SEROTONIN 1A RECEPTOR MEDIATED NEUROGENESIS IN THE DEVELOPING HIPPOCAMPUS

by

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ABSTRACT

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The importance of the brain serotonin 1A receptor (5-HT_{1A}-R) during postnatal brain development has been established, but the mechanism of its action in brain neurons remains unclear. It is currently known that the 5-HT_{1A}-R plays a crucial role in the brain by regulating mood and behavior and 5-HT_{1A}-R stimulation in adult mice has been suggested to induce neurogenesis in the adult neurogenic niches such as the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. In mice, absence of the 5-HT_{1A}-R during development results in heightened anxiety and depressive behavior. However, the 5-HT_{1A}-R mediated signaling activity that is responsible for its role during development is unknown. Among the hippocampal signaling pathways stimulated by the agonist-bound 5-HT_{1A}-R, the mitogen activated protein kinase (MAPK) pathway is an important regulator of both division and survival of neuronal cells in the brain. Additionally, the Protein Kinase C (PKC) isozyme PKC**ε** is an important signaling molecule that is highly expressed during early postnatal brain development particularly postnatal day 2-6 (P2-P6). Here we show that neurogenesis in the developing hippocampus at P6 in mice is dependent on both MAPK and PKC**ε**. Our

initial experiments use pharmacological inhibitors to confirm that PKCε mediates 5-HT_{1A}-R-linked activation of MAPK. We then demonstrate that neurogenesis is increased upon stimulation of this 5-HT_{1A}-R➔➔PKCε➔➔MAPK pathway both in a hippocampal-derived neuronal cell line stably expressing the 5-HT_{1A}-R (HN2-5) and in the DG of P6 mice. Further, inhibition of either MAPK or PKCε considerably disrupts the burst in bromo-deoxy-uridine (BrdU) labeling and Ki-67 staining, showing neuroblast number, in the DG. As for a downstream signal that relays the proliferative signal from MAPK, we have identified the Retinoblastoma protein (Rb) as a potential target of MAPK, and shown that its phosphorylation dynamics may be tightly regulated in response to 5-HT_{1A}-R stimulation. Therefore, our findings reveal a novel pathway involving PKCε, MAPK, and Rb through which the 5-HT_{1A}-R potentially regulates neurogenesis during early postnatal hippocampal development.

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ABBREVIATIONS

5-HT_{1A}-R- serotonin 1A receptor

SVZ- subventricular zone

DG- dentate gyrus

Trk- tyrosine kinase receptor

Rb- retinoblastoma protein

P-Rb-S^{780}- retinoblastoma protein phosphorylated at Ser^{780}

GCL- granular cell layer

SGZ- subgranular zone

NMDA- N-methyl D aspartic acid

CDK- cyclin dependent kinase

TPH- tryptophan hydroxylase

PDB- protein data bank

PLC- phospholipase C

MARCKS- myristoylated alanine-rich C-kinase substrate

MLP- muscle LIM protein

GAP-43- growth associated protein 43

Grb2- Growth factor receptor-bound protein 2

SOS- Son of Sevenless

SAP- sphingolipid activator protein

SP-1- Stable Protein 1

GFAP- Glial fibrillary acidic protein

BrdU- 5-bromo-2-deoxyuridine

^{3}H-8-OH-DPAT- tritiated 8-hydroxy-2-(di-n-propylamino)tetralin

MAPK- Mitogen-Activated Protein Kinase

DMEM- Dulbecco's Modified Eagle Medium

FBS- fetal bovine serum

DFBS- dialyzed fetal bovine serum

EGTA- ethylene glycol tetraacetic acid

EDTA- ethylenediaminetetraacetic acid

PBS- phosphate buffered saline

TCA- trichloro acetic acid

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

PMSF- phenylmethanesulphonylfluoride

Na_3VO_4- Sodium Vanadate

WAY- 5-HT_{1A}-R antagoninst (WAY 100635)

U – MEK inhibitor (U0126)

RACK- Receptor for Activated C-Kinase

GFX- protein kinase C inhibitor (GF109203X)

MGBSS- modified Grey's balanced salt solution

EBSS- Earle's balanced salt solution

NaHEPES- {2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic Acid Sodium Salt-

P-PKC- phospho protein kinase C

P-ERK- phospho extracellular regulated kinase

NeuN- neuronal nuclei

PROX 1- Prospero-related homeobox 1

CHAPTER 1

1.1 Background

In most regions of the mammalian brain neurogenesis ceases upon birth. But in the dentate gyrus (DG) and sub ventricular zone (SVZ) lining the lateral ventricles proliferation continues onto adulthood and persists through the entire life of the animal. During embryonic hippocampal development, granule cell precursors that originate from the wall of the lateral ventricles migrate along radial glial fibers to the developing hippocampus. From the late embryonic period through the first postnatal week in the rat, granule neurons are produced from a pool of precursor cells in the hilus of the DG. These cells divide and migrate along radial glia that extend from the hilus to the developing granular cell layer (GCL), the majority of granule cells are produced during this time (Rickmann 1987, Schlessinger 1975, Altman 1990). In adulthood, granule cells originate from precursor cells that are found primarily in the subgranular zone (SGZ) which is the region between the GCL and hilus (Cameron 1993). These precursor cells divide and produce daughter cells that differentiate into granule neurons that become incorporated into the GCL. Within three weeks of DNA synthesis, the cells in the GCL express markers of mature granule neurons, including neuron specific enolase (NSE), calbindin, the NMDA receptor subunit NR1, and Neuronal Nuclei (NeuN) (Cameron 1993, Kuhn 1996). Cells produced in adulthood extend projections through the mossy fiber pathway while receiving input from the entorhinal cortex (Kaplan 1984, Kaplan 1977, Stanfield 1988).

This phenomenon of post-natal neurogenesis has been shown to occur in many

species of mammals from shrews, marmosets, macaques, to humans (Gould 1997, Gould 1998, Gould 1999b). Studies performed initially using tritiated thymidine, BrdU labeling and most recently, retroviral labeling of early neuronal progenitors have shown that thousands of new cells are produced each day in the dentate gyrus of young and middle-aged adults. The majority of these cells are believed to differentiate into mature neurons. The substantial number of new granule neurons produced in both early and adult hippococampal development and the conservation of this process across mammalian evolution strongly suggests a function for these adult-generated neurons, though this function is yet to be identified (Gould 1999a, Gould 1999b).

It is believed that macroneurogenesis or the proliferation of preneuronal cells reaches a maximum at embroyonic day 17 (E17) in the mouse hippocampus, following which glial proliferation takes over and attains a peak at post-natal day 2-3 (P2-P3). These preneuronal cells yield mainly pyramidal neurons. Neurogenesis does not stop at E17 and others and we have observed widespread "microneurogenesis" (yielding granule cells) to be operative in the dentate gyrus of the hippocampus at postnatal day 6 (P6) (Morgane 2002, Mehta 2007). Such post-natal microneurogenesis has also been observed in human hippocampal development and continue through the neonatal stages (Morgane 2002). In mice, this microneurogenesis reaches a peak at P6-P7 and is followed by synaptogenesis. Although now adult neurogenesis is widely accepted, mechanisms regulating post-natal neurogenesis and development of the hippocampus have remained obscure because of the scarcity of appropriate signaling studies.

The role of the molecule serotonin, or 5-hydroxytryptamine (5-HT), as a neurotransmitter has been studied for decades. Work done in the 1990's have suggested a

new role for 5-HT in regulating the structure of the brain both during development and in adulthood (Mazer 1997, Yan 1997, Watanabe 1992). These studies have primarily focused on the role of serotonin in sculpting of the adult brain. Recent studies have identified a new role for 5-HT in directly mediating granule cell neurogenesis in the adult brain. Serotonin has been shown to stimulate cell proliferation in nonneuronal systems (Fanburg 1997, Takuwa 1989). Serotonin being a hydrophilic molecule exerts its effects by binding to 14 distinct G protein-coupled receptors (GPCRs), among which particularly the 5-HT_{1A}-R seems to be important in regulating neurogenesis in early development.

In-situ hybridization and immunohistochemistry have shown dense expression of the 5-HT_{1A}-R receptor in the DG, which receives serotonergic innervation from the median raphé nucleus of the brainstem (Patel 1996, Patel *et al.* 1996, Azmitia *et al.* 1996, Azmitia 1996). Conditions that cause a decrease in granule cell genesis, such as malnutrition, aging, high corticosterone, stress, and NMDA receptor activation, also decrease the density of serotenergic fibers or 5-HT_{1A} receptors, or inhibit the release of serotonin in the DG. Furthermore, conditions that enhance granule cell genesis, such as seizures, adrenalectomy, and NMDA receptor antagonist treatment, also increase the density of 5-HT_{1A} receptors or the release of 5-HT in the DG. Studies in which 5-HT levels in the hippocampus are increased using fenfluramine or specific stimulation of the 5-HT_{1A}-R have shown a simultaneous increase in proliferation of granule cell precursors (Gould 1999a). But the connection between receptor stimulation and enhanced neurogenesis especially during early postnatal development has not been established and no signaling studies have looked for the signal transduction pathway responsible for this effect.

On the whole, proliferation of most hippocampal and other cells are believed to be regulated through some fundamental mechanisms. The most common pathway includes mitogens and growth factors activating receptors leading to induction of the cell cycle checkpoint proteins, termed Cyclins, which combine with and activate kinases, dubbed Cyclin-dependent kinases (CDKs). The Cyclin-CDK complexes then catalyze phosphorylation-mediated regulation of downstream processes, thereby prompting the cells to transition through checkpoints between the G1-S and S-G2 phases, and eventually into the M phase or mitosis (Galderisi 2003). In non-neural cells, the G1-S transition is promoted by the formation of Cyclin D1-CDK4/6 and Cyclin E-CDK2 complexes (Lundberg 1998), but the mechanism seems to be more complex in the neural cells (Galderisi 2003). In the PC12 cell line it has been shown that Cyclin D1 induction is associated with cell cycle arrest and differentiation without any increase in CDK activity (Xiong 1997, Yan 1995). However, in mouse cortical neural progenitors, CDK4/6 have been shown to regulate cell cycle progression and this function is totally dependent on the phosphorylation dynamics of the retinoblastoma protein (Rb) (Ferguson 2000). The cell-cycle regulator Rb is phosphorylated downstream of the initial signals *via* Cyclins or other promoters of proliferation (e.g. the MAPK family proteins ERK1/2). Rb, in its unphosphorylated state, remains bound to the transcription factors E2F1, E2F2, or E2F3. Phosphorylation of Rb at several amino acid residues induces conformational changes resulting in destabilizing the Rb/E2F complex, thus setting free the E2F protein. Rb-free E2F enters the nucleus as a heterodimer with its binding partner, DP-1, to cause induced expression of genes that are linked to DNA synthesis and cell proliferation (Trimarchi 2002, Du 2006). Therefore Rb is an important cell cycle regulator, deletion of

which causes uncontrolled proliferation of trophoblast cells and disruption of the normal labyrinth architecture in the placenta, thereby leading to embryonic death (Wu 2003). As for these activating E2Fs (1,2,3), there is some redundancy, and deletion of all three, causes disruption of cell cycle progression (Wu 2001).

Intriguingly, given that two Rb residues that are phosphorylated (Ser^{780} and Ser^{788}- for human Rb), are possibly substrates of ERK1/2, we addressed the possibility of Rb being involved in neurogenesis downstream of activated ERK1/2. We examined the Rb phosphorylation status at these residues to check if Rb was regulated by the MAPK pathway. Additionally, we examined the possible regulation of E2F and its target genes and the dependence of 5-HT_{1A}-R mediated neurogenesis on both MAPK pathway and PKCε. Altogether, we have used pharmacological and immunohistochemical approaches to provide a novel, signaling pathway for 5-HT_{1A}-R mediated neurogenesis.

1.2 Significance of the proposed research

Early brain development is associated with an initial burst of cell division. At a certain stage of brain development, the preneuronal cells receive signals for them to either divide or migrate to their respective destinations in the brain. During and after migration, these cells receive molecular signals to differentiate into mature neurons with appropriate synaptic connections. Therefore, a receptor-mediated signaling pathway that promotes early cell division as well as survival of differentiating and post-differentiation neurons would be of prime importance in early brain development. Our studies have revealed that 5-HT_{1A}-R-mediated signaling causes stimulation of the MAPK pathway,

which plays an important role in both division as well as survival of hippocampal neurons (Adayev 1999, Mehta 2007).

Though it has been long speculated that serotonin and the serotonergic system play an important role in brain development and function, mechanistic studies relating 5-HT to cell proliferation are rare and few. Further, though our knowledge on cell cycle progression and exit has been growing in the recent years there are yet many unanswered questions on how neuronal cells (specifically hippocampal cells) regulate cell cycle progression to determine their eventual fates. Consequently, understanding the mechanisms through which such cells regulate cell cycle progression will provide us with the conceptual understanding to realize rational therapeutic strategies in targeting and treating the numerous diseases linked to aberrant neuronal proliferation in the limbic system during early development.

1.3 The 5-HT_{1A}-R and its possible involvement in brain development:

It is believed that abnormalities occurring during early brain development lead to impaired neuronal connections, which in turn result in behavioral abnormalities, such as depression, chronic anxiety, aggression, etc. Such emotional states are regulated by a neuronal communication existing between the limbic structures like hippocampus-amygdala and forebrain (Drevets 1992, Drevets 1999). The neuronal signals that build such connections are transmitted *via* neurotransmitters and their receptors. The serotonergic circuitry constitutes a widespread neurotransmitter system in this brain region. Among the 5-HT receptors involved, the 5-HT_{1A}-R, which occurs abundantly in

the limbic system as well as the forebrain and cerebral cortex plays a pivotal role in animal behavior.

1.4 History of research on serotonin synthesis, storage, and uptake.

Work on 5-HT dates back to more than 60 years ago when Rapport and collaborators isolated it from serum, demonstrated its contractive action on smooth muscle cells, and dubbed it as the 'tonic' substance in serum, hence serotonin. The structure of 5-HT consists of a hydroxyl group in the 5 position of the indole nucleus with a primary amine nitrogen serving as a proton acceptor at physiological pH. This gives the 5-HT molecule a hydrophilic character, hence it does not pass through the blood brain barrier. This is important functionally as early studies identifying its presence in the brain meant that it had to be synthesized within the brain. Currently serotonin and the serotenergic system is one of the most thoroughly studied neurotransmitter systems in the brain. The synthesis of serotonin in the brain is primarily restricted to the raphe nuclei of the brain stem. This is a result of the specific expression of tryptophan hydroxylase (TPH)- the rate-limiting enzyme in the synthesis of serotonin- in these neurons (Wood 2001). The specific action of TPH is in the conversion of tryptophan to 5-hydroxtryptophan. The second enzyme responsible for conversion of 5-hydroxytryptophan to serotonin (5-hydroxy tryptamine) is aromatic L-amino acid decarboxylase (AADC).

During early embryonic development (around E13 in rats and at six weeks of gestation in humans) a large group of these multi-polar neurons known collectively as

raphe neurons, which are located along the midline of the brain stem, differentiate and mature to form serotonin synthesizing neurons. They are among the first neurons to differentiate and almost immediately start sending projections to different areas of the brain (Wallace 1983). The timing and arrival of these projections have suggested their importance in numerous development processes including neurogenesis, migration, neurite outgrowth, neurophil formation, axon mylenation, synapse formation and maturation (Lauder 1982, Whitaker-Azmitia 1996). During early postnatal development, mature serotonin intervention of the cortex and hippocampus is apparent and serotonin levels are suggested to increase two-fold during this important development time frame, further there is a massive increase in the density of the axon terminals of these neurons. Also, it has been shown that depletion of serotonin by PCPA (Para-chlorophenylalanine) treatment hinders the differentiation of these neurons and inhibits neurogenesis, more importantly removal of serotonin has been shown to have long lasting and profound effects on brain maturation, development and synaptic plasticity (Lidov 1982, Whitaker-Azmitia 2001). Upon synthesis serotonin in the cytoplasm is stored in small synaptic vesicles through active transport by a proton-ATPase. Depolarization induced by an influx of Ca^{+2} in response to a change in electrochemical gradients, releases serotonin through exocytosis, which in turn diffuses across the synaptic cleft. The amount of serotonin in the synaptic cleft is currently thought to be regulated by SERT (serotonin transporter) that functions by reuptaking serotonin from the synaptic cleft back into the neuron through dynamic conformational changes involving K^+, Na^+, and Cl^- ions. Monoamine Oxidase (MAO) converts serotonin to 5-hydroxy indole acetaldehyde, which

is in turn converted by aldehyde dehydrogenase to 5-hydroxy indole acetic acid, which is a one of the major excretory metabolites of serotonin (Siegel 1996).

1.5 Serotonin receptor subtypes

Serotonin mediates its function by coupling to 14 different receptor subtypes forming the largest GPCR family known to date. The current classification is based on the affinity for different ligands, its primary sequence and the major signal transduction pathways they are believed to couple with. The largest subdivision compromises three families constituting of the 5-HT_1 family, 5-HT_2 family and a family that includes 5-HT_4, 5-HT_6, and 5-HT_7 the only distinctive member is the 5-HT_3 receptor that is a ligand gated ion channel.

The serotonin 1A receptor is a GPCR with the characteristic seven transmembrane domains. It couples to different heterotrimeric G-proteins, among these the Gi/Go are believed to inhibit adenylyl cyclase, while the Gβγ complex is known to activate multiple effector molecules or pathways, such as the phospholipase C β/ ERK 1/2 pathway or the PI-3 kinase (PI-3K) pathway (Stoyanov 1995, Della Rocca 1999, Adayev 1999). Among these signaling molecules stimulated by the 5-HT_{1A}-R, the MAPK isozymes extracellular signal regulated kinases 1 and 2 (ERK1/2) stand out as important regulators of both division and survival of neuronal cells in the brain (Adayev 1999, Bonni 1999, Jin 2002, Stork 2002). The signal transduction pathways of the 5-HT_{1A}-R are shown in **Fig. 1**.

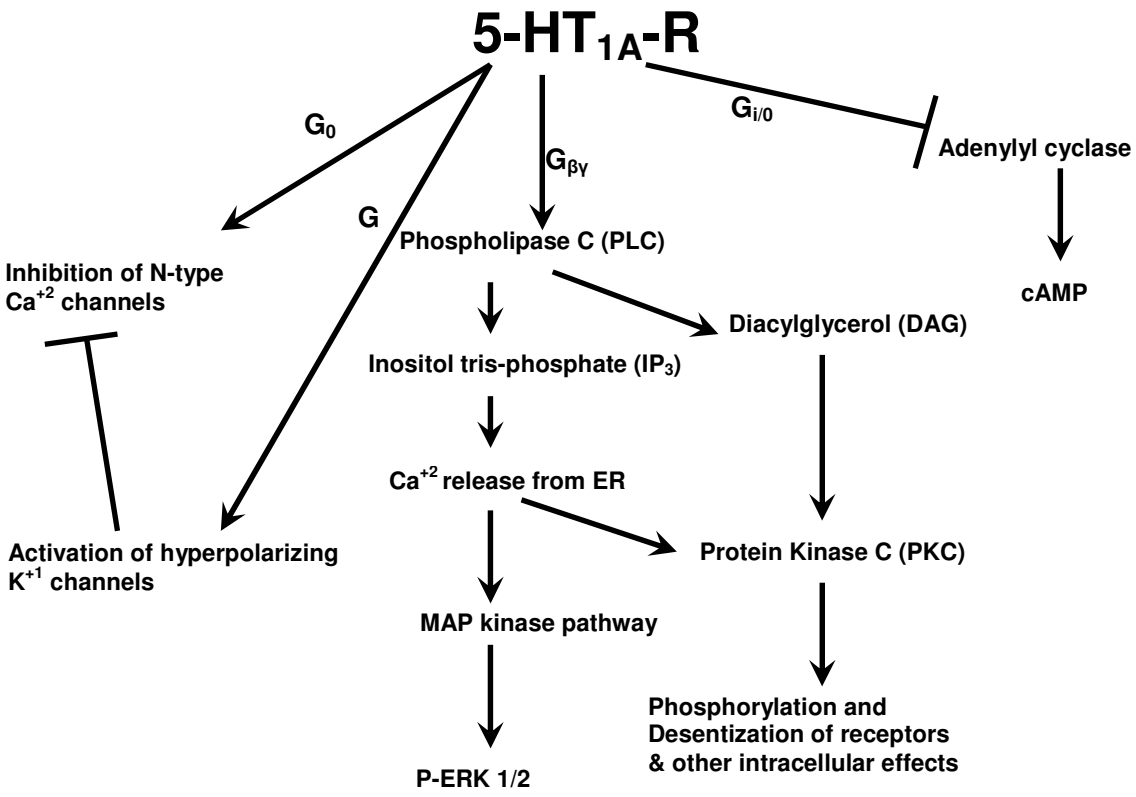


Fig.1.- Signal transduction pathways mediated by the 5-HT_{1A}-R: (Adayev 2005)

1.6 Expression and function of the 5-HT_{1A}-R during brain development

Hillon and coworkers have shown that the mRNA for this receptor is first detected in the rat brain at embryonic day 12 (E12), following which it undergoes a dramatic increase between E14 and E16 to finally decrease to a lower level at E18 (Hillion 1993). It is also believed that during E14-19, the neuronal cells complete their migration and start the process of maturation into fully branched neurons (Lidov 1982). But, does 5-HT_{1A}-R expression reach a peak at this point? On the contrary, 5-HT_{1A}-R expression begins after the peak of 5-HT_{1A}-R mRNA synthesis. The ligand (^{125}I-MPPI)-bound receptor is first observed at E17, reaches a slightly higher level at P5, and then plateaus at adult levels after P21 (Gross 2002). 5-HT_{1A}-R knockout mice [5-HT_{1A}-R (-/-)] including 5-HT_{1A}-R conditional knockouts engineered to express the receptor only after postnatal day 21 (controlled by an inducible promoter) display elevated levels of anxiety (Ramboz 1998, Parks 1998, Heisler 1998, Gross 2002). Further 5-HT_{1A}-R conditional knockouts engineered to express the receptor only until postnatal day 21 (and blocking its expression thereafter) yields normal adult mice. Thus, these studies suggest important changes that occur in the early postnatal hippocampus may indeed be regulated by signal transduction pathways directly or indirectly involving the 5-HT_{1A}-R. But how does 5-HT_{1A}-R signaling during this period regulate brain development?

Previous work done in our lab has shown that agonist activation of the 5-HT_{1A}-R results in slow activation of the mitogen-activated protein kinase (MAPK) pathway and protein kinase C (PKC), which protects post-mitotic, neuronal HN2-5 cells against caspase-3-promoted apoptosis (Adayev 1999).

1.7 Protein kinase C family

The PKC family consists of 11 different serine/threonine kinases that are subdivided into three groups based on sequence homology, as well as activator and cofactor requirements. These groups include the conventional (cPKC α, β, and γ), the novel (nPKC δ, ε, θ, μ, and η), and the atypical (aPKC λ and ζ) isoforms (Mellor 1998, Viveiros 2003). The conventional PKC isotypes are activated by phosphatidylserine (PS), $Ca^{2+}$and diacylglycerol. The novel PKCs are not Ca^{2+}-dependent, but are still activated by diacylglycerol and PS. The atypical PKCs are lipid-dependent, but they are neither Ca^{2+}-sensitive, nor do they respond to diacylglycerol (Newton 2001, Mellor 1998, Ventura 2001). Ca^{2+}-dependent isotypes have different substrate specificity and phospholipid dependency compared to the Ca^{2+}-independent isoforms. The Ca^{2+}-independent PKCs are suggested to be involved in different cellular functions than the Ca^{2+}-dependent isozymes (Konno 1989).

Despite having closely related structures, specific PKC isozymes appear to be localized differentially in cells and their roles are specific to individual species, tissues, and possibly the stage of development (Majewski 1998). Specific signal transduction pathways stimulated at various stages of brain development, cause activation of the different isotypes of PKC.

