NEONATAL INVOLVEMENT OF THE SEROTONERGIC SYSTEM IN HIPPOCAMPAL WIRING: UNRAVELING ITS ROLE IN GENDER-SPECIFIC MOOD DISORDERS

by

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

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Abstract

Neonatal Involvement of the Serotonergic System in Hippocampal Wiring: Unraveling its Role in Gender-Specific Mood Disorders

by

Sreyashi Samaddar

Advisor: Dr. Probal Banerjee

The hippocampus has been linked to a plethora of mood disorders. The monoamine neurotransmitter serotonin (5-HT) plays a critical role in the development of several of these mood and neuropsychiatric disorders. Serotonergic signaling *via* serotonin 1A receptor (5-HT_{1A}-R) is crucial during the early postnatal days for later-life behavior, like anxiety and depression. Specifically, the forebrain 5-HT_{1A}-R heteroreceptors have been implicated in several mood disorders. Intriguingly, the incidence of mood disorders is two-fold higher in women than men. Furthermore, the level of the serotonin 1A receptor (5-HT_{1A}-R) is significantly higher in the brain of women than men, suggesting that the women may be more sensitive to a deficiency in 5-HT_{1A}-R signaling. Taken together, all these studies also suggest that the serotonergic system operating *via* 5-HT_{1A}-R in brain development may determine behavioral manifestation of mood disorders in adulthood. However, the mechanistic details of the 5-HT_{1A}-R signaling pathway, especially how it operates in early developmental stages, are still unclear. The current study is aimed at bridging this gap.

Current findings reveal that 5-HT_{1A}-R signaling acting through PKC epsilon and Erk1/2 augments neuroproliferation and neurogenesis in the dentate gyrus (DG) in mice at postnatal day-6 (P6), which marks the peak of postnatal neuroproliferation. However, only the basal level of neuroproliferation was significantly stunted in the female but not male 5-HT_{1A}-R (-/-) (KO)

mice at P6. Subsequently, the neuroproliferation in the KO female mice could be restored to almost wild-type (WT) levels through the stimulation of the downstream PKC epsilon molecule using a selective activator, DCP-LA. Using Timm staining, a significant increase was observed in the arborization of the DG granule cell-derived mossy fiber (MF) axons and their connectivity with the CA3 pyramidal cells in the *Stratum Oriens* (SO) region in the female KO mice at P18. Such increased MF connectivity could lead to increased excitation and elevated anxiety. Confirming the importance of the identified signaling activity through PKC epsilon, this increased MF connectivity was restored to normal levels in the KO females treated with DCP-LA from P6-14. Finally, this treatment eliminated the significantly elevated anxiety levels in the adult female KO mice. Thus, a sex-specific effect of serotonergic signaling via the 5-HT_{1A}-R plays an important role in hippocampal development and later-life behavior, which can be corrected by targeting a downstream signaling molecule, PKC epsilon. New-born and immature granule cells of the DG are resistant to inhibition by gamma-aminobutyric acid (GABA), and each granule cell-derived MF axon connects with about 50 inhibitory inter-neurons, which cause inhibition of the CA3 pyramidal neurons. In contrast, each MF axon connects directly with only 10-14 CA3 pyramidal neurons. Thus, the MF axons, especially the excitatory immature granule neurons, produce more feed-forward inhibition than excitation, which allows for only a limited level of activation of the CA3 neurons. Decreased neuroproliferation at P6 would yield less GABA-insensitive new granule neurons in the DG, which may then lead to a decrease in feed-forward inhibition, thereby eliciting an overall increase in excitation of the CA3 pyramidal neurons and the downstream Schaffer Collateral pathway of the hippocampus.

While this may explain the extension of MF collaterals in the SO region, the sex-specific effect of 5-HT_{1A}-R deficiency on neuroproliferation remains to be explained. Although sex-based

differences have yet to be recorded for steroid levels in the neonatal hippocampus, it is known that neonatal neuroproliferation in the DG is significantly higher in male mice, and estradiol treatment boosts neuroproliferation only in the females. This study introduces the serotonergic system as a second signaling scheme with pronounced sex-specific effects in the neonatal DG and suggests a possible crosstalk between brain steroids and 5-HT during brain development.

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Table of Content

Table of Figures

Abbreviations:

SGZ: Sub-Granular Zone

DG: Dentate Gyrus

CA1-CA4: *Cornu Ammonis* regions 1-4

MF Pathway: Mossy Fiber Pathway

EC: Entorhinal Cortex

Sub: Subiculum

SL: Stratum lucidum

SO: Stratum oriens

SR: Stratum radiatum

5-HT: 5-hydroxytryptophan or Serotonin

5-HTR: Serotonin receptor

5-HIAA: 5-hydroxyindoleacetic acid

MAO: Monoamine Oxidase

SERT: Serotonin Transporter

SSRIs: Selective Serotonin Reuptake Inhibitors

GPCRs: G-protein coupled receptors

HPA axis: Hypothalamo-Pituitary-Adrenal axis

PKC: Phospho kinase C

PKCε: Phospho kinase C epsilon

DAG: Diacylglycerol

PS: Phosphatidylserine

ERK1/2: Extracellular-signal-regulated Kinase

MAPK: Mitogen activated protein kinase

MEK: MAPKK or MAP2K, Mitogen activated protein kinase kinase

BrdU: 5-bromo-2'-deoxyuridine, proliferation marker.

8-OH-DPAT: (±)-8-hydroxy-2-(di-N-propylamino) tetralin, 5-HT_{1A}-R agonist

WAY 100635: N-[2-[4-(2-methoxy-phenyl)-1-piperazinyl] ethyl]-N-(2-pyridinyl) cyclohexane-carboxamide, selective 5-HT_{1A}-R antagonist

Myr-εV1-2 (N-Myr-EAVSLKPT): Selective PKCε inhibitor

GFX: *Bis-indolylmaleimide*, the general PKC inhibitor

PD: PD98059, 2'-Amino-3'-methoxyflavone, the MEK1 inhibitor

U0126: 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene, MEK1 and MEK2 inhibitor

DCP-LA: 2-[(2-pentylcyclopropyl)methyl]cyclopropaneoctanoic acid, selective activator of PKCε

VEGF: Vascular Endthelial Growth Factor

Flk1: Fetal Liver kinase 1/KDR, VEGFR type2

KDR: Kinase Insert domain receptor, also designated as CD309, cluster of differentiation 309.

Flt1: Human gene encoding VEGFR type1

SU5416**:** 1,3-Dihydro-3-[(3,5-dimethyl-1H-pyrrol-2-yl)methylene]-2H-indol-2-one, Flk1 specific kinase inhibitor

NeuN: Neuronal Nuclei

DCX: Doublecotin

GFAP: Glial Fibrillary Acidic Protein

AP1: Activator Protein 1

siRNA: small interfering RNA

shRNA: short hairpin RNA

HN2-5 cells: Hippocampal neuron derived cells

1. Chapter 1

Introduction

The hippocampus in one of the most intensely studied parts of the brain. Over the years researchers have tried to unravel all aspects of hippocampus starting from its anatomy, physiology, biochemistry, its diverse roles in emotion, mood, anxiety, stress, learning, memory, its effect on other regions of the brain as well as its connectivity with other regions of the central nervous system. The hippocampus is surrounded by the entorhinal, parahippocampal and perirhinal cortices and is connected to cortical and sub-cortical parts of the brain. Majority of the hippocampus's neocortical inputs come from the perirhinal and the parahippocampal cortices through the entorhinal cortex and its neocortical outputs project through subiculum, which again project back to the entorhinal cortex (Burgess 2008). The perirhinal cortex plays an important role in representing complex objects and the parahippocampal cortex is responsible for visuospatial information (Burgess 2008).

British neuroscientist and psychologist, David Courtnay Marr has performed pioneering studies to explain how the hippocampus operates in relation to other parts of the brain. His computational model, otherwise known as Marr's model, describes the hippocampus as the 'convergence zone', as the hippocampus encodes the multi-modal information represented by the cortical areas. The hippocampus is thus the hub of brain activity constantly receiving and processing information and also influencing other brain regions (Fig.1). Hippocampus is also connected to the pre-frontal cortex and amygdala (Leuner and Shors). The hippocampal CA1 region sends GABAergic projections to the hypothalamus thus exerting an inhibitory effect on the latter which in turn regulates the HPA axis and secretion of glucocorticoids (Vale 2006). The hypothalamus in turn secretes important hormones like oxytocin, which again plays a crucial role

in emotion and social behavior. An understanding of the hippocampus and its development has therefore become a prerequisite to determine the etiology of the several mood disorders.

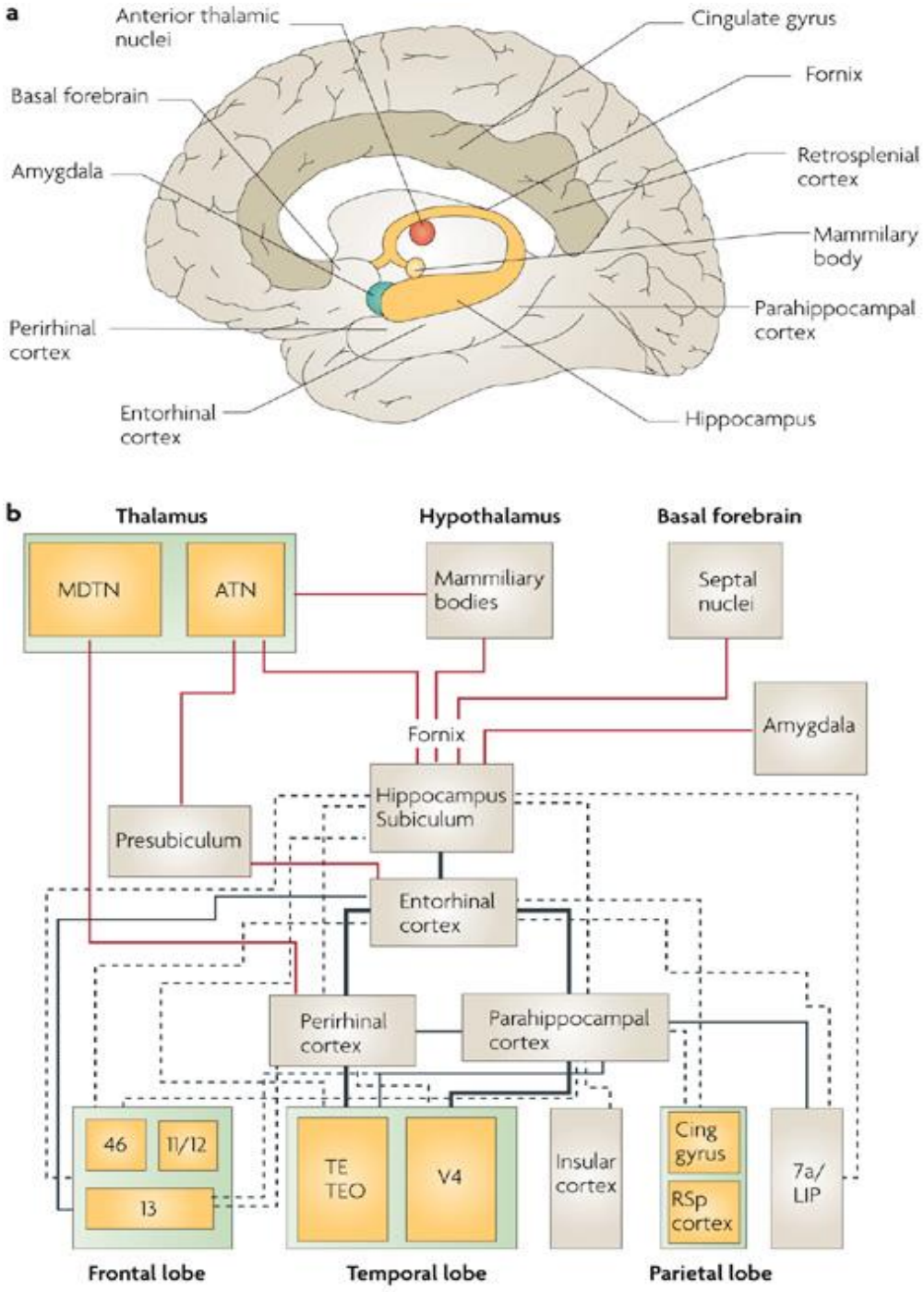


Figure 1-1 Connectivity of the hippocampus to other brain regions

Adapted from Chris M. Bird and Neil Burgess, Nature Reviews, Neuroscience 2008.

Overall anatomy of the hippocampus:

The hippocampus is a major component of the limbic system. The mammals have one hippocampus in each hemisphere of the brain. Hippocampus means 'Seahorse' in Latin. The presence of different neuron types and its multifaceted role make hippocampus an important scientific research model (Fig 1.1-1.3)

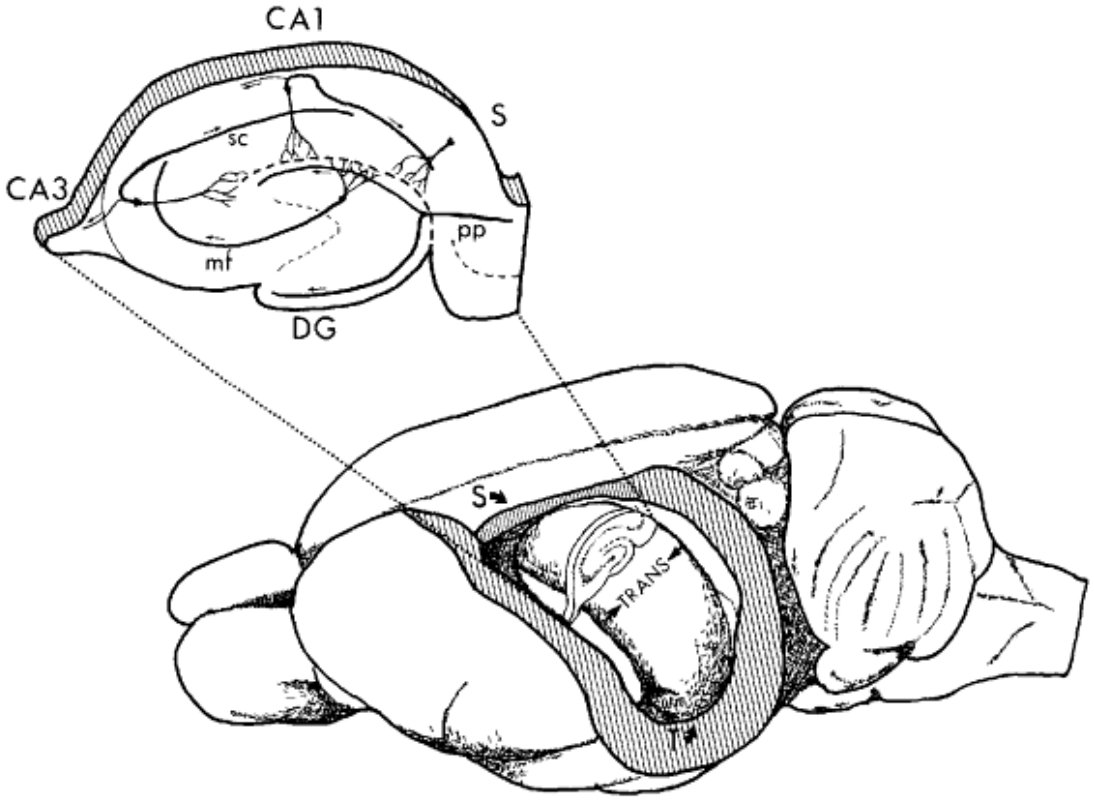


Figure 1-2 Location of the hippocampus in the brain

Adapted from the 'Synaptic Organization of the Brain' by Daniel Johnston and David G. Amaral

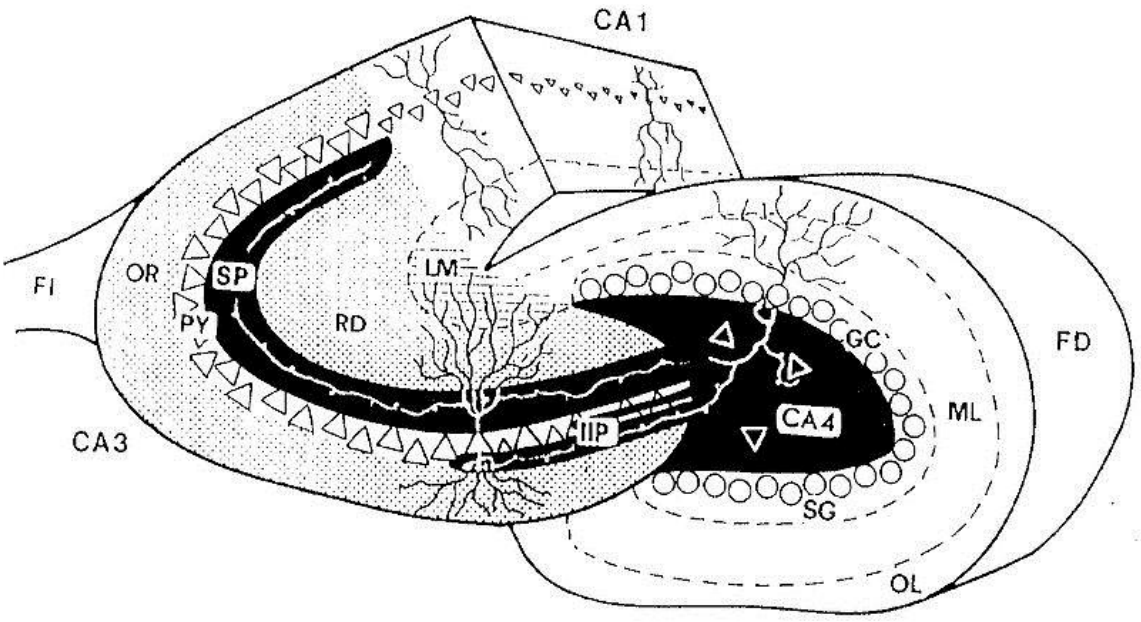


Figure 1-3 Cell layers in a cross-section of the hippocampus

The hippocampus is differentiated along its length, the dorsal part being involved in learning and memory and the ventral part being associated to emotionality. This topological difference is linked to different functions: The ventral hippocampus receives inputs from the rostromedial entorhinal cortex, and provides output projections to the prefrontal cortex, amygdale, and nucleus accumbens. On the other hand, the dorsal hippocampus receives input signals from the lateral and caudomedial entorhinal cortex, and has efferents to the dorsal lateral septum and mammillary complex.

Anatomy of the hippocampus: The hippocampal region is composed of the *hippocampus proper* (*area dentata*, *Ammon's horn* and the *subiculum*) and the *retrohippocampal areas* (*presubiculum*, *parasubiculum*, *area retrosplenialis e* and *area entorhinalis*). The dentate area, more recently called as dentate gyrus (DG), is composed of *fascia dentate* and the *hilus fasciae dentatae*. The *fascia dentate* is in turn composed of molecular layer and the granular layer. The *fascia dentate* has a characteristic U-shape. Its two limbs, the *medial* and the *lateral blades*, join posteriorly at the *dentate crest*. The cell bodies of the granule cells, the principal neurons of the

dentate area form the granular layer, while the dendritic trees of these granule cells radiate into the molecular layer (Gaarskjaer 1986).

The Ammon's horn is composed of pyramidal cells, whose apical and basal dendrites are dispersed over other layers. This pyramidal layer is divided into four sub-regions *CA1-CA4* (Lorente de No 1934). 'CA' stands for *Cornu Ammonis*, or the Horn of Ammon, with reference to the ram's horn of the Egyptian God *Ammon*. *CA1 region* is the part of Ammon's horn closest to subiculum and consists of *small pyramidal cells*. *CA2 region* consists of both *small* and *large pyramidal cells*. *CA3 region* is adjacent to the *dentate area* and consists of *large pyramidal cells*, which make synaptic connections with the *mossy fibers* (explained later) (Fig. 1.3). The *CA4 region* corresponds to the *hilus of the dentate area* and contains *large mossy cells* (Lorente de No 1934). Nevertheless, Ammon's horn has been best described according to *Cajal*, as *regio superior*, corresponding to CA1 and *regio inferior* corresponding to CA2 and CA3 (Cajal 1893).

Hippocampal circuits:

The hippocampus is well known for its tri-synaptic loop. The tri-synaptic loop is the circuit of synaptic transmission in the hippocampus. The first projection comes from the cells of the entorhinal cortex, which make connections with granule cells of the dentate gyrus called the 'Perforant pathway'. The dentate gyrus then synapses with the pyramidal cells of the CA3 region via the 'Mossy fiber pathway'. The CA3 ultimately sends projections to the CA1 pyramidal cells through the 'Schaffer collaterals', which are carried out through the fornix into the deep layers of the entorhinal cortex. However, recent studies have hinted towards other synaptic connections apart from these three already known connections. It is believed that projections from the layer III of the entorhinal cortex make direct connections with the CA1 region.

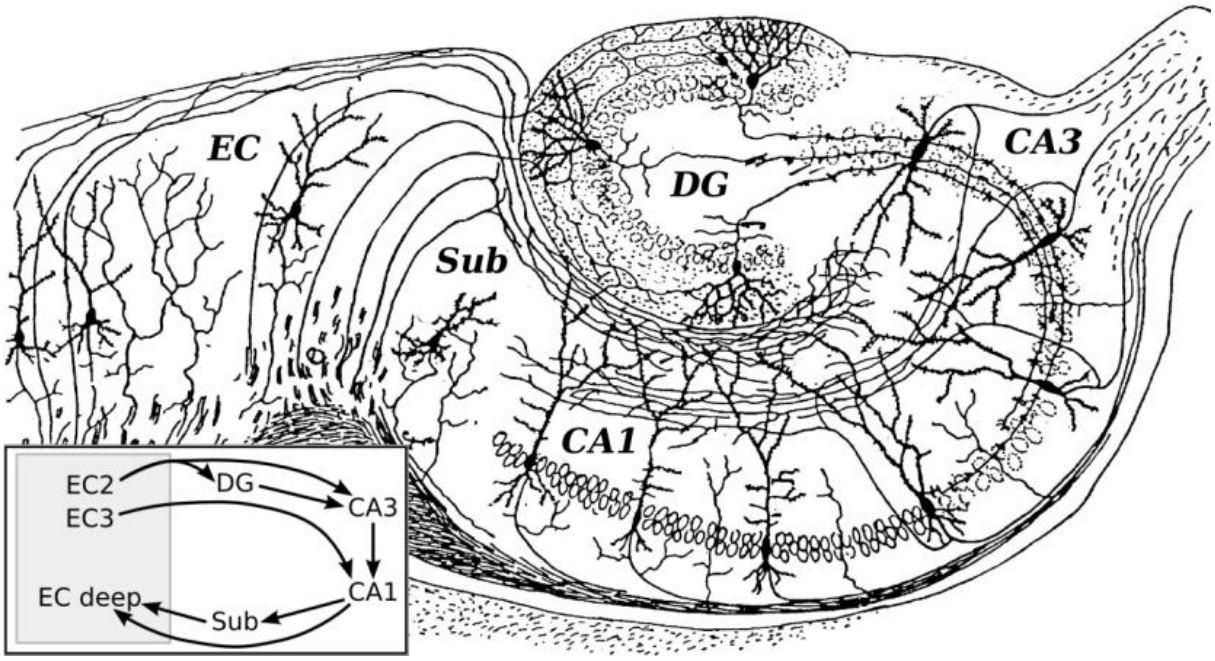


Figure 1-4 The tri-synaptic circuitry of the hippocampus

Circuits of the hippocampus, modified from a drawing of *Santiago Ramon Y. Cajal*.

