SEROTONIN-1A RECEPTOR MEDIATED SIGNALING IN NEONATAL HIPPOCAMPAL DEVELOPMENT.

By

MUKTI MEHTA

A dissertation submitted to the Graduate faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

SEROTONIN-1A RECEPTOR MEDIATED SIGNALING IN NEONATAL HIPPOCAMPAL DEVELOPMENT.

By

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Adviser: Professor Probal Banerjee

The brain serotonin $_{1A}$ receptor (5-hydroxytryptamine-$_{1A}$-R) plays a crucial role in the modulation of emotional processes and is a target of medications used in the treatment of psychiatric disorders. Ablation of this receptor causes heightened anxiety in mice. Further studies have shown that the presence of post-synaptic hetero-5-HT_{1A}-R (mainly in the hippocampus, septum and cortex), and not the autoreceptors (in the raphé) is essential between postnatal day-5–21 (P5–21) for the expression of normal anxiety levels in adult mice. Additionally, our earlier studies in differentiated HN2-5 cells (derived from hippocampal neurons) have shown that a 5-HT_{1A}-R→ MAPK (Mitogen activated protein kinase) causes protection of these cells against apoptosis. Based on such observations and also considering that MAPK is linked to cell division we have postulated that the 5-HT_{1A}-R→ MAPK cascade plays an important role in regulating neurogenesis and strengthening of synapses in the hippocampus during early post-natal development. Using cultured hippocampal slices from mice as a model, we studied 5-HT_{1A} receptor- mediated specific responses during neonatal development. Our studies have revealed that at postnatal day-6 (P6) and day-15 (P15), 5-HT_{1A}-R agonist (8-OH-DPAT) treatment causes activation of the MAPK isozymes ERK1/2 (extracellular signal-regulated kinases 1 and 2). Intriguingly, at P6, a PKC isozyme (probably protein kinase

C-epsilon) was involved upstream of ERK1/2, whereas at P15, PKC-alpha was stimulated downstream of ERK1/2. Thus, the 5-HT_{1A}-R-mediated stimulation of ERK1/2 in the hippocampus undergoes a transition between P6 and P15. At P6, a PKC isozyme is required for the 5-HT_{1A}-R→ERK1/2 cascade, that upregulates cell division in the dentate gyrus. In contrast, at P15, PKCα participates downstream of ERK1/2 to mainly augment synaptic transmission through the Schaffer Collateral pathway. This temporal switch in the 5-HT_{1A}-R signaling uses PKC isozymes differentially, first boosting the cell division to form new hippocampal neurons to regulate neurogenesis at P6 and then undergoing a timely transitions in mechanism at P15 to strengthen synaptic connections that are probably essential for securing synaptic connections. Our overall objective has been to delineate transitions in 5-HT_{1A}-R mediated signaling cascades and their functional effects on neonatal brain development.

This thesis is dedicated to my parents especially my mother (Dr.Geeta Mehta) for her support and encouragement.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
5-HT1A-R	5-hydroxytryptamine 1A receptor
Akt	Protein kinase B, PKB
BSA	Bovine serum albumin
Ca	Calcium
CREB	cAMP response element binding protein
DAG	Diacylglcerol
DMEM	Dulbecco's modified Eagle's medium
EPSP	Excitatory postsynaptic potential
ERK 1/2	Extracellular signal regulated kinase 1/2, MAPK
FBS	Fetal bovine serum
GABA	Gamma (γ) aminobutyric acid
GFX	Bisindoylmaleimide I
GPCR	G protein coupled receptor
LTP	Long term potentiation
MAPK	Mitogen activated protein kinases
MAO	Monoamine oxidase
MEK	MAPK kinase
PBS	Phosphate-buffered saline
PDK-1	Phosphoinositide-dependent kinase-1
PI-3K	Phosphoinositide *tris*- phosphate kinase
PIP2	Phosphoinositide *bis*- phosphate

PKC	Protein kinase C
PL-Cβ	Phospholipase Cβ
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphotidylserine
RIPA	Radioimmune precipitation buffer
5-HTT	5-HT transporter, SERT
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TPH	Tryptophan hydroxylase

CHAPTER 1

INTRODUCTION

SEROTONIN

Synthesis, Storage and Reuptake

Serotonin, one of the major modulatory neurotransmitters, has been implicated in a wide range of physiological, behavioral, and pharmacological effects including mood, emotions, sleep, appetite, and temperature regulation. These effects are mediated by serotonin receptor subtypes, but the precise, underlying mechanisms are unknown.

Serotonin (5-hydroxytryptamine, 5-HT) is so named because it was isolated, purified and identified as a tonic substance in "serum" (sero= serum, tonin= tonic). The hydroxyl group in the 5 position of the indole nucleus and a primary amine nitrogen atom, which serves as a proton acceptor at physiological pH, renders 5-HT hydrophilic. As such, it does not cross the lipophilic blood brain barrier. Thus, its discovery in the brain indicated that 5-HT is synthesized in the brain, where it might play an important role in brain function. Now it is well established that 5-HT –a monoamine neurotransmitter is involved in regulating various central nervous functions and behaviors. It has been implicated in more behaviors, physiological and diseases than any other neurotransmitter of the nervous system.

Serotonin biosynthesis is restricted to serotonergic neurons in the raphé nuclei of the brain stem. This is largely due to the cell type specific expression of tryptophan hydroxylase (TPH) in these cells (Wood, 2001). The initial step in the synthesis of serotonin is the facilitated transport of the amino acid L-tryptophan from blood into the

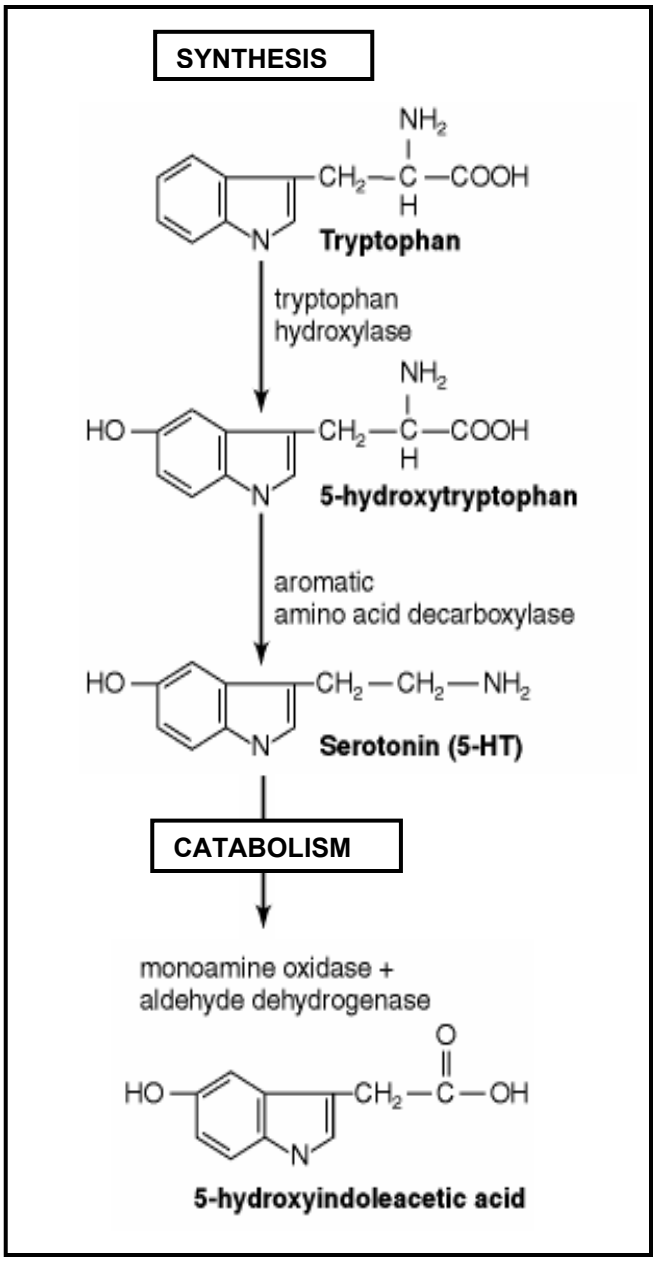


Figure 1.1. Serotonin biosynthesis and catabolism.

Tryptophan (Trp) hydroxylase catalyses, the first rate controlling reaction converting Trp to 5-hydroxytryptophan (5-HTP). The second enzyme 5-HTP decarboxylase converts 5-HTP to 5-Hydroxytryptamine (5-HT) which is converted to 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme monoamine oxidase.

brain. The dietary proteins are the primary source of tryptophan. In the brain, the enzyme L-tryptophan-5-monooxygenase, more commonly termed tryptophan hydroxylase, converts tryptophan to 5-hydroxytryptophan (5-HTP). Another enzyme involved in the synthesis of serotonin, aromatic L-amino acid decarboxylase (AADC) converts 5-HTP to 5-HT (Siegel, 1998) [Figure 1.1]. Post-translational regulation of TPH is responsible for the control of serotonin synthesis. Another mechanism for the control of serotonin levels involves a negative feedback loop via activation of presynaptic 5-HT1 autoreceptors, which causes an inhibition in neuronal firing and suppression of serotonin release (Wood, 2001) .

Once synthesized serotonin is stored by neurons in small synaptic vesicles from where it can be quickly and easily released. Serotonin is stored in vesicles by its active transport from the cytoplasm driven by a vesicular transporter that uses an electrochemical gradient generated by a vesicular H^+-ATPase. In the processes a cytoplasmic amine is exchanged for a proton; that is, uptake of 5-HT is coupled to the efflux of H^+ [Figure 1.2]. Serotonin is released by exocytosis, thus, depolarization -induced influx of Ca^{2+} into the neuronal terminal causes a discharge of the entire content of the individual vesicles from the synaptic terminal. An increase in raphé cell firing enhances the release of 5-HT in terminal fields and vice-versa.

The binding of 5-HT to specific transporter proteins on presynaptic neurons terminates the synaptic effect of this neurotransmitter. A serotonin transporter (SERT), which is located on serotonergic neurons, at the level of soma, dendrites, axons and terminal (Cour, 2001) regulates the concentration of 5-HT in the synapse. The current model of how these transporters work posits that one Na^+, one Cl^- and one protonated

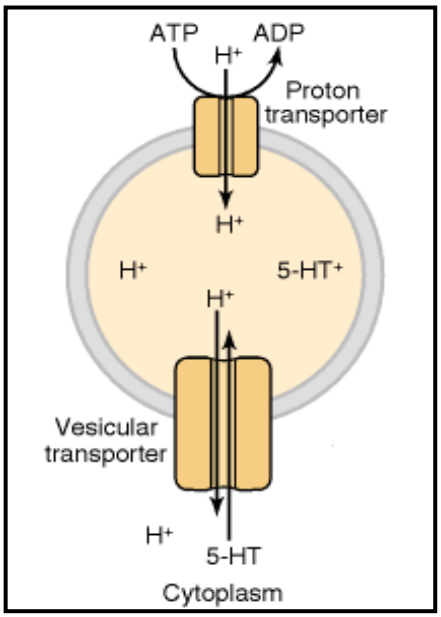


Figure 1.2. Serotonin vesicle storage.

Uptake of 5-HT in the vesicle is coupled with efflux of H^+ proton which generates electrochemical gradient by a vesicular H^+-ATPase (Adapted from Siegel, 1998).

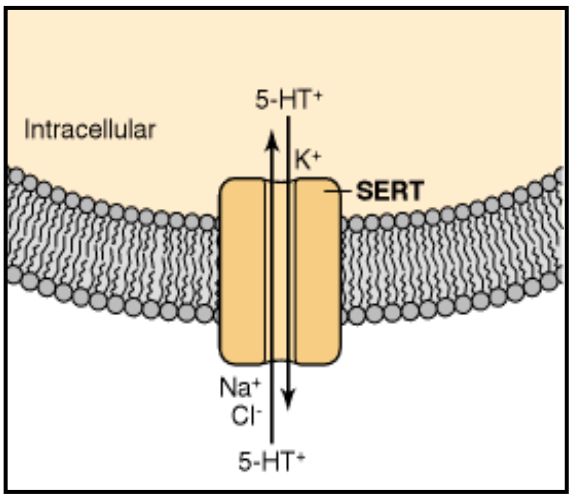


Figure 1.3. Reuptake of serotonin into serotonergic terminals.

One of each Na^+, Cl^- and one protonated 5-HT bind to the transporter extracellularly that subsequently undergoes a conformational change to release the neurotransmitter and translocate the ions into the cytoplasm where K^+ associates with SERT to promote reorientation of the transporter (Adapted from Siegel, 1998).

5-HT bind to the transporter extracellularly to form a quaternary complex that subsequently undergoes a conformational change to release the neurotransmitter and translocate the ions into the cytoplasm. In the cytoplasm, K^+ associates with the SERT to promote reorientation of the unloaded carrier for another transport cycle [Figure 1.3].

Serotonin is converted to 5-hydroxyindoleacetaldehyde by monoamine oxidase, and this product is oxidized by an NAD^+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA). Thus, 5-HIAA is the major excreted metabolite of serotonin [Figure 1.1] (Siegel, 1998).

Development and distribution of serotonin in the brain neurons

During development serotonergic neurons are formed from a large group of multipolar neurons located along the midline of the brainstem, collectively known as raphé nuclei. Raphé (serotonergic) neurons are among the first to differentiate during embryonic development. In humans, they emerge at about six weeks of gestation and stabilize by 15 weeks, in rats it is first observed at E13. These neurons synthesize serotonin soon after their last cell division and the arrival of their projections in other brain regions coincides with ongoing differentiation and growth events (Wallace, 1983). Thus, they play an important role in various neurodevelopmental processes including neurogenesis, differentiation, neuropil formation, migration, neurite outgrowth, growth cone motility, axon myelination, synapse formation and maturation (Lauder, 1978; Lauder, 1982; Whitaker-Azmitia, 1996). Mature serotoninergic innervation of the cortex and hippocampus occurs during the first two postnatal weeks, a process that is accompanied by a twofold increase in serotonin, and it has been postulated to play a role in synaptogenesis (Chubakov, 1986; Emerit, 1992).

Thus, experimental evidence has indicated that 5-HT plays atleast two distinct roles in development prior to assuming its role as a neurotransmitter in the brain-

1) 5-HT plays a role as an autoregualtor in the maturation of the post synaptic areas to which serotonergic neurons project. Thus, depletion of 5-HT synthesis by p-chloro-phenylalanine (PCPA) administrated to a pregnant dam delays the time course of neurogenesis and retards onset of differentiation in brain regions containing the 5-HT terminals. Conversely, when 5-HT is added to the culture medium of developing hippocampal sections, the neurons show earlier development of interneuronal synaptic connections (Chumasov, 1978).

2) 5-HT can regulate the direction and extent of growth of selected neurons. In a culture of helisoma neurons, 5-HT inhibits the motility of growth cones and synptogenesis during early development (Haydon, 1984).

These tropic as well as trophic influences of serotonin occurs at critical periods when removal of serotonin causes long lasting effects on development, synaptic plasticity and brain maturation (Lidov, 1982; Whitaker- Azmitia, 2001).

In the adult mammalian CNS, the serotonin system is organized into two subsystems, a rostral division with cell bodies localized in the midbrain and rostral pons, providing projections to the forebrain, and a caudal division located primarily in the medulla oblongata with descending projections to the spinal cord and brainstem nuclei. Thus although serotonergic cell bodies are restricted to discrete clusters or groups of cells in the raphé nuclei, their axons innervate nearly every area of the CNS. In all species studied so far, neurons containing 5-HT consists of a morphologically heterogeneous population (Jacobs, 1992; Steinbusch, 1981; Tork, 1990). In 1964, Dahlstrom and Fuxe

described nine groups of serotonin-containing cell bodies, which were designated B_1 through B_9, and which corresponded for the most part to the raphé nuclei [Table 1.1]. The largest group of serotonergic cells is B_7, which is continuous with a smaller group of serotonergic cells, B_6. Groups B_6 and B_7 often are considered together as the dorsal raphé nucleus where B_6 is the caudal extension. The group B_8 corresponds to the median raphé nucleus. The group B_9, which is a part of the ventrolateral tegmentum of the pons and midbrain, forms a lateral extension of the median raphé. Serotonergic ascending projections innervating the cerebral cortex and other regions of the forebrain arise primarily from the dorsal raphé, median raphé and B_9 cell groups. The two main ascending serotonergic pathways emerging from the midbrain raphé nuclei to the forebrain are the dorsal periventricular path and the ventral tegmental radiations. The median raphé projects heavily to hippocampus, septum and hypothalamus, whereas the dorsal raphé innervates predominantly striatum and frontal cortex (Azmitia, 1978; Vertes, 1999). The dorsal and median raphé nuclei send overlapping neuronal projections to the neocortex. Within the dorsal and median raphé, cells are organized in particular zones or groups that send axons to specific areas of the brain. The axons from the median raphé nucleus are type M and look relatively coarse with large spherical varicosities whereas axons from the dorsal raphé are type D, appear very fine, and typically contain small, pleomorphic varicosities. The other raphé nuclei, B_1 to B_4, are situated more caudally in the midpons to caudal medulla and contain a smaller number of serotonergic cells. These cell groups give rise to serotonergic axons that project within the brainstem and to the spinal cord (Dahlstrom, 1964; Siegel, 1998) [Figure 1.4]. The central 5-HT system is now commonly named after the cytoarchitectonic groups- the rostral group (B4-B9 nuclei),

which projects mainly to forebrain regions, and the caudal group (B1-B3 nuclei) that projects to the spinal cord (Tork, 1990). .

Table 1.1. Serotonergic cell body groups classification.

Groups of serotonin containing neurons	Anatomical Structures	Projections
B1	Raphé pallidus nucleus	Caudal projections to brain stem and spinal cord.
B2	Raphé obscurus nucleus	Caudual projection to lamina IX of the ventral horn of the brain stem and spinal cord.
B3	Rostral ventrolateral medulla and lateral paragigantocellular reticular nucleus	Caudal projections to laminae I and II of the dorsal horn of the brain stem and spinal cord.
B4	Raphé obscurus nucleus	Dorsolateral extension.
B5	Median raphé nucleus	Caudal extension
B6	Dorsal raphé nucleus	Caudal extension.
B7	Dorsal raphé nucleus	Rostral (ascending) extension to cerebral cortex and forebrain mainly striatum.
B8	Median raphé nucleus/ Nucleus central superior	Rostral main part, projects to hippocampus, septum and hypothalamus; caudal linear nucleus.
B9	Lateral extension of medial raphé	Ascending to cerebral cortex and forebrain.

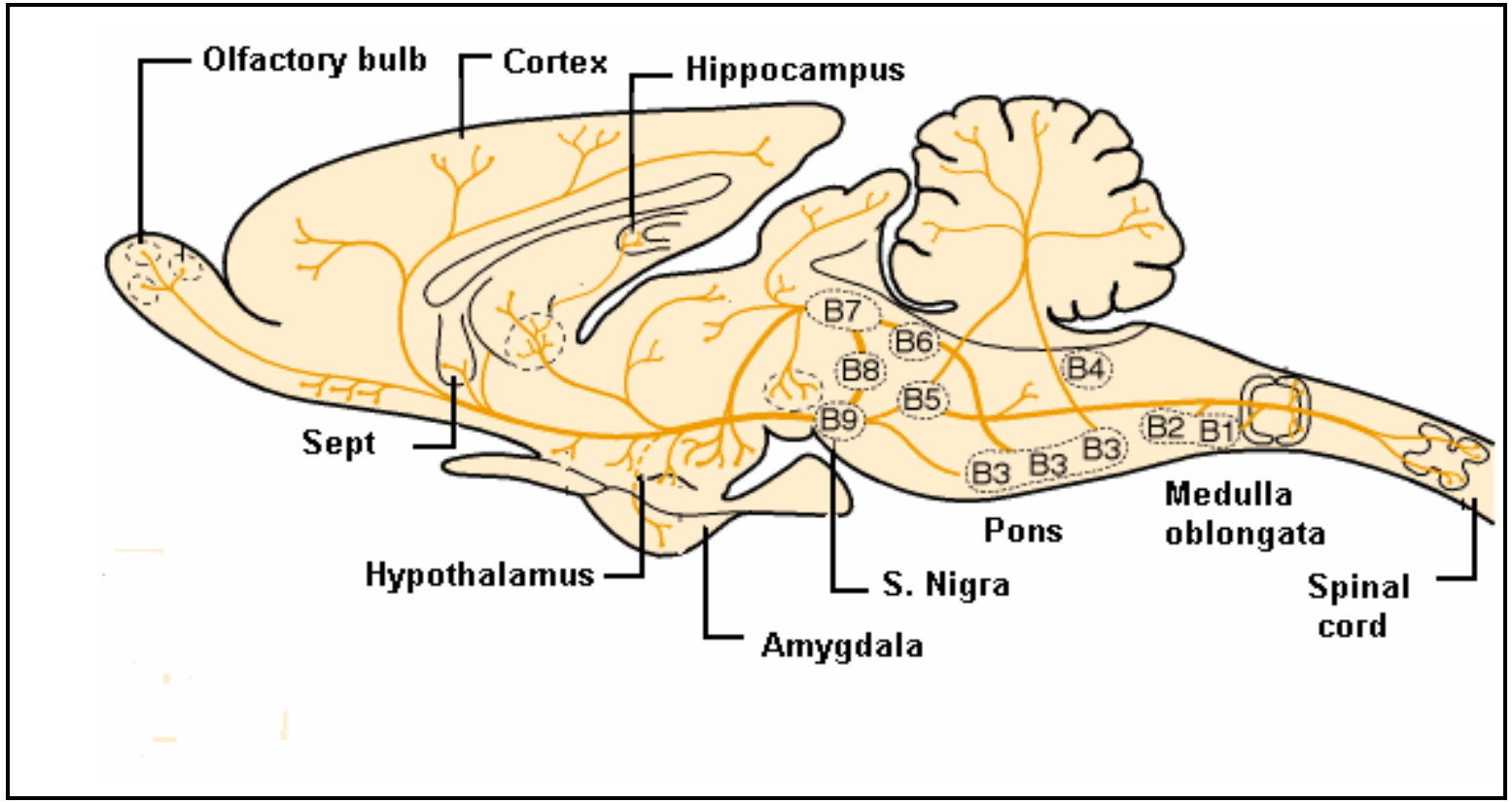


Figure 1.4. Serotonergic cell body groups in a sagittal section of the rat CNS and their major projections. S. Nigra = substantia nigra, Sept = Septum (Modified from Siegel, 1998).

Serotonin receptor subtypes

Serotonin mediates a wide range of physiological functions by interacting with at least, seven distinct receptor subfamilies (5-HT1-5-HT7), containing at least 14 different receptor subtypes. The current classification is based not only on operational criteria, such as drug-related characteristics, but also on information about intracellular signal transduction mechanisms and amino acid sequence of the receptor protein. The three serotonin receptor subfamilies, the 5-HT_1 family; the 5-HT_2 family; and the family that includes the 5-HT_4, 5-HT_6 and 5-HT_7 receptors, represent the three major classes of serotonin receptors that are members of the G protein-coupled receptor superfamily or seven transmembrane-spanning receptors. The 5-HT_3 receptor is a ligand-gated ion channel and is a separate subfamily [Table 1.2]. Thus, the diversity of the effect produced by serotonin is expanded not only by including as many as fourteen receptor subtypes but also by coupling to different G-proteins.

Structure and Mechanism of Serotonin (G-protein coupled) receptors (GPCRs)

Receptors that are coupled to GTP -binding and hydrolyzing proteins are termed G (guanine nucleotide binding)-protein coupled receptors (GPCRs). GPCRs are composed of seven transmembrane-spanning α-helical domains (H1-H7) with an intracellular carboxy-terminus and an extracellular amino-terminus. The transmembrane domains of G protein-coupled receptors are the most highly conserved regions of these proteins. The ligand binding site including for the natural biogenic amine ligand, serotonin, lies within a cavity formed by the bundling of seven transmembrane regions with a specific amino acid side chain (Wang, 1993). The interaction of the receptor with the G-protein is thought to occur through the intracellular loops and the C-terminal tail.

Table 1.2. Serotonin receptor subtypes.

5-HT Receptors Subtypes	**Distribution**	**Type**	**G-protein Coupling**	**Effector Mechanism (Mainly Neuronal cells)**	**Physiological/ Behavioral effect**
5-HT_1		**GPCR**		(Hannon, 2002; Serres, 2000)	Neuronal hyperpolarization/ Inhibition.
5-HT_{1A}	Presynaptic- Raphé nuclei (autoreceptors). Postsynaptic- Hippocampus (CA1, dentate gyrus), amygdala, septum, entorhinal cortex, hypothalamus, spinal cord (heteroreceptors) (Bonasera, 2000).		Gi/ Go/ Gz	i) Inhibition of adenylyl cyclase. ii) Opening of inwardly rectifying K^+ channels. iii) Inhibits both N-type and P/Q-type of Ca^{+2} channels (Kushwaha, 2005). iv) Inhibition/ activation of PLC. v) Stimulation/ inhibition of MAPK (ERK1/2). (Adayev, 2003; Kushwaha, 2005; Mukhin, 2000). vi) PKC activation (Raymond, 1991).	Implicated in anxiety, depression, mood disorders. Involved in sexual behavior, appetite control, neuroendocrine function- thermoregulation, cardiovascular function, immune function, developmental diseases.

5-HT_{1B}	Presynaptic- Cingulate cortex, entorhinal cortex, Postsynaptic-hippocampus, striatum, substantia nigra, global pallidus, dorsal subiculum, purkinje cells (Bonasera, 2000)		Gi / Go	Inhibition of adenylyl cyclase (Hen, 1992).	Motor control. and aggression.
5-HT_{1D}	Substantia nigra, basal ganglia, superior colliculus		Gi / Go	Inhibition of adenylyl cyclase.	Involved in neurogenic inflammation and nociceptive.
5-HT_{1E}	Caudate putamen, parietal cortex, olfactory tubercle.		Gi / Go	Inhibition of adenylyl cyclase.	?
5-HT_{1F}	Cerebral cortex, striatum, hippocampus, olfactory bulb.		Gi / Go	Inhibition of adenylyl cyclase.	Role in vascular contraction. Possible involvement in migraine, dural inflammation.
5-HT_2		**GPCR**			Neuronal Depolarization
5-HT_{2A} (Kurrasch -Orbaugh, 2003)	Claustrum, cerebral cortex, olfactory tubercle, striatum, nucleus accumbens.		Gq	i) Stimulation of phosphoinositide-specific phospholipase C (PLC) (Hen, 1992). ii) Closing of K^+ channels. iii) Stimulation of	Involved in Sleep, thermoregulation, hypertension and vasoconstrictor effects, and hallucinogenic behavior.

				phospholipase A_2. iv) Stimulation of ERK1/2 and p38	
5-HT_{2B}	Cerebellum, septum, hypothalamus, amygdale.		Gq	i) Stimulation of phosphoinositide-specific phospholipase C. ii) Stimulation of MAPK.	Grooming, artery contraction in hypertension.
5-HT_{2C} (Before 1C)	Choroid plexus, globus pallidus, cerebral cortex, hypothalamus, septum, substantia nigra, spinal cord.				Suggested to be involved in regulation of cerebral spinal fluid, food intake and cognitive impairment.
5-HT_3	Hippocampus, entorhinal cortex, amygdala, nucleus accumbens, solitary tract nerve, trigeminal nerve, motor nucleus of the dorsal vagal nerve, area postrema, spinal cord.	**Ion-Channel**		Opening of a channel for cations (Na^+, Ca^{++} influx, K^+ efflux).	Membrane depolarization. Seems to be involved in cardiac, lung, intestine function. Induce pain, vasodilation and sensitization of nociceptive neurons.
5-HT_4	Hippocampus, striatum, basal ganglia, nucleus accumbens, olfactory tubercle, substantia nigra.	**GPCR**	Gs	Stimulation of adenylyl cyclase	Cognition enhancing effects, controversial involvement in anxiety.

5-HT$_5$ 5-HT$_{5A}$ 5-HT$_{5B}$	Hippocampus, cerebral cortex, granular layer of the cerebellum, olfactory bulb, hypothalamus, corpus calloscum (Bonasera, 2000). Also on glial cell.	**GPCR**	G	?? Stimulation / inhibition of adenylyl cyclase	?
5-HT$_6$	Olfactory tubercle, cerebral cortex, nucleus accumbens, striatum, hippocampus and the molecular layer of the cerebellum, hippocampus, amygdale(Kohen, 2001).	**GPCR**	Gs	Stimulation of adenylyl cyclases	?
5-HT$_7$	Cerebral cortex, septum, thalamus, hypothalamus, amygdala, superior colliculus	**GPCR**	Gs	i) Stimulation of adenylyl cyclase ii) Stimulation of MAPK/ERK1/2.	Implicated in the control of circadian rhythm and smooth muscle relaxation.

GPCR= G protein coupled receptors, PLC= Phospholipase C, MAPK= Mitogen activated protein kinase, ERK= Extracellular regulated kinase.

A GPCR can be rendered constitutively active by introducing a mutation in its third cytoplasmic loop (Kjelsberg, 1992). In the serotonin 1A receptor (5-HT_{1A}-R) this alters ligand response and G protein coupling (Malmberg, 2000). Site directed mutagenesis has shown that a conserved aspartic acid residue in the third transmembrane domain (TMD3) is an agonist (5-HT) binding site for 5-HT_{1A}-R, whereas a conserved aspartic acid in the second transmembrane domain (TMD2) participates in the generation of second messenger responses (Kroeze, 2003). It has also been reported that a single conserved threonine residue (Thr 149) in the second intracellular loop is directly involved in G-protein coupling to Gβγ– induced pathways, but coupling to Gαi-mediated inhibition of cAMP remains intact (Albert, 1998; Lembo, 1997) [Figure 1.5].

The mechanism of action of GPCR is best described by the ternary complex model. In this model, the receptor exists in the ground state (R) and also a partially activated state (R*) that is able to couple to a G-protein. The agonist stabilizes the R* and R*G to form the active state AR*G, the so called ternary complex (Barr, 1997). In the ternary complex, GDP bound to the G-protein is exchanged for GTP and the complex then dissociates, releasing α and βγ subunits of the G protein. These subunits then modulate the activity of different effector enzymes and ion channels (Malmberg, 2000). A single G protein-coupled receptor signal is amplified by regulating different effector systems signaling through multiple subtypes of heterotrimeric G proteins.