PKCδ has shown to be expressed in most differentiated cell types while PKCα, λ and ζ are expressed in stem cells and newly arising cell types. In contrast, PKCβII has

been shown to be abundant only in the somata of only 2.5% of all neurons whereas PKCε is localized in the somata and the axon (Oehrlein 1998).

Studies done on mRNA and protein expression for the PKC isozymes in mouse preimplantation development showed that PKCα and PKCδ proteins are most likely synthesized during oogenesis for use during embryogenesis whereas PKCβ was not detected at all before or during embryonic development (Pauken 2000). PKCγ and PKCζ transcription is probably initiated during or immediately after fertilization. Of particular importance is the increased expression of PKC ε during early development.

1.8 Role of Rb/E2F in cell cycle progression

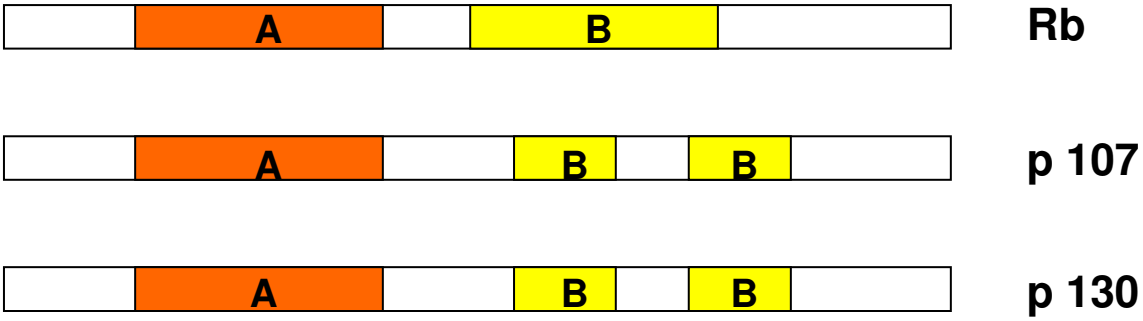
Rb, p107, and p130 belong to the same family and are collectively known as pocket proteins because their main sequence similarity is in the pocket domain that binds to other cellular proteins. Their importance is illustrated by their evolutionary conservation in diverse species such as maize and mammals (Claudio 2002). The Rb proteins differ in the classes of E2F each protein binds to. Classically, Rb is known to act on activator E2F's 1-3, while p107 and p 130 acts on repressor E2Fs 4-5, though this may vary from one cell line to another (Zhu 1993). Each E2F family member can heterodimerize with two DNA-binding protein partners (DP1 and DP2). Rb affects the cell cycle progression by regulating the activity of E2F/DP complexes, (which regulate the expression of many genes that are essential for cell cycle progression and S phase entry) (Dyson 1994, Dyson 1998, Frolov 2004, Lundberg 1998). Rb contains a 379

residue N-terminal domain of unknown function, a 406 residue pocket domain which is best conserved among the Rb family proteins which functions in binding and blocking the E2F transactivation domain, and a 143 residue C-terminal domain whose function is to enhance the E2F –DP binding (Rubin 2005, Flemington 1993, Helin 1993b, Helin 1993a)

The classical view mostly from work done on cell lines, identifies the regulation of Rb activity by a cascade of phosphorylation events during different phases of the cell cycle that are mediated mainly by kinases associated with Cyclins D and E (Sherr 1999, Lundberg 1998, Harbour 1999, Knudsen 1996). The Cyclin D-CDK4/6 complex phosphorylates Rb during early G1 phase and results in its disassociation with E2F/DP, thus allowing the transcriptional activation of proteins including Cyclin A/E helping the cell progress through the cell cycle. In late G1 phase, Cyclin E/CDK2 complex further phosphorylates Rb, keeping it further from binding to E2F/DP, which allows the expression of essential proteins required for G1/S transition. During S phase, Cyclin A-CDK2 activity is responsible for maintaining Rb phosphorylation (Sherr 1999). As a result through 16 putative phosphorylation sites Rb regulates cell cycle progression.

The problem with this view is that most of this information has been based on experiments done using over-expression systems or cancer cell lines in which the Rb regulation system may already be altered. As a result it is therefore unclear as to what regulates Rb phosphorylation *in vivo* following a mitogenic stimulus for normal cell cycling.

A)



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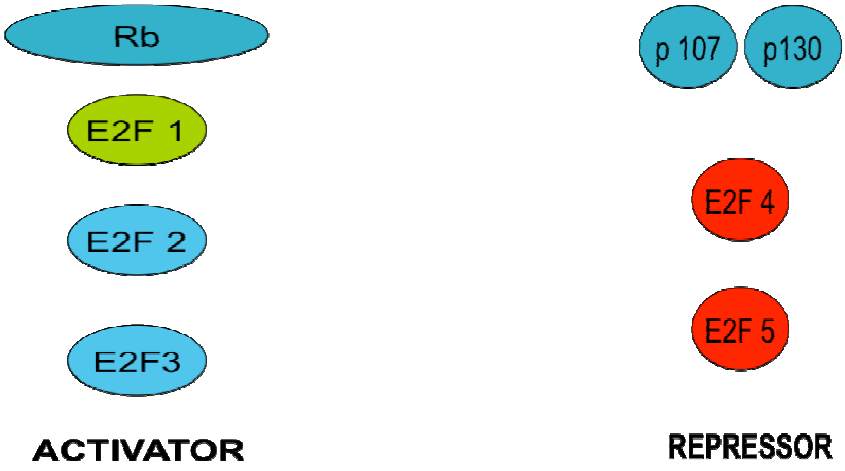


Fig.2. A) Rb Pocket proteins and its homologs (p107, p130), A and B refer to the E2F binding pocket domains. B) E2F transcription factors activated by Rb and its homologs (Du 2006).

CHAPTER 2

5-HT_{1A}-R Mediated Proliferation of HN2-5 Cells

2.1 Introduction:

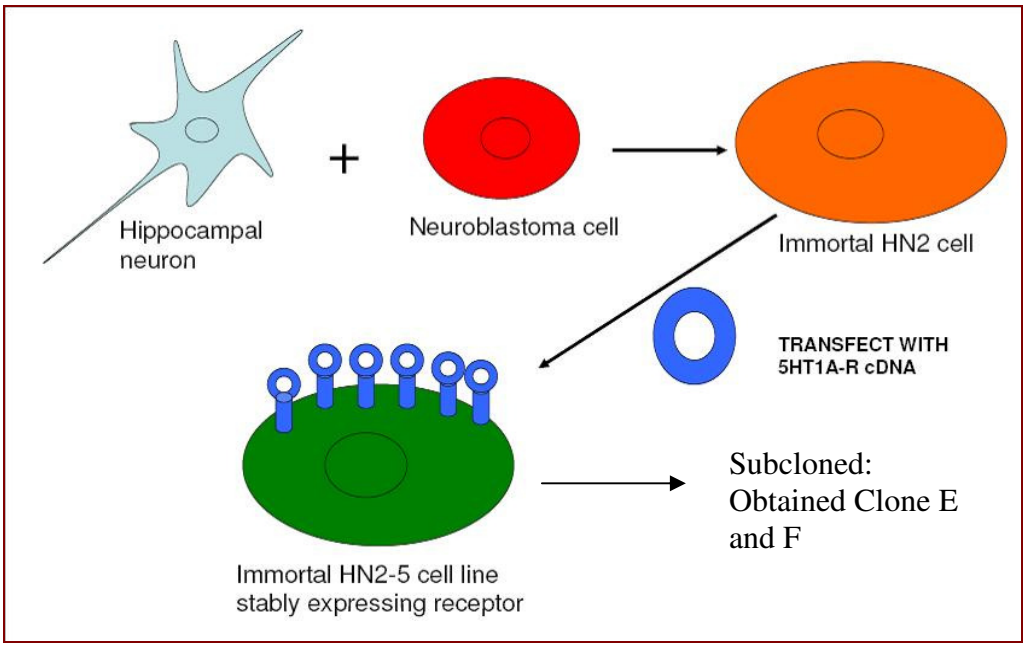
Increased expression of the 5-HT_{1A}-R in the hippocampus during early postnatal development coincides with the period of enhanced neurogenesis in the dentate gyrus. This suggests that the 5-HT_{1A}-R might play an important role in neurogenesis. Work done on adult neurogenesis has shown that stimulation of the 5-HT_{1A}-R leads to a higher proportion of BrdU positive cells in the adult proliferative niches: the dentate gyrus and the subventricular zone. Currently there are only a limited number of studies reported on this phenomenon, none particularly during early postnatal development in the first week following birth. So the question remains on the functional significance of the 5-HT_{1A}-R expression during this critical time frame. Further, no studies have been done to delineate the signal transduction pathway responsible for this enhanced neurogenesis. In order to establish the role of the 5-HT_{1A}-R during early postnatal development of the hippocampus and to identify the molecular players involved in a systematic manner devoid of the complexity of the brain, we first established a model cell line- the HN2-5 cell line.

The HN2 cell line is a hybridoma cell line prepared by the fusion between a mouse neuroblastoma cell (N18) and a hippocampal neuron (**Fig. 3A**). The stable transfection of the 5-HT_{1A}-R gene into an HN2 cell constitutes the HN2-5 cell line. The HN2-5 cell line has been thoroughly characterized as an acceptable model for hippocampal neurons

expressing the 5-HT_{1A}-R (Banerjee 1993). Cells of neuronal lineage in the dentate gyrus maintain their proliferative capacity till they exit the cell cycle and differentiate into mature neurons. As a result a better representation of proliferating neurons would be undifferentiated cells actively cycling through the cell cycle.

In our initial experiments we first established the presence of the functional receptor in HN2-5 cells by a radio-labeled receptor binding assay. The membranes prepared from the transfected cells are treated with ^{3}H-8-OH-DPAT (tritiated agonist of the 5-HT_{1A}-R) in the presence and absence of excess 5-HT. ^{3}H-8-OH-DPAT cannot bind to membranes already treated with a much higher concentration of 5-HT (10 μM). Such competitive binding allows us to compare individual clones and identify the clones with the highest total counts measured by the difference between the radioactive counts in the presence (non-specific) and absence (specific) of 5-HT. This enables us to identify the clones where the functional receptor is expressed to the greatest extent (clone# E) **(Fig. 3B**). These clones are then cultured and used for all subsequent experiments.

A)



B)

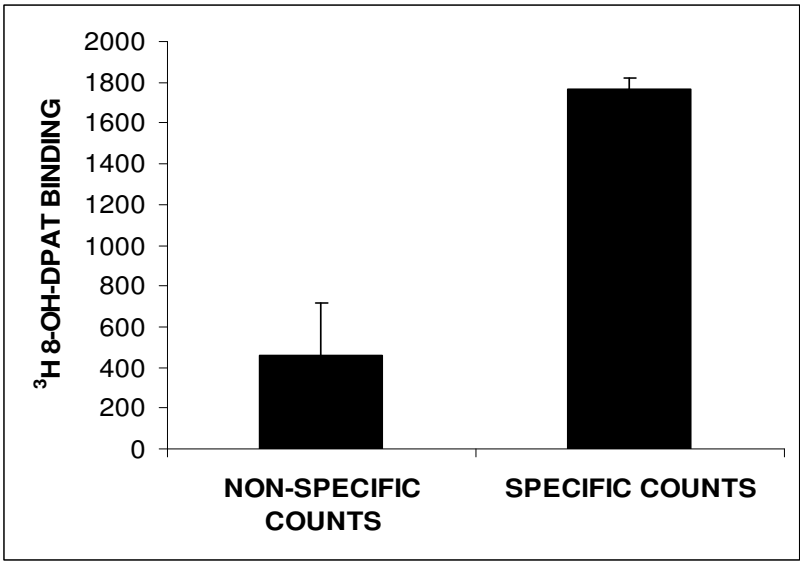


Fig. 3. The functional 5-HT$_{1A}$-R receptor is expressed in HN2-5 cells A) The stable transfection of the 5-HT$_{1A}$-R gene into an HN2 cell constitutes the HN2-5 cell line. B) in the HN2-5 cells were further subcloned to obtain clone# E, which contained the functional 5-HT$_{1A}$-R as evident from ^{3}H-8-OH-DPAT binding assay. Membranes prepared from different stably transfected clones were pulsed with ^{3}H-8-OH-DPAT in the presence (non specific) and absence of serotonin (specific). The difference between the two counts gives an indication of the functional receptor expression.

To test if indeed stimulation of the receptor causes increased cell proliferation we devised three distinct measures of cellular proliferation. Using all three methods, we were able to show convincingly, that in response to receptor stimulation, cell proliferation increased significantly. Earlier research performed in our lab on differentiated HN2-5 cells has suggested that 5-HT_{1A}-R-mediated signaling causes activation of the MAPK pathway as well as PKC. Based on this observation, we next asked the question if these pathways were also involved in 5-HT_{1A}-R mediated increase in proliferation in our model cell line. Using inhibitors specific for both ERK1/2 and PKCε, we were able to identify the PKC isozyme involved as PKCε, and show that the increase in cell proliferation was dependent on both ERK1/2 and PKCε. Furthermore, we looked for the specific phosphorylation status of each protein in order to establish the hierarchy of activation. Our results demonstrate that in the HN2-5 cells PKCε appears to be upstream of ERK1/2 activation. We conclude that in the HN2-5 model cell line, proliferation increases in response to 5-HT_{1A}-R stimulation through a pathway that includes both PKCε and ERK1/2 where PKCε is located upstream of ERK1/2.

2.2 Materials & Methods

Cell culture

The mouse hippocampal neuron-derived HN2–5 cells were cultured to about 70–80% confluence in DMEM (Dubelco Modified Eagles Media) containing either 10% FBS (Fetal Bovine Serum), 10% DFBS (dialyzed fetal bovine serum, GIBCO 10,000 mw cutoff), or 5% HS (Horse Serum) and 1% penicillin–streptomycin (PS) in poly-L-lysine-coated plates. For BrdU/HOECHST proliferation assays and trypan blue exclusion cell counting experiments, 96-well plates were used while for all other experiments 24 well plates were used. Prior to drug treatment, cells were synchronized by subjecting them to serum-free conditions for 24 h. Synchronization is temporary withdrawal from the cell cycle and is essential when looking for expression profile of proteins involved in cell cycle regulation. Serum starvation for 24 h is an accepted method of synchronizing cells in culture (Chou 2003). We also established this protocol for HN2-5 cells by noting no significant change in cell number after 24 h under serum-free conditions (data not shown).

Membrane Preparation & Ligand Binding Assay

To look for functional receptor expression membranes were prepared from harvested HN2-5 cells grown to confluence. The pellet obtained was washed once using a cold (4 °C) swelling buffer [10 mM Tis-HCL (pH 7.4) and 5mM EGTA] and homogenized using a Potter-Elvehjem homogenizer on ice. The lysate was then centrifuged at 300,000 g for 5 min. The supernatant was discarded and the process

repeated two more times. After the final wash the pellet was resuspended in buffer A1 [50 mM Tris-HCL (pH 7.4) and 5 mM $MgCl_2$] by Potter-Elvehjem homogenization and stored at -80 °C before being used for radioligand binding assays. Tubes in duplicate with 200 μg of total protein in a volume of 1 ml buffer B (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) were incubated at 37 °C for 5 min in the presence of 5 mM ^{3}H-8-OH-DPAT. The binding reaction was terminated by rapid filtration under vacuum in a Brandel cell harvester (Gaithersburg, MD) through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0-mm pore size), which were presoaked in 3% (w/v) polyethylenimine. The filters were then washed three times with 3 ml of cold water (4 °C) and dried, and the retained radioactivity was measured in a Beckman liquid scintillation counter using 3 ml of scintillation fluid. Nonspecific binding was determined in the presence of 10-μM 5-HT.

Cell counting using Trypan Blue Exclusion

One hundred nanomolar 8-OH-DPAT was added to synchronized HN2-5 cells growing in varying concentrations of DFBS (4,8,10%). After 0, 40, 50, 64, 74, 88 h, cell number was counted by the Trypan blue exclusion method using a hemocytometer. Experiments were performed in triplicates and repeated three times.

[^{3}H] Thymidine incorporation assay

Varying concentrations of 8-OH-DPAT and 1 mCi [^{3}H] thymidine (NEN #NET-027Z) were added to each well of HN2-5 synchronized cell cultures in 24-well plates and incubated for 64 h. Media was aspirated and washed in 1 ml of ice cold PBS. Ice cold 5%

TCA (tri-chloro acetic acid) (1 ml) was added to each well and incubated at 4 °C. After 30 min the wells were washed once with PBS. To each well 0.5 ml 0.5N NaOH / 0.5% SDS was added, and the solubilized matter from each well was transferred into 3 ml of scintillation fluid. The scintillation vials were read on a Beckman scintillation counter.

BrdU/HOECHST proliferation assay

BrdU (10 μM) and 8 OH-DPAT at varying concentrations were added to synchronized cultures. When using inhibitors the following concentrations and time periods were used: WAY (4 μM, overnight), U (10 μM, 30 min) and M (400 nM, 30 min). After 24, 48 and 72 h, DNA was denatured by a 10-min incubation with 4 N HCl, followed by two rinses with 0.1 M sodium tetraborate, pH 8.5. After rinsing with PBS, cells were incubated with an anti-BrdU antibody (clone BU-33, Sigma; St Louis, MO) diluted 1:1000 in PBS with 0.2% Tween for 2 h at room temperature. Plates were washed 3 times with PBS with 0.2% Tween, and incubated with 1: 400 of anti mouse AlexaFluor 568 secondary antibody (Invitrogen) for 1 h. Cells were then labeled with HOECHST33342 (10 μM) in PBS for 30 min. After 2 washes in PBS the fluorescence intensity was measured using a fluorescence plate reader (excited at 350 nm and 560 nm for HOECHST and BrdU respectively) at each respective maximum emission wavelength λ= 460 nm and λ= 610 nm for HOECHST and BrdU, respectively. For microscopy, pictures were taken under different windows using an inverted NIKON florescence microscope and visualized using the SPOT software.

SDS-PAGE and Immunoblotting analysis:

To determine the expression levels of P-PKCε and P-ERK 1/2, cell lysates were prepared in RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4, protease inhibitor cocktail, PMSF). Aliquots containing 25-µg protein were mixed with SDS-PAGE treatment buffer and boiled for 5 min. Proteins were resolved on a 10% SDS-PAGE gel and then transferred to nitrocellulose membrane. Blots were blocked overnight with 5% nonfat dry milk in 0.1% Tween-TBS and subsequently incubated overnight with P-PKCε (1:1000) or P-ERK 1/2 antibody (1:2000 dilution). Following three washes with 0.1% Tween-TBS, blots were incubated for 1 h with horseradish peroxidase-labeled goat-anti-rabbit IgG (1:20,000). Protein bands were detected using Super signal West Pico kit (Pierce). For normalization, blots were stripped using a stripping buffer (0.25 M glycine, pH 2.0) for 1 h at room temperature, re-blocked and probed with monoclonal PKCε or ERK 2 antibody (Santa Cruz, biotech, CA) in a 1:10,000 dilution with overnight incubation. After subsequent washes, blots were incubated with horseradish peroxidase-labeled goat-anti-mouse IgG (1:50,000).

2.3 Results & Discussion

Increased cell proliferation of HN2-5 cells in response to 5-HT_{1A} receptor activation through ^{3}H-thymidine incorporation

To test if receptor stimulation increases cell proliferation, we used ^{3}H-thymidine incorporation. ^{3}H-thymidine is a radioactive analog of thymidine, which is incorporated into DNA during replication (the S phase of the cell cycle). The resulting radioactive counts show the proportion of cells undergoing DNA replication, which in turn correlates directly with cell proliferation. Initially we wanted to establish a proper growth medium for the culture of HN2-5 cells in order to determine if 5-HT_{1A}-R stimulation leads to increased proliferation. HN2-5 cells appear to be a robust cell line that can be cultured in the presence of a variety of sera. Fetal Bovine Serum (FBS) is normally used for the culture of these cells. Since FBS contains 5-HT and other growth factors, we first evaluated the incorporation of ^{3}H-thymidine in HN2-5 cells in the presence of Fetal Bovine Serum (FBS), Dialyzed fetal bovine serum (DFBS), and Horse serum (HS) in response to 8-OH-DPAT (5-HT_{1A}-R agonist), 5-HT, and WAY (a 5-HT_{1A}-R antagonist) (**Fig. 4**). The most significant change in 8-OH-DPAT-evoked ^{3}H-thymidine incorporation occured in the presence of 10% Dialysed FBS (DFBS). This suggests that increased cell proliferation occurs primarily in response to 5-HT_{1A}-R stimulation. In both 10% fetal bovine serum (10% FBS) and Horse Serum (5% HS) cultured cells there appeared to be no significant change in response to receptor stimulation, suggesting that there may already be exogenous levels of serotonin present in these media. Additionally,

WAY (an antagonist of the 5-HT_{1A}-R) blocked the 8-OH-DPAT-evoked increase in ^{3}H-thymidine incorporation. Furthermore, WAY treatment caused suppression of ^{3}H-thymidine incorporation below the control levels in all samples. This effect could possibly be attributed to the inverse agonist effect, which has been reported earlier for WAY (Abbas 2007) . On this same basis, the lowering of ^{3}H-thymidine incorporation in the serotonin-treated samples in comparison with the 8-OH-DPAT-treated samples in DFBS suggests the possibility that serotonin activates another serotonergic receptor/receptors pathway that causes the suppression of the 5-HT_{1A}-R mediated proliferation (Fig. 4).

Based on this preliminary set of experiments, DFBS was chosen as the medium for assessing changes in cell proliferation and hence used in the subsequent experiments. This is in line with the observation that DFBS does not contain 5-HT and the 8-OH-DPAT-evoked cell proliferation can be attributed primarily to the activation of the 5-HT_{1A}-R.

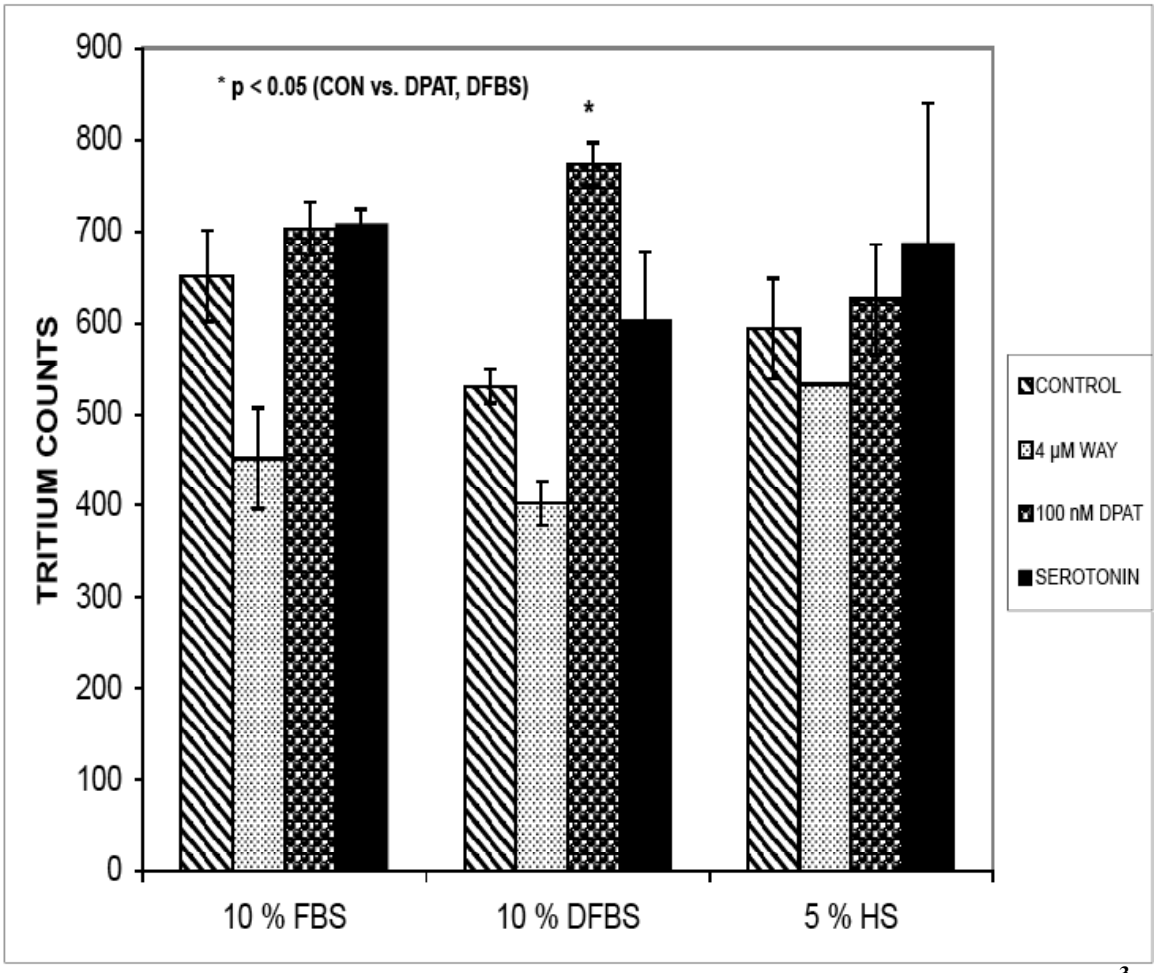


Fig. 4. In proliferating HN2-5 cells, maximum increase in incorporation of ^{3}H thymidine – a marker of DNA synthesis- occurs in the presence of Dialysed Fetal Bovine Serum (DFBS). Synchronized HN2-5 cells growing in different media were pulsed with ^{3}H thymidine followed by either 4 µM WAY, 100 nM 8-OH-DPAT or 10 µM 5-HT. After 64 h, total radioactive counts were measured. Results presented are the mean (±S.D.) of three independent experiments.