Neurogenesis in the Hippocampus:

Adult neurogenesis occurs in adults throughout life. Adult hippocampal neurogenesis is down regulated by stress, aging, glucocorticoid hormones and drugs of abuse, whereas it is upregulated by exposure to an enriched environment, exercise, hippocampal-dependent learning, estrogen, and antidepressant drugs.

Postnatal neurogenesis is restricted to two distinct locations in the brain, a) Sub-Ventricular Zone (SVZ) and the b) Sub-Granular Zone (SGZ). The progenitor cells grow in close apposition to vascular and endothelial cells, and VEGF secreted by these cells has been shown to enhance neurogenesis. The progenitors are present in the sub-granular zone and they can mature into granule cells whenever required. Apart from several growth factors like Epidermal Growth Factor (EGF), and Transforming Growth Factor (TGFα), and the neurotransmitter Serotonin (5-

HT), have been shown to play key roles in granule cell proliferation. There is also evidence of the existence of serotonin receptors (5-HTRs) type 1A (discussed later) and release of 5-HT in the dentate gyrus (Cameron and Gould 1994; Elizabeth Gould 1994; Gould 1999).

Hippocampal Development:

Hippocampal development has been studied since a long time using ^{3}H-thymidine autoradiography. Joseph Altman and Shirley Bayer have been studying the development of the hippocampus from the embryonic period. Hippocampal regions start forming from E15-E21 and continue forming postnatally. Though the hippocampal regions are fully formed by P22, new neurons are added to the granular layer of the dentate gyrus throughout life. Hippocampal development involves multiple steps like cell proliferation, cell maturation, radial migration and cell survival, which are also genetically controlled by genes like Wnt3a, Imx2, and Reelin (S.M. Lee 2000; H. Abraham 2003)

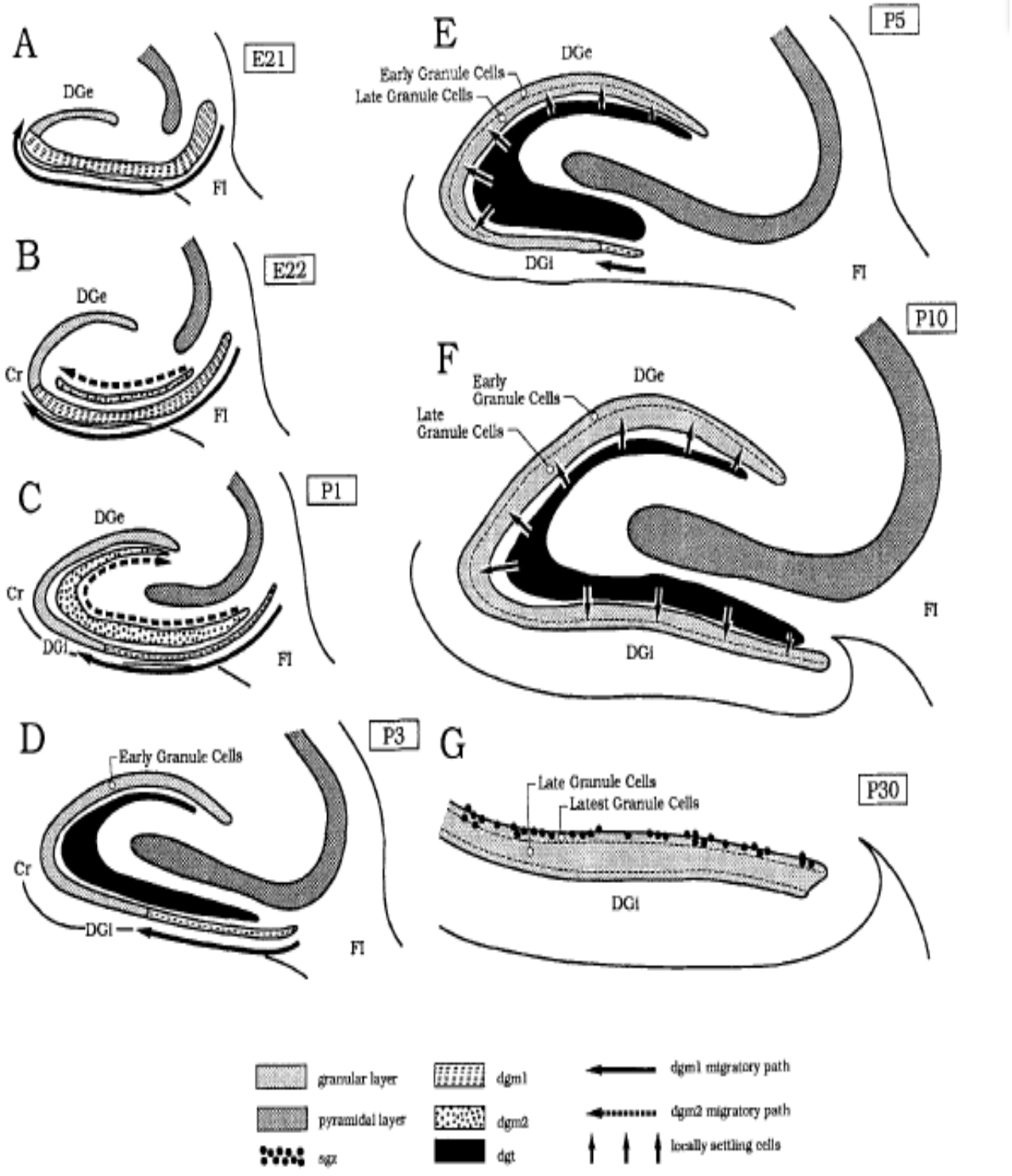


Figure 1-5 Schematic representation of hippocampal development

Adapted from J. Altman and S.A.Bayer, 1990

Schematic representation of hippocampal development. E18, marks the beginning of the differentiation of the neuroepithelium and they migrate to form granule neurons (arrows showing the trajectories). By P20, the dentate gyrus and the Ammon's Horn and almost fully formed. At P30, the sub-granular zone is still present and will be present from neurons will be formed later in adulthood.

The hippocampal epithelium consists of three layers: the evaginated component adjacent to the subiculum, the putative Ammonic neuroepithelium, and the invaginated putative primary dentate neuroepithelium. By E18, the dentate neuroepithelium is much reduced and there are fewer mitotic cells. On the days E19 and E20, a stream of spindle shaped cells starts extending to the primordial dentate gyrus. E20 and E21 marks the abundance of proliferative cells in the dentate gyrus area. By E22, there is already a small but distinct external limb of the dentate gyrus (Fig 1.5). From birth to P5, proliferative cells keep migrating towards the internal limb and towards the external limb in a proximal-to-distal direction. As they mature, proliferating cells get incorporated in the granular layer of the dentate gyrus. The radial migration along the horse-shoe shaped scaffolding gives rise to a neurogenic gradient such that the oldest cells are at the tip of the upper external blade or limb and the youngest cells are at the tip of the lower blade or the internal limb of the dentate gyrus (Guangnan Li 2005). The Ammon's neuroepithelium also at around the same time forms the Ammon's horn. At around P22, there exists hardly any difference between the granule cells of the external and the internal limb of the dentate gyrus. (Bayer 1990; Joseph Altman 1990).

Serotonin:

Serotonin belongs to a class of biogenic amine neurotransmitters known to regulate not only brain functions but are also active in the peripheral nervous system. Serotonin, or 5-hydroxytryptamine (5-HT), is localized within neurons in the raphe region of the brain stem, which sends projections to various regions of the forebrain and other parts of the brain. The raphe neurons have been classified into nine groups named B1-B9. Nerve fibers arising from the caudal groups of serotonergic neurons (B1-B4) form a descending system directed to the spinal cord and also project to cerebellum, pontine and midbrain structures, whereas ascending fibers originate from the rostral groups of serotonergic neurons (B5-B9) and innervate almost all brain areas.

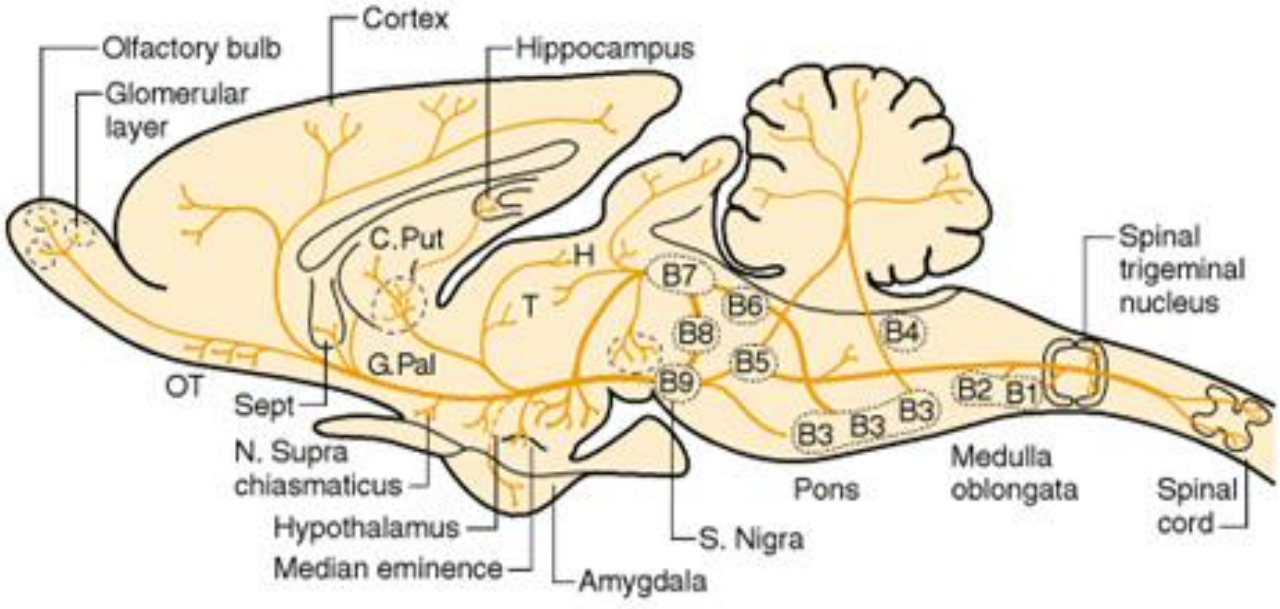


Figure 1-6 The Raphe nuclei.

Serotonin (5-HT) is synthesized from the amino acid tryptophan, an essential dietary requirement and hydroxylated to 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase, through the rate-limiting step for 5-HT synthesis. Then 5-hydroxytryptophan undergoes enzymatic decarboxylation to form serotonin. It is ultimately metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme monoamine oxidase (MAO).

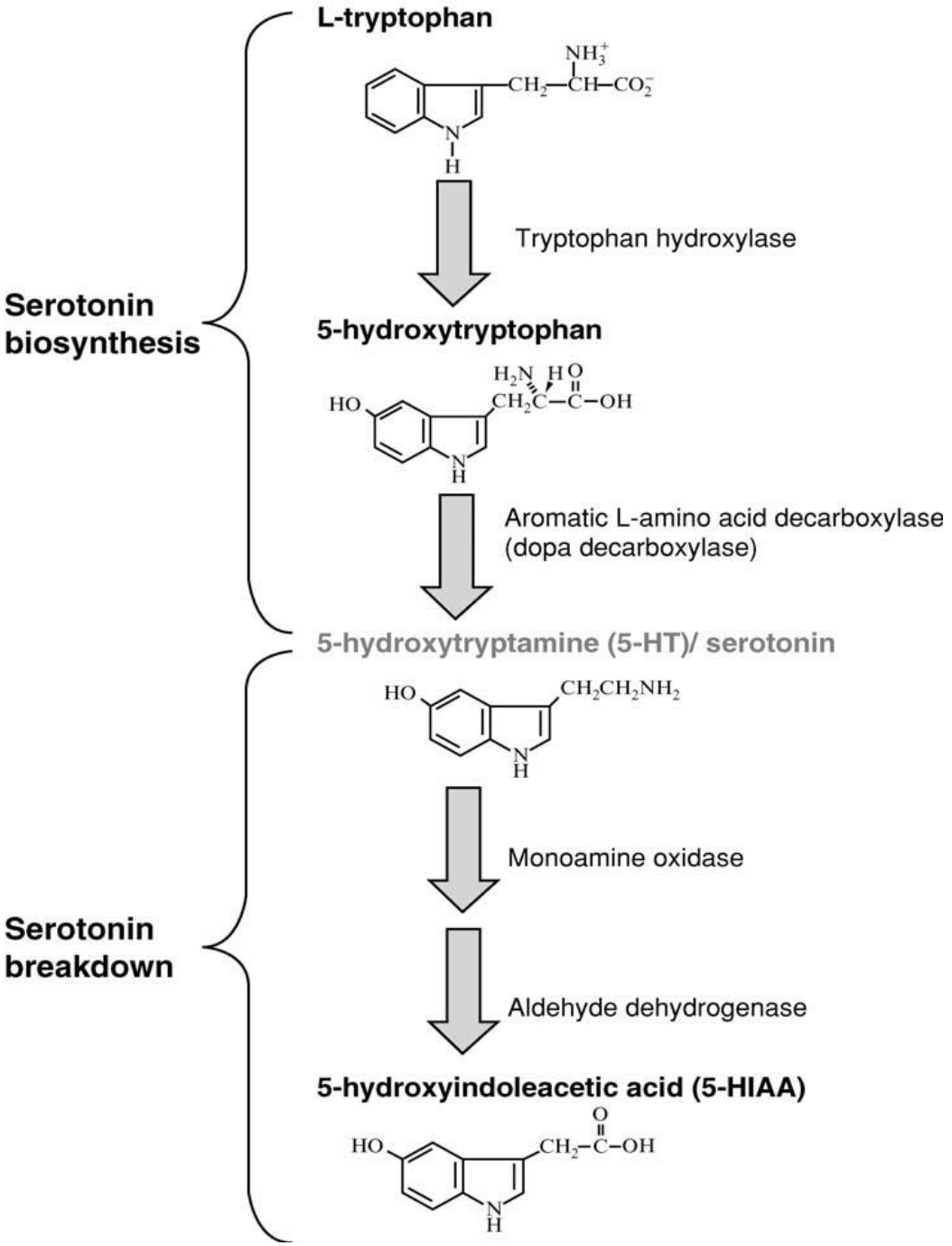


Figure 1-7 Biosysthesis and degradation of serotonin

Serotonin (5-HT) occupies a very important place in the field of neuropsychology, because a large number of anti-psychotic drugs that are used to treat depression and anxiety are targeted towards the serotonergic system. The postsynaptic effect of 5-HT is regulated by the

uptake of the same into the presynaptic nerve terminals *via* a specific serotonin transporter (SERT). Most of the antidepressant drugs are selective serotonin reuptake inhibitors (SSRIs) that inhibit SERT. Serotonin receptors have been implicated in behaviors like emotions, circadian rhythms, motor behaviors, and state of mental arousal. Thus impairment in the function of these receptors have been responsible for numerous biological and psychiatric disorders like depression, anxiety, aggression, appetite, cognition, learning, memory, mood, nausea, sleep, and thermoregulation. Serotonin is also secreted from the enterochromaffin cells in the gut, where it regulates intestinal movements.

The Serotonin 1A Receptor:

The 5-HT receptors are a group of G protein-coupled receptors (GPCRs), with the exception of only one, which is a ligand-gated ion channel, and are distributed in the central and peripheral nervous systems, intestinal and blood cells. They mediate both excitatory and inhibitory neurotransmission. The serotonin receptors are activated by the 5-HT, which acts as their natural ligand. There are seven classes of receptors 5-HT_1 to 5-HT_7, out of which only the 5-HT_3 receptor, with its three subtypes (5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}), is a ligand-gated ion channel. All the others are metabotropic G protein-coupled receptors (GPCRs) that activate intracellular second messenger cascades to produce excitatory or inhibitory responses. The 5-HT-1 subfamily consists of five GPCRs that are coupled to G_i/G_o and mediate inhibitory neurotransmission, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}. The 5-HT2 family three subtypes (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}) are coupled to the G_q- protein and stimulate phospholipase C (PLC), thereby causing excitatory neurotransmission. The 5-HT_4, 5-HT_6, 5-HT_7, are coupled to G_s, and mediate excitatory neurotransmission by increasing cAMP synthesis. The 5-HT_5 receptor

subtypes (5-HT_{5A}, 5-HT_{5B}), have been shown to cause both inhibition as well as excitation of adenylyl cyclase (Hannon 2002). The 5-HT_{1A} receptor is the most widespread of all the 5-HT receptors. In the central nervous system, 5-HT_{1A} receptors exist in the cerebral cortex, hippocampus, septum, amygdala, and raphe nucleus in high densities, and in low amounts in the basal ganglia and thalamus. The 5-HT_{1A} receptors in the raphe nucleus are largely somatodendritic autoreceptors, whereas those in the various target regions of the brain like forebrain and corticolimbic regions are postsynaptic heteroreceptors. This receptor couples to the $G_{i/o}$ proteins, causing inhibition of adenylyl cyclase, while the βγ complex elicits activation of the PLCβ effector protein (Adayev 2003).

Serotonin 1A Receptor and Brain Development:

The Serotonergic neurons originate in the raphe nucleus of the brain stem and project to different parts of the brain. The dorsal raphe nucleus sends its projections to the cortex, whereas the median raphe nucleus sends its projections to the hippocampus (Fig 1.8).

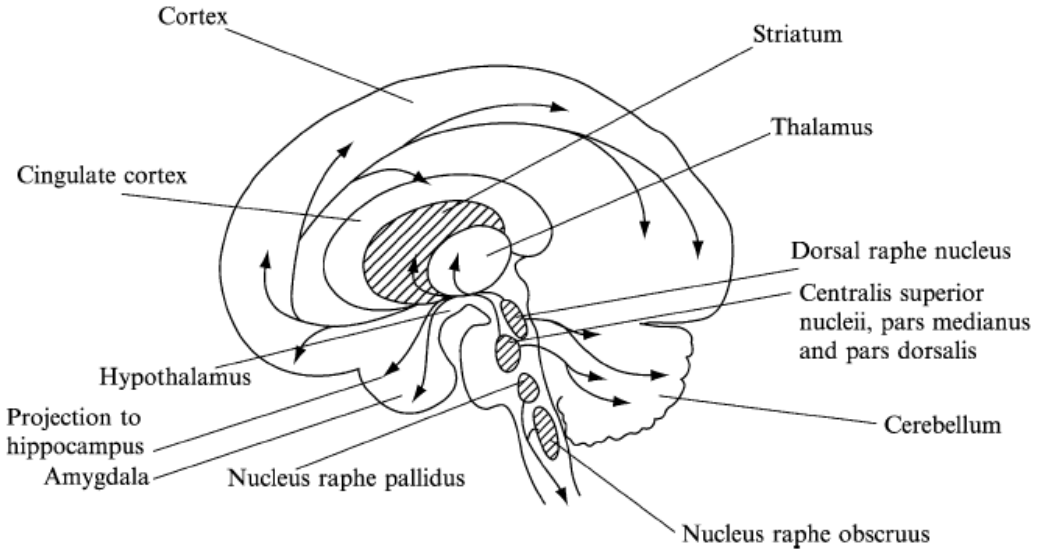


Figure 1-8 Serotonergic projections in the brain.

Preceding its role as a neurotransmitter in the adult brain, 5-HT functions as a major regulator of early brain development. There is evidence of the existence of 5-HT in the brain much earlier than any other monoamine neurotransmitter, indicating that it plays an important role in brain development (Sodhi, Sanders-Bush et al. 2004). Serotonin influences neurogenesis, cell migration, dendritic refinement and synaptic plasticity. At a later developmental stage, it is shown to influence length of dendrites, formation of dendritic spines and dendritic branches into hippocampus and cortex (Sodhi, Sanders-Bush et al. 2004).

Several developmental disorders like Autism and Down Syndrome (DS) have been linked to serotonin. Autistic individuals have been reported to have increased levels of blood serotonin. PET studies have revealed decreased serotonin synthesis in the cortex and thalamus but increased synthesis in the dentate nucleus. Studies with postmortem autistic brains have shown decreased dendritic branching in the CA1 and CA4 regions indicating delayed or lack of maturation and also decreased hippocampal volume. DS has also been long associated to

serotonin due to lower levels of serotonin in postmortem brains, CSF and blood. In DS, the serotonin 1A receptor (5-HT_{1A}-R) in particular peaks earlier but then decreases below normal levels before birth (Whitaker-Azmitia, Christian et al.; Whitaker-Azmitia 2001).

The hippocampus has been linked to various mood disorders like anxiety, depression, stress and panic disorder to name a few. Genes and environment play a synchronous role in the development of anxiety. The developmental approach of anxiety is very crucial for anxiety since it is one of the earliest psychiatric disorders to manifest, even before the age of 11 years (Kessler, Berglund et al. 2005). The 5-HT_{1A}-R has been implicated in the development of anxiety and depression. Mice deficient of 5-HT_{1A}-R have been shown to display anxiety-like phenotype. A tissue-specific conditional rescue strategy has been designed to express the 5-HT_{1A}-R in the hippocampus and cortex, but not in the raphe nuclei, showing the importance of the expression of this receptor in early postnatal stages to rescue anxiety-like behavior (Gross 2002). Antidepressants act through the serotonergic pathway to increase neurogenesis in the hippocampus and attenuate anxiety-like behavior (Santarelli 2003). Selective desensitization of the 5-HT_{1A}-R autoreceptors in raphe facilitates antidepressant action (Lemonde, Du et al. 2004). Serotonergic signaling *via* 5-HT_{1A}- receptor during the critical postnatal period is important for the developmental programming of anxiety-related behavior (Lo Iacono and Gross 2008). These studies indicate that the serotonergic system acting *via* 5-HT_{1A}- receptor plays a crucial role in manifestation of anxiety and depression in adulthood.