G-protein-coupled receptors (GPCRs) are found in coated vesicles and various intracellular vesicles associated to endocytic pathways. Studies (with the β-adrenergic receptor in particular) have delineated a general pathway for agonist-mediated internalization/ desensitization. The agonist-induced activation of receptor leads to

receptor phosphorylation and the subsequent binding of proteins such as arrestin to phosphorylated receptor. Arrestin binding appears to facilitate translocation of phosphorylated GPCRs to clarthrin-coated pits and the eventual internalization of GPCRs via the endosome pathway (Bhatnagar, 2001). Continued exposure to an agonist results in down regulation of the receptor (Kobilka, 1992). Later information suggests that receptor signaling and desensitization are in reality two intimately linked aspects of receptor function and that mechanisms previously viewed as “desensitizing” with respect to one signaling pathway may be “activating” with respect to another (Lefkowitz, 1998).

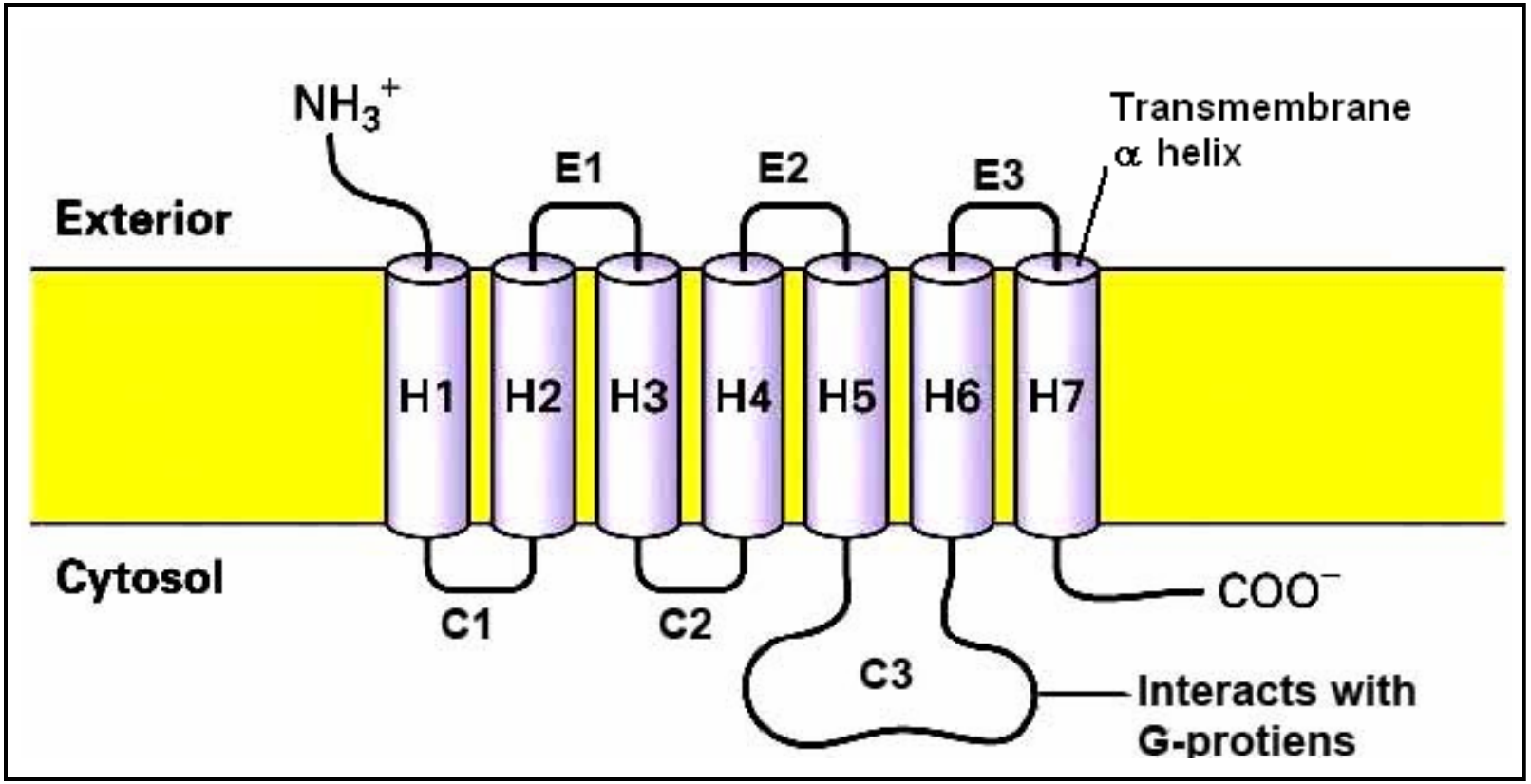


Figure 1.5. Structure of G protein coupled receptor (GPCRs)/ Serotonin 1A receptor.

Conserved aspartic acid residue in helix 3 (H3) is an agonist binding site whereas the second messenger response binding is in the second helix (H2) of the seven transmembrane structure. E1-E3= Extracellular loops, C1-C3= Cytoplasmic loops, NH_3^+= amino terminal, COO^-= Carboxy terminal.

SEROTONIN 1A RECEPTOR

5-HT_{1A}-R signaling in neurons

Out of all the serotonin receptors, serotonin-1A receptors have gained maximum focus because of its central role in various neuronal functions and pathological conditions. The heptahelical, serotonin 1A receptor couples mainly to a variety of effectors via activation of heterotrimeric, pertussis toxin (PTX)-sensitive G-proteins, such as Gi and Go. The G protein interaction with the receptors results in the release of Gα and the heterodimer G-protein βγ complex. The G-protein α subunit couples to an effector molecule—e.g. Gαi couples negatively to adenylate cyclase, Gαs couples positively to adenylate cyclase, and Gαq couples positively to PLCβ (Adayev, 1999; Fargin, 1989; Stoyanov, 1995). By contrast, the Gβγ can activate multiple effector molecules or pathways, such as the phospholipase Cβ––ERK1/2 pathway or the PI-3 kinase (PI-3K) pathway (Raymond, 1999). Among the signaling pathways activated by agonist-bound 5-HT_{1A} receptor, ERK 1/2- members of the mitogen activated protein kinase (MAPK) pathways are important for both proliferation of pre-neuronal cells and survival of post-mitotic neurons in the brain. It has been shown in neuronal cells that activation of the 5-HT_{1A} receptor causes inhibition of apoptosis and caspase-3 via ERK1/2 activation, which further stimulates protein kinase C (PKCα) (Adayev, 2003). This pathway seems to be different and does not involve phosphoinositide-3-kinase (PI-3K), which often plays a role in the activation of MAPK (Lopez-llasaca, 1997). In transfected, Chinese hamster ovary (CHO) fibroblast cells, the 5-HT_{1A} receptor mediates activation of ERK1/2, through a Giβγ- Ras-dependent mechanism, and NF-κB, through a

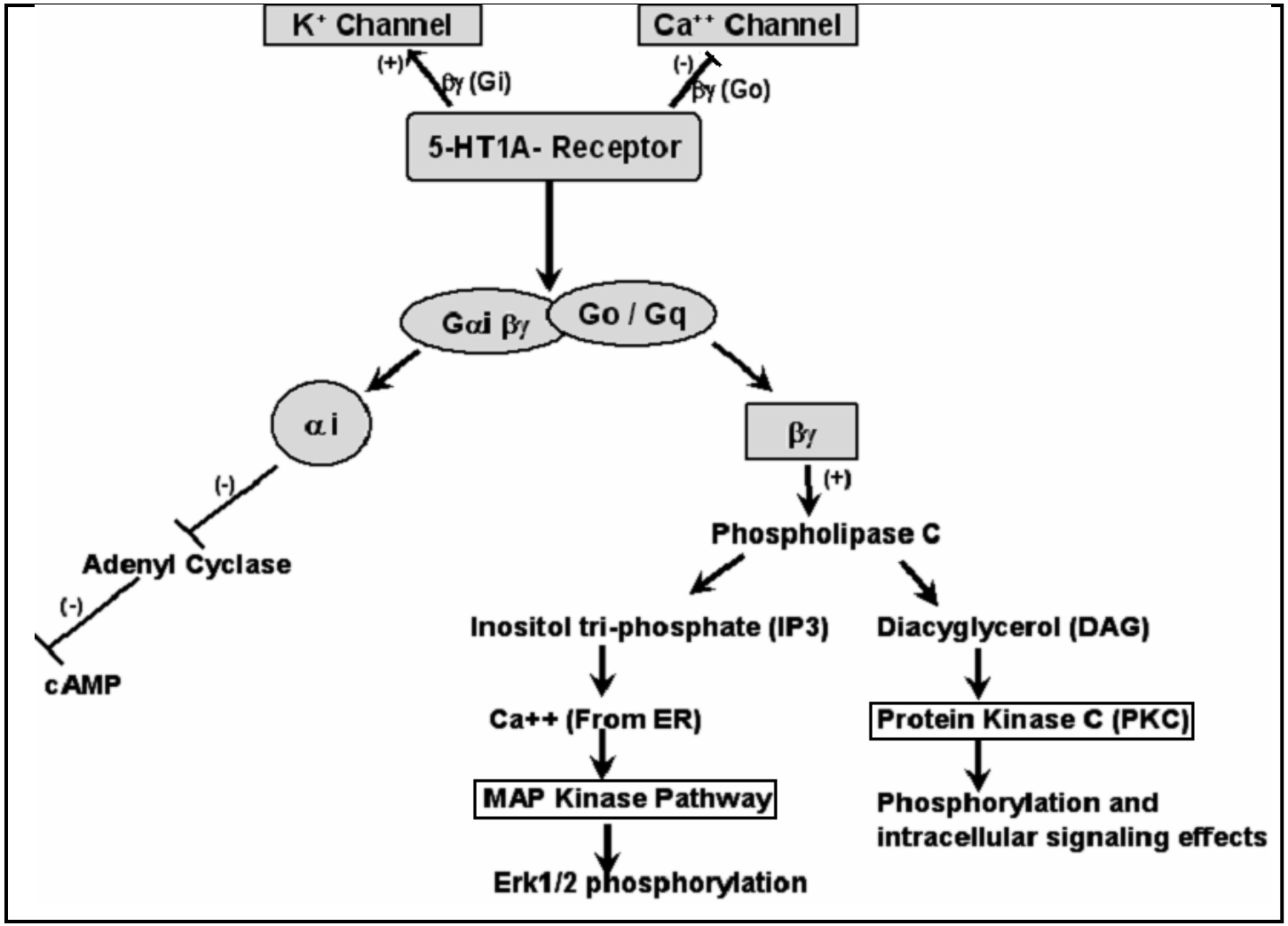


Figure 1.6. Serotonin-1A receptor signaling.

Gi/ Go signaling pathway (Cowen D. S., 1997; Cowen, 1996; Della Rocca, 1999; Garnovskaya, 1996; Mukhin, 2000). In addition, agonist binding to this receptor causes inhibition of N-type Ca^{2+}-channels (Bayliss, 1997) In many central neurons, 5-HT_{1A} receptors activate an inwardly rectifying K^+ current leading to hyperpolarization of the hippocampal, raphé, and the neonatal pyramidal neuronal cells (Bonasera, 2000; Jeong, 2001; Katayama, 1997). It also mediates agonist- induced acceleration of Na^+/ H^+ exchange (Garnovskaya, 1997). [Table 1.1] [Fig 1.6]. Thus, it is important to emphasize that, depending on the type of effectors that are present in the 5-HT_{1A} responsive cell, receptor activation can lead to opposite, cell type-specific response and cellular activity. Although it has been often reported as inhibitory in neurons, the same receptor stimulates a number of signaling molecules, causing calcium mobilization and cell proliferation in different cell types (Albert, 1998).

5-HT_{1A}-R ligands, antidepressants, and neurogenesis

Serotonin neurons and receptors are the targets of a wide variety of therapeutic drugs among which the most widely used class of antidepressant drugs, is commonly referred to as the tricyclic antidepressants. The 5-HT_{1A} receptor has gained maximum attention due to its role in the action of the most frequently used antidepressants- the selective serotonin reuptake inhibitors (SSRIs), which act on the 5-HT transporter (5-HTT/ SERT). It is also well established that antidepressant drugs (such as monoamine oxidase inhibitors- MAOIs, the SSRIs, and tricyclic antidepressants- TCAs), exert their therapeutic effect through the enhancement of 5-HT neurotransmission in brain regions such as hippocampus and cerebral cortex. Experimentation in animals has indicated that at the onset of these treatments, there is an increase in extracellular 5-HT, which activates

5-HT_{1A} autoreceptors located on the soma–dendrites of 5-HT neurons, which in turn inhibits firing and 5-HT release from the synaptic terminals of those neurons. Repetitive treatments causes gradual removal of this inhibition through desensitization of the 5-HT_{1A} autoreceptors, thus causing enhanced 5-HT neuron firing, increased extracellular 5-HT, and hence the desired antidepressant effect (Blier, 1987; Czachura, 2000; Le Poul, 1995). Thus, in the absence of the 5-HT_{1A} autoreceptors, the released serotonin binds only to the post-synaptic 5-HT_{1A} receptors, thereby eliciting the anxiolytic effect of the SSRIs. As mentioned before, one of the mechanisms leading to desensitization of G protein-coupled receptors is their internalization. That the 5-HT_{1A} autoreceptors internalize (in the nuclei of raphé dorsalis) but the heteroreceptors do not has been demonstrated following acute treatment with the specific 5-HT_{1A} receptor agonist, 8-OH-DPAT, and also after acute treatment with the antidepressant SSRI (fluoxetine). The total number of receptor binding sites was not changed, although there was an increase in the density of the receptor in the cytoplasm and a decrease in labeling of plasma membrane receptor. This indicates that there is mobilization of pools of autoreceptors following agonist and acute SSRI treatments (Kushwaha, 2005; Riad, 1994; Riad, 2004; Zimmer, 2004). In addition, the desensitization of somatodendritic 5-HT_{1A} autoreceptors in the dorsal and median raphé following chronic SSRI treatment appears to be due to a reduced capacity of the 5-HT_{1A} receptor to activate G-proteins rather than a significant change in receptor number. By contrast, changes in postsynaptic 5-HT_{1A} receptors most likely occurs distal to receptor-G-protein interactions, perhaps at the level of effector, or involving changes in neuronal function at the system or circuit level (Hensler, 2002). Serotonin concentration were increased in extracellular areas and decreased inside the cell of the

SERT (-/-) mice. 5-HT also down regulated function of 5-HT_{1A} and 5-HT_{1B} autoreceptors on serotnergic neurons in these mice. The increase in 5-HT synthesis and turnover in SERT(-/-) mice occurs in the absence of any evident change in TPH enzyme activity or MAO inhibition (Kim, 2005). In contradictory results, another group has shown in vivo as well as vitro that long treatments with SSRIs (sertraline, fluoxetine) augments serotonin synthesis via upregulation of gene expression and protein levels of TPH, the rate-limiting enzyme in serotonin synthesis (Kim, 2002).

It requires several weeks of antidepressant treatments to observe any effects. This suggests that slow neurochemical and structural changes take place within the limbic target areas of monoaminergic projections following such treatments. Santarelli and co-workers have shown that the behavioral effects of chronic antidepressants are caused by a stimulation of hippocampal neurogenesis and this is mediated by the 5-HT_{1A}-R (Santarelli, 2003). Various antidepressant treatments, including fluoxetine, increase neurogenesis in the dentate gyrus and hilus of the rat hippocampus, as evident by an increase in the number of progenitor cells that incorporate the DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU) and then differentiate into mature neurons (Jacobs, 2000; Malberg, 2000).

The 5-HT_{1A} receptor agonists have been clinically used in conjunction with antidepressant drugs and also in the treatment of Schizophrenia (Burnet, 1997). They have been also reported to exert anxiolytic and antidepressive activity (Blier, 2003). The 5-HT_{1A} receptor antagonists have been suggested to have therapeutic utility in diseases such as depression, anxiety, drug- and nicotine-withdrawal, Alzheimer's disease and other diseases with associated cognitive dysfunction (Schechter, 2002; Nishi, 1999). The

5-HT_{1A} receptor blocker WAY100635 would mask the inhibitory 5-HT_{1A} autoreceptors and thereby, bolster release of 5-HT from these neurons, thus augmenting the anxiolytic effect. The combined treatment with an SSRI and a 5-HT_{1A} receptor antagonist increases the extracellular concentration of 5-HT more than that caused by SSRI alone. Thus, treatment of patients with major depression with an SSRI and a 5-HT_{1A}-R antagonist (such as pindolol) have been shown to markedly reduce the latency of the antidepressant response in previously untreated patients and also induce a rapid response in treatment-resistant patients (Artigas, 1996).

DEVELOPMENT and 5-HT$_{1A}$ RECEPTOR

Development of the Nervous system

Early brain development is associated with an initial burst of cell division where, uniform populations of neural progenitors (the cells of neural plate) are recruited (Zigmond, 1999). After this the preneuronal cells acquire properties of immature neurons or glial cells. The immature neurons then receive signals to either divide further or migrate to their respective destinations in the brain. These cells then receive molecular signals to differentiate into mature neurons with appropriate synaptic connection. The formation of contacts between the growing axons and their target cells then initiates a process of selective synapse formation during which some synaptic contacts are strengthened and others eliminated, thus controlling patterns of connectivity. So, the first phase of brain development requires rapid cell division and the final stage requires survival of the differentiated neuronal cells while they are in the process of building appropriate neuronal contacts and also strengthening the right connections (Kandel, 2000; Purves, 1997). Therefore, a receptor-mediated signaling pathway that could assist early cell division as well as survival of differentiating neurons and strengthening of synapses would be of great significance in brain development.

Developmental role of the 5-HT$_{1A}$ receptor

In general, 5-HT upregulates the monoaminergic neuronal phenotype. Such upregulation is mediated by the 5-HT$_{1A}$ receptor, the principal receptor involved in neurotrophic effects (Azmitia, 1997).

The 5-HT$_{1A}$ receptor has been shown to control branching of cortical neurons in culture (Sikich, 1990). Specifically, addition of the 5-HT$_{1A}$ agonist 8-OH-DPAT to

dissociated cultures of embryonic septal cholinergic neurons promotes neurite outgrowth and branching (Riad, 1994). In recent years, a number of studies have focused on the role of 5-HT_{1A} receptor during hippocampal development. Specifically, neonatal serotonin depletion or 5-HT_{1A} receptor blockade can impede normal development of pyramidal and granule cell dendrites resulting in reduced branching (Borella, 1997), spine density, and synapse formation (Wilson, 1998). There are reports showing that the dentate gyrus is very sensitive to serotonin (Yan, 1997) and 5-HT_{1A} receptor activity. Hippocampal serotonin depletion during early postnatal development leads to decreased synaptic densities in the hippocampus and spatial learning deficits in adult rats (Mazer, 1997). These effects of serotonin deletion on hippocampal synaptogenesis are mediated via the 5-HT_{1A} receptor (Daval, 1987; Nishi, 1996). Furthermore, the effects of 5-HT depletion on dendritic spine density and synapse formation can be blocked by systemic administration of the 5-HT_{1A} agonist buspirone (Yan, 1997). Finally, it has been reported that 8-OH-DPAT mediated 5-HT_{1A} activation can stimulate the proliferation of granule cells in the dentate gyrus (Radley, 2002). Blockade of this receptor permanently reduces numbers of dendritic spines and overall synaptogenesis in postnatal rat pups (Andrews, 2004; Faber, 1999). Evidence from other studies indicates that this receptor is centrally involved in cerebellar development (del Olmo, 1994). It has been shown that the loss of dendritic spines caused by a 5-HT_{1A}-R antagonist (NAN-190) is a function of decreased stimulation of 5-HT_{1A} receptors (Yan, 1997). Lastly, adrenalectomy (ADX) evoked loss of adult neuronal morphology in granular neurons of the hippocampus was reversed by treatment with a 5-HT_{1A}- receptor agonist. This again provides evidence for the trophic importance of the receptor in the brain (Huang, 1997).

Development and distribution of the 5-HT_{1A}-R

The 5-HT_{1A}-R is expressed early in mammals and other animals during development (Azimita, 2001). High densities of 5-HT_{1A} receptor binding, in excess of adult levels, have been reported in human fetal cortex and hippocampus between the 16th and the 22nd week of gestation (Bar-Peled, 1991; del Olmo, 1998). The 5-HT_{1A}-R mRNA is first detected in the embryonic rat brain neurons as early as day 12 (approximately corresponds to E12 in mice). Its concentration increases to a maximum at ED15 (the highest levels are found at ED14 and 15) and then decreases progressively to very low levels just before birth (ED20) strongly suggesting that it is developmentally regulated (Emerit, 1992; Hillion, 1994; Hillion, 1993). More recent data indicate that an increase in 5-HT_{1A}-R expression begins after the peak of 5-HT_{1A}-R mRNA synthesis. Thus, using ^{125}I-MPPI (a 5-HT_{1A} antagonist) binding to brain slices and autoradiography, Gross and coworkers have shown that low but significant level of expression of the functional receptor is first observed at E17 in the forebrain of wild-type mice, after which the expression gradually increases to adult levels during the postnatal period. On the other hand, receptor expression in 5-HT_{1A} (-/-) mice under the control of an inducible promoter first becomes detectable in the hippocampus and cortex at P5, and reaches significant levels at P15 (Gross, 2002). In the neonatal rat brain, 5-HT_{1A} binding sites have been identified in the brainstem, hippocampus, and cortex (Daval, 1987). Andrews and coworkers have found that 5-HT_{1A} receptor mRNA was present in the hippocampal CA1 subfield and dentate gyrus (DG) by gestation day 40 in fetal guinea pig hippocampus (term = 70 days). Receptor mRNA levels in the DG, but not the CA1, increased with the progression of gestation. But near term, levels of 5-HT_{1A} receptor

mRNA were similar in both limbic regions (Andrews, 2004). Patel and coworkers have shown that 5-HT_{1A}-R is expressed in all populations of hippocampal neurons as they accumulate in their appropriate layers during embryonic and postnatal development in rats. The 5-HT_{1A} receptor begins to be expressed as early as E16. The expression of the 5-HT_{1A}-R is initially (through P10) localized to the cell body and gradually shifts to the dendrites during postnatal development as the neurons mature (Patel, 2005). This is supported by the distribution of the receptor along the apical and basal dendrites and their protruding spines in adult rat hippocampus (Riad, 2000). In some areas, hippocampus, cerebral cortex, septum, and brainstem, the density of 5-HT_{1A} receptor mRNA has been shown to increase throughout development to reach a maximum at the adult stages. On the contrary, some other areas, cerebellum, inferior colliculi, and thalamus, appear to transiently express this mRNA, especially in the cerebellum where the peak is reached from postnatal day 3 to 8 followed by a decrease (Matthiessen, 1993; Miquel, 1994). Thus, a high rate of expression of the 5-HT_{1A}-R during a critical period in early life (P5-P21 in mice) supports the hypothesis that this receptor is involved in a trophic action of serotonergic neurons during brain development. Therefore, a study of the profile of 5-HT_{1A}-R mediated signaling activity during this period could yield important information on brain development.

In adult mammalian brain, the 5-HT_{1A} receptor occurs in two different neuronal cell populations:

1. On 5-HT neurons of the midbrain raphé nuclei (Autoreceptors)
2. On neurons postsynaptic to 5-HT nerve terminals (cortico-limbic areas)

The 5-HT_{1A} receptor has a widespread distribution in the brain of various mammalian species. In the serotonergic nuclei of the raphé, where the receptor is also abundantly expressed, it is located in the somato-dendritic regions and functions as an autoreceptor of these neurons. Activation of theses autoreceptors reduces the firing of 5-HT neurons, suppresses 5-HT synthesis, and reduces 5-HT turnover. Thus the autoreceptors provide feedback regulation of the serotonergic system throughout the brain (Kreiss, 1994; Kusserow, 2004; Sotelo, 1990; Sprouse, 1987). Highest receptor densities are found in the hippocampus, septum and cortex whereas the lowest levels are observed in the cerebellum (Celeda, 2004; Parsey, 2005). Santana and coworkers report that ~60% of glutamatergic cells express the 5-HT_{1A}-R transcript and ~25% of GAD (enzyme synthesizing GABA, glutamic acid decarboxylase) -expressing cells contain the 5-HT_{1A}-R mRNA (Santana, 2004). In cortex and hippocampus the receptor localizes extensively in both pyramidal cells and calbindin- and parvalbumin-positive cells, which in these areas mostly define two non-overlapping and well-characterized sub populations of interneurons with distinct functions (Aznar, 2003). In prefrontal, insular and occipital cortex, the 5-HT_{1A} receptor mRNA is expressed in pyramidal neurons of layer 2 whereas in the striate and ventral occipital cortex, receptor mRNA is present within layers 5 and 6 in pyramidal neurons. Strong expression of both 5-HT_{1A} receptor mRNA and protein has been observed in the amygdale of the rat (Chalmers, 1991; Zhou, 1999). In the hippocampus, the 5-HT_{1A} receptor is strongly expressed in the CA1 region, at moderate levels in the dentate gyrus and at low levels in the CA3 region. In cortical and limbic brain regions, the expression of the 5-HT_{1A} receptor occurs on neurons of pyramidal

morphology (Palchaudhuri, 2005). Thus, hippocampus could be an important model to study the activity of these receptors.

5-HT$_{1A}$-R in developmental disorders

Serotonergic neurons play a major role in the modulation of emotion and behavior. There is also evidence that serotonin can modulate anxiety and depression in opposite manners, with high serotonergic activity associated with anxiety and low activity with depression (Graeff, 1996). The 5-HT$_{1A}$ receptor have been long been implicated in the above effects of serotonin of anxiety, depression and suicide (Santana, 2004). Knockout studies have revealed a role for 5-HT$_{1A}$ receptor not only in anxiety related behavior (Heisler, 1998; Parks, 1998; Ramboz, 1998) but also in hippocampal dependent learning in water and Y-maze (Sarnyai, 2000). Transient overexpression of this receptor during early postnatal period (P1.5) has been reported to have enduring effects on emotional behavior in terms of reduced anxiety (Kusserow, 2004) whereas transient overexpression during embryonic and perinatal development (E12 to P1.5) has detrimental effects on water-maze performance at adult stages (Bert, 2005). The existence of two distinct 5-HT$_{1A}$ receptor population subtypes produces opposing effects on anxiety regulation. For example, the stimulation of 5-HT$_{1A}$ autoreceptors is believed to produce anxiolytic effects via suppression of serotonergic neuronal activity, with a consequent decrease serotonin release in diverse projection areas (Sotelo, 1990). Whereas in post-synaptic region, there are contradictory reports regarding the amount of serotonin released in anxiogenic effects (File, 1996; Sprouse, 1987) . It is therefore possible that the enhancement of anxiety in 5-HT$_{1A}$ receptor mutant mice reflects a disinhibition of serotonergic neuronal activity. To resolve the role of the pre- and post-synaptic 5-HT$_{1A}$-

receptor in anxiety-like behavior Gross and coworkers created a tissue specific, conditional expression of the receptor in otherwise of 5-HT_{1A}-/- mice. They found that inducible expression of 5-HT_{1A} receptors primarily in the forebrain and hippocampus but not in raphé nuclei, between postnatal days 5 and 21 was sufficient to reverse the anxiety-like behavior to normal (Gross, 2002), indicating the importance of developmental regulation of 5-HT_{1A} heteroreceptors. Rescue experiments in these knockout mice revealed that the expression of 5-HT_{1A} receptor during the early postnatal period (P5-P21), but not in adult, is crucial for the establishment of normal anxiety-like behavior in the adult. Transgenic mice overexpressing the 5-HT_{1A} receptor exhibits decreased anxiety like behavior (Stockmeiwe, 1998) aggressiveness (Kusserow, 2004; Palchaudhuri, 2005) than normal. Thus, above results raise the importance of the 5-HT_{1A}R and/or its downstream signaling pathway during development to establish normal anxiety-like behavior later in life. In addition, it also suggests that the normal role of the serotonin1A receptor during development may be different from its function when this receptor is activated by therapeutic intervention in adulthood.

Recent positron emission tomography (PET) studies using the 5-HT_{1A} antagonist WAY100635 suggest that 5-HT_{1A} receptors may be reduced in patients with bipolar and major depression rather than unipolar depression. The ligand binding studies have shown that serotonin-1A receptors were increased significantly in the midbrain dorsal raphé of suicide victims with major depression as compared to psychiatrically normal control subjects (Stockmeiwe, 1998). There is also evidence for alteration of the activity of downstream signaling targets of 5-HT_{1A} receptor signaling, such as Akt and ERK1/2 (Hsiung, 2003). In patients with Schizophrenia, post-mortem studies have reported an

increase in 5-HT_{1A} receptor density but not G-protein coupling in prefrontal and limbic areas (Bantick, 2001; Burnet, 1997; Joyce, 1993; Kasper, 2002; Simpson, 1996; Sumiyoshi, 1996). Production of an ontogenic map from postmortem cerebellum showed that human neonatal cerebellum acquired dense 5-HT_{1A} receptors, most of which were eliminated by early childhood. However, in chronic schizophrenia, these receptors were not eliminated (Slater, 1998). 5-HT_{1A} receptor binding in temporal cortex has been inversely correlated with aggressive behavior in Alzheimer diseases (AD) in postmortem studies (Lai, 2003). The alterations in receptor/G-protein coupling has been also implicated in different sets of AD patients (Weinstein, 1996). In addition, receptor binding analyses for the 5-HT_{1A} receptor in mice revealed significant reductions in 8-OH-[3H] DPAT binding in several hippocampal and cortical regions of a Huntington model (Yohrling, 2002). Lastly, 5-HT_{1A} receptor has been involved in alcoholism and locomotory effects of cocaine (Hofmann, 2002; Muller, 2002; Zhou, 1998). These disorders have serious effects on neuronal development.

Thus, to understand the etiology of many developmental disorders of the brain and also to make a headway into 5-HT_{1A}-R-based drug development, it is essential to study the profile of signaling activity of this receptor in the developing brain.

KINASES

Mitogen Activated Protein Kinase (MAPK)

Many receptors that couple to heterotrimeric guanine-nucleotide binding proteins (G proteins), which are pertusis toxin-sensitive, have been shown to mediate activation of mitogen-activated protein kinases (MAPK's also known as extracellular regulated protein kinases or ERK's), including, 5-HT_{1A} receptors. Unique to the eukaryotes, the mitogen-activated protein kinases (MAPK) are signal transducing enzymes that play an important role in many facets of cellular regulation. They are a group of serine/threonine kinases that are activated in response to a variety of extracellular stimuli and represent a point of convergence for cell surface signals that regulate cell growth and division. Several mammalian MAPKs have been identified and classified into groups [Table 1.3].