Previous studies had suggested 100 nM 8-OH-DPAT as the optimal concentration for activation of the 5-HT_{1A}-R in differentiated HN2-5 cells (Adayev 2003). In order to establish if a similar profile was evident in actively dividing HN2-5 cells, we performed a dose response with synchronized HN2-5 cells in order to identify the optimum concentration of 8-OH-DPAT needed for this increase in ^{3}H-thymidine incorporation. Our results indicate that similar to the differentiated HN2-5 cells, the actively dividing HN2-5 cells also showed the maximum increase in ^{3}H-thymidine incorporation over control in the presence of 100 nM 8-OH-DPAT **(Fig. 5)**. Furthermore, the decreased response with concentrations above 100 nM suggests possible receptor desensitization.

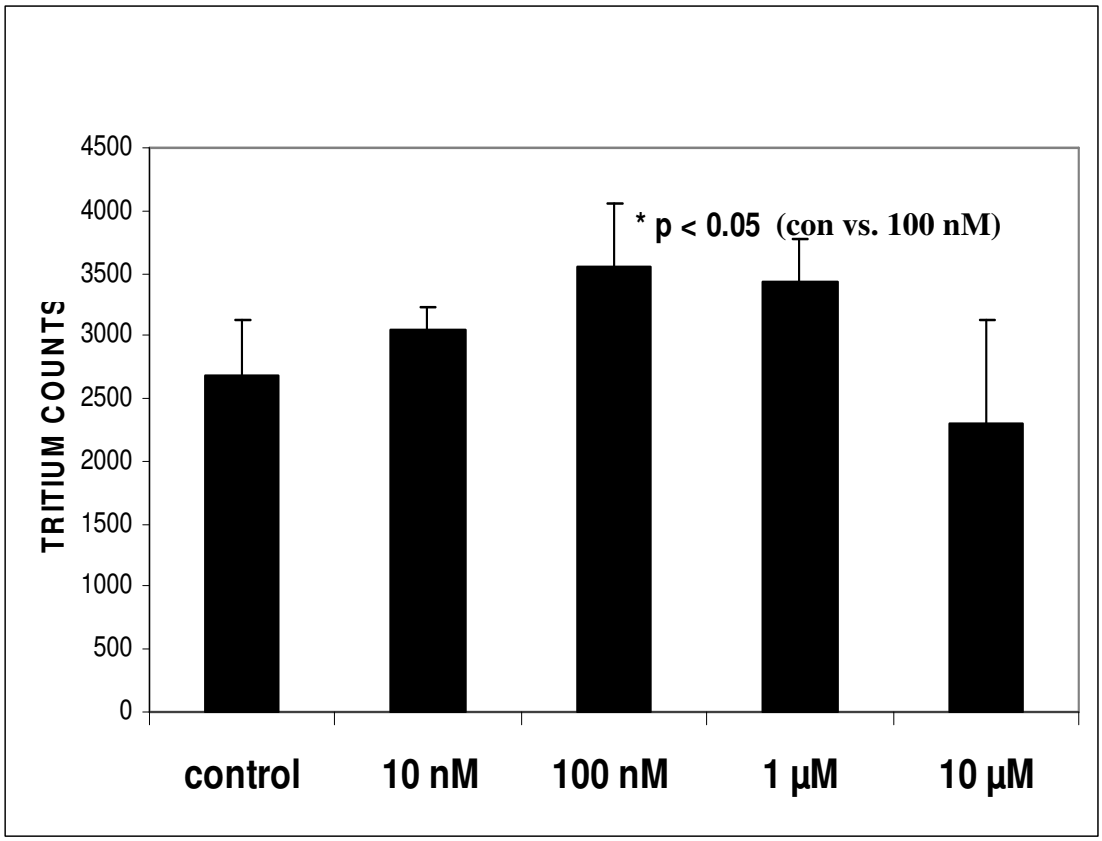


Fig. 5. In actively dividing HN2-5 cells, the maximum increase in ^{3}H thymidine incorporation occurs in the presence of 100 nM 8-OH-DPAT. Synchronized HN2-5 cells were pulsed with ^{3}H thymidine in varying concentrations of 8-OH-DPAT, after 64 h total radioactive counts were measured. Results presented are the mean (±S.D.) of three independent experiments.

Measurement of increased cell proliferation of HN2-5 cells in response to 5-HT_{1A} receptor activation by Try pan Blue Exclusion cell counting

We next assessed the actual increase in cell number in response to 5-HT_{1A}-R stimulation by using trypan blue exclusion cell counting. Trypan blue is incorporated into dying cells and by counting for non- trypan blue stained cells over time one can measure the increase in cell number. Our results show that for all concentrations of DFBS used (4%, 8%, and 10%), stimulation of the 5-HT_{1A}-R by 8-OH-DPAT caused a general trend of increase in the number of cells (**Fig. 6**). The observed decrease in cell number between 0 and 40 h in 4% DFBS is possibly due to apoptosis of a significant proportion of cells. Also at lower serum concentrations (4% DFBS) there was evidence of differentiation of cells based on the morphology of flattened cells with extended processes. On a similar basis, the increase in cell number in 8-OH-DPAT-treated 4% DFBS samples at later time points can possibly be attributed to protective mechanisms activated by 5-HT_{1A}-R stimulation, as previous studies have identified anti-apoptotic pathways activated in differentiated HN2-5 cells in response to 5-HT_{1A}-R stimulation. Based on these studies we chose a serum concentration of 10% DFBS for all future studies concerning cell proliferation.

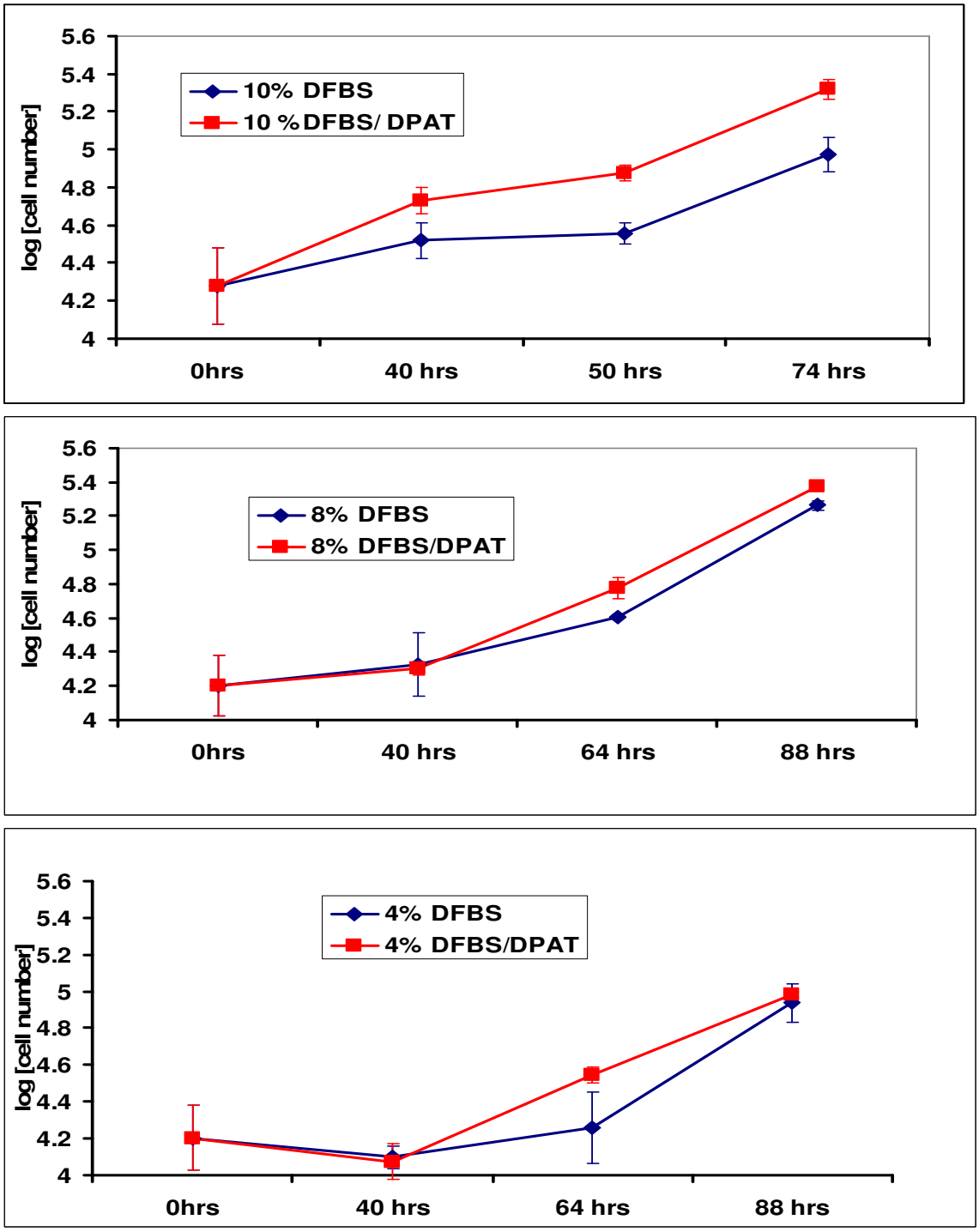


Fig. 6. Proliferation of HN2-5 cells in culture increases over time upon 5-HT$_{1A}$-R stimulation at all DFBS concentrations used. Synchronized HN2-5 cells were treated with varying concentrations of DFBS in the absence or presence of 8-OH-DPAT and cell number was counted at varying time points using the Trypan blue exclusion method. Results presented are the mean (±S.D.) of three independent experiments.

Increased proliferation of HN2-5 cells in response to 5-HT_{1A} receptor activation through a BrdU/HOECHST proliferation assay

Finally, to measure cell proliferation in a more systematic and convenient manner, we developed a florescence-based proliferation assay. BrdU (bromo-deoxy uridine) is an analog of thymidine incorporated into proliferating cells during the S phase of the cell cycle, and BrdU staining is an accepted proliferation marker. By using an anti-BrdU primary antibody and then a secondary antibody attached to a flourophore, we were able to identify cells that had incorporated BrdU. To normalize for total cell number, we stained all cells with the DNA binding dye HOECHST33342 (HOECHST) and measured the florescence intensity of each fluorophore at their respective emission maxima using a florescence plate reader **(Fig 7**). The BrdU florescence intensity was divided by the intensity of HOECHST and the results were expressed as % carrier. Cells were synchronized by serum starvation before drug treatment. We conducted both a time course and a dose response in order to obtain the optimum condition for cell proliferation. We observed the maximum increase in relative florescence intensity at 24 h after treatment with 100 nM DPAT (**Fig. 7 and Fig. 9**). The results were compared with images captured from an inverted florescence microscope to evaluate for consistency.

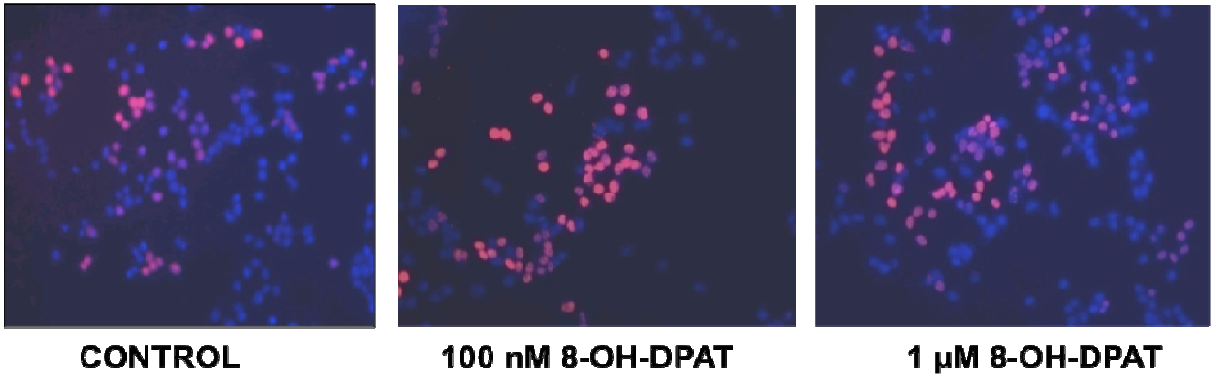


Fig. 7. Stimulation of the 5-HT$_{1A}$-R in undifferentiated but synchronized HN2-5 cells causes increased BrdU labeling. HN2-5 cells were synchronized by serum starvation (24 h) and then treated with BrdU (10 µM) and 8-OH-DPAT (D) for 24 h, followed by anti-BrdU, and HOECHST33342 (10 µM) staining.

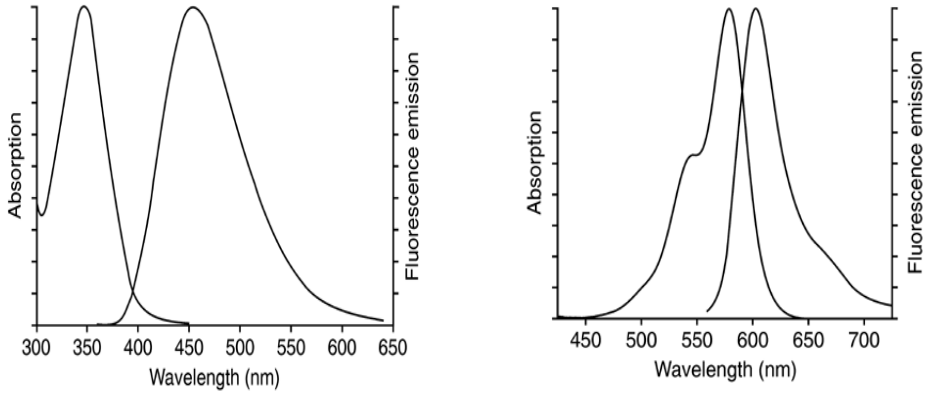


Fig. 8. Emission spectra of HOECHST33342 and Alexa flour 568.

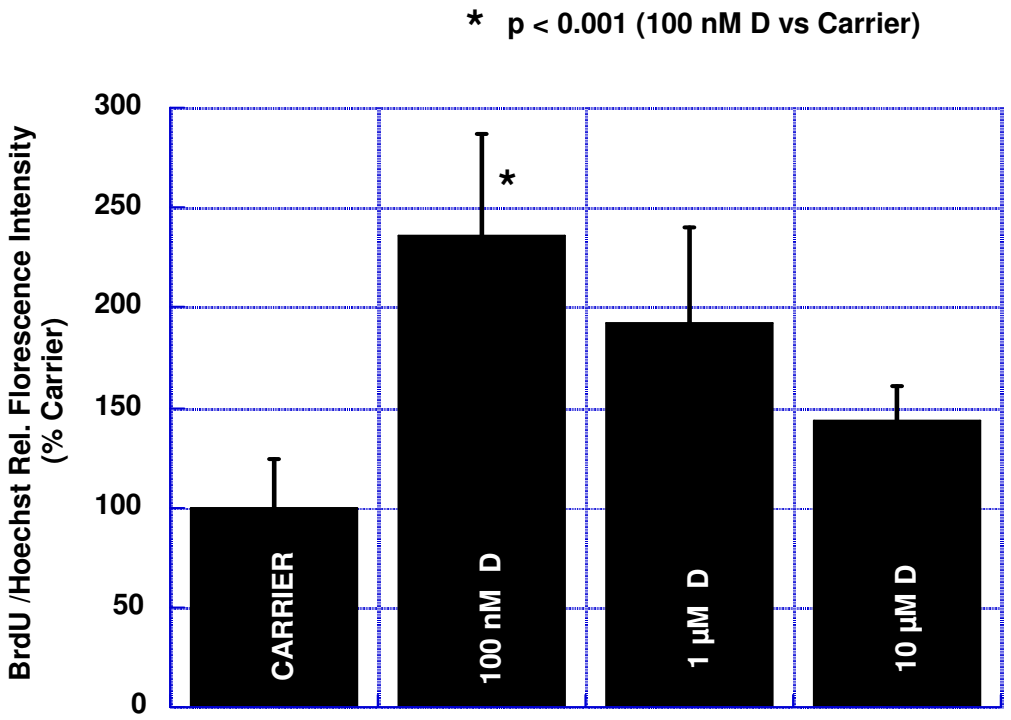


Fig. 9. Stimulation of the 5-HT_{1A}-R HN2-5 cells with 100 nM 8-OH-DPAT (D) for 24 h causes increased BrdU labeling. Synchronized HN2-5 cells in culture were treated with varying concentrations of 8-OH-DPAT (D) in the presence of BrdU (10 µM) for 24 h. After labeling with anti-BrdU, and HOECHST33342 (10 µM) fluorescence intensity for each fluorophore was measured. BrdU fluorescence intensity (645 nm) was normalized to HOECHST fluorescence intensity (460 nm) and expressed as % carrier. Data obtained from quintuplicate samples were expressed as mean (±S.D.)

Proliferation in HN2-5 cells occurs through a pathway that involves the 5-HT_{1A}-R, PKCε and MAPK.

Previous research on differentiated HN2-5 cells had demonstrated 5-HT_{1A}-R mediated activation of the MAPK pathway and PKC. Also, work done on the expression of different PKC isozymes during development had revealed that the novel PKC isozyme PKCε was particularly important, as its expression high in the P6 (post natal day 6) dentate gyrus during the same time frame when 5-HT_{1A}-R-linked neurogenesis reaches a maximum (Purkayastha 2009). Based on these studies we next asked if the observed increase in cell proliferation in our model HN2-5 cells could possibly be mediated by a pathway involving MAPK and PKCε in response to 5-HT_{1A}-R stimulation.

U0126 is a potent and well-characterized inhibitor of MEK that prevents the activation of ERK1/2 by preventing the ability of MEK to phosphorylate ERK1 and 2 at threonine 185/202 and tyrosine 187/204 respectively and activate them. As MEK is suggested to be the sole activator of ERK1/2, the inhibition of MEK abolishes ERK1/2 activation. Furthermore, the novel PKC isozyme, PKCε, contains a v1 hinge region, characterized by a stretch of 8 amino acids, necessary for its binding to RACK proteins allowing eventual activation by removal of the pseudosubstrate at intracellular membranes. The PKCε inhibitor M is a myristylated inhibitor peptide identical to the v1 hinge region that competes with PKCε and hinders it from binding to RACK proteins. This inhibits PKCε activation (Johnson 1996).

Using the BrdU/HOECHST proliferation assay, we then measured the increase in cell proliferation of HN2-5 cells in response to 5-HT_{1A}-R stimulation in the presence of the 5-HT_{1A}-R antagonist WAY, the MEK inhibitor U0126, and the PKCε inhibitor M.

Our results show that the 5-HT_{1A}-R antagonist WAY, the MEK inhibitor U0126, and the PKCε inhibitor M block the 5-HT_{1A}-R-mediated increase in cell proliferation (**Fig. 10**), suggesting that that both the MAPK pathway and PKCε are involved in this 5-HT_{1A}-R mediated signal transduction pathway that regulates cell proliferation in HN2-5 cells.

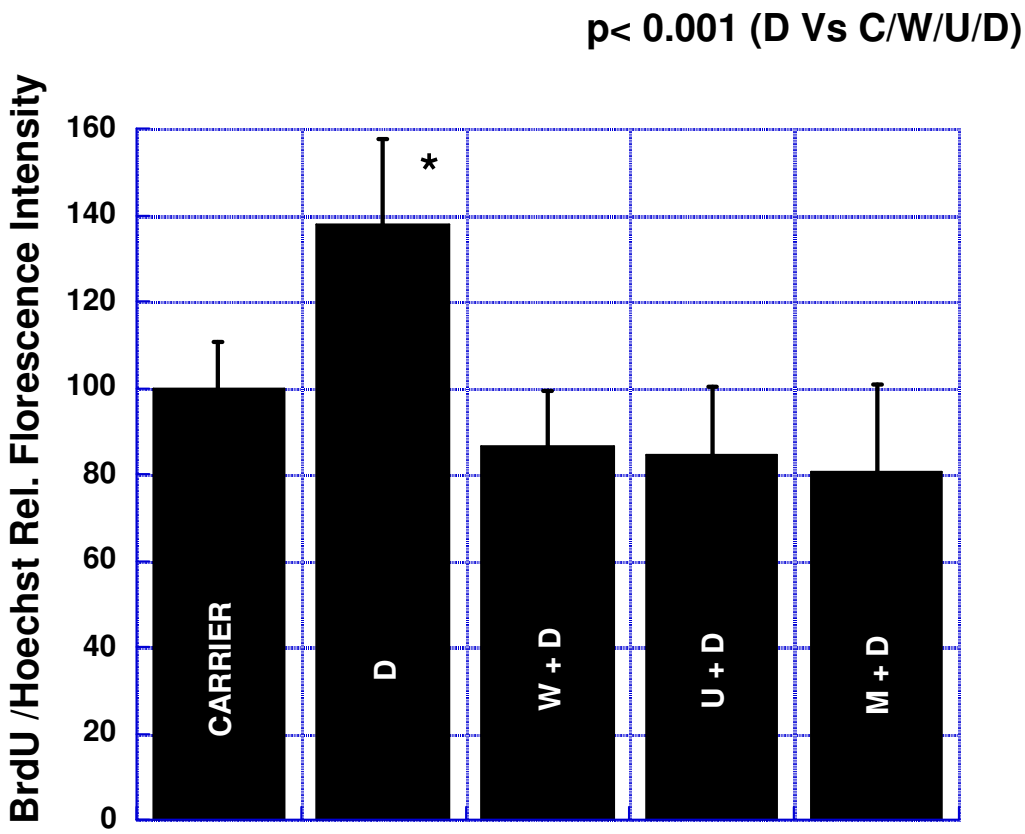


Fig. 10. Serotonin 1A receptor-mediated proliferation in HN2-5 cells requires PKCε, and MAPK. Synchronized HN2-5 cells in culture were treated with WAY (4 μM, overnight), U (10 μM, 30 min), and M (400 nM, 30 min), followed by 100 nM of 8-OH-DPAT (D) in the presence of BrdU (10 μM) for 24 h. After labeling with anti-BrdU, and HOECHST (10 μM), fluorescence intensity for each fluorophore was measured. BrdU fluorescence intensity (645 nm) was normalized to HOECHST fluorescence intensity (460 nm) and expressed as % carrier. Data obtained from quintuplicate samples were expressed as mean ±S.D. Statistical analysis was performed using ANOVA, with Bonferroni *ad hoc* analysis.