Protein Kinase C (PKC):

Protein Kinase C (PKC), is a family of serine/threonine protein kinases. They are known to play important roles in cell proliferation, differentiation and development. The PKC family of protein kinases consists of several isoforms, which are divided into three broad classes depending on the type of second messenger used for its activation. They are a) conventional: α, γ and alternatively spliced βI and βII, which require Ca^{2+}, Diacylglycerol (DAG) and phosphatidylserine for activation b) novel: δ, ε, η/L, θ , requiring DAG and phosphatidylserine for activation and c) atypical: ζ and ι/λ isoforms, requiring only phosphatidylserine for its activation. There is some debate over two isoforms μ and ν since some believe that they belong to a fourth class of the PKCs, while others hold that they should be categorized as a distinct family called Protein Kinase D (Newton 2001).

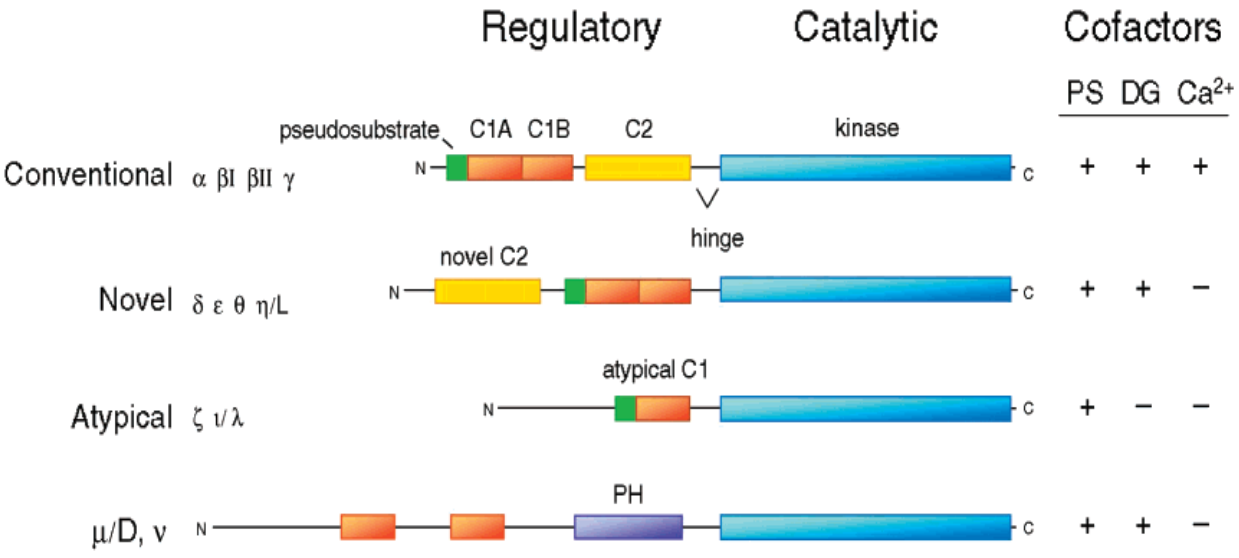


Figure 1-9 The PKC isoforms

Adapted from Alexandra C. Newton *Chem. Rev.* 2001

A physiologically functional PKC requires a series of phosphorylation, proper conformational change and a definite subcellular localization. PKCs are phosphorylated at three different positions, the activation loop, the turn motif and the hydrophobic motif. Of these the

phosphorylation at the turn motif is the most important because it is crucial for the activation of PKC. The hallmark of PKC activity is its translocation to the plasma membrane (Newton 2001). The mature phosphorylated form of PKC translocates to the membrane. DAG and phorbol esters act as molecular glue by increasing the affinity of the PKC s to the membranes. Phosphatidylserine is present in the membrane where the PKC binds and Ca^{2+} also acts to increase the affinity towards the membrane in case of conventional PKCs (Newton 2001; Newton 2003). The amino acid sequences of the PKCs are very crucial for its phosphorylation as well the translocation of the kinase. Manipulation of amino acids has been used extensively to regulate the activity of the kinase in several studies.

Earlier studies from our lab have shown the differential expression and localization of the different isoforms of PKC in neonatal mouse hippocampus. The novel PKC esozymes, ε and θ were expressed earlier during post natal day 2-6 (P2-6), with expression of PKCε high around P2, and underwent a further increase at P15 (peak of synaptogenesis). The expression of the classical isoforms like α, β, γ, δ isoforms were low during the early post-natal days (P2-6), with their expression increasing from P8-10 and reaching a peak around P15-20. This suggested that PKC ε and θ were probably involved in early development (neuroblast proliferation). and the isoforms α, β, γ, and δ were involved later in synaptic plasticity (Purkayastha, Fernando et al. 2009). Study of the subcellular localization of these isoforms (ε and α) at the two important time points P6 (peak of cell proliferation) and P15 (peak of synaptogenesis) revealed that PKCε protein was expressed in the nuclei of the cells in the sub-granular zone (SGZ) at P6, and was co-localized with Ki67. At P15, PKCε was no longer expressed in the nuclei, rather it was expressed in the cytoplasm. The co-localization of the PKCε with Ki67 at the early developmental stage could be due to its role in neuroblast proliferation. On the other hand immunoreactivity for

PKCα was low at P6, and was abundant at P15, when it was localized in the neuronal processes like apical dendritic regions and thin axonal processes, suggesting its involvement in the formation of synapses at a later developmental stage (Purkayastha, Fernando et al. 2009). The involvement of PKCα in synaptogenesis was indeed confirmed with later studies in the lab with P15 mouse hippocampus (Mogha 2012).

The fact that an isoform of PKC and ERK was involved in the 5-HT_{1A}-R mediated signaling pathway was confirmed from studies with organotypic cultures of hippocampal slices (4DIV) treated with different concentrations of the 5-HT_{1A} receptor full agonist 8-OH-DPAT for various time periods to determine the optimum conditions in the presence of 5-HT_{1A} receptor antagonist WAY 100635 (WAY) (4 μM), the general PKC inhibitor *bis-indolylmaleimide* (GFX, 2 μM), and the Erk1/2 kinase (MEK) inhibitor PD98059 (PD) (50 μM) to analyze the level of Erk1/2 activation. The maximal activation of Erk1/2 was obtained with 100 nM of 8-OH-DPAT at about 20 min, which persisted for at least one hour. The activation was blocked in the presence of WAY, GFX and PD98059, confirming that a 5-HT_{1A}-R- mediated signaling pathway caused activation of MEK and Erk1/2 and it also involved a PKC upstream of MEK and Erk1/2 (Fig 1.10). Although the PKC isozyme involved was not confirmed yet, the hierarchy of PKC and Erk1/2 was established in this study.

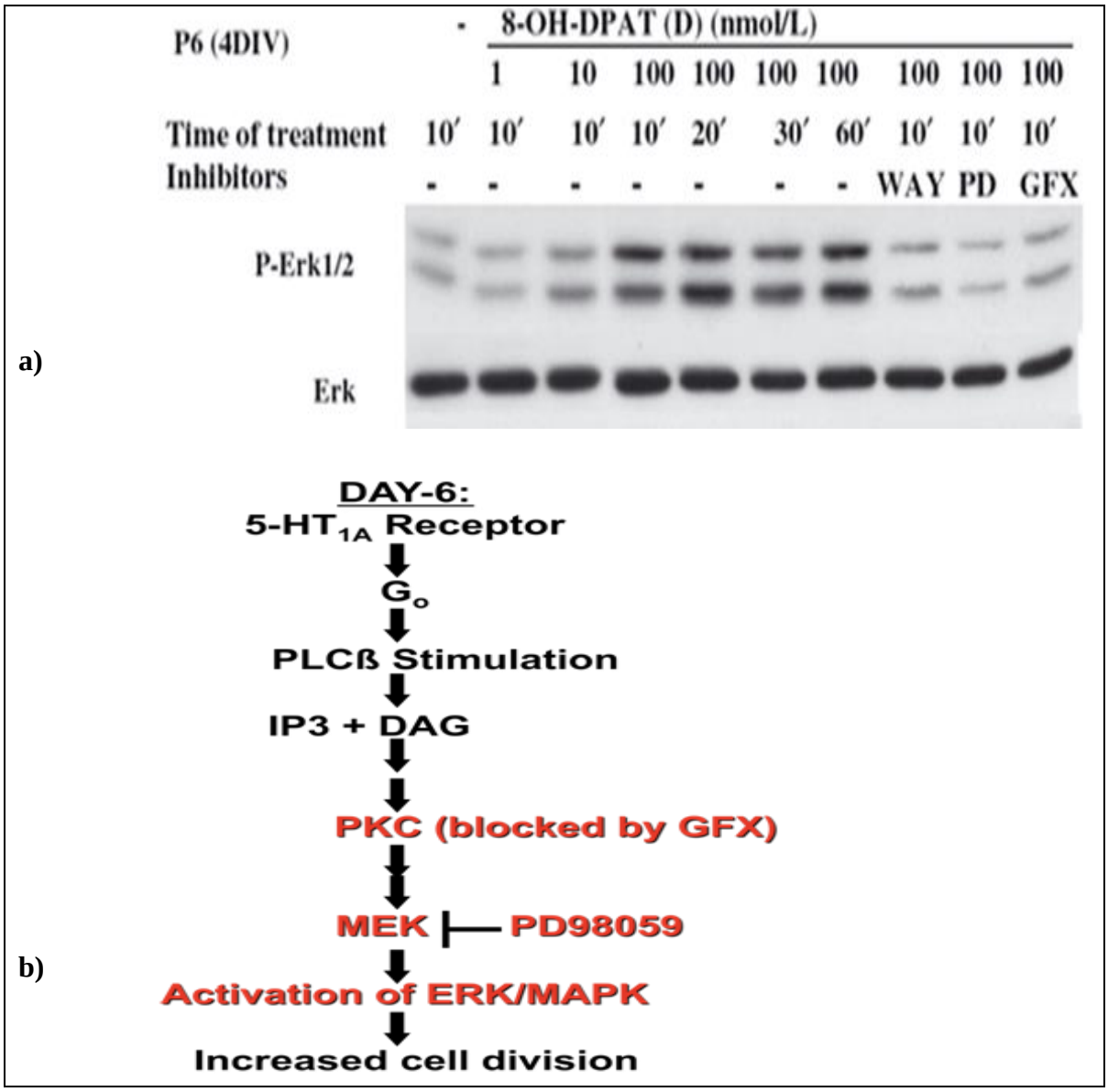
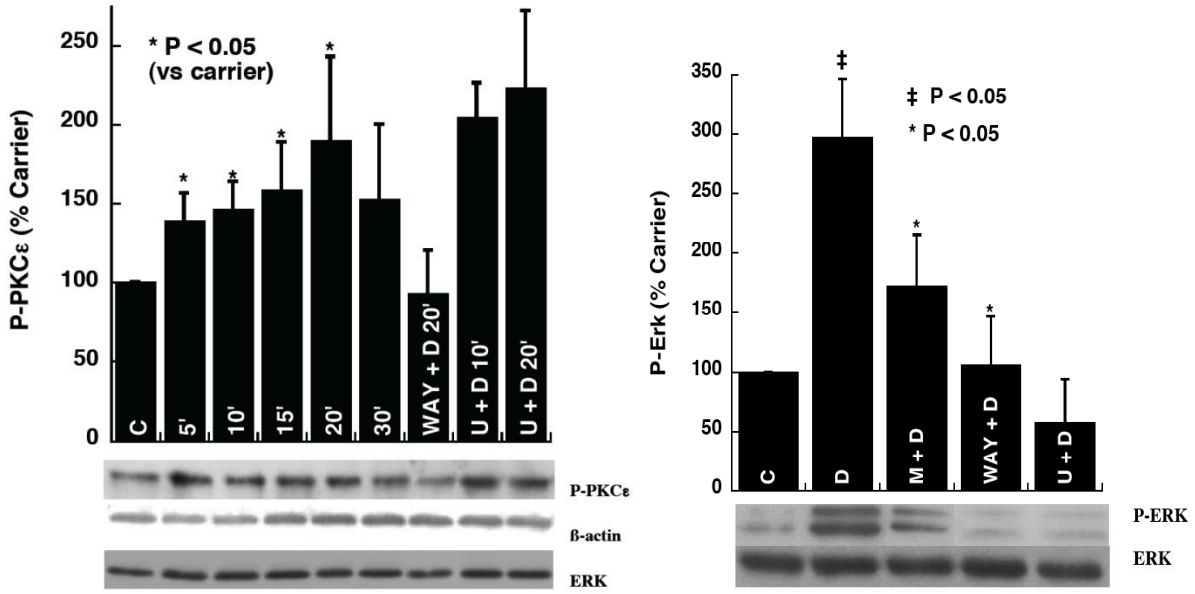


Figure 1-10 Agonist stimulation of the 5-HT1A-R causes activation of Erk1/2 through a PKC isozyme.

a) Western blotting was performed to measure the optimal concentration and time of 8-OH-DPAT treatment required for the activation of Erk, in the absence and presence of WAY100635 (WAY), GFX and PD98059 (PD). **b)** A schematic representation of 5-HT_{1A}-R mediated signaling at P6. (Mehta *et.al*. (2007) J. Neurochem., 101, 918-928).

Earlier studies also used a Pan-P-PKC antibody to identify a ~80-kDa PKC, which was activated by 5-HT_{1A}-R signaling in organotypic cultures of hippocampal slices. This corresponded to PKCε (since the other isoforms were in the range of 60-70 kDa), thereby suggesting the involvement of PKCε in 5-HT_{1A}-R-mediated signaling in P6 hippocampal slices (Mehta 2007; Purkayastha 2009). At the outset of the subsequent *in vivo* analysis we re-verified the involvement and positioning of PKCε in 5-HT_{1A}-R signaling in a 5-HT_{1A}-R-expressing model hippocampal neuron-derived cell line (HN2-5) created by us earlier (Banerjee 1993; Singh 1996; Singh 1996; Adayev 1999; Adayev 2003). Agonist (100 nM 8-OH-DPAT) treatment of proliferating HN2-5 cells caused a stimulation of PKCε, as measured using a P-Ser^{729}-PKCε (Santa cruz Biotechnology, Santa Cruz, CA) (Fig 3.2a). This activation of PKCε was eliminated in the presence of the 5-HT_{1A}-R antagonist WAY100635 (4 μM) but not in the presence of the inhibitor of the MEK inhibitor (U0126) (10 μM). In sharp contrast, 5-HT_{1A}-R-mediated activation of Erk1/2 was blocked in the presence of the selective inhibitor of PKCε Myr-εV1-2 (M; a PKCε inhibitor) (Johnson, Gray et al. 1996). Therefore, PKCε was located upstream of MEK in 5-HT_{1A}-R-mediated stimulation of Erk1/2.



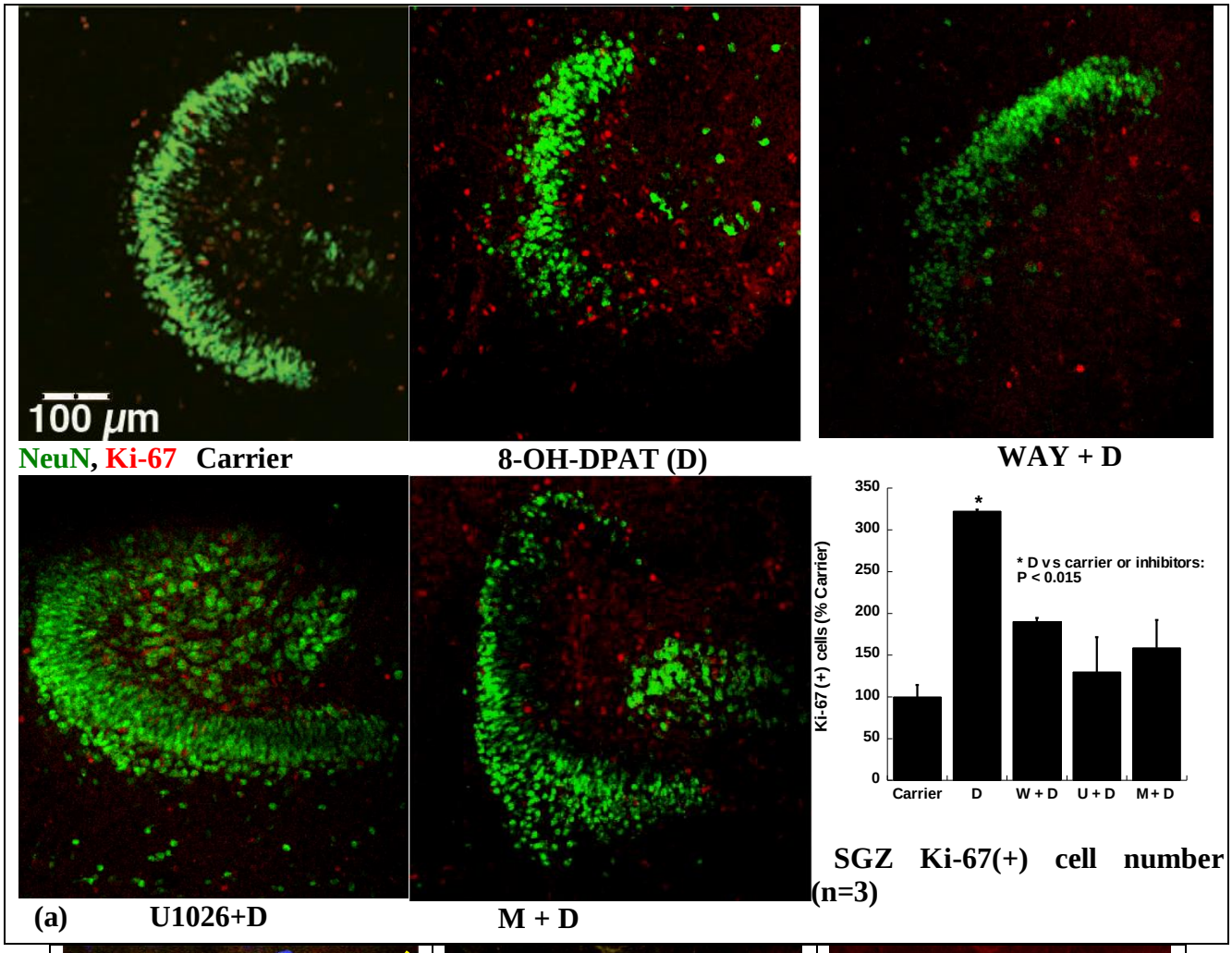
(a) **(b)**

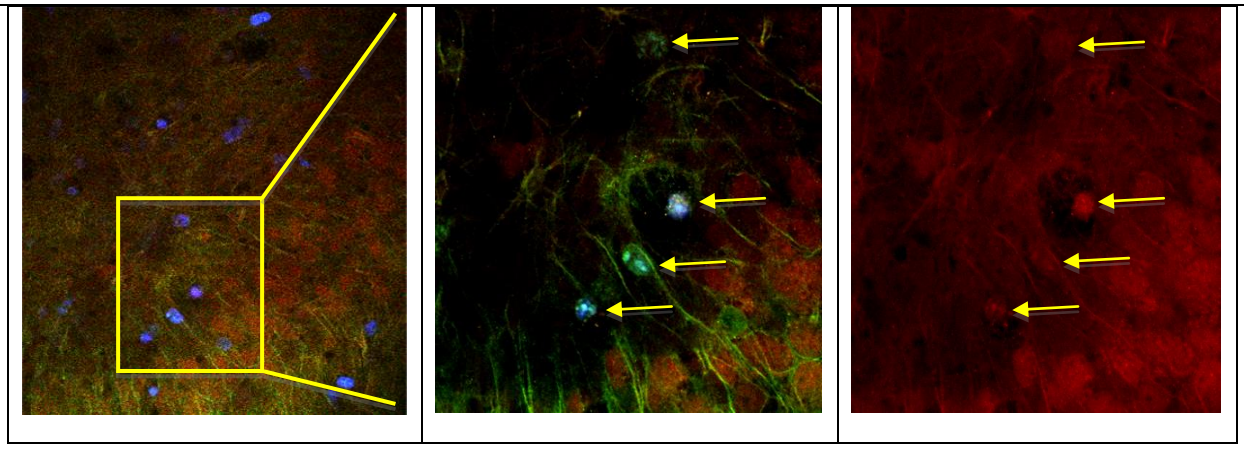
Figure 1-11 PKCε is upstream of Erk1/2 in 5-HT1A-R-mediated signaling in proliferating hippocampal neuron-derived HN2-5 cells.

a) Relative to carrier (C) treatment, agonist (8-OH-DPAT, D) (100 nM) treatment caused maximal activation of PKCε in 20 min (measured using P-Ser^{729}-PKCε antibody detection), which was eliminated in the presence of WAY (10 µM), but not in the presence of the MEK inhibitor U0126 (U) (10 µM). **(b)** Relative to carrier treatment (C), 8-OH-DPAT (D) treatment (100 nM) caused a dramatic increase in Erk1/2 activity in 30 min (measured using a P-T^{202},Y^{204}-Erk antibody), and this activation was blocked in the presence of the PKCε inhibitor (M) (400 nM) and also U0126 (10 µM).

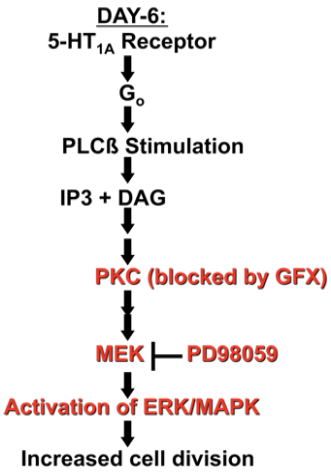
(Samaddar, S., Schroder, R., Marsillo, A., Debata, P.R., Ranasinghe, B., Purkayastha, S., Diallo, S., Kerr, D., Chanthrakumar, P., Tantry, S.J., and Banerjee, P. *Gender-specific Effect of Aberrant Serotonergic Signaling on Neonatal Hippocampal Development Makes Females Prone to Later-life Mood Disorders*. Manuscript in preparation).