MAPK Signaling

The MAP Kinases can phosphorylate many different proteins, including transcription factors that regulate expression of important cell-cycle and differentiation-specific proteins in the nucleus (Norum, 2003; Daub, 1996). All MAPKs recognize similar phosphoacceptor sites composed of serine or threonine followed by a proline, and the amino acids that surround these sites further increase the specificity. Full specificity is ensured through a docking interaction mediated by another site on the kinase that recognizes a distinct site on the substrate (Chang, 2001). MAPK signaling cascades are organized hierarchically as shown in the Figure 1.7 (Pearson, 2001). In response to the stimulus, the activated Ras-GTP molecule binds directly to a serine-threonine kinase, Raf (MAP kinase kinase kinase-MAPKKK), forming a transient membrane-anchoring signal. Activated Raf kinase phosphorylates a dual-specificity kinase, MEK (MAPKK /ERK

Table 1.3. Mammals express at least four distinctly regulated groups of MAPKs. (Pearson, 2001)

MAPK (Chang, 2001)	**1. Extracellular signal-related kinase (ERK1/2 + ERK 3)**	**2. c-Jun kinase activated protein kinase- Jun amino-terminal kinase/ stress activated protein kinases (JNK1/2/3 or SAPKγ/β/α)**	**3. p38 MAPK (α,β,γ,δ)**	**4. ERK 5**
A) Activated in Response to- (Hill, 1995)	G-protein-coupled receptors (GPCRs), serum, growth factors, cytokines etc.	Agents that interfere with DNA and protein synthesis, heat shock, stresses, or inflammatory cytokines.	Inflammatory cytokines, hormones, GPCRs, osmotic and heat shock, endotoxins, and osmotic stress.	Epidermal growth factors (EGF), Nerve growth factors (NGF)
B) MEKK	Rafs, Mos, MEKK1-4	MLK3, MEKK1-4, DLK	TAO1-2, ASK, MEKK1-4	(?) MEKK3
C) MAPKKs, MKK or MEK activator	MEK 1/2	MKK 4/7	MKK 3/6	MEK 5

D) Transcriptional Factor Target (Treisman, 1996)	Ribosomal protein S6 kinase (RSK), the transcription factor ELK1/ CREB, and the proapoptotic Bcl-2 family protein Bad (Hill, 1995; Chang, 2001).	Elk-1 and c-Jun.	(?)	(?) c-Jun.
E) Functional role	Cell proliferation, cell survival	Cell death/ induction of apoptosis.	Induction of apoptosis.	

kinase), either at serine^{218} or threonine^{222} and activates it. Finally, MEK binds to ERK (MAPK) and phosphorylates first the tyrosine^{185} residue followed by the threonine^{183} residue and then dissociates from ERK. The monophosphorylated ERK then rebinds to an active MEK1 for dual phosphorylation and complete activation.

The major targets of activated ERKs are p90 ribosomal S6 kinase (Rsk) and the cytoplasmic phospholipase A_2 (Della Rocca, 1999). The ERKs also translocate to the nucleus to phosphorylate the transcription factor Elk-1 (on serine^{383} and serine^{389}). Most MAPKs phosphorylate Ets transcription factors that are involved in the induction of fos genes, whose products heterodimerize with Jun proteins to form activation protein 1 (AP-1) complexes. Although transcription factors are important MAPK targets, only a part of the active MAPK pool translocates to the nucleus and much remains in the cytoplasm and other subcellular compartments where they regulate post-transcriptional mechanisms involving cytoplasmic targets. Activated ERK can also phosphorylate the transcriptional factor CREB (cAMP response element protein) at Ser 133, which not only promotes neuronal survival, proliferation and differentiation but also plays a vital role in neuronal plasticity (Ginty, 1994; Lonze, 2002; Xing, 1996). Given the ubiquitous expression of the MAPK pathway, the high degree of evolutionary conservations, and the wide range of the cell surface stimuli that trigger ERK activation, it is not surprising to find that ERK is involved in a vast number of cellular functions including proliferation, differentiation, survival, migration etc. Earlier studies in a neuron-derived cell line have demonstrated that agonist stimulation of the 5-HT_{1A} receptor causes upregulation of the MAP kinase pathway, which results in an inhibition of the pro-apoptotic protein caspapse-3 (Adayev, 1999).

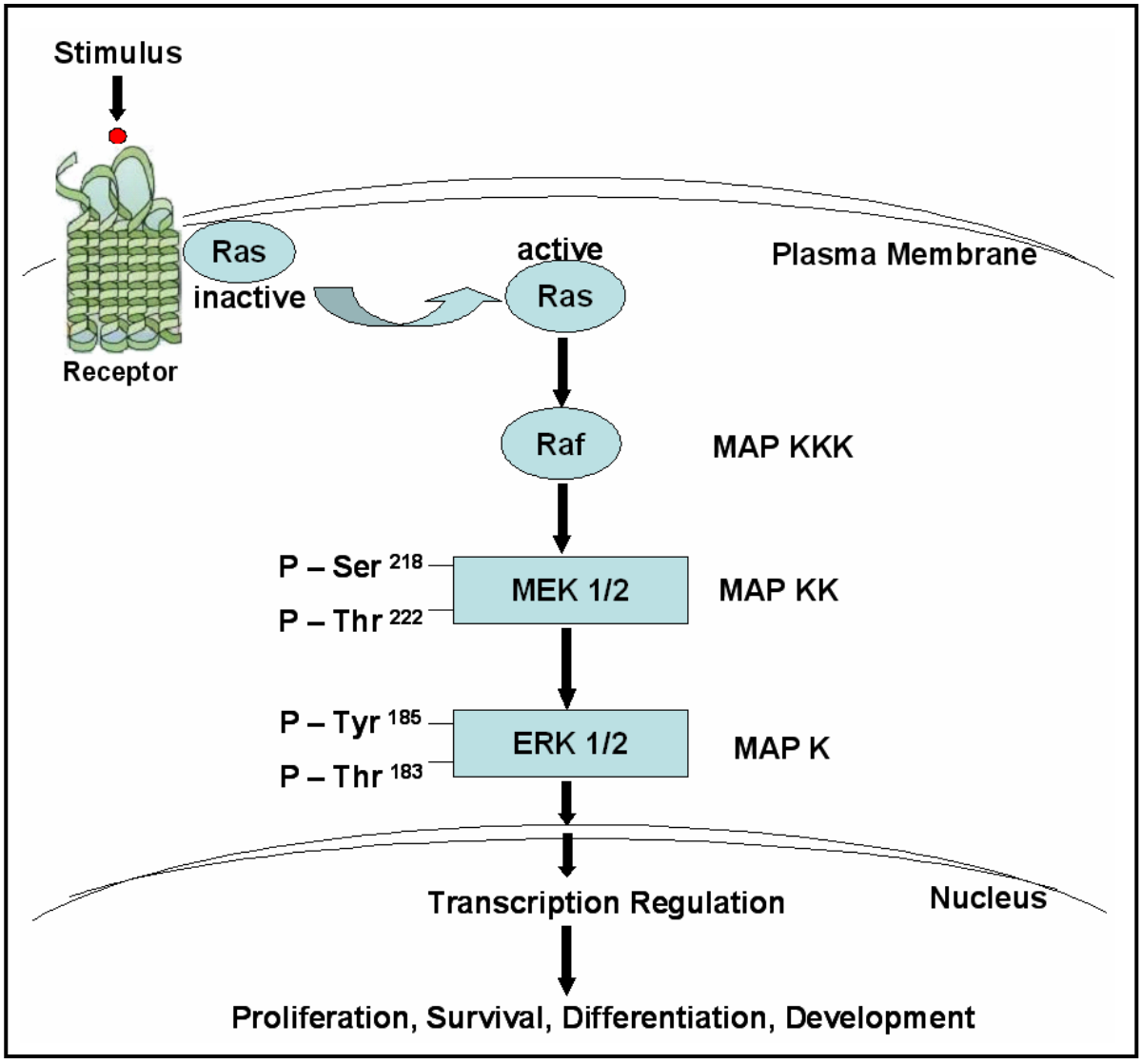


Figure 1.7. Schematic representation of stimulus induced signal transduction through MAPK pathway.

In response to the stimulus, the activated Ras-GTP molecule binds directly to Raf, causing its activation. Raf kinase phosphorylates MEK that in turn phosphorylates ERK 1/2, which further acts on downstream targets to result in stimulus-specific effects.

A) ERK signaling in neuronal proliferation and survival

Several lines of evidence have suggested that activation of the ERK cascade promotes proliferation and survival of cells. However, more direct evidence using mutations of the various signaling components has also been used to link the cascade to cellular proliferation. The activation of not only ERK1/2 but also ras and raf (the two proto-oncogenes, which encode two upstream activators of this signaling) is found to be essential for a cell to propagate growth and differentiation signals (Pages, 1993). Bonni and coworkers have shown that the MAPK signaling pathway promotes cell survival by a dual mechanism that modulates the cell death machinery directly by phosphorylating and thereby inhibiting the pro-apoptotic protein BAD, and by inducing the expression of prosurvival genes in a CREB-dependent manner (Bonni, 1999; Jin, 2002). Graves and coworkers have shown that activation of the MAPK cascade causes regulation of DNA synthesis through phosphorylation of carbamoyl phosphate synthetase II, a rate-limiting enzyme in pyrimidine nucleotide biosynthesis, which is required for mammalian cells to proliferate *in vivo* (Graves, 2000). The ERK/MAP kinase pathway signaling cascade has been described to be involved in the induction of the immediate-early gene product c-Fos *via* phosphorylation and activation of the ternary complex factor ELK1 as well as the Ets family and STAT family proteins. Alternatively it has been also shown that the Fos-related gene products Fra-1 and Fra-2 are highly upregulated after induction of MEK1 via a transcriptional factor AP-1 (Treinies, 1999). The AP-1 activity is also induced by heterodimerization of Fos and Jun proteins as mentioned before, which induces expression of cyclin D1 (Chang, 2001). Also, p42 MAPK is activated downstream of the Cyclin-dependent kinase 2 (Cdc2) during mitosis in *Xenopus* oocytes, that the mitotic

state is actively maintained during the period after Cdc2 inactivation, and p42 MAPK activity is essential for this maintenance (Gudagno, 1998). In addition, the ERKs, probably through (MAPK activated protein kinases) MAPKAPKs such as RSK, promote cell-cycle progression by inactivating MYT1, a cell-cycle inhibitory kinase (Palmer, 1998). Several studies have also linked ERK1/2 to the inhibition of apoptosis (Xia, 1995).

B) ERK signaling in neuronal plasticity

ERK signaling is involved in synaptic plasticity, specifically LTP, which is the best studied synaptic plasticity in the central nervous system (Thomas, 2004). Many forms of synaptic plasticity, such as NMDA receptor-dependent independent forms of LTP in the hippocampal area (CA1), LTP in the dentate gyrus, fear-dependent LTP in the amygdala and spatial learning, involve ERK (Thomas, 2004). Furthermore, inhibition of the ERK cascade markedly attenuates the induction of LTP but has no effect on the expression of established LTP in the hippocampus (English, 1997). It has been also shown that ERKs induce immediately early genes, and are involved in the late phase of long-term potentiation (LTP) and memory consolidation (Schafe, 2000). ERK activation is required for associative learning, memory, facilitation of synaptic plasticity and visual cortical plasticity in mammals (Atkins, 1998; Di Cristo, 2001). In addition to ERK1/2 (Atkins, 1998), their downstream targets, Elk-1 and p90Rsk-1 (Sananbenesi, 2002) are involved in associative learning and fear conditioning in mice.

ERK activation is also correlated with and is essential for structural plasticity, such as spine formation. Studies using time-lapse imaging in cultured hippocampal neurons have shown that ERK activation causes formation of new spines and filopodia. The ERKs also phosphorylate voltage-dependent K+ channels (Thomas, 2004).

These limited examples of the importance of the ERK signaling pathway illustrate the fact that it is a part of a complex signaling network, which finely regulates a variety of biological functions.

Protein Kinase C (PKC)

The protein kinase C (PKC) isozymes constitute a family of serine/threonine kinases, which play a major role in signal transduction of a cell to regulate cell division, proliferation, and apoptosis. Activation of the PKC signaling pathway could occur through either Gβγ or Gα subunit-mediated coupling to Phospholipase Cβ (PLCβ) with or without the involvement of PI-3K. Phospholipase Cβ, activated by the stimulation of a G protein-coupled receptor, cleaves phosphoinositide-bis-phosphate (PIP2) into two second messengers, diacyglycerol (DAG) and inositol-tris-phosphate (IP3). Cytosolic IP3 binds to the IP3 receptors on the endoplasmic reticulum (ER), which then releases Ca^{2+} from its storage. The DAG and Ca^{2+}, as discussed later in this section, are second messengers that activate PKC. In a widely accepted pathway of PKC signaling, it is activated upstream of MAPK by GPCRs. In our studies, we have observed participation of PKC in a more general and novel pathway with respect to MAPK via 5-HT_{1A} receptor activation.

Each PKC isozyme consists of a single polypeptide chain having two structurally well-defined domains: the amino-terminal regulatory domain and the carboxyl-terminal catalytic domain (Pears, 1995; Ron, 1999). The regulatory and the catalytic domains are connected by a hinge region that is highly sensitive to proteolytic cleavage by cellular proteases. The PKC isoforms can be divided into three basic groups on the basis of sequence homology of basic domains of the regulatory moiety (described below) and biochemical activity [Figure 1.8]. The two basic target modules are the C1 and C2 domains, and each comes either in a form that binds to a ligand (such as phorbol ester) or

in a form that lack determinants for ligand binding. The C1 domain is the DAG sensor and the C2 domain is the Ca^{2+} sensor.

1. The conventional/ classical PKCs- cPKCs (α, ßI, ßII and γ) contain functional C1 and C2 domains and respond to diacylglycerol, and Ca^{2+} signals.
2. The novel nPKCs (δ, ε, θ and η) contain a functional C1 domain and a non-ligand binding C2 domain: these isoenzymes respond to diacylglycerol, but not Ca^{2+} signals.
3. The atypical aPKCs (ζ and λ) contains a non-ligand-binding C1 domain and no C2 domain and, as a consequence, respond to neither diacylglycerol nor Ca^{2+}.

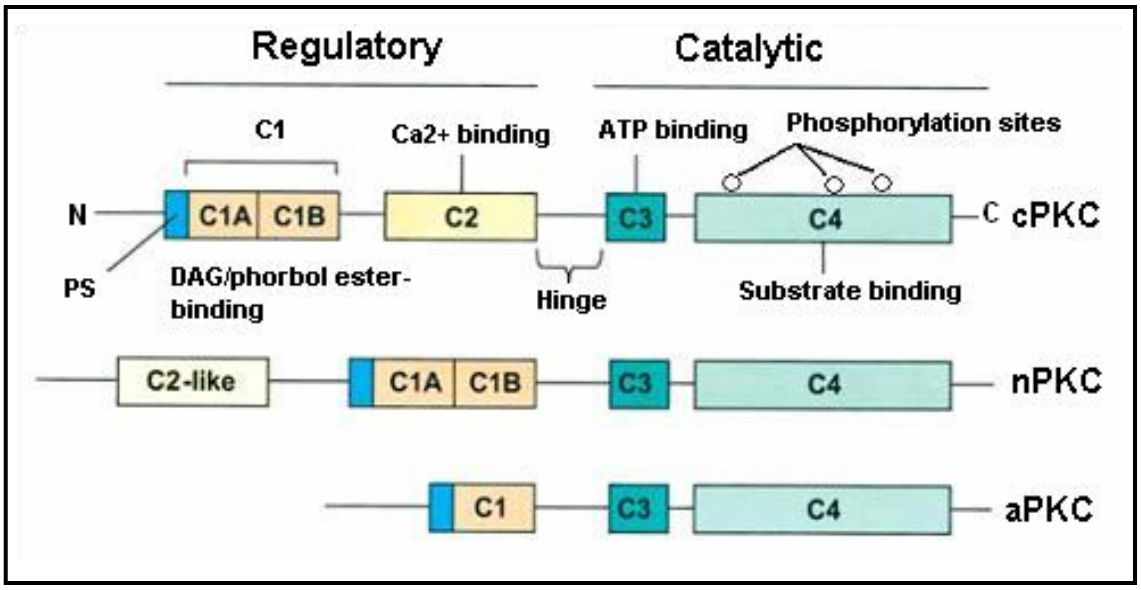


Figure 1.8. Schematic of primary structure of protein kinase C family members showing domain composition. PS= pseudosubstrate binding site, N= N terminal, C= C terminal (Modified from Tan, 2003).

The Regulatory domain: The regulatory region possesses motifs involved in the binding of DAG, Ca^{2+} and participates in protein–protein interactions that regulate PKC activity and localization. The regulatory domain of conventional PKCs is composed of two conserved membrane-targeting modules, C1 and C2 domains, as well as a pseudosubstrate region and variable regions. The Cys-rich C1 domain is present in all PKC isoenzymes but conventional and novel PKCs have two C1 domains- C1A and C1B. The ligand-binding pocket of the C1 domain is impaired in atypical PKCs, and these isoenzymes do not respond to either diacylglycerol or Ca^{2+} (Hurley, 1997). The C1 region contains the binding site for DAG in a phosphatidylserine (PS) dependent mechanism, whereas the C2 region interacts with anionic phospholipids (e.g. PS) whose binding is allosterically facilitated by the association of Ca^{2+} with the same region (Johnson, 2000; Oancea, 1998). At the tip of typical C1 domains, there is a hydrophilic ligand-binding cleft that is surrounded by hydrophobic residues. Binding of the ligand-diacylglycerol, caps the hydrophilic cleft and generates a continuous hydrophobic surface, which enables the C1 domain to penetrate membrane without any conformational change (Zhang, 1995; Newton, 2001). The C2 domain is present in conventional and novel PKCs but the novel C2 domain lacks key residues involved in Ca^{2+} binding as a consequence they lack Ca^{2+} binding. Important Aspartic residues required for Ca^{2+} binding are not present in the C2-like domain of nPKCs. It is believed that this domain in nPKCs may be involved in phospholipid binding and regulates lipid activation of nPKCs (Mellor, 1998). The C2 domain acts as a membrane docking module, where the Ca^{2+} ions and basic residues contribute to electrostatic membrane binding. There is a pseudosubstrate site or autoinhibitory domain which has all the sequence characteristics

of a substrate for PKC but it lacks the serine or threonine residues to act as a phosphate acceptor and instead it contains a conserved alanine residue. In the inactive state, the autoinhibitory sequence is thought to bind to the active site and prevent substrate access. Thus, PKC is maintained in an inactive conformation by binding of the pseudosubstrate sequence to the substrate-binding cavity (Pears, 1990).

The regulatory domain serves two key functions:

i) It targets the kinase to appropriate locations in the cell

ii) It regulates the kinase with its autoinhibitory/ pseudosubstrate unit. (Newton, 2003)

The Catalytic domain: It is the kinase domain and includes motifs involved in ATP and substrate binding. It contains three conserved phosphorylation sites without which the kinase is catalytically inactive and immature.

1. The activation loop site- It has a threonine residue which is different for different kinases in their activation loop segment. It must be phosphorylated by a novel kinase-PDK-1 (phosphoinositide dependence kinase 1). Hence, it is an upstream kinase for conventional, novel and atypical PKC family members. PKC becomes a substrate for PDK-1 when it is in the membrane-bound 'open' conformation in which the pseudosubstrate does not occupy the substrate-binding cavity (Newton, 2003).
2. The turn motif- The turn motif is a conserved phosphorylation site in a segment of the C-terminus. Phosphorylation at the activation loop triggers rapid auto-phosphorylation of a motif in a Pro-rich domain.
3. The hydrophobic motif- The third conserved auto-phosphorylation site contains a

serine or threonine residue flanked by hydrophobic residues. It makes stable contacts with the activation loop segment. It also provides a docking site for PDK-1. This site is exposed for PDK-1 binding in the unphosphorylated form of protein kinase C but becomes masked in the phosphorylated (and inactive) conformation (Gao, 2001). Mutation of this site does not affect the kinase activity of PKC but is necessary for stabilization and sub-cellular localization of the enzyme (Edwards, 1997). There is evidence suggesting that this site is autophosphorylated (Behn-Krappa, 1999).

The Life Cycle of PKC: The intrinsic function of protein kinase C isozymes is regulated by three mechanisms:

i) phosphorylation of the enzyme, which primes it for catalysis,

ii) cofactor binding (such as DAG, Ca^{2+}), which allosterically activates the enzyme, and

iii) interaction with targeting proteins that positions it near its regulators and substrates.

Newly synthesized PKC associates with the membrane in an open conformation in which the pseudosubstrate is released from the substrate-binding pocket and in which the C-terminus is exposed to allow PDK-1 to bind (Dutil, 2000) [Figure 1.9]. Before PKC is ready to respond to second messengers, it must first be phosphorylated at three conserved positions: the activation loop and two positions (turn motif, hydrophobic motif) at the carboxyl terminus of the protein. PDK-1 phosphorylates the activation loop of PKC isozymes, and this phosphorylation is essential for the maturation of PKC isozymes. This

phosphorylation does not activate PKC, but rather, it serves to trigger the two carboxy-terminal (auto) phosphorylations that are required to lock PKC in a catalytically competent conformation (Dutil, 1998). This phosphorylation event does not, however, regulate the maximal catalytic activity of the mature enzyme. The phosphorylated enzyme is released into the cytosol where it is maintained in an inactive conformation by the bound pseudosubstrate. The activity of all isozymes of protein kinase C is regulated by phosphatidylserine, an aminophospholipid found exclusively on the cytoplasmic leaflet of membranes. Binding studies revealed that, in the presence of diacylglycerol, protein kinase C binds phosphatidylserine-containing surfaces with much higher affinity than membranes composed of other anionic lipids (Johnson, 1998). The receptors that trigger activation of phospholipase C lead to the generation of two second messengers, diacylglycerol (DAG) and inositol trisphosphate. Inositol trisphosphate elevates intracellular $Ca^{2+}$by binding to its intracellular receptors. The increased intracellular Ca^{2+} and membrane DAG promote targeting of conventional PKCs to the plasma membrane and its subsequent activation (Kandel, 2000). It has been also proposed that the protein initially binds to the membrane surface via Ca^{2+}-dependent PS binding of the C2 domain, enhancing hydrophobic interactions and membrane affinity. Once bound to PS-containing membranes, the protein undergoes conformational changes that include the insertion of C1A domain into the membrane causing its activation (Medkova, 1999). This membrane penetration allows for optimal DAG binding and drives the release of pseudosubstrate region from the active site, thereby allowing downstream signaling. Thus, the conventional PKC isozymes have two regulatory switches: phosphorylation of the activation loop, which renders the enzyme catalytically competent and cofactor binding,

which removes the autoinhibitory domain from the active site. In contrast, the atypical PKC isozymes are regulated by phosphorylation of the activation loop. This serves as a direct 'on–off' switch for catalysis (Dutil, 1998). In the case of novel PKCs, which contain a Ca^{2+}- independent C2 domain in the amino terminus, followed by the C1A and C1B domains in the regulatory region, membrane targeting and activation is regulated by phosphorylation, DAG and other lipids, and adaptor proteins. For example, the differential activation mechanisms of PKCε and PKCβ are based on different structural and functional properties of their C1 domains. Due to high DAG affinities and conformational flexibility, both C1A and C1B domains of PKCε are involved in the membrane binding whereas only C1A domain is involved in the case of PKCβ (Stahelin, 2005).

In its active conformation, PKC is rapidly dephosphorylated (Newton, 2003). When extracellular signals are turned off, the enzyme returns to the cytoplasm by a process that is dependent on the autophosphorylation status of PKC. The hypothesis that autophosphorylation of PKC keeps the enzyme from associating with the plasma membrane and thus retains the enzyme in the cytoplasm is further supported by the fact that PKC autophosphorylation (for PKC βII at Ser ^{660}) provides a further force for reverse translocation and signal termination (Feng, 2000). PKC is then degraded by a novel E3 ubiquitin ligase named RING finger (Violin, 2003).

The preferential occurrence of different PKC isoforms in the central nervous system (CNS) suggests that these enzymes are involved in a variety of neuronal functions.

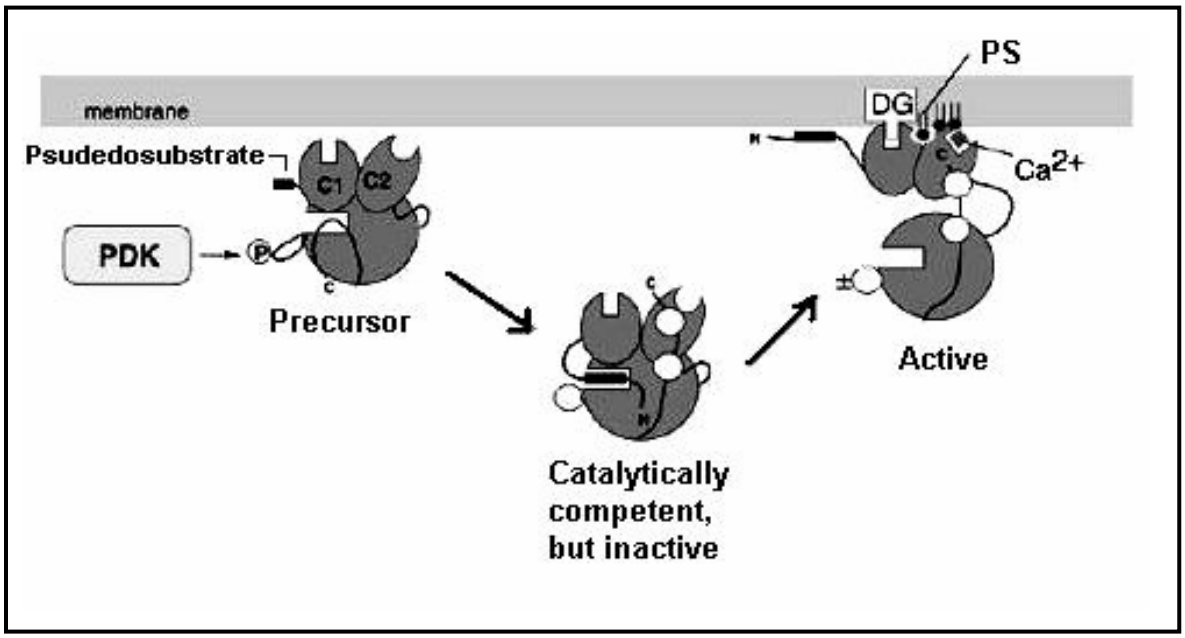


Figure 1.9. Model showing activation and recruitment of PKC to the plasmamembrane. PS= Phosphatidylserine.

A) PKC and Neuronal functions- PKCs play an important role in cell proliferation, differentiation, development, apoptosis and tumor promotion. In addition, neuronal functions of PKC include modulation of ion channels and receptor regulation by serotonin (5-HT) and noradrenaline (NA) transporters, use-dependent synaptic plasticity (long-term potentiation), and presynaptic release of neurotransmitters (Popoli, 2000; Hussain, 2003; Linden, 1989). Earlier studies have also shown that PKC activation blocks apoptosis (Adayev, 1999; Tan, 1999). In general, it has been suggested that PKC is a promoter of spreading and migration in many cell types. In particular PKCα and PKCε have been suggested to promote neuronal and glial cell migration, respectively (Besson, 2002; Larsson, 2006) . There are also many studies indicating important roles for novel PKC isoforms in promoting neurite outgrowth (Larsson, 2006).

B) PKC and Development- Signaling events regulate many processes that initiate the program of early development. Within the cell many of these changes are mediated by the activation or inactivation of kinases and phosphatases. The activation of PKC also plays an important role during development. It has been shown to be involved in at least two developmental transitions during early development, fertilization and embryonic compaction. Each cell contains more than one isotype of PKC in a distinct spatial pattern. Thus, depending on the signaling cascades stimulated in the cell, different PKC isozymes mediate distinct signaling pathways. Different isozymes of PKCs are developmentally regulated, which is discussed in detail later in the discussion. Moreover, PKCs are predominant in certain types of tissues and they are concentrated in different subcellular compartments within the cell, suggesting that they may function within specific regions of the cell (Pauken, 2000) [Table 1.4].

Table 1.4. PKC isozymes can be divided into four groups, expressed differentially during development.

ISOFORMS OF PKC	SUB TYPES	CO-FACTORS	PRE-DOMINANT TISSUE LOCATION	ROLE IN CELLULAR RESPONSES (Tan, 2003)	DEVELOPMENTAL PROFILE
I. CONVENTI-ONAL/ TYPICAL	α	PS, DG, Ca^{2+}.	Brain	i) B-cell development. ii) Promoter of cell spreading and migration. iii)Cell survival and suppression of apoptosis.	A) In human CNS, (Sposi, 1989) - gene products detected 6 weeks of PC, increases until 9 weeks. B) In Chick, - low levels during embryogenesis. C) In Rats, (Raz, 1998; Yoshida, 1988), - low at 1 week and increases between 2-3 weeks postnatal. - embryogenesis (metaphase II eggs), mRNA, and protein detected. D) In Mouse, - decrease in concentration during embryogenesis.
	β I β II	PS, DG, Ca^{2+}.	Brain	T-cell migration during inflammation.	A) In Humans CNS, (Sposi, 1989) - 6 weeks PC, increases until 9 weeks. B) In Chick, - low levels during embryogenesis. C) In Rats, (Raz, 1998; Yoshida, 1988) - increase post-natal (3days-3week) and constant thereafter. - embryogenesis metaphase II eggs, mRNA,

					and protein detected. D) In Mouse, (Pauken, 2000) - embryogenesis (metaphase II egg), protein detected.
	γ	PS, DG, Ca^{2+}.	Brain, Spinal cord.		A) In Human CNS, (Sposi, 1989) - postnatal and adults life. B) In rats, - embryogenesis (metaphase II eggs), mRNA, and protein detected. - increase post-natal (3days-3week) and constant thereafter. C) In Mouse, - present during embryogenesis.
II. NOVEL	δ	PS, DG.	Brain, Lymphoid tissue.	Regulating B-cell immunity.	A) In Rats, - expressed post-natal. - embryogenesis (metaphase II eggs), mRNA, and protein detected. B) In Mouse, - decrease in con. during embryogenesis.
	ε	PS, DG	Brain, Epithelial tissue	i) In Macrophage activation. ii) Glioma cell migration. iii)Neurite outgrowth.	A) In Chick, - predominant and higher from E6. B) In Rats, - positive mRNA, protein expression in embryonic brain.
	η	PS, DG	Brain, Lymphoid tissue.		A) In Rats, - expressed post-natal.

