). The other two phosphorylation sites T^{638} and S^{657} correspond to the turn motif and the hydrophobic site, respectively, and are considered to be autophosphorylation sites.

GPCR-mediated regulation by co-factors

Phospholipase Cβ, activated by the stimulation of a G protein-coupled receptor (Figure 1.6), catalyzes hydrolysis of phosphoinositide-*bis*-phospate (PIP2), which results in the production of diacylglycerol (DAG) and inositol-*tris*-phosphate (IP3). Cytosolic IP3 binds to the IP3 receptor on the ER that gates Ca^{2+} release from storage. The C2 domain of cytosolic PKC is sensitive to changes in intracellular Ca^{2+} concentrations, and quickly binds Ca^{2+}. Now calcium-bound C2 domain has increased affinity to the anionic phospholipids that result in the lateral movement of a PKC molecule along the membrane in search for DAG. Phosphatidylserine (PS) and PLCβ-partitioned DAG anchor PKC to membranes via C1 domain binding. The pseudosubstrate, expelled form anchored PKC molecule, frees the catalytic domain for substrate binding and phosphorylation.

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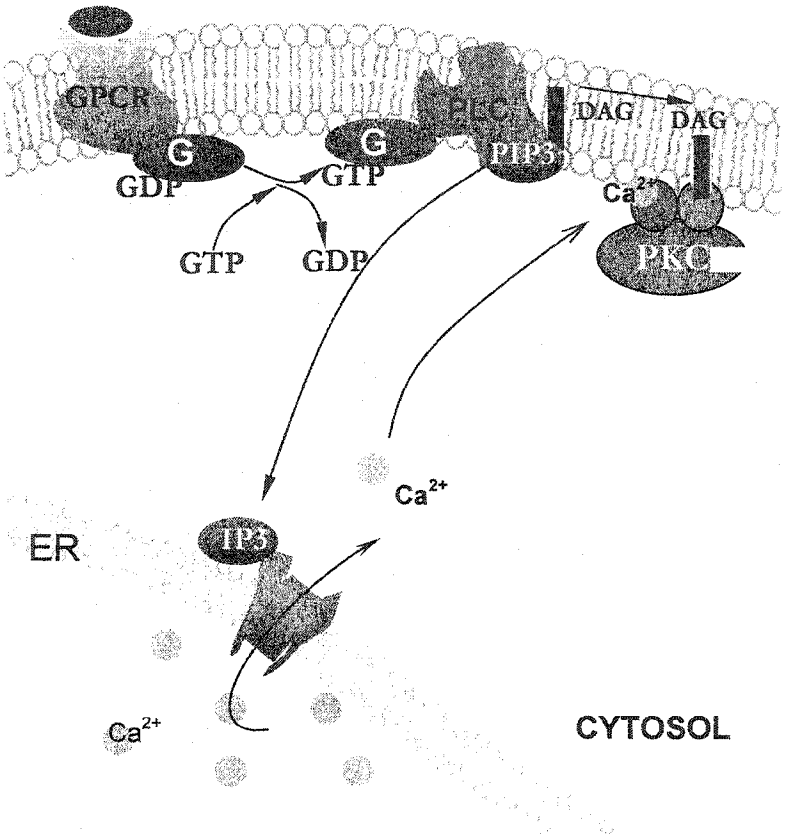

Figure 1.6 GPCR mediated PKC activation in the presence of co-factors.

In conjunction with 5-HT_{1A}-R mediated suppression of caspase-3, protein kinase C appears to be a plausible candidate which could play a crucial role in the neuroprotective pathway.

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OBJECTIVE OF THIS STUDY

Identification of key events in 5-HT_{1A}-R mediated neuroprotective signaling independent of ion channel regulation is set as the main focus of this study.

This study identifies neuroprotective 5-HT_{1A}-R signaling to be effective against apoptotic changes at and downstream of mitochondria. It establishes PKCα as a novel link between the MAP kinase pathway and caspase-3 (MAPK →PKCα→Caspase-3 inhibition) in 5-HT_{1A} receptor-evoked caspase-3 inhibition. It also makes suggestions on the regulation PKCα by MAPK through direct interactions between these two kinases detected in a membrane bound state.

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Chapter 2

Materials and Methods

MATERIALS

The anti-active MAPK antibody, anti-phospho- PKC (pan) antibody and anti-phospho – PKCα (Thr^{638}) were obtained from New England Biolabs (MA); the ERK-2, PKCα, PKCβ, PI-3K, Gβγ reactive antibody as well as secondary antibody for western blotting were obtained from Santa Cruz Biotechnology (CA). Protein A Sepharose beads used for immunoprecipitations was obtained from Sigma (MO). The vectors pZipneo, pZipneoN17Ras and pNMC, pNMCRaf301 were, respectively, kind gifts from Dr. D. Foster (Hunter College, CUNY) and S. Gutkind (NIH). The vectors for PKCalpha transfection studies, SRDpkcαKN, SRDpkcαWT and SRDpkcαACT were obtained from Dr. T. Biden and Dr. M. Reyland (Carpenter et al., 2001; Matassa et al., 2001). Active Caspase-3 in western blotting and in immunocytochemistry was detected with CM1 antibody (IDUN pharmaceuticals). The CM1 staining was visualized with Alexa Fluor 568 (red-orange) antibody from Molecular Probes (OR). In immunoblotting analysis, the protein bands were detected by enhanced chemiluminescence using the Super Signal kit from Pierce (IL). The caspase substrate, acetyl-DEVD-(amino-4-methylcoumarin) (AcDEVD-AMC), the caspase inhibitor, acetyl-DEVD-aldehyde (AcDEVD-CHO) as well as pertussis toxin (PTX), WAY100635, wortmannin and 8-OH-DPAT were obtained from Sigma/RBI. An inhibitor of PL-Cß U73122, Gö6976 (inhibitor of Ca-dependent

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PKCα and PKCβ), Bisindolylmaleimide I (GFX, a general PKC inhibitor), and PD98059 (an MEK inhibitor) were obtained from Calbiochem (CA). Repinotan hydrochloride (BAY x 3702) was obtained from Bayer Corporation (CT).

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METHODOLOGICAL APPROACHES

Cell culture

The mouse hippocampus derived cell line, HN2-5, obtained by engineered expression of human serotonin$_{1A}$ receptor gene (via stable transfection of G-21pBCI construct) was used for all signaling studies (Banerjee et al., 1993). The HN2-5 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS) with the supplement of 200μg/ml of Neomycin in 100-mm tissue culture dishes. Cultures were kept at 37° C, 5% CO_2 tissue culture incubator and split every 5 days (at about 70-80% confluence). For experimental protocols cells were seeded in poly-L-lysine-coated plates, allowed to grow to 60-70% confluency, and then differentiated by 16-h (overnight) treatment with either phosphate-free Kreb's Ringer Buffer (KRB) (25 mM HEPES, 20 mM glucose, 5 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 145 mM NaCl, pH 7.4) or 5 μM retinoic acid in DMEM containing 1% FBS, 1% PS for 24 h. Both processes confer to the cells neuronal morphology and strong immunoreactivity to the neurofilament protein-antibody SMI33 (Singh et al., 1996). Following differentiation, the medium was changed to serum-free DMEM and incubated for at least 1 hour prior to any experimental manipulations; when needed, cultures were supplemented with drugs (8-OH-DPAT, BAY x 3702, antagonists, inhibitors etc.), incubated for indicated time as described in experiments, and then lysed for further analysis (see also: caspase activity assay, MAPK assay, cytosol/ membrane sample prep for PKCalpha studies).

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MAPK assays

For MAP kinase (ERK-2) assays, 60-70% confluent, 10-cm plates of HN2-5 cells were differentiated, the medium changed to serum-free DMEM followed by drug-treatment as described in the figure legends. Following treatment the medium was aspirated and cells lysed with ice-cold RIPA buffer (PBS pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), lysate supplemented with protease inhibitor cocktail (Boeringer) and phosphatase inhibitors. Samples were kept on ice or at 4°C thereafter. Cell lysates were immuno-precipitated with 1mg/ml of rabbit polyclonal ERK-2 antibody, and protein A-sepharose immunoprecipitate conjugates were resolved on 7-16% SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-active MAPK antibody at concentration 1:1000. Blocking and antibody dilution was done in 5% dry milk in 0.1% Tween-20 TBS (TBST). For detection of each primary antibody the corresponding anti-mouse HRP-linked IgG secondary antibody (1:1000) was used. Bands visualized with SuperSignal chemiluminiscence detection kit (Promega). For band normalization original immunoblots were stripped (0.2 M Glycine solution pH 2.5, 1 hour at room temperature) and re-probed with the precipitating antibody.

Immunoprecipitations

All experiments that involved immunoprecipitations (IP) were carried out in a standardized manner. Prior to IP, cells were treated in accordance with experimental design; cellular lysates were obtained, passed through a 21 gauge needle and then treated with 1 mg/ml of the immunoprecipitating antibody of choice (anti-

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Gβγ, anti-ERK-2, anti-PKCα or anti-PKCβ). Following 1 hour incubation at 4°C, 40 µl of reconstituted protein A-Sepharose was added to each sample and IP was allowed to proceed over night at 4°C with agitation. The IP pellets, that were obtained after centrifugation at 8 000 rpm, were further washed three times with 200 µl of ice-cold RIPA buffer. After a final wash is removed, IP pellets were either used for further experiments (see PKC enzyme assay section) or mixed with 100 µl of "Treatment buffer" and set aside for SDS-PAGE.

Anoxia and H_2O_2 induction of apoptosis

Anoxia conditions were generated as described in our previous report (Singh et al., 1996*b*; Adayev et al., 1999). In short, HN2-5 cells were first subjected to differentiation, followed by change of medium to serum-free DMEM. The amount of medium introduced depended on the size of the experimental plate: for 10 cm plates – 8 ml per plate; for 24-well plate – 1 ml per well. The medium was further supplemented with agonist, antagonist, inhibitors etc or with carrier for the control. Cells were further subjected to anoxia in the Gas-Pak chamber (Becton and Dickinson, MD) for 10-cm plates or in sealed Biobags (Becton and Dickinson, MD) for multi-well plates. For Biobag-anoxia 'generator' ampoule of $NaBH_4$ (provided with the kit) is cracked in the jacket of water to provide a source of hydrogen. The Biobag is also equipped with palladium catalyst that aid the reaction of oxygen and hydrogen to produce moisture, thus resulting in atmosphere of hydrogen, nitrogen, 5% carbon dioxide and moisture within 30-40 minutes. Anoxic conditions were maintained for 6 hours followed by 16 hours (overnight) of reoxygenation in tissue culture incubator.

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For hydrogen peroxide-induced apoptosis experimental plates of differentiated HN2-5 cells were additionally supplemented with hydrogen peroxide from freshly diluted and sterile filtered stock solution prepared in serum-free DMEM. The concentrations and duration of H_2O_2 were determined experimentally and tailored to the cell line. Typically, 200 or 500 μM H_2O_2 treatment was carried out for 16 hours before analyzing caspase-3 activity using either a fluorescent substrate or immunocytochemistry.

Caspase-3 assay

Fluorescent substrate-based assay

Caspase assays were performed using monolayers of HN2-5 cells at 80% confluence. Cell lysates were prepared from hydrogen peroxide-treated or normoxic and anoxic cell pellets obtained after two washes with ice cold PBS. Cells were next lysed in 0.5 ml of Yama buffer (10 mM HEPES pH 7.5, 142 mM KCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 0.1 mM PMSF) (Deveraux et al., 1997). Each sample was homogenized with a Potter Elvehjem homogenizer and then ultracentrifuged at 100, 000 x g for 5 min. The supernatant obtained was assayed for protein concentration, which was adjusted to 2μg/μl by diluting with Yama buffer and then an aliquot containing 300 μg protein was added to each well of a 96-well plate. This was followed by addition of 1.5 μl of 10 mM stock AcDEVD-AMC in oxygen-free water (final concentration 100 μM). The released fluorescence (due to the fluorescent group, AMC, that is cleaved by the caspases) was monitored after 30 min incubation

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using a fluorescence plate reader set at 360/460 nm excitation/emission wave length. Results from multiple caspase assays, each performed with triplicate samples, were combined after conversion into percentage of control caspase activity. Statistical analysis and determination of p values were carried out using student's t-test.

Anti-active caspase-3 immunoblotting

Samples for immunoblotting of anti-active caspase-3 were prepared in the same way as for fluorescent substrate-based assay described in the previous section. An aliquote of the supernatant containing 100 μg of protein was mixed with 2X Treatment buffer, boiled for 5 minutes and resolved on 7-16% SDS-PAGE. Following transfer to nitrocellulose, the membrane was blocked in 5% non-fat dry milk in 0.05% Tween-20 –PBS and incubated in polyclonal CM1 antibody 1:1000 over night at 4°C. The immunoreactive, processed 17-kDa fragment of CPP-32 (caspase-3) was detected via goat anti-rabbit IgG coupled to HRP and SuperSignal chemiluminescence substrate exposure. The CM1 antibody specifically reacts with processed caspase-3 and does not bind the full length, unprocessed 32 kDa caspase-3 protein.