In HN2-5 cells, PKCε functions upstream ERK 1/2.

Knowing that both MAPK and PKCε were involved in the signal transduction pathway, we next looked to examine the hierarchy of their activation profiles. We had previously observed an increase in phosphorylation/activation of ERK1/2 in response to 5-HT_{1A}-R stimulation in differentiated HN2-5 cells. In order to determine if a similar activation occurred in undifferentiated HN2-5 cells actively cycling through the cell cycle, we initially looked for ERK1/2 phosphorylation / activation in response to 5-HT_{1A}-R stimulation using HN2-5 cell lysates to probe for phosphorylated ERK1/2 at various time points. We observed that the time course of 5-HT_{1A}-R-mediated phosphorylation / activation of ERK1/2 attains a maximum at 20 min post-100 nM 8-OH-DPAT treatment **(Fig. 11)**.

To confirm the involvement of PKCε and determine its position in the pathway, we next looked for ERK1/2 activation/phosphorylation in response to inhibition of PKCε (using the PKCε inhibitor M). Our results show that in HN2-5 cells, 5-HT_{1A}-R-mediated ERK1/2 activation is suppressed in presence of the PKCε inhibitor (M), (**Fig 11**), indicating that ERK1/2 activation occurs through PKCε activation.

The signaling hierarchy of PKCε in this pathway was further confirmed by monitoring phospho-Ser^{729} PKCε formation, which is the hallmark of its activation. Our results show that the MEK inhibitor U0126 does not block the 5-HT_{1A}-R mediated activation of PKCε, (**Fig 12**), thus confirming that the MAPK pathway lies downstream of PKCε in the proliferating HN2-5 cells.

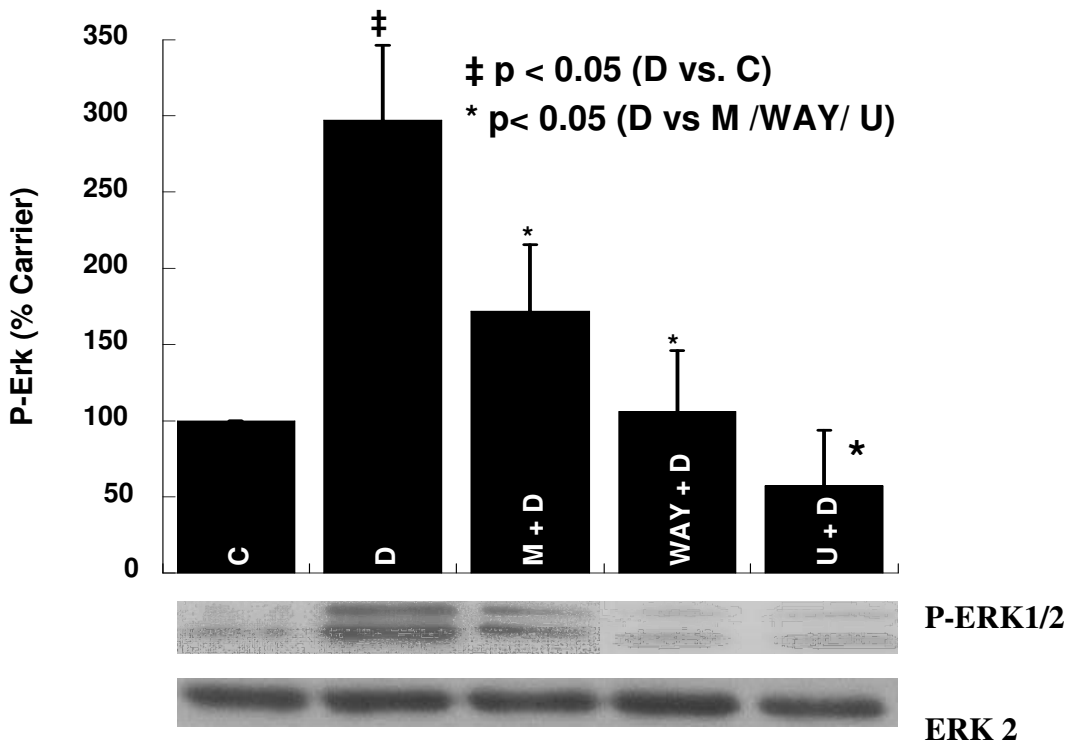


Fig. 11. In proliferating hippocampal neuron-derived HN2-5 cells, ERK 1/2 undergoes 5-HT_{1A}-R-and PKCε-dependent stimulation. Synchronized HN2-5 cells in culture were treated with WAY (4 μM, overnight), U0126 (10 μM, 30 min) and M (400 nM, 30 min) followed by 100 nM of 8-OH-DPAT (D) for 20 min. Cell lysates were examined for expression of P-ERK1/2 and ERK2 using SDS-PAGE followed by immunoblotting. P-ERK band intensities were normalized to ERK2. Results presented are the mean (±S.D.) of three independent experiments.
(Purkayastha. S, and Banerjee. P, unpublished data)

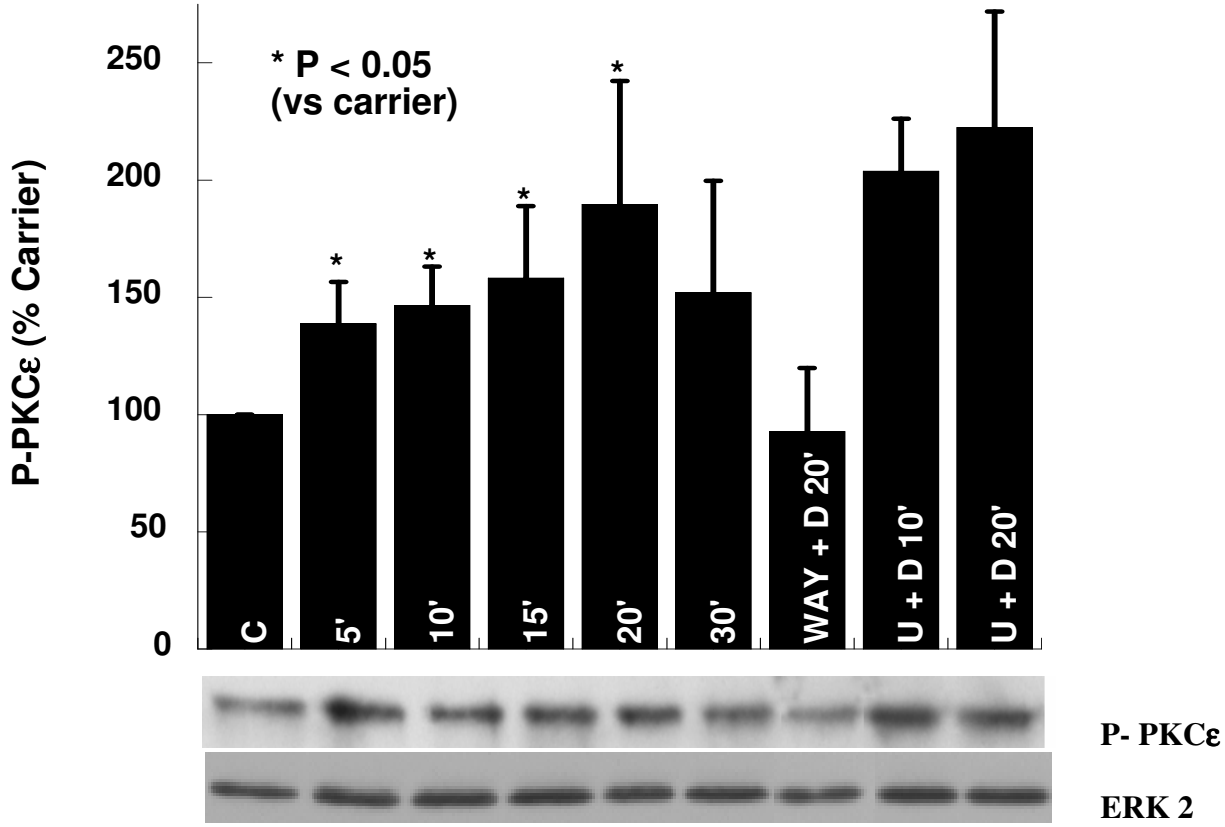


Fig. 12. In proliferating hippocampal neuron-derived HN2-5 cells, PKCε undergoes 5-HT_{1A}-R-dependent, but ERK1/2-independent activation. Synchronized HN2-5 cells in culture were treated with either 100 nM of 8-OH-DPAT (D) for varying times or WAY (4 μM, overnight), and U0126 (10 μM, 30 min) followed by 100 nM of 8-OH-DPAT (D) for 10 or 20 min. Cell lysates were examined for expression of P-PKCε and ERK2 using SDS-PAGE followed by immunoblotting. P-PKCε band intensities were normalized to ERK2. Results presented are the mean (±S.D.) of three independent experiments.
(Purkayastha. S, and Banerjee. P, unpublished data)

CHAPTER 3

5-HT_{1A} –R Mediated Neurogenesis in the P6 Dentate Gyrus

3.1 Introduction:

Based on our studies on the cell lines we next sought to investigate the mechanisms operating during enhanced neurogenesis in the DG of the postnatal day-6 hippocampus. Previous studies had demonstrated that in adult animals, conditions that cause a decrease in granule cell genesis, such as malnutrition, aging, high corticosterone, stress, and NMDA receptor activation, also decrease the density of 5-HT_{1A} receptors (Gould 1999a). Conversely, conditions that enhance granule cell genesis, such as seizures, adrenalectomy, and NMDA receptor antagonist treatment, increase the density of 5-HT_{1A} receptors. Furthermore, stimulation of the 5-HT_{1A}-R in adult rats cause a simultaneous increase in proliferation of granule cell precursors in the DG (Gould 1999a). Based on these studies we sought to investigate the previously unexplored phenomenon of 5-HT_{1A}-R-mediated early postnatal neurogenesis with particular emphasis on the signal transduction pathway. We first needed to demonstrate increased neurogenesis during early postnatal development using a proper model system, which allowed us to control the environment of the dividing cells of the DG. This model system had to allow us to also use proper manipulation in order to inhibit potential signaling proteins in the pathway while allowing identification of their activation profiles.

The standard interface method for organotypic slice cultures have been standardized and studied extensively (Stoppini 1991, Sakaguchi 1994). Previous studies

reported by other groups as well as ours have confirmed the preservation of morphologies and localization of all types of neurons and glia in our slice culture model, comparable to that of the living animal (Gahwiler 1984, Dailey 1994). Further studies have shown that neurons in slice culture maintain their physiological membrane properties and synaptic transmission as well as several forms of short and long term plasticity even after several weeks in culture (Gahwiler 1997). As a result of these observations organotypic hippocampal slice cultures have been used extensively in studies on physiology, morphology and plasticity of the hippocampus (Kamada 2004). Furthermore, the ability of hippocampal slice cultures to generate new neurons has been documented by identification of neuronal progenitor-like cells in the slice culture (Miyaguchi 1997). It has been also shown that transplanted embryonic stem cells differentiate into neurons in slice cultures (Benninger 2003). More recently neurogenesis in slice cultures have been established through BrdU labeling and retrovirus vector transduction methods (Raineteau 2004, Kamada 2004). These studies also reported that hippocampal slice cultures retain endogenous neural progenitors in the DG throughout the culture period. Additionally such progenitor cells were shown to maintain their potential to develop into new neurons and become incorporated into the normal architecture of the granular cell layer in the hippocampus (Kamada 2004).

Using this *ex-vivo* system we first asked if the signal transduction pathway responsible for increased cell proliferation in HN2-5 cells might also play a role in increased neurogenesis in the postnatal day-6 DG. We followed the same methodology, first establishing increased neurogenesis through BrdU and Ki-67 labeling. We then verified if this phenomenon was regulated by the 5-HT_{1A}-R. We observed increased

neurogenesis as a result of 5-HT_{1A}-R stimulation. Next we used specific inhibitors of ERK1/2 and PKCε in order to verify the involvement of these proteins in this signal transduction pathway. And finally, we sought to identify the hierarchy of the activation of these proteins. Overall, these studies show that like HN2-5 cells, neurogenesis in the post natal day-6 DG is regulated by the 5-HT_{1A}-R, through a pathway involving ERK1/2 and PKCε, where PKCε is possibly upstream of ERK1/2.

3.2 Materials & Methods

Animals

C57Bl/6 mice were used for all experiments. Mice were obtained from Taconic and kept in the CSI Animal Facility.

Organotypic culture of hippocampal slices

Preparing organotypic cultures of hippocampal slices have been described in our earlier publications (Mehta 2007). Briefly, mouse pups of specific ages are anaesthetized with xylazine (20 mg/ Kg) and ketamine (100 mg/ Kg) and decapitated. Under sterile conditions, the brains are isolated and then cut at 60 ° from the longitudinal fissure at the top using a hippocampal cutter to expose the hippocampus. The hemispheres containing the hippocampi are then placed in modified Grey's balanced salt solution (mGBSS) for 30-45 min that had been pre-chilled to 4 °C and bubbled vigorously with a 95% O_2/5% CO_2 gas mixture. Individual hippocampus is isolated using dissection tools and then 400µm thick transverse slices are prepared using a tissue chopper (Stoelting, IL). The slices are placed in ice-cold mGBSS and inspected using a dissection microscope for the presence of uninterrupted bright, transparent neuronal layers characteristic of the hippocampal structure. Only such slices are plated onto Millicell CM filters (Millipore, Bedford, MA). Up to three slices are placed on each filter and the filters are placed in a six-well dish. For the first two days, the slices are kept in high potassium culture medium (25% horse serum, 50% Basal Essential Media-Eagles, 25% Earle's balanced salt

solution (EBSS), 25 mM NaHEPES, 1 mM glutamine, 28 mM glucose, pH 7.2). The medium is placed underneath the filter such that the slices remain in contact with the medium. After incubation at 32 °C in a 5% CO_2 atmosphere, the culture medium is changed to physiological potassium slice culture medium (20% horse serum, 5% Basal Essential Media-Eagles, and EBSS modified to adjust the potassium concentration to 2.66 mM. After two days, the medium is changed again to 5% horse serum in physiological potassium culture medium. Before drug treatment, the slices are placed in serum-free medium (the same medium as above, but without serum) for 1 h. This is followed by treatment with the inhibitors and antagonists for 30 min, (except WAY which is done overnight) followed by treatment with the agonist for the specified time periods. After drug treatment, the slices are fixed in 4% paraformaldehyde and then washed several time in phosphate buffer (PB) without salt (salt often causes disintegration of the tissue). mGBSS (in mM): $CaCl_2$ (1.5), KCl (4.9), KH_2PO_4 (0.2), $MgCl_2$ (11.0), $MgSO_4$ (0.3), NaCl (138), $NaHCO_3$ (2.7), Na_2HPO_4 (0.8), NaHEPES (25), glucose 6% (w/v), pH 7.2.

Immuno-histochemistry

The cultured and drug-treated slices were washed quickly with chilled 10-mM PB and then fixed overnight at 4 °C in 4% paraformaldehyde. The sections were then removed from the membrane with a brush and placed in a 96-well plate in PBS. This was followed by 2–3 washes (15 min each) with 1X PBS. For BrdU immunofluorescence staining, free-floating sections were first incubated for 30 min in 2 N HCl at 37°C, and then rinsed 3X (15 min each) with PBS. Sections were then blocked in PBS-X (PBS-

0.1% Triton X-100 – 10% serum from the animal used to raise the secondary antibody that was going to be used later) overnight at 4°C. This was followed by the treatment with primary antibody in PBS-0.1% Triton X-100 – 2% serum from the animal used to raise the secondary antibody, for 48 h at 4 °C with gentle rocking. Antibody concentrations used: BrdU (1:500), P-PKCε (1:50), P-ERK 2 (1:400), P-Rb-S^{780} (1: 200), NeuN (1:150), Ki-67 (1:150), and PROX-1 (1:200), Doublecortin (1:200). The sections were next washed 3 X 15 min at 23 °C with PBS and then treated with fluorescent, secondary antibodies covalently linked to AlexaFluor488 (green) (1:200), AlexaFluor568 (red) (1:200) AlexaFluor633 (cherry red- pseudo color blue) (1:200). After 24 h of secondary antibody treatment at 4°C, the sections were washed in PBS (3X 15 min) and then mounted on slides with ProLong Gold antifade reagent (Molecular Probes, Eugene, OR, USA) for visualization and photography using a laser confocal microscope. Controls for each primary and secondary antibody were performed in parallel to visualize for non-specific binding.

Confocal Microscopy

The 3D stacks were analyzed using the Imaris 6.0.2., 64-bit version (Bitplane AG, Saint Paul, MN, www.bitplane.com) software, using the entire confocal stack by measuring the intensity of each label in each voxel. An iterative procedure determined an intensity threshold (in the 0–255 pixel intensity range) (the background) for each of the three labels (Marvizon 2007, Costes 2004). Voxels with intensities above this threshold were automatically background adjusted and quantified. The image was reconstructed in each channel with identical signal intensity (quality 10) and cells with intensity above

threshold and 0.9 μm in diameter are quantified. BrdU, Ki-67 and P-RB-S^{780} positive cell numbers were divided against NeuN cell numbers and normalized to carrier and expressed as % carrier. Statistical significance was determined using ANOVA with bonferronni post-hoc test.

Signal Intensity measurements using Image J

Confocal images of neurons were obtained using a Zeiss 63X and 100X (NA 1.4) objective with sequential acquisition settings at a resolution of 1024 X 1024 pixels. The confocal microscope settings were kept the same for all scans when fluorescence intensity was compared. All measurements were performed using ImageJ (NIH) software. For P-ERK1/2 and P-PKCε quantification, outlines of cells from control and drug treated slices were taken from random regions of the DG. Individual cells were carefully traced. Fluorescence intensity was then determined for the traced areas (10 pictures per slice and 10 cells per picture). Staining intensity measurements are expressed in arbitrary units of fluorescence. All values in figures and text refer to mean ± SD unless otherwise stated. Statistical analysis was performed using ANOVA.

3.3 Results & Discussion:

Serotonin 1A receptor-mediated increase in cell proliferation in the DG occurs through a pathway involving PKCε and ERK1/2.

As mentioned earlier around P6 there is a sudden burst in neurogenesis in the dentate gyrus and during this time the 5-HT_{1A}-R expression in the hippocampus reaches a level that is easily detected from ligand binding (Gross 2002). Studies have shown that the 5-HT_{1A}-R expression is crucial during this developmental time frame and mice lacking the receptor show heightened anxiety-like behavior. Therefore, we asked if stimulation of the receptor in an organotypic slice culture model would indeed increase neurogenesis. Using BrdU as a marker for cell proliferation we measured the number of cells that were BrdU labeled in response to 5-HT_{1A}-R stimulation. Cultured slices were given a pulse of BrdU (10 μM) along with 100 nM 8-OH-DPAT for a period of 24 h. Fixed sections were then immunostained for cells that incorporated BrdU, the post mitotic marker NeuN was used to identify neuronal cells.

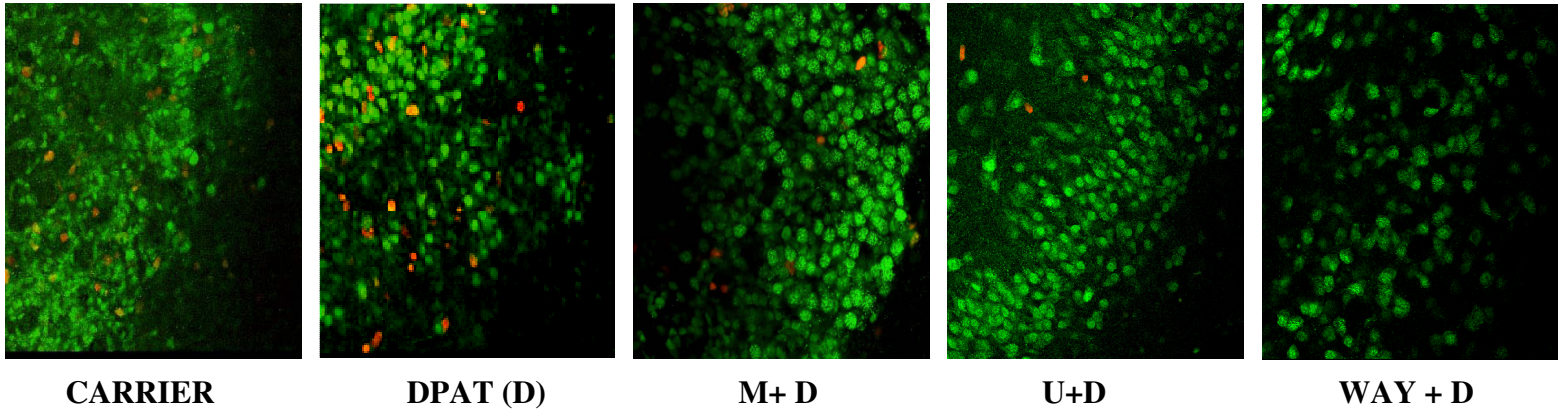
We reconstructed the three-dimensional structure of the DG for each slice using the IMARIS software and analyzed all labeled cells in the entire volume for both green (NeuN) and red (BrdU) channels. The post mitotic neuronal marker (NeuN) was also used to normalize for total cell number because sections, though initially sectioned at 400 μm, would have different degrees of thinning after seven days in culture.

After labeling, confocal microscopy, and slice rebuilding using IMARIS software, we found a significant proportion of BrdU-labeled in 8-OH-DPAT treated slices in

comparison to slices treated with carrier (PBS) alone **(Fig 13**). This increase in the proportion of BrdU labeled cells was blocked in the presence of WAY (5-HT_{1A}-R antagonist), confirming that indeed the 5-HT_{1A}-R was involved in the pathway leading to cell proliferation. Although we did see BrdU labeled cells in the sub granular zone of the DG, we consistently observed the presence of a few proliferating cells also in other hippocampal areas. This is quite interesting due to the fact that neurogenesis is suggested to occur exclusively in the sub granular zone of the dentate gyrus, and whether this is due to the slice culturing procedure has yet to be determined.

As for the HN2-5 cell lines, we next set out to ask if PKCε and ERK1/2 were involved in this pathway, which induces cell proliferation in response to 5-HT_{1A}-R stimulation. Using the inhibitors of MEK (U0126) and PKCε (M) we looked to see how the number of BrdU labeled cells changed in response to these inhibitors. Our results show a pronounced decrease in the proportion of BrdU-labeled cells in both U0126- and M- treated slices **(Fig 13B**). This suggests that in the DG of the postnatal day-6 hippocampus, 5-HT_{1A}-R stimulation leads to an increase in proliferation through a signal transduction pathway involving both PKCε and ERK1/2.