	θ	PS, DG	Brain, Lymphoid cells.		A) In Rats, - expressed post-natal.
III. ATYPICAL	ζ	PS	Brain	Normal function of B-cells.	A) In Chick, - low levels during embryogenesis. B) In Rats, - positive mRNA, protein expression in embryonic brain.. C) In Mouse, - embryogenesis (metaphase II egg), protein detected.
	λ	PS	Brain	Activation of signaling cascade during early B-cell development.	A) In Rats, - positive mRNA, protein expression in embryonic brain. B) In Mouse, - embryogenesis (metaphase II egg), mRNA, protein detected. - present during embryogenesis
	PKD μ, ν	PS	Brain		A) In Rats, - expressed post-natal. - positive mRNA, protein expression in embryonic brain. B) In Mouse, - metaphase II egg, mRNA, protein detected. - present during embryogenesis

CNS= Central Nervous System, PS = Phosphotidylserine, DG = Diacylglycerol, PC= Post conceptational.

CELL CYCLE:

Generally, signals such as mitogens, growth factors, cytokines can direct cells to enter the cell cycle. The activation of ERK/MAP kinase pathway has been identified as a major signaling cascade that enables cells to leave the quiescent state (G0) and pass through G1/S transition of the cell cycle.

In an organism, the rate of cell division is a tightly regulated process that is intimately associated with growth, differentiation and tissue turnover. Resting cells are said to be in the G0 phase (quiescence) of the cell cycle. The mammalian cell cycle consists of four discrete phases: S phase, in which DNA is replicated; M phase, in which the chromosomes are separated into two new nuclei in the process of mitosis. These two phases are separated by two so called "Gap" phases, G1 and G2, in which the cell prepares for the upcoming events of S and M, respectively. At a point in late G1, termed the restriction point (R), cells become largely refractory to the growth factors and once past R they are committed to completing the mitotic cycle. When a cell is in any phase of the cell cycle other than mitosis, it is often said to be in the interphase. The passage of a cell through the cell cycle is controlled by various proteins in the cytoplasm [Figure 1.10].

1. Cyclins: A class of proteins found in eukaryotic cells that fluctuate through the cycles of synthesis and degradation during the cell cycle. When synthesized, cyclins activate cyclin-dependent protein kinases by binding to them. Cyclins are synthesized or degraded according to the cell's readiness to move into the next stage of the cell cycle and as such they do not have enzymatic activity of their own. There are numerous cyclin proteins each of which is associated to a certain stage of the cell cycle.

2. Cyclin-dependent kinases (Cdks) - Cyclin dependent kinases (Cdks) are serine/threonine kinases involved in the regulation of the cell cycle. As their name suggests, the Cdks require associating with activator proteins called cyclins for their activity. They are regulated by phosphorylation and dephosphorylation events. Different cyclin-Cdk complexes are activated at different points in the cell cycle. Each cyclin associates with two or more Cdks and most Cdks associate with one or two cyclins (Murray, 2004). Their levels in the cell remain fairly stable, but each must bind the appropriate cyclin (whose levels fluctuate) in order to be activated. They add phosphate groups to a variety of protein substrates that control processes in the cell cycle [Figure 1.10].

- **G_1 Phase** - Three Cyclins D-D1, D2, D3 combine with Cdk4 and Cdk6 to signal the cell to prepare the chromosomes for replication.
- **S-phase Promoting Factors (SPF) -** Cyclins E and Cyclin A associate with Cdk2 to enter the nucleus and prepare the cell for duplication of its DNA (and its centrosomes).
- **Mitotic cyclins includes Mitosis promoting factors (MPF)-** They initiates assembly of the mitotic spindle, breakdown of the nuclear envelope and condensation of the chromosomes **and Anaphase promoting factors** allows the sister chromatids to separate and move to the poles **-** Cyclins B associates with Cdk1 and A associates with Cdk 2 or Cdk1 respectively.

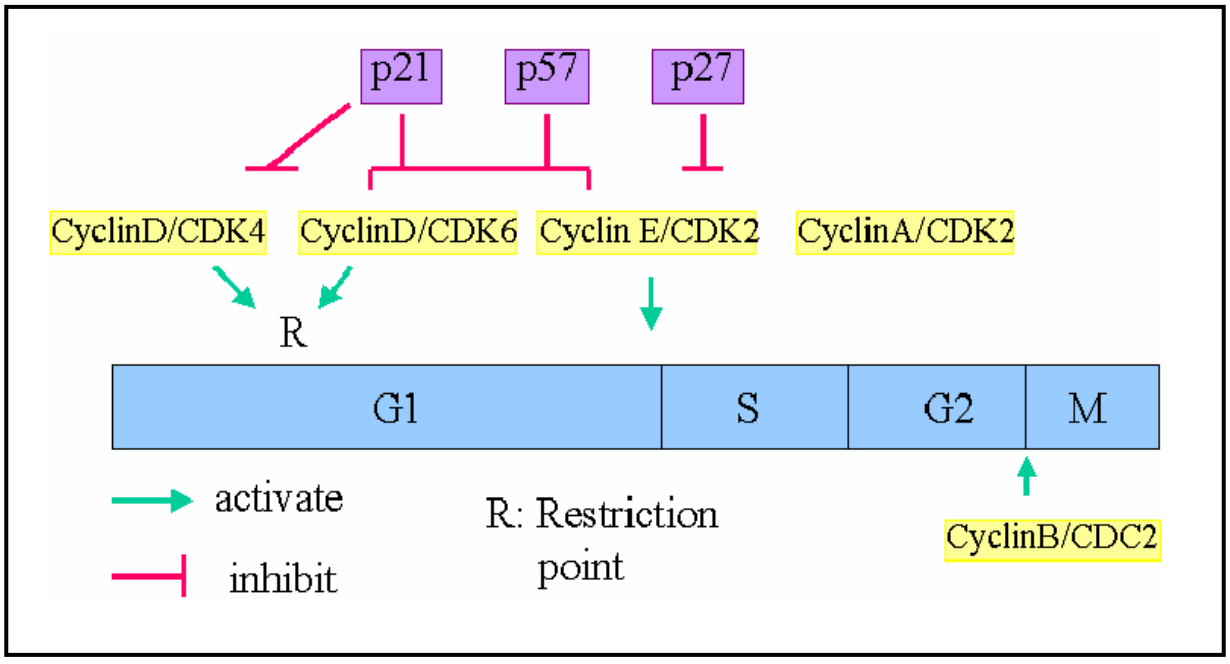


Figure 1.10. Schematic representation of regulation of cell cycle by various cyclins and cyclin-dependent kinases (CDKs).

A) Cell cycle and Development- In the early embryonic cell cycles, there are three cyclins and two Cdks. Two cyclins, A and B, rise during interphase and fall during mitosis, whereas the third, cyclin E, remains constant. Cyclin E supports DNA replication and centrosome duplication. Cyclin A, which is nuclear, supports both of these processes and mitosis, and cyclin B, which is cytoplasmic, supports mitosis alone (Pines, 1991; Strausfeld, 1996). Cdk1 binds cyclins A and B and Cdk2 binds cyclins A and E. Also, in early embryonic cell cycles, DNA replication begins as soon as cells leave mitosis whereas after embryonic development, animal cells spend appreciable time in G1 phase due to inhibitory factors. In animal cells, post-embryonic development requires Cyclin D-Cdk4/Cdk6 complexes to promote G1 progression by partially phosphorylating the retinoblastoma tumor-suppressor-protein (Rb), a regulator of G1 exit. This process is completed by the CyclinE-Cdk2 complex, thereby inactivating Rb's ability to act as a transcriptional repressor in a complex with transcriptional factor E2F, which in turn activates S-phase genes. Mitogen-induced Ras signaling promotes induction of the cyclinD1 gene via activating E2F family members in a similar fashion as described above. This eventually induces cyclin-D1 expression and cell cycle. Further details are included in the discussion. Inactivation of Ras in cycling cells, causes a decline in CyclinD1 protein levels, accumulating the hypophosphorylated growth-suppressive form of Rb and G1 arrest (Peeper, 1997). It also regulates cyclin D1 expression as well as association with cdk for complex formation during the cell cycle (Lavoie, 1996; Weber, 1997). Assembly of newly synthesized cyclin D1 with CDK4 also depends on the same kinase cascade (Cheng, 1998). The activity of G1 cyclin-Cdk complexes is regulated, in part, by the CDK inhibitors p16, p21 and p27. The CDK inhibitor- p16 directly inhibits

the activity of cyclin D-CDK4/ CDK6 and both p21 and p27 inhibits the cyclin E-CDK2 holoenzymes. (Lundberg, 1998). Thus Cyclin D expression plays a crucial role in regulating cell growth and proliferation (Sherr, 1999).

With developmental progression, the subcellular localization of most cell cycle proteins has been shown to be increasingly shifted from nuclear to the cytoplasmic compartment. However, even in the adult, cell cycle-related proteins have been found in terminally differentiated pyramidal and granule neurons, suggesting supplementary functions in differentiated neurons that might be associated to neuronal plasticity (Schmetsdorf, 2005).

All known cyclins are targeted to the proteasome by the addition of a chain of ubiquitins, but the details of this conjugation differ for the different cyclins. G1 cyclins are ubiquitinated by the SCF (Skp1-Cdc53-F-box protein) complex, whereas mitotic cyclins are ubiquitinated by the anaphase-promoting complex.

OBJECTIVE OF THIS STUDY:

The serotonin-1A receptor is required for normal neonatal development of the mouse brain. Based on earlier studies performed in our laboratory as well as other groups, we have postulated that 5-HT_{1A}-R→ MAPK signaling plays a bivariate role by stimulating neurogenesis during early postnatal stages and then switching in its function to cause increased strengthening of synaptic connections in the hippocampus during the later stages.

To achieve this, we have monitored activation of MAPK isozymes ERK1/2 via 5-HT_{1A}-R signaling and its effect on cell division and maturation at two developmental stages (P5 and P15). We have also revealed involvement of different PKC isozymes at these stages and their transition in 5-HT_{1A}-R signaling.

Thus, the major purpose of this project is to detect and measure 5-HT_{1A}-R mediated activation/ inhibition of key signaling molecules which will have therapeutic importance in developmental disorders and may shed new light on the etiology of multiple brain disorders.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Mice (Age: Postnatal day 6-P6 and postnatal day 15-P15) of C57 black wild type were used for the experiments. Animals were kept in a 12 hour light/ dark cycle with libitum access to food and water.

MATERIALS

The antibodies to P-Erk, P-PKCα, P-Akt, Cyclin-D1, and P-CREB were obtained from Cell Signaling (Beverly, MA, USA). P-PKCε, Erk1/2 and the horse radish peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-ß-actin antibody, BrdU antibody, 8-OH-DPAT, WAY100635, and PD98059 were obtained from Sigma Chemicals (St. Louis, MO, USA). Bisindolylmaleimide or GF109203X (GFX) was purchased from Calbiochem (La Jolla, CA, USA). The NeuN antibody was procured from Chemicon (Temecula, CA, USA). The Alexafluor- labeled fluorescent secondary antibodies and the fluorescent Nissl stain were obtained from Molecular Probes (Eugene, OR, USA).

METHODOLOGICAL APPROACHES

Organotypic culture of hippocampal slices

The procedure of isolation of mouse brain slices for culture was adopted from various publications (Stoppini, 1991; Xiang, 2000) . Hippocampal transverse slices (400μ thick) were prepared from C57 wild type mice pups of postnatal d6 and d15. Briefly, mouse pups of specific ages were anesthetized with ketamine (100 mg/kg) and decapitated. Under sterile conditions, the brains were isolated and then cut at 60° from the longitudinal fissure at the top using a hippocampus dissecting tool to expose the hippocampus. The two hemispheres containing the hippocampi were then placed for 30–40 min in modified Gey's balanced salt solution (mGBSS) (pre-chilled to 4°C) while bubbling a mixture of 95% O_2 and 5% CO_2. mGBSS composition (in mmol/L): $CaCl_2$ (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.

Individual hippocampi were isolated using dissection tools and then 400-μ thick transverse slices were prepared using a tissue chopper (Stoelting, IL, USA). The slices were placed in ice-cold mGBSS and inspected using a dissection microscope for the presence of uninterrupted neuronal layers- characteristic of the hippocampal structure. Only such slices were placed on Millicell CM filters (Millipore, Bedford, MA, USA). Up to five slices were placed on each filter and the filters were placed in a six-well dish with 1 mL of medium [Figure 2.1]. Thus, slices were maintained in culture at the interface between air and a culture medium containing high potassium (25% horse serum, 50% basal essential media-Eagles, 25% Earle's balanced salt solution (EBSS), 25 mmol/L Na-HEPES, 1 mmol/L glutamine, 28 mmol/L glucose, pH 7.2) for the first 2 days. After

incubation at 32°C in a 5% CO_2 atmosphere, the culture medium was changed to physiological potassium medium (20% dialyzed fetal bovine serum, 5% basal essential media-Eagles, and EBSS modified to adjust the potassium concentration to 2.66 mmol/ L). After 20% dialyzed fetal bovine serum treatment for 2 days, and before drug treatment, the slices were placed in serum-free medium (the same medium as above, but without serum) for 1 h then slices were treated with drugs.

Note: These tissue samples were isolated from post-synaptic regions, therefore, would not synthesize serotonin. In addition, they were cultured in the absence of serotonin by using dialyzed serum (serum, dialyzed for 12-18 h to get rid of all small molecules below MW 1000 kDa is available from Omega Scientific, CA) which was tested by HPLC for the absence of serotonin.

Drug treatment of slices

The slices were routinely treated with drugs on the fourth day of culture. The inhibitors were added 30 min before the agonist (100 nM 8-OH-DPAT). The concentrations of antagonists and inhibitors were as follows: WAY100635 (4-10 μM), PD98059 (25-50 μM), GFX (2-4 μM). After drug treatment, the slices were either fixed in 4% paraformaldehyde or lysed as discussed in the following sections.

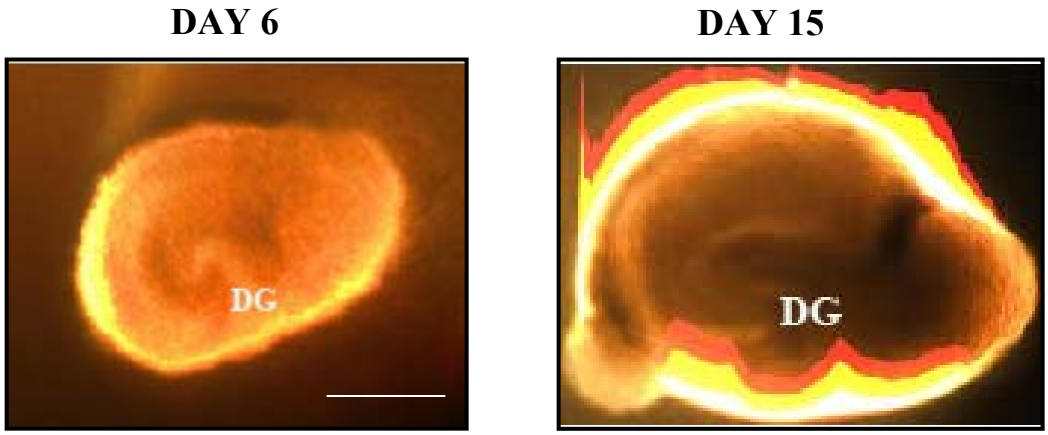


Figure. 2.1. Organotypic cultures of hippocampal slices.

The hippocampal morphology is retained in the cultured slices obtained from mice at various postnatal stages. Mouse pups are anesthetized by ketamine injection (100 mg/Kg) then the brains are dissected out, the hippocampi separated and sliced to 400-μ thickness using a tissue slicer. The slices are cultured on membranes at the interphase of medium and air in inserts placed in six-well plates. Pictures above show such cultured slices in inserts at 40X magnification. This figure also shows that the dentate gyrus (DG) structure becomes better defined at d15. Scale bar, 200 micron.

Western Blotting

The drug-treated slices were lysed in 1 ml RIPA buffer (PBS containing 1% NP_4O, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4 plus freshly added protease inhibitor cocktail; Boeringer), the lysate (10 µg protein) was resolved on a 7-16% gradient acrylamide gel, protein bands transferred to nitrocellulose membrane, blocked in 5% solution of dry milk and then probed with an ERK1/2 antibody (1: 500) followed by treatment with HRP-linked goat anti-rabbit IgG (1:50,000). Following probing with ERK1/2 antibody, the blot was stripped by incubating for 1 hour at room temperature in 0.2 M glycine (pH 2.5), and then blocked in 5% dry milk and reprobed using a monoclonal, phospho-ERK specific (anti-active) antibody at 1: 1000 dilution and then with horse radish peroxidase (HRP)-labeled anti-mouse IgG (1: 5000). The immunoreactive bands were visualized using the Supersignal luminal kit (Pierce) and incubation with an X-ray film. The ERK1/2 bands were used to confirm that the increase in P-ERK bands were not due to an increase in the amount of ERK1/2 proteins (a loading control). Since P-ERK, P-CREB, and P-PKCα antibodies (Cell Signaling, CA) could not be stripped off in successive stripping/reprobing, they were always used in the final probing. Antibody concentrations used for the other antigenic markers/signaling proteins were as follows: P-CREB (1:1000), P-PKCα (1:1000), P-PKCε (1:1000), P-Akt (1:5000), Cyclin D1 (1:1000), ß-actin antibody (1: 10,000).

Immunostaining of Slices

The cultured and drug-treated slices were washed quickly with chilled 10 mM phosphate buffer (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 48-well

plate in TBS. This was followed by 2-3 washes (15min each) with 1X TBS. For BrdU staining, free-floating sections were first incubated for 30 min in 2N HCl at 37 °C, and then rinsed 3X (15' each) with TBS. Sections were then blocked in TBS-X (TBS-0.1% Triton X-100-3% serum from the animal in which secondary antibody was raised) for 1 hour at room temperature (RT). This was followed by treatment with primary antibody in TBS for 48 hours at 4 °C with gentle rocking. Antibody concentrations used: P-ERK (1:400), BrdU (1:500), NeuN (1:150). The sections were next washed 3 x 15' at room temperature with TBS and then treated with fluorescent, secondary antibodies covalently linked to AlexaFluor 488 (green) (1:200), or AlexaFluor 568 (red) (1:200). After 48 hours of secondary antibody treatment at 4 °C, the sections were washed in TBS and then mounted on slides with ProLong Gold antifade reagent (Molecular Probes, Eugene, CA) for visualization and photography using a Laser scanning- confocal microscope.

Confocal microscopy, cell counting, and statistical analysis of the immunstained slices

Using a Nikon C1-LU3 laser scanning confocal system and 488 nm exciting wavelength for NeuN and 568 nm for BrdU labeled cells, the slices were viewed at 4x and 20x. The EZC1-system software was used to determine the total thickness of each slice after adjusting channels to obtain pictures from each exciting wavelength separately while blocking the laser beam of the other, exciting wavelength. Approximately 1-μ thick layers were used (adjusted by software) to obtain Z-stacked images. Subsequently, superimposed images were created from the individual colored images. Cell counting was carried out using the software "ImagePro", included in the software package that was

obtained with the confocal microscope. Statistical analysis was carried out using ANOVA with Bonferroni post-hoc test.

Recording fEPSP from acutely isolated hippocampal slices

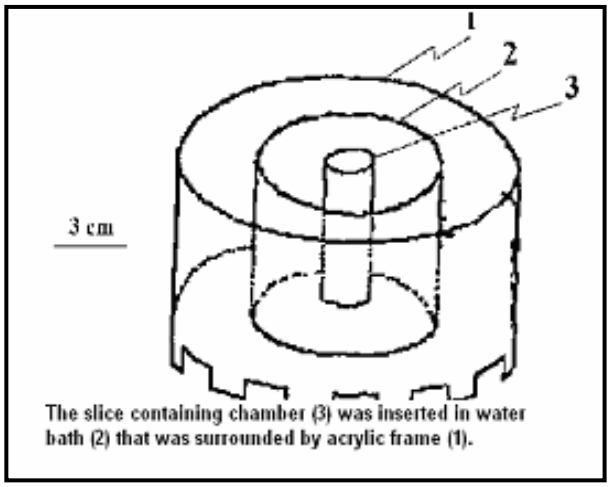
Recording from acutely isolated hippocampal slices from mice at P6 and P15 was performed according to standard procedures used earlier (Wieraszko, 2004). Following decapitation, the brain was removed from the skull and the two hippocampi were dissected out into the ice-cold Ringer solution containing (in mM): NaCl-124, KCl-3.1, KH_2PO_4-1.3, $MgSO_4$-1.3, $CaCl_2$-3.1, $NaHCO_3$-25.5, glucose-10.0. Both hippocampi were sliced into 350-400μ sections using a manual tissue chopper and the sections placed in an incubation chamber containing Ringer solution (at 33 °C), which was constantly oxygenated with O_2/CO_2 (95:5). Following a 1 hour incubation period, the sections were transferred to the interface of buffer and air, and the recording chamber was maintained at 33°C constantly oxygenated with O_2/CO_2 (95:5) [Figure 2.2a]. A bipolar, stimulating electrode was placed on Schaffer collateral-commissural fibers (Schaffer Collateral) near the CA3 region and the recording electrodes was guided into the radiatum dendritic layer of CA1 to record EPSP [Figure 2.2b]. fEPSP is the potential induced outside the post-synaptic neuron by the excitatory post-synaptic potential generated within the post-synaptic cell. The initial slope of the negative wave was taken as a measure of field EPSP (fEPSP). The strength of the stimulation was adjusted to obtain potential in the range of 30-40% of the maximal response. The potential was monitored at this level for the next 15-20 minutes, and experiments were performed only on slices that showed stable responses to low frequency (0.03 Hz) stimulation. The slices that did not show the stable response were discarded. Then the agonist (8-OH-DPAT-100 nM) was added to the

recording chamber. The recording was continued for at least another 40 minutes although the agonist-triggered response was observed 25 min after drug addition. The antagonists and inhibitors were added at the same time as the agonist. Data were presented as mean ± S.D. Statistical analysis was carried out using student's t- test.

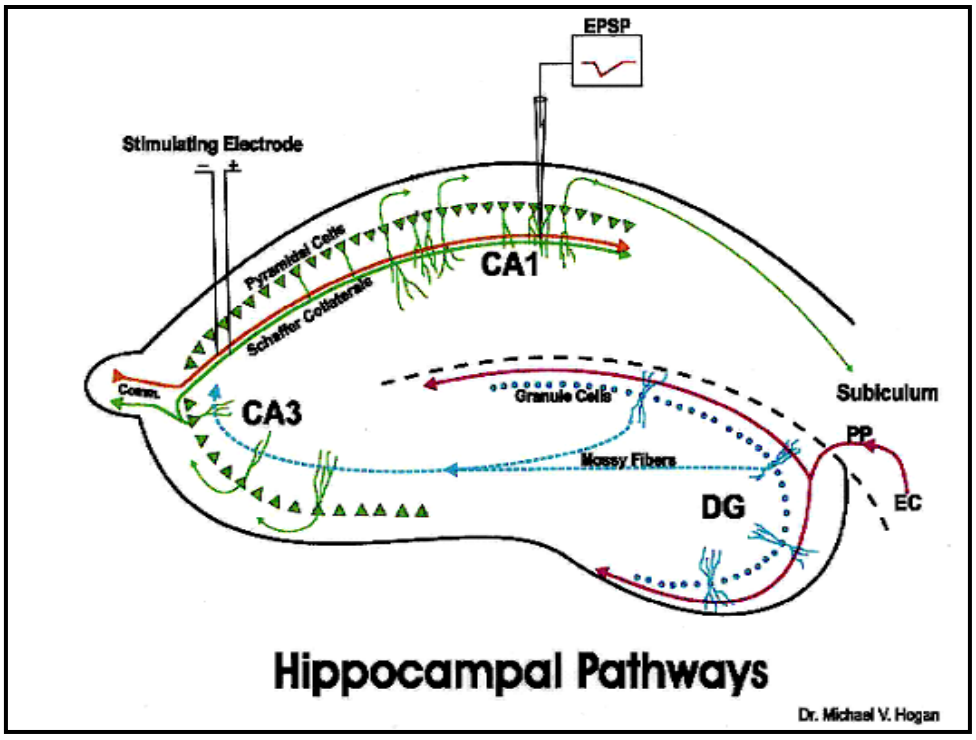
A change in this response represents either a change in the degree of synaptic activation, or an alteration in intrinsic excitability of the neuron (Anderson, 1971). A response recorded from the dendritic region (fEPSP) reflects the current generated by excitatory synaptic events (Lomo, 1971; Bliss, 1993). Any change in this response represents a real change in the potency of the synapses being activated which also reflect alteration in the neurotransmission process.

Statistical analysis

Statistical analysis of sets containing more than two groups was carried out using One Way ANOVA with Bonferroni Post Hoc Test ($\alpha = 0.05$). Paired t-test was used to compare between two sets (e.g. with and without 8-OH-DPAT treatment) from triplicate or multiple repeats of the same experiment.



(a)



(b)

Figure 2.2. 5-HT_{1A}-R mediated augmentation of fEPSP was measured in acutely isolated hippocampal slices at P6 and P15. **(a)** The recording chamber was maintained at 33°C with constant oxygen **(b)** The stimulatory pulse is applied to the Schaffer collateral axons near the CA3 region and the post-synaptic effect (fEPSP) of this pulse was recorded at the pyramidal cell dendrites at the CA1 layer, which form synaptic contacts with the Schaffer collaterals.

CHAPTER 3

EXPERIMENTAL RESULTS

Agonist stimulation of the 5-HT_{1A} receptor causes prolonged activation of ERK1/2 in hippocampal cultured slices from P6 (4 Days *in vitro*- 4DIV)

Earlier studies from our laboratory had demonstrated 5-HT_{1A} receptor-mediated activation of ERK1/2 in the differentiated, mouse hippocampus-derived cell line HN2-5 (Adayev, 2003). To test the presence of the same pathway in mice, cultured hippocampal slices from day 6 pups (cultured four days *in vitro*- 4DIV) were treated under serum-free conditions with different concentrations of the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT). A dose–response analysis (1 nmol/L to 1 μmol/L) for 8-OH-DPAT revealed that 100 nmol/L concentration of this 5-HT_{1A} receptor agonist was optimal for the activation of ERK1/2 (measured by double phosphorylation of each of the isozyme at T^{202} and Y^{204}). Maximum activation occurred after 10 min of agonist treatment. The activation of ERK1/2 was prolonged, as it persisted for the entire period of measurement (60 min) [Figure 3.1a and Figure 3.1b]. The 8-OH-DPAT-evoked activation of ERK1/2 was reversed in the presence of 4 μmol/L WAY100635 (a 5-HT_{1A}-R antagonist), 50 μmol/L PD98059 (an inhibitor of MEK, immediate upstream factor of ERK1/2 in the MAPK pathway), and 2 μmol/L GFX (a general PKC inhibitor). The P-ERK1/2 band intensities were normalized to the corresponding ERK1/2 band intensities. Statistical analysis of normalized P-ERK band intensities by ANOVA revealed significant activation of ERK1/2, which was reversed in the presence of WAY100635, PD98059, and GFX [Figure 3.1a and Figure 3.1b]. Immunostaining showed similar

activation of P-ERK at 10’ and 20’ upon 100 nM 8-OH-DPAT treatments, as analyzed by Western blotting [Figure 3.1c].

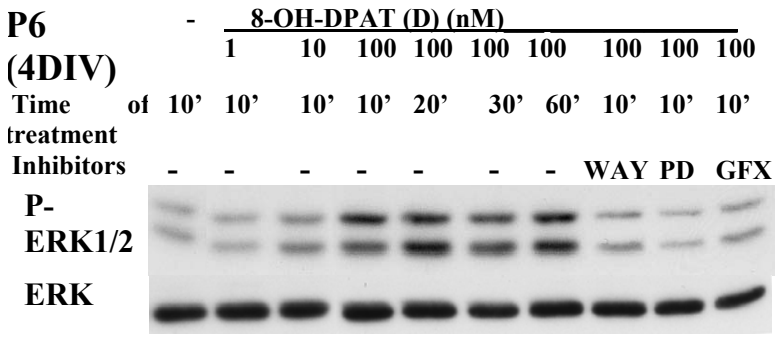


Figure 3.1(a)

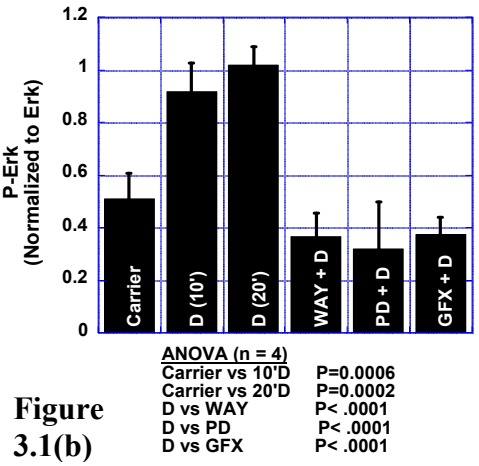


Figure 3.1(b)

Figure. 3.1. In P6 slices, 5-HT$_{1A}$-R-mediated signaling causes prolonged activation of ERK1/2 and is dependent on PKC-

Cultured hippocampal slices (4DIV) from P6 mice were placed in serum-free medium and then appropriate wells were treated for 30 min with the indicated inhibitors (WAY, PD, and GFX). Subsequently, 100 nM 8-OH-DPAT was added to all except the control wells. After incubation for the indicated time periods, **(a)** the agonist and inhibitor-treated slices were lysed and the proteins were subjected to SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of ERK1/2. Equal loading of lanes (10 µg protein/ lane) was verified by probing with an ERK1/2 antibody. **(b)** The P-ERK band intensities were normalized to the intensity of the corresponding ERK bands and data obtained from four independent experiments have been presented after statistical analysis using ANOVA, with Bonferroni post-hoc test. **(c) (Next page)** Immunostaining

shows activation of ERK at 10’ and 20’ with 100 nM 8-OH-DPAT (D) treatment in dentate gyrus (DG) of hippocampus. Nissl (red) and P-ERK (green) colocalized, which yielded yellow fluorescence. This activation was reversed by the MEK inhibitor PD98059. Scale bar, 50 micron.