Staining of anti- active caspase-3 (immunocytochemistry)

In experiments requiring immunostaining, the cells were grown in 24-well plates, co-transfected as described in "Transfection of PKCα vectors..." (see below), differentiated, and then subjected to 200 μM hydrogen peroxide treatment for 16 h. Subsequently, the cells were fixed in freshly prepared 3% paraformaldehyde for 30 min at room temperature, washed two times with PBS, and

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then blocked in PBS containing 2% bovine serum albumin (Fraction V), 2% normal goat serum and 0.4% Triton X-100 for 1 hour at room temperature. Next, the blocking solution was replaced with 1° antibody (CM1) diluted 1: 3000 in blocking solution. After overnight treatment at 4 °C, the cells were washed three times with wash buffer (0.2% Tween-20 in PBS) at room temperature (5 minutes per wash), and then incubated with the 2° antibody (goat anti-rabbit IgG-Alexa Fluor 568) (1: 2500) in blocking solution for 1 h at room temperature. After three washes with the wash buffer and one wash with PBS, the cells were visualized by fluorescence microscopy using filters with emission wavelengths of 580 nm (for Alexa Fluor 568) and 510 nm (for viewing a green fluorescent protein co-transfectants). Digital images were collected and analyzed. The levels of CM1 immunoreactivity were assessed only for green fluorescent protein (GFP) positive cells.

Protein kinase C enzyme assays

Differentiated HN2-5 cells were treated for 30 min with 1 μM phorbol-myristate (PMA- a positive control), carrier or 1 μM 8-OH-DPAT in the absence and presence of 25 μM PD98059, following which the cells were lysed in RIPA buffer as described in MAPK assays. PKCα and PKCß were immunoprecipitated from the cell lysates using the respective antibodies (Santa Cruz Biotech, CA). Further immunoprecipitates were subjected to PKC enzyme assay with PKC-Assay Kit (Pierce) in accordance with manufacturer's protocol with some modifications. In short, the immunoprecipitates were washed thoroughly, and then resuspended in 10 μl PKC dilution buffer as provided in the PKC-Assay Kit. This suspension was supplemented on ice with 15 μl of a pre-mixed reaction mixture containing equal

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volumes of 5x reaction buffer, 5x activator solution (sonicated in a bath sonicator prior to addition), PKC substrate which also contained 5 μCi/ tube of γ-P^{32} ATP. Myelin basic protein (MBP) peptide supplied with the kit was used as substrate. The final volume with the protein A-sepharose beads was 30 μl. The tubes were vortexed and then incubated at 30 °C for 30 min., followed by microcentrifugation and transfer of 25 μl of the supernatant to SpinZyme columns. The columns were microcentrifuged to elute the aqueous phase. The non-phosphorylated peptide was next washed off by addition of 250 μl of Phosphopeptide Binding Buffer (provided in the kit), incubation for 3 min, followed by centrifugation for 1 min at 6000 rpm. This procedure was repeated once for a total wash of 500 μl, and then the column was placed in a fresh tube before elution of the phosphopeptide adhering to the column. For the elution, 250 μl of Phosphopeptide Elution Buffer (provided in the kit) was applied to the column, followed by incubation for 3 min and centrifugal elution. This process was repeated once to obtain a total elution volume of 0.5 ml. Next, 20 μl of each eluate was mixed with 3 ml of scintillation fluid and subjected to scintillation counting using a Beckman scintillation counter.

Transfection of PKCα vectors and pEGFPC1 into HN2-5 cells

The pEGFPC1 (2 μg) alone or 1 μg each of pEGFPC1 and SRDpkcαKN or pEGFPC1 and SRDpkcαWT (Carpenter et al., 2001; Matassa et al., 2001) were cotransfected into HN2-5 cells in 24-well plates. Transfection was carried out using the Superfect Transfection kit from Qiagen, Inc optimized for transfection of HN2-5 cells in 24 well plates without pre-plating. The cells ($1x10^5$ per well) were incubated for five hours with the transfection complexes containing 2 μg of total

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DNA and 5μl of SuperFect reagent. Medium was then changed to normal growth medium (10% FBS, 1% penicillin-streptomycin in DMEM) and cells were allowed to recover for 19-24 h before differentiation. Differentiation was carried out in 5 μM retinoic acid in 1% FBS-containing DMEM for 24 h, following which the medium was changed to serum-free DMEM and appropriate plates were treated with carrier or 8-OH-DPAT in the absence or presence of 25 μM PD98059. H_2O_2 was added to a final concentration of 200 μM and the cells were incubated for 16 h in a tissue culture incubator as previously described.

Preparation of membrane bound and cytosolic fractions

Differentiated HN2-5 cells in 10 cm dishes were used for experimental manipulations in signaling. After drug exposure according to experimental design plates were quickly rinsed on ice with ice-cold PBS and scraped in presence of 0.5 ml of PKC homogenization buffer supplemented with protease inhibitors cocktail and phosphatase inhibitors. Membrane/cytosol fractions were prepared in accordance with protocol for PKC enzyme assay (Pierce, IL). In short, cell lysates were kept on ice at all the time. Following Teflon-glass homogenization samples were ultracentrifuged at 50 000 g for 20 minutes. Supernatant was removed into a fresh vial and constitute cytosolic portion of the preparation. The pellet is further resuspended in detergent containing buffer (RIPA) and constitute a membrane bound protein portion of the prep. After protein estimation of both fractions 50-100 μg of each samples (membrane and cytosol) were resolved on 7-16% SDS-PAGE directly or after immunoprecipitation with ERK-2 or PKCα antibody (from 300 μg of cytosol or membrane bound fractions). Western blots of those gels were then

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immunoblotted with variety of antibodies (ERK-2, p42/p44- ERK, P-PKC (pan), P-PKCa (Thr 638), PKCα, PKCβ).

Transfections of Ras and Raf-1 vectors

The dominant negative constructs of pZip-neoN17Ras and pNMCRaf301 (pZip-neo and pNMC used as respective controls) as well as pEGFP (which encodes for photoenhanced mutant of green fluorescent protein used as transfection efficiency control) were cotransfected into 10^7 HN2-5 cells by means of electroporation. Salmon sperm carrier DNA and 10 μg of each plasmid DNA were delivered via electroporation at 200 V and 1000 μF in serum-free DMEM. Following electroporation cells were seeded in normal growth media 10% FBS, 1% Pen-Streptomycin DMEM and allowed 24-48 hours for recuperation. Approximately 60-80% transfection efficiency was recorded. Experimental manipulations were carried out within 96 hours of transfection. Transfectants were seeded in poly-lysine precoated multiwell plates, differentiated, drug treated according to figure legend and subjected to anoxia/re-oxygenation in BioBag (see 'Anoxia...' section for details), following which viability of cells was assessed through Hoechst 33342 staining.

HOECHST 33342 staining to assess apoptosis

Controlled treatment of cells with the membrane-permeable dye Hoechst 33342 has been shown to effectively stain apoptotic nuclei and detect chromatin condensation (Dive et al., 1992; Ham et al., 1995). HN2-5 cells were transfected with control vectors (pZipneo and pNMC) and those harboring the dominant negative mutants of Ras (N17Ras) and Raf-1 (Raf301), differentiated, treated with the agonist,

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and then subjected to hypoxia and reoxygenation. Following this the cells were fixed, permeabilized and then assessed for chromatin condensation and disintegration of the nucleus by Hoechst staining (Dive et al., 1992). A 1-μM concentration of Hoechst33342 was used to label the cells for 15 min, following which the dye was washed out with 10 mM PBS and then pictures shot using a 360-nm excitation/ 420-nm emission filter. The apoptotic cells were identified by their disintegrated nuclear structure and then counted to assess the proportion of healthy and apoptotic cells in multiple fields.

Statistics

All statistical analysis and determination of significance (p value) were carried out using Sigma Plot student's t-test.

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Chapter 3

Experimental findings

Stimulation of the 5-HT_{1A}-R by agonists causes time-limited and concentration dependent activation of the MAPK.

Differentiated mouse hippocampus derived cell line HN2-5 was treated under serum free conditions with either one of selective 5-HT_{1A}-R agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) or repinotan (BAY x 3702). Consistent with an earlier report on HN2-5 cells, 1 μM concentration of 8-OH-DPAT produced optimal activation of ERK 1/2 in 30 minutes (Figure 3.1a), which further persisted for at least 3 hours (Adayev et al., 1999). Similar analysis for BAY x 3702 agonist and its potency in activation of ERK 1/2 revealed that 100 nM BAY x 3702 elicited a prominent increase in 5 minutes of agonist treatment, that also reached a maximum at 30 minutes (Figure 3.1b & c), as seen with 8-OH-DPAT treatment. However, following this, it decreased dramatically to basal levels in 3 hours (Figure 3.1 c). Such difference from the prolonged activation of ERK observed in 8-OH-DPAT-treated HN2–5 cells was attributed to the lesser chemical stability of BAY x 3702 in aqueous solutions. Concentration-dependent analysis revealed that BAY x 3702-evoked ERK 1/2 activation was significant already at 1 nM agonist concentration (Figure 3.1d, lane 3). Similar to the dose–response curve of 8-OH-DPAT, the ERK 1/2 stimulation reached a maximum at 100 nM BAY x 3702 and then remained unchanged up to at least 1 μM.

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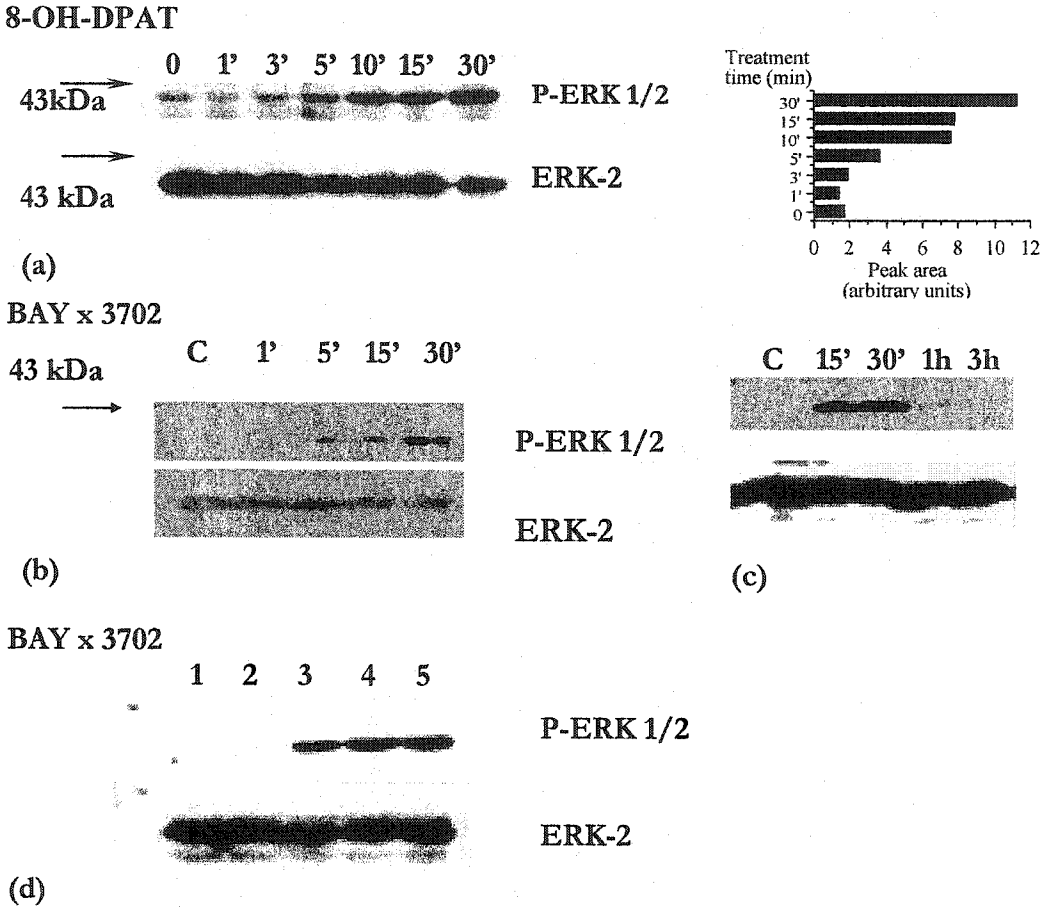

Figure 3.1 Agonist stimulated 5-HT_{1A}-R mediated MAPK activation profile

(a) Time course of 8-OH-DPAT - mediated ERK-2 stimulation. Differentiated HN2-5 cells were treated with 1μM 8-OH-DPAT for the indicated time periods. **(b)** Time course of BAY x 3702-evoked ERK-2 stimulation. Differentiated HN2-5 cells were treated with BAY x 3702 (100 nM) for the indicated time periods. **(c)** Extended time course. The control was carrier-treated for 3 h, whereas the other samples were treated for the indicated time periods with BAY x 3702 (1 μM). **(d)** Dose dependence of BAY x 3702-evoked ERK-2 stimulation. Lanes: 1. Control, 2- 5. Treatment for 30 min with the following concentrations of BAY x3702: 5 pM (lane 2), 1 nM (lane 3), 100 nM (lane 4), and 1 μM (lane 5). Data shown represent two independent experiments.

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Serotonin$_{1A}$ receptor agonists activate ERK 1/2 pathway independent of PI-3Kγ.