A)



B)

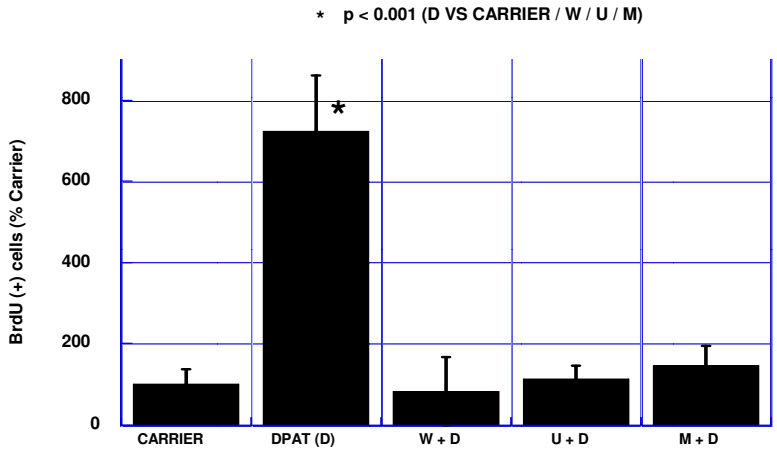


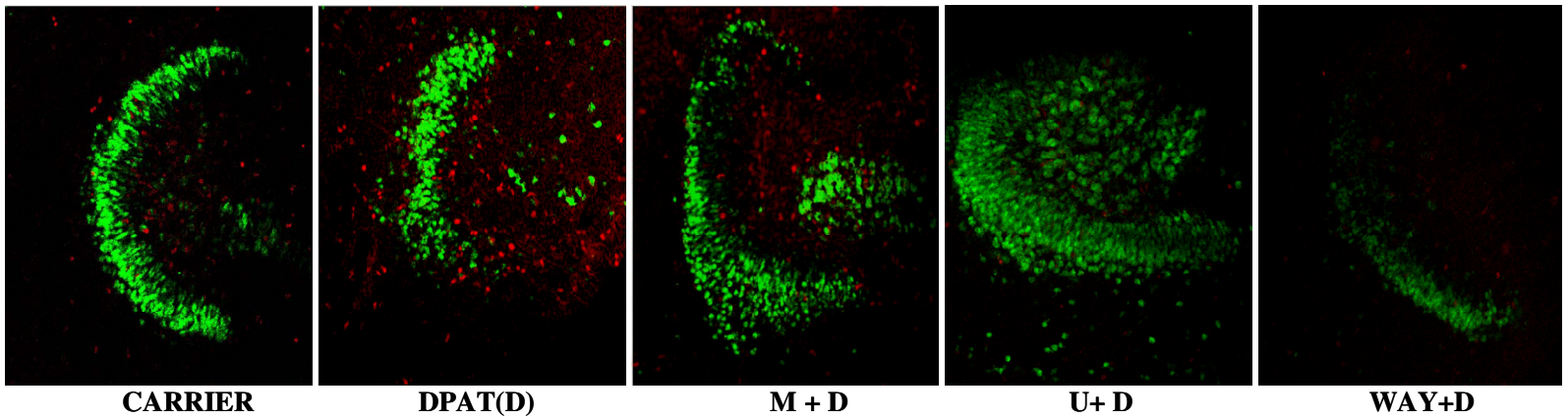
Fig. 13. The stimulation of the 5-HT$_{1A}$-R causes increased BrdU labeling in the P6 dentate gyrus, which is inhibited in the presence of U (MEK/ERK 1/2 inhibitor, 10 µM), M (PKCε inhibitor, 400 nM), WAY (5-HT$_{1A}$-R antagonist, 4 µM). (A) Immunostaining for BrdU (red) and NeuN (green) shows increased BrdU labeling in response to 8-OH-DPAT (D). **(B)** Statistical analysis of intensity measurement results using IMARIS software shows the mean of three independent experiments each performed with triplicate samples.

Neurogenesis itself is ambiguous and poorly defined. Moreover, the validity of BrdU incorporation, as a proliferation marker is questionable as it is suggested by others to also occur during DNA repair. In order to overcome this controversy, we monitored how the neuroblast number changed during this time frame. Neuroblasts are believed to be the third lineage of cell type from the original progenitor cell and are suggested to undergo an additional cell division before exiting the cell cycle and expressing proteins distinct to the mature neurons of the dentate gyrus. During neurogenesis it has been suggested that the most distinct changes occur in these neuroblasts, which are also named as D3 cells (Kempermann 2004).

As mentioned earlier, the highest level of granule cell proliferation occurs around embryonic day six and our quantification of actively dividing D3 cells should reflect the rate of neurogenesis. To achieve this we used a marker for neuroblasts, Ki-67, which also is a marker for cells actively cycling through the cell cycle, i.e. all cells that have not entered quiescence are labeled with anti-Ki-67. Using the same methodology, we sought to determine if 5-HT_{1A}-R stimulation does affect the proportion of neuroblasts present in the organotypic slices.

Our results show that 5-HT_{1A}-R stimulation by 8-OH-DPAT boosts the number of neuroblasts and this effect is blocked by the 5-HT_{1A}-R antagonist WAY **(Fig 14 A)**. Furthermore, similar to what was observed with BrdU staining, this increase in Ki-67 positive cells is blocked in slices treated with either M (PKCε inhibitor) or U0126 (MEK inhibitor) **(Fig 14 B)**. The overall trend is distinctively similar to that observed with BrdU staining and thus confirmed that active cell proliferation in organotypic slice cultures was enhanced by 5-HT_{1A}-R stimulation through PKCε and ERK1/2.

A)



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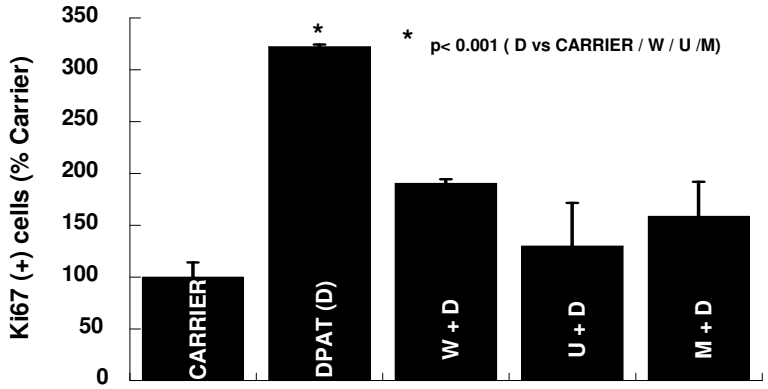


Fig. 14. Stimulation of the 5-HT$_{1A}$-R causes increased Ki-67 labeling in the P6 DG, which is inhibited in the presence of U (MEK inhibitor, 10 µM), M (PKCε inhibitor, 400 nM), WAY (5-HT$_{1A}$ inhibitor antagonist, 4 µM). (A) Immunostaining for Ki-67 (red) and NeuN (green) shows increased Ki-67 labeling in response to 8-OH-DPAT (D). **(B)** Statistical analysis of intensity measurement results using IMARIS software shows the mean (±S.D.) of three independent experiments each performed with triplicates samples.

In the pathway linking 5-HT_{1A}-R stimulation to increased neurogenesis in the P6 DG, PKCε possibly operates upstream ERK 1/2

In order to test if a similar mechanism as occurring in the HN2-5 cells was operative in the hippocampal slices, we looked specifically at the phosphorylation status of PKCε and ERK1/2, since both proteins are activated by phosphorylation. Our studies show consistently that at 10 min, post-DPAT treatment in organotypic cultures of hippocampal slices, PKCε phosphorylation/activation is seen most densely in the dentate gyrus region of the hippocampus (**Fig 15A**). At higher magnification we see the localization of PKCε mainly in the cytosol and plasma membrane confirming the suggestion that PKCε gets activated at the membranes. However, nuclear stained cells are also observed, which could possibly be proliferating neurons. More importantly this induction in expression is blocked in the presence of M, the PKCε inhibitor, and the 5-HT_{1A}-R antagonist WAY, confirming the importance of the 5- HT_{1A}-R pathway in PKCε activation (**Fig 15B**). Finally, we observed no inhibition in the activation/phosphorylation of PKCε in the presence of U0126 (MEK inhibitor), which suggests that similar to what was observed in the HN2-5 cells, PKCε participates upstream of the MAPK pathway at the early postnatal stage (**Fig 15B**). In a different note we wanted to determine if the some of the cells expressing P-PKCε were of neuronal lineage and if they were also undergoing cell division. In order to confirm this we used Doublecortin, a marker of immature neurons, along with Ki-67 and PKCε (**Fig 17**). Our data show that most proliferating cells (Ki-67 positive cells) are of neuronal morphology (Doublecortin positive) and also express P-PKCε. We also observed that P-PKCε was expressed in most

granular cells of the dentate gyrus in response to 5- HT_{1A}-R stimulation, though the significance of P-PKCε expression in post-mitotic neurons is yet to be investigated.

In order to further establish the hierarchy of PKCε and ERK1/2 in this signal transduction pathway, we looked for ERK1/2 phosphorylation /activation in response to 5-HT_{1A}-R stimulation and inhibitors of MAPK and PKCε. Our results confirm that ERK1/2 phosphorylation activation peaks at 10 min post-DPAT treatment. The staining is strongly cytoplasmic and is most dense in the DG. (**Fig 16**) This induced expression is blocked in the presence of WAY, confirming 5-HT_{1A}-R mediation. More importantly the staining was very weak in both U0126- and M-treated hippocampal slices. This corroborates the earlier studies and confirms that in this 5-HT_{1A}-R mediated signaling pathway, MAPK operates downstream of PKCε, and activation of PKCε phosphorylation/activation is necessary for ERK1/2 phosphorylation. This also fits in with the work done on proliferating HN2-5 cells, suggesting that a similar pathway operates both in the cell lines and organotypic slice cultures.

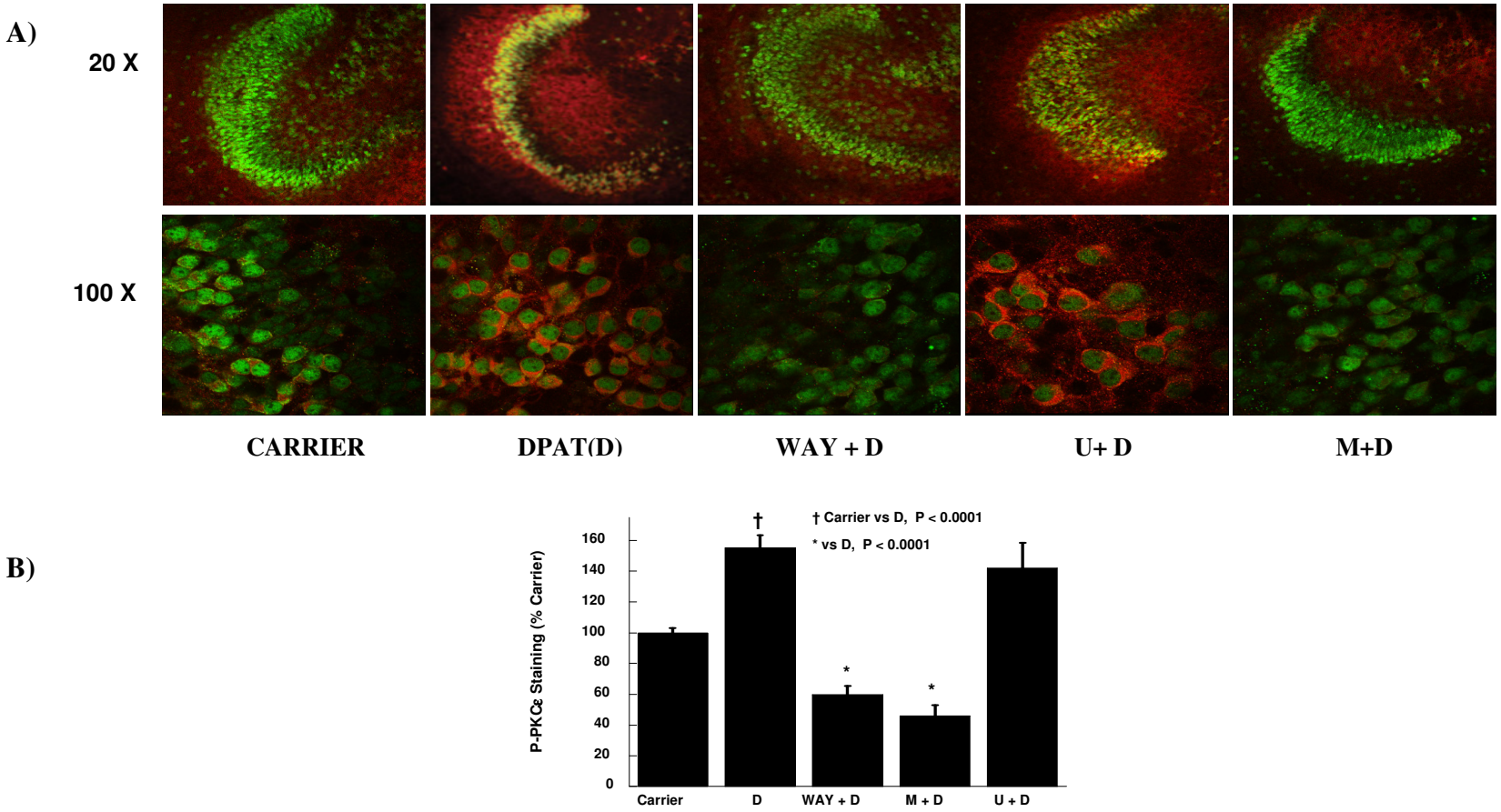
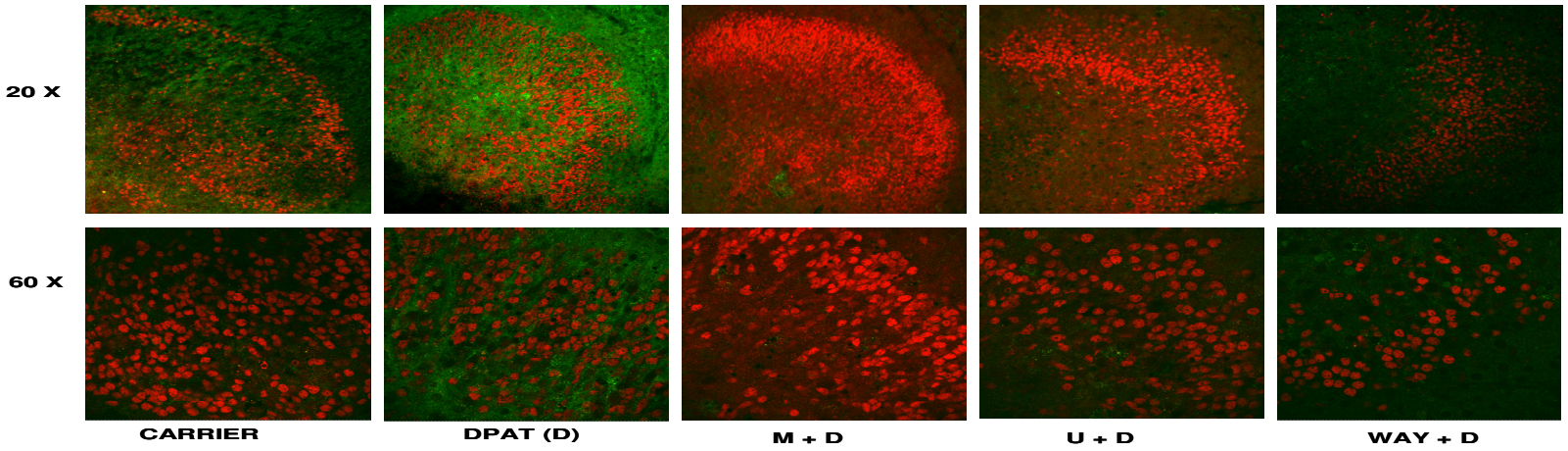


Fig. 15. The 5-HT$_{1A}$-R-mediated stimulation of PKCε at P6 occurs in an ERK 1/2 -independent manner mainly in the dentate gyrus, which harbors proliferating neuroblasts. (A) Immunostaining for P-PKCε (red) shows activation of PKCε in the dentate gyrus. NeuN staining (green) shows the granular cell layer. Magnified images show cytosolic activation of PKCε. **(B)** Densitometry was performed using the Image J software (NIH) and statistical analysis (ANOVA) was conducted using data from three independent experiments, each performed with triplicate samples.

A)



B)

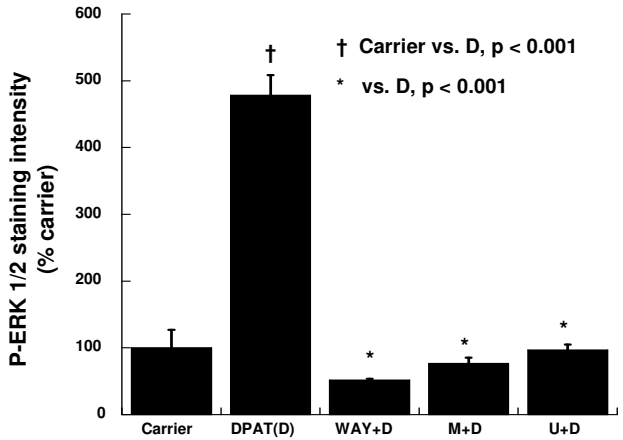


Fig. 16. The 5-HT$_{1A}$-R-mediated stimulation of ERK1/2 at P6 occurs in a PKCε-dependent manner mainly in the dentate gyrus (A) Immunostaining for P-ERK1/2 (green) shows activation of ERK1/2 in the dentate gyrus. PROX-1 staining (red) shows the granular cell layer. Magnified images show cytosolic activation of ERK1/2. **(B)** Densitometry was performed using the Image J software (NIH) and statistical analysis (ANOVA) was conducted using data from three independent experiments, each performed with triplicate samples.

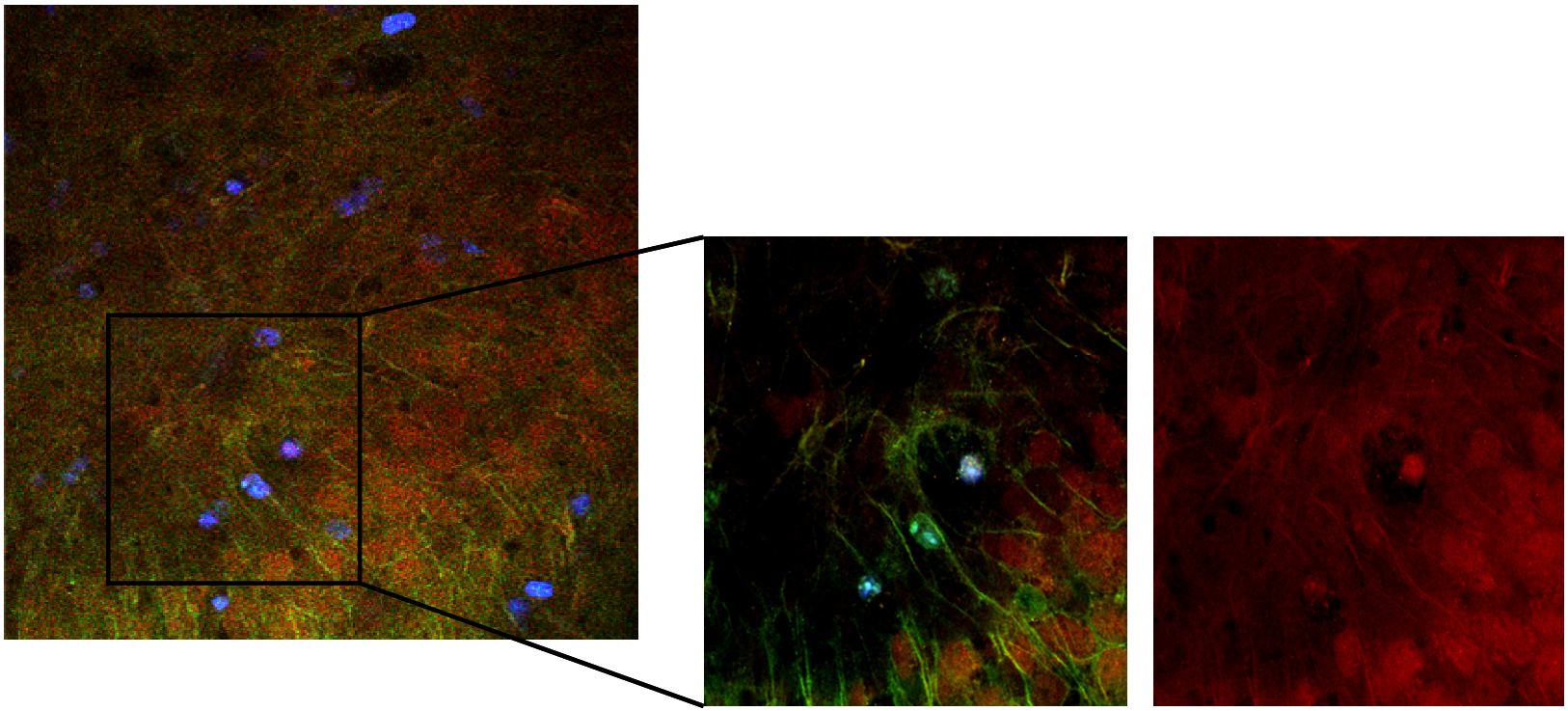


Fig. 17. Proliferating cells of neuronal origin also express P-PKCε- Slices treated with 8-OH-DPAT were stained for P-PKCε (red), Ki-67 (blue) and Doublecortin (green). Co-localization of all three signals are visible in certain proliferating cells in the sub granular zone, suggesting proliferating neuroblasts also express activated/phosphorylated PKCε.

CHAPTER 4

5-HT_{1A}-R Mediated Rb Activation in the P6 Dentate Gyrus

4.1 Introduction

There is evidence that neural progenitor cells might regulate cell cycle progression and terminal differentiation through classical cell cycle machinery (e.g. CDK 5). Yet the exact regulators of neurogenesis with respect to cell cycle dynamics is currently unknown as we are yet to clearly identify any molecular mechanisms involved in neurogenesis. Our earlier data is in line with other studies suggesting that cell cycle machinery is tightly regulated in proliferating neuronal cells as evident through immunohistochemical labeling with both BrdU and Ki-67. Both these markers of cell proliferation show that the dividing cells within the dentate gyrus appear in clusters, suggesting the likelihood that these cells cycling at a faster rate through the cell cycle in order to induce neurogenesis. Similar morphology has been demonstrated in most studies done on neurogenesis in both neurogenic niches in the adult brain.

Based on these observations we asked the question if ERK1/2 could indeed regulate a cell cycle protein that will enable the cells to proliferate at a higher rate. The retinoblastoma protein (Rb) is a classical orchestrator of cell cycle machinery. Extensive work done mostly on cell lines in the last two decades has demonstrated and identified Rb as a major regulator of cell cycle progression with most cell cycle proteins converging on either activation/deactivation of Rb through phosphorylation. The dynamic regulation of Rb and its upstream signals has been thoroughly sought after and remains imperative

particularly in the field of cancer research. Further, although the immunoreactivity of the retinoblastoma protein in progenitor cells of the dentate gyrus has been shown, suggesting its importance in neurogenesis (Okano 1993), surprisingly no studies have revealed the role of Rb in neurogenesis, more importantly, work done on intestinal cells have suggested the possibility of direct regulation of Rb by ERK1/2.

Using this information we sought to investigate the possibility of Rb regulation downstream of ERK1/2. We first carried out bioinformatic analysis in order to confirm the presence of ERK1/2 phosphorylation sites on Rb. From the two phosphorylation sites the phosphorylation at ser 780 was particularly suggested to be important in releasing E2F-the transcription factor associated with Rb. Using crystal structures from the protein data bank we docked the two proteins together and showed a favorable interaction between them. In order to confirm the significance of this possible interaction in the dentate gyrus of the hippocampus, we then used lysates prepared from the P6 dentate gyrus to immunoprecipitate with ERK2 and probed the immunoprecipitates for Rb phosphorylated at ser 780. We showed not only do Rb and ERK2 interact in hippocampal slice cultures, but also the phosphorylation at ser 780 in Rb seems to increase in response to 5-HT_{1A}-R stimulation. In order to investigate this further using immunohistochemistry we labeled cells for P-Rb-S^{780}, Ki-67, and NeuN in hippocampal slices, rebuild the images using IMARIS software and measured the cells positive for each channel. Our results show that the number of P-Rb-S^{780}-positive cells increases in response to 5-HT_{1A}-R stimulation in line with the co-immunoprecipitation data. Further we examined using immunoblotting analysis if this increased phosphorylation of Rb at ser 780 was dependent on PKCε and ERK 1/2 since earlier we had demonstrated the involvement of

these proteins in a pathway activated in response to 5-HT_{1A}-R stimulation resulting in enhanced neurogenesis. Our data suggests that in the presence of PKCε and ERK 1/2 inhibitors, phosphorylation of Rb at ser 780 in response to 5-HT_{1A}-R stimulation is suppressed, suggesting that Rb regulation occurs through a pathway that involves PKCε and ERK 1/2 downstream of 5-HT_{1A}-R stimulation.