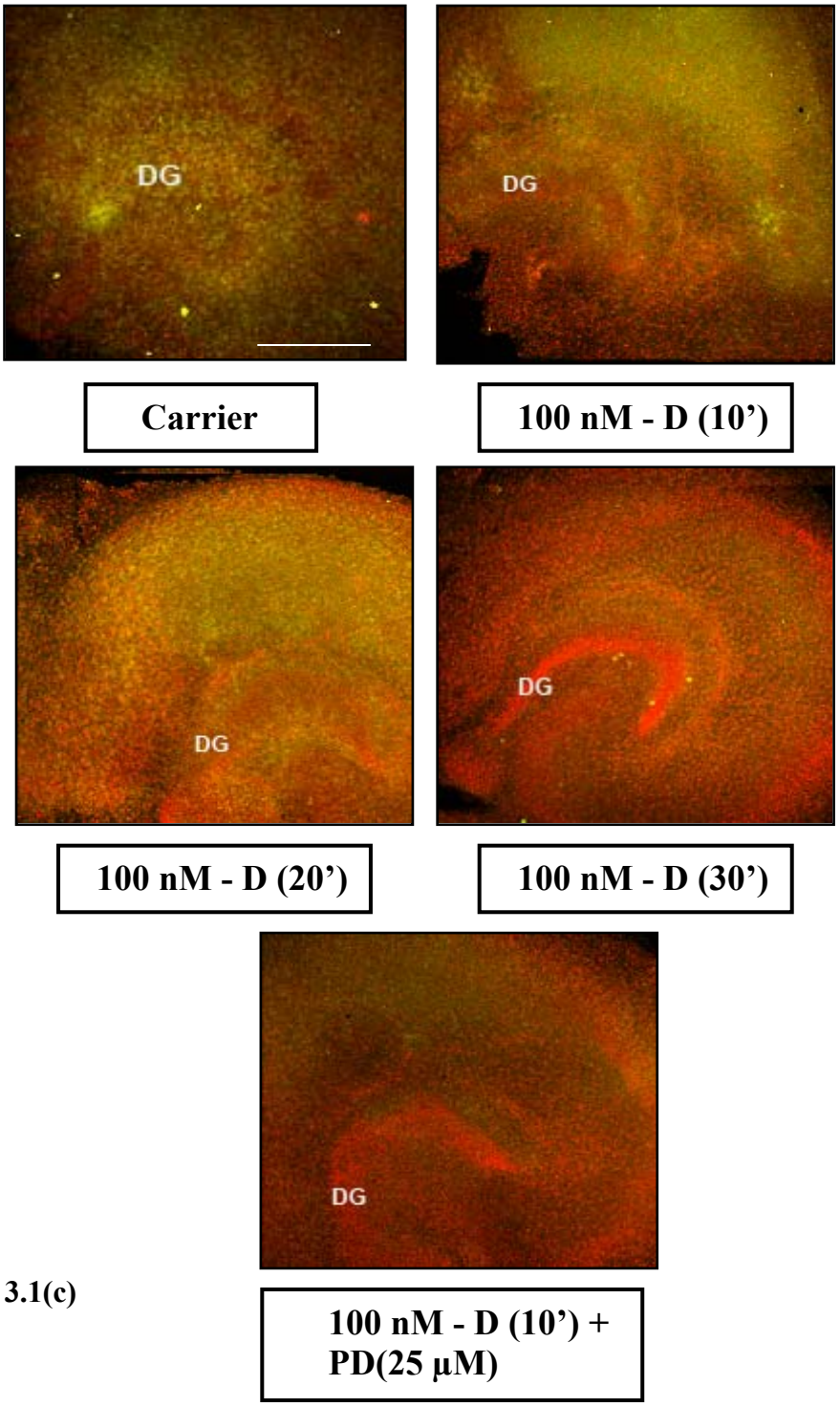


Figure 3.1(c)

Serotonin-1A receptor-mediated activation of ERK1/2 is PKC-dependent but PKCα is not involved in this pathway at P6

Since agonist exposure between 10 to 20 minutes caused optimal activation in our sliced cultures, all consequent studies were performed with 10 minutes of exposure to 100 nM of 8-OH-DPAT. As seen in Figure 3.1 a, the activation of ERK1/2 was blocked by a general inhibitor of PKC isozymes (GFX). Thus, ERK1/2 activation by 5-HT_{1A} receptor is PKC dependent at this stage and this PKC molecule is involved upstream of ERK1/2. The activation profile for PKCα, as shown by increased phosphorylation of PKCα at T^{638}, displayed no similarity to that for ERK1/2 [Figure 3.2]. Only a mild stimulation was observed for PKCα after about 60 min of 8-OH-DPAT treatment and statistical analysis of data obtained from four experiments (n = 4) failed to show any significant change in 5-HT_{1A}-R-mediated activation of PKCα in the P6 slices. These data showed that PKCα was not involved in 5-HT_{1A} receptor activated ERK1/2 pathway however stimulation of ERK1/2 was blocked in the presence of the PKC-inhibitor GFX. Thus, the 5-HT_{1A}-R-mediated activation of ERK is probably dependent on a different PKC isozyme at P6.

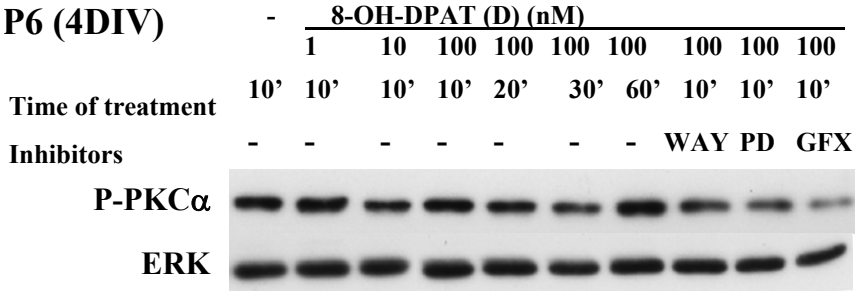


Figure. 3.2. In P6 slices, PKCα is not involved in 5-HT_{1A}-R→ ERK1/2 signaling pathway.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 minutes with the inhibitors, and processed as described in Figure 3.1. The agonist and inhibitor-treated slices were lysed and the proteins were subjected to SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of PKCα. Equal loading of lanes (10 μg protein/ lane) was verified by probing with an ERK1/2 antibody.

Serotonin-1A receptor agonist causes activation of PKCε at P6

The PKCα, which is a Ca^{+}-dependent/conventional PKC isozyme, was not involved in 5-HT_{1A}-R→ ERK signaling. Further analysis revealed that a relatively high molecular weight PKC isozyme was activated by 8-OH-DPAT, which was later identified as PKC-ε. The peak of the activation of this enzyme (20 min) corresponded well to the activation profile of ERK1/2 [Figure 3.3 a]. Statistical analysis by paired t-test revealed a significant increase in the normalized P-PKC-ε band intensity compared with β-actin after 20 minutes of 8-OH-DPAT treatment [Figure 3.3 b].The time course of activation of PKC-ε was parallel to the activation profile for ERK1/2 in the P6 slices. Thus, it is hypothesized that the PKC isozyme involved upstream of ERK1/2 signaling at P6 seems to be PKC-ε which is also activated by 5-HT_{1A}-R at P6 [Figure 3.4].

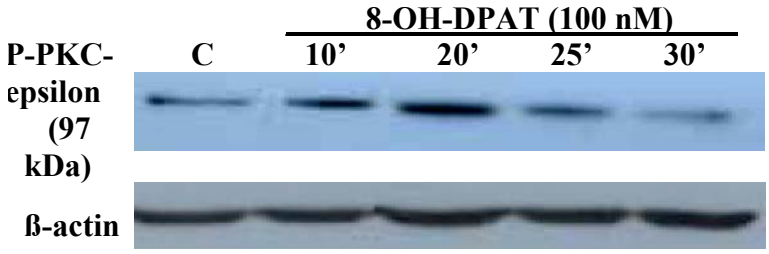


Figure 3.3(a)

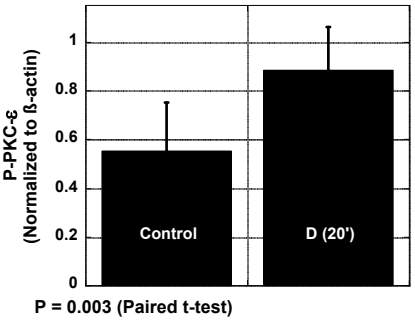


Figure 3.3(b)

Figure. 3.3. In P6 slices, PKCε is activated by agonist of 5-HT_{1A}-R.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 min with the inhibitors, and processed as described in Figure 3.1. **(a)** The agonist- treated slices for indicated time were lysed and the proteins were subjected to SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation profile of PKCε. Equal loading of lanes (10 µg protein/ lane) was verified by probing with a β–actin antibody. **(b)** The band intensities were normalized to the intensities of the corresponding ß-actin bands. Agonist treatment at 20minutes caused significant activation of PKCε. Statistical analysis of the normalized P-PKC-ε band intensities obtained from three experiments (n=3) was performed using students' t-test of paired samples (P = 0.003 for 20 min agonist treatment) (Fernando, S., and Banerjee, P., unpublished).

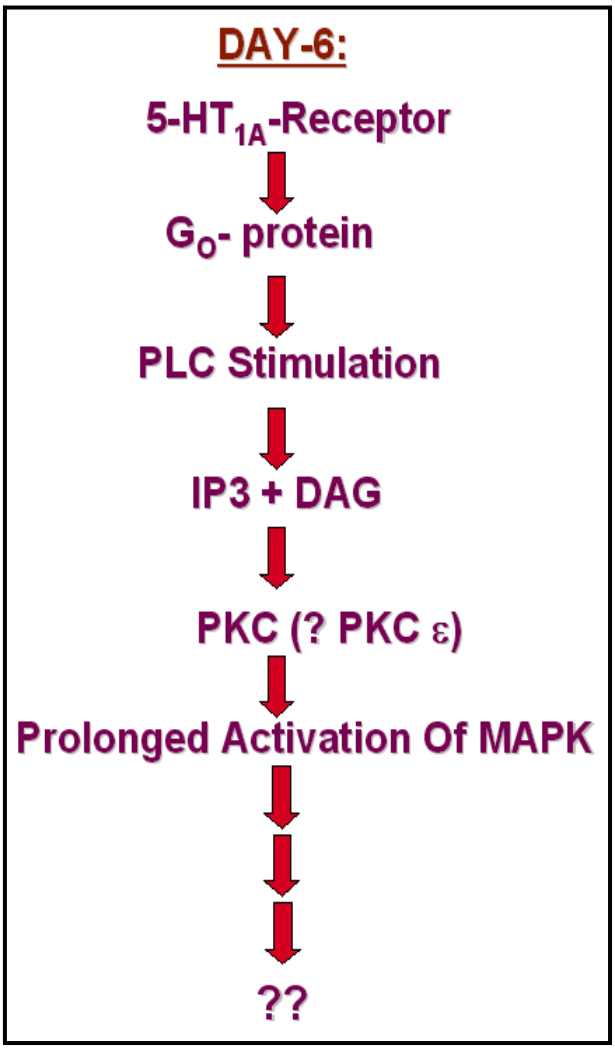


Figure. 3.4. Possible model of 5-HT_{1A} receptor signaling where PKC isozyme, PKC-epsilon may be involved upstream of ERK1/2 activation at P6.

Serotonin-1A receptor agonist activation of ERK1/2 pathway does not involve signaling molecules such as Akt or CREB at P6

The receptor→PI3-K→Akt pathway has been implicated in numerous physiological functions including activation of the ERKs via the 5-HT_{1A} receptor (Cowen, 1996). Therefore, we monitored the activation of Akt by measuring phosphorylation of this protein at Serine 473 by agonist treatment of 8-OH-DPAT. There was a prominent activation of Akt after 60 minutes of 8-OH-DPAT treatment but the activation profile did not correlate with the activation of ERK1/2 [Figure 3.5a and 3.1a]. Thus, the activation of Akt was probably not involved in the 5-HT_{1A}-R→ ERK1/2 signaling pathway but could channel through some other signaling mechanism.

Serotonin1A receptor-activated ERK1/2 can also phosphorylate the transcriptional factor CREB (cAMP response element binding protein) at Ser 133, which not only promotes neuronal survival, proliferation and differentiation but also plays a vital role in neuronal plasticity (Ginty, 1994; Xing, 1996; Lonze, 2002). Therefore, we monitored activation of CREB at Serine 133 following 8-OH-DPAT treatment. There was no change in the profile of activation of CREB with the 8-OH-DPAT treatment [Figure 3.5 b]. Thus, CREB is not involved in this 5-HT_{1A}-R → ERK 1/2 pathway.

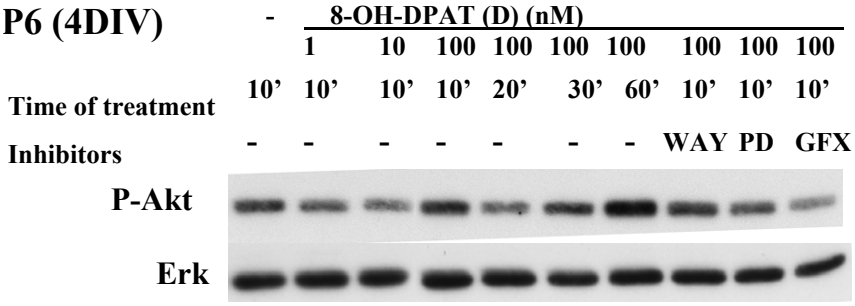


Figure 3.5(a)

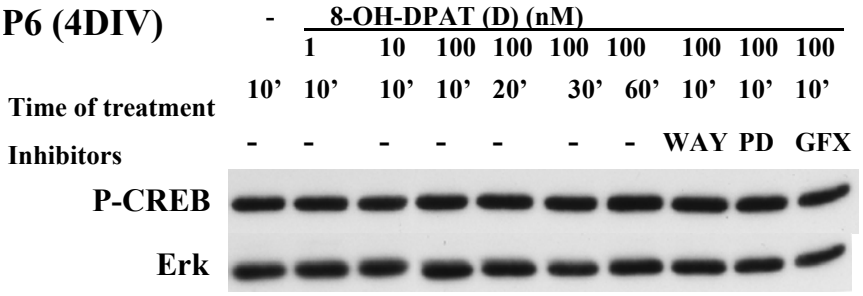


Figure 3.5(b)

Figure. 3.5. In P6 slices, neither Akt nor CREB is activated by 5-HT_{1A}-R→ Erk1/2 signaling pathway.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 minutes with the inhibitors, processed as described in Figure 3.1. The agonist and inhibitor-treated slices were lysed and the proteins were subjected SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of **(a)** Akt and **(b)** CREB. Equal loading of lanes (10 μg protein/ lane) was verified by probing with an Erk1/2 antibody.

Agonist stimulation of the 5-HT_{1A} receptor causes a dramatic increase in cell division in the P6 slices (4DIV)

The prolonged activated MAP kinase isozyme ERK1/2 at P6 could translocate to the nucleus and cause an increase in cell division and neurogenesis in the developing brain (as discussed in the introduction). To check this possibility, the cultured hippocampal slices were first kept in serum free medium for an hour after which they were treated first with bromo-deoxyuridine (BrdU) (50 μM) and carrier (PBS) or 100 nM 8-OH-DPAT. The slices were fixed after 16 hours of treatment. Immunostaining of slices using a BrdU antibody showed a significant increase in staining (red, which appears yellow when overlapped with NeuN, green) in slices treated with 8-OH-DPAT. The 8-OH-DPAT-evoked increase in BrdU labeling was reversed in the presence of WAY100635, PD98059, and GFX [Figure 3.6 a]. Only those BrdU (+) cells that were closely apposed to one another (either clearly dividing or divided) were considered, thus avoiding the possibility of counting cells that were BrdU-labeled through DNA repair. Statistical analysis using ANOVA revealed a highly significant elevation in the number of BrdU (+) cells in the 8-OH-DPAT (D)-treated slices as compared to the control, D+WAY100635-treated, D+PD98059-treated and D+GFX-treated slices ($P<0.0001$ for each comparison). BrdU labeling was not significantly different among the group of control slices and the groups that were treated with D plus the inhibitors ($P = 1$) [Figure 3.6 b].

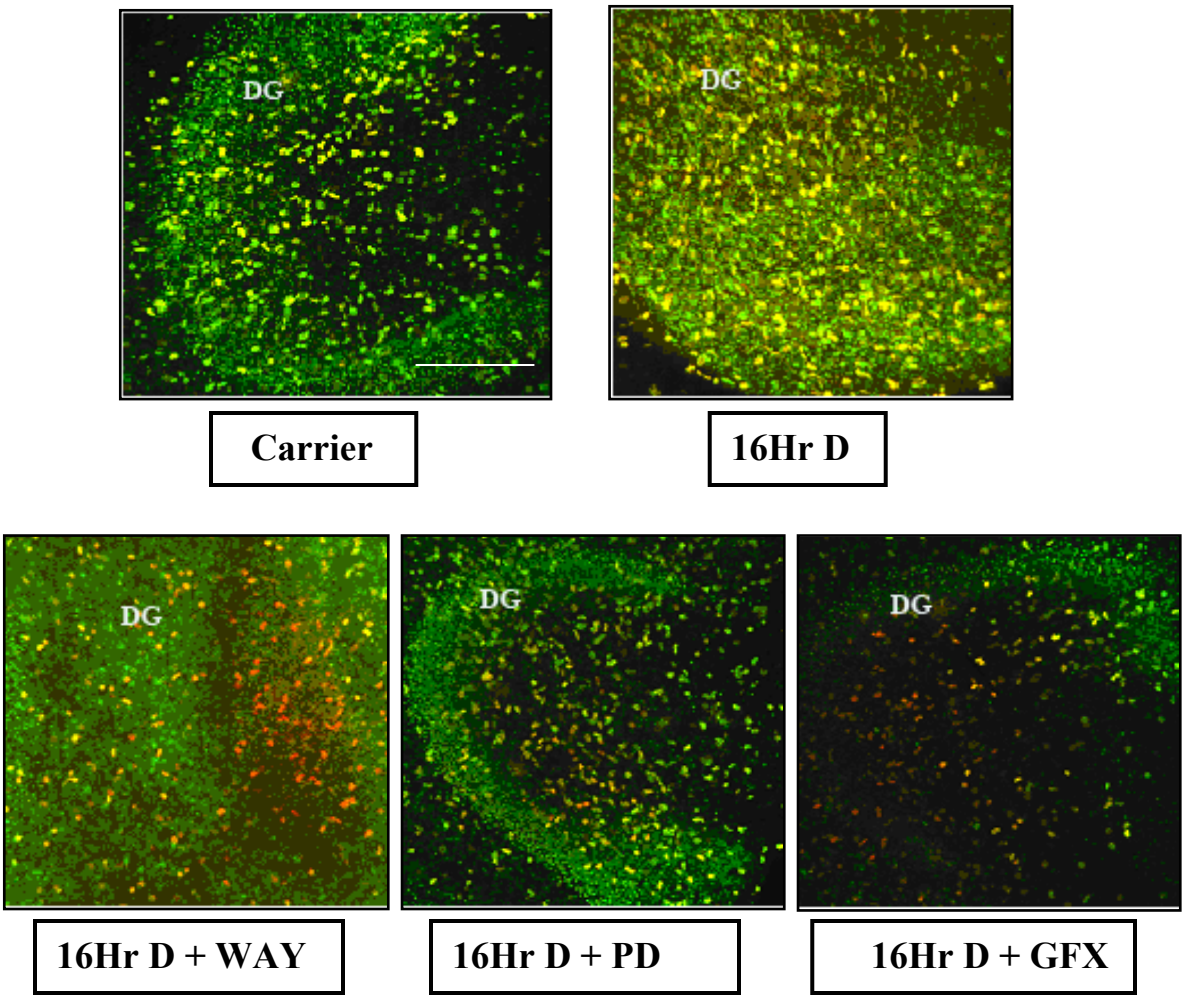


Figure 3.6(a)

Figure. 3.6. In P6 slices, agonist treatment causes increased cell division in the dentate gyrus (DG) region of hippocampus.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 min with the indicated inhibitors (WAY, PD, and GFX). Subsequently, 100 nM 8-OH-DPAT was added to all except the control wells. Next, all the slices were incubated for 16 h with 5-bromo-2'-deoxyuridine (BrdU-5 µg/ml), washed, and fixed for immunostaining. **(a)** Confocal images of cells, stained with anti-BrdU (red) and/or anti-NeuN (green), were obtained at 20X magnification from individual excitation beams as described in the methods. Scale bar, 100 micron. (Continue on Next page).

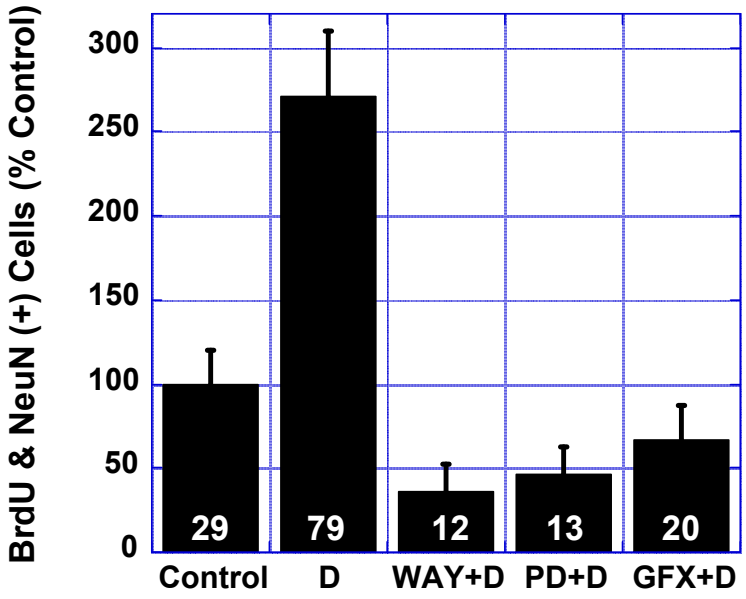


Figure 3.6 (b)

(b) The red and green images were superimposed (yellow) and subjected to Z-stack analysis and automatic cell counting to obtain the number of anti-BrdU stained cells in the dentate gyrus (DG) region of each slice. Data were obtained from three independent experiments, each of which included 5-10 hippocampal slices for each condition of treatment. The numerical figure shown in each bar shows the average cell number per hippocampus as obtained from multiple experiments. Statistical analysis was performed using One Way ANOVA with Bonferroni Post Hoc test.

Agonist stimulation of the 5-HT_{1A} receptor stimulates cell division and causes induction of Cycliln-D1 in the P6 slices (4DIV)

As mentioned before, activated ERK1/2 is known to translocate to nucleus where it can phosphorylate and activate the transcription factor Elk-1, which increases expression of c-Fos. Once induced, c-Fos combines with existing c-Jun molecules to yield elevated levels of the dimeric transcription factor AP-1 (Karin, 1995). The AP-1 then induces cyclin D1 expression (cell cycle protein) that could cause increase in the cell division (Bakiri, 2000; Shaulian, 2001) observed at P6 [Figure 3.6 b].

These experiments were done in a similar fashion where the cultured, hippocampal slices from P6 mouse brain were placed in serum-free medium and then treated for 16 hours with 8-OH-DPAT in the absence and presence of a 5-HT_{1A}-R antagonist (WAY100635, 4 μM), or the MEK inhibitor PD98059 (25 μM), or a PKC inhibitor (GFX, 2 μM). These, data show that the 5-HT_{1A}-R mediated induction of cyclin D1 also requires protein kinase C (PKC), because, in addition to WAY100635 and PD98059, the PKC inhibitor GFX also causes reversal of this 5-HT_{1A}-R mediated induction of cyclin D1 [Figure 3.7 a and b]. Thus at P6 the increase in cell division could be mediated by the 5-HT_{1A}-R→ PKC-ε → Erk1/2 → Cyclin D1 pathway.

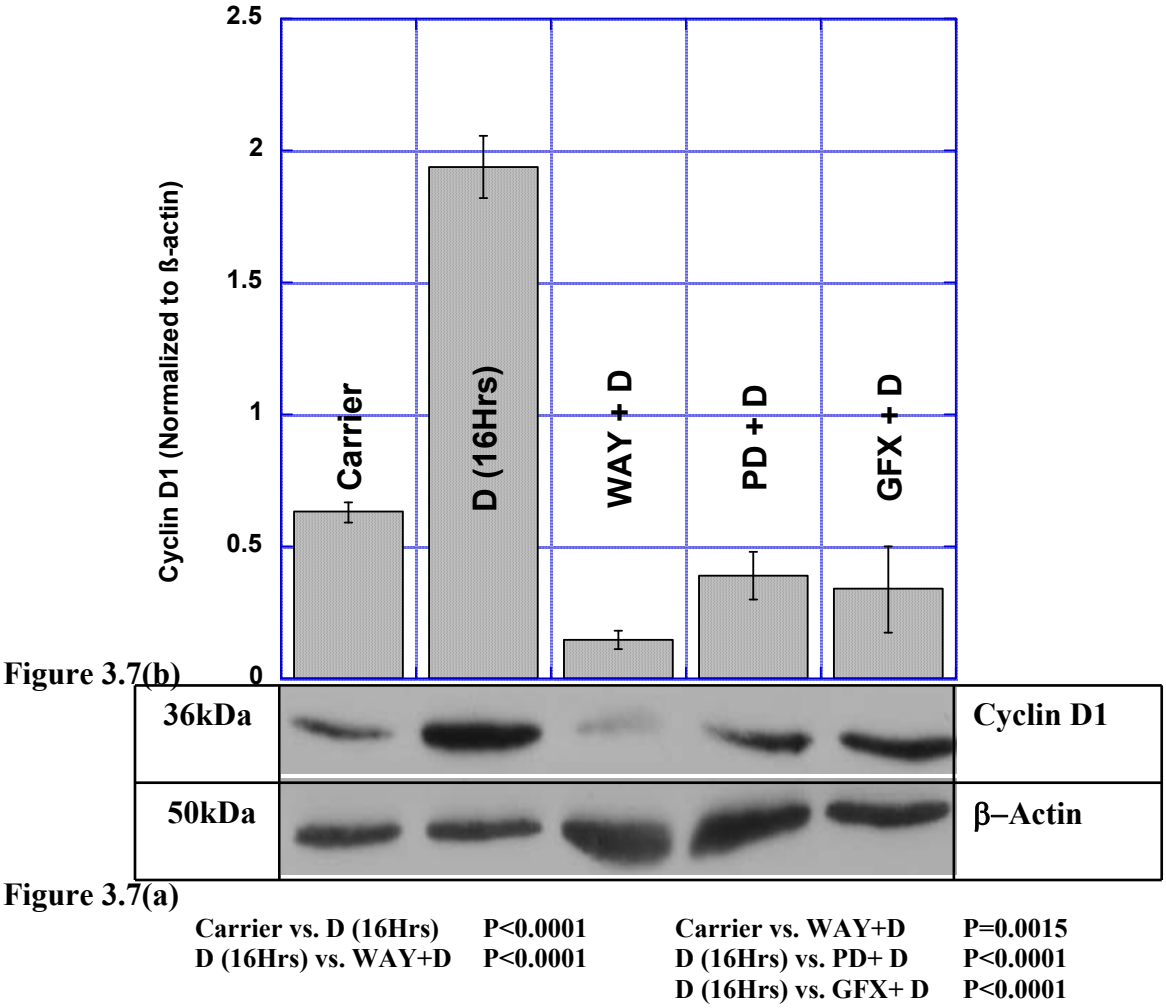


Figure. 3.7. In P6 slices, serotonin 1A receptor stimulates induction of Cyclin D1.

Cultured, hippocampal slices from P6 mouse brain were placed in serum-free medium and then treated for 16 hours with 8-OH-DPAT (D) in the absence and presence of a 5-HT_{1A}-R antagonist (WAY100635, 4 μM), or MEK inhibitor PD98059 (25 μM), or a PKC inhibitor (GFX, 2 μM). **(a)** The post-treatment slices were lysed in RIPA buffer and then 10-μg aliquots of protein were analyzed for cyclin D1 and ß-actin levels by SDS-PAGE and Western blotting. **(b)** The experiment was repeated three times (n=3) and the data were presented as mean ± standard deviation.

Agonist stimulation of the 5-HT_{1A} receptor causes transient activation of ERK1/2 in cultured hippocampal slices from P15 mice (4 DIV)

These experiments were designed to study the activation profile of ERK1/2 at P15. The hippocampal cultured slices from day 15 pups (4DIV) were treated under serum free conditions with different concentrations of the 5-HT_{1A} receptor agonist 8-OH-DPAT. Similar results, as obtained for the P6 slices (4DIV), showed that 100 nM 8-OH-DPAT was optimal for the activation of ERK1/2 [Figure 3.8 a]. The P-ERK1/2 levels in the P15 slices (4DIV) reached a peak at about 10– 20 minutes and then decreased to a lower level after 30 minutes of 8-OH-DPAT treatment. Thus, unlike P6 where ERK1/2 was activated for prolonged time, there was only a transient activation of ERK1/2. Statistical analysis of normalized P-ERK band intensities showed a significant increase in P-ERK1/2 following 8-OH-DPAT treatment, which was reversed in the presence of WAY100635 and PD98059 but not GFX [Figure 3.8 b]. This means that ERK1/2 activation was independent of PKC activation. PKC could have been activated downstream of ERK1/2. Statistical analysis of normalized P-ERK band intensities showed a significant increase in P-ERK1/2 following 8-OH-DPAT treatment, which was reversed in the presence of WAY100635 and PD98059 but not GFX. Immunohistochemistry revealed a similar profile for Erk (green) activation between 10' and 20’ in the P15 slices. Counter-staining with Nissl (red) confirmed the identification of neurons, many of which also showed P-ERk labeling [Figure 3.8 c].

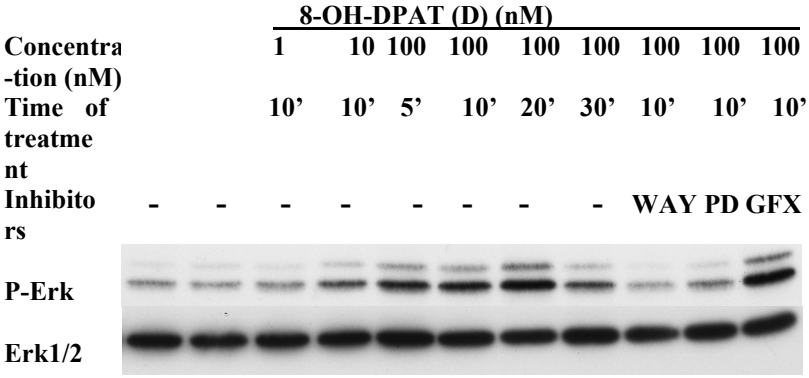


Figure 3.8(a)

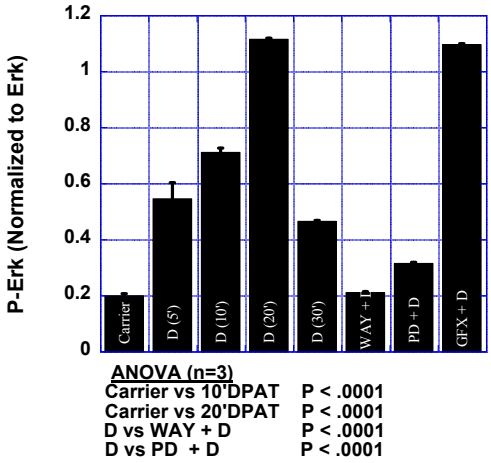


Figure 3.8(b)

Figure. 3.8. In P15 slices, 5-HT_{1A}-R mediated signaling causes transient activation of ERK1/2 and is not dependent on PKC.