The proliferative potential of this 5-HT_{1A}-R-linked, PKCε-mediated Erk1/2 activation was also tested in cultured hippocampal slices. Agonist (8-OH-DPAT)–evoked increase in cell proliferation in the DG was measured by Ki-67 staining was eliminated in the presence of the PKCε inhibitor M and also the MEK inhibitor U1026 (Fig 1.12a). Statistical analysis revealed a significant difference in the number of Ki67 positive cells between 8-OH-DPAT-treated sections with those treated with carrier and inhibitors ($p < 0.015$). Ki67 was not neuron specific, but it colocalized with double-cortin (DCX), a neuroblast marker, to confirm that the cells proliferating under the influence of 8-OH-DPAT were indeed neuroblasts (Fig 1,12b). This *in vitro* study also confirmed the hierarchy of the signaling molecules in the pathway.


100 μm
NeuN, Ki-67 Carrier
8-OH-DPAT (D)
WAY + D
(a) U1026+D
M + D
Ki-67 (+) cells (% Carrier)
350
300
250
200
150
100
50
0
*
* D vs carrier or inhibitors:
P < 0.015
Carrier
D
W + D
U + D
M + D
SGZ Ki-67(+) cell number (n=3)



b)



(c)

Figure 1-12 Serotonin 1A receptor mediated signaling causes boosted cell proliferation in the DG of cultured P6 hippocampal slices.

a) Cell proliferation, as marked by the cell proliferation marker Ki67 in cultured hippocampal slices was monitored after stimulating with 5-HT_{1A}-R agonist 8-OH-DPAT (D), in the absence and presence of WAY, U and M. Statistical analysis revealed a sharp increase in the Ki67 positive cells (red) which was blocked in the presence of WAY, U, and M ($p < 0.015$; One-way ANOVA). **b)** Organotypic culture of P6 hippocampal slices were D-treated for 16 hrs, following which they were fixed and stained using antibodies against P-PKCε (red), Ki-67 (purple) (a proliferation marker in the nucleus), and doublecortin (green) (cytosolic stain for proliferating neuronal progenitors) to show the proliferating neuroblasts. **c)** Schematic representation of the pathway and the hierarchy of the signaling molecules operating *in vitro.*

(Samaddar, S., Schroder, R., Alexandra Marsillo, Debata, P.R.[1], Ranasinghe, B., Purkayastha, S., Diallo, S., Kerr, D., Chanthrakumar, P., Tantry, S.J., and Banerjee, P. *Gender-specific Effect of Aberrant Serotonergic Signaling on Neonatal Hippocampal Development Makes Females Prone to Later-life Mood Disorders*. Manuscript in preparation).

Protein Kinase C epsilon (PKCε):

As a novel PKC, protein kinase C Epsilon (PKCε) can be activated by the second messenger diacylglycerol (DAG), but not calcium. PKCε phosphorylates a variety of protein targets and has been identified to participate in diverse cellular signaling pathways, such as neuron channel activation, apoptosis, cell proliferation, differentiation, migration, secretory vesicle trafficking, endocytosis, exocytosis and other nervous funtions (Dorota Garczarczyk 2009). It serves as the receptor for phorbol esters, a class of tumor promoters. Changing some amino acids has led to formation of constitutively active PKCε (changing the Alanine to Glutamic acid A/E at the 159 position and, removing the pseudosubstrate binding region renders the kinase catalytically active) (Dorota Garczarczyk 2009). Additionally, translocation inhibitors have been designed to block the activity of PKCε. As discussed before, following activation, translocation of PKCε is a requisite for the kinase to be functional. This translocation requires the binding of the activated protein kinase to specific anchoring proteins such as RACKs (Receptor for Activated C Kinases). Peptides like εV1–2 (EAVSLKPT) can selectively block the translocation (Johnson, Gray et al. 1996). A Myr group was added to the N-terminal end of the oligopeptide, Myr-εV1-2 to aid the entry of the oligopeptide into cells (Johnson, Gray et al. 1996). PKCε can be selectively activated by a fatty acid derivative called DCP-LA.

DCP-LA, also formerly known as FR236924 is a selective activator of PKCε. Essential fatty acids have long being used as raw materials for bioactive lipid mediators, and one such attempt is the formation of this compound. FR236924, is a derivative of lenoleic acid where the *cis*-double bonds have been replaced with more stable cyclopropane rings due to their structural similarity (Tanaka and Nishizaki 2003). The compound is also known by its synonymous IUPAC names: 2-[(2-pentylcyclopropyl)methyl]cyclopropaneoctanoic acid or 8-[2-(2-pentyl-

cyclopropylmethyl)-cyclopropyl]-octanoic acid. Reverse phase High Performance Liquid Chromatography (HPLC) has revealed that DCP-LA activates PKC in a concentration-dependant manner, and is blocked with GFX (a general PKC inhibitor) and also with specific peptides for inhibiting novel PKCε (Kanno 2006). Similar inhibition of activation of PKC was seen when siRNA against PKCε was used in HEK-293 cells. It has been suggested that DCP-LA activates PKCε selectively about >7 fold higher than the other isoforms, by binding to its phosphatidylserine binding site (Kanno 2006).

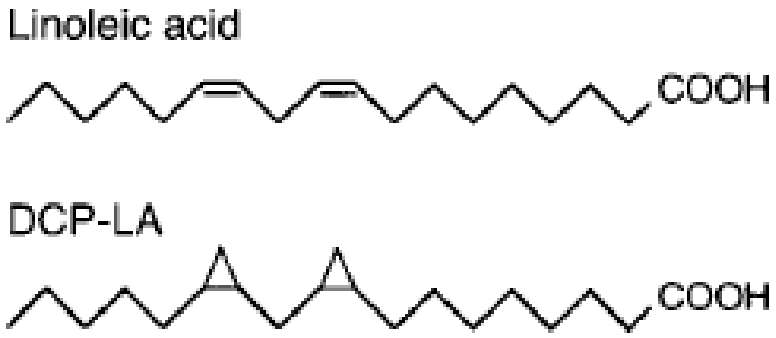


Figure 1-13 Chemical structure of DCP-LA

DCP-LA has been shown to enhance nicotinic α7 ACh receptor activity, facilitating hippocampal neurotransmission by enhancing glutamate release via a PKC pathway (Tanaka and Nishizaki 2003). DCP-LA shortened the prolonged latency to reach the hidden platform in the water maze test in senescence-accelerated mice thus exerting a beneficial action on age-related disorders of spatial memory (Takahiro Yaguchi 2005). As mentioned before, PKCε plays a key role in neurite growth, synapse formation and neurotransmitter release. It is also well known that synaptic loss is an important finding that has been related to the loss of memory in Alzheimer's Disease. DCP-LA has been shown to prevent amyloid plaque formation and restore synaptic connections in mouse model of Alzheimer's along with improvement of spatial maze learning and memory (Hongpaisan, Sun et al. 2011). DCP-LA also activated PKCε and stimulated release

of Serotonin, Glutamate and Dopamine (Tadashi Shimizu 2011). All these reports have successfully hinted towards the corrective role of DCP-LA in various models and tests, morphological and behavioral with special correspondence to spatial memory and hippocampal synaptic transmission. This led us to use this activator to test its effects on anxiety in the 5-HT_{1A}-R-deficient mice during developmental stages.

The Mossy Fiber Pathway:

In the hippocampus, the axons emerging from the basal portions of the granule cells of the dentate gyrus form distinctive unmyelinated axons projecting all the way to the stratum lucidum of the CA3 region. This is called the mossy fiber pathway. Granule cell synapses have been shown to be glutamatergic (e.g. excitatory), and also GABAergic (Sandler and Smith 1991; Gutierrez 2005) Moreover, there is growing evidence for co-localization of GABAergic and glutamatergic neurotransmitters within mossy fiber terminals, though the functional consequence of this colocalization is unclear.

This pathway was so named by Ramon y Cajal because the axons display bulbous swellings all along their lengths, giving them a "mossy" appearance.

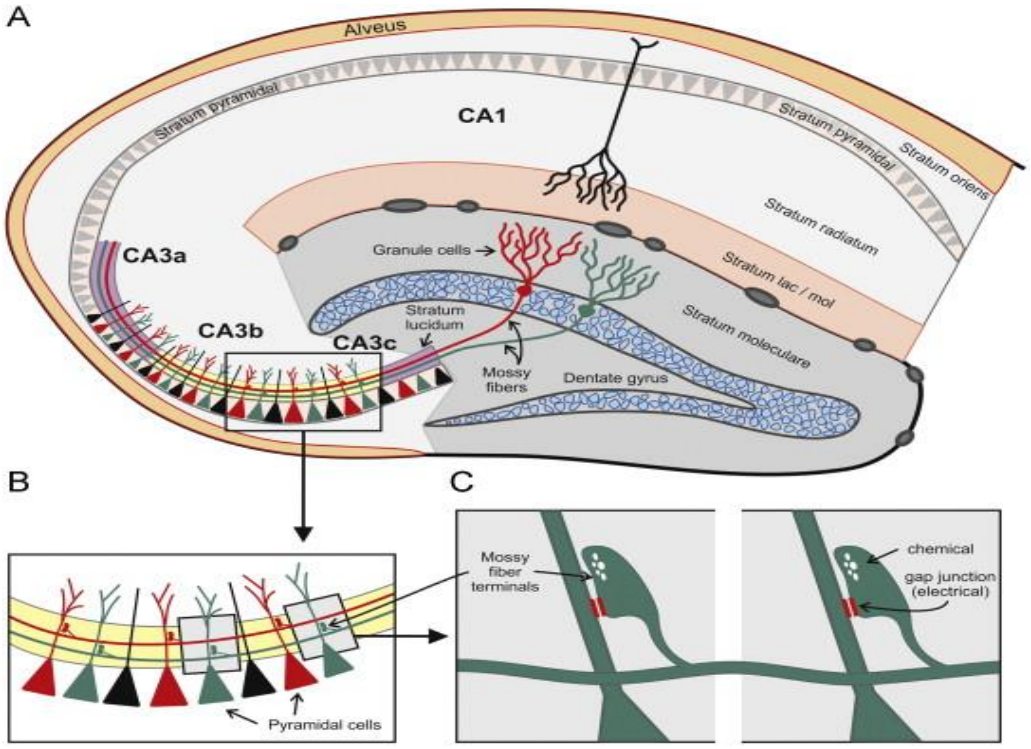


Figure 1-14 The Mossy Fiber Pathway.

Axonal projections of the granule cells in the area dentate that make synaptic connections with the pyramidal cells of the CA3 region are called the 'mossy fibers'. Mossy fibers are thin unmyelinated axons with a diameter of 0.1-0.7 µm and are studded with giant boutons, which can be up to 10 µm. These boutons are invaginated by giant spines or excrescences of the pyramidal cells of the *regio inferior*. Timm's sulfide silver method is a sensitive method for demonstrating the histological distribution of laminated pattern of the hippocampus. Timm staining of the mossy fibers is a very reliable method for defining the mossy fibers. The intense staining is due to the presence of a large amount of zinc, and it is associated with neuronal excitability and also with neurotrophic nerve growth factor (Gaarskjaer 1986). The outgrowth of the mossy fibers stars prenatally. The mossy fibers first originate from the granule cells which are located more laterally, followed by the ones located medially. At about postnatal day 24, all granule cells have mossy fibers projecting to the region inferior (Gaarskjaer 1986).

In the hilus, mossy fibers are believed to make synaptic connections with basket cells, mossy cells, oviform cells and long-spined multipolar cells, of which the functional consequence of the synapses with the basket cells and the mossy cells are of importance in this study. Evidences have shown that the dendrites of the hilar basket cells are potential targets of the mossy fiber boutons. Thus the mossy fibers also excite the basket cells apart from the exciting the pyramidal cells of the CA3 region. These basket cells in turn form axo-dendritic, axo-somatic and axo-axonic synapses on the granule cells. These latter synapses are inhibitory in nature, demonstrated by the presence of the inhibitory neurotransmitter GABA and its synthesizing enzyme, glutamate decarboxylase. These synaptic connections between the granule cells, and the basket cells form a inhibitory feed-back circuit (Gaarskjaer 1986).

The connections that the mossy fibers make with the pyramidal cells are therefore regulated by an intrinsic inhibitory feed-back mechanism, ensuring there is no scope of any redundancy. Aberrancies with this auto-regulatory mechanism might lead to development of unnecessary mossy fiber sprouting. This hypothesis is well supported by some learning studies done with some status epilepticus rats where a negative correlation was observed between the score of mossy fiber sprouting and the latency of learning. Thus fewer mossy fiber terminals aided better learning compared to more number of terminals (De Oliveira, Fischer et al. 2008). In the light of this controlled connectivity of the mossy fibers in learning, mossy fiber connectivity might be equally important in the development of anxiety-like behavior and depression in adulthood.

Purpose of the study:

As mentioned earlier, the hippocampus has been extensively studied for various mood disorders in adults. But the role hippocampal development in several early developmental neuropsychiatric disorders is not clear. Aberrant intrahippocampal connectivity early on in life might be causal to anomalies in brain development and later-life manifestations of anxiety and depression in adulthood. To study the details of neontal hippocampal development and the role of the 5-HT_{1A}-R in the sculpting of this structure, we have used wild type and 5-HT_{1A}-R-deficient mice as model systems. In the process we have been able to delineate a pathway and specific target molecules, which could yield potential therapeutic strategies for infants at risk of developing later-life mood disorders.

Importance of the study for the general audience:

The current work is of high importance due to the fact that the brain is not just a conglomerate of several structures morphologically, but it critically depends on the functional association of these structures. Thus proper development of the brain is not just about cells, their numbers, sizes, and location, but also about their connectivity, their ability to talk to one another at the right time and with correct intensity to have appropriate information flow. Events in the childhood leading to any anomaly in this information flow may leave a profound blueprint for adult-life activity. Therefore, it is of utmost importance to understand the developmental period in order to correct later-life mood disorders.

2. Chapter 2

Materials and Methods

Animals: Mice (C57BL/6) were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee. Some of the Wild type (WT) (C57BL/6) mice were obtained from Taconic and bred in CSI Animal Care Facility. The 5-HT_{1A}-R (-/-) pups used were generated by crossing 5-HT_{1A}-R (+/-) mice (Het) against the C57BL background obtained from Dr. Lawrence Tecott (Heisler 1998). The WT, Het, and 5-HT_{1A}-R (-/-) mice of the F1 generation were genotyped according our earlier report (Mogha 2012), and the 5-HT_{1A}-R (-/-) mice were paired to obtain pups (F2 generation), which were used in our experiments. Animals were kept in a 12-hour light/dark cycle with *ad libitum* access to food and water. Mice were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee.

Materials: The following reagents and antibodies were used for the study: 5-bromo-2'-deoxyuridine (BrdU), 8-OH-DPAT, WAY 100635, and Pargyline were obtained from Sigma Chemicals (St Louis, MO, USA). Myr-εV1-2 (N-Myr-EAVSLKPT) was prepared by solid-phase synthesis using a peptide synthesizer according to published reports (Johnson 1996; Chen 2001; Teng 2008). U0126 was purchased from Calbiochem (La Jolla, CA, USA). DCP-LA (formerly known as FR236924) was obtained from Tocris (R&D Systems, Ellisville, MO). The NeuN antibody was procured from Millipore (Billerica, MA, USA). The Alexafluor- labeled fluorescent secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Ketamine was obtained from Hospira Inc. (Lake Forest, IL), xylazine from Sigma Chemicals (St Louis,

MO). Anti- P-T^{202},Y^{204}- Erk antibody was obtained from Cell Signaling (Beverly, MA, USA). Antibodies against P-Ser^{729}-PKCε and Doublecortin (DCX) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, and GFAP from Santa Cruz Biotechnology, Santa Cruz, CA.

BrdU injection: For all the neuroproliferation and neurogenesis studies, the pups were injected with BrdU (100 mg/kg) intra-peritoneally 2 h prior to intra-hippocampal or intra-peritoneal injection of the drugs. It is done for all neuroproliferation and neurogenesis experiments. The proliferating cells are then detected with antibodies against BrdU, and counter probed with antibodies against doublecortin (DCX), NeuN, or glial fibrillary acidic protein (GFAP).

Intra-hippocampal infusion: Mouse pups at post-natal day 6 (P6) were anaesthetized with a mixture of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg), placed in a steretaxic set-up, and then stereotaxically infused with the 5-HT_{1A}-R agonist (±)-8-hydroxy-2-(di-N-propylamino) tetralin (8-OH-DPAT) (D) (final conc in the hippocampus: 100 nM) in the absence and presence of the 5-HT_{1A}-R antagonist N-[2-[4-(2-methoxy-phenyl)-1-piperazinyl] ethyl]-N-(2-pyridinyl) cyclohexane-carboxamide (WAY 100635) (final conc: 10 μM), PKC epsilon pharmacological inhibitor Myristoyl-EAVSLKPT (Myr-εV1-2) (M) (final conc: 400 nM), and MAPK kinase (MEK) inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) (final conc: 10 μM), selective PKC epsilon activator, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) (3 mg/kg) (Johnson 1996; Chen 2001; Teng 2008). The stereotaxic coordinates used were as follows: (V) 2 mm under the external surface of the scalp skin in the frontoparietal area of the right hemisphere, (L) 2 mm from the

midline in the lateral-medial plane, and (AP) 3 mm from the junction of the sagittal and lamboid sutures in the rostrocaudal plane.

Drug Concentrations: Average hippocampal volume of a P6 C57BL6 mouse was obtained as 5 µl by isolating hippocampi, weighing, and then using mean brain tissue density as 1.02 mg/ml. Drug concentrations were made for the observed hippocampal volume of 5 µl of a P6 hippocampus. The total volume of the infusate was 0.5 µl for all the drugs or carrier (0.1 M PBS plus 0.5 % DMSO). Stock solutions were made as follows:

8-OH-DPAT: 1 µM stock solution in PBS for the final concentration of 100 nM in the hippocampus.

WAY 100635: 100 µM stock solution in PBS for the final concentration of 10 µM in the hippocampus.

Myr-εV1-2: 4 µM stock solution in PBS for the final concentration of 400 nM in the hippocampus.

U0126: From a stock solution of 20 mM in DMSO, 0.5 µl was diluted in 99.5 µl of PBS to obtain a 100-µM working stock (containing 0.5% DMSO). Next, 0.5 µl of this working stock was infused into the hippocampus to obtain the final concentration of 10 µM of U0126 in the hippocampus.

DCP-LA: Intrahippocampal infusion: 3 µl of 1 mg/ml of DCP-LA in DMSO was dissolved in 1 ml PBS to obtain a working stock of 30 ng/µl (0.3% in DMSO) and then 0.5 µl of this solution was injected in the hippocampus to obtain the desired concentration of 3 ng/ µg (the same as the recommended dose of 3 mg/Kg).

Systemic (I.P.) infusion: Based on the body weight, 9 µl (for P6, 3 g), 15 µl (for P10, 4.6 g), 20 µl (for P14, 7 g) of a 1 mg/ml DCP-LA solution in DMSO was dissolved in 160 µl of PBS to obtain a working stock (10% in DMSO). The entire volume of this solution was injected into each pup to achieve the final concentration of 3 mg/Kg of body weight for DCP-LA (Hongpaisan 2011) (final systemic DMSO concentration of 1.6%). The systemic injection of carrier also contained the same volume of DMSO.

Maintaining DMSO concentrations in the intrahippocampal infusions: Each infusate (0.5µl), whether 8-OH-DPAT, WAY100635, M, U0126, DCP-LA, or carrier contained the same volume of DMSO, which yielded a final intrahippocampal concentration of $\leq 0.05\%$ DMSO.

Processing of the brains: For neuroblast proliferation and neurogenesis studies, following injection, the mouse pups were allowed to recover from anaesthesia and returned to their mother. For the analysis of neuroproliferation, after 24 hours of drug treatment, the pups were perfused through the heart with 4% paraformaldehyde, the brains isolated, and placed in 4% paraformaldehyde. After at least 24 hours of post-fixing, the brains were fully soaked in 30% sucrose (approximately in 24 hours or more). Following this, the brains were cryosectioned into 30-µm coronal sections, and then subjected to immunohistochemical staining. For the analysis of neurogenesis, the pups were allowed to develop until P36 and then perfused with 4% paraformaldehyde. The perfused brains were subjected to similar treatments as for cell proliferation studies and 30-µm coronal brain sections were subjected to immunohistochemical staining. For the analysis of 5-HT_{1A}-R-mediated signaling through PKCε and Erk1/2, the pups were perfused and brains removed 1 hour after the intra-hippocampal drug injection. The brains were fixed, sucrose-treated, cryosectioned, and the sections subjected to immunohistochemical

staining. Before each experiment, one pup was injected with Coommassie blue to confirm intra-hippocampal injection with the coordinates mentioned above (Mogha 2012).

Cryosectioning of brain and Immunostaining of brain sections: The two hemispheres were separated using a surgical blade (World Precision Instruments), and each hemisphere mounted for cryosectioning using dry ice, and Cryomold Intermediate (Tissue-Tek) on a metal pedestal and sectioned using a cryostat (Vibratome, ULTRApro 5000, SIMS Corporation, South Korea). The 30-µm coronal sections were stored at 4 °C in floating condition in 1x PBS solution containing 0.01% sodium azide in six-well plates for future use. The sections slated for immunostaining were selected using a microscope making sure that all the sections contained the hippocampal structure from both ventral as well as dorsal halves of the hippocampus. The selected slices were treated with 50% formamide in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate buffer) for 2 h at 65 °C. After washing with 2x SSC for 5 min, they were treated with 1N HCl for 10 min on ice, followed by 2 N HCl for 20 min at 37 °C, neutralized by 0.1 M Boric acid for 10 min, and then rinsed 3 times with 1x TBS buffer (10 min each). Sections were then blocked with a solution of 3% normal goat serum/0.25%Triton X-100/1x TBS for 30 min. This was followed by treatment with primary (1°) antibody in 2% normal goat serum and 0.25% Triton X-100 in 1x TBS for 1 overnight at 4 °C. Antibody concentration used were: NeuN (Millipore, Temecula, CA) (1:500), DCX (Santa Cruz Biotechnology, Santa Cruz, CA) (1:500), BrdU (Sigma, St. Louis, MO, USA) (1:250), GFAP (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1000), P-T^{202},Y^{204}- Erk antibody (Cell Signaling Technology, Boston, MA) (1:600), VEGF (Millipore, Temecula, CA) (1:300). The sections were then washed with 1x TBS, 1x PBS, and then treated with fluorescent secondary (2°) antibodies covalently linked to Alexafluor 488 (green) (1:500) or

Alexafluor 568 (red) (1: 500) or Alexafluor 633 (blue) (1:750). After incubating overnight in the 2° antibodies, the sections were washed and then mounted on slides with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR, USA) covered with cover glasses for visualization under a laser confocal microscope.