Since prolonged agonist exposure caused pronounced activation of MAPK (as seen in Figure 3.1a-c) all consequent studies were performed with 30 minutes agonist exposure generalized for convenience in use with both agonists (8-OH-DPAT (D) and BAY x 3702 (BAY)). The 8-OH-DPAT mediated activation of MAPK in the same cell line has been shown to be independent of PI-3Kγ pathway (Adayev et al., 1999). The next experiment was carried out to question the dependency of the repinotan–mediated phosphorylation of ERK 1/2 kinases on the PI-3K pathway. Upon close examination of MAP kinase activity in anti-ERK-2 immunoprecipitated fractions by Western blotting it became apparent that both agonists mediate MAPK activation, that is receptor-specific, because blocking of the receptor with an antagonist WAY 100635 (WAY) reverses the agonist-mediated MAP kinase (Figure 3.2, lanes 3 & 5) activity to nearly control levels. The 5-HT$_{1A}$-R mediated ERK 1/2 stimulation was eliminated in the presence of the MEK inhibitor PD98059 (Figure 3.2, lane 9), which inhibits this immediate upstream factor of ERK 1/2 in MAPK pathway. However, phosphorylation levels of ERK 1/2 was not affected by the PI-3Kγ inhibitor wortmannin (Figure 3.2, lane 7), confirming that both agonists (8-OH-DPAT and repinotan BAY x 3702) stimulate 5-HT$_{1A}$-R in a similar fashion by coupling the receptor to a Pertussis toxin sensitive G-protein (Figure 3.2, lane 11).

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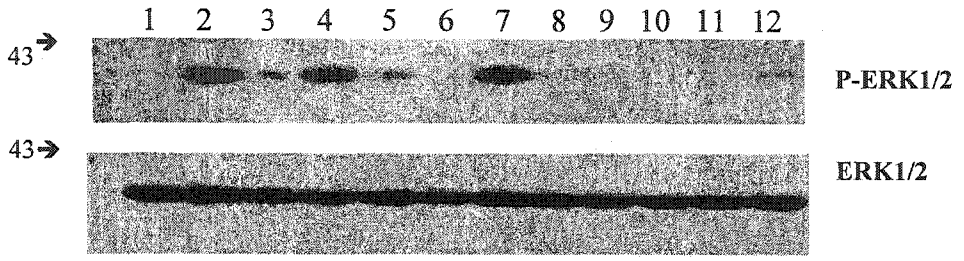

Figure 3.2 BAY x 3702 (repinotan)-mediated ERK 1/2 activation is not dependent on PI-3Kγ kinase activity.
Western blot of anti-ERK-2 immuniprecipitation products resolved by 7-16% SDS-PAGE and probed with anti-phospho-ERK 1/2 antibody. Agonist exposure for all the samples was 30 minutes.
Lane 1. Carrier treated control,
Lane 2. 1 μM 8-OH-DPAT,
Lane 3. 1 μM WAY100635 + 1 μM 8-OH-DPAT,
Lane 4. 1 μM BAY x 3702,
Lane 5. 1 μM WAY100635 + 1 μM BAY x 3702,
Lane 6. 1 μM WAY100635,
Lane 7. 0.1 μM Wortmannin + 1 μM BAY x 3702,
Lane 8. 0.1 μM Wortmannin,
Lane 9. 25 μM PD98059 + 1 μM BAY x 3702,
Lane 10. 25 μM PD98059,
Lane 11. 100 ng/ml Ptx + 1μM BAY x 3702,
Lane 12. 100 ng/ml Ptx.

Thus, 5-HT_{1A}-R mediates activation of ERK 1/2 kinases, in response to treatment by both agonists, completely independent of PI-3Kγ activation. Upon stimulation of the receptor by agonists, PI-3Kγ is activated, but it enroutes its signaling most likely through other signaling targets, such as Akt, since 8-OH-DPAT-triggered phosphorylation of Akt was indeed sensitive to the PI-3Kγ inhibitor, wortmannin. (Adayev et al., 1999). This also shows the efficacy 0.1 μM wortmannin in causing complete inhibition of PI-3K. Moreover, previous studies also showed that PI-3Kγ is not involved in the 8-OH-DPAT mediated suppression

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of caspase-3 activity; however ERK 1/2 played a key role in this inhibition of caspase-3 (Adayev ey al., 1999).

An inhibitor of PLCβ effectively blocks ERK 1/2 activation and Caspase-3 inhibition

Agonist stimulation of the 5-HT_{1A} receptor also causes a simultaneous increase in intracellular inositol phosphates (IP_3), which is a direct measure of PLC activity (Adayev et al., 1999). This increased PLC activity could be essential for the observed activation of ERK 1/2, and this is supported by earlier studies confirming that ERK stimulation by other G protein-coupled receptors requires activation of PLCβ upstream of Ras (Della Rocca et al., 1997). Elimination of the 5-HT_{1A}-R mediated activation of both ERK 1/2 (Figure 3.3, lane 8) and inhibition of caspase-3 (Figure 3.4 a & b) by the PLCβ inhibitor U73122 (U) confirmed the involvement of PLCβ upstream of ERK 1/2 (Della Rocca et al., 1997).

Activation of PLC results in an increase in intracellular calcium, released from the endoplasmic reticulum (ER), and also generates diacylglycerol (DAG). Both, Ca^{2+} and DAG are the co-factors for activation of PKC. Furthermore, PKC is known to phosphorylate and activate the signaling protein Raf-1, which stimulates ERK 1/2 via the signaling protein MEK (Daum et al, 1994). Therefore, a PKC isozyme could relay the signal from the activated PLCβ further to lead to the activation of ERKs (see Figure 1.4 in introduction chapter). This possibility was tested in the next experiment.

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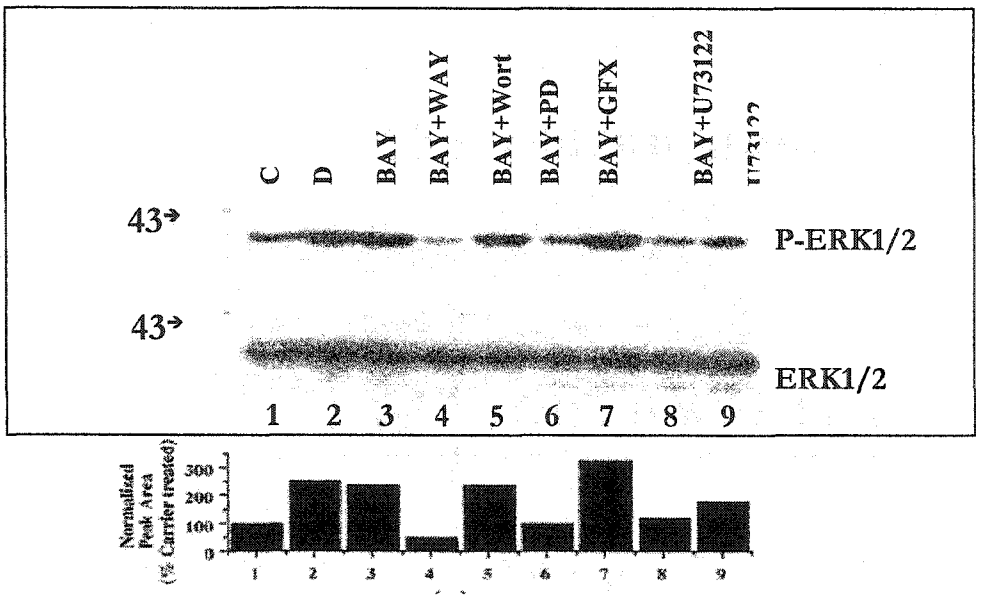

Figure 3.3 PL-Cß, but not PKC, is involved in 5-HT_{1A} receptor-mediated activation of ERK1/2.

Differentiated HN2–5 cells were treated for 30 min with either carrier (lane 1), or 1 μM 8-OH-DPAT (D) (lane 2), or 1 μM BAY x 3702 (BAY) in the absence (lane 3) or presence of 1 μM WAY100635 (WAY) (lane 4), 0.1 μM wortmannin (Wort) (lane 5), 25 μM PD98059 (PD) (lane 6), 2 μM GFX (lane 7), or 1 μM U73122 (U) (lane 8). In a separate set, the cells were treated with 1 μM U73122 alone (lane 9). Next, the cells were lysed and subjected to immunoprecipitation using an ERK1/2 antibody followed by Western blotting using an anti-active ERK1/2 antibody.

PKC is not involved in 5-HT_{1A}-R-mediated MAP Kinase activation, but is required for agonist-mediated inhibition of Caspase-3.

Pretreatment of cultures with a general PKC inhibitor (GFX) prior to agonist exposure had no effect on the levels of ERK 1/2 phosphorylation (Figure 3.3, lane 7). Therefore, at least the part of the pathway, before MAP kinase, does not depend on the activation of PKC or PI-3K.

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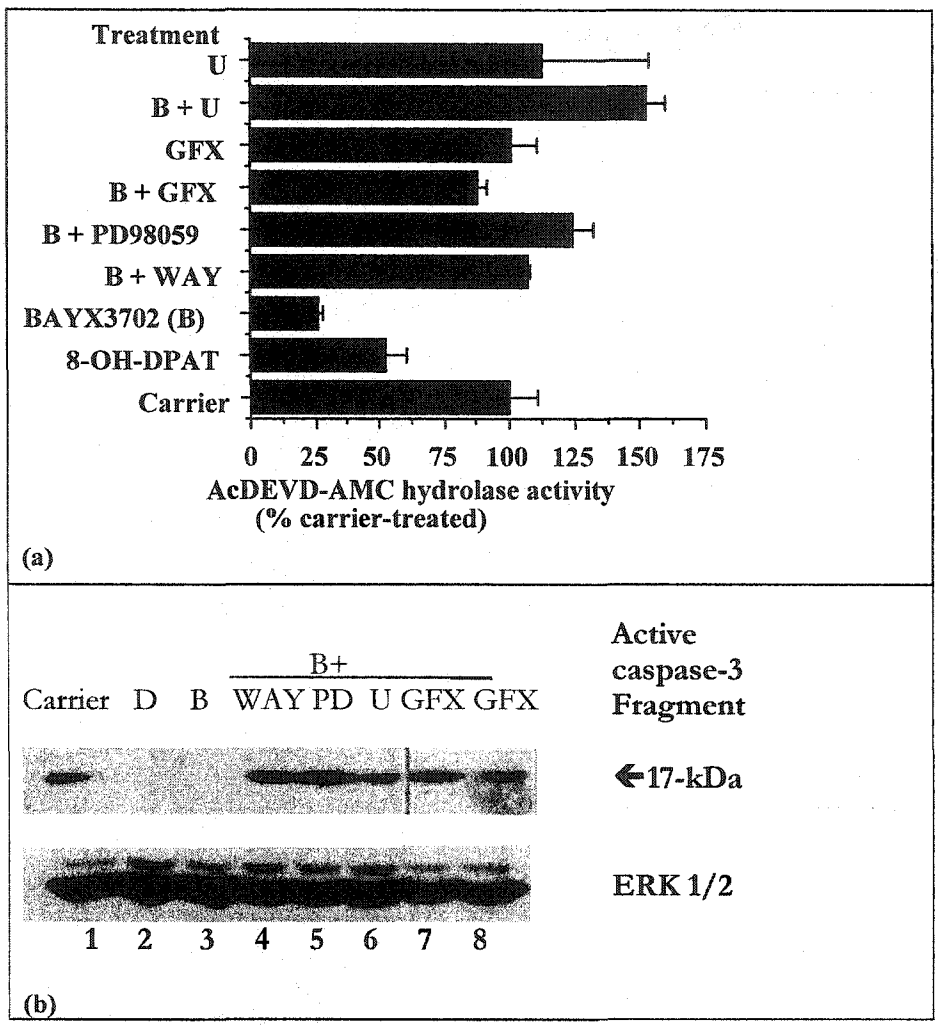

Figure 3.4 The 5-HT_{1A} receptor-mediated protective pathway causes caspase-3 inhibition in PLCβ, PKC, and ERK1/2 dependent way.

Differentiated HN2-5 cells were treated for 30 min with carrier, or 8-OH-DPAT (1 μM) (D) or BAY x 3702 (1 μM) (B) in the absence and presence of GFX (2 μM) (GFX), or PD98059 (25 μM) (PD), WAY100635 (1 μM) (WAY), or U73122 (1 μM) (U). Next, cells were subjected to 6 h hypoxia and 16 h reoxygenation, following which cell lysates were either **(a)** subjected to caspase assay or **(b)** analyzed by Western blotting using the anti-active caspase-3 antibody (CM1). Decreased presence of the 17-kDa fragment of active caspase-3 indicates inhibition of caspase-3 activity. Data presented are the mean (±S.D.) of three independent experiments carried out with triplicate samples.

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Next, the involvement of PKC in 5-HT_{1A}-R mediated neuroprotection was examined with respect to caspase-3 inhibition. Although, blocking of PKC activity with GFX (general PKC inhibitor) had no effect on MAP kinase activity it had a severe effect on cell survival and literally annuls the 5-HT_{1A} agonist-evoked protection. Receptor-mediated inhibition of the Caspase-3 activity was reversed almost to control levels in cells treated with GFX + agonist or GFX alone prior to the onset of anoxia (Figure 3.4 a & b). This runs in parallel with the result of PD-mediated inhibition of anti-apoptotic effect of the receptor agonists. Thus, the data points to the requirement of PKC for successful inhibition of Caspase-3 activity by the 5-HT_{1A} agonist. Western blot analysis of cytosolic extracts, obtained from the cells after anoxia and re-oxygenation treatment, for the presence of the processed caspase-3 revealed the pattern of bands supporting the data obtained in caspase activity assays (Figure 3.4b). Samples that received only agonist treatment had lowest caspase activity and showed no active caspase-3 fragment when immunoblotted with anti-active CMI antibody. Blocking the receptor with an antagonist (WAY100635, WAY) or inhibition of PLCβ, MEK and PKC with U73122, PD98059 and GFX, respectively, in combination with agonist treatment, shows the presence of the active caspase-3 band only at levels observed in the carrier-treated control. Thus, PKC is essential in the 5-HT_{1A}-R mediated inhibition of Caspase-3 processing and activity in anoxia-induced apoptosis.