Cultured P15 slices (4DIV) were placed in serum-free medium and then treated for 30 minutes with the inhibitors, processed as described in Figure 3.1. **(a)** the agonist and inhibitor-treated slices were lysed and the proteins were subjected to SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of ERK1/2. **(b)** The P-ERK band intensities were normalized to the intensity of the corresponding ERK bands. Data obtained from three independent experiments have been presented after statistical analysis using ANOVA, with Bonferroni post-hoc test. **(c) (Next page)** Immunostaining shows activation of ERK at 10' with 100nM 8-OH-DPAT (D) treatment in dentate gyrus (DG) of hippocampus. Nissl (red) and P-Erk (green) were colocalized, which yielded yellow fluorescence. Scale bar, 50 micron.

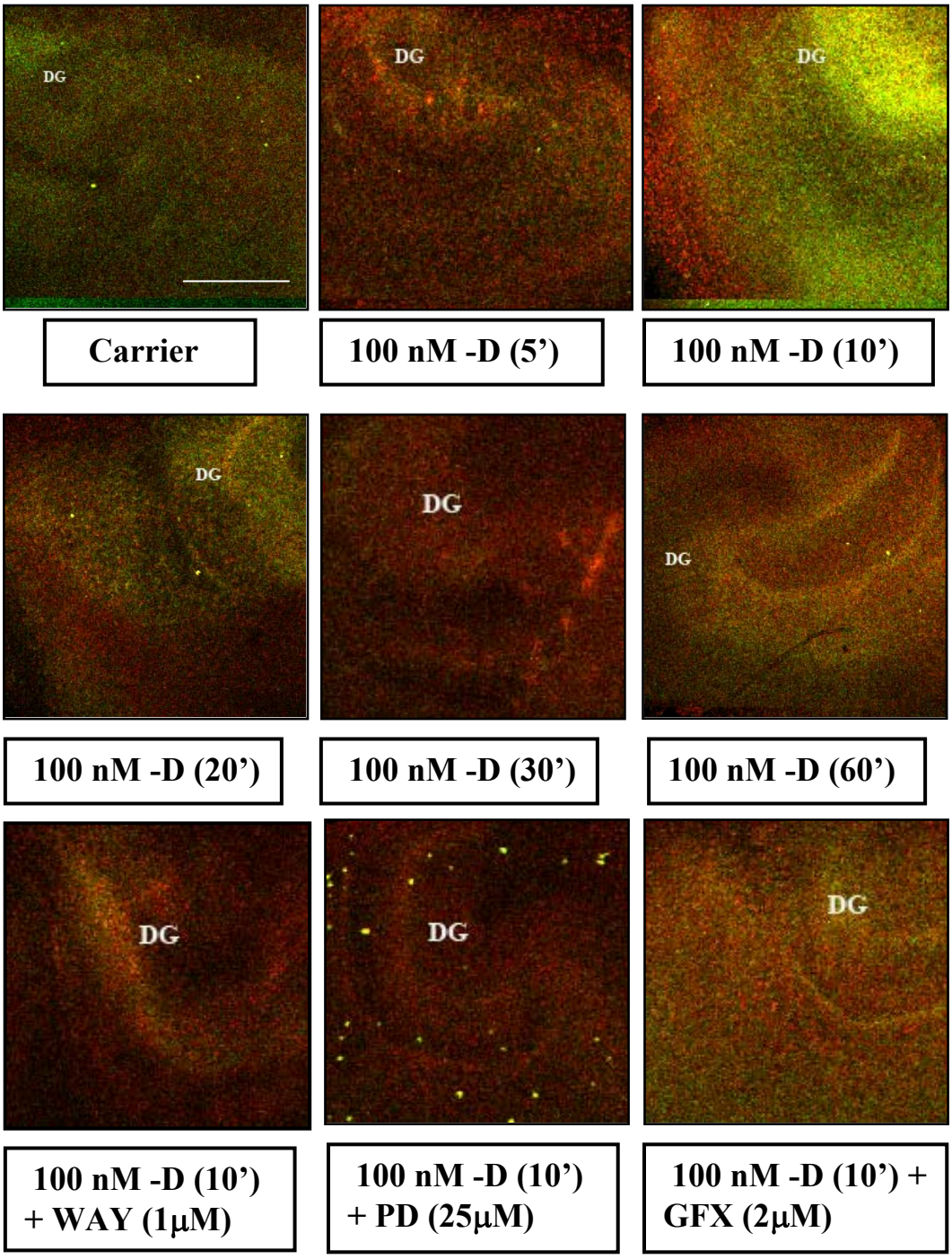

DG
DG
DG
Carrier
100 nM -D (5')
100 nM -D (10')
DG
DG
DG
100 nM -D (20')
100 nM -D (30')
100 nM -D (60')
DG
DG
DG
100 nM -D (10') + WAY (1μM)
100 nM -D (10') + PD (25μM)
100 nM -D (10') + GFX (2μM)

Figure 3.8(c)

Serotonin-$_{1A}$ receptor mediated activation of ERK1/2 is independent of PKC activation and leads to PKCα activation at P15

In sharp contrast to the lack of correlation between the activation profiles of PKCα and ERK1/2 in the P6 slices (4DIV), both ERK1/2 and PKCα showed almost parallel activation profiles in the 4DIV P15 slices [Figure 3.9 a]. The PKCα activation was reversed by WAY100635 and PD98059. Thus, PKCα activation was dependent on the 5-HT$_{1A}$-R→ ERK1/2 pathway [Figure 3.9 b]. Furthermore, the activation of ^{638}T-PKCα was not inhibited by the general PKC inhibitor GFX, which indicates that autophosphorylation was probably not the mechanism of activation of PKCα. Therefore at P15 the 5-HT$_{1A}$-R stimulates the pathway 5-HT$_{1A}$-R→ ERK1/2→ PKCα in which PKCα is positioned downstream of ERK1/2. This is in sharp contrast to our observation for the P6 slices in which a different PKC isozyme was positioned upstream of ERK1/2. Previous studies from our lab have confirmed the presence of the latter pathway (5-HT$_{1A}$-R→ ERK1/2→ PKCα) in the hippocampal neuron-derived cell line HN2-5 where it has neuroprotective role (Adayev, 2003).

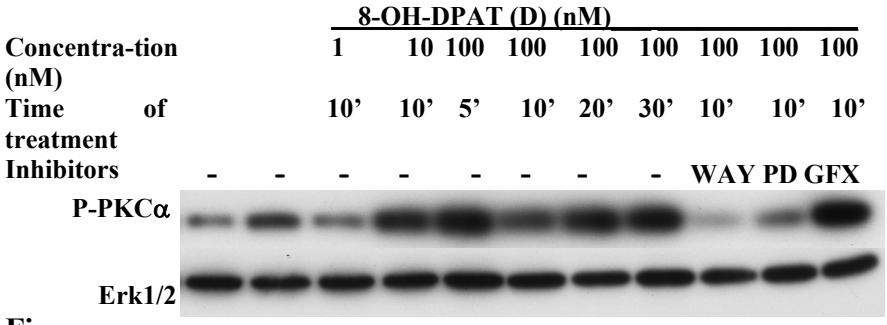


Figure 3.9(a)

Figure. 3.9. In P15 slices, PKCα is involved in 5-HT_{1A}-R→ ERK1/2 signaling pathway.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 minutes with the inhibitors, processed as described in Figure 3.1. **(a)** The agonist and inhibitor-treated slices were lysed and the proteins were subjected to SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of PKCα. Equal loading of lanes (10 μg protein/ lane) was verified by probing with an ERK1/2 antibody.

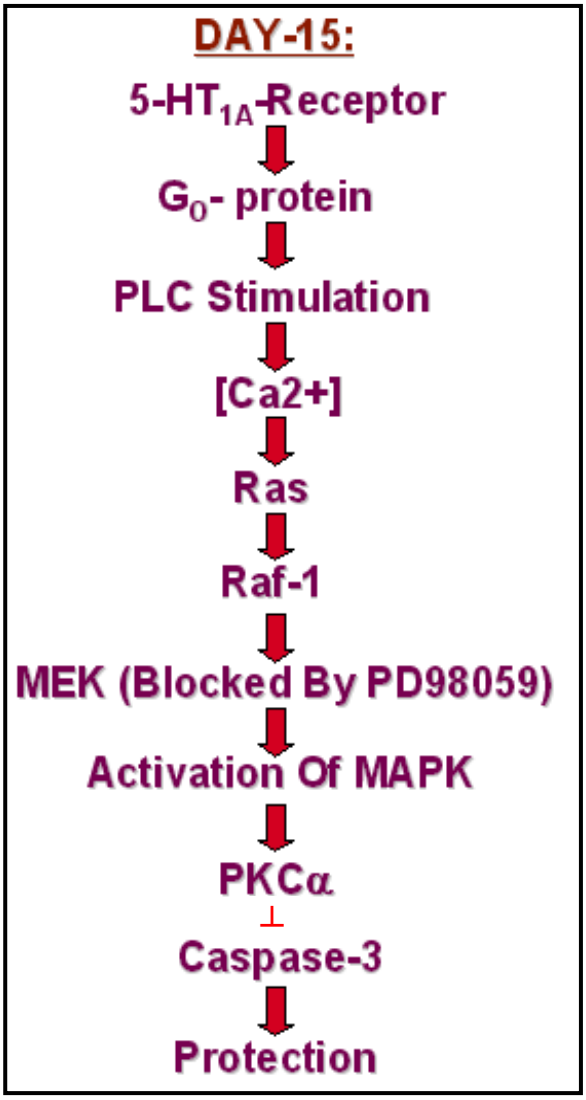


Figure 3.9(b) Possible model of 5-HT_{1A} receptor signaling where a PKC isozyme PKC-alpha is involved downstream of ERK1/2. (Adopted from Adayev, 2003).

Serotonin-$_{1A}$ receptor mediated activation of ERK1/2 does not involve signaling molecules such as Akt or CREB at P15

Once again, the temporal profile of activation of Akt did not correspond with that of ERK, therefore Akt did not seem to be involved in this pathway. Also, 8-OH-DPAT treatment yielded no change in phosphorylation of CREB at S^{133} at P15 [Figure 3.10 a, b].

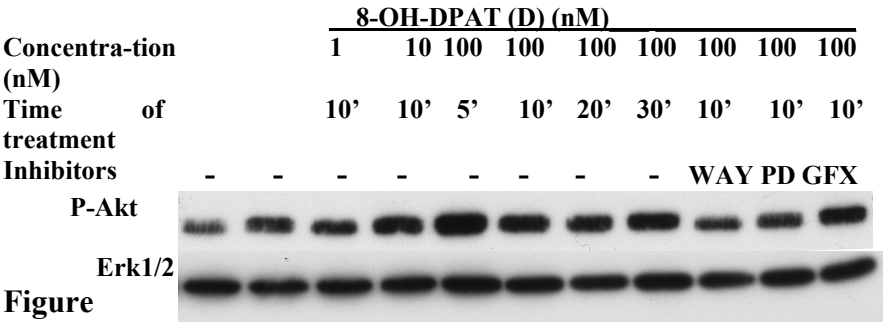


Figure 3.10(a)

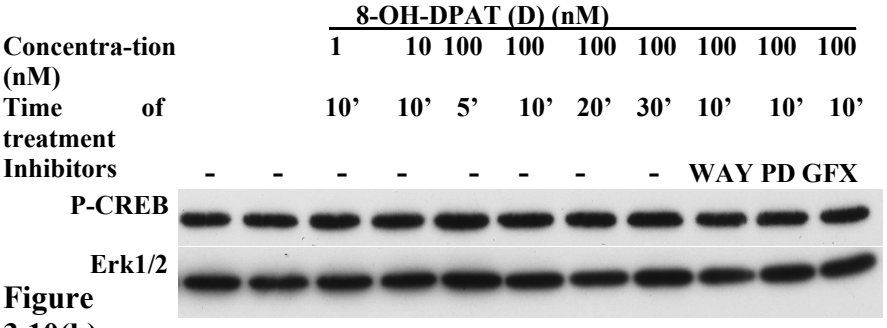


Figure 3.10(b)

Figure. 3.10. In P6 slices, neither Akt nor CREB is activated by 5-HT$_{1A}$-R→ ERK1/2 signaling pathway.

Cultured P6 slices (4DIV) were placed in serum-free medium and then treated for 30 minutes with the inhibitors, processed as described in Figure 3.1. The agonist and inhibitor-treated slices were lysed and the proteins were subjected SDS-PAGE and Western blotting using appropriate antibodies to monitor the activation of **(a)** Akt and **(b)** CREB. Equal loading of lanes (10 µg protein/ lane) was verified by probing with an ERK1/2 antibody.

In the P15 slices (4DIV), agonist activation of the 5-HT_{1A} receptor caused no increase in cell division

At P6 5-HT_{1A}-R→ ERK1/2 caused an increase in cell division. To test if 5-HT_{1A}-R mediated activation of ERK1/2 was also linked to increased cell division in the P15 hippocampus, we performed BrdU treatment in the absence or presence of 8-OH-DPAT as described earlier for the P6 hippocampal culture. In the 4DIV P15 slices, 8-OH-DPAT treatment for 16 hours elicited no significant increase in the number of BrdU (+) cells [Figure 3.11 a]. The overall number of BrdU labeled cells was significantly reduced at P15 compared with P6. Also, there was a wide variation in basal level of cell division in the P15 slices and panels presented, which show the upper and lower limits of BrdU labeling in both control as well as 8-OH-DPAT treated slices [Figure 3.11 a]. This accounted for the large standard deviation shown in Figure3.11 b. This wide variation was observed with or without 8-OH-DPAT, which demonstrates that 8-OH-DPAT does not have any significant effect on BrdU labeling in the P15 slices after 16 h treatment with 8-OH-DPAT. However, this does not rule out the possibility that cell cycle at this stage could have been shortened and cell division could be increased at different time points than 16 h of agonist treatment.

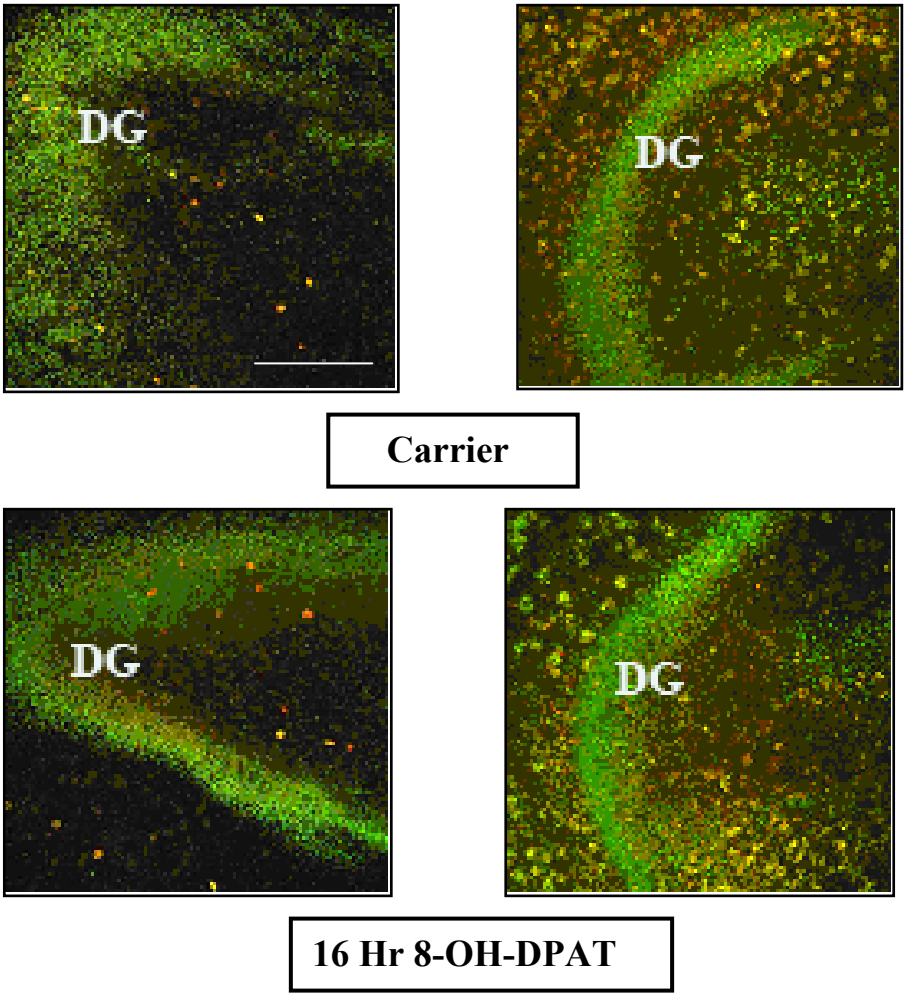


Figure 3.11(a)

Figure. 3.11. 8-OH-DPAT does not cause increased cell division in P15 hippocampus.

(a) The cultured P15 hippocampal slices (4DIV) were placed in serum-free medium and then processed for immunostaining for BrdU (red) and NeuN (green), Z-stack analysis and cell counting as described in Figure 3.6. Scale bar, 100 micron. (Continue on Next page).

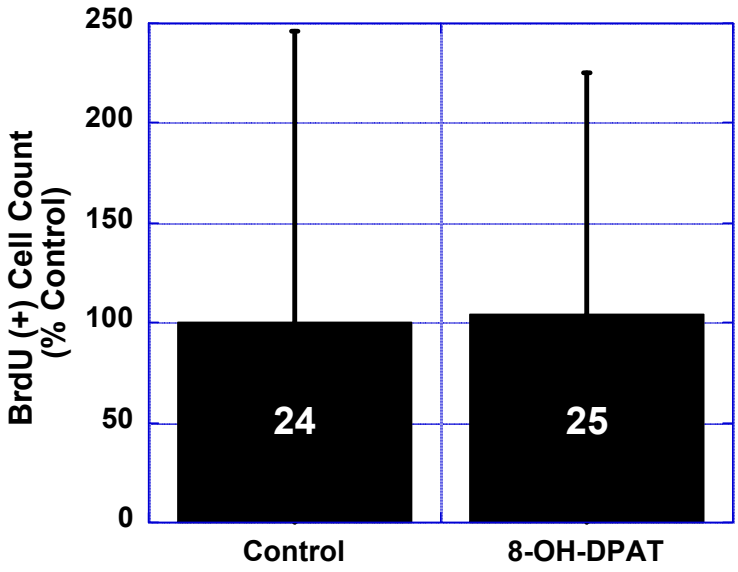


Figure 3.11(b)

(b) Data processed for statistical analysis were obtained from four independent experiments, each of which included 5-10 hippocampal slices for each condition of treatment.

Agonist activation of the 5-HT_{1A} receptor caused ERK-dependent augmentation of fEPSP at both P6 and P15, but this effect was two-fold higher at P15 than at P6

So far, we have shown that in neonatal mouse hippocampus, the 5-HT_{1A}-R→ ERK1/2 cascade is a major signaling pathway which undergoes profound developmental changes. We had hypothesized that during the later stages (P15) the 5-HT_{1A}-R would be linked more to secure synaptic connections. Testing this hypothesis required measuring synaptic strengths at both P6 and P15. We achieved this by monitoring excitatory postsynaptic potential (EPSP) in acutely isolated hippocampal slices. EPSP measures the effect of presynaptic excitation in generating a change in postsynaptic membrane potential. The change in membrane potential in turn induces a change in potential outside the cell, which was recorded and termed as field EPSP or fEPSP. Increased synaptic strength was evidenced by an increase in fEPSP near the dendritic terminals of the CA1 neurons upon electrical excitation of the presynaptic CA3 neurons in the Schaffer collateral pathway [Figure 2.2 b]. fEPSPs generated at both P6 and P15 were further augmented upon 100 nmol/L of 8-OH-DPAT treatment of the slices [Figure 3.12 a and b] and this increase was reversed in the presence of WAY100635 ($P < 0.0001$ for Day6 and $P = 0.0004$ for Day15), PD98059 (an inhibitor of the Erk pathway) ($P = 0.0027$ for Day6 and $P = 0.003$ for Day15), and the PKC inhibitor GFX ($P = 0.0031$ for Day6 and $P = 0.0109$ for Day15) [Figure 3.12 c]. In corroboration of our hypothesis, the 8-OH-DPAT-evoked increase in fEPSP was two–fold higher at Day15 than at Day6 ($P = 0.016$) [Figure 3.12 d], thus confirming a greater efficacy of 5-HT_{1A}-R mediated signaling in strengthening of synapses during the later stages of brain development (Day15). It should be noted that in the Day15 hippocampal slices (4DIV), the 5-HT_{1A}-R mediated

activation of ERK1/2 was not GFX-sensitive, but its downstream effect, an increase in fEPSP, was sensitive to both PD98059 as well as GFX. Thus, this increase in fEPSP was dependent on 5-HT_{1A}-R→ ERK1/2→ PKCα signaling cascade.

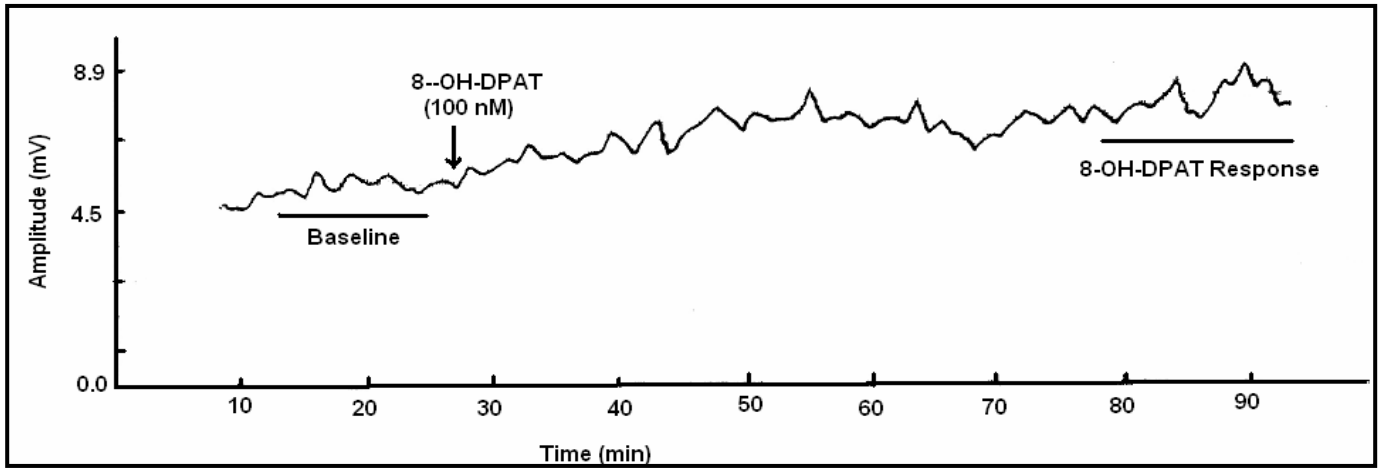


Figure 3.12(a) Agonist activated fEPSP at P6.

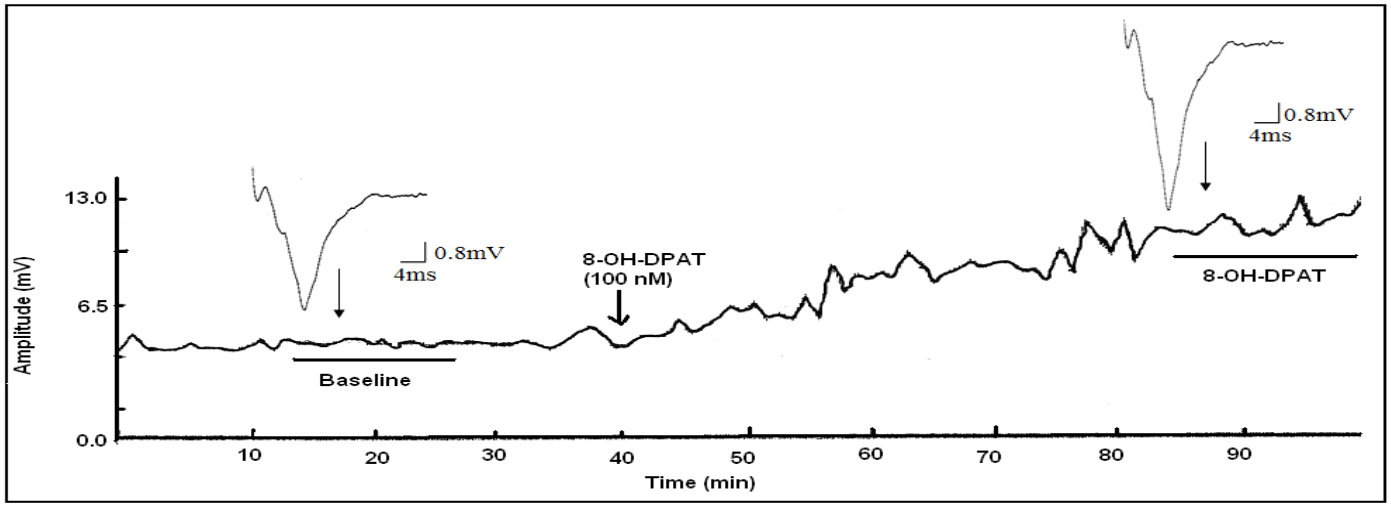


Figure 3.12(b) Agonist activated fEPSP at P15.

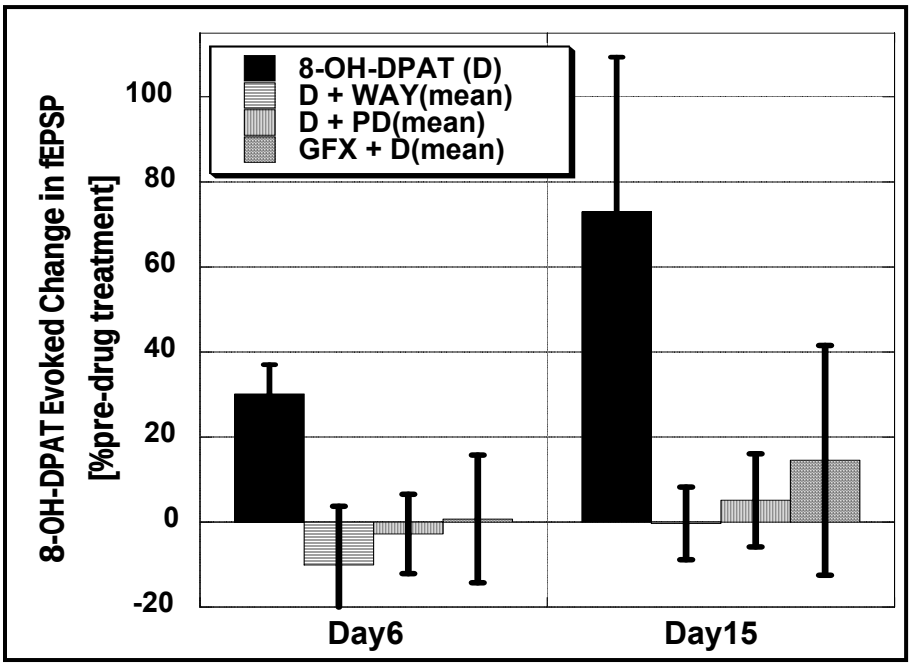


Figure 3.12(c)

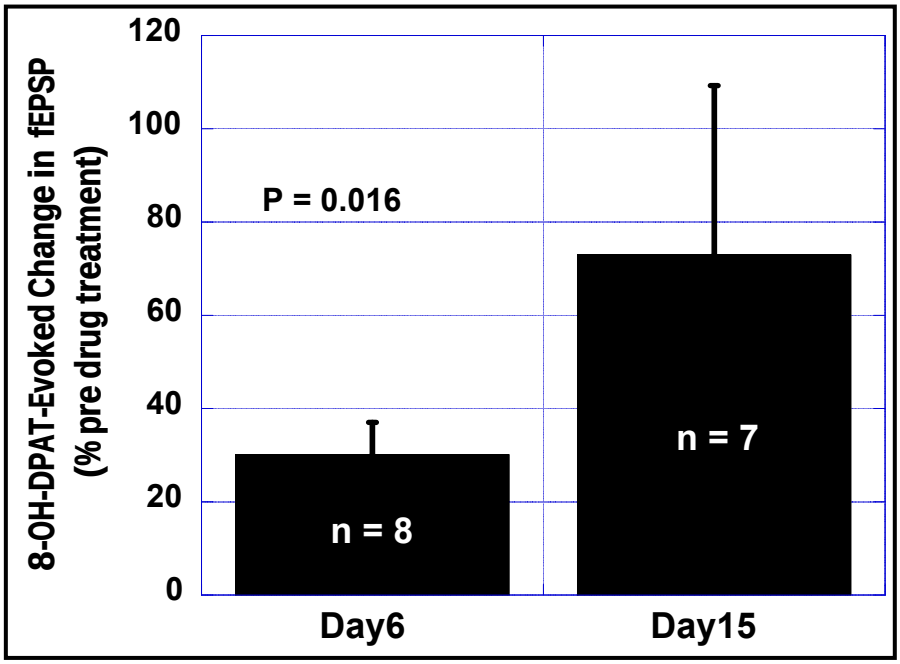


Figure 3.12(d)

Figure 3.12. At both P6 and P15, 5-HT$_{1A}$ receptor-mediated augmentation of fEPSP requires activation of an ERK-dependent pathway and this effect is higher in the P15 slices.

The experiments were done on acutely isolated hippocampal slices. **(a, b)** Each experiment started with stimulus recording for 15 minutes till the recording showed stability in the fEPSPs, following which 8-OH-DPAT (100 nmol/L) was added to the recording chamber. The recording was continued for another 40 minutes, although the agonist-triggered response was observed 25 minutes after drug addition. The antagonists, inhibitors, and the agonist were added at the same time. **(c)** Data have been presented as mean ± SD. Statistical analysis among the groups was performed using One-Way ANOVA. **(d)** Statistical comparison of ΔfEPSPs elicited by 100 nmol/L 8-OH-DPAT in P6 and P15 hippocampal slices was performed using paired t-test.