4.2 Materials & Methods

Bio-informatics-based docking

Retinoblastoma protein crystal structure from the Protein Data Bank (PDB entry 1gux) was obtained and residues of chain E were removed to visualize pocket domains A and B. Similarly phosphorylated ERK 2 crystal structure was obtained from the Protein Data Bank (PDB entry 2ERK). The two molecules were docked by using two different docking servers Grammx docking server (http://vakser.bioinformatics.ku.edu/resources/ gramm/ grammx) and the ClusPro docking server with the ZDOCK algorithm and the present default parameters. We followed the recommended default procedure in which 10 models were retained after initial docking and ranked based on desolvation energies and electrostatics. We ran the docking search twice with the same input structures, in the two different servers and compared the best model from each which was essentially identical. Grasp 2 from http://trantor.bioc.columbia.edu and Pymol from www.pymol.org were used to visualize the crystal structures.

SDS PAGE and Immunoblotting in slices

The dentate gyrus of each hippocampal slice was removed using a tissue punch and a dissection microscope. The isolated dentate gyrus was then lysed in 1 mL RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4 plus freshly added protease inhibitor cocktail; (Roche Diagnostics GmbH, Mannheim, Germany), the lysate (10 μg protein) was resolved on a 4–10% gradient

acrylamide gel, protein bands transferred to nitrocellulose membrane, blocked in 5% solution of dry milk dissolved in 0.1% tween in TBS (20 mM Tris–HCl, pH 7.4, 0.8% NaCl) (t-TBS). The membranes were then probed with a P-Rb-S^{780} antibody (1: 1000) (Santacruz Biotechnology, CA), followed by treatment with HRP-linked goat anti-rabbit IgG (1: 2000). The immunoreactive bands were visualized using the Supersignal luminol kit (Pierce) and incubation with an X-ray film. The β-actin bands were used to normalize for total protein content.

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Co-immunoprecipitation

Lysates of the dentate gyrus, each containing 200 μg of protein, were diluted to a final volume of 1 mL in RIPA buffer. Two micrograms of mouse anti-ERK was added and allowed to incubate in the reaction mixture for 48 h with constant rotation at 4 ºC. Protein A-agarose beads were washed three times in and reconstituted in ice-cold PBS; 50 μL of the reconstituted Protein A-agarose beads was added and allowed to incubate

for 24 h to harvest the immunoprecipitate. Following incubation, the agarose pellet was washed in ice-cold RIPA buffer and boiled for 5 min in Laemmli buffer. The immunoprecipitate was resolved overnight on 7-16% gradient SDS-PAGE gels. Protein bands were transferred to nitrocellulose membranes, blocked in blocking solution for at least 1 h, probed overnight with P-RB-S^{780} antibody (1:1000) (Santacruz biotechnology, CA), which was detected with a HRP-conjugated anti-rabbit IgG (1:2000) (Santacruz biotechnology, CA). All IP bands were visualized and densitometrically quantified using NIH image software.

4.3 Results & Discussion

Using Bioinformatics to study possible interactions between ERK2 and RB

In order to approach the possibility of ERK1/2-mediated phosphorylation of Rb, we first investigated the presence of putative ERK phosphorylation sites on Rb using the program Clustaw2 and found two distinct ERK1/2 phosphorylation sites on Rb **(Fig 18).** The two putative sequences PXS/TP and PXXS/TP both occur with 100% complementarity on the primary structure of Rb (Wu 2009, Lange 2004, Caunt 2006). Two of these sequences map to ser 780 and ser 788 on human Rb, which in turn corresponds to ser 773 and ser 781 on the mouse homolog. This was particularly important, as ser 780 has been identified earlier as a possible phosphorylation site involved in the detachment of E2F. As ERK is a well-characterized protein believed to have numerous substrates, its docking and recognition sequences in its hydrophobic core have been well characterized. We then looked for specific docking sequences, which are of 2 kinds the D domain with the sequence, K/R_{2-3}-X_{1-6}-L/I-X-L/I and the DEF domain with the sequence F/Y-X-F/Y-P (Caunt 2006). Further, examination revealed that there was a docking site and a possible but not 100% identical DEF-domain usually present in most ERK1/2 substrates **(Fig 18)**.

Fig. 18. Primary structure of Human Rb harboring three potential ERK1/2 phosphorylation sites and one ERK1/2 docking site. The putative ERK phosphorylation sites are PXT/SP (Caunt 2006) and PXXSP (green) (Wu 2009, Lange 2004), and the ERK docking site is KKVYRLAYLRL (red). Ser^{780} corresponding to Ser^{773} in mouse is marked in the Figure.

A prerequisite for Erk-mediated phosphorylation of Rb is the presence of physical interactions between these two proteins, and we explored the possibility of such interactions using bio-informatics. We obtained the crystal structures of` human ERK2 and Rb from the protein data bank (PDB) and analyzed them. The E7 oncogene of the Rb protein used for crystallization was removed in order to obtain the structure of the A/B pocket domains. Finally, these proteins were docked together using the Cluspro and GRAMMX docking server and the most stable structures were analyzed using Pymol and GRASP-2 software. The default parameters of the Cluspro docking server are set as such to produce viable computational structures only under accepted salvation energies. As a result all of output models are theoretically stable and arranged in the order of decreasing stability. The picture presented is the most stable structure based on salvation energies. Our results indicate that the two proteins bear complimentarity and dock together forming a stable structure based on their solvation energies **(Fig 19).** Though theoretically the Ser^{780} residue was expected to be in the interface of interaction between the molecules, it appeared that this serine residue was at a different site which might be attributed to dynamic conformational changes that result in phosphorylation at Ser^{780} or even possibly the necessary interaction of other proteins in the ERK2/Rb complex.

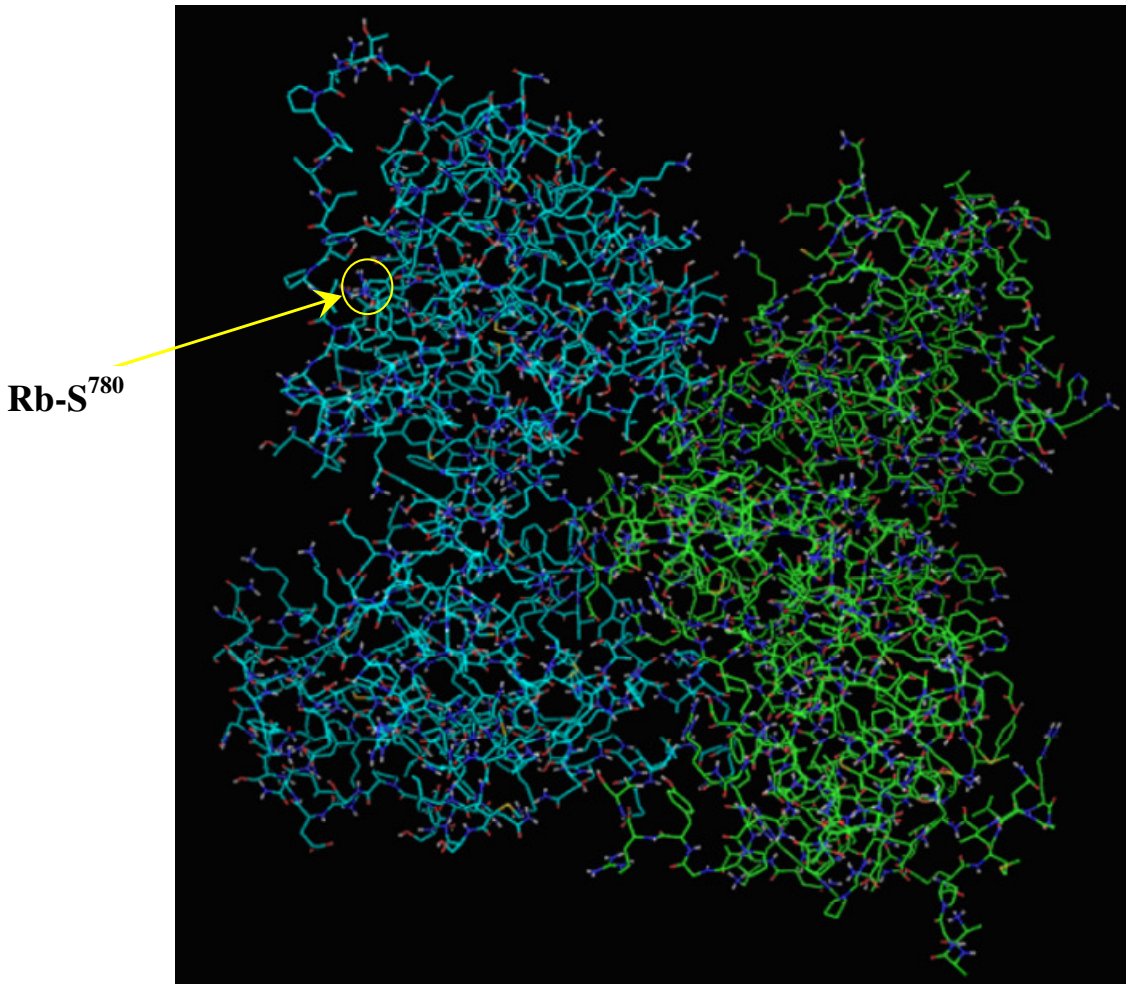
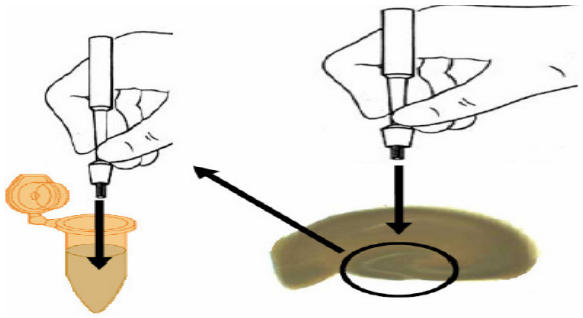
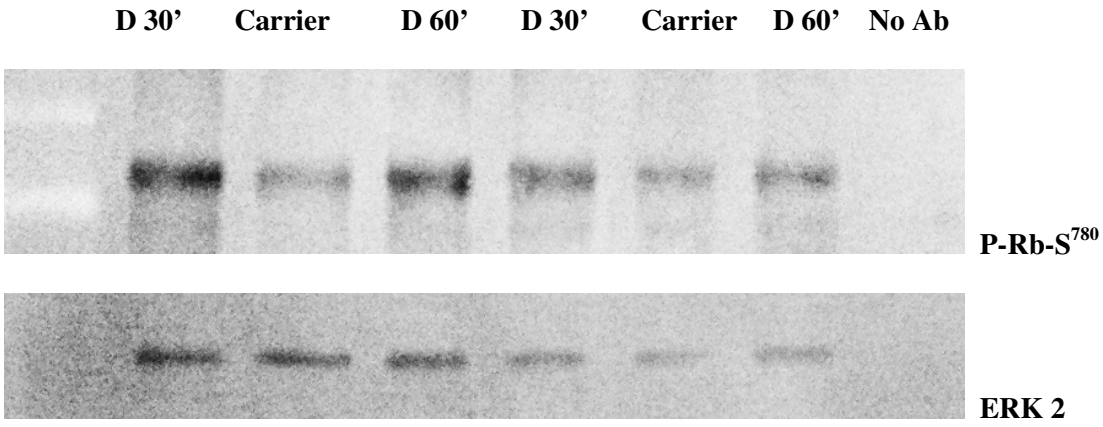


Fig. 19. The cell cycle inhibitor Rb docks stably with ERK 2. The crystal structures of the two proteins were obtained from the PDB. Docking was performed using the Cluspro and Gramxx docking servers. The structure presented is the most stable structure based on solvation energies and visualization was achieved using Pymol and GRASP-2 software. Rb is shown in Cyan on the left and ERK2 is shown in Green in the right. Ser^{780} residue is labeled in the figure.

In the P6 DG, Rb undergoes 5-HT_{1A}-R-mediated phosphorylation in association with ERK2.

To demonstrate the actual interaction of ERK1/2 and P-Rb we performed immunoprecipitation on lysates of the dentate gyrus excised from hippocampal slice cultures. We initially immunoprecipitated using the ERK2 antibody and probed for P-Rb-S^{780} in the immunoprecipitates. Our results show that ERK2 and P-Rb-S^{780} coimmunoprecipitate, which is indicative of strong intermolecular interactions in the dentate granule cells. We then looked to determine if the expression of P-Rb-S^{780} co-immunoprecipitated changed significantly in presence of 8-OH-DPAT. We found that at 30 min and 60 min post 8-OH-DPAT treatment there was an increase in the intensity of the P-Rb-S^{780} band when normalized to ERK2 levels, suggesting that 5-HT_{1A}-R stimulation causes an increase in ERK2/P-Rb-S^{780} interaction **(Fig. 20)**. This validates the bio-informatics data and indicates that phosphorylation of Rb at Ser^{780} may occur through ERK2.





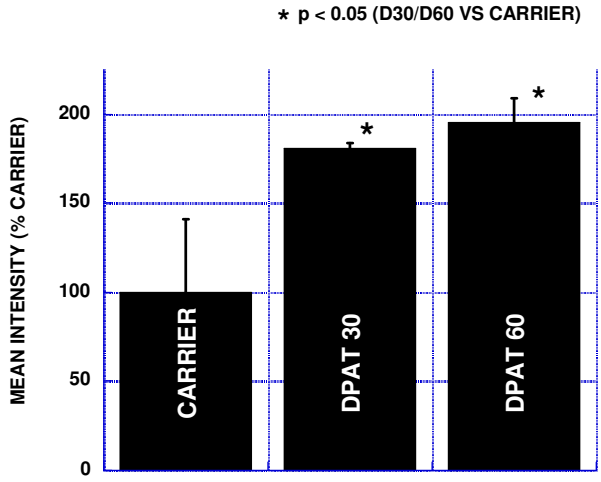
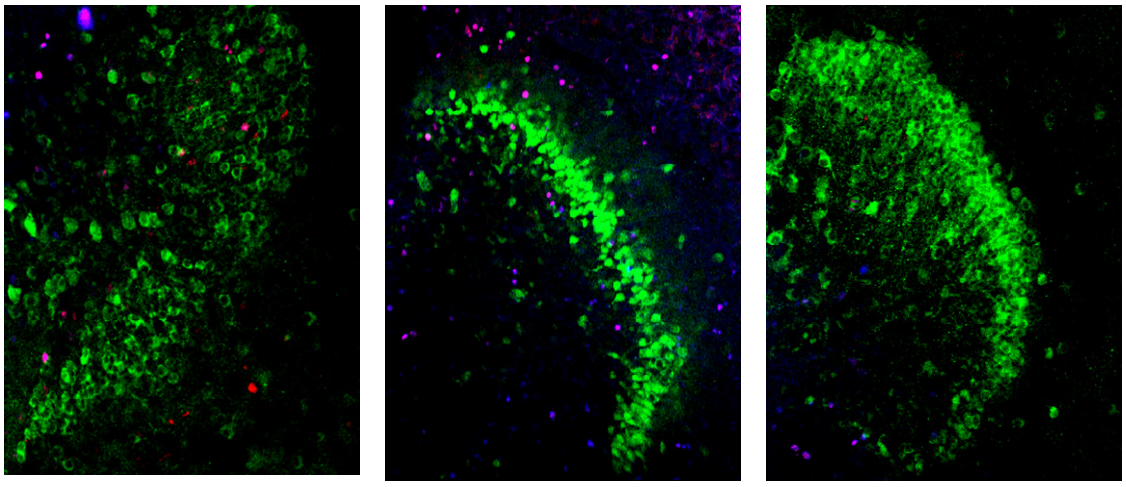


Fig. 20. In the P6 dentate gyrus, Rb undergoes 5-HT$_{1A}$-R-mediated phosphorylation in tight association with ERK. Upon 30- and 60-min treatment of organotypic slices with 8-OH-DPAT the dentate gyrus was excised using a tissue punch. Lysates prepared from the dentate gyrus was first immunoprecipitated with ERK2 antibody. The immunoprecipitates were resolved on a 4-16% gradient gel and probed for P-Rb-S^{780}. ERK 2 was used for normalization and densitometry was performed using NIH image. Statistical analysis was performed using ANOVA. Results presented are the mean (±S.D.) of two independent experiments.

In the P6 DG, Rb undergoes 5-HT_{1A}-R-mediated phosphorylation through PKCε and ERK 1/2.

Equipped with the molecular models and the possible interaction of ERK2 / Rb in the dentate gyrus,. we next investigated further the possibility that the 5-HT_{1A}-R➔ PKCε➔Erk1/2 pathway causes phosphorylation of Rb at Ser^{780}. As mentioned earlier Rb is a dynamic molecule, regulated by up to 16 distinct phosphorylation sites. Among these sites Ser^{780} in human that correspond to Ser^{773} in mouse has been suggested to be possibly important in detachment of E2F. We first looked to see how phosphorylation of Rb at $Ser^{780/773}$ phosphorylation status varied in hippocampal slices in response to 5-HT_{1A}-R stimulation. Our earlier data suggested ERK 1/2 phosphorylation could trigger hyperphosphorylation of Rb particularly at Ser^{780}. This is in line with earlier studies that have shown that hyperphosphorylated Rb detaches from its inhibitory interactions with the transcription factors E2F1, 2, 3, which then bind to promoters to cause induced expression of cell cycle genes, such as Cyclins D and E. Using immunohistochemostry and the organotypic slice culture model we first sought to confirm the increase in phosphorylation of Rb at Ser^{780} in response to 5-HT_{1A}-R stimulation. Comparison of the proportion of P-Rb-S^{780} labeled cells in the dentate gyrus between carrier, 8-OH-DPAT, and WAY-treated slices showed us an increase in phosphorylation of Rb at Ser^{780} upon 5-HT_{1A}-R simulation with 8-OH-DPAT, which was blocked in the presence of WAY **(Fig. 21).**



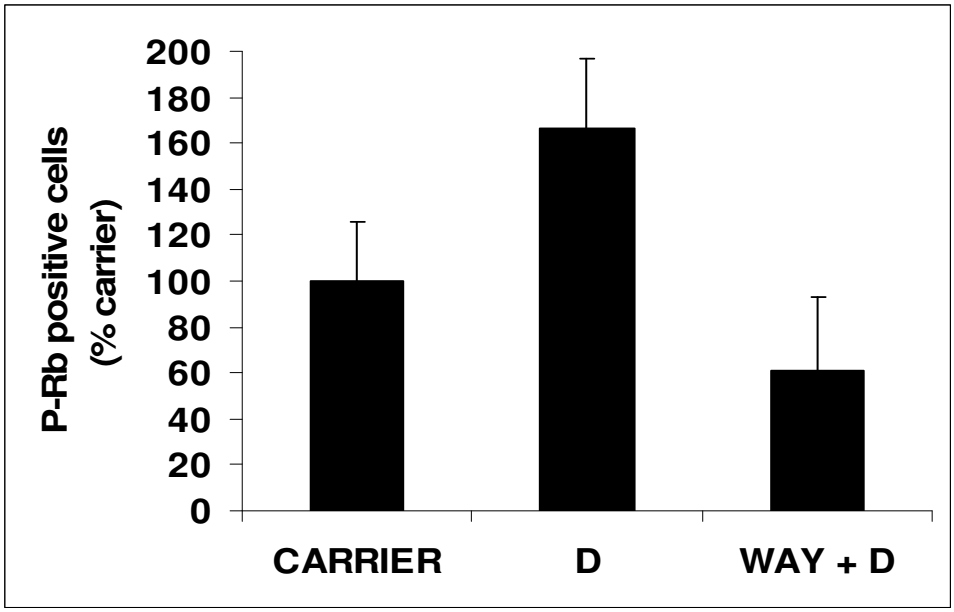


Fig. 21. Stimulation of the 5-HT$_{1A}$-R in P6 dentate gyrus causes phosphorylation of Rb at Ser^{780}. The 8-OH-DPAT (D)-evoked increase in P-Rb-S^{780} antibody labeling (blue) is blocked in the presence of the 5-HT$_{1A}$-R antagonist WAY (4 µM). (**B**) Statistical analysis of the intensity measurement results using IMARIS software shows the mean of three independent experiments, each performed with triplicate samples. The dentate gyrus cell layer was labeled with a NeuN antibody (green), proliferating cells are labeled with Ki-67 (red) and pictures were shot at 20x using a Zeiss confocal microscope.

On a different note, we observed colocalization of P-Rb-S^{780} with Ki-67 labeled cells in all slices examined suggesting that actively proliferating cells of the dentate gyrus expressed P-Rb-S^{780} **(Fig. 22)**. This could serve as a potentially interesting observation as this fits in with the presumption that Rb phosphorylation at Ser^{780} is needed in order to transition cells from G1 to S phase and enhance proliferation.

As our earlier studies indicated the involvement of PKCε and ERK1/2 in a pathway leading from 5-HT_{1A}-R simulation to enhanced neurogenesis, we sought to determine if a similar pathway may also regulate 5-HT_{1A}-R mediated Rb phosphorylation at Ser^{780}. Using SDSPAGE combined with immunoblotting analysis on cell lysates from the postnatal day 6 dentate gyrus we looked for the expression of P-Rb-S^{780} in response to 5-HT_{1A}-R simulation and the differential expression in the presence of ERK1/2 inhibitor U0126 and PKCε inhibitor M. Our results demonstrate as observed using immunohistochemistry that 5-HT_{1A}-R simulation using 8-OH-DPAT induces Rb phosphorylation at Ser^{780} and this effect is blocked in the presence of the WAY **(Fig. 23 B)**. More importantly we observe a decrease in Rb phosphorylation at Ser^{780} in the presence of ERK1/2 inhibitor U0126 and PKCε inhibitor M, suggesting the involvement of these proteins in the pathway that leads from 5-HT_{1A}-R simulation to Rb phosphorylation at Ser^{780}. In this set of studies we also used SB-269970 (SB), a 5-HT_7 receptor antagonist as a positive control in order establish if the observed effect can be attributed to the stimulation of the 5-HT_{1A}-R alone. Our data show that in the presence of SB there is no suppression of Rb phosphorylation at Ser^{780} as observed in the presence of WAY, suggesting that the phosphorylation of Rb at Ser^{780} occurs independent of the 5-HT_7 receptor.