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8-OH-DPAT treatment attenuated H_2O_2-induced caspase-3 activity in a MAPK- and PKC-dependent pathway

Oxidative stress-associated increase in intracellular levels of reactive oxygen species (ROS), including superoxide and hydrogen peroxide, are frequent and unbidden companions of numerous neurodegenerative conditions. Hydrogen peroxide (H_2O_2) is one of the agents that induce oxidative damage and is commonly used in cell cultures to generate hydroxyl radicals shortcutting directly to the mitochondrial changes in apoptosis leading to activation of caspase-3 (Bhat & Zhang, 1999; Laszkiewicz et al., 1999; DiPietrantonio et al., 1999). Given that 5-HT_{1A}-R mediated signaling attenuated anoxia-induced apoptosis through reduction of the caspase-3 activity, an experiment was set up to test the generality of this mechanism and its effectiveness against the oxidative injury. Differentiated cultures of HN2-5 cells were subjected to oxidative stress through exposure to increasing concentrations (0.1 mM to 1mM) of hydrogen peroxide. The optimal experimentally determined H_2O_2 concentrations were 200 μM and 500 μM producing on average a 4 to 6 times increase in detected caspase-3 activity (Figure 3.5a). Such H_2O_2 concentrations also was found to be comparable with increase in caspase-3 activity observed during anoxia/re-oxygenetion (Das et al., 2001). Higher concentrations of H_2O_2 resulted in poor sample recovery with respect to caspase-3 that could be explained by severity of the oxidative stress that can lead to a shift from apoptosis to necrosis. At concentrations of H_2O_2 higher than 500 μM the cell membranes were ruptured causing a loss of cytosolic caspase-3 to the surrounding media. In at least ten experiments performed with H_2O_2-induced apoptosis the magnitude of the caspase-3 increase produced with 500 μM H_2O_2 varied depending on the confluence

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of the plates. Pretreatment of the differentiated HN2-5 cells with the 5-HT_{1A}-R agonist 8-OH-DPAT (D) attenuated the caspase-3 activity by at least 40% (Figure 3.5b). Attenuation of the caspase-3 activity was not detected if the receptors were previously blocked with the antagonist WAY100635 (WAY) (Figure 3.5a) Consistent with the data obtained from the anoxia/re-oxygenation caspase-3 assay, caspase-3 inhibition was eliminated by the MAPK kinase inhibitor PD98059 (PD) and PKC inhibitor GFX (Figure 3.5b). Therefore, agonist-mediated inhibition of caspase-3 activity in anoxia/re-oxygenation- and in H_2O_2-induced apoptosis is most likely to occur through the same neuroprotective pathway.

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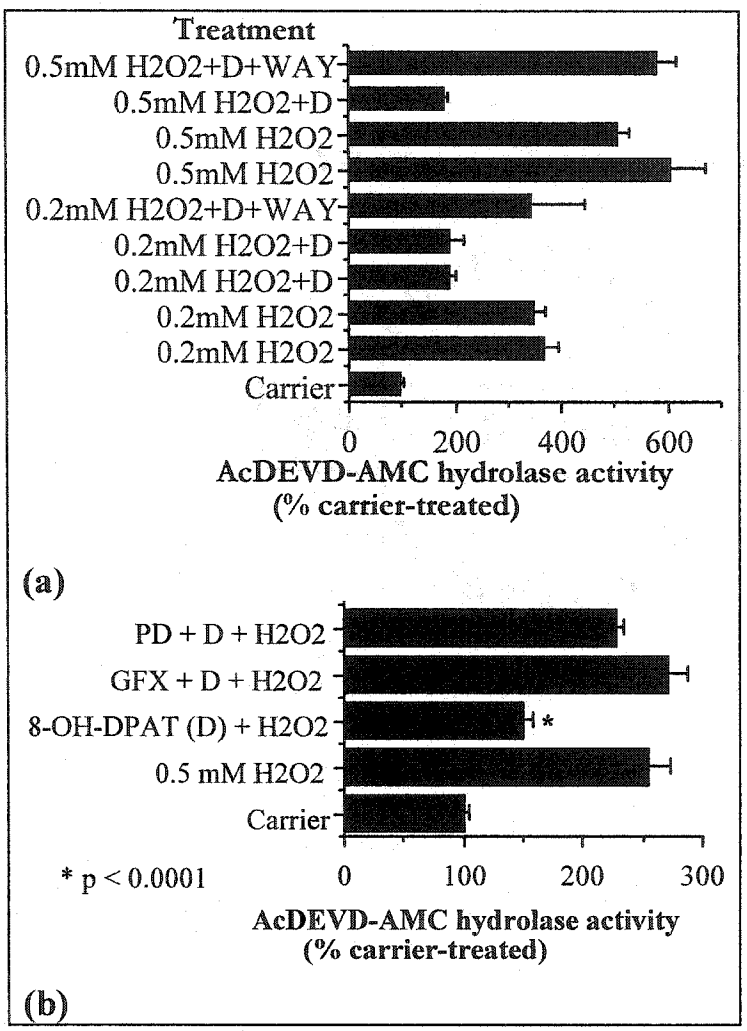

Figure 3.5. Oxidative stress causes an increase in caspase-3, which is also reversed by a 5-HT_{1A} agonist through MAPK-, and PKC- dependent pathway. Differentiated HN2-5 cells were treated 8-OH-DPAT (1 μM) in the absence and presence of **(a)** WAY100635 (1 μM), or **(b)** GFX (2 μM), PD98059 (25 μM), for 30 min. This was followed by addition of H_2O_2 from a stock solution in serum-free DMEM to the indicated concentrations. Next, the cells were incubated at 37 °C for 16 h and then lysed for caspase assay. Each bar presented is the mean (±S.D.) of triplicate samples.

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Serotonin$_{1A}$ receptor-mediated protection of HN2–5 cells against anoxia-triggered apoptosis involves Ras and Raf-1

It was reported earlier that for some GPCRs (adrenergic receptors α1B or α2A), which signal either through Gα or Gβγ subunits, activation of ERK 1/2 pathways converge at the level of phospholypase C and then proceed through Ras-dependent activation of Raf-1 and ERK 1/2 (Della Rocca et al., 1997; Luttrell et al., 1999; Maudsley et al., 2000). The link between the G protein-coupled 5-HT$_{1A}$ receptor and ERK1/2 was not clear. Additionally, Ras dependent activation of Raf-1 was reported to be insensitive to PKC inhibitors in adrenergic receptor mediated activation of ERKs. Thus one possibility was that the 5-HT$_{1A}$ receptor was connected to MEK and ERK1/2 through Ras and Raf-1. Involvement of Ras in the 5-HT$_{1A}$ receptor-mediated protection of HN2–5 cells was tested by selective inhibition of Ras and Raf-1 activity through transient expression of the corresponding dominant inhibitory mutants, N17Ras and Raf301, respectively. Cotransfection of a photoenhanced mutant of the green fluorescent protein in pEGFP vector was used to confirm the transfection efficiency (60–80%).

The transfected and differentiated HN2–5 cells were subjected to anoxia-induced apoptosis. Those cultures appeared to be more fragile to anoxia and reoxygenation than the differentiated, untransfected HN2–5 cells. In contrast to only 20–30% of apoptosis usually recorded in the untransfected HN2–5 cells (Adayev et al., 1999), about 50–80% apoptosis was observed in the transfected HN2–5 cells. Control transfection was achieved using the vectors without the insert cDNAs (pZipneo and pNMC) and showed inhibition of apoptosis by BAY x 3702 in a dose-

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dependent manner (Figure 3.6a & b). This BAY x 3702-evoked protection was completely eliminated in the presence of either N17Ras (Figure 3.6a) or Raf301 (Figure 3.6b), thus confirming the involvement of Ras and Raf-1 in 5-HT_{1A} agonist-evoked protection of anoxic HN2–5 cells.

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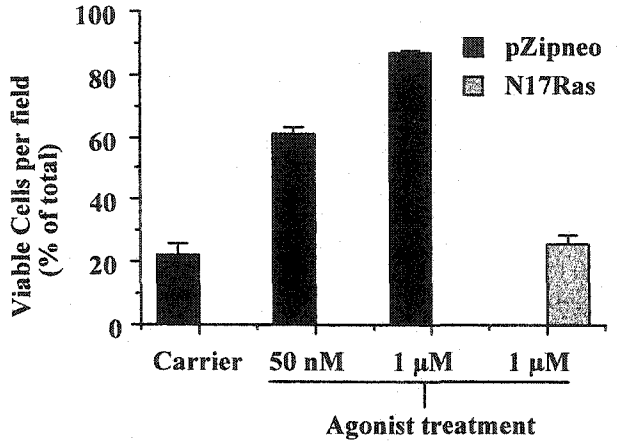
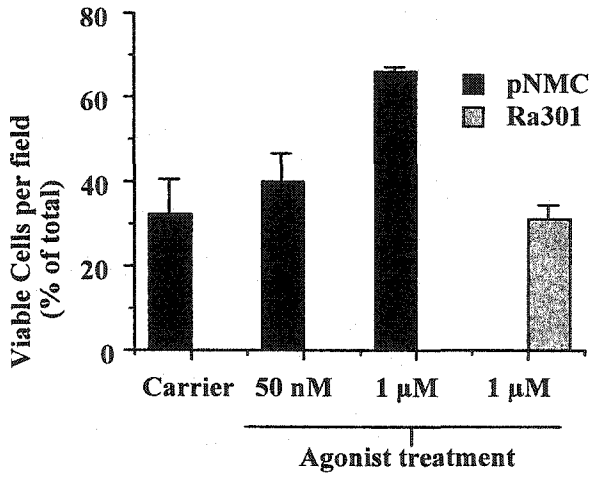


(a)



(b)

Figure 3.6 Involvement Ras and Raf-1, in serotonin 1A Receptor-mediated protection of anoxic HN2-5 Cells. HN2-5 cells transfected with **(a)** pZipneo or pZipneo-N17Ras or **(b)** pNMC or pNMC-Raf-301, were differentiated and then treated with carrier or the agonist (BAY x 3702), and then subjected to 6h of anoxia and 16 hours reoxygenation. The cells were then fixed, permeabilized and stained with HOECHST33342 (1μM) to assess cell viability by fluorescence microscopy. Data presented are the mean (±S.D.) of two independent experiments.

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MAPK-mediated Caspase-3 inhibition occurs through a calcium-dependent PKC isozyme.

In the family of PKC's only the so-called major isoforms (α, β_I, β_{II}, and γ) actually require calcium ions for activation together with diacylglycerol. The minor forms of PKC lack the calcium-binding domain, and therefore their activity is independent of calcium. Since the intracellular level of Ca^{2+} increases as the result of PLCβ action, the chances of involvement of a Ca-dependent PKC isozyme are much greater. Using the selective inhibitor of calcium dependent PKC (α and β) – Gö6976 (Martiny-Baron et al., 1993), an attempt was made to narrow down the possible candidates of PKC family (Figure 3.7). The IC_{50} of Gö6976 in inhibition of PKC is 2.3 nM for PKCα and 6.2 nM for PKCβ (Martiny-Baron et al., 1993). It was found that Gö6976, even at very small concentrations ranging 1-5nM, was extremely efficient in inhibiting the effect of 1μM 8-OH-DPAT (Figure 3.7a) and of 1μM BAY x 3702 (Figure 3.7b).

Therefore, the PKC molecule that mediates the neuroprotection via Caspase-3 inhibition is one of the calcium-dependent isozymes.

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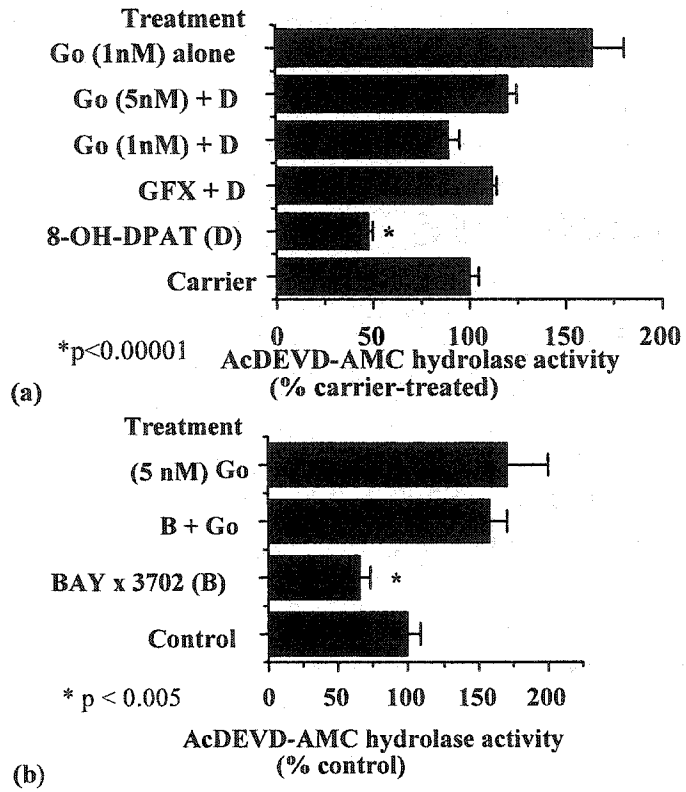

Figure 3.7. A calcium-dependent PKC isozyme is involved in the 5-HT_{1A} receptor-mediated inhibition of caspase-3-like activity. Differentiated HN2-5 cells were treated with 1 μM 8-OH-DPAT **(a)** or 1μM BAY x 3702 **(b)** in the absence or presence of 2 μM GFX or increasing concentrations of Gö6976 (Go) and further subjected to anoxia and re-oxygenation. Cell lysates for fluorescent substrate-based caspase-3 assay were prepared as described in methods. Results represent the mean (±S.D.) of two discrete experiments performed with triplicate samples.