TUNEL Assay of Tissue sections: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with control and 8-OH-DPAT-treated slices to determine the number of apoptotic cells during both neuroproliferation and neurogenesis (Adayev 1999). The ApopTag®Plus Fluorescein *in situ* Apoptosis Detection Kit was used (Millipore, S711) for the experiment. The protocol followed was for 'Fluorescence Staining of Tissue Cryosections'. The fixed brains were cryosectioned and the sections kept in PBS. They were rinsed in 3 changes of PBS (10 min per rinse), and then post-fixed in pre-cooled Ethanol: Acetic Acid = 2:1 for 5 min at -20 °C. Sections were again washed in 2-3 changes of PBS (10 min each). After aspirating, "Equilibration buffer" (in the kit) was applied directly on the sections followed by incubation for 5-10 min at room temperature. The solution was aspirated and the sections immediately treated with TdT enzyme and incubated at 37 °C for 1 h according to the kit protocol. The solution was aspirated and STOP/WASH buffer was applied, the mixture gently hand-mixed for 15 sec and then incubated for 10 min at room temperature. The slices were then washed in PBS three times (5-10 min each), and the excess solution aspirated and treated with room temperature ANTI-DIGOXIGENIN CONJUGATE and incubated in a humidified chamber for 30 min at room temperature, avoiding exposure to light (covered with foil). The sections were rinsed 4 times (5-10 min each) at room temperature. The sections were then stained with 10 µg/ml HOECHST33342 in PBS at room temperature for 45 min for better

visualization of the granular cell layer of the hippocampus. The sections were mounted with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR, USA) with coverslips for visualization using a laser confocal microscope (Leica Microsystems, Heidelberg, Germany).

Confocal Microscopy of Immunostained slices and Quantification: The immunostained sections were imaged with a confocal microscope (Leica Microsystems, Heidelberg, Germany). For neurogenesis experiments both DPAT-evoked and DCP-LA rescue experiments, we looked for BrdU (proliferation marker) and Doublecortin (DCX) (neuroblast marker) double-labeled cells. For neurogenesis experiments, we looked for BrdU (proliferation marker) and NeuN (marker of mature neurons) double-labeled cells. The TUNEL-stained sections were images in the similar way, but in this case we looked for cells, which were stained green. At least 3 sections were imaged and analyzed for each treatment and at least 3 such sets of experiments were performed for proper statistical analysis. Sequential single images with all required wavelengths were taken 6-7 per slice and also z-stacks were done. Volume-rendered images were created from the Z-stacks, which were used for analysis. 3D- animation videos were created to exactly view the positions of the cells proliferating and also the mature neurons. BrdU and DCX double-labeled cells were counted with ImageJ point selection tool, and then plotted in GraphPad Prism or Excel for bar graph construction and statistical analysis. Student t-test and One-way ANOVA were used to calculate the statistical significance.

Treatment of WT and KO mouse pups and Timm staining: C57BL/6 pups were injected intraperitoneally with 3 mg/kg DCP-LA at P6, P10, P14. For basal level comparison of the mossy fiber pathway, WT pups were not injected (serving as WT un-injected controls) and the 5-

HT_{1A}-R (-/-) KO pups were injected with vehicle (PBS+DMSO as discussed above) (serving as knockout injected controls). On day 18, the mice are anesthetized and perfused first with Na_2S and then with glutaraldehyde and processed for Timm staining as described below. The brains were divided into left and right hemispheres, embedded in Tissue-Tek and sectioned with a cryostat (30 µm thickness). The sections were collected over gelatin-coated slides and allowed to stick on the gelatin coated slides for at least overnight. Next day, the slices were developed as described the following sections.

Timm Staining (Timm 1958) : The staining has been performed based on an earlier protocol from Dr. Tammy Ivanco with a few modifications (Ivanco Tammy L 2002). Each mouse was first perfused with a freshly made solution of 2.9 g Na_2S in 100 ml of 0.12 M monobasic sodium phosphate and then with freshly made 50% glutaraldehyde in 0.12 M monobasic sodium phosphate.

Preparing gelatin-coated slides: First the slides were cleaned with a mixture of 95% ethanol (300 ml) and 8 ml of acetic acid. The glass slides were placed in a Wheaton dish and incubated in the cleaning solution for 5 min, followed by drying in an oven. High-grade gelatin (2.5 g of 100 bloom) was dissolved in 500 ml of deionized water by heating to 55 °C with stirring, and then solution cooled to 21 °C. This solution was further supplemented with chromium potassium sulphate (0.25 g) (added with mixing). The slides were placed in this gelatin solution for 5 min. Excess gelatin was removed from the slide rack and then the rack with slides placed in a 40-°C oven and dried overnight. After drying, the slides are placed in slide boxes.

Gum Arabic (GA) solution is made by stirring 50 g (GA) in 100 ml of deionized water overnight at room temperature and then strained through a piece of cheesecloth. The solution is divided in 120-ml portions and frozen at -20 ^{o}C.

A developer solution was freshly made by mixing 2 M citrate buffer (20 ml), 0.5 M Hydroquinone (60 ml), 1 M silver Nitrate (1 ml) (protected from light), and freshly thawed Gum Arabic solution (120 ml) for each Wheaton dish. (for 8-10 slides).

The sections on slides were placed in glass rack in a Wheaton dish and re-hydrated by treating serially with 100% ethanol for 20 min, 95% ethanol for 10 min, 70% ethanol for 10 min, 50% ethanol for 10 min, and deionized water twice (10 min each). The rehydrated slides with the sections were next developed in a dark room by adding the developer mix and incubating for 45-60 min. The slides with sections were next rinsed by gently passing tap water through the Wheaton dish for 45 min. The tissue sections were then overlayered with a few drops of 90% glycerol and 10% 1X PBS and then mounted with coverslips for imaging.

Imaging and Quantitative Analysis: The slides are viewed using an upright microscope (Diaphot Nikon, Tokyo Japan). Images were captured at 2X magnification. Timm staining or Timm's sulfide silver staining has been used to visualize a variety of metals in brains and other tissues. This method, originally developed by Timm (Timm 1958), was later modified (Danscher 1981). The principle of the technique is based on sulphide-precipitation of metals in tissue followed by a physical development. During the later stage, the silver sulphide formed is reduced to silver metal in the presence of reducing agents. Analysis and measurements of Timm(+) fibers in the *Stratum Oriens* (SO) were performed as described by Ivanco and Greenough, 2002 (Ivanco Tammy L 2002). For all groups, matching sections from identical planes along the anterior-posterior axis were selected for densitometric analysis. Densitometry

measurements for analyzing the synaptic connections and also measurements of total distance of the connections were performed on images (5 in x 13 in) of 4X magnification in Photoshop. To prepare for analysis, images were first inverted and then converted to greyscale. Cursors (open circles of 0.5 inch diameter) were consecutively placed along the length of the stratum oriens (to measure the density of Timm granules); and along the stratum radiatum (to measure the background density). Photoshop's histogram tool was then used to obtain the mean density within the area of each cursor position. These values were then imported into Excel. For each ventral section, 7 measurements were obtained from the SO, and 4 from the *Stratum Radiatum* (SR). For each dorsal section, 16 measurements were obtained from the SO, and 8 from the *Stratum Radiatum* (SR). The relative optical density (R.O.D.) ratio of each cursor position along the SO was calculated by dividing the mean density of that cursor position by the average background density (i.e. the SR cursors) of the same sample. The radial distance in inches (in the 5 in x 13 in image) of each cursor from the bifurcation of the collateral in the hilus (position ZERO) for a section with coordinates (x, y) was calculated by the standard Euclidean distance formula: $d = \sqrt{(x_n - x_1)^2 + (y_n - y_1)^2}$. The R.O.D values were plotted against the radial distances of the cursors to compare the extensions of Timm stained MF fibers in the SO among WT, KO, and DCP-LA-treated KO.

Behavioral testing: Mice were generally tested in the light phase of their cycle. Before each testing session, mice were placed into acclimation in a dark room, adjacent to the testing room, for at least one hour so that they would be fully awake and active for testing. All of the behavioral tests were conducted in a sound-proof behavioral room, maintained at a constant temperature of 23–25 °C, located in the animal facility (close to the animal colonies). In order to

decrease stress levels, behavioral testing was started after the experimenter had extensively handled every mouse (5 minutes a day for one week prior to testing). In order to prevent inconsistencies in testing conditions that could potentially influence the animals' performance, the same experimenter conducted all behavior testing. Furthermore, for each test, all groups were tested on the same day at approximately the same time. All mice were tested under an overhead light of 300-lux intensity. During testing, videos were recorded by an overhead camera, and simultaneously transferred to a nearby computer using the video capture software Capture Wizard (CapWiz). Additionally, a white noise generator was used to block out any extraneous noises that might cause distraction. After each trial, the apparatus was thoroughly cleaned with 70% ethanol in order to prevent odor cues left behind by the previous mouse and picked up by the next mouse. Videos from the CapWiz software were then run through motion-tracking software called ANY-Maze. Data collected from ANY-maze were exported into Microsoft Excel and analyzed using statistical methods. For behaviors that could not be detected by ANY-Maze, a blinded observer scored the videos manually. Statistical analyses were carried out using unpaired, two-tailed Student's t-test or analysis of variance (ANOVA). Significant main effects or interactions were followed up with *Tukey's HSD* (honestly significant difference) post hoc test. Data was presented as mean ± s.e.m. Statistical significance was defined as $p<0.05$.

General Test Principles:

***Elevated-Plus Maze for Anxiety Measures*:** This test was conducted with reference to previously published procedures (Walf A 2007). The elevated plus-maze used consisted of two opposing open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15 cm), which joined at a square central area (5 cm × 5 cm) to form a plus sign. The maze floor was constructed of wood covered with vinyl that was painted white. The covering provided enough traction for mice

to move comfortably without falling over while peering over the edges of the open arms. The walls of the closed arms (15 cm high) were also made of wood that was painted black. The entire apparatus was elevated to a height of 45 cm above the floor by four supports, or legs, at the end of each arm. Mice were initially placed in the center of the maze, and exploratory behavior on the plus-maze was recorded for ten minutes. Time in the open arms and number of entries to the open arms were measured by the ANY-maze recording software, and they can be used to relate effects in anxiety and motility.

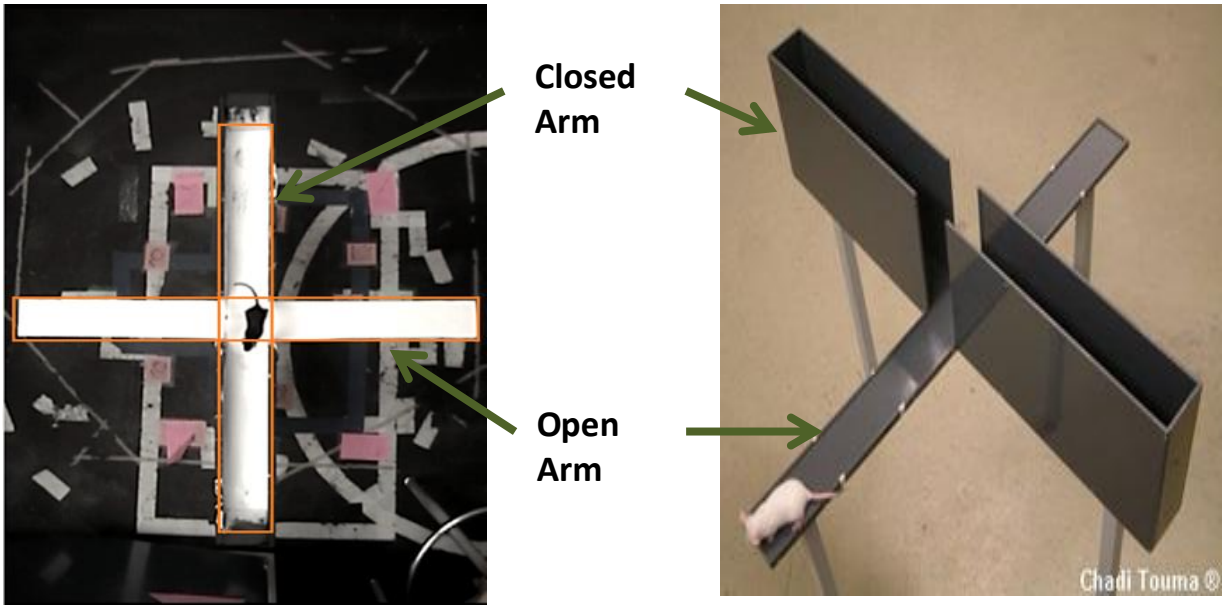


Figure 2-1 Elevated Plus Maze.

Left: The orange lines indicate the boundaries of the different zones (as marked in AnyMaze during analysis. The two vertical rectangles are the closed arms, the two horizontal rectangles are the open arms, and the square in the center is the center zone.

***Light-Dark test*:** The Light-Dark test was conducted similarly to published protocols (Amani, Samadi et al.) with some modifications. The light-dark test apparatus consisted of a plastic box (20x40x25 cm) equally divided into two chambers by a wall with a small, open

window. The light chamber was transparent and illuminated by an overhead fluorescent light (300 lux at cage floor), while the dark chamber was painted black with an opaque ceiling and walls. During the test, mice were individually placed in the center of the light chamber and subsequently recorded for 10 min as they moved freely between the two chambers. The total number of transitions, the time spent in each chamber, and the latency to enter the light chamber was recorded manually by a blinded observer.

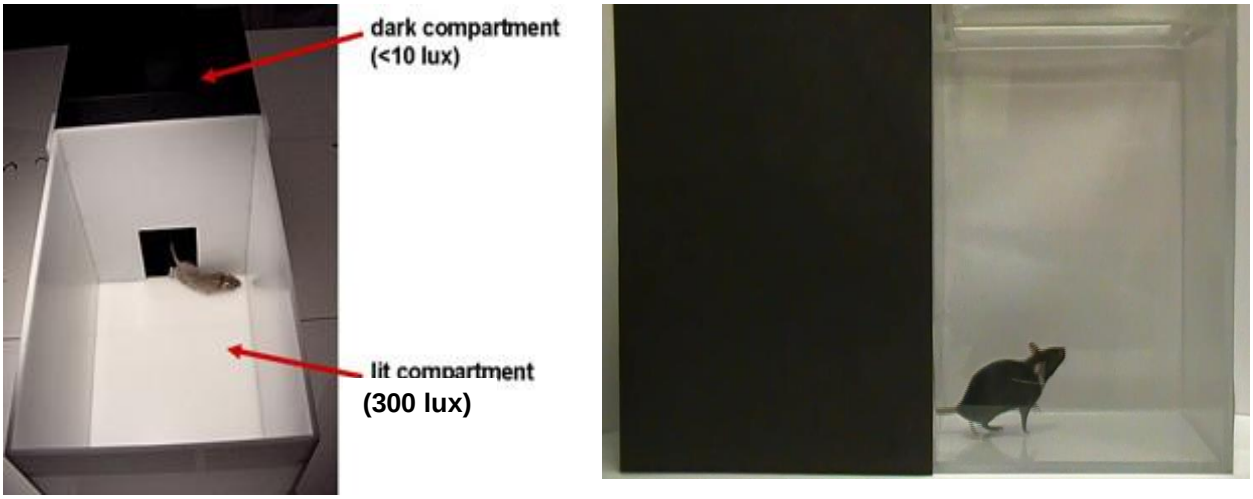


Figure 2-2 Light-Dark test for measuring anxiety

Left: Overhead view of the apparatus showing two compartments light and dark separated by a partition with a small outlet to allow the mouse move freely between two chambers. Right: Front-view of the apparatus used for the test.

***Open Field Test for locomotor activity and anxiety measures*:** This test was conducted with reference to previously published papers (Kovacsics and Gould) with minor modifications. The test consists of placing the mouse in a large, rectangular arena, from which escape is prevented by surrounding walls. In such a situation, mice generally prefer the periphery of the apparatus to the center of the open field, as open spaces correspond to areas where, in the wild, predators may easily spot the mice. Animals were individually placed in the center of the arena,

and video was recorded for ten minutes by an overhead camera positioned above the center of the arena. To characterize spontaneous locomotor activity, the total distance traveled across time and average speed were recorded by the ANY-maze analysis software. Locomotor activity was examined to provide insight into anxiety behaviors. Factors such as hyperactivity, for example, could skew results collected to measure anxiety. The open-field apparatus consisted of a rectangular arena (56 cm x 39 cm) with a wooden floor that was painted white in order to provide contrast with the black-coated mice, and four wooden walls surrounding the arena (22.86 cm high). Total distance traveled over time, number of line crossings, time spent mobile, and average speed were used to characterize spontaneous locomotor activity. Total distance traveled over time, number of line crossings, time spent mobile, and average speed were used to characterize spontaneous locomotor activity.

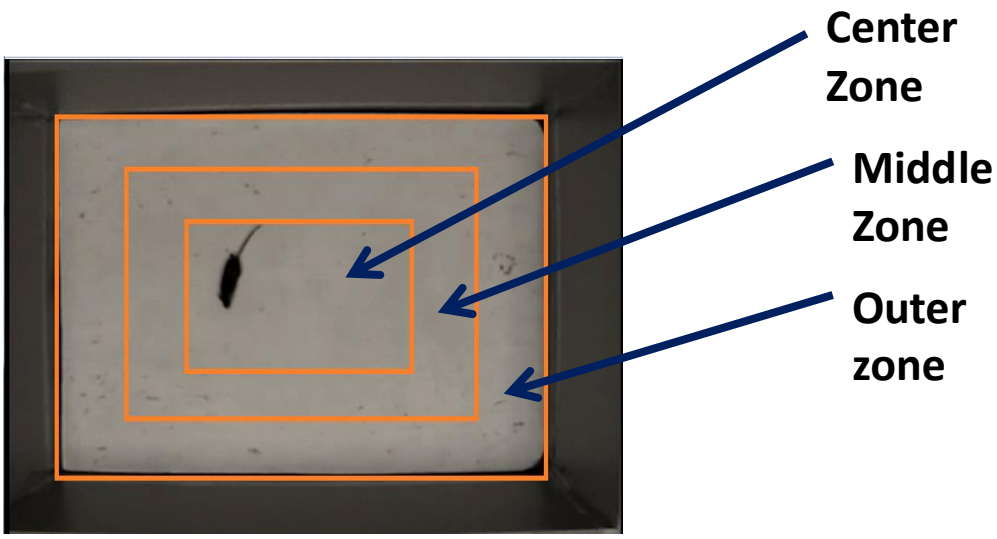


Figure 2-3 Open Field.

Overhead view of the Open Field test while it is running. The orange lines indicate the boundaries of the center zone, the middle zone, and the outer zone. When a mouse is in a specific

zone, information about its anxiety can be collected. On the left is an orthographic projection of the Open Field test.

Enzyme-linked Immunosorbent assay (ELISA assay): The pups at post natal days 6 and 15 (P6 and P15) were anesthesized (as mentioned before) and then sacrificed an hour later. The brains were isolated and the hippocampi taken out and suspended in 1X PBS and pargyline solution (1X solution in PBS) and kept on ice. The hippocampi were then slowly triturated with glass Pasteur pipette followed by centrifugation at 7500 rpm for 10 min. The supernatant was collected and stored in aliquots in -80°C. The pellets were also stored in – 80°C for western blots or other experimental purposes. The assay was performed using a kit from Genway (Serotonin ELISA, ref # 40-371-25002). The following dilutions were first made prior to the assay.

Preparation of Lyophilized and concentrated components: Control samples 1+2 which were provided with the kit as lyophilized products were diluted with 0.5 ml of bidistilled water and let stand stand for 15 min. Aliquots were made and stored at -20°C. 15 ml of Assay buffer was diluted with 150 ml of bidistilled water in a ratio of 1:10. 15ml of Wash buffer was diluted with 300ml of bidistilled water in a ratio of 1:20. Enzyme-Conjugate concentrate supplied 60 µl was diluted with diluted assay buffer in the ratio of 1:101 and this was prepared fresh and used only once.

Acylation of the samples: The samples and the controls are acylated, since the Standards have already been acylated when provided. 20 µl of each control and brain homogenate samples were pipetted into glass test tubes and 100 µl of diluted Assay buffer was added to each tube. It was mixed by vortex. 25 µl of Acylation Reagent was then added to each tube and immediately vortex after pipetting. The tubes were covered and incubated for 15 min in a 37^{o} C waterbath. 2

ml of diluted Assay buffer is pipetted in each tube and vortex. The tubes are then centrifuged for 10 min at 1500×g. The prepared samples were assayed immediately since the supernatant is stable only for 1 h at 18-25^{o} C.

Assay Procedure: The long overnight incubation version of the assay was performed. On the first day, 50 µl of each Standard, acylated control, and acylated sample is pipetted into the respective wells in a microtiter plate. Serotonin Biotin (50 µl) is then pipetted into each well. Serotonin Antiserum (50 µl) is pipetted into each well on top of the Serotonin Biotin. The plate is covered with adhesive foil, and shaken carefully to mix. The plate is then incubated overnight at 4^{o} C. On the next day, the adhesive foil is first removed and then the incubation solution discarded. The plate is washed three times with 250 µl of diluted Wash Buffer. The excess solution is removed by tapping the inverted plate on a paper towel. Freshly prepared Enzyme Conjugate (150 µl) is then pipetted into each well. The plate is again covered with adhesive foil and incubated for 60 min at room temperature (18-25) ^{o} C on an orbital shaker (500 rpm). After one hour, the adhesive foil is first removed and then the incubation solution discarded. The plate is then washed three times with 250 µl of diluted Wash Buffer. The excess solution is removed by tapping the inverted plate on a paper towel as earlier. PNPP Substrate Solution (200 µl) is then pipetted into each well and incubated for 30 min at room temperature (18-25) ^{o}C in an orbital shaker (500 rpm) as before. The substrate reaction is stopped by adding 50 µl of PNPP Stop Solution into each well. The contents are mixed briefly by gently shaking the plate. For adding the Substrate and the Stop Solution extra care was taken. Pipetting was carried out in such a way, so that same time intervals are maintained for Substrate and the Stop Solution in the wells. Formation of air bubbles is also avoided. The optical density is measured with a photometer at 405 nm within 60 min after pipetting the Stop Solution. The obtained OD of the

Standards was plot against their concentrations. The concentration of the samples can be directly read from the standard curve.