CHAPTER 4

DISCUSSION

The initial development of the nervous system from neural precursor cells depends on the intricate interplay of cellular movements and inductive signals. It has been concluded that the fate of each individual precursor cell is not determined by its mitotic history; rather, the information required for differentiation arises largely from the interaction between the developing cell and its local environment. All of these events are also dependent on the same categories of molecular and cellular phenomena and cell-cell signaling, transcriptional regulation and, ultimately, gene expression. These key signaling events early in neural development are special points of focus that can determine the generation of a normal nervous system.

Serotonergic neurons are among the first neurons to differentiate. They can play an important role in various neurodevelopmental processes including neurogenesis, differentiation, neuropil formation, migration, neurite outgrowth, growth cone motility, axon myelination and maturation (Lauder, 1978). Our results presented here support the hypothesis that 5-HT_{1A}-R signaling mediates some of these crucial trophic effects of serotonin in mouse brain development. Our studies are the first to address the possibility that 5-HT_{1A}-R signaling is involved in some of the temporal changes that drive normal hippocampal development.

Intriguingly, the 5-HT_{1A}-R-mediated signaling mechanism observed in these studies at P15 matches with the corresponding biochemical cascade observed earlier in the hippocampal neuron-derived cell line HN2-5 (Adayev, 2003). Considering the fact

that the HN2-5 cells were originally obtained from E17 hippocampal neurons, it could be expected that signaling mechanisms operating in this cell line would be similar to biochemical cascades observed in the hippocampus at P6 rather than P15. However, it should be also noted that all studies performed earlier involved fully differentiated HN2-5 cells. Under such conditions the 5-HT_{1A}-R→ MAPK pathway was independent of PKC activity and functioned through PKCα to cause suppression of apoptosis. Therefore, it is not surprising that the signaling mechanism observed at P15, when the neurons were more differentiated, was identical to that operating in the differentiated HN2-5 cells.

Another important aspect of the study is that the 5-HT_{1A}-R→ MAPK→ PKCα signaling cascade, which was originally delineated in the homogenous HN2-5 cells, was also observed in the heterogeneous population of hippocampal neurons at P15. This underscores the possibility that the observed 5-HT_{1A}-R-mediated signaling plays a key role within the hippocampus to produce a widespread effect on a variety of neurons.

Hippocampal development and brain disorders

Formation of the dentate gyrus in the rat begins at embryonic day 21 and the proliferation of granule neurons proceeds laterally from the suprapyramidal limb (adjacent to the hippocampal fissure) to the infrapyramidal limb (next to the deep roots of the alveus) (Altman, 1990). This early stage of granule cell proliferation is essentially completed by postnatal day 5 (P5) and is replaced by a prominent, larger, second proliferative zone in the polymorph layer (CA4 or hilus area of the dentate gyrus) beginning at P3. This broad proliferation zone of the hilus becomes confined to a narrow subgranular zone at the base of the granular layer by P15, which is the source of granule cell proliferation until adult age (Altman, 1990; Reznikov, 1991). New proliferative

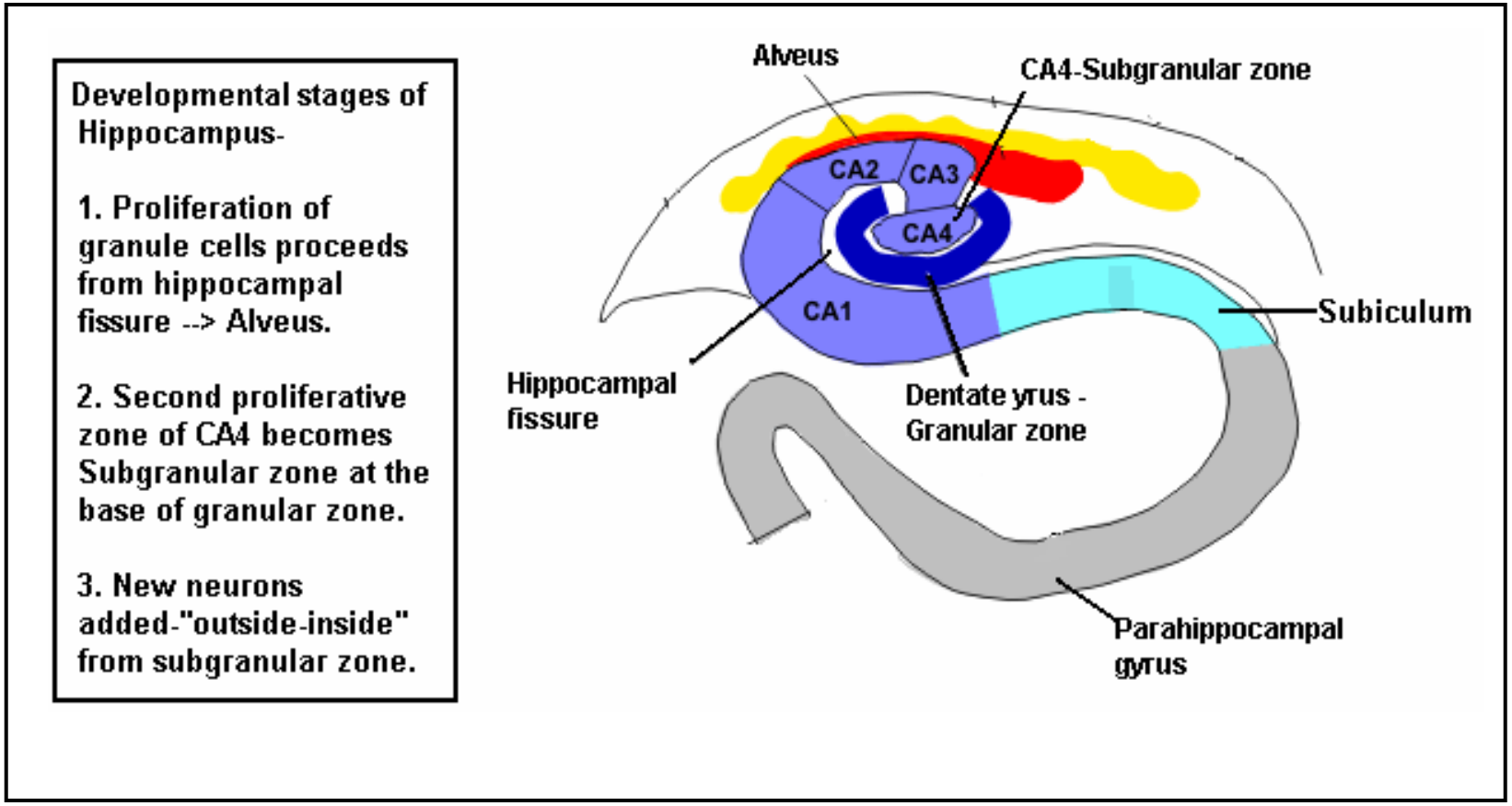


Figure. 4.1. Schematic representation of structure and steps involved in development of the hippocampus.

neurons of granular origin proceed in an ‘‘outside–inside’’ order, with the newer neurons added to the inner edge of the granular cell layer from a proliferative zone of germinal cells in the subgranular zone [Figure 4.1] (Angevine, 1965; Greaves, 2005). In the pyramidal and granular cell layers, it is only after settling into their appropriate laminar positions that hippocampal neurons begin to extend neurites and gradually assume their mature morphological and phenotypic characteristics. Pyramidal cell apical dendrites, for example, undergo their most rapid rate of growth between P0 and P10 (Loy, 1980). Thus, we have selected P6 and P15 not only because of high receptor expression, but also because the development of the hippocampus is at its peak during this period.

The hippocampus has long been associated with learning and memory processes, but now there is increasing evidence that this structure is also involved in the modulation of emotional responses (McNaughton, 1997). Lesions of the ventral hippocampus or local administration of pharmacological agents results in altered behavior in a number of rodent models of anxiety (Menard, 1998). It has been also suggested that there is a functional differentiation of the hippocampus along its dorsoventral axis. Manipulations of transcription or neurotrophic factors in this structure can also produce an antidepressant-like effect (Shirayama, 2002). Santarelli and coworkers have shown that hippocampal neurogenesis is required for the behavioral effects of antidepressants (Santarelli, 2003). In further support of the involvement of the hippocampus in mood regulation, Sheline and coworkers have reported that subjects with a history of major depression have smaller left and right hippocampal volumes (Sheline, 1996). Ashtari and coworkers have also shown relation between hippocampal volume and depression in the elderly (Ashtari, 1999). In addition, it has been shown that p44/42 MAP kinase activity

was significantly decreased in the prefrontal cortical areas (Brodmann's areas 8, 9 and 10) and the hippocampus of depressed suicide subjects without any change in the cerebellum (Dwivedi, 2001). It has also been demonstrated recently in postmortem brain tissues that the catalytic activity of protein kinase C (PKC) is altered in suicidal subjects and patients with affective disorders (Dwivedi, 2001).

The 5-HT_{1A}-R plays a crucial role in postnatal brain development (P5-P21) in mice such that its absence results in heightened anxiety later in life. The 5-HT_{1A}-R (-/-) mice show higher levels of anxiety-related behaviors in various conflict tests in adulthood, while displaying the same level of basal neurogenesis as wild-type (Santarelli, 2003). This finding implies that those behavioral anomalies are due to failures in neuronal connectivity during development. Therefore, our studies were performed during the critical postnatal period (P6 and P15) in the important hub, the hippocampus, where the receptor expression is at its peak during this time. Here, the receptor appears to modulate neuronal development in such a way that it produces lifelong effect.

The organotypic hippocampal slices have the ability to retain the hallmark cytoarchitectural organization of the hippocampus, whilst allowing environmental manipulation of the culture. In addition, the cultured brain tissue derived from newborn or early postnatal animals develops in much the same way as if it had remained *in situ* in the intact animal. This is true for most of the morphology, synaptogenesis, electrophysiology, and biochemistry of the tissue (Gahwiler, 1997). Several studies have shown that cultured hippocampal slices preserve the morphological and physiological features of the hippocampus of live animals for several weeks in rodents (Jahnsen, 1999; Kamada, 2004). Buckby and coworkers have validated the slice cultures as a relevant

model by comparing the levels of synaptic proteins in the cultures with those from acutely isolated hippocampal slices (Buckby, 2004). Therefore, our studies with cultured slices at P6 and P15 after 4DIV are appropriate to demonstrate the functional changes that occur during neonatal brain development. Also, our preliminary data (not shown here) and other studies show that *in vivo* studies of cellular signaling in the hippocampus can be difficult to interpret. The 5-HT_{1A}-R agonist induces hormonal changes (Vicentic, 1998) that could possibly alter the activity of ERK1/2 and other signaling molecules. Additionally, acting through pre-synaptic autoreceptors, 5-HT_{1A}-R agonists cause a reduction in synaptic 5-HT concentrations. Such inhibition could mask 5-HT_{1A}-R-mediated specific response. *In vivo* studies would show an overall effect of signaling by both pre-synaptic and post-synaptic 5-HT_{1A}-Rs and would not reveal the effect of 5-HT_{1A}-R-mediated signaling specifically in the post-synaptic neurons. Thus, slice cultures have been a good model to study the specific effects of this receptor signaling in the post-synaptic region such as the hippocampus where the presence of the receptor is crucial for the development of normal anxiety like behavior (Djavadian, 2005)

5-HT_{1A}-R activates ERK1/2 signaling during mouse brain development

In the present studies we observed that 5-HT_{1A}-R mediated activation of ERK1/2 was a widespread phenomenon in the hippocampus. The average time for peak activation of ERK was found to be 10 to 20 minutes from western and immunostaining analysis. The immunostaining data showed strong anti-P-ERK staining all over the dentate gyrus (DG) and the CA1-CA3 areas, which represent the spatial profile of ERK1/2 activation [Figure.3.1 and 3.8]. Thus, in developing mouse brain, the peak of 5-HT_{1A}-mediated ERK1/2 stimulation seems to be around 10-20 minutes in most regions of the

hippocampus. Previous studies have reported a rapid and transient activation of ERK1/2 within 3 minutes, which returned to basal levels within barely 30 minutes. However, those studies were done in non-neuronal cell lines, in which phosphoinositide-tris-phosphate kinase (PI-3 K) often played an essential role in the 5-HT_{1A}-R-mediated activation of ERK1/2 (Cowen, 1996; Garnovskaya, 1996; Lopez-llasaca, 1997; Della Rocca, 1999). In contrast, previous studies in our laboratory in hippocampal neuron-derived HN2-5 cell lines had also showed a 5-HT_{1A}-R-mediated, slow but prolonged activation of ERK1/2 independent of PI-3K signaling. Thus, the long-lasting component of MAPK was the major focus of this study as it proved to be part of a pathway stimulating neurogenesis as well as enhancing synaptic strength.

These studies involving the 5-HT_{1A}-R point to the dual role of the ERK1/2 pathway where different PKC isozymes lead to functional changes during hippocampal development of the mouse brain. The cellular state of differentiation declares a shift from active cell division to greater neuronal communication with little or no cell division. Such functionally different outcomes could be also due to a difference in the time of activation of MAPK. At P6 (4DIV), the 5-HT_{1A}-R-mediated activation of ERK1/2 is mediated by a PKC isozyme; however, PKCα was not found to be involved in this pathway. Further analysis showed that another isozyme, which is expressed at a higher level during the earlier stages of development, PKC-ε, is activated after 20 minutes of 8-OH-DPAT treatment at P6, and this profile corresponded well to the temporal profile of ERK1/2 activation, as shown in Figure 3.3. Since PKC-ε activation is not Ca^{2+}-dependent, the 5-HT_{1A}-R-mediated activation of this PKC isozyme could occur only through phospholipase C (PLC), which leads to the formation of diacylglycerol and IP_3 (Adayev,

1999; Adayev, 2003). The second messenger, DAG, can cause stimulation of PKC molecules. The activated PKC may then regulate Raf through direct phosphorylation (Kolch, 1993). The activation of PLC as a result of agonist treatment of the 5-HT_{1A}-R has been demonstrated in hippocampal HN2-5 cells (Adayev, 1999). Upstream involvement of PKC in the activation of the MAPK pathway has also been reported earlier (Ping, 1999; Cheng, 2001). PKCε-mediated activation of the MAPK pathway has been reported (Xuan, 2005). Hamilton and coworkers have shown that PKC-ε phosphorylates Raf-1 and thereby increasing its activity (Hamilton, 2001). Therefore, at P6, such PLC -mediated activation of PKC-ε could be linked to the stimulation of ERK1/2.

At P15 (4DIV), the temporal profiles for ERK1/2 and PKC-α were very similar and GFX- a general PKC blocker, did not cause inhibition of either ERK1/2 or PKC-α but the stimulation of PKC-α was reversed in the presence of PD98059 (MEK inhibitor) [Figure 3.9]. This suggests that at P15 PKC was not upstream of the 5-HT_{1A}→ERK1/2 signaling, but PKC-α was stimulated downstream of ERK1/2. This pathway had been discovered earlier in the hippocampal neuron-derived HN2-5 cells (Adayev, 2003). As discussed earlier activation of PKC-α requires initial phosphorylation at Thr 497 by PDK-1. Phosphorylation by PDK1 is followed by autophosphorylation of two additional sites in the sequence, namely,Thr-638 and Ser-657 for PKC-α. The present data also suggests that at P15, the phosphorylation of PKC-α at T638 might not have been carried out due to autocatalysis. Rather ERK1/2 could have actually caused phosphorylation-mediated activation of PKC-α, which has a consensus ERK1/2 phosphorylation site (PVL-^{638}T-P) at ^{638}T [Figure 4.2] (Adayev, 2003).

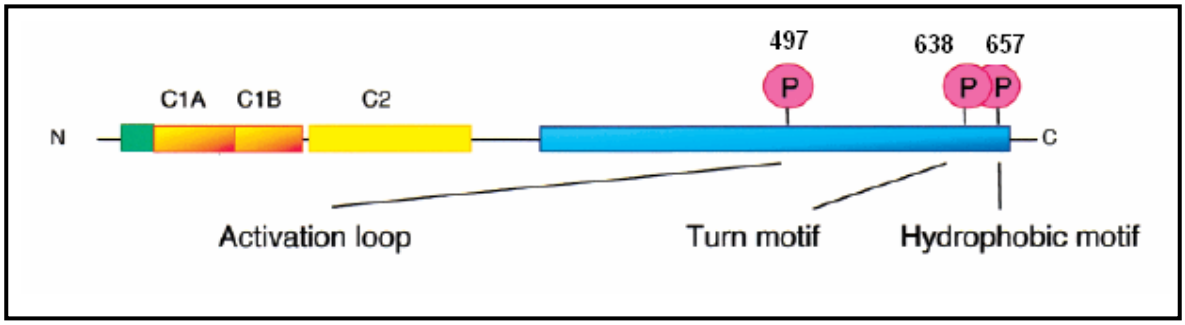


Figure. 4.2. Phosphorylation sites on PKC-α.

Activation loop, T-497: Phosphorylation is essential for the translocation and maturation of PKC. Lack of phosphorylation at this position yields non-phosphorylated PKC in the detergent-insoluble fraction. Thus, T-497 phosphorylation by PDK-1 is the first essential step in the activation of PKC. **Turn motif,** T-638: Dephosphorylation at this position yields inactive PKC. **Hydrophobic motif,** S-657: Phosphorylation at this position is not required for its activity, but is important for the stability of the PKC molecule.

In contrast to 5-HT_{1A}-R mediated activation of MAPK in post-synaptic neurons, Kushwaha and coworkers have reported that agonist activation of the 5-HT_{1A}-R in the raphe´-derived cell line RN46A causes a dramatic inhibition of the basal MAPK activity (Kushwaha, 2005). The biochemical signaling effects in pre-synaptic and post-synaptic receptors (autoreceptors and heteroreceptors respectively) have been hypothesized to be different as a result of their differences in G-protein coupling (Blier, 1993). Recent studies performed with subtype-specific anti-G-protein antibodies have indicated that native 5-HT_{1A}-Rs are coupled to Giα in dorsal raphé nuclei (DRN) and to Gio in the hippocampus (Mannoury la Cour, 2001). Two classical hypotheses have been proposed to explain the distinct pharmacological properties of pre-synaptic and post-synaptic 5-HT_{1A}-Rs: 1) greater receptor reserve in somatodendritic than in post-synaptic areas (Yocca, 1990) and 2) different pre-synaptic and post-synaptic receptors (de Montigny, 1992a, 1992b). This has been shown by different coupling of several agonists and antagonists. Also, antidepressants induce desensitization of pre-synaptic but not post-synaptic (shown in CA3) 5-HT_{1A}-mediated responses (Blier, 1994; Pineyro, 1999). Electron microscopic studies of the ultrastructural anatomy of 5-HT receptors have also shown that 5-HT_{1A}-Rs in the dorsal raphé nucleus and septum are localized to both dendritic processes and somata, whereas in the dorsal hippocampus 5-HT_{1A}-Rs are found primarily on dendritic spines (Kia, 1996). Thus, the difference in outcome of these two signaling activities could be explained by the differences in the coupling and desensitization properties of the same receptor in addition to the spatial environment in two distinct areas of the brain.

Discrete PKC isozymes are activated in different hierarchies at two developmental stages in mouse development

The most striking aspect observed here is a clear switch in the hierarchy of PKC in the 5-HT_{1A}-R➔➔ERK1/2 pathway between P6 and P15. Being a member of the G protein-coupled receptor family, the 5-HT_{1A}-R signals through Gα and Gβγ subunits. At P6 (4DIV), PKC (probably PKC-ε) relays 5-HT_{1A}-R signaling to the MAPK pathway. In contrast, at P15 (4DIV), the 5-HT_{1A}-R-mediated activation of ERK1/2 leads to the activation of PKC-α [Figure 3.4 and 3.9b]. Thus, PKC-ε could be located upstream of ERK1/2 at P6 and PKC-α is involved downstream of ERK1/2 at P15. It has been shown in several studies that PKC is an upstream mediator of ERK activation, which in turn is involved in the proliferation of various cell types (Liao, 1997; Ping, 1999; Cheng, 2001; Hattori, 2001). Studies have proved that overexpressed PKC-ε is a powerful growth stimulus (Acs, 1997). Also 5-HT_{1A}R-mediated stimulation of PKC has been demonstrated in non-neural cells (Raymond, 1991). Essentially, 5-HT_{1A}R-mediated activation of PLC leads to the formation of diacylglycerol and IP_3, as mentioned before (Adayev, 1999; Adayev, 2003). The other second messenger IP_3, interacts with its receptors on the endoplasmic reticulum (ER) to elicit release of Ca^{2+} into the cytosol. This increase in $[Ca^{2+}]_i$ together with DAG causes stimulation of conventional Ca^{2+}-dependent PKC-α molecules. Activated PKC molecules can consequently phosphorylate receptors and their downstream signaling molecules as suggested by a study in which coupling of the 5-HT_{1A}-R to N-type Ca^{2+} channels was examined (Wu, 2002).

PKC-α and PKC-ε have been shown to sequentially activate Raf/ERK1/2 in which PKC-α contributes to the early, and PKC-ε to the late but sustained, phase of

ERK1/2 activation in endothelial cells (ECs) (Cheng, 2001). In our studies, we observed a sustained activation of ERK1/2 *via* PKC (possibly PKC-ε) at P6. Corroborating reports show that PKC-ε activates p44/p42 MAPK in the cytosol, which subsequently translocates to the nucleus (Ping, 1999). Not only may some PKC isoforms be active, and others not, for a given response, but the actions of different isoforms may even be antagonistic.

This intriguing switch in the positioning of PKC in the 5-HT_{1A}R→ ERK1/2 pathway could be caused by the differential involvement of PKC isozymes. Another speculation for this observed shift in signaling could also reflect a response to changes in the mode of serotonergic transmission. For example, during development, the somatic pattern of expression could be viewed as being consistent with a general role of 5-HT_{1A}-R as a mediator of trophic or paracrine hormone-like activity on processes such as cell growth and gene transcription. On the other hand, the dendritic localization of 5-HT_{1A}-R in mature hippocampal neurons could be viewed as being consistent with the known effects of 5-HT_{1A} on the modulation of neurotransmission *via* inhibition of neuronal excitability. Preferential targeting of the receptors to the dendrites would allow such modulation to occur at or near dendritic sites where excitatory signals are integrated (Patel, 2005).

PKCε is the predominant isozyme during early development

The ε isozyme of PKC is expressed predominantly in the nervous system with only trace amounts detected in non-neuronal tissues (Koide, 1992). It has been shown to be particularly abundant in the hippocampus, olfactory tubercle, and layers I and II of cerebral cortex in the rat brain (Saito, 1993). It has been suggested that PKC-ε plays an

important role in cell proliferation (Chang, 2002). In addition, in developing chick brain, PKC-ε is the major isozyme found in non-dividing and differentiating neurons (Mangoura, 1993). Hundle and coworkers have found that overexpression of PKC-ε enhanced neuronal growth factor (NGF) -induced activation of MAP kinases and NGF-induced neurite outgrowth. Thus, in this study, PKC-ε was suggested to enhance MAP kinase activation by decreasing Ras-GAP activity or, alternatively, by activation of kinases downstream of Ras that lead to activation of MAP kinases (Hundle, 1995) or by direct phosphorylation of Raf-1, as suggested before.

One possible explanation of the observed switch in the hierarchy of PKC could be that the profile of expression of PKC isozymes in the hippocampus undergoes a dramatic change between P6 and P15. The underlying mechanism is largely unknown, but it is possible that PKC-ε is the most abundant form of PKC in the hippocampus at P6. It is thus more easily available for interaction with ERK1/2 *via* Raf cascade. By contrast, at P15, PKC-α could interact with ERK1/2 and participate in the reaction because that is the predominant isoform later in the development. One of the very few studies done in cultured hippocampal neurons reports that the conventional PKC isozymes (α, ß, γ) are expressed at very low levels during the initial stages, after which their expression increases. By contrast, the less conventional isozymes (δ, ε, and ζ) are expressed at higher levels initially, for the first three days in culture, after which their expression declines progressively (Tejero-Diez, 1995). In a similar study, it was found that PKC-ε was the predominant isoform and was expressed from E6 onward in all brain regions of the chick embryos. PKC-α/β and -ζ isoforms were expressed at lower levels prior to PKC-ε expression and throughout embryogenesis (Mangoura, 1993). In another study,

the gene expression of PKC (ε, μ, λ) was shown to be positive and more evident in the embryonic brains of rats, and other isozymes occurred postnatally (Minami, 2000). Also, such PKC-ε-mediated activation of the MAPK pathway has been demonstrated earlier (Xuan, 2005). Complete explanation of the observed effect is most likely to be found in the nature of the interaction between ERK and PKC molecules.

5-HT_{1A}- R→ ERK1/2 signaling causes increased cell division at P6 with no effect at P15

The observed mechanistic change in the 5-HT_{1A}-R→ERK1/2 pathway could be responsible for the concomitant switch in the functional effect of this pathway from causing increased cell division at an earlier stage (P6) [Figure 3.6a and 3.6b] to evoking a greater level of synaptic activity (measured by fEPSP increased synaptic strength) at P15 [Figure 3.12b]. Several studies in adult rat show that 5-HT_{1A}-R agonists increase cell proliferation in the hippocampal dentate gyrus (Haleem, 1990; Wilson, 1998; Radley, 2002; Santarelli, 2003; Greaves, 2005). We monitored cell division with BrdU, which is a thymidine analog that is incorporated into the DNA during the S phase of the cell cycle prior to the division of the cell (Del Rio, 1989). Our results are unlikely to result from BrdU labeling of neurons undergoing DNA repair or abortive mitosis, because we observed a typical morphological characteristics of dividing cells (either cells were posed next to one another or were in the processes of dividing). Next, the cell cycle marker cyclin D1 was induced, as discussed in the following section.

The increase in cell division at P6 could be due to prolonged activation of MAPK, compared to transient activation at P15. The prolonged activation has been reported to be required for mediating cell growth. This cell growth was shown to be indeed dependent

on ERK1/2 activation through calcium independent PKC *via* pertusis toxin sensitive, G protein coupled receptors (Sellers, 1999).

At P6, the prolonged activation of MAPK could be significant, as the MAPK isozymes ERK1/2 are known to phosphorylate and activate the transcription factor Elk-1, which in turn causes induced expression of the protein c-Fos. Previous studies have demonstrated that 5-HT agonists increase the expression of c-Fos in cortical and other forebrain regions of the rat *via* 5-HT_{1A}-R (Hajo´s-Korcsok, 1999). Upon translocation to the nucleus, c-Fos combines with a pre-existing protein, c-Jun, to form the dimeric transcription factor AP-1. Further phosphorylation of both subunits of AP-1 by the kinases JNK and FRK causes complete activation of AP-1 (Karin, 1995; Karin 1997), which is essential for DNA binding. In addition to c-Fos, other Fos homologs, such as Fos-B, Fra1, or Fra2, are also induced upon activation of ERK1/2 (Chang, 2001). The activated transcription factors could then upregulate the expression of cell cycle proteins (such as cyclin D1), thus causing increased proliferation of pre-neuronal cells. In support of this signaling, it has been also reported that ERK1/2 cascade is not only required for cyclin D1 transcription and protein synthesis, but it is sufficient by itself (Lavoie, 1996). Cyclin D1, in association with cyclin-dependent kinase (CDK), phosphorylates the retinoblastoma protein (RB), blocking its growth inhibitory activity and promoting the release of bound E2F transcription factor (Sherr, 1999; D'Abaco, 2002; Olashaw, 2002). In its hypophosphorylated state, RB is bound to the E2F transcription factor, which represses transcription of target genes that are required for progression through the S phase of the cell cycle. Phosphorylation of RB leads to its inactivation and de-repression of E2F, facilitating DNA replication [Figure 4.3] (Weinberg, 1995). Also, these events

facilitate the activation of the cyclin E-CDK2 complex, which is required for the completion of RB phosphorylation and entry into and completion of the S phase (Stacy, 2003). The elevated levels of cyclin D1 are maintained through the G1 phase and are required for the initiation of the S phase, at which time cyclin D1 levels are automatically reduced to low levels. The decision to continue cell cycle progression takes place in the G2 phase, when cellular Ras induces the elevation of cyclin D1 levels. The reduction of cyclin D1 to low levels during the S phase is required for DNA synthesis and forces the cell to induce high cyclin D1 levels once again when it enters the cell cycle (Stacey, 2003). Hoshino and coworkers have shown that MEK inhibitors induce a remarkable G1 cell cycle arrest (Hoshino, 2001). The ERK pathway has been shown to regulate positively the expression of cyclin D1 (Lavoie, 1996). These results are consistent with the idea that activation of the ERK pathway is essential for cells to pass through the G1 restriction point (Pages, 1993). It has recently been reported, however, that the ERK pathway functions not only in the G1/S transition but also in the transition from G2 to M phase in mammalian fibroblasts (Wright, 1999). The inducibility and rapid turnover of D-type cyclins thus integrates growth factor signaling with the cell cycle machinery, allowing the availability of extracellular growth factors to control the progression of cells through G1. In this way, cyclin D1 is proposed to serve as an active and critical switch function in the regulation of continued cell cycle progression.