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Although the PKC involvement in 5-HT_{1A}-mediated neuroprotection was experimentally proven, it was unclear whether MAPK- and PKC-mediated signaling are synergistic effects of different pathways (Figure 3.8) or MAPK and PKC are active players in the same pathway (Figure 3.8, Mechanism 1). Activation of PKC is not an unlikely event, since the activity of the PLCβ results in the accumulation of DAG and an increase in intracellular Ca^{2+} -- the necessary co-factors for PKC activation (Figure 3.8, Mechanism 2). On the other hand, if MAPK and PKC are members of the same signaling cascade and PKC is involved downstream of MAPK then its activity would totally depend on activity of MAPK.

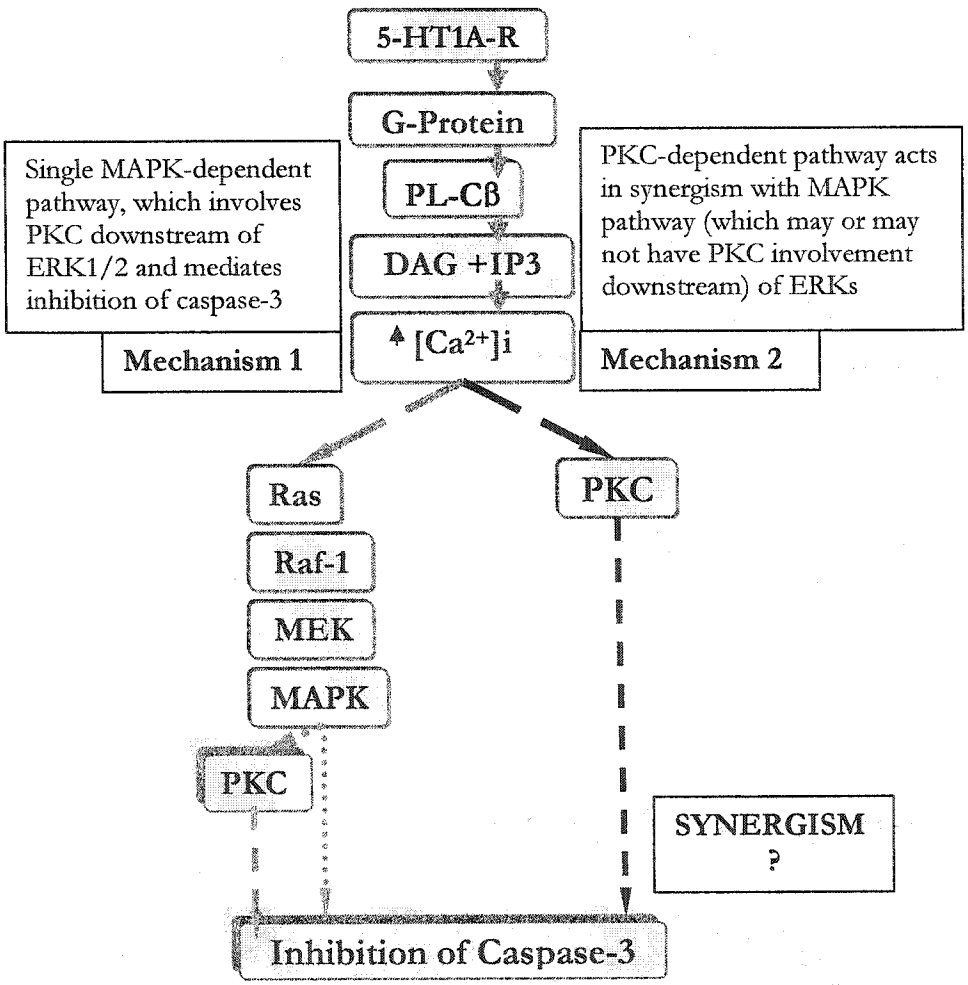

Figure 3.8 Possible mechanisms of 5-HT_{1A} receptor-mediated inhibition of caspase-3.

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The PKCα isozyme is activated in the MAPK-dependent manner.

Next experiment addresses the issue of synergism and investigates which isozyme is showing the MAPK dependency. Since the neuroprotection was particularly sensitive to the Gö6976 inhibitor of calcium-dependent isozymes PKCα and PKCβ, selective immunoprecipitation of these isozymes was performed from the lysates of the 5-HT_{1A} agonist-stimulated and carrier-treated control HN2-5 cells. The immunoprecipitates were next tested for protein kinase C activity using a PKC-assay kit with MBP peptide as substrate. Only PKCα isozyme but not PKCβ showed 8-OH-DPAT-mediated increase in kinase activity (Figure 3.9, obtained in collaboration with Dr. I. Ray). Moreover, agonist-mediated stimulation of PKCα was eliminated in the presence of MEK inhibitor, PD98059 (Figure 3.9, (D+PD)). Thus, PKCα is most likely to be the isozyme involved in the 5-HT_{1A}-R-mediated signaling to caspases-3. It was activated downstream of the 5-HT_{1A}-R-ERK 1/2 pathway, such that blocking ERK 1/2 activation eliminated the stimulation of PKCα. The data supports **mechanism 1** as shown in Figure 3.8 and provides no indication for synergistic action, since MAPK inhibitor did not just reduce the activity of PKCα but brought it down to the level of control. Therefore the increase in PKCα activity was totally MAPK-dependent.

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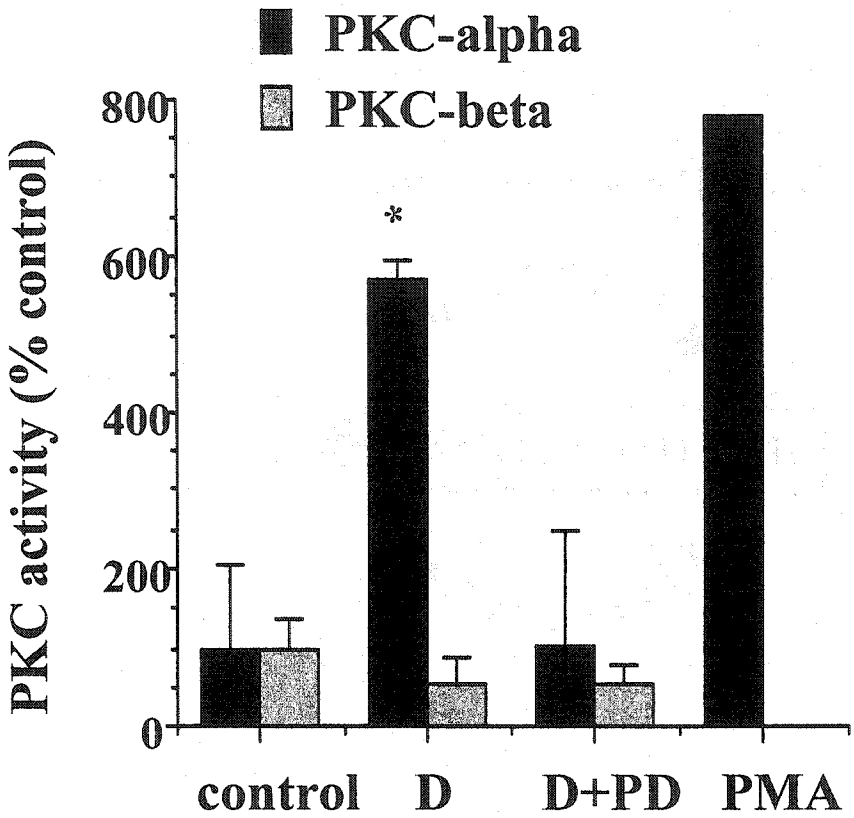

Figure 3.9. Serotonin$_{1A}$ receptor agonist causes MAPK-dependent stimulation of PKCα but not PKCβ. Differentiated HN2-5 cells were treated with either carrier (control), 1 μM PMA or 1 μM 8-OH-DPAT (D) in the absence and presence of PD98059 (PD) for 30 min. Next, the cells were lysed and PKCα and PKCβ immunoprecipitated from the lysates. The IP products were subjected to PKC enzyme assay. The experiment was carried out two times with quadruplicate samples (* $p<0.0005$).

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Kinase negative PKCα mutant eliminates 5-HT_{1A}-R-mediated inhibition of caspase-3 activity under H_2O_2-induced conditions.

Mutant or wild type vectors of PKCα (Carpenter et al., 2001; Matassa et al., 2001) were transiently transfected into the HN2-5 cells to confirm that PKCα is indeed the isoform required to relay the signal from the agonist stimulated 5-HT_{1A} receptor to cause a reduction of caspase-3 activity in the cytosol of stress-compromised HN2-5 cells. Transfection was carried out in the presence of pEGFP-C1 vector (encoding the green fluorescent protein, GFP) for internal transfection control that enables the visualization of the transfected cells by fluorescence microscopy (upper panel, Figure 3.10). At 24 hours post-transfection, experimental plates were differentiated as described in Methods and then subjected to oxidative stress with 200 μM H_2O_2 for 16 hours. Post-transfection cells appeared to be more sensitive to the stress and thus required lesser concentrations (200 μM instead of 500 μM) of hydrogen peroxide to produce a significant apoptotic effect. Treatment of transfectants with 500 μM H_2O_2 produced more pronounced late apoptotic cellular changes that would make the assessment more difficult. Taking into consideration the transfection efficiency (Figure 3.10) it was decided to assess the caspase-3 activity through immunocytochemical staining with the same anti-active caspase-3 (CM1) antibody that was used for Western blot analysis previously (Figure 3.4b). In contrast to caspase-3 enzyme activity assay with a fluorescent substrate that gives the quantitative evaluation, immunostaining for anti-active caspase-3 allowed for an effective and detailed examination of the 5-HT_{1A} agonist effect on suppression of the caspase-3 activity.

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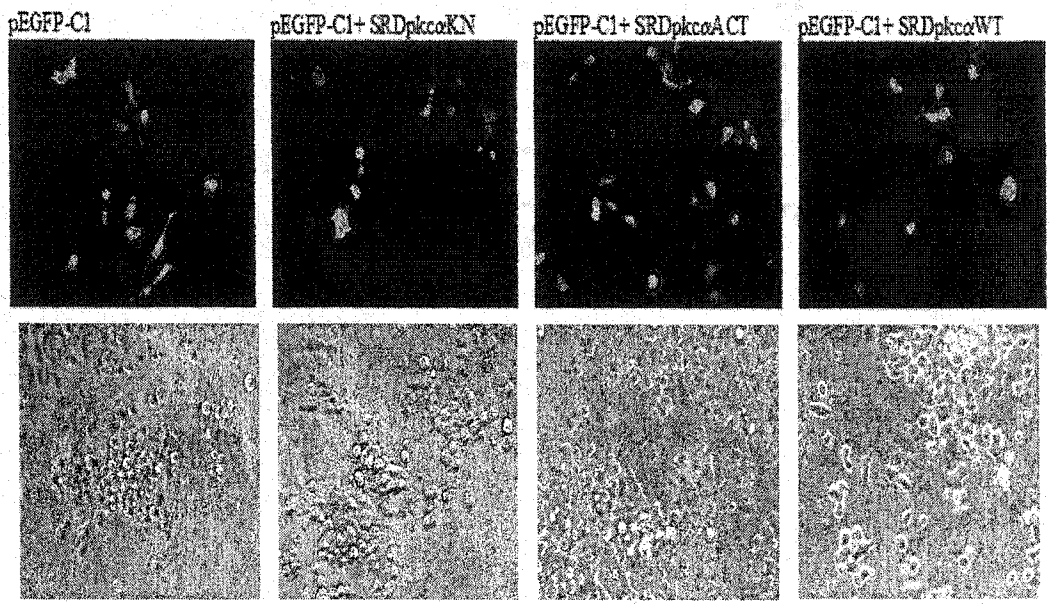

Figure 3.10 Overview of the GFP co-transfections with different PKCα vectors 24 hours after the transfection. HN2-5 cells were transiently transfected with GFP encoding vector alone (pEGFP-C1) or in combination with kinase-negative mutant (SRDpkcαKN), constitutively active PKCα mutant (SRDpkcαACT), or a wild type PKCα encoding vector (SRDpkcαWT). Transfection was carried out using 1 μg DNA of each vector and 5 μl of the SuperFect reagent (Qiagen Inc.) in 24-well plate without preplating. Digital images of GFP (upper pannel) expression 24 hours after transfection were visualized by fluorescence microscopy using a 510 nm filter (presented in the upper panel), with the corresponding phase contrast image (at the lower panel).

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Overall, consistent with the caspase-3 activity assay (Figures 3.4a & 3.9), agonist-evoked effect on caspase-3 did not eliminate active caspase-3, but rather inhibited it, so residual staining was detected to some extent in all the cells and characterized by low-intensity diffused staining (Figure 3.11 column 1). Cells showing such low-intensity staining were considered to be negative in terms of caspase-3 activation. The experimental wells that did not receive agonist, or where the action of the agonist was blocked by the MEK inhibitor PD98059 or by the expression of kinase-negative mutant of PKCα had majority of the cells with very intense and bright, cytoplasmic and perinuclear (Figure 3.12A column 1 and 3) staining profile of the anti-active caspase-3. Figure 3.11 consists of representative images collectively presenting the range of anti-active caspase-3 staining from negative to positive cells (positioned left to right).