Genotyping of C57BL/6 5-HT1A-R (-/-) (KO) mice: Mouse pups were anaesthetized as mentioned before with a mixture of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg). 2 mm of tail was removed from each mouse and pla[ced into a 1.5 ml Eppendorf tube and kept on ice until digestion. The mice were ear-tagged for identification. To each tube, 500 µl of SNET buffer was added (20 mM Tris-Cl (pH 8), 5 mM EDTA (pH8), 400 mM NaCl, 1% SDS) and 10 µl of 20 mg/ml i.e. 400 µg/ml Proteinase K (Invitrogen, Cat. # 25530-0-15) was added and then incubated at 50-55 °C overnight. The next day, 500 µl of freshly mixed phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1 was added and incubated for 30 minutes at room temperature. To separate the organic and the aqueous phase, the tubes were centrifuged for 5 minutes in a micro-centrifuge (Eppendorf) at 14,000 rpm after which the upper, aqueous phase was removed from the organic layer and placed in a fresh 1.5 ml tube. The DNA was precipitated by adding 500 µl of 100% isopropanol and centrifuged at 13,200g for 15 minutes at 4 °C. The isopropanol was carefully removed and the DNA pellet was washed twice with 70% ethanol by centrifuging each time at 14,000 rpm for 5 min. The ethanol was removed and the pellet air-dried. The pellet was dissolved in 30 µl of TE (Tris-EDTA) buffer and left overnight on a shaker at 4 °C for proper dissolving. The DNA concentration was measured by NanoVue (GE Healthcare, Life Sciences). For the PCR reaction 50ng of DNA was used. To each 0.2ml tube (USA Scientific, 1402-8100) 2 µl 10x PCR Buffer, 2mM $MgSO_4$, 0.25 mM dNTPs, 500 nM of both forward and reverse primers, 0.1 µl of Platinum Taq Polymerase (Invitrogen, 10966-034)

The volume was brought to 20 µl with H_2O. The reaction was run in a thermo cycler (Applied Biosystems).

The wild type primers used were:

Htr1a Forward: CTGCTCATGCTGGTCCTCTATG

Htr1a Reverse: TAGGAGGTAGCTCCTGATTCGC

The product size was 323 bp.

The Knock out primers used were

NeoD (forward): CACCTTGCTCCTGCCGAGAAA

NeoH (reverse): AGAAGGCGATAGAAGGCGATG

The product size was 464 bp.

PCR Conditions maintained were:

1. 94 °C for 3 minutes

2. 94 °C for 30 seconds

3. 65 °C for 30 seconds

4. 68 °C for 30 seconds

5. Step (2-5) 29 more times (total 30cycles).

6. 68 °C for 7 minutes

7. Hold at 4 °C

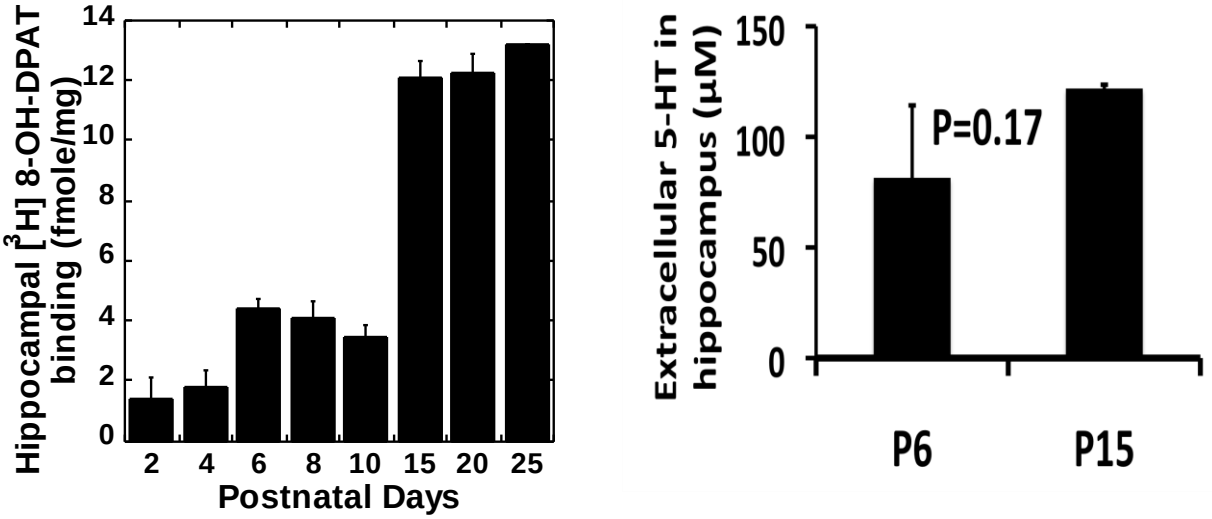
The PCR product was run for 1 hour at 100 volts on a 1.5 % Agarose gel in TBE (Tris-Boric acid-EDTA) buffer. A WT sample, KO sample and No-template sample were also run in the gel as controls. The gel was stained with Ethidium bromide and visualized under UV light.

3. Chapter 3

Experimental Results

Expression of 5-HT1A-R and extracellular serotonin in P6 mouse hippocampus:

Hippocampal lysates subjected to [^{3}H] 8-OH-DPAT binding assays showed a two-fold increase in the G-protein coupled form of the 5-HT_{1A}-R between P4 and P6 (time window for neuroproliferation), and another increase between P10 and P15 (peak time for synaptogenesis). Measurement of extracellular serotonin levels from hippocampal lysates by ELISA assays showed significant and comparable levels of the monoamine neurotransmitter Serotonin (5-HT) at P6 and P15, which would trigger 5-HT_{1A}-R signaling.



(a) **(b)**

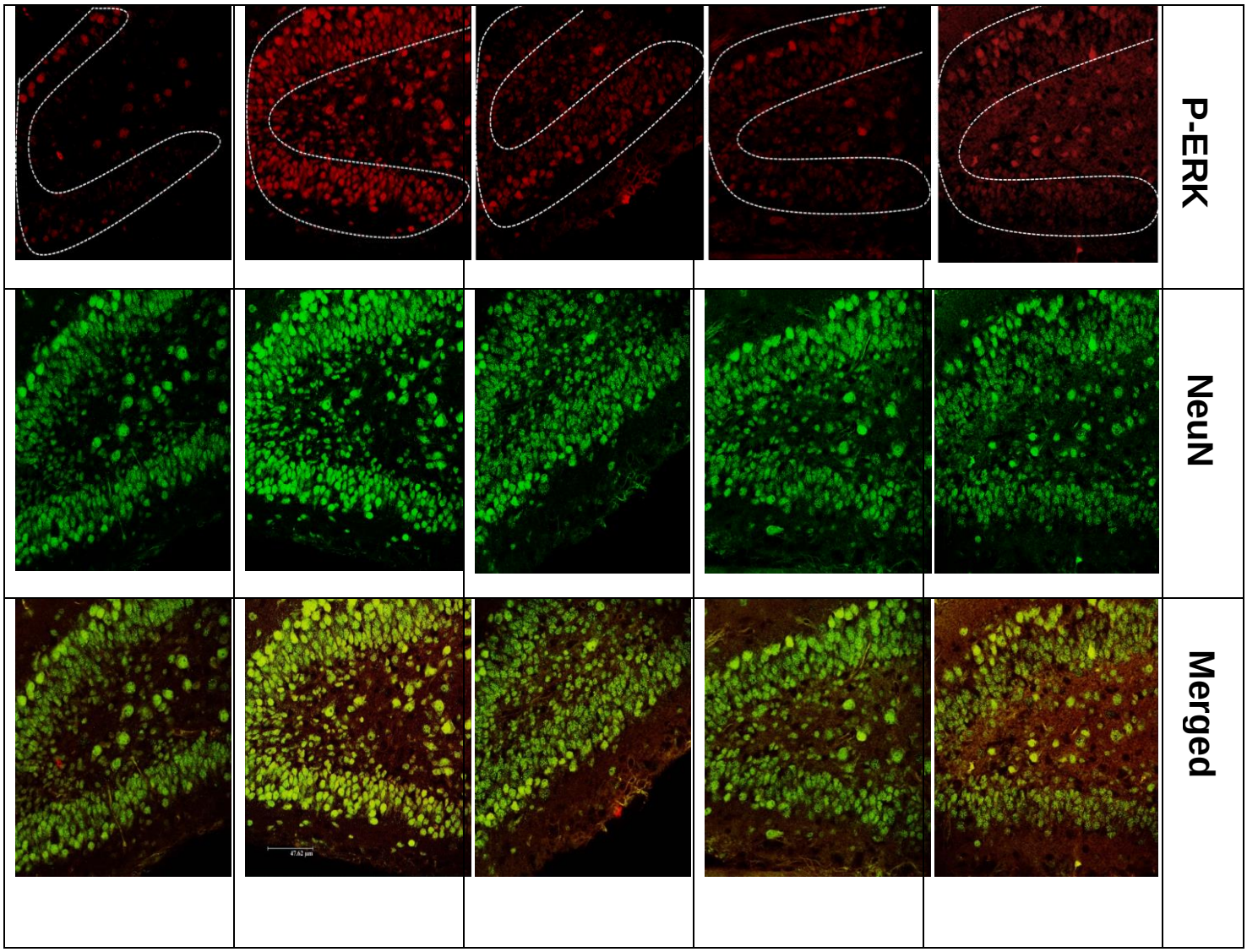
Figure 3-1 Levels of 5-HT and 5-HT1A-R in developing mouse hippocampus:

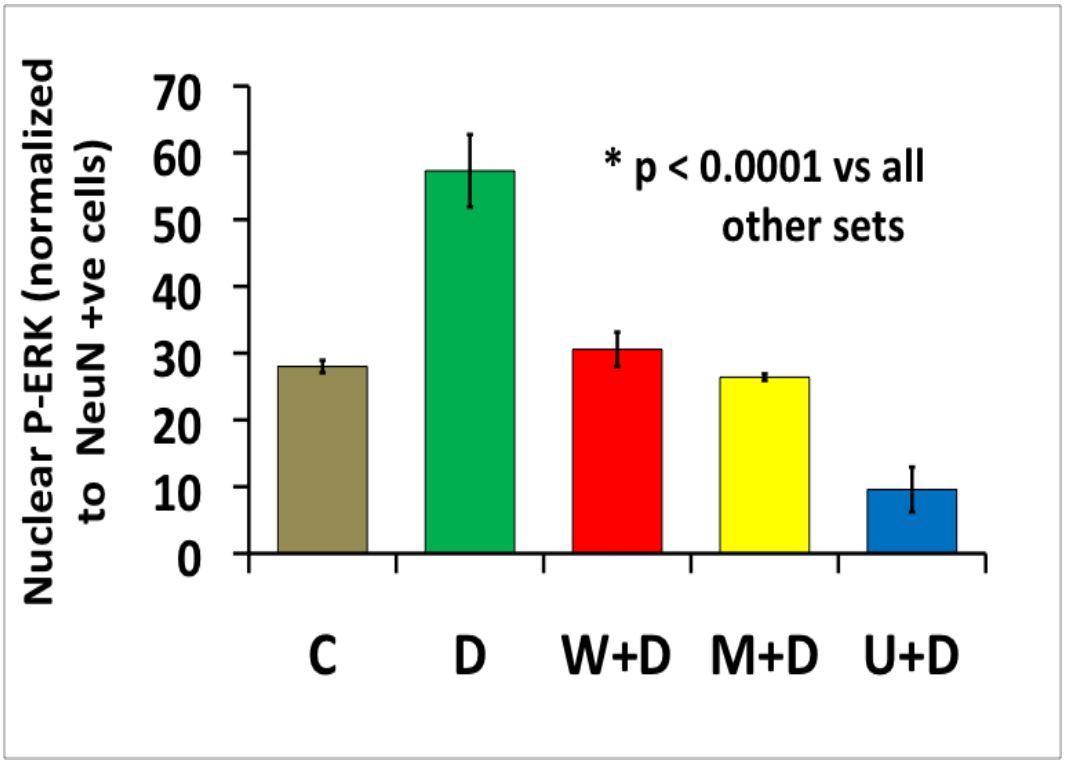
a) Hippocampal lysates were prepared from P2 to P25 mice and 100-µg protein aliquots in triplicate were subjected to [^{3}H] 8-OH-DPAT binding assays to determine the levels of the G protein-coupled 5-HT_{1A}-R (Singh 1996). The peak of postnatal neuroproliferation at P6 and the

postnatal peak of synaptogenesis at P15 (Mogha 2012) are marked by two increases in expression of the functional 5-HT_{1A}-R. **b)** Hippocampal lysates were prepared from different postnatal stages to determine the level of extracellular serotonin which would bind to the post-synaptic heteroreceptors in the hippocampus. The levels at P6 (peak of neuroproliferation) and P15 (peak of synaptogenesis) were comparable. (Samaddar, S., Schroder, R.[1], Marsillo, A., Debata, P.R., Ranasinghe, B., Purkayastha, S., Diallo, S., Kerr, D., Chanthrakumar, P., Tantry, S.J., and Banerjee, P. *Gender-specific Effect of Aberrant Serotonergic Signaling on Neonatal Hippocampal Development Makes Females Prone to Later-life Mood Disorders*. Manuscript in preparation).

Agonist stimulation of the 5-HT1A-R causes increased expression of nuclear P-ERK downstream of PKCε:

As shown in Fig. 1.12a neuroproliferative 5-HT_{1A}-R-mediated signaling operates in cultured hippocampal slices *via* PKCε and ERK (5-HT_{1A}-R➔➔PKCε ➔➔ERK). With the objective of testing the role of this pathway *in vivo* in neonatal neuroblast proliferation in the P6 hippocampus and to study its effect on brain development, we infused the 5-HT_{1A}-R agonist 8-OH-DPAT (D) in the absence and presence of WAY100635 (W), M (Myr-εV1-2), and U0126 (U) into the right hippocampus of P6 C57BL/6 mouse pups (drugs in 0.5 µl carrier). After 1 h, the pups were perfused and brain sections subjected to immunohistochemistry using antibodies against NeuN (green) and P-Erk (red) (Fig. 3.2a). Increase in nuclear P-Erk was indicative of activation, since nuclear entry is a crucial sequel to the activation of Erk (Smith, Cai et al.). This activation was eliminated in the presence of the PKCε activator M (Fig. 3.2a), thereby confirming that even *in vivo*, PKCε was positioned upstream of Erk in 5-HT_{1A}-R-mediated signaling.


P-ERK
NeuN
Merged
CARRIER
8-OH-DPAT (D) (100 nM)
WAY+D
M (Myr-εV1-2)+D
U0126+D



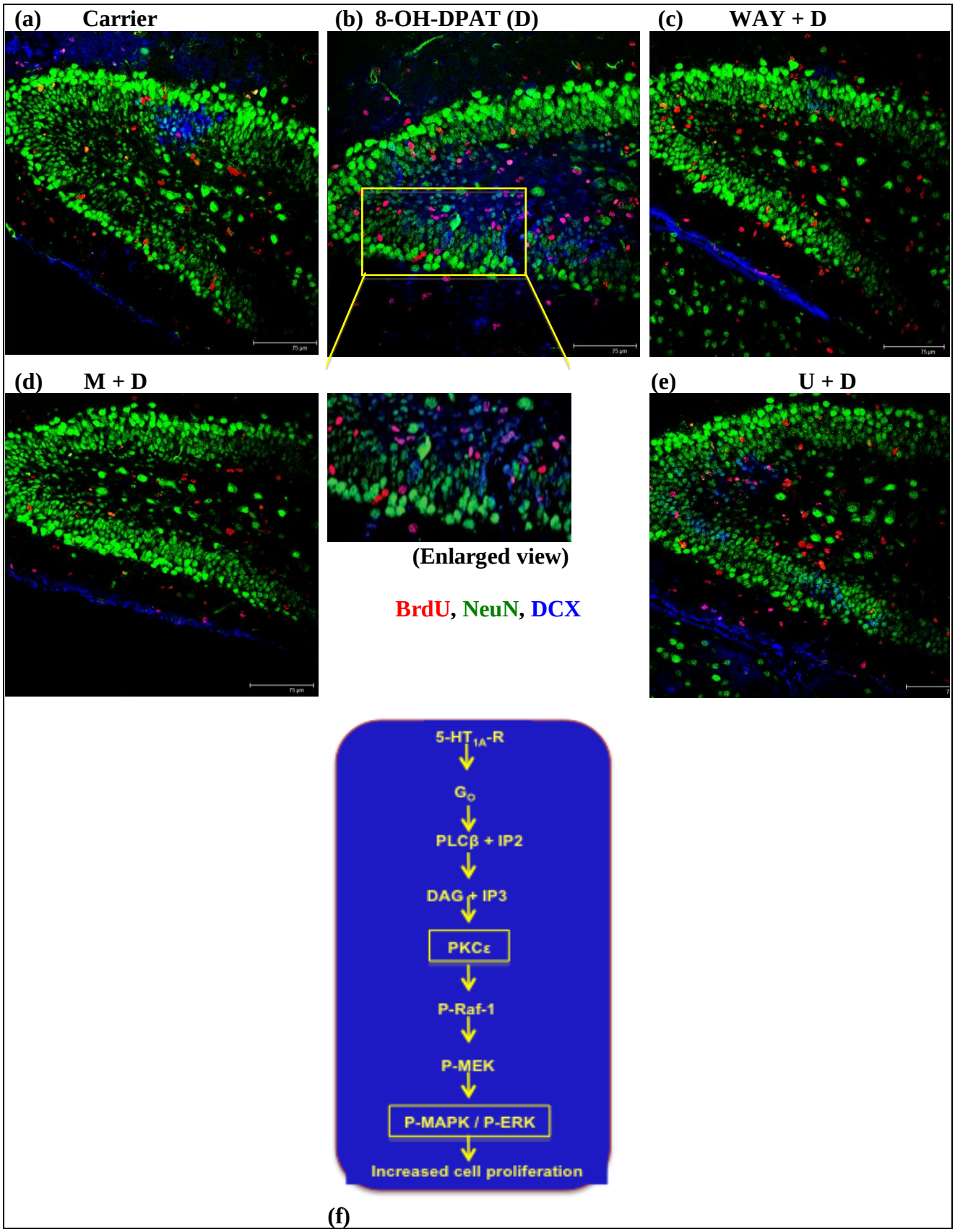
(b)

Figure 3-2 The 5-HT1A-R→ →PKCε→ →Erk pathway also functions in vivo.

a) One hour after infusion of drugs into the right hippocampus of P6 pups, the pups were perfused with paraformaldehyde and the ipsilateral hemispheres of the brains were cryosectioned and processed forimmunostaining with P-T^{202},Y^{204}- Erk (P-Erk) (red), NeuN (green). 8-OH-DPAT (D) treatment caused an increase in nuclear P-ERK (red), which was suppressed in the presence of the MEK inhibitor U0126 (U), and also the PKCε inhibitor (Myr-εV1-2) (M). Note: M, U, and WAY100635 (W) block D-evoked activation of Erk (marked by an increase in nuclear translocation of P-Erk). **b)** Graphical representation and statistical analysis of Nuclear P-ERK positive cells showed that the P-ERK was significantly higher in the D evoked group ($p < 0.0001$)

Agonist stimulation of 5-HT1A-R mediated signaling causes induced neuroblast proliferation in P6 mouse hippocampus.

The role of 5-HT_{1A}-R mediated signaling in neuroblast proliferation in neonatal dentate gyrus (DG) was next studied *in vivo* using systemic (i.p.) injection of BrdU followed by intrahippocampal infusion of 8-OH-DPAT in the absence and presence of WAY100635 (W), M (Myr-εV1-2), and U0126 (U) into the right hippocampus. Immunohistochemical staining of cryosections of the ipsilateral lobe revealed a marked increase in the number of cells that were double positive for BrdU (red) and DCX (blue) in the 8-OH-DPAT-treated brain sections ($p < 0.05$) (Fig. 3.3). This proliferative effect was blocked in the presence of WAY, M, and U0126, revealing that this 5-HT_{1A}-R signaling activity was mediated by PKCε and ERK1/2. This demonstrated that the identified 5-HT_{1A}-R mediated signaling pathway plays a key role in neuroblast proliferation in the SGZ of the DG. We did observe a few cells, which were double positive for NeuN (green) and BrdU (red), but this was indicative of DNA repair occuring in some mature granule neurons of the dentate gyrus (DG).


(a) Carrier
(b) 8-OH-DPAT (D)
(c) WAY + D
(d) M + D
(e) U + D
(Enlarged view)
BrdU, NeuN, DCX
5-HT1A-R
Gο
PLCβ + IP2
DAG + IP3
PKCε
P-Raf-1
P-MEK
P-MAPK / P-ERK
Increased cell proliferation
(f)

(g)

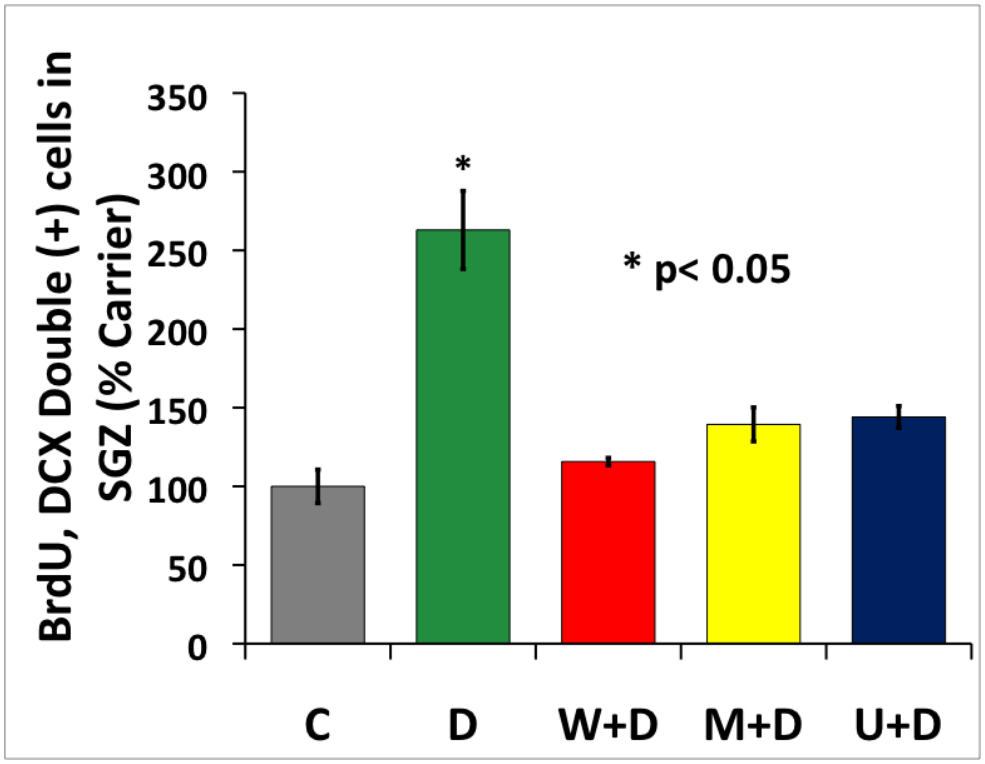


Figure 3-3 Signaling through 5-HT1A-R, PKCε, and Erk1/2 causes induced cell proliferation in P6 hippocampus.

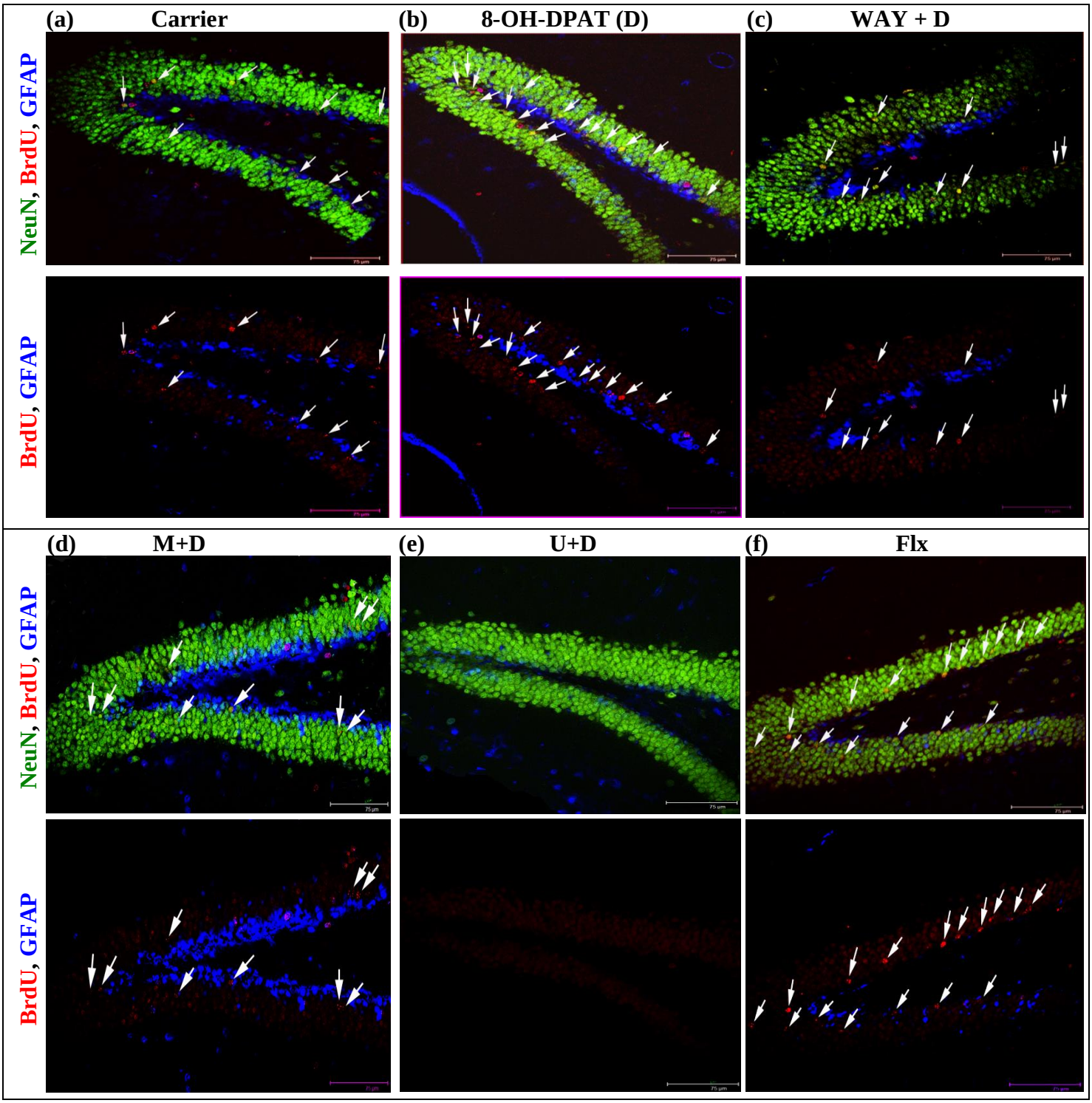
(a-e) Number of neuroblasts proliferating in P6 dentate gyrus (DG) when subjected to intra-hippocampal injection with carrier or 8-OH-DPAT (D) in the absence (b) and presence of 5-HT_{1A} receptor antagonist WAY100635 (W) (c), PKC epsilon inhibitor (Myr-εV1-2) (M) (d), and MEK inhibitor U0126 (U) (e) as shown by the co-localization of BrdU (red) and DCX (blue). (f) A diagrammatic representation of the neurogenic 5-HT_{1A} receptor signaling cascade showing the hierarchy of the key players PKCε and Erk1/2. (g) Stimulation of the 5-HT_{1A}-R with intrahippocampally infused DPAT (D) caused a significant increase in neuroblast proliferation, and this neurogenic effect was blocked in the intrahippocampal presence of WAY 100635, Myr-εV1-2 and U0126. ($p<0.05$, $n=3$). Scale bar: 75 µm.

Agonist stimulation of the hippocampal 5-HT_{1A}-R causes increased neurogenesis in the DG.

Earlier reports have demonstrated that 5-HT_{1A}-R regulates neurogenesis in adult mouse hippocampus (Santarelli 2003). Having confirmed that supra-basal activation of the 5-HT_{1A}-R causes increased neuroproliferation in P6 mouse hippocampus, we investigated if this boosted neuroproliferation at P6 could eventually lead to an increase in neurogenesis. We used the strategy shown in Fig. 3.3, and performed BrdU infusion (i.p.) two hours before intracranial infusion of 8-OH-DPAT into the right hippocampus in the absence and presence of WAY100635, M, or U0126. However, in order to analyze mature neurons, we waited for 30 days after this treatment and then perfused, isolated, and then cryosectioned the brains for immunostaining. Triple immunostaining with NeuN (green), BrdU (red), and Glial Fibrillary Acidic Protein (GFAP) (blue) showed increased formation of BrdU, NeuN double-positive new neurons (Fig. 3.4). This increase in neurogenesis was blocked in the presence of WAY, M, or U0126, confirming the involvement of the same 5-HT_{1A}-R-mediated signaling pathway as shown in Fig. 3.3 in this boosted neurogenesis. This study also revealed few cells, which were double stained with GFAP and BrdU indicating gliogenesis, but no significant difference was observed in the number of glial cells among the different treatment groups (data not shown here).

This experiment also included a sixth group from intrahippocampal fluoxetine (Prozac- a serotonin reuptake inhibitor)-treatment, which would be expected to increase extracellular serotonin in the hippocampus. Fluoxetine treatment yielded a similar increase in neurogenesis as observed after 8-OH-DPAT treatment, suggesting that the natural serotonin-mediated signaling in the hippocampus may operate through the 5-HT_{1A}-R-mediated signaling pathway delineated here. However, the supra-basal stimulation of the 5-HT_{1A}-R may not represent the effect of

tonic or basal activation of this receptor *in vivo*. Therefore, further experiments were required to address this functional aspect of the receptor.


(a) Carrier
(b) 8-OH-DPAT (D)
(c) WAY + D
NeuN, BrdU, GFAP
BrdU, GFAP
(d) M+D
(e) U+D
(f) Flx
NeuN, BrdU, GFAP
BrdU, GFAP

(g)

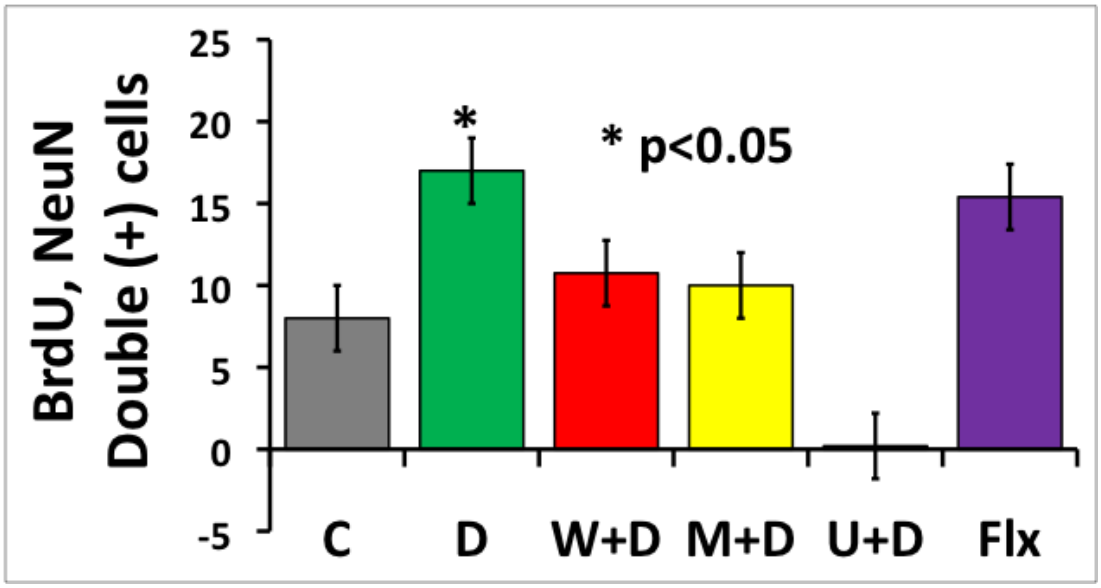


Figure 3-4: 5-HT1A-R-mediated neurogenesis in the dentate gyrus (DG) monitored by triple staining for BrdU (cell division marker, red), NeuN (mature neurons, green), and GFAP (astrocytes, blue).

Wild type C57BL/6 P6 pups were injected (i.p.) with 5-bromo-2'-deoxyuridine (BrdU) 2 h before intra-hippocampal infusion of 8-OH-DPAT (D) (100 nM) in the absence and presence of antagonist and inhibitors WAY, M, and U. One brain in each experiment was dedicated for Fluoxetine (Flx) injection alone (Mogha 2012). After 30 days, the mice were perfused, brains cryosectioned and subjected to IHC. (a-f) Confocal images show co-localization of BrdU (red) with NeuN (green) indicating increased neurogenesis when treated with D which was eliminated in the presence of antagonist WAY (c), M (d), and U (e). (Arrows show some of these BrdU and NeuN double-positive cells). Treatment with Fluoxetine alone increased the level of neurogenesis close to that achieved with 8-OH-DPAT (f). Additionally, some BrdU (red) cells co-localized with GFAP (blue) (especially in sections from WAY or M treatment) indicating 5-HT1A-R-independent gliogenesis (Samaddar 2011). (g) Quantification from 3 experiments (n =

3) showed that the D and Flx groups were significantly higher than all other groups. ($p < 0.05$). Scale bar: 75 µm.

The increase in the number of Neuroblasts or Neurons is not due to a decrease in apoptosis.

As shown in earlier studies, the observed 5-HT_{1A}-R-mediated increase neuroblast proliferation and neurogenesis as shown in Fig. 3.3 and 3.4 could be the result of inhibited apoptosis. To address this possibility, we used the Millipore Apoptag TUNEL ASSAY KIT to monitor apoptosis in the carrier and 8-OH-DPAT-treated hippocampal sections. As shown in Fig 3.5, neither 24h or 30-day 8-OH-DPAT (D) treatments yielded any decrease in TUNEL (+) apoptotic cells. Intriguingly, D treatment completely blocked apoptosis in the subventricular zone (SVZ) in the 30-day treatment (ventral to the DG). Thus supra-basal activation of the 5-HT_{1A}-R was not neuroprotective; rather it was actually inducing the formation of more neurons.

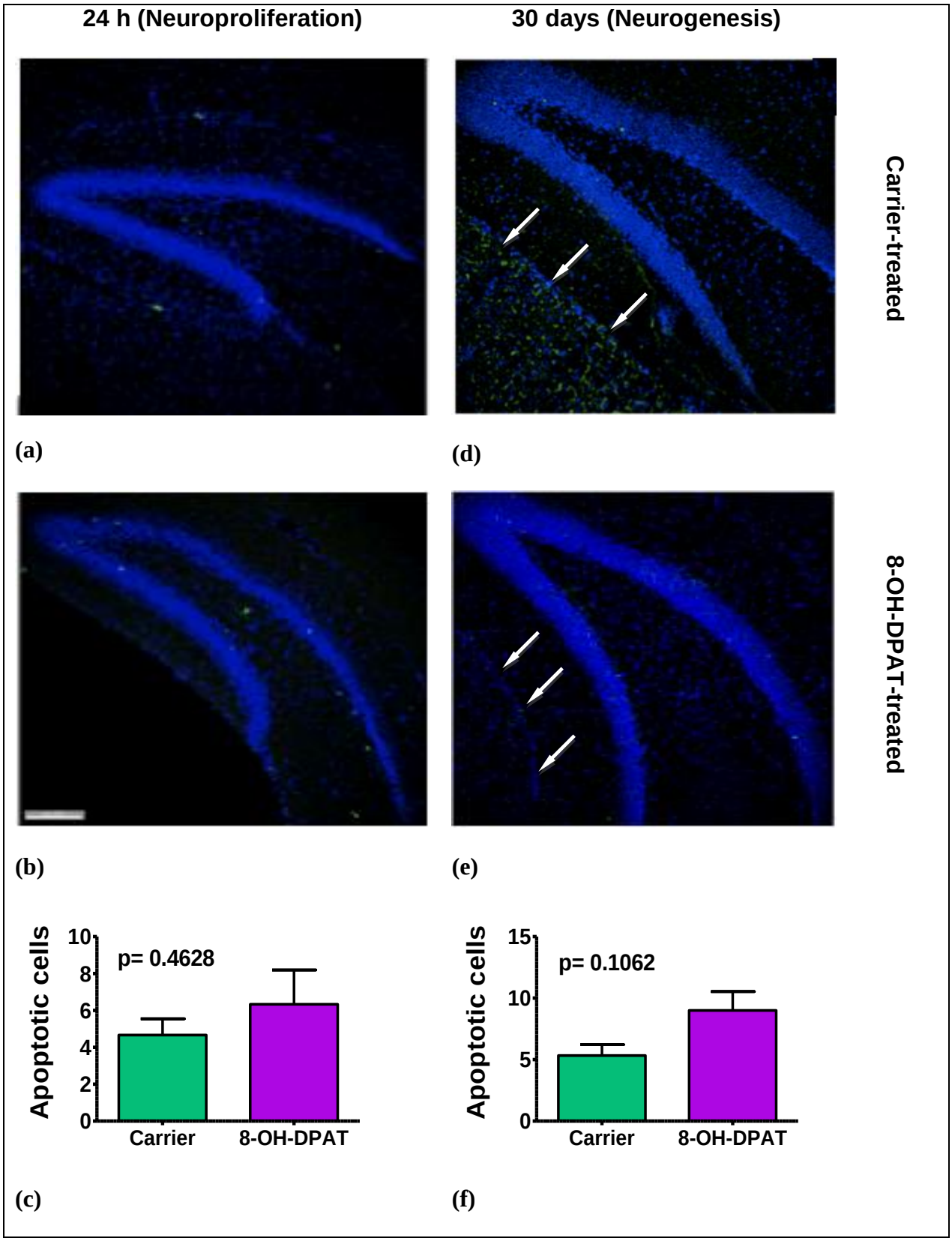
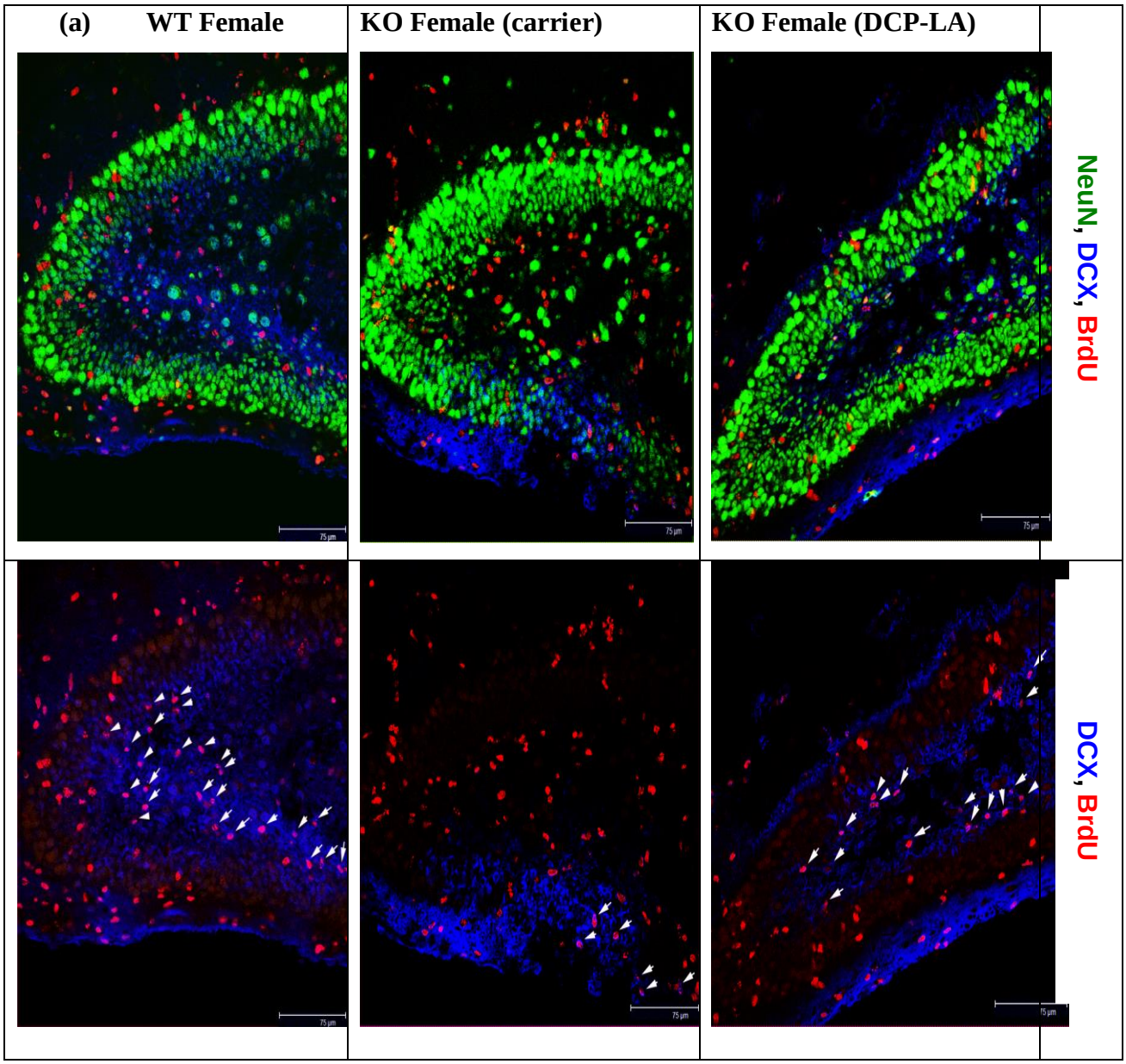

24 h (Neuroproliferation)
30 days (Neurogenesis)
Carrier-treated
8-OH-DPAT-treated
(a)
(d)
(b)
(e)
Apoptotic cells
p= 0.4628
Carrier
8-OH-DPAT
(c)
Apoptotic cells
p= 0.1062
Carrier
8-OH-DPAT
(f)

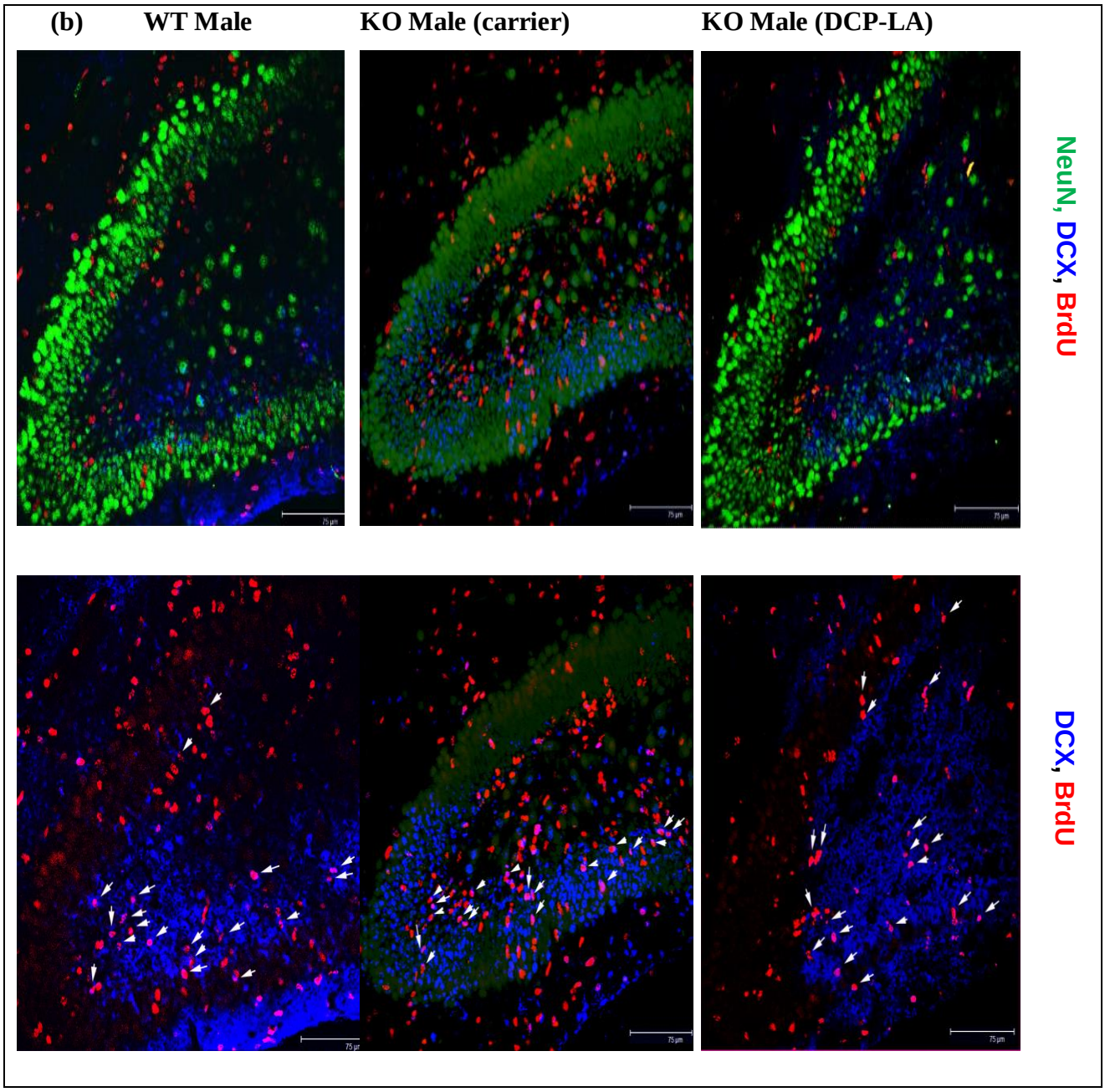
Figure 3-5: TUNEL assays reveal that 8-OH-DPAT treatment in vivo does not block apoptosis in the D.