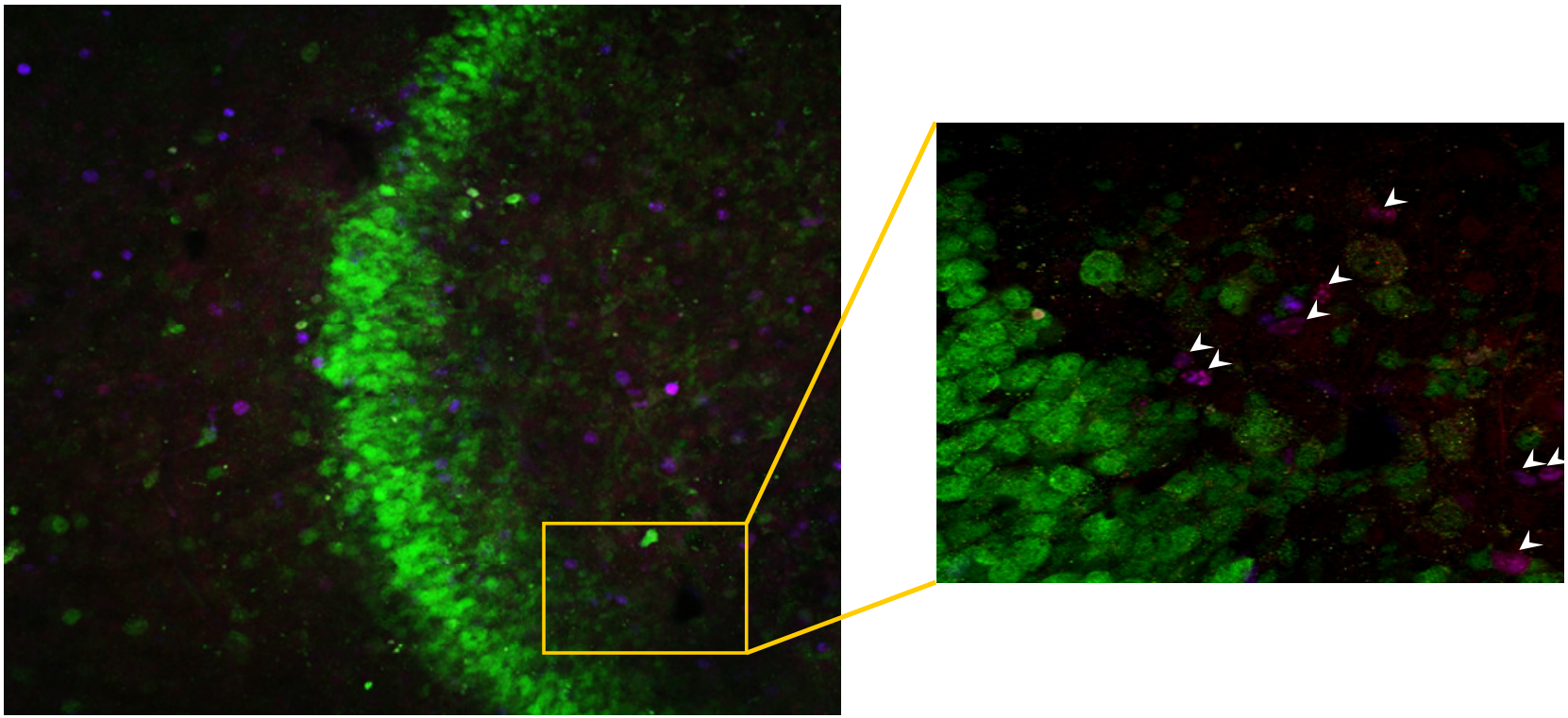
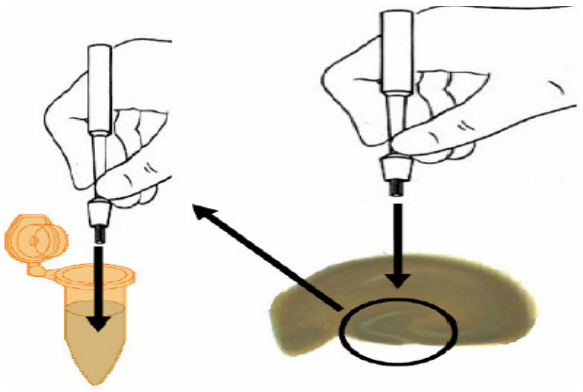


Fig. 22. Proliferating cells in the P6 dentate gyrus express nuclear P-Rb-S^{780}- Slices treated with 8-OH-DPAT were stained for Ki-67 (red), P-Rb-S^{780} (blue) and NeuN (green). Co-localization of Ki-67 and P-Rb-S^{780} are visible in certain proliferating cells (marked) in the sub granular zone suggesting proliferating neuroblasts also express Rb phosphorylated at Ser^{780}.

A)



p < 0.05 (D vs Carrier)

B)

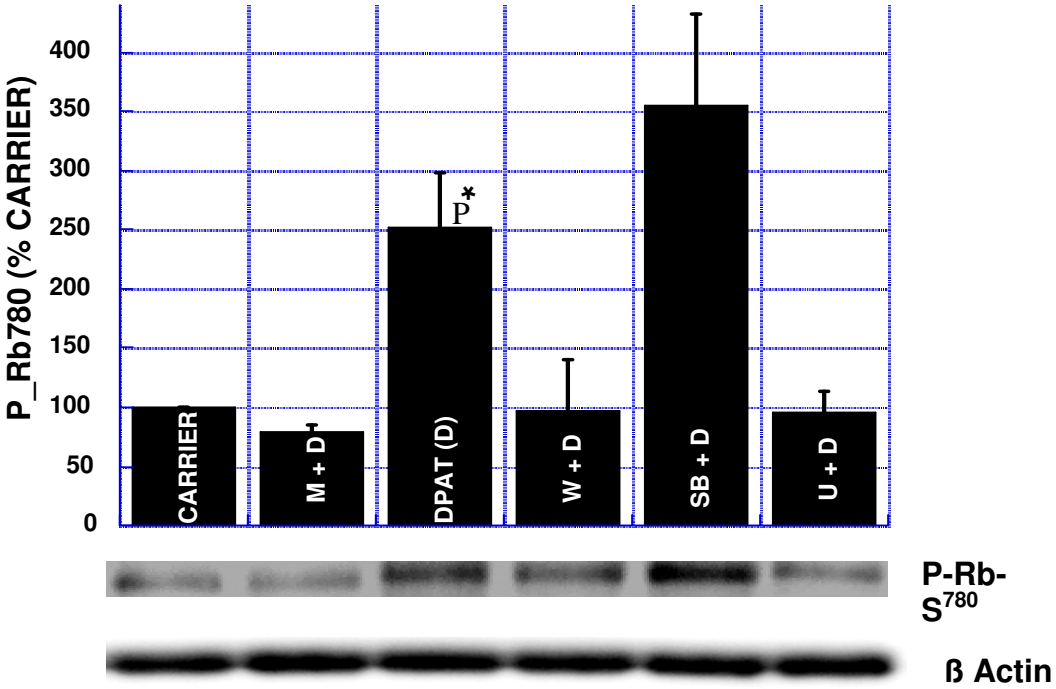


Fig. 23. In P6 dentate gyrus, Rb undergoes 5-HT$_{1A}$-R-mediated phosphorylation through a pathway involving PKCε and ERK 1/2. Organotypic slice culture were treated with WAY (4 μM, overnight), U (10 μM, 30 min), M (400 nM, 30 min) and SB (10μM, 30 min) followed by 100 nM of 8-OH-DPAT (D). A) Upon treatment the dentate gyrus was excised using a tissue punch. B) Lysates prepared from the dentate were run on a 4-16% gradient gel and probed for P-Rb-S^{780}. β actin was used for normalization of protein and densitometry was performed using NIH image. Statistical analysis was performed using ANOVA. Results presented are the mean (±S.D.) of three independent experiments.

CHAPTER 5

5.1 Summary of Findings

Prior studies from our group had shown that the agonist-stimulated 5-HT_{1A}-R results in the activation of the MAPK isozymes Erk1/2 *via* a PKC isozyme (Mehta 2007). With the intention of understanding the molecular players activated in this biochemical cascade, the first part of our studies was performed using the actively cycling, hippocampal neuron-derived HN2-5 cells. These experiments used pharmacological inhibitors and the results strongly indicated that this PKC isozyme was PKCε and that it caused stimulation of ERK1/2. We next asked if the activation of the MAPK pathway caused an increase in cell proliferation, since classically MAPK has been linked to proliferation of neurons. Using simple cell counting, tritiated thymidine assays, and finally by developing a HOECHST/ BrdU proliferation assay we showed that cell proliferation increases in response to 8-OH-DPAT (100 nM) treatment, and this effect was dependent on both PKCε and MAPK. The next goal of our study was to determine if the mechanism we observed in our cell lines also operated in the hippocampus during development. Using a slice culture model where manipulation of the media allowed us to control the activation and expression of particular proteins in the signal transduction pathway we were able to show that a similar mechanism to which was observed in HN2-5 cells did exist in the regulation of neurogenesis, specifically in the dentate gyrus of the P6 hippocampus. We were also able to show that in this signal transduction pathway, PKCε operated as observed in cell lines, upstream of MAPK, and were able to directly relay 5-HT_{1A}-R stimulation to enhanced neurogenesis in the dentate gyrus using both

BrdU, a proliferation specific marker, and Ki-67, a marker of proliferating neuroblasts. We observed that in both cases the labeled cells appeared as clusters, suggesting that the cells were possibly passing through the cell cycle at an increased rate. Using this information we next set out to determine the cell cycle proteins downstream of ERK1/2.

Earlier studies have demonstrated immunoreactivity of Rb in hippocampal neurons, and also suggested the cell cycle inhibitor Rb to be a possible substrate of ERK1/2. Since phosphorylated Rb is released from its inhibitory attachment to the cell cycle-promoting transcription factors E2F1/2/3, this Erk1/2 phosphorylation could be the mechanism by which a 5-HT_{1A}-R agonist regulates cell cycle. Using bioinformatics we identified ERK2 recognition and docking sites on Rb. With the help of modified crystal structures from the protein data bank we docked the two proteins together and showed that they did indeed interact to form a stable structure with minimum free energy. To understand if ERK1/2 and Rb actually interact in tissue of the dentate gyrus we performed immunoprecipitation and showed that Rb immunoprecipitates with ERK1/2, and also showed that with 8-OH-DPAT treatment there is an increase in the amount of Rb phosphorylated at Ser^{780} that coimmunoprecipitated, suggesting that ERK1/2 possibly phosphorylates Rb downstream of 5-HT_{1A}-R stimulation. Other studies have also suggested that among the 16 distinct phosphorylation sites of Rb, Ser^{780} (human) that corresponds to Ser^{773} in mouse is important in causing release of E2F. E2F can then cause up regulation of genes such as Cyclin E1, which are needed for transition from the G-phase to the S-phase of the cell cycle. In order to explore this phenomenon, we further examined the phosphorylation status of Rb in response to 8-OH-DPAT treatment. We showed that in response to 5-HT_{1A}-R stimulation a larger number of cells were labeled

with an antibody against Rb phosphorylated at Ser^{780} (P-Rb-S^{780}). SDSPAGE followed by immunoblotting analysis using lysates of the dentate gyrus further showed an increase in the Rb protein concentration phosphorylated at Ser^{780} in response to 5-HT_{1A}-R stimulation. More importantly we showed that this increased expression was mediated by the same signal transduction pathway involving PKCε and ERK1/2.

Overall these studies identify a novel pathway that occurs in proliferating neurons of the dentate gyrus essential for proper development (**Fig. 24)**. In the rest of the discussion I plan to address specific aspects of the pathway and comment on the current understanding and finally on possible future directions.

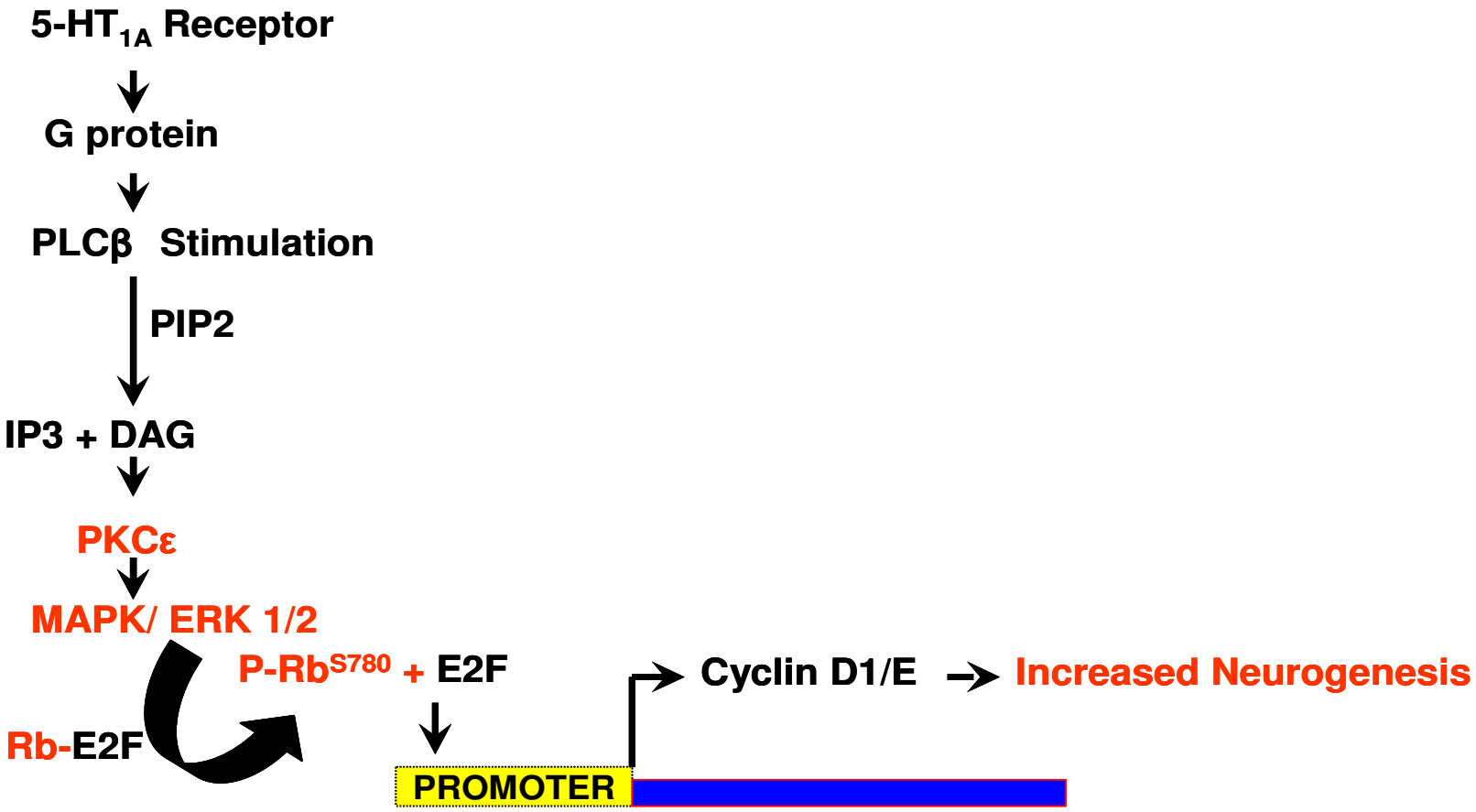


Fig. 24. Proposed pathway based on our studies- the molecules explored in the current study are marked in red.

5.2 Discussion

5-HT$_{1A}$-R

As mentioned earlier molecular cloning studies over the last few decades have identified 14 distinct serotonin receptors, each encoded by a different gene. Among these the 5-HT$_{1A}$-R is one of most well characterized receptor subtypes. The gene is intronless and the protein is expressed highly in the brain, spleen, and neonatal kidney. The 5-HT$_{1A}$ receptors are found both pre and postsynaptically in the brain and in both cases their activation leads to hyperpolarization in the neuron, resulting in slower firing. Functionally, there are two different types of 5-HT$_{1A}$ receptors: the autoreceptors, found presynatically in the cells of the dorsal and median raphé nuclei, inhibit cell firing by acting as somatodentritic autoreceptors. As these raphé neurons innervate large areas of the brain, a decrease in their firing results in lower levels of serotonin biosynthesis and release throughout the projections of the serotonergic system. The post-synaptic 5-HT$_{1A}$-R is expressed highly in the limbic system particularly in the CA1, CA3 and the dentate gyrus of the hippocampus (Barone 1994). The 5-HT$_{1A}$-R was first described as growth-stimulatory in glial cells expressing the 5-HT$_{1A}$-R endogenously. This was suggested to occur through the S100β protein known to promote growth of serotonergic neurons (Azmitia 1991). Initially three different transfected cell lines were used to study the link between the 5-HT$_{1A}$-R and proliferation. Abdel-Baset et al (1992) first transfected BALB/C3T3 fibroblasts with the 5-HT$_{1A}$-R and showed that there was an increase in incorporation of tritiated thymidine. Another group showed a similar but weaker effect in NIH3T3 cells. Lam et. al. in 1996 showed that expressing the 5-HT$_{1A}$-R in rat fibroblasts

also increased cell proliferation. *In vivo* studies done on adult animals have shown increased neurogenesis in both the SVZ and dentate gyrus- the neurogenic regions of the adult brain- in response to activation of the 5-HT_{1A} heteroreceptor (Gould 1999b). Further groups have shown similar effects in shrews, opossums and other mammals. Though the mitogenic response for serotonin seems to have been established, the signal transduction pathway responsible for this effect is not known. Additionally, most of the studies have focused on adult neurogenesis and though postnatal and adult neurogenesis might have some overlap, the two processes are distinctively different and therefore may involve different signal transduction pathways. Serotonin's role during brain maturation is less defined, although many groups have suggested its importance. The primary focus of our studies is to identify the role of the 5-HT_{1A}-R during early postnatal development. In this perspective, work done on 5-HT_{1A}-R knockout models have shown that these mice exhibit heightened anxiety and increased responsiveness to stress. They also display a range of behaviors that include tendency to avoid novel or fearful situations and prefer to escape from stressful situations (Heisler 1998, Parks 1998). Experiments performed using conditional 5-HT_{1A}-R knockout mice have further shown that the rescue of this anxiety phenotype is possible with the expression of the receptor in the hippocampus and cortex during development and not in the adult stage (Gross 2002). Moreover, the expression of the receptor in the raphé neurons during development does not rescue the anxiety phenotype visible in the knockouts (Gross 2002). Also studies performed using x-ray irradiation to ablate neurogenesis in the dentate gyrus of the hippocampus have shown that most of the visible behavioral effects of decreased 5-HT_{1A}-R expression can be attributed to a decrease in neurogenesis, though direct concrete evidence in humans is

still lacking. This suggests that early postnatal neurogenesis that may be regulated by the 5-HT_{1A}-R possibly has an important role in modulating adult behavior.

G- protein coupling and PLCβ

Once serotonin binding induces a conformational change in the serotonin receptor, which is transferred to intracellular loops 2 and 3 where heterotrimeric GTP-binding proteins are present. G-proteins are compromised of Gα subunit (and a dimeric Gβγ cluster) that occurs in four classes: Gαs, Gαq, Gαi and Gαo. In its inactive state the Gα subunit within the Gαßγ complex is bound to GDP. Upon activation by a ligand the receptor undergoes a conformational change in the G-protein that triggers the replacement of GDP with a GTP molecule. The GTP binding induces conformational changes within the switch regions of the Gα subunit causing its disassociation from Gβγ. The two subunits, Gα and Gßγ, can then act upon a variety of effectors within the cell. The hydrolysis of GTP to GDP by an intrinsic GTPase terminates the activation and brings the G-protein back to the original confirmation in which Gα is bound to Gβγ. These pathways have been discovered primarily by work done with pertusis toxin-, which prevents the disassociation of Gα and Gβγ. The 5-HT_{1A}-R is coupled to Gi/Go its final outcome is membrane hyperpolarization and reduced firing either by inhibiting adenylate cyclase or modestly stimulating PLC. In recognizing the mitogenic response to serotonin in neurons the pathway of importance is activation of PLC.

PLC's are Ca^{2+} dependent enzymes that catalyze the breakdown of phosphatidyl inositol 4,5 *bis* phosphate (PI (4,5) P2) to form inositol 1,4,5 *tris* phosphate (Ins (1,4,5) P3) and diacylglycerol (DAG). Ins (1,4,5) P3 binds to its receptors in the endoplasmic

reticulum triggering the opening of Ca^{2+} channels, hence increasing the Ca^{2+} concentration in the cytosol. Both Ca^{2+} and DAG are secondary messengers that can in turn activate PKC.

Currently there are six known families of PLC consisting of 13 different isoforms that vary in structure, activation and tissue-specific expression. The general structure of PLC's is composed of a N-terminal pleckstrin homology (PH) domain, followed by four EF hands, a catalytic domain and a C2 domain. Gαq is the major activator of PLCβ'S. PLCβ'S are composed of four distinct family members β1, β2, β3, β4, each varying in the degree to which they are activated in response to Gαq, the order from largest to smallest is β1, β4, β3 and β2 (Smrcka 1993). Furthermore, PLCβ2 and PLCβ3 is activated by Gβγ (Camps 1992).

Protein Kinase C (PKC)

PKC isozymes participate in diverse signaling pathways that critically regulate brain development and function. During rat brain development, PKC activity has been shown to increase with age (Hashimoto 1988). As mentioned earlier the expressions of various PKC isozymes are differentially controlled during rat development (Yoshida 1988). In rat brain, both PKCß and PKCα are found to increase progressively from 3 days before birth up to 2-3 weeks of age and remain constant thereafter (Huang 1989). However, the expression of PKCγ, which is found solely in the brain, is low initially.

PKCs also regulate MARCKS (myristoylated alanine-rich C kinase substrate), which is a key controller of embryonic cortical neurogenesis and despite negligible MARCKS phosphorylation, embryonic day 16 (E16) rat brain extracts contain both

MARCKS as well as PKCγ, δ, ε and λ. In the embryonic rat brain, while PKCγ and ε are present at very low levels, the α and ß isoforms are undetectable. Another neuronal differentiation regulator, GAP-43, is also activated by the PKCs. McNamara and Lenox have shown that the mRNAs for the various PKC substrates (MARCKS, MLP, GAP-43, RC3), which have different subcellular and regional localizations in the hippocampus at several time points (6 h, 12 h, 18 h, 24 h, 48 h, 5 days, or 15 days), exhibit unique expression profiles and regulation in the different cell fields of the mature hippocampus following kainic acid seizures and during subsequent synaptic reorganization (McNamara 2000). PKCδ has been shown to negatively regulate polysialyltransferase activity in the rat brain during development and also in the hippocampus during memory consolidation (Gallagher 2001).

Nicotinic cholinergic modulation of synaptic transmission in the hippocampus is also found to be mediated by PKCs (Braga 2004). Additionally, PKCs have been suggested to play important roles in various signaling pathways involving: growth factors, cytokines, and hormones (Redig 2007). It is evident that PKCs control numerous signaling cascades by the virtue of their ability to phosphorylate target proteins that may include other kinases which might play an essential role in early development of nervous system (Spitzer 2006). The extensive involvement of PKC-mediated activity makes it likely that it is required at all developmental stages as a necessary partner with transcriptional genetic controls. This also suggests that posttranslational modification of the kinases is extremely important for every stage of neuronal development, from initial proliferation and differentiation of progenitor cells, to migration and synapse formation.

Activation of the PKCs is critical for neuronal cell survival (Lin 1998), and is

essential for proper neural tube closing. Although all known PKC isoforms are detected in the closing neural tube, the use of chemical PKC inhibitors has identified a particular requirement for 'conventional' PKC isoforms. Intriguingly, the neurulation stage is critically dependent upon PKCß1 and γ, whereas other PKCs (α, ßII, δ, and ε) are thought to be dispensable (Cogram 2004). These studies reveal the importance of specific isoforms of PKCs during neuronal ontogenic development. Studies undertaken on mouse hippocampal tissue lysates have shown that with the exception of PKCε and PKCθ, the other PKC isozymes (α, ß, γ, δ) undergo a dramatic increase between P6 and P15. In contrast, both PKCε and PKCθ are already expressed at relatively high levels at P6 and undergo only a 1.5-2.0-fold increase between P6 and P15. This strongly suggests that the signaling activity of the isozymes α, ß, γ, δ is likely to be essential during the later stages of neonatal development, whereas the isozymes ε and θ are required at both early as well as later stages of hippocampal development (Purkayastha 2009). These results in mouse hippocampus are consistent with many of the earlier studies either in total brain or in cerebellum (Hashimoto 1988, Yoshida 1988, Huang 1989). Of the two novel PKC isozymes that are present at a relatively higher level even at P6 (PKCε and θ), PKCε is suggested to be the likely molecule that links the 5-HT_{1A}-R to the MAPK pathway and proliferation of preneuronal cells at P6 (Mehta 2007). Expression of both PKCε protein and mRNA has been shown by us and others to occur predominantly in the hippocampus, olfactory tubercle, and layer 1 and 2 of celebral cortex in the rodent brain (Purkayastha 2009, Koide 1992, Saito 1993). In a similar study performed in chick embryos it has been shown that PKCε is the predominant isoform and was expressed in all brain regions

of the embryo beginning at embryonic day 6 (E6). Furthermore, other studies have suggested that PKCε is an important mediator of cell proliferation (Chang 2002).