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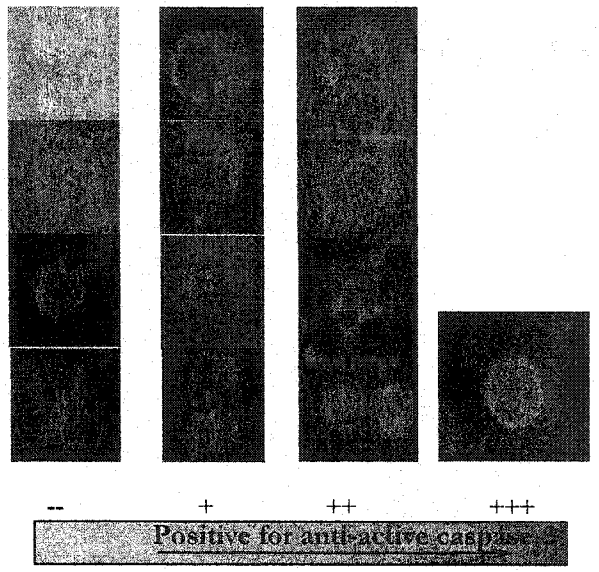

Figure 3.11 Representative images of the anti-active caspase-3 staining.

Population of H_2O_2 –treated post-transfection cells were immunostained for anti-active caspase-3 (CM1) antibody and visualised with AlexaFluor 568 (Molecular Probes) secondary antibody via fluorescent microscopy and 580 nm filter. Caspase-3 negative "-" cells (column 1) show diffuse, low intensity staining with poorly visible nucleus; Cells that were considered caspase-3 positive "+"(columns 2-4) present various degree of caspase-3 activation specified by corresponding intensity of the staining.

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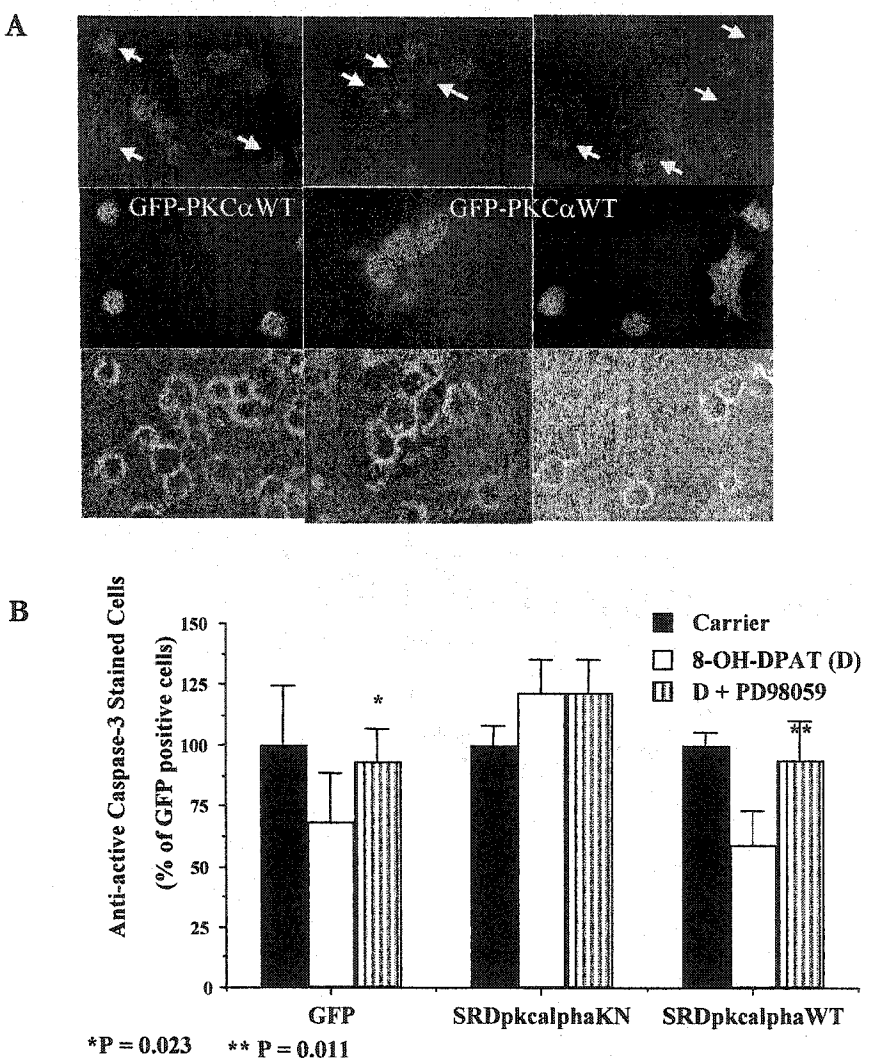

Figure 3.12. Kinase negative mutant of PKCα reversed the 8-OH-DPAT mediated suppression of caspase-3. HN2-5 cells were transfected with pEGFP alone or co-transfected in the presence of either SRDpkcalphaKN (kinase negative mutant of PKCα) or SRDpkcalphaWT (wild type of PKC α) in duplicates. The cells were differentiated 24 hours after the transfection and then treated with carrier, 1μM 8-OH-DPAT in the presence of (or without) 25 μM PD98059. All experimental wells were

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further subjected to 200 μM H_2O_2 treatment for 16 hours, followed by fixing and anti-active caspase-3 antibody staining (CM1), and then visualized with AlexaFluor 568 (orange-red) goat anti rabbit antibody, examined under a fluorescent microscope. Activation of Caspase-3 was assessed from the proportion of GFP cells ((A) row 2) that also show CM1-positive staining specific for presence of the processed caspase-3. (**A**) Representative images show punctate cytoplasmic, perinuclear anti-active caspase-3 staining (row 1), indicative of proteolytic cleavage of caspase-3. Reduced caspase-3 activity appears as low-intensity, diffused staining and considered negative for statistical analysis. (**B**) Data obtained are respresentative of three independent experiments, each performed with duplicate wells in which cells were counted in six fields. The 8-OH-DPAT-evoked inhibition in caspase-3 staining varied between 30 and 50% of carrier-treated wells in the GFP- and SRDpkcαWT-transfectants.

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Thus, cells transfected with GFP alone or in combination with wild-type (SRDpkcαWT) PKCα vector showed the expected inhibition of CM1 antibody staining upon agonist pretreatment. In contrast, cells cotransfected with the kinase-negative mutant of PKCα (SRDpkcαKN), did not show 8-OH-DPAT-evoked attenuation of anti-active caspase-3 staining (Figure 3.12B). Elimination of the 8-OH-DPAT-evoked suppression of caspase-3 was also characteristic of the cells pretreated with MEK inhibitor PD98059.

Cells cotransfected with GFP and costitutively active PKCα (SRDpkcαACT) responded to agonist treatment in the way similar to that observed with GFP alone and wild-type PKCα cotransfections. (Figure 3.13) Presence of the active PKCα at all the time, although resulted in the greatest number of attached cells as seen 24 hours after transfection (Figure 3.10, pEGFP + SRDpkcaACT), did not render neuronal HN2-5 cells increased survival potency. Thus, it is not the presence of the active PKCα itself but rather its regulated participation in the signal transduction that seems to be important for the neuroprotection.

Collectively, the data presented in figures 3.5; 3.9; and 3.12 confirmed that the general, 5-HT_{1A}-R-mediated protective pathway observed in the HN2-5 cells undeniably involve PKCα downstream of ERK 1/2 as suggested in the Figure 3.8 (mechanism 1).

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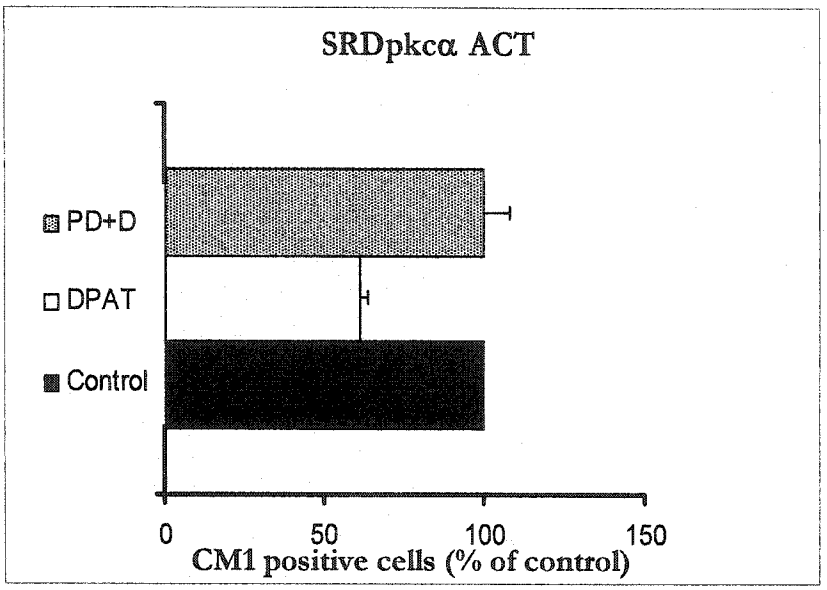

Figure 3.13 8-OH-DPAT mediated inhibition of caspase-3 in cells transfected with PKCα kinase-active mutant. HN2-5 cells cotransfected with GFP plus constitutively active mutant SRDpkcalphaACT were subjected to 8-OH-DPAT (DPAT) pretreatment and H_2O_2-induced oxidative stress. The agonist inhibited the activity of caspase-3. Cells pretreated with MEK inhibitor PD98059 in combination with the 8-OH-DPAT (PD+D) eliminated the agonist-mediated reduction of processed caspase-3, as assessed via immunostaining with CM1 antibody for active caspase-3. Data presented here is the average of three independent experiments and presented as the percentage of CM1 positive cells with respect to carrier-treated control.

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Increase in Phospho-PKCα at the membrane in a MAPK-dependent manner

It was established that the PKCα isozyme was involved in the neuroprotection, and its enzymatic activity was MAPK-dependent. As a highly regulated kinase, PKC undergoes a whole sequence of regulatory modifications. Phosphorylation of PKC is probably the first of the regulatory events, followed by cofactor (Ca^{2+}) binding to a fully phosphorylated cytosolic PKC species, and further translocation of the kinase to membrane. Next experiment was designed to test whether agonist exposure of the HN2-5 cells would have an effect on membrane translocation of PKCα. Selective isolation of the cytosolic and membrane-associated fractions revealed a MAPK-dependent increase in PKCα phosphorylated (P-PKCα) at Serine 657 (Figure 3.14). This phosphorylation site (the hydrophobic motif) is the last of the three ordered phosphorylations that give the PKC molecule catalytic competence. Following this last phosphorylation event, PKC becomes cytosolic and then the fully phosphorylated PKCα translocates to the membrane. The membrane-associated fraction isolated from the 8-OH-DPAT stimulated cells signifies activity-associated membrane anchoring (Figure 3.14, membrane 1h DPAT). Such increase in P-PKCα was not detected in the samples pretreated with either 5-HT_{1A}-R antagonist (WAY) or the MEK inhibitor PD98059 (PD) in combination with 1 hour of 8-OH-DPAT treatment.

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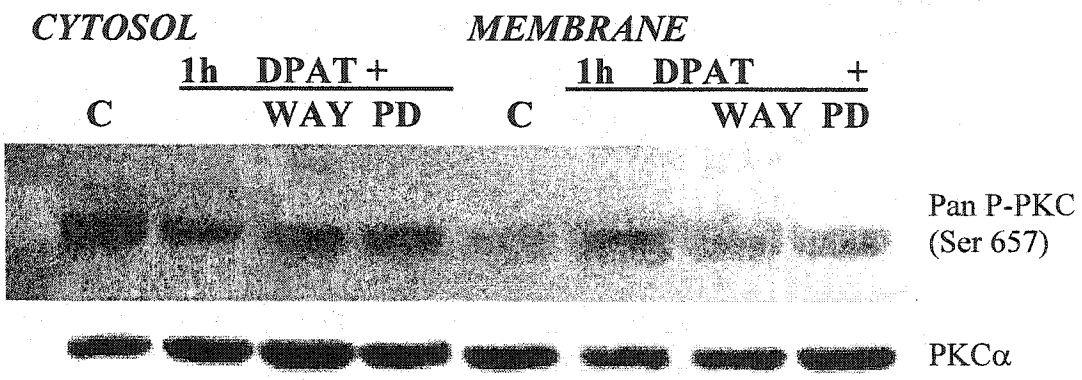

Figure 3.14. In agonist–treated HN2-5 cells P-PKCα sequestered to the membrane in a MAPK dependent way. Following 15 minutes of WAY or MEK inhibitor (PD) pretreatment, cells were treated for 1 hour with carrier or 8-OH-DPAT, then homogenized in PKC homogenization buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, plus protease and phosphatase inhibitors) and centrifuged at 50,000 x g at 4 °C for 20 min. The membrane pellet was suspended in detergent-containing buffer. Immunoprecipitates obtained using PKCalpha antibody from cytosolic and membrane fractions were analyzed by SDS-PAGE and immunoblotted with PKCα antibody (Santa Cruz, CA), shown in the lower panel, then the blot was stripped and re-probed with Pan P-PKC antibody (Cell Signal, MA) (upper panel).