TUNEL stained cells were marked green within the dentate gyrus granule cells labeled with Hoechst33342. TUNEL assays were performed with sections of brains, which were treated with (a) carrier or (b) 8-OH-DPAT (D) for 24 h (to analyze apoptosis during neuroproliferation) and (d) carrier or (e) D for 30 days (to analyze apoptosis during neurogenesis). (c,f) Neither experiment recorded a decrease in TUNEL(+) cells in the DG. Intriguingly, apoptosis of a large number of cells in the subventricular zone (SVZ) (white arrows) during neurogenesis appeared to be blocked in the 8-OH-DPAT-treated brains that were being monitored for neurogenesis (d and e).

The 5-HT1A-R deficient mice show a significant but gender-specific decrease in neuroproliferation, which can be partially corrected by using a selective activator of PKCε.

Supra-basal activation of the 5-HT_{1A}-R signaling in P6 mouse hippocampus was used to initially delineate a neurogenic 5-$HT_{!A}$-R signaling pathway, but our primary objective was to modulate the basal level of this signaling activity to regulate neuroproliferation. With this objective, we performed BrdU infusion into wild type and 5-HT_{1A}-R (-/-) (KO) mice at P6 followed by DCX and NeuN staining of brain sections after 24 hours. Surprisingly, we observed a dramatic, but gender-specific suppression in BrdU, DCX double labeling in the SGZ of female mice ($p=0.003$) (Fig. 3.6 a, b), thereby confirming a decrease neuroblast proliferation in the P6 SGZ. The absence of the 5-HT_{1A}-R did not cause any significant change in the basal level of neuroblast proliferation in the male P6 mice (Fig. 3.6c, d). An increase of neuroproliferation was also observed following intrahippocampal infusion of DCP-LA (Fig 3.7), but because this molecule selectively activates the identified hippocampal neurogenic signaling pathway, the less invasive systemic infusion of this reagent was used to achieve the same result (Fig. 3.6 a, b). Systemic infusion of a selective activator of PKCε, DCP-LA, partially corrected the suppressed neuroblast proliferation observed in the P6 SGZ of female mice (Fig. 3.6b), ($p=0.007$) further confirming the importance of the identified 5-HT_{1A}-R signaling pathway. Therefore, even in the absence of the upstream 5-HT_{1A}-R molecule, the selective PKCε activator DCP-LA would activate the downstream section of the identified pathway to boost neuroblast proliferation in the hippocampus.


(a) WT Female
KO Female (carrier)
KO Female (DCP-LA)
NeuN, DCX, BrdU
DCX, BrdU


(b)
WT Male
KO Male (carrier)
KO Male (DCP-LA)
NeuN, DCX, BrdU
DCX, BrdU

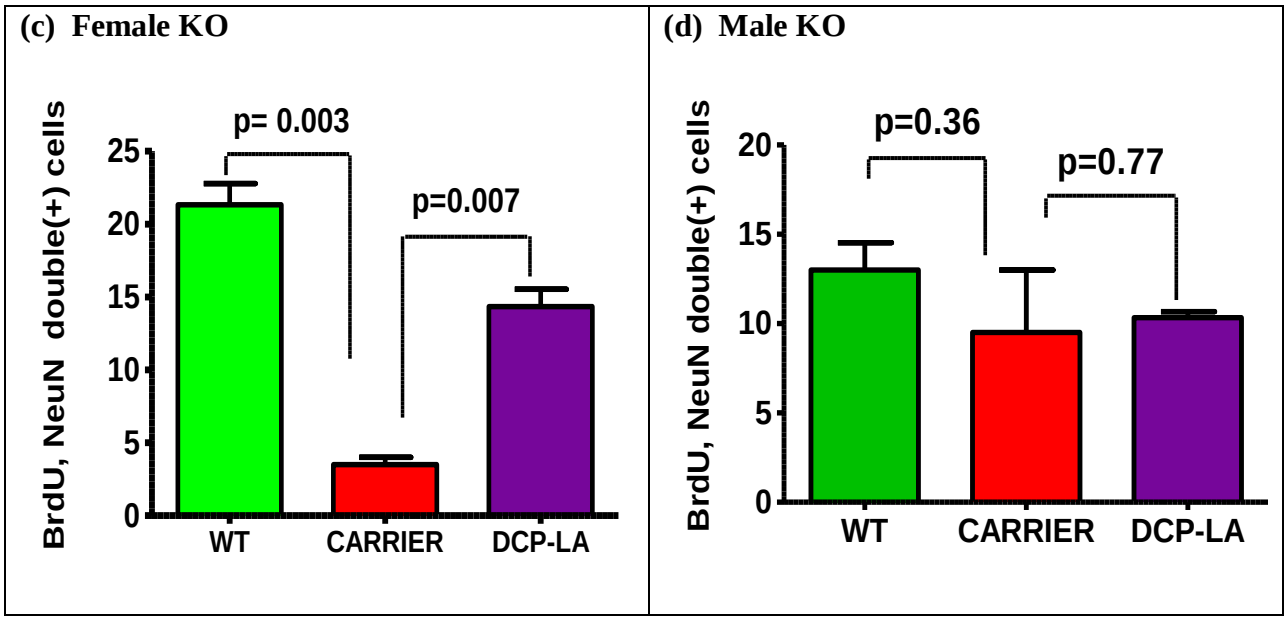


Figure 3-6: Female but not male KO mice at P6 show reduced neuroblast proliferation in the DG, which is partially corrected in the presence of a selective stimulator of PKCε.

Proliferating neuroblasts co-labeled with antibodies against BrdU (red) and DCX (blue) have been shown with white arrows after hiding the green (2nd row) NeuN staining in the images of DG. Partial correction of neuroblast proliferation was seen in the female KO mice in the presence of a selective stimulator of PKCε (a,c). No significant decrease in neuroproliferation of Male KO or correction with DCP-LA was observed (b,d). (c,d) Graphical representation and statistical analysis of the observed data.

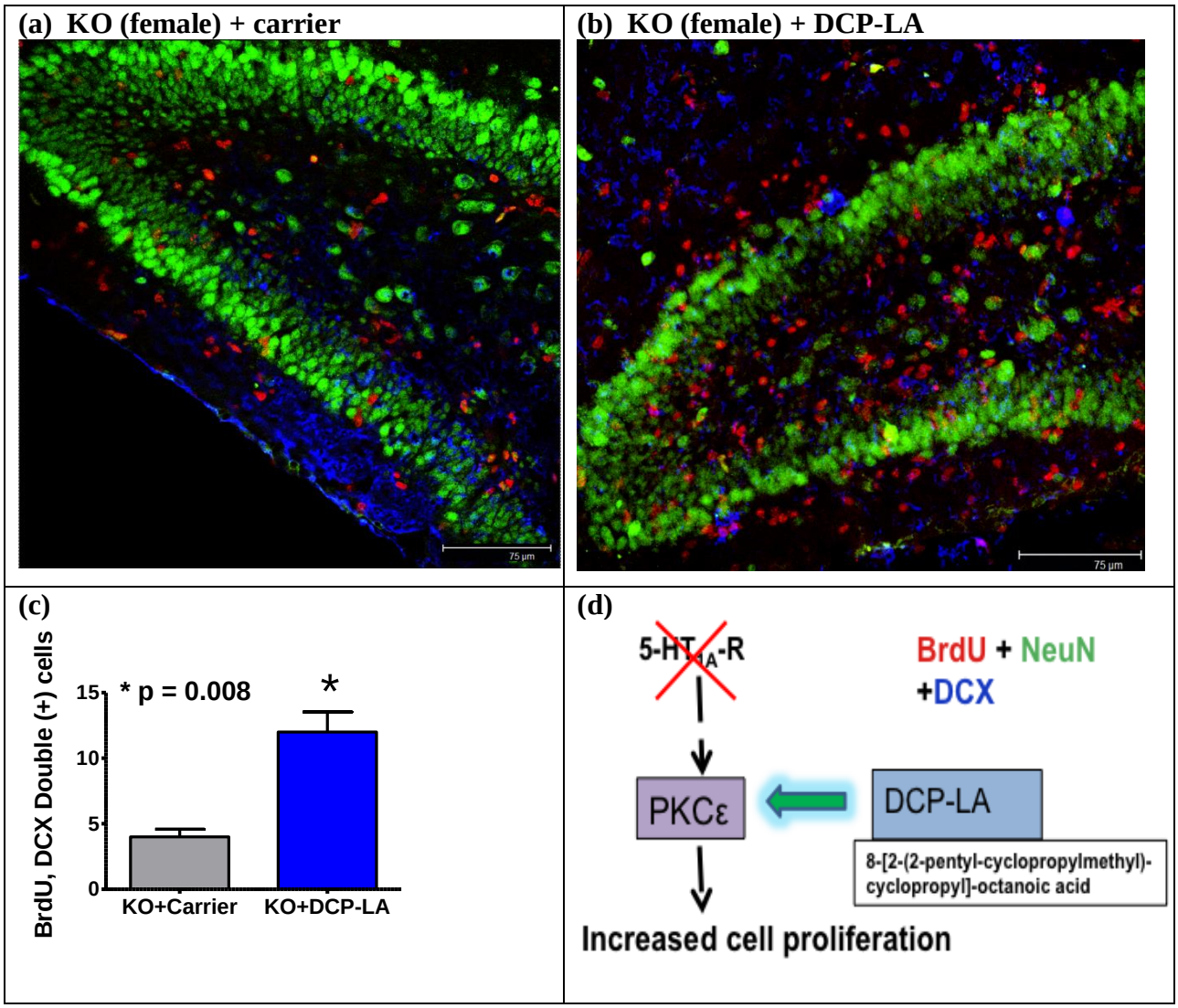


Figure 3-7: Intrahippocampal infusion of DCP-LA at P6 causes increased neuroblast proliferation in KO females.

P6 KO mouse pups were infused I.P. with BrdU and after 2 hours, carrier or DCP-LA were infused into the hippocampus as described in the methods. After 24 hours, the mice were perfused and the brains sectioned and immunostained for NeuN (green), DCX (blue), and BrdU (red). (a and b) DCP-LA treatment caused a dramatic increase in purple BrdU, DCX double positive cells in in the SGZ in these 5-HT_{1A}-(-/-) mice. (c) Quantification and statistical analysis

revealed a significant increase in the proliferation in DCP-LA treated group ($p=0.008$). (d) Schematic representation of the strategy used in this experiment.

The 5-HT1A-R deficient mice show a significant but gender-specific aberrancies in mossy fiber connections which can be partially corrected by using a selective activator of PKCε.

There is a strong possibility that stunted neuroproliferation in the DG of KO mice could result in anomaly in a few crucial connections in the Mossy Fiber pathway. The Mossy Fiber axons originating in the granule cells of the DG harbor high levels of zinc, which can be detected using a silver precipitation-based histochemical procedure, termed Timm Staining. The observed gender-specific decrease in neuroblast proliferation in P6 female KO mouse DG was also accompanied by an increase in Timm stained, Mossy Fiber (MF) collaterals in the CA3 *stratum oriens* (SO) at P18 (Fig. 3.8). As expected, i.p. infusion of DCP-LA into female KO mice at P6, P10, and P14 corrected this aberrant increase in MF collaterals at P18 almost to the levels of the WT mice (Fig. 3.8 d and f). Further Timm-staining images and analyses of sections from different parts of the hippocampus have been shown **(a-c)** and **(d-f).** Graphical representation and statistical analysis is shown (Fig 3.8 g and h) for respective parts of the hippocampus **(a-c,g)** and **(d-f, h).**

Thus, the overall maturation of the hippocampus and its neuronal framework appear to be delayed and aberrant in the KO female mice.

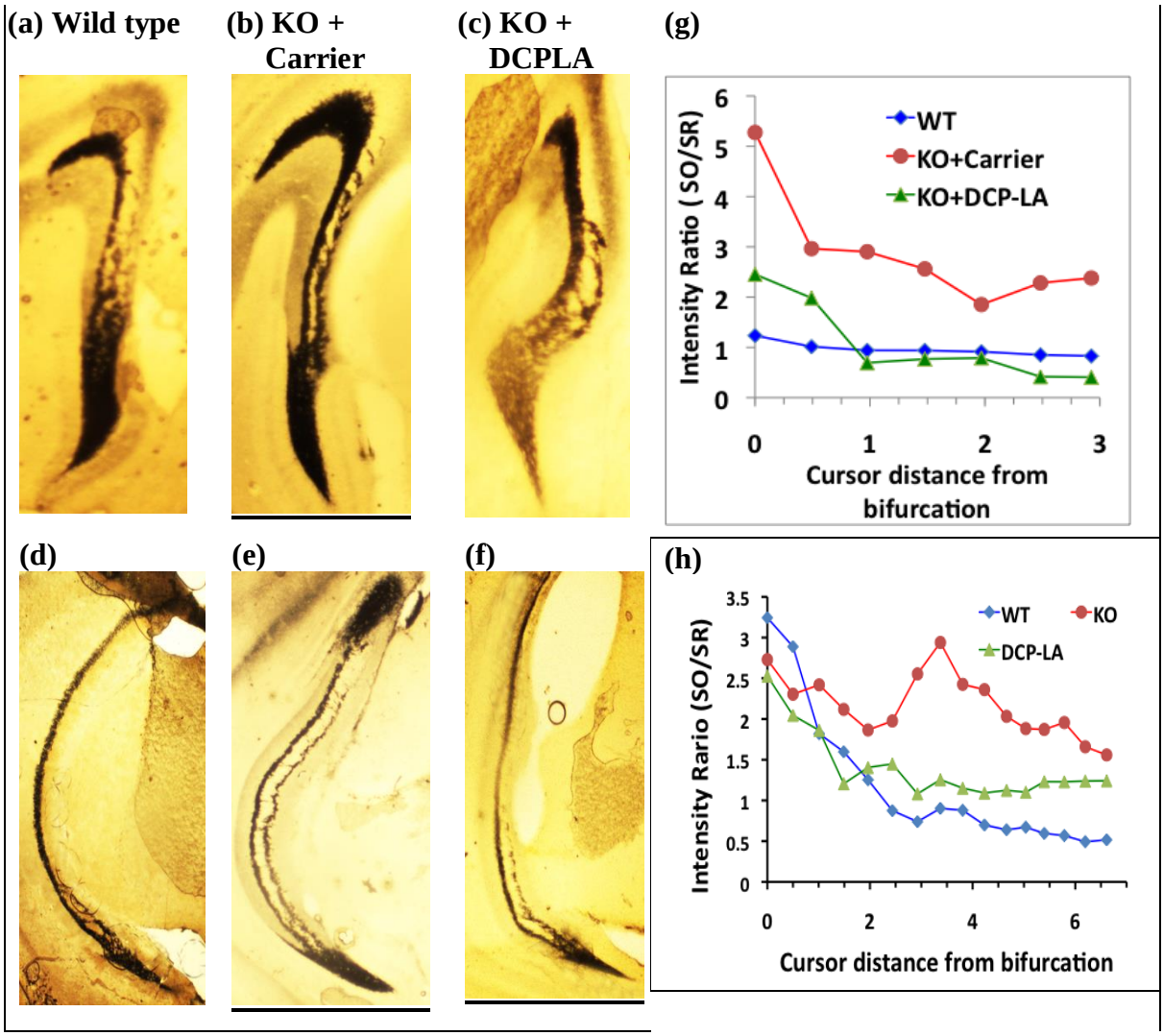


Figure 3-8: Female KO mice at P6 show aberrancies in the mossy fiber connection in the hippocampus, which is partially corrected in the presence of a selective stimulator of PKCε.

(a-c) and (d-f) Sections from different parts of the hippocampus. (g,h) Graphical representation of densitometry analysis of the Timm stained terminals. The x-axis shows cursor distance in inches from the bifurcation in enlarged images, each of which has the dimensions of 13 in (height) x 5 in (width).

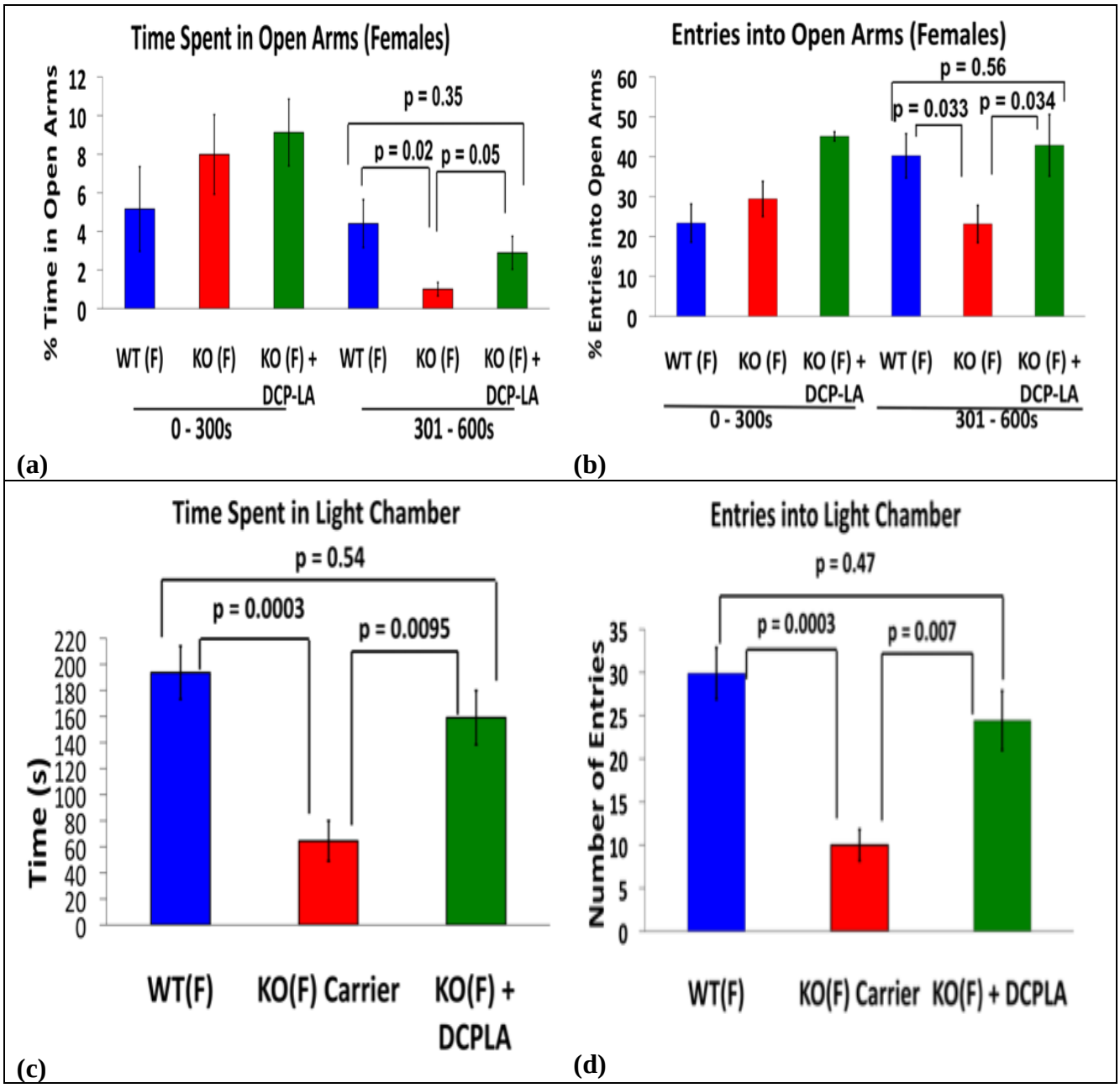
Suppressed neuroblast proliferation at P6 in female KO mice is associated with heightened anxiety at P60, which is corrected in DCP-LA-treated KO mice.

The female but not male KO mice (Fig 3.9e) at P60 displayed elevated anxiety in the elevated-plus maze (EPM) (Fig. 3.9a, b). Intriguingly, this heightened anxiety was partially or fully corrected in the DCP-LA-treated mice (Fig. 3.9a, b).

Additionally, the female but not male KO mice (Fig. 3.9f and g) showed an even more robust sign of hyper-anxiety in Light-Dark tests, with a significant decrease in entry as well as time spent in the light chamber (Fig. 3.9 c, d). Once again, the anxiety level in the DCP-LA-treated female KO mice appeared to be in the range of the wild-type mice (Fig. 3.9 c and d), thereby strongly indicating a causal link among neuroblast proliferation at P6, MF connectivity in the SO at P18, and anxiety in adulthood (P60) in the female mice.

A decrease in entries and time spent in the open arms of the elevated plus maze, as well as entries and time spent in the lit chamber of the light dark test, can also be a consequence of locomotor hypoactivity, as opposed to anxiety. To rule out this possibility, we tested all groups in the open field test (OFT). The OFT is a standard measure of exploratory behavior and general activity in rodents (T.D. Gould 2009), and is sensitive to motor dysfunction as well as cerebellar and/or basal ganglia damage (Chetrit 2009; Bauer 2011). Although an increase in time spent in the center can be an indicator of anxiolysis, the OFT alone does not always claim predictive validity for anxiety disorders (Belzung 2002). Therefore, whereas performance in the elevated plus maze and light-dark tests (EPM and LD, respectively) are generally considered as the gold standards for assessing anxiety (Bailey 2009), the OFT may be better suited for assessing locomotor activity. In light of this, since we observed no significant differences between groups in any of the aforementioned parameters in the OFT (Fig 3.9 h), the differences observed in the

EPM and LD tests can most likely be attributed to an anxiety phenotype and not locomotor hypoactivity.