Wheeler and coworkers demonstrated that overexpression of PKCε in mouse epidermis results in a spontaneous myeloproliferative-like disease (Wheeler 2005). Overexpression of PKCε in NIH-3T3 fibroblasts elicited an increase in cell proliferation along with increased densities and confluence. *In vivo*, 100% of mice injected with these PKCε overexpressing cells developed tumors suggesting an important role for this isozyme in cell proliferation (Mischak 1993). PKCε's involvement specifically in cell cycle regulation has been suggested through work done in NSCLC cells where cells treated with a dominant negative form of PKCε or RNAi against PKCε resulted in a decrease in G1-S cell transition and overall decrease in both cell proliferation and anchorage-independent growth (Bae 2007).

In line with observations that suggest an important proliferative role for PKCε, PKCε is found to be expressed in the cell bodies of the neurons of the dentate gyrus layers where dividing cells are located and also in the interneurons of the hilus. Thus, as suggested by our studies, in order to play a crucial role in the proliferation of neuronal progenitor cells in the dentate gyrus, the abundant PKCε molecules may couple at the early stage of P6 to the 5-HT_{1A}-R (Mehta 2007). However, its expression in the CA3 and CA1 cell layers as well as in the interneurons of the *stratum radiatum* may serve some function, which has yet to be determined. Several studies have pointed to the link between PKCε and the MAPK pathway. Overexpression of PKCε in rat fibroblasts, rat colonic epithelial cells, and COS cells is known to cause an increase in Raf-1 kinase

activity (Cai 1997). PKCε co-immunoprecipitates with Raf-1, which suggests that these two proteins physically interact with one another (Cacace 1996). Furthermore, introduction of dominant negative Raf-1 reversed the oncogenic effect of PKCε in rat fibroblasts and colonic epithelial cells (Perletti 1996), and overexpression of PKCε reversed this growth inhibitory effect caused by dominant negative Ras in NIH3T3 and rat colonic epithelial cells (Perletti 1998, Cai 1997). Moreover, a direct link between ERK and PKCε has been suggested by showing that overexpression of PKCε enhances nerve growth factor induced phosphorylation of ERK in PC12 cells (Hundle 1995). Also, expression of dominant negative PKCε inhibited ERK activation by constitutively active Raf-1 (Kampfer 2001). These results suggest a direct link between PKCε and ERK activation. Further *in vivo* studies need to be conducted in order to show actual interaction between PKCε and MAPK signaling enzymes in tissue. Our work corroborates these observations and elucidates a novel role for PKCε in a 5-HT_{1A}-R mediated neurogenetic pathway where it is positioned upstream of ERK and most likely downstream of PLCβ.

MAPK pathway

MAPK's are evolutionarily conserved proline-targeted serine threonine kinases. In humans currently there are 11 members in the MAPK superfamily, which, based on sequence homology, can be divided into six sub-groups: the extra-cellular signal regulated kinases (ERK 1 and ERK2); c-Jun N-terminal kinases (JNK 1, JNK 2 and JNK3); P38'S (α, β, γ, δ); ERK5; ERK3'S (ERK3, p97 MAPK, ERK4) and ERK7'S (ERK 7 AND ERK8), (Schaeffer 1999, Pearson 2001). Each of the different MAPKs can

be stimulated by a distinct cascade that generally follows the sequential activation of a specific MAPK kinase kinase (MAPKKK) (which could be Raf-1), a MAPK kinase (MAPKK- also named as MEK) and finally a MAPK (Pearson 2001).

Each MAPK is composed of two domains an N-terminal domain formed primarily of beta sheets with two helices and a C-terminal domain that is mostly helices with four short beta-strands (Cheng 2001). The catalytic site is located between the two domains. The MAPK kinases bind to their substrates mainly through their DEF docking sequence FXFP and D-domain with the consensus sequence K/R (2-3)-X (1-6) L/I-X-L/I, these have been identified primarily by site directed mutagenesis studies (Caunt 2006).

The mammalian ERK MAPK pathway has been implicated in numerous responses to growth factors including cell proliferation, differentiation and survival (Pearson 2001). ERKs have been a hot research topic in the last few decades, primarily since their activation can lead to both normal and cancerous growth (Mansour 1994).

ERK1/2 were the first MAPKs to be described in mammals and are expressed ubiquitously in mammalian cells (Rossomando 1989). The two isoforms, ERK 1 (P44) and ERK 2 (P42), show 83% sequence identity. Many growth factors and signal transduction pathways are believed to involve ERK 2, which is the better-characterized member of the two. Growth factors binding to receptors induce conformational changes that activate and recruit a multitude of signaling proteins including Grb2. Grb2 then binds to SOS, which in turn activates Ras by allowing the exchange of GDP to GTP. Activated Ras sequentially activates Raf, MEK1/2 and finally ERK 1/2 by phosphorylation of the Thr and Tyr residues within its activation loop (Pearson 2001). This is one of the well-characterized activation pathways of ERK 1/2, though there are

indeed other activators. Mutations in genes coding for Ras resulting in constitutive activation of ERK has been reported in numerous human cancers (Hoshino 1999). ERK's upon activation phosphorylates numerous substrates with the consensus sequence P-X-S/T-P (Caunt 2006). ERK's are also known to directly activate and regulate gene expression through number of nuclear transcription factors. Among those transcription factors regulated by ERK1/2 are SAP1A, ELK1, SMAD1, SMAD2, 3, SMAD4, MAF-A, p53, c-Myb, SP1, c-Myc and c-Fos (Turjanski 2007).

The involvement of ERKs in cell proliferation was inferred by observing their phosphorylation in response to mitogenic stimuli (Nakamura 1983, Cooper 1984). Subsequent studies revealed a direct correlation between ERK 1/2 activation and DNA synthesis (Meloche 1992). A recent study reported the necessity of sustained ERK 2 activation until late G1 phase of the cell cycle for successful entry into the S-phase (Yamamoto 2006). Additionally, overexpression of catalytically inactive mutants of ERK 1 or antisense RNA was shown to block activation of endogenous ERK 1/2, thereby causing suppression of fibroblast proliferation, which could be fully reversed by co-transfection with a sense ERK1 construct (Pages 1993). Also, in the same way, synthetic MEK1/2 inhibitors which prevent ERK 1/2 activation were shown to inhibit proliferation in different cell types, including fibroblasts, T lymphocytes, smooth muscle cells, hepatocytes and epithelial cell lines (Meloche 2007). Many important aspects arise from studies performed using knockout mice. ERK1 knockout mice develop normally and are fertile, although keratinocytes isolated from these mice show reduced proliferation in response to mitogens when compared to wild type cells (Pages 1999). ERK1/2 knockouts are embryonic lethal and die in the utero at around E7.5, possibly due to a defect in

tropoblast development (Yao 2003). Analysis of chimeric embryos suggests an important role for ERK2 in the development of the ectoplacental cone and extra-embryonic ectoderm since no proliferation of polar trophectoderm cells is observed in ERK2 homozygous mutants (Saba-El-Leil 2003). Together this suggests distinctive roles for ERK 1 and ERK 2 during development though proper molecular signaling studies will need to be done in order to find their exact functions.

The importance of MAPK in cell proliferation has linked it to the retinoblastoma protein (Rb), which is believed to regulate cell cycle progression. Work done on intestinal crypt cells have shown evidence of phosphorylation at key residues in the retinoblastoma protein by activated ERK 1/2. To the best of our knowledge we are the first to report of this possible interaction in hippocampal tissue.

Retinoblastoma Protein (Rb)

Majority of cancers occur either by: mutation or deletion of the *Rb* gene, or by alterations in the Rb regulators such as Cyclin-dependent kinases (CDKs), Cyclins, and CDK inhibitors. This suggests a very important role for Rb in cell proliferation (Sherr 1996, Weinberg 1995). Rb is also inactivated by DNA tumor viruses such as the human papillomavirus (HPV), adenovirus and the simian virus 40 (SV40), all of which express proteins that induce S phase entry by binding to Rb and releasing activator E2F's (Nevins 1994, Rubin 2005).

Rb's importance in development was first shown when Rb knockout embryos were shown to die between embryonic day 13 and 15 (Lee 1992). The defects were suggested to be clearly visible particularly during differentiation and growth of neurons

and erythrocytes (Iavarone 1994). Also it was shown that abnormal mitosis and apoptosis occurred in the intermediate zones of the developing neural tube along with a decrease in the expression of a number of neuronal markers including TrkA, Trk B, and p75 during this stage of development (Lee 1994). Later, using a neuron specific promoter driving the LacZ gene, it was shown that numerous abnormalities of the developing nervous system occurred when Rb expression was blocked and these effects were also observed in the olfactory epithelium and the retina (Slack 1998). Additionally, a conditional knockout model specific for the telencephalon suggested a crucial role for Rb in neuronal migration (Ferguson 2002).

The cell cycle as we know is tightly regulated. Among the different stages associated with the cell cycle of particular importance is the first part of the intermediate phase referred to as G1 phase. This phase can be further divided into two stages of which the first is the growth factor dependent stage. During this stage the cell makes a decision to divide primarily due to an external stimulus such as certain growth factors. This stage begins at the end of mitosis and can last up to 3-4 h, ending approximately around the first restriction point. During this stage signal transduction pathways converge on activating Cyclin D1, which pushes the cell on to the second part of the G1 phase also referred to as growth factor independent phase. Rb is suggested to become hyperphosphorylated at this stage and, based on the availability of nutrition; the cell decides to continue with DNA synthesis. This stage starts just after the first restriction point and can last anywhere from 1-10 h ending with entry into the S phase. The protein primarily up regulated during this stage is Cyclin E1, which is a major target gene of E2F1.

Rb is suggested to become hyperphosphorylated at the late G1 phase causing its functional inactivation, thereby releasing E2F. It remains hyperphosphorylated and functionally inactive through S, G2, and M phases (Buchkovich 1989) before becoming hypo-phosphorylated and active at early G1. Rb is particularly hypophosphorylated or even unphosphorylated in cells that have exited the cell cycle and moved onto G0. It has been shown, that injecting purified Rb into early G1 cells stops progression of the cell into the S-phase, whereas injecting Rb during late G1 has no evident effect on cell cycle progression (Goodrich 1991), which reflects the point of possible regulation of Rb by mitogens during early G1.

Even though Rb is regulated in numerous processes including development, most of the data are from *in vitro* studies of cancerous cell lines where possibly Rb regulation is altered due to the morphology of the cells. The *in vivo* mechanisms of Rb regulation remain rudimentary because of the lack of appropriate signaling studies. In this perspective, our studies remain a novel approach in understanding the Rb-regulated signal transduction pathways.

E2F and its target genes

E2F was first described as a cellular activity required by adenovirus E1A proteins for transactivating the adenovirus e2 promoter (Kovesdi 1986). Subsequent studies showed that Rb interacted with E2F and suppressed its transcriptional activity suggesting a model for cell cycle control. E2F overexpression or Rb inactivation is sufficient to induce an entry into S-phase suggesting the importance of the Rb-E2F pathway in G1 to S transition (Weinberg 1995, Dyson 1998). Now we know that the RB/E2F complex

consists of three pocket proteins (Rb, P107, and p130) and eight E2F family members (E2F 1, 2, 3, 4, 5, 6, 7, 8), each of which can heterodimerize with two DNA binding partners (DP1 and DP2) to bind 12 different DNA-binding transcriptional regulators (Cam 2003). The E2F family can be divided into 2 classes: activator E2F's (E2F1, 2, 3) and repressor E2F's (E2F4, 5). E2F 6, is known to be an independent E2F and the recently identified E2F 7, 8 are believed to be Rb and homolog-independent repressors (Di Stefano 2003). Activator E2F's use Rb as their binding partner whereas the repressor E2F's use p107, and p130. The changes in functionalities of the two major classes are based on structural differences. Activator E2F's have a nuclear localization signal that is exposed upon disassociation from Rb, hence they move into the nucleus and activate transcription. In contrast, repressor E2F's (E2F4, 5) bind Rb pocket homologs p107 and p130 and the entire complex acts as a repressor of transcription, upon activation, their nuclear export signal is exposed which translocates the E2F's out of the nucleus.

Genes regulated by the two classes of E2F's are different. Also their expression pattern varies across the different stages of the cell cycle. E2F 1, 2, 3 become more abundant during late G1 and S phase whereas E2F 4, 5 are more prevalent in early G1 and particularly Go cells that have exited the cell cycle. Among the activator E2F's, E2F 1 mRNA is abundantly expressed in the brain *in vivo* (Helin 1992). *In situ* hybridization studies also revealed an increase in E2F 1 mRNA in the neuronal precursors in the neural tube (Dupont 1998). Based on these studies and others it is highly possible that E2F 1 is the activator E2F of interest in neurogenesis.

Large scale DNA oligonucleotide microarrays and chromatin immunoprecipitation assays using E2F specific antibodies have identified numerous E2F

target genes that include genes that regulate processes such as DNA damage/repair, apoptosis, differentiation and most importantly development (Bracken 2004).

Cyclin E

Cyclin E was first discovered by its ability to compensate the problems in proliferation associated with cyclin-deficient yeast cells (Koff 1991). It has been identified as an important component of the Rb pathway. In mammals, 20 cyclins and 10 cyclin-dependent kinases have been identified. The two most important cyclin/ CDK complexes at the G1/S transition are the cyclin D/CDK4/6 complex and the cyclin E/CDK2 complex. The activity of cyclin E/CDK2 is highest in cells transitioning through the G1/S interphase and lowest in quiescent cells in Go (Dulic 1992, Koff 1992). Other studies have shown that overexpression of cyclin E enables cells to have a much shorter G1 phase as compared to control cells (Resnitzky 1994). Still others have shown that inhibition of cyclin E/CDK2 activity prevented cells from entering the S-phase (Tsai 1993).

Though the classical model identifies cyclin E upstream of Rb, studies have shown that cyclin E is indeed a prominent E2F target gene and others and we have shown that the cyclin E promoter contains defined E2F binding sites (Ohtani 1995, Geng 1996). Conditions that are believed to inactivate Rb and release E2F increase cyclin E expression. The regulation of Cyclin E by E2F is evolutionarily conserved and studies performed in drosophila have shown that Cyclin E is required for E2F -induced progression of cells from G1 to S (Knoblich 1994, Duronio 1996). The exact role of cyclin E and E2F in the mammalian cell cycle is less clear and some studies have

suggested a pathway that elevates cyclin E levels irrespective of E2F activation (Lukas 1997).

5.3 Future directions

While working on the thesis I came across new sets of questions that might be relevant to this project. As shown below I have presented some questions followed with some logical experimental setup that can be used to answer some of these questions. I would like to stress that the appropriateness of these techniques and ideas have been by no means confirmed.

Questions

1) How do we explain the Rb independent cell proliferation observed in HN2-5 cell lines?

In identifying Rb as a potential target of ERK1/2 we first looked to see if this regulation did occur in our model HN2-5 cell line. Our data (not shown) demonstrated that there was no differential regulation of total Rb and Rb phosphorylatated at residue numbers 780,807, 811. This suggests two possibilities. First, as the HN2-5 cell line also has a cancer origin and Rb regulation is lost in a majority of cancers, cell proliferation might be occurring through a pathway independent of Rb. Secondly, the characterization of Rb and the significance of each phosphorylation site are far from complete and the possibility of phosphorylation regulation occurring through a site distinct from the ones we examined might also be possible. In order to resolve this dichotomy a thorough

evaluation of all 16 phosphorylated sites should be performed using antibodies specific for each site. In order to confirm Rb mediated regulation, one can also first establish if there is indeed a regulation of E2F and its target genes downstream of Rb using EMSA (electrophoretic mobility shift assay) or CHIP (Chromatin immunoprecipitation) assays.

2) Why do tritiated thymidine incorporation and BrdU incorporation peak at two different time points?

Thymidine incorporation into HN2-5 cells showed the most significant difference between control and 8-OH-DPAT treated samples at 64 h post DPAT. In cortrast, BrdU incorporation peaked at 24 h. While we are unable to offer a comprehensive answer for this observation, studies have suggested that tritiated thymidine could itself inhibit cell cycle progression. As a further note from counting cell number we realized that the actual log phase in culture may occur at different time points based on the available area for cells to grow. Based on this, we can possibly infer that the observed difference can be due to different plates (24 well and 96 well) used for the two different procedures. In order to thoroughly understand this further, a systematic study using different concentrations of tritiated thymidine and BrdU in different culture plates will have to be performed.

3) Why do we observe a consistent decrease in NeuN staining when the 5-HT_{1A}-R is inhibited?

Of particular interest was the fact that we observed slightly irregular patterns of NeuN staining in organotypic slices treated with the 5-HT_{1A}-R antagonist WAY. Though a 48-h treatment, in theory, should not significantly affect neuronal maturation, this

consistent observation suggested to us that it could be a possibility. In order to answer this question I would propose the following: organotypic slice cultures should be treated with WAY for differing time points and NeuN labeled while simultaneously looking through SDS-PAGE and immunoblotting for expression of the maturation marker NeuN, if there is indeed a difference in NeuN expression in response to WAY this will suggest that the 5-HT_{1A}-R is also possibly involved in terminal differentiation and neuronal maturation.

4) What percentage of these proliferating cells are neurons? Glia?

The lineage of the progenitor cells as they mature has not yet been established, though some reports claim that 80% of the cells undergoing proliferation in the subgranular zone of the dentate gyrus have neuronal morphology. To answer this question in our slice culture model we will have to use specific markers for each cell type. Some possibilities are as follows: in the hippocampus, the putative stem cell, also called the RA cell, is stained with GFAP (Glial fibrillary acidic protein). However, GFAP also identifies mature glial cells (Kempermann 2004). But by using the mature glial marker S-100β we will be able to distinguish between the mature glia, which will be positive for both GFAP and S-100β, and the putative stem cells that will be negative for S-100β. As for the neurons, nestin- a marker of cells early along their lineage – combined with doublecortin, a marker of immature neurons, will help us identify the proportion of maturing cells that might actually become neurons. This will also help us identify the population of progenitor cells responsible for the increase in neurogenesis we observe.

5) Does the neurogenesis observed in HN2-5 cell lines and ex-vivo organotypic slice cultures apply to what is observed in the live animal?

Though our studies are based on two model systems similar to the live animal, our observations are most relevant in the context of the live animal. In order to apply our studies in the actual context of the developing brain there are some experiments could be undertaken. First, intraperitonial injection of some drugs, such as 8-OH-DPAT, in the adult animal has been shown to enhance neurogenesis in the proliferative niches (Banasr 2004). However, some drugs used test the signaling pathway may not cross the blood-brain barrier. To overcome this problem and in order to use other inhibitors like U0126 and M, direct action is required since these drugs have the potential of being metabolized in the live animal. Thus, one could use direct intrahippocampal injections and eventual processing of tissue to study changes in proliferation. Standardization of such a method would not only validate our studies but also lay the groundwork for identifying other proteins potentially involved in similar signal transduction pathways.

6) Is proliferation possible in areas distant from the subgranular layer of the dentate gyrus?

One interesting observation we noted while performing our studies was the finding of proliferative neuronal cells in other areas of the dentate gyrus beyond the classical subgranular layer and the hilus region. While we understand this may be a direct result of our slice culture technique there is also the distinct possibility that this observed proliferation is indeed real and typical to the postnatal day-6 hippocampus. To test this possibility one could undertake direct administration of BrdU into postnatal day-6

hippocampus *in vivo* followed by immunohistochemical identification of cells that are triple stained for BrdU, Ki67, and double cortin.

7) How is colocalization of NeuN with BrdU possible just after 24 h?

One heavy criticism that has been leveled against our studies involves the observation of cells that are both BrdU and NeuN positive. Classically, neuronal cells are not expected to express NeuN till they have undergone terminal differentition, which presumably could not occur within 24 h. The problem with such a view is that the exact mechanism that occurs between neurogenesis and terminal differential remains unknown and there is a possibility of identifying neuronal cells down the lineage that might incorporate BrdU and start expressing NeuN within a 24-h time frame. Furthermore, since BrdU incorporation is suggested to occur during DNA repair, it has been suggested that the BrdU staining might be actually an artifact due to repair mechanisms. The latter of these questions we believe have been answered through Ki-67 (another proliferation marker) staining, which follows a similar profile to what is observed with BrdU, double cortin, that is expressed by neuroblasts. In order to answer these questions experimentally we can use a similar approach to that presented in question 3 & 4 but also additionally infect these cells with a green fluorescent protein (GFP)-expressing construct at different time points of growth and maturation and simultaneously follow the expression of NeuN and BrdU in order to determine if these cells can indeed start expressing NeuN within 24 h of their final mitotic division.

8) Can ERK1/2 directly phosphorylate Rb *in vitro*? If so, at what residues?

In order to bio-chemically establish ERK mediated Rb phosphorylation, one can obtain purified unphosphorylated Rb (phosphate groups removed by treating with phosphatases) and incubate it in the presence of an ERK-active site mutant that has been engineered so it can only bind an analog of a radiolabeled ATP (cyclo-pentyl ATP), which is is not recognized by the wild type Erk. By allowing interaction of the two proteins under appropriate conditions in the presence of the radiolabeled ATP analog one could confirm that Erk indeed phosphorylates Rb (Eblen 2003). Finally, Rb can be analyzed for the presence of specific phosphorylated residues by NMR. Another alternative method will be to synthesize peptides representing segments of interest on the Rb protein and look for ERK mutant mediated phosphorylation using autoradiography.

9) How do we confirm the involvement of Rb and E2F in this pathway?

In order to establish the functional importance of Rb/E2F pathway in neurogenesis, it is essential to ask if inhibition of this pathway has any effect on cell proliferation and neurogenesis. As far as we know, currently there are no inhibitors of Rb and some studies have focused on site-directed mutagenesis to form dominant negative mutants of Rb. The problem with this picture is that Rb as mentioned earlier has up to 16 putative phosphorylation sites essential for varying degrees in its physiological functions. The exact significance of each site still remains unclear though it has been suggested that the sites close to the c-terminal end of the AB pocket domain are specifically important for interactions with E2F. Using siRNA, antisense technology or even retroviral techniques to inhibit Rb it might be firstly important to look for effects in neurogenesis in

neurospheres and eventually organotypic slice cultures. Such a study would add to our claim for Rb's significance in neurogenesis. Another way to test the claim of RB/E2F interaction in hippocampal slices in response to 5-HT_{1A}-R stimulation is to analyze coimmunoprecipitates by first immunoprecipitating with an E2F antibody and then probing for Rb in these immunoprecipitates. According to the current hypothesis presented here, stimulation of the 5-HT_{1A}-R should cause activation of E2F, thus reducing the availability of E2F for interaction with Rb. If this were indeed the case, then a time course analysis of Rb immunoprecipitates obtained following 5-HT_{1A}-R stimulation should have decreasing amounts of E2F. In order to address E2F's involvement in this pathway it will be useful to perform Chromatin Immunoprecipitation studies (CHIP) in order to analyze transcriptional regulation of Cyclin E and Cyclin D genes by E2F. By showing that stimulation of the 5-HT_{1A}-R causes more E2F binding to the promoter of these target genes we would establish this pathway. Finally, it might be interesting to analyze the RNA levels for Cyclin E and Cyclin D in response to 5-HT_{1A}-R stimulation since the degradation and stability of the mRNA have been suggested to be the main regulatory step for Cyclin D/E expression.

10) Are pharmacological inhibitors sufficient to show the action of a particular protein with regard to cell proliferation?

The experimental setup presented here has been primarily based on the assumption that pharmacological inhibitors are relevant tools to stop the activation/phosphorylation of a particular protein. Though our work shows consistency in this approach in order to confirm the importance of any particular protein, one must confirm such results through

multiple inhibitors or in a better scenario, through a different experimental setup. If the question is regarding 5-HT_{1A}-R mediated neurogenesis, one could examine neurogenesis in organotypic slice cultures made from 5-HT_{1A}-R knockout animals. Another technique that could be used is actively preventing the expression of a particular protein through gene expression in the slice cultures. The problem with such an approach is that if our interest is activation through phosphorylation, then the presence of basal levels of proteins might enable effective activation through phosphorylation. To overcome this dominant negative constructs can be used which have site directed mutagenesis that prevents them from getting activated through phosphorylation at specific residues. Since transfection in organotypic slice culture models remains as an obstacle, the proper standardization of retrovirus mediated gene transfer will first have to be established before pursuing such a complex experiment.

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