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PKCα amino acid sequence analysis

The previous experiments established that PKCα is a downstream target of ERK 1/2, its activity is regulated by the MAPK, and it is required for neuroprotection. To address the possibility of direct interaction of ERK 1/2 and PKCα in the signaling pathway, the amino acid sequence of PKCα was analyzed first. If PKCα is a direct target of ERK 1/2, then at least one phosphorylation site of the PKCα should conform to ERK 1/2 recognition sequence (Figure 3.15). Indeed, such a site was found within the catalytic domain of the PKCα sequence. Out of three phosphorylation sites (**bold** face in Figure 3.15) required for proper functional maturation of the PKCα molecule only the turn motif site - Threonine[638] was within MAPK consensus recognition region PXXS/TP.

Thus, amino acid sequence analysis of PKCα identified the turn motif phosphorylation site (Thr638) as a possible target of ERK 1/2 for direct phosphorylation. The possibility that the two kinases, ERK 1/2 and PKCα, form a physical complex was addressed in the next experiment by screening the ERK-2 immunoprecipitation products for the presence of phosphorylated PKCα.

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MADVYPANDSTASQDVANRFARKGALRQKNVHEV
KDHKFIARFFKQPTFCSHCTDFIWGFGKQGFQCQVC
CFVVHKRCHEFVTFSCPGADKGPDTDDPRSKHKFKI
HTYGSPTFCDHCGSLLYGLIHQGMKCDTCDMNVHN
QCVINDPSLCGMDHTEKRGRIYLKAEVTDEKLHVTV
RDAKNLIPMDPNGLSDPYVKLKLIPDPKNESKQKTK
TIRSNLNPQWNESFTFKLKPSDKDRRLSVEIWDWDR
TTRNDFMGSLSFGVSELMKMPASGWYKAHNQEEGE
YYNVPIPEGDEEGNMELRQKFEKAKLGPVGNKVISP
SEDRKQPSNNLDRVKLTDFNFLMVLGKGSFGKVML
ADRKGTEELYAIKILKKDVVIQDDDVECTMVEKRVL
ALLDKPPFLTQLHSCFQTVDRLYFVMEYVNGGDLM
YHIQQVGKFKEPQAVFYAAEISIGLFFLHKRGIIYRDL
KLNNVMLNSEGHIKIADFGMCKEHMMDGVTTR**T**[497]
FCGTPDYIAPEIIAYQPYGKSVDWWAYGVLLYEMLA
GQPPFDGEDEDELFQSIMEHNVSYPKSLSKEAVSICK
GLMTKQPAKRLGCGPEGERDVREHAFFRRIDWEKL
ENREIQPPFKPKVCGKGAENFDKFFTRGQPVL**T**[638]PP
DQLVIANIDQSDFEGF**S**[657]YVNPQFVHPILQSAV

Figure 3.15. PKCα amino acid sequence Phosphorylation sites are shown in underlined bold face. Turn motif phosphorylation site - Threonine 638 (**T**[638]) corresponds to MAPK consensus recognition region PXXS/TP.

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Phospho-PKCα co-immunoprecipitates with ERK-1/2 only from membrane bound cellular fractions.

The next set of experiments sought to identify the approximate site of ERK-1/2 and PKCα association. Since MAPK dependent variations in the phospho-PKCα levels were detected in the membrane fractions of HN2-5 cells following the agonist exposure, membrane-bound and cytosolic fractions of agonist-treated cultures were subjected to immunoprecipitation with anti-ERK-1/2 antibody. Interestingly enough, only membrane-bound detergent-soluble fractions of the cells produced positive immunoreactivity on Western blot when probed with anti-phospho-PKCα (Thr638) antibody (figure 3.16). Furthermore, the detected phosphorylation profile of the PKCα turn motif followed the phospho-ERK 1/2 profile. The levels of P-PKCα and P-ERK1/2 (compared to the carrier-treated **C** lanes, Figure 3.16) increased steadily after 30 and 60 minutes of agonist (8-OH-DPAT, **D**) exposure and this increase was effectively blocked by the antagonist WAY 100635 (**W**) and the MEK inhibitor PD98059 (**PD**). The levels of membrane-bound P-PKCα were unaffected by the general PKC inhibitor (GFX). Thus, MAPK in the membrane-bound fraction showed association with PKCα and their phosphorylation states parallel each other. As reported earlier for MAPK, in response to 5-HT_{1A}-R stimulation, PKCα showed delayed but sustained activation and also association with MAPK in a cellular membranes. The association, membrane sequestering and the turn motif phosphorylation state of PKCα were all dependent on the 5-HT_{1A} receptor-mediated MAPK activation.

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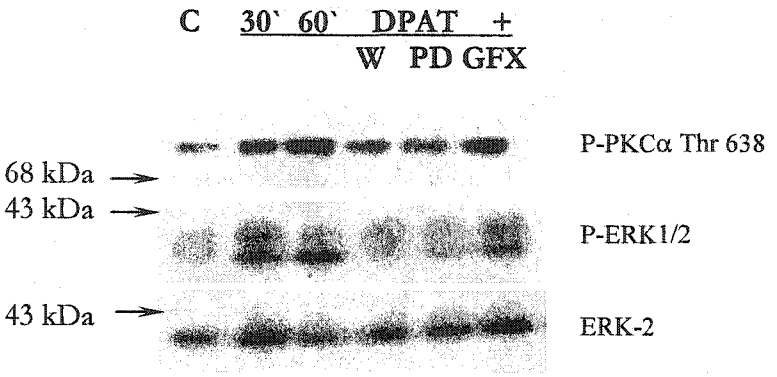

Figure 3.16 PKCα Thr638 phosphorylation profile of ERK-2 co-IP products shows phospho-ERK1/2 phosphorylation profile. Differentiated HN2-5 cells were treated with carrier, agonist (DPAT) for 30 or 60 minutes alone or in combination with 15 minutes pretreated with antagonist WAY 100635 (W), MEK inhibitor PD98059 (PD) or PKC inhibitor (GFX). Cytosolic and membrane fractions were isolated and subjected to immunoprecipitation (IP) with anti-ERK-2 antibody. The IP pellets were then resolved on 7-16% SDS-PAGE and immunoblotted with anti-phospho-PKCα (P-PKCα Thr 638, row 1), anti-phospho-ERK1/2 (P-ERK1/2, row 2) and anti-ERK-2 (ERK-2, row 3) antibodies. Only membrane associated cellular fraction showed co-immunoprecipitation of the phosphorylated PKCα and ERK-2. No complexes were detected in the cytosolic fraction.

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Chapter 4

Discussion

The loss of the neurons by means of apoptotic cell death is associated with a number of pathological conditions. It is also a part of normal physiology of development. Neuronal apoptosis is an outcome of the battle between the pro-apoptotic and anti-apoptotic signaling, where multiple factors play their discrete roles. These factors grossly could be divided into two categories, which are (i) signal-related (the strength of the signal, its duration and persistence), and (ii) cell-related, as determined by the state of the cell at the time of signal arrival and the cellular immediate environment. The internal and external environments of a cell play specific roles in evaluating the incoming signal, and accentuating or inhibiting it depending on the cellular state. The overall sum of those signals determines the fate of the cell. The more specialized, or differentiated the cell becomes, the more discrimination is exerted in the process of signal evaluation. The ease with which a cell can be replaced is yet another aspect that is involved in the equation of fate of the cell.

This study is focused on the neuroprotective signaling by 5-HT_{1A} receptor agonist(s) that mediates its effect via extracellular-signal regulated kinases-1 and 2 (ERK ½ or ERKs). This pathway takes an unconventional path through a PKCα-dependent anti-apoptotic pathway. Existence of such a neuroprotective signaling pathway, mediated by the 5-HT_{1A} receptor, effective against anoxia-induced, or oxidative stress-evoked apoptosis, independent of the

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activity of the plasma membrane ion channels (voltage gated Calcium channels), holds promise in the treatment of stroke patients to prevent neuronal loss from the receptor-bearing postsynaptic areas. The ERK-mediated neuroprotection is thought to function side by side with ion channel-associated anti-apoptotic function of the 5-HT_{1A}-R where receptor action can be accounted for more than 40% reduction of Ca influx (receptor activation lowers the effect of excitatory signaling from massive, stroke-induced, depolarized pre-synaptic neurons to post-synaptic regions). Thus, postsynaptic regions could be also salvaged by the receptor-mediated recruitment of the MAP Kinase signaling pathway that is independent of regulation of the Ca ion channels and interfere with apoptotic machinery at the later stages.

Recent studies in the same cell line involving the 5-HT_{1A} receptor points in fact to a biphasic nature of MAPK pathway, where initial stimulation causes acute activation of ERKs, reaching a peak of activity within a few minutes (data not presented here). Such activation is consistent with the reports for non-neural cells, although biphasic nature of the MAPK in hippocampal regions was also reported as necessary component for retention of inhibitory avoidance in rats. (Walz et. Al., 2000). In the course of the current study, the initial stimulation of the 5-HT_{1A} receptor by agonists is followed by another slow, sustained and persistent signal to MAPK that is receptor-specific rather then agonist specific as it showed the same profile for different agonists (Figure 3.1, and Adayev et. al., 1999). That long-lasting component of the MAPK was the major focus of this study as it proved to be part of a neuroprotective pathway.

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As a member of the G protein-coupled receptor family, 5-HT_{1A}-R uses G-proteins to channel its signaling through Gα and Gβγ subunits. Cellular state of differentiation that declares shift from active cell division and proliferation to assumption of more neuronal characteristics with little or no cell division and pronounced neuronal phenotype results also in the shift of functionally different outcome of the MAPK stimulation. In undifferentiated HN2-5 cells agonist promoted stimulation of the receptor does not result in such pronounced increase of phospho-MAPK levels as compared to highly elevated basal levels of MAPK activity; however the agonist-mediated increase in thymidine incorporation was noted (Banerjee, unpublished data). Shift in the Gβγ binding partners of the G-protein, coupled to the receptor, results in the re-direction of the signal to MAPK through PI-3Kγ-independent pathway in differentiated cells (Figure 3.2 & 3.3) that participates in neuroprotection.

Stimulation of the receptor in differentiated HN2-5 cells, under stressful conditions, generated by either anoxia or hydroxyl radical formation, following the H_2O_2 treatment, rescues the cells from apoptosis in PLC_β- and PKC-dependent manner. Unconventional participation of protein kinase C downstream of the ERK1/2 in the signaling pathway certainly draws much attention (Figures 3.3 & 3.4). Initial non-involvement of PKC in Ras-dependent activation of Raf-1 and further MAP kinase pathway components, however required the involvement of a calcium-dependent PKC downstream for successful suppression of caspase-3 activity (figure 3.7). Dominant negative mutants of Ras and Raf annulled the effect of the agonist (BAY x 3702) on

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sustaining cell viability during anoxia (Figure 3.6), thus confirming involvement of certain upstream elements of the MAPK pathway important in neuroprotection.

The neuroprotection offered by the receptor turned out to be as effective for H_2O_2-induced as for anoxia-induced apoptosis (Figure 3.4 & 3.5). Under both conditions, generation of hydroxyl radicals will disrupt the mitochondrial membrane potential and inevitably lead to cytochrome c spill, followed by assembly of all necessary components for caspase-9 activation and consequent processing of its downstream target procaspase-3. In both cases downstream elements were effectively compromised in their activity in the presence of the receptor agonist. Thus, the 5-HT_{1A}-R-mediated neuroprotection does not interfere with the events that led to generation of hydroxyl radicals but rather likely to act at elements that are involved in apoptosis at the level of mitochondria. This makes it into a general neuroprotective pathway that will be effective in reversing numerous pathological conditions (Figure 1.3). In addition, since expression of the 5-HT_{1A} receptor starts early during development, alterations of this pathway at early stages of brain development could be the basis of some developmental pathology.

The PKCα is one of the kinases that take part in the endogenous anti-apoptotic signaling, which normally functions to endure cell survival. Conditions under which it exerts its action stretch from routine maintenance of cellular integrity to coping with the changes in the state of distress. Highly regulated signaling through this enzyme is crucial for cells. Elevated PKCα signaling, which has been reported in a number of transformed cells, is typical of cellular

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proliferation in malignant tissue. However, this kinase is also a crucial and active player in the normal signal transduction pathways. For instance, neuronal cells, PC12, HN2-5, RN46A, harbor very high basal levels of fully phosphorylated, cytosolic, activatable PKCα. Clearly the function of this enzyme is directed not only on driving a survival signaling, but in many other cellular processes.

In this particular study, PKCα activity which was totally dependent on MAPK (Figure 3.9), when removed from the equation of anti-apoptotic signaling (Dominant negative PKCα transfection, Figure 3.12) initiated by the 5-HT_{1A}-R agonist, removed its effect on caspase-3 activity.

The ERK 1/2 and PKCα duet, as a key element in the pathway were reported to mediate an opposite effect in renal cells in cisplatin-induced apoptosis, where mitochondrial damage was promoted by ERK and PKCα-dependent activation of caspase-3, and ERKs activity, once again, was independent of PKCα (Nowak, 2002). The fact that the effect from ERK1/2-PKCα duo swings both ways supports not coincidental discovery of such interactions. The difference in the outcome of the signaling for sure is not determined solely by those two molecules but could be explained by the differences in the nature of a stimulus itself, the pathway, and tissue and cell-type specificity.

Transfection of the PKCα active isoform (SRDpkcαACT, Figure 3.13) of the kinase yielded somewhat unexpected data from caspase-3 processing analysis. The agonist treatment showed the same profile with respect to caspase-3 inhibition as observed for GFP alone and SRDpkcαWT transfectants (Figure

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3.12). Complete explanation to the observed effect is most likely to be found in the nature of interactions between ERK and PKCα, although it is apparent that presence of the increased amount of the active PKCα is not enough to provide for the neuroprotection. Activation of PKCα in the course of the 5-HT_{1A}-R signaling, possibly, also strategically locates it near its target(s) and increases the opportunity for further interactions. Transient transfection of PKCαACT simply increases basal levels of the PKCα activity but does not guarantee a short cut in neuroprotection. So, agonist-mediated suppression of the caspase-3 processing observed in PKCαACT transfection is likely to be attributed to recruitment of endogenous PKCα.