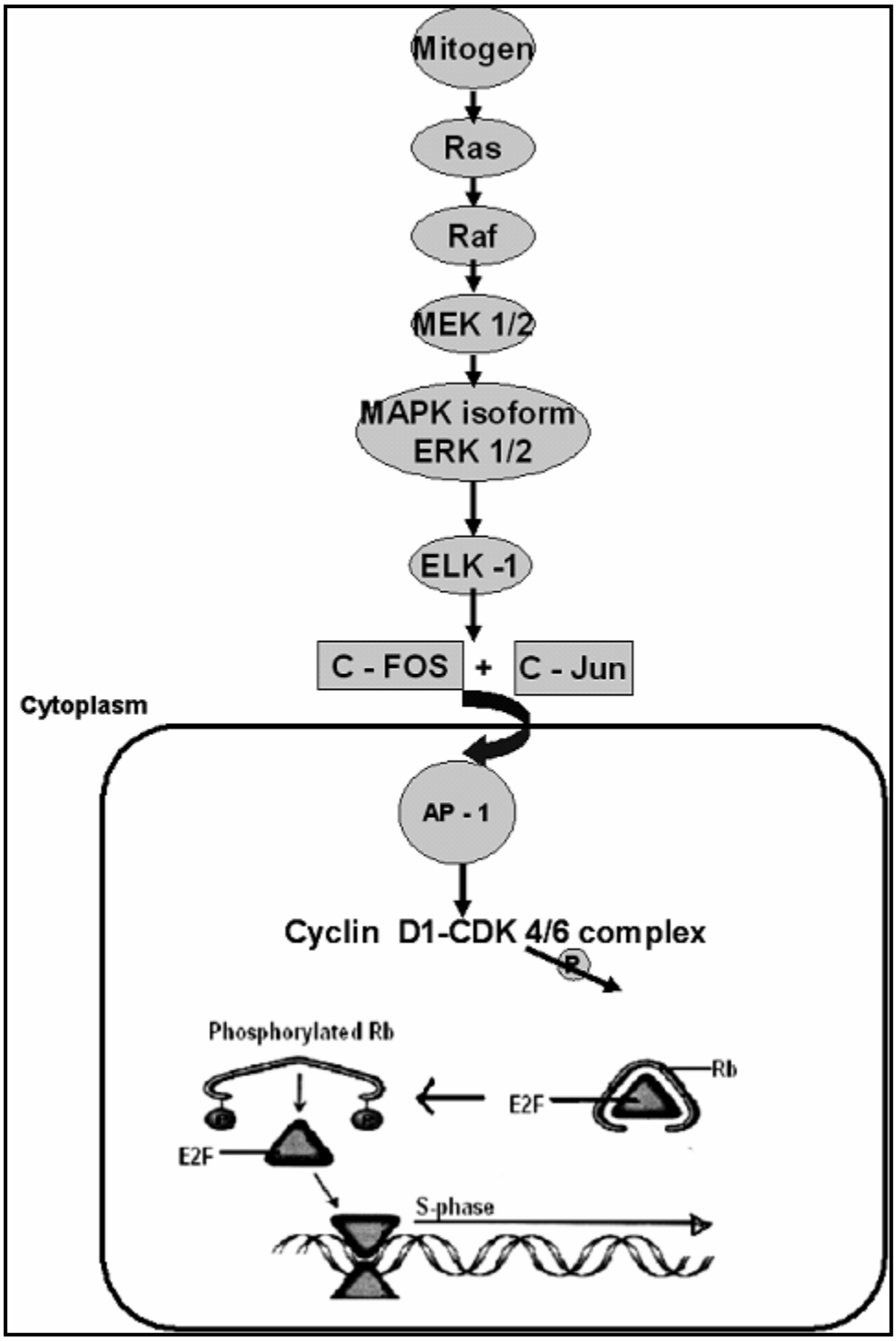


Figure. 4.3. Model showing induction of Cyclin D1through ERK1/2 cascade.

Indeed, at P6, we observed induction of cyclin D1 after treatment for 16 hours with 8-OH-DPAT. Also, the majority of (75% ± 5%) of BrdU-positive cells expressed the neuronal marker (NeuN), and a much smaller number of cells, which were not labeled with NeuN, could be either glial cells or quiescent preneuronal cells. Such rapid generation of neuronal cells during the early neonatal stages, followed by maturation and formation of increased synaptic connections during the later stages, is also expected to be crucial outside the hippocampus for the maintenance of its connections to the amygdala, prefrontal cortex, and other regions. Impairment of this neuronal circuit is believed to cause major emotional disturbances in human, such as anxiety, obsessive, compulsive disorder, and also other affective disorders. It has also been well demonstrated that the glutamatergic transmission inhibits cell proliferation in the DG *via* NMDA receptors (Cameron, 1995; Nacher, 2001), which are expressed by granule cells. The 5-HT_{1A}-Rs have also been shown to be expressed on these granular cells and thus their activation would be expected to counterbalance the NMDA receptor-mediated suppression of cell proliferation.

It can be argued that any change in cell cycle time from P6 to P15 (4DIV) could be responsible for the observed lack of 5-HT_{1A}-R-mediated stimulation of BrdU at P15. Although we have checked for a 2 hour time point in addition to 16 hours, the possibility of change in cell cycle time point can not be completely ruled out. Even though the cell cycle time in the DG at P15 is not known, a thorough analysis of cell cycle has been reported for rats (Cameron, 2001;Vaccarino, 2001). This study showed that even in adult rats the cell cycle time is 24 hours in the DG. In agreement with this observation, our data show significant levels of spontaneous cell division in the P15 slices (4DIV), but 8-OH-

DPAT treatment does not alter the rate of this process. Future experiments must be conducted to more thoroughly monitor cell cycle times at P15.

5-HT_{1A}-R→ ERK1/2 signaling causes greater increase in fEPSP at P15 than at P6

At P15, 8-OH-DPAT causes a significant increase in fEPSP in the hippocampus. fEPSP is the potential induced outside the post-synaptic neuron by the excitatory post-synaptic potential generated within the postsynaptic cell, thus showing the summed result with the effect of inhibitory neurons. Serotonin (5-HT) containing fibers originate in the brain stem raphé nuclei and innervate the hippocampus extensively. These innervations are supposed to modulate long-term potentiation in the perforant pathway, which in turn regulates learning and memory acquisition in hippocampus (Bliss, 1983; Vanderwolf, 1987). It has been shown that serotonin releasers (e.g. fenfluramine) increases the dentate gyrus population spike response to perforant path stimulation. This indicates that serotonin acts in the hippocampus to enhance afferent stimulation (Segal, 1988).

The Schaffer collaterals innervate apical dendrites of pyramidal cells and interneurons in area CA1 *via* glutamatergic mechanisms. Furthermore, postsynaptic 5-HT_{1A}-R are found mainly in the dendritic compartments, associated with dendritic spines (Kia, 1996) in which glutamate receptors are also concentrated, raising the possibility that 5-HT_{1A}-Rs may exert some of their functions by modulating or increasing glutamatergic signaling (Yuen, 2005), which could further enhance fEPSP.

Previous data indicate that the 5-HT_{1A}-R directly modulates the activity of GABAergic neurons. The stimulation of this receptor reduces the firing activity of inhibitory interneurons in the hippocampal CA1 neuronal slices (Palchaudhuri, 2005). A reduced GABAergic inhibition of the principal cells increases neuronal activity of the

hippocampus (Gulyas, 1999) and thus could help in increasing fEPSP [Figure 4.4]. Suppression of GABAergic transmission by reducing both spontaneous and evoked IPSP (inhibitory postsynaptic potentiation) has an important impact in enhancing neuronal excitation following tetanic stimulation in the CA1 region of hippocampal slice preparation from guinea pig. These experiments also demonstrated that postsynaptic GABA transmission efficacy is highly modifiable by a variety of physiologically active substances at both extracellular and intracellular membrane sites. These modifications have been suggested to contribute to long-term increase in neuronal excitation (long-term potentiation) (Stelzer, 1988). Another example is in the entrohinal cortex, where 5-HT_{1A}-R mediated signaling causes modulation of excitatory postsynaptic currents (EPSCs) recorded in putative GABAergic neurons (Schmitz, 1998). Interestingly, GABAergic cells also express 5-HT_{1A}-R mRNA, and 5-HT increases EPSCs in dorsal raphé 5-HT neurons *in vitro* by a disinhibitory mechanism (Santana, 2004). Studies have also suggested that the antidepressant effects of serotonergic drugs involve GABA-ergic mechanisms, and that chronic antidepressant treatment alters GABA receptor number (Lloyd, 1985) and receptor-mediated responses (Beck, 1997).

The dynamic interaction between the relative expression of EPSPs and IPSPs within the hippocampus can directly influence the input/output relationship. Hence, pharmacological manipulations of either transmission can markedly alter behavioral responses *in vivo*. The hippocampal computations can be understood in terms of a simple trisynaptic circuit, which is a purely feed-forward network and is considered to be excitatory. The activity of the principal cells in the hippocampus is under the control of GABAergic interneurons (Freund, 1990). Whole cell recordings from hippocampal and

prefrontal cortex (PFC) slices mostly show a hyperpolarizing, inhibitory effect of 5-HT_{1A} agonists on glutaminergic neurons (Be´ique, 2004;Yuen, 2005). Rather than performing recording from individual cells, we monitored the overall fEPSP generated in the CA1 neurons. Both excitatory (glutamatergic) and inhibitory, feed-forward GABAergic outputs contribute to such summed input into the CA1 neurons (to both dendrites and soma). We consistently observed a 5-HT_{1A}-R mediated enhancement of fEPSP in the CA1 neurons generated by electrical stimulation of the CA3 neurons of the Schaffer collateral pathway. This could be explained by a 5-HT_{1A}-R mediated suppression of the inhibitory interneurons that release GABA to generate IPSP at the CA1 neurons. Stimulation of CA1 neurons by inhibiting these feed-forward, GABAergic interneurons has already been demonstrated in the Schaffer collateral pathway (Pouille and Scanziani 2001; Maccaferri and Dingledine 2002). It has been shown atleast in pre-synaptic nerve terminals that the 5-HT_{1A} agonist causes an inhibition of GABAergic mIPSCs by directly acting on GABA-releasing processes independent of K^+ and Ca^{2+} channels (Koyama, 1995). Thus, it has been suggested that the GABAergic system receives balanced modulation *via* both NMDA receptor and 5-HT_{1A} receptors in the dentate gyrus. In conclusion, GABAergic interneurons, which reciprocally communicate with main excitatory neurons, send projections to each other and also receive serotonergic fibers from the raphé nuclei, thereby providing feedback and feed-forward inhibition of the principal excitatory circuit in the dentate gyrus (Matsuyama, 1997). Thus, the 5-HT_{1A}-R could decrease feed-forward inhibition in the hippocampus, resulting in increased excitability and thus increased fEPSP [Figure 4.4]. Bailey and coworkers have gone further to show that 5-HT_{1A}R-deficient mice against Swiss Webster genetic background

display a reduced expression of the main GABA-A receptor subunits during postnatal development (Bailey, 2004). Gene expression for the GABA-A receptor subunits is known to be sensitive to regulation by mitogen-activated protein kinases (MAPK) (Bulleit, 2000), which was also shown to be activated in our studies.

The hyperpolarizing effect of 5-HT_{1A}-R has been hypothesized as a result of activation of inwardly rectifying K^+ channels. In this regards, one curious observation has been that the kinetic characteristics of regulation of these channels by receptors are markedly different in transfected cells vs. in cells that natively express this channels and synaptic receptors. In addition, in previous studies a decrease in EPSP was observed only with high concentration of 5-HT or 8-OH-DPAT (≥ 100 μM) in Schaffer collateral (Schmitz, 1995). These studies were performed on adult rats, and the results were clearly separate from those observed at lower concentrations of agonist. In fact, Colino and coworkers have reported that 100 to 200 nM of 8-OH-DPAT antagonizes the hyperpolarizing effect, revealing depolarizing action in CA1 neurons (Colino, 1987). *In vivo* concentrations of 5-HT have been estimated by a variety of methods to be in the nanomolar, low micromolar range (O'Connor, 1991), which was the concentration used in our studies.

Thus, the possible model for 5-HT_{1A}-R-mediated increase in fEPSP is illustrated in Figure 4.4. The 5-HT_{1A}-R → MAPK→ PKC pathway can cause hyperpolarization by activating K^+ channels in Schaffer collateral-commissural fibers (Schaffer collateral) near the CA3 region where a stimulatory electrode was placed. This in turn can reduce not only excitatory postsynaptic potential (EPSP) at the stimulatory site, but can also reduce the inhibitory postsynaptic potential (IPSP) in the GABAergic interneurons. Thus, the

overall effect of excitation and inhibition can be summed up in increasing the excitation at the dendritic layer of the CA1 where the recording electrode was placed, thus showing depolarization in the dendrites of the CA1 region.

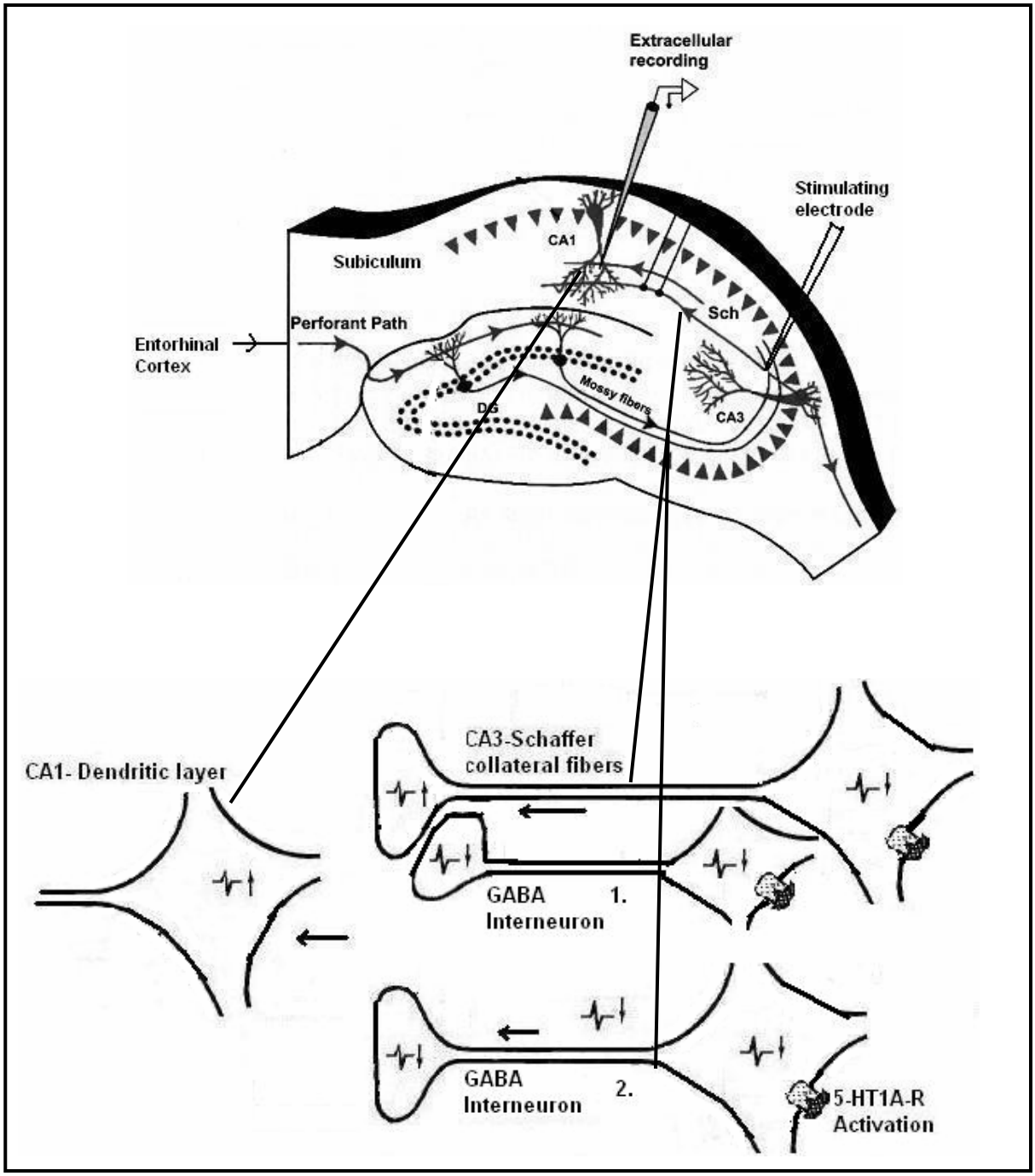


Figure. 4.4. Possible Model for $5HT_{1A}$-R mediated increase in fEPSP.

Activation of the 5-HT_{1A}-R on GABAergic interneurons causes hyperpolarization-mediated inhibition of these neurons. Since the GABA neurons generally suppress the excitatory effect the Glutamate neurons, inhibition of these GABA

neurons would result in excitation of post-synaptic neurons linked to the CA3 dendrites. Thus, 5-HT_{1A}-R-mediated signaling could cause suppression of (1) GABAergic interneurons that inhibit the Glutamate and (2) GABAergic interneurons that cause inhibition of CA3 target neurons directly. Further experiments will verify the possibilities.

PKCα harbors a ERK1/2 consensus phoshorylation site -

Previous studies from our laboratory have used immunoprecipitation experiments to show that 8-OH-DPAT treatment of hippocampal HN2–5 cells causes activation of PKC-α *via* ERK1/2 (Mehta, 2007). The three phosphorylated residues in PKC-α (Thr^{497}, Thr^{638}, and Ser^{657}) maintain the protein in a closed, active conformation that is functionally suppressed through the occupation of the substrate-binding site by its pseudosubstrate domain (House, 1990; Pears, 1990). The absence of phosphate at any one of these phosphorylation sites promotes an open conformer that is phosphatase-sensitive, protease-sensitive and sensitive to oxidation (Bornancin, 1996, 1997). This phosphorylation thus acts in a cooperative fashion to maintain the active (latent) conformation. The turn-motif phosphorylation site, Thr638, on PKC-α is within a consensus ERK1/2 phosphorylation sequence (PVLT638P) and is crucial for the activation of PKC-α. Therefore, although ERK1/2-mediated phosphorylation of PKC-α has not been reported so far, our data from P15 hippocampal slices and the HN2-5 cells propose this interesting possibility that it is regulated by ERK1/2 [Figure 4.2]. Alternatively, activated ERK1/2 could phosphorylate and stimulate another kinase that could cause phosphorylation of PKC-α at T638 and in this way the phosphorylated PKC-α molecule can reenter the signaling pool. Further *in vitro* experiments must be performed to address this possibility.

At P15, the 8-OH-DPAT-evoked activation of PKC-α is ERK1/2-dependent [Figure 3.9]. Our earlier studies have established that the same 5-HT_{1A}-R→ERK1/2→PKCα pathway, which causes stimulated fEPSP in the P15 hippocampal slices, is effective in blocking apoptosis in the hippocampal neuron-derived HN2-5 cells

(Adayev, 1999; Adayev, 2003). Thus, this ERK1/2→PKCα signal could also be involved in causing protection of maturing neurons that require trophic support while they form and stabilize their synaptic connections in the postnatal period P15-21.

Possible downstream target involvement in the 5-HT_{1A}-R→ ERK1/2 cascade

Studies undertaken in multiple laboratories have concurrently assigned a pivotal role for CREB in neuronal plasticity (Lonze, 2002). CREB also regulates cell proliferation, differentiation, and survival responses in a range of cell types in developing vertebrates. Josselyn and coworkers have shown that long-term memory in rats is facilitated by the overexpression of CREB in the amygdala (Josselyn, 2001). Sanyal and coworkers have demonstrated that CREB functions downstream of the transcription factor AP-1 to increase synaptic strength but not bouton number in the neuromuscular junction in Drosophila (Sanyal, 2002). CREB activity (i.e. anti-P-CREB staining) has been shown to co-localize with BrdU staining in adult mice (Nakagawa, 2002). Thus, CREB activity could also be involved downstream of ERK1/2 in regulating cell proliferation and stabilization and consolidation of synapses (Zhang, 2000). Also, prolonged 8-OH-DPAT treatment has been shown to produce a widespread effect on CREB phosphorylation in the hippocampus (Dulawa, 2002). Further, CREB is highly expressed by the granule cell layers (GCLs) of the dentate gyrus (DG) (Nakagawa, 2002). Also, when CREB is eliminated beginning postnatally in a *Crem* null background, the result is a profound degeneration of the CNS that occurs progressively throughout adult life and affects regions including the cortex, hippocampus, and striatum (Mantamadiotis, 2002). It is now recognized from the protein phosphorylation studies that multiple phosphorylation is the norm rather than the exception. However, 5-HT_{1A}-R is coupled to

Gi, and activation of the receptor reduces cAMP/PKA. Probably, these two opposing effects resulted in virtually no change in ^{133}S-CREB in our experiments [Fig. 3.5b and 3.10b]. Our earlier studies in the hippocampal HN2-5 cells showed that the 5-HT_{1A}-R➔ERK1/2 pathway is dependent on PLCß activity and calcium-calmodulin (CAM) (Adayev, 1999; Adayev, 2003). In addition to phosphorylation at Ser-133, calcium-activated phosphorylations at ^{142}S and ^{143}S plays a major role in CREB-mediated gene expression through an alternative, CREB binding protein (CBP)-independent pathway (Kornhauser, 2002). This could be a possible target for future studies.

In addition, another transcription regulatory factor, NF-kB, which is an important player in proliferation and protection, has been shown to be activated by 5-HT_{1A} agonist in non-neuronal and neuronal cell lines (Cowen, 1997). The trimeric NF-κB is normally retained in the cytosol in an inactive form, bound to IkB. Dissociation of IκB occurs concomitantly with phosphorylation and ubiquitination by IκB kinase (Koide), releasing NF-κB to enter the nucleus and find its way to several genes that it regulates (Beg, 1993; Mattson, 2000). In non-neuronal cells, NF-κB activity is increased following ectopic expression of constitutively active forms of ras, raf, or mitogen-activated protein kinase (Hirano, 1996) also PKC (PKC) causes activation of NF-κB (Hida, 2000). In contrast, a study from our laboratory has shown that 5-HT_{1A}-R activation of NF-κB does not require MAPK, PKC and cAMP in a hippocampal neuron-derived cell line (Sobocki, 2007).

Protein kinase B (PKB) or Akt-1 is a signaling protein that links the 5-HT_{1A}-R to anti-apoptotic signals in non-neural cells (Cowen, 2005; Hsiung, 2005). By monitoring activation of Akt-1 using a P-Akt antibody, we observed little or no correlation between the activation profiles for ERK1/2 and Akt-1 [Figure. 3.5a and 3.10a]. Thus it is unlikely

that Akt-1 plays an important role in 5-HT_{1A}→ERK1/2 signaling in the hippocampus. In support of this assumption, studies from our laboratory and others have shown that Akt is activated by the Phosphoinositol-3kinase (PI-3K) pathway (Cowen, 2005), which further activates Ca^{2+}/CaM kinase II (CaMKII) (Sobocki, 2007). Thus, the 5-HT_{1A}-R→ PI-3K→ Akt signaling probably has different functions in hippocampal neurons.

The 5-HT_{1A}-R activation of the MAPK pathway has been observed in both neuronal and non-neuronal cell lines. However, this is the first demonstration of 5-HT_{1A}-R→ERK signaling in slice cultures, which also reveals the functional significance of this pathway. This makes it a general pathway that could be used as a therapeutic target in numerous drug actions. In addition, by studying how this pathway affects brain development, we could also understand the basis of some developmental pathologies.

One assumption has remained implicit in the above experiments. It is expected that proliferation is the major event occurring during the early stages (P6-P10) and differentiation and synapse formation are the main events that take place during the later stages (P15-P20). On the contrary, proliferation and differentiation may occur simultaneously during the entire period of brain development. One indication of this possibility was obtained from the fEPSP measurement shown in Figure 3.12. Although the 8-OH-DPAT-evoked increase in fEPSP was greater at P15, the basal fEPSP was not largely different (e.g., by an order of magnitude) at these two stages. This meant that at both P6 and P15 there were comparable levels of synaptic transmission *via* the Schaffer collateral pathway.

The P10-P12 period is an important milestone in mouse development, with several important physiological changes occurring simultaneously around this time (e.g.,

eye opening, synaptogenesis, and cell proliferation). Thus, it is not completely surprising that signaling by the serotonin 1A receptor is different before and after this time. Thus, the major purpose of this project has been to detect and measure 5-HT_{1A}-R mediated activation/inhibition of key signaling molecules that will have therapeutic importance in developmental disorders and may shed new light on the etiology of multiple brain disorders.

This study further opens the opportunity to investigate in several areas:

1) Involvement of different G-protein subtypes participating in the switch of PKC hierarchy at these two developmental stages.
2) Other members of the 5-HT_{1A}-R → MAPK cascade upstream of ERK1/2, such as Pyk, Src.
3) Study of developmental profiles of PKC isozymes during the same postnatal period in mouse brain, which may explain the switch in PKC isozyme observed in this study.
4) Whether the observed 5-HT_{1A}-R-mediated increase in fEPSP was due to an effect of neurotransmitter release or receptor number and sensitivity, or alterations in postsynaptic morphology that involve permanent synaptic changes. To further analyze the long-term effect of this increase in hippocampal slices, a study of long term potentiation (LTP) induction and maintenance (activity-dependent synaptic plasticity that may underlie learning and memory) along with the corresponding behavioral implications, need to be performed. This is in view of the fact that signaling kinases such as MAPK and PKC have been shown to be necessary for the induction of LTP in the Schaffer collateral pathway to CA1 (SC-CA1)

(Linden, 1989; Hussain, 2003).

5) The nature of the interaction between ERK1/2 and PKC-α also opens a whole new area of research, which could explain another means of PKC regulation.
6) One final question that needs to be asked is "what is the approximate developmental stage at which these mechanistic transitions occur?" A possible explanation is that during neonatal stage (P5-P8) the 5-HT_{1A}-R expression is low, whereas during the later stages (P15) it is relatively high. Stiochmetric compensation is probably made through a PKC isozyme (E.g. PKC-ε) that is presumably expressed at higher level during the early neonatal stages. Thus, during P5-P8 5-HT_{1A}-R signaling could involve PKC-ε whereas during the later stages, around (P15), when 5-HT_{1A}-R as well as PKC-α are relatively abundant, then the PKC-α isozymes was used in the signaling cascade. Our preliminary studies have indicated that there is dramatic increase in [^{3}H]-8-OH-DPAT binding around P8-P10. Based on this, we predict that the mechanistic transition occurs around the same time of postnatal development, i.e. around P8-P10.

The interplay between 5-HT_{1A}-R signaling and other transduction pathways during the plasticity of development is an area of daunting complexity, but one that will also need to be dissected.

On the surface it may seem unlikely that a single chemical could play two such entirely different roles in the brain [Figure 4.5]. However, when one considers how the mammalian brain develops, it is not so improbable. The possible activation of PKC-ε and then PKC-α in a temporal manner in the hippocampus indicates that PKC molecules are sequestered in the cytosol as a sequel to the agonist activation of a receptor. The current

literature is replete with examples of such channeled transmission of molecular signals in the neural and other cell types. Thus, this project initiates a new process of interconnecting multiple effectors of the same receptor to a conceptual model of receptor-mediated regulation of early brain development.

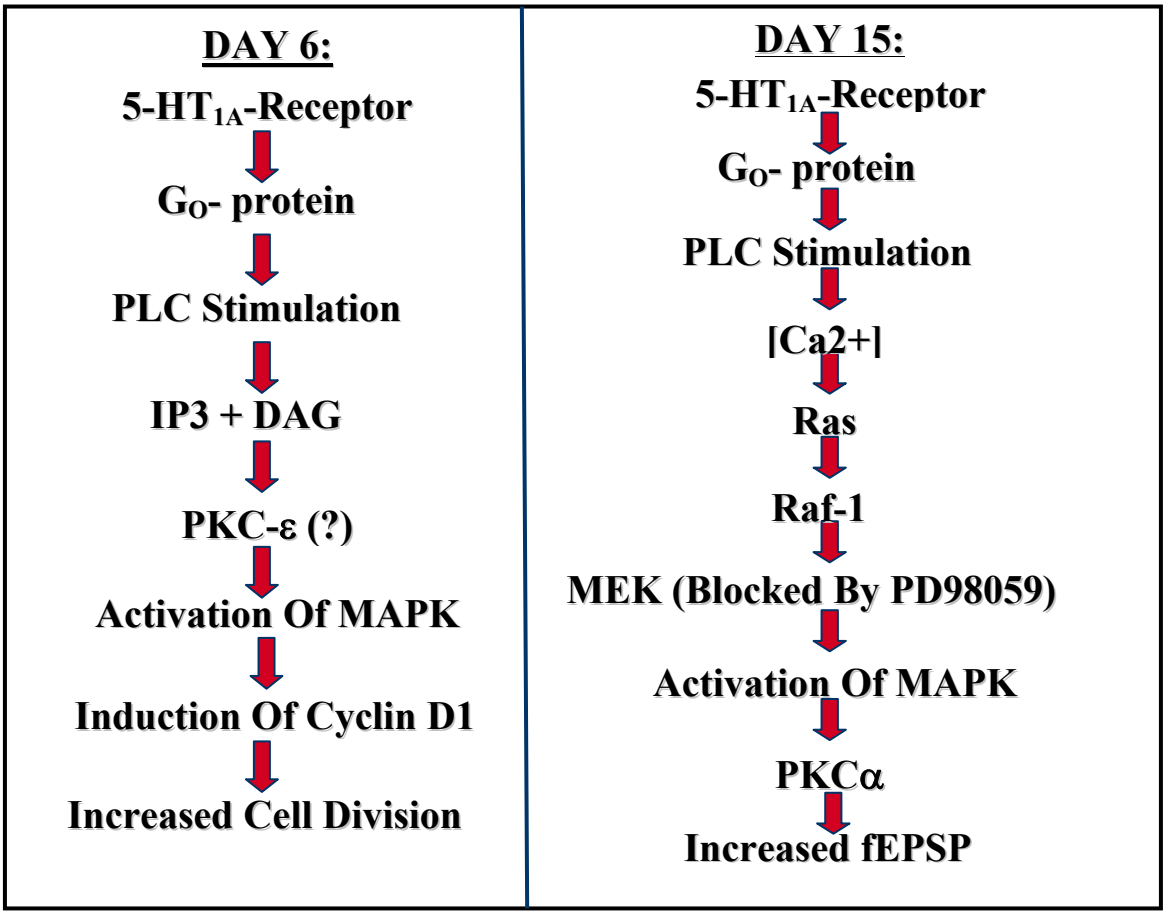


Figure 4.5. A possible transition in the mechanism of 5-HT_{1A}-R during signaling neonatal brain development.

CONCLUSION:

Previous studies have strongly suggested that 5-HT_{1A}-R plays an important role in the regulation of emotion, which is blueprinted during development by the action of some crucial signal transduction pathways. Our study sheds new light on the role of this receptor in brain development and constructs a mechanistic framework for its action. Unraveling of such mechanisms could help in the design of new therapeutic strategies to treat developmental brain disorders that are linked to 5-HT_{1A}-R-mediated signaling.

Although our studies were mainly focus on the mechanistic analysis of the anomalous brain development that could lead to anxiety and depression, the crucial role the 5-HT_{1A}-R→ MAPK pathway plays in neurogenesis can be linked to many other developmental disorders of the brain. For example, aberrant cell division and synaptogenesis could be the cause of variety of other disorders such as Autism, Schizophrenia, Attention-deficit-hyperactivity disorder (ADHD), Obsessive compulsive disorder (OCD), and many others.

Most of the signaling studies have focused on adult animals, leaving an important gap in our knowledge of prenatal factors. These prenatal factors are thought to play an important role in the normal development of the brain and also in the etiological processes leading to mental disorders involving the hippocampus.

Beyond clinical implications, understanding how the brain is wired provides a fundamental insight into what it means to be human. As Descartes said, "I think, therefore I am."

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