The agonist → ERK 1/2-dependent increase of the levels of fully phosphorylated species of the PKCα at the membrane (Figure 3.14) supports the idea of highly regulated participation of the PKCα in the pathway, where MAPK activity plays a novel regulatory role. Differential separation of cytosolic and membrane associated PKCα allows to discriminate between mature (fully phosphorylated and activatable) found in cytosol and activated PKCα molecules recruited to the membrane. Immature PKCα usually would lock the phosphate groups and remain in detergent-insoluble membrane fractions. Since the phosphorylation at Serine 657 is the last to occur during maturation of the PKC molecule, coinciding with the release of mature PKC to cytosol, the membrane associated (detergent-insoluble) fraction is unlikely to contain a fully phosphorylated PKC species. The PKCα found in the cytosolic fraction appears (Figure 3.14, lanes1-4) to be also positive for presence of the p-Ser657. The

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agonist (8-OH-DPAT) treatment reduces PKCα phosphorylation levels at Ser657 in cytosolic fractions. Such reduction could be attributed to two possible events: 1) activity-related translocation of PKCα to the membrane or 2) reduction in the levels of cytosolic PKCα pSer657 due to the action of phosphatases. The increase in pSer657 level observed in the membrane-associated fraction after agonist treatment supports the idea of PKCα translocation to the membrane. If simultaneous increase would be observed in both fractions after the agonist treatment, than the greater would be the likelihood of ERK 1/2-mediated maturation of PKCα. However, the levels of pSer657 of PKCα decrease in cytosol and increase in membrane fraction are indicative of, most likely, common pool of the PKC that just translocates or associates more with membrane in response to 8-OH-DPAT treatment.

From amino acid sequence analysis of PKCα (Figure 3.15) it appears that the turn motif phosphorylation site (Thr638) that is within ERK 1/2 recognition sequence potentially undergoes a MAPK-dependent regulation. In addition, increase in P-PKCα at the membrane (Figure 3.14) and requirements of its function for neuroprotection, all depends on MAPK activity, and this led to the discovery that PKCα (phosphorylated particularly at Thr638 site) is co-immunoprecipitated with ERK-2 from detergent-soluble membrane fractions (Figure 3.16). Thus, in the course of $5HT_{1A}$-R agonist-mediated activation of ERKs, at some point, ERKs form a complex with PKCα, and this is found only in membrane bound state and not in the cytosol.

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A few possibilities exist on the exact location of MAPK regulation of PKCα (Figure 4.1). If MAPK function is partially directed at phosphorylation of PKCα turn motif (only partially, because cytosol- as well as membrane-associated fractions are both positive for presence of activated ERKs, figure not shown), then this phosphorylation might take place either during initial maturation of the PKCα (Figure 4.1, 1), or the activated ERKs could re-phosphorylate the PKCα that had not yet enter a degradation pathway (Figure 4.1, 3).

If MAPK would play a role in maturation of PKCα then greater amounts of the mature PKCα should be detected in the cytosol. However such increase was not detected (Figure 3.14). The turn motif Thr 638 phosphate is all that is absolutely essential for the catalytic function of mature PKC (Newton A., 2001). High levels of the basal activity of PKCα, commonly present in the cells, are a constant subject to the action of phosphatases. De-phosphorylated PKC is less stable, usually rapidly ubiquitinated and enter the degradation pathway. Recent research by Newton's group (Gao and Newton, 2002; Newton, 2003) reported specific binding of the heat shock protein Hsp70 to de-phosphorylated PKC, which contributes to the stability of PKC allowing the re-phosphorylation to occur. Although initial phosphorylation of the turn motif site is thought to be a primarily autophosphorylation event, re-phosphorylation could very well be accomplished by a different kinase. Since ERK-P-PKCα complexes are detected in the membrane fraction, this increases the possibility that the other kinase could be ERK.

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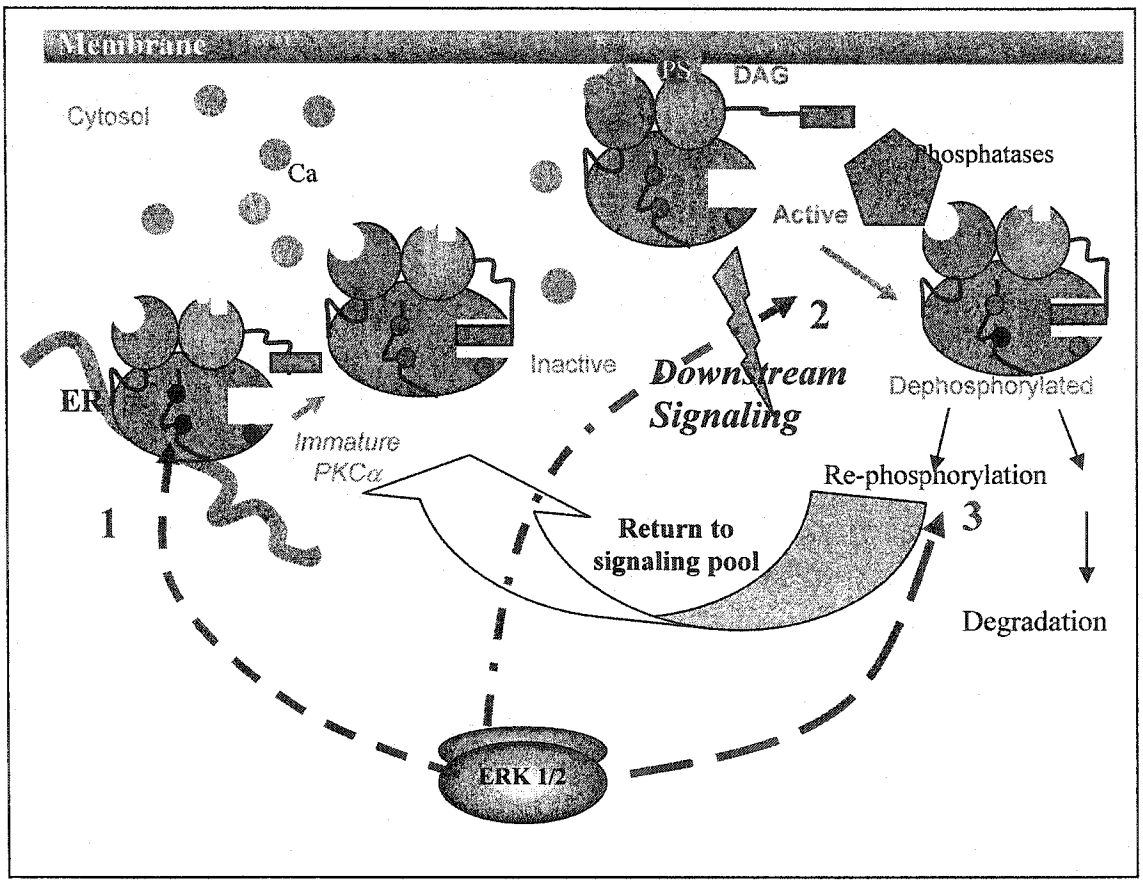

Figure 4.1 Possible sites of PKCα regulation by ERK 1/2. The ERK 1/2 enzymes, activated via $5HT_{1A}$-R stimulation by the agonists, might be involved in novel regulation of PKCα in at least three instances (marked by dotted lines 1-3): (1) Initial phosphorylation of the turn motif site during maturation of the PKCα molecule. (2) Interference with the action of phosphatases due to shielding of the turn motif site by ERK 1/2-PKCα complex. (3) Re-phosphorylation of the turn motif site of PKCα providing its re-entrance back into the signaling pool.

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The complex formation between ERK and P-PKCα could also serve a different purpose. By forming the complex, the turn motif phosphate could be protected form the action of phosphatases, which would prolong the life time of active PKCα (Figure 4.1, 2). In this case the ERKs activity will be required more for the formation of the complex than for actual phosphorylation of the turn motif site.

Independent of the exact role of MAPK in phosphorylation-related regulation of the PKCα, the complex formation itself could be another way for regulation of PKCα action, because it might strategically locate the PKC near its target in a timely fashion. To support this idea – transfection of constitutively active PKCα isoform showed no increase in neuroprotective signaling over GFP vector transfected alone.

Signaling events that might lead to anti-apoptotic effect downstream of PKCα are summarized in Figure 4.2. The activity of PKCα can lead to phosphorylation of the proapoptotic protein Bad thus rendering it inactive and thereby promoting the action of antiapoptotic Bcl-2. Next, the processing of caspase-9 can be inhibited by phosphorylation (Cardone et al., 1998) which would lead to a decrease in processing of caspase-3. Caspase-3 itself could become a target of phosphorylation that can potentially inhibit either its processing or its function. Protein kinase C activity could also be involved in the activation of NFκB, which could cause transcriptional upregulation of the inhibitor of apoptosis proteins, the IAPs (Robertson et al., 2000; Abdouh et al., 2004). The IAPs would eventually suppress the activity of caspase-3.

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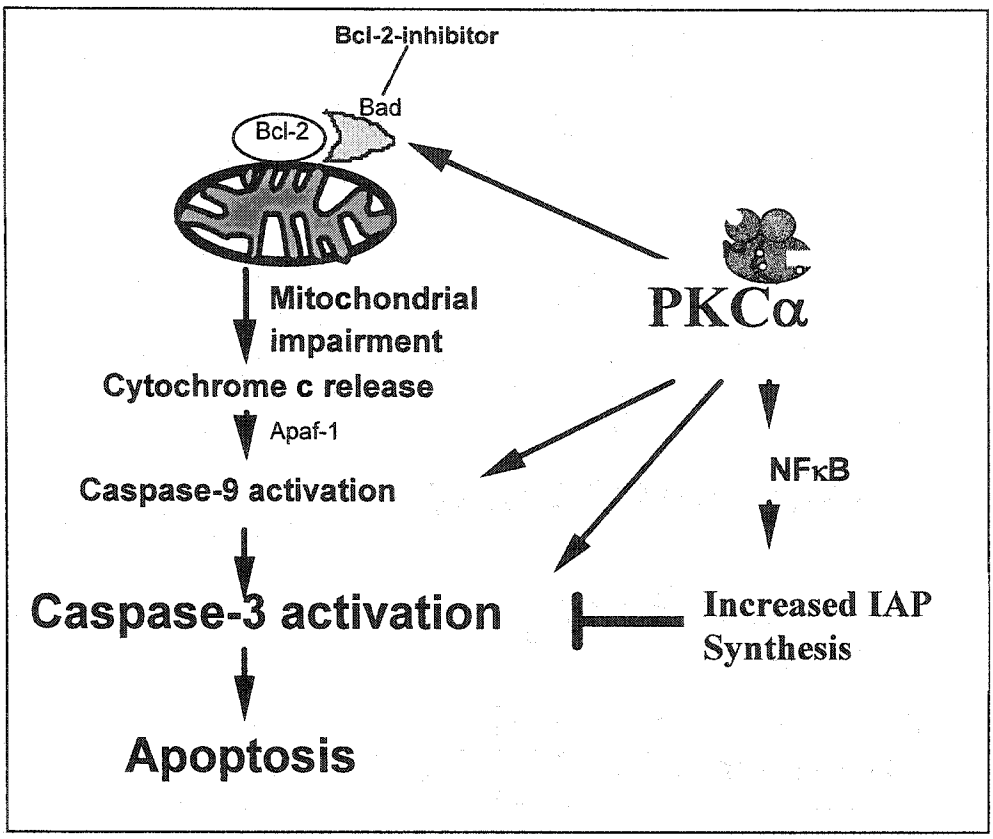

Figure 4.2. Possible targets of a kinase in relaying 5-HT_{1A}-R-mediated, protective signaling. A kinase, which could be either PKCα, or more likely, a downstream target of PKCα, could phosphorylate to inactivate proapoptotic proteins. Alternatively, PKCα-catalyzed phosphorylation might trigger the formation of anti-apoptotic proteins that cause caspase-3 inhibition.

Interestingly, the turn motif site of protein kinase C in addition to its role in maturation and stability may also act as a docking site for protein-protein interactions that could be a target of phosphorylation-state-dependent regulation. The sequence surrounding the turn motif conforms to binding site for the antiapoptotic protein 14-3-3 (Yaffe et al., 1997).

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The novel effect discovered in the current study includes 5-HT_{1A}-R mediated prolonged activation of ERK 1/2, which in turn physically interacts with and regulates PKCα. The activated PKCα then quite intriguingly leads to the inhibition of the proapoptotic enzyme caspase-3. Further research is required to sift through the alternative pathways described in the previous paragraph and finally delineate the complete protective cascade initiated by the 5-HT_{1A} receptor in the hippocampal neuron-derived HN2-5 cells.

This study further opens the opportunity to investigate the connectivity and hierarchy of the 1) upstream members of the G protein-coupled receptor-activated MAPK cascade, such as Pyk2, Src, or CaM (using MAPK activity as a readout point), 2) anti-apoptotic pathway downstream of PKCα leading to caspase-3 inhibition (making use of caspase-3 activity assay as the next readout). Neither of these steps has been clarified yet. The nature of interactions between ERKs and PKCα, by itself, opens a whole new area of research, interesting from a purely biochemical stand point, which could explain yet another means of Protein Kinase C regulation.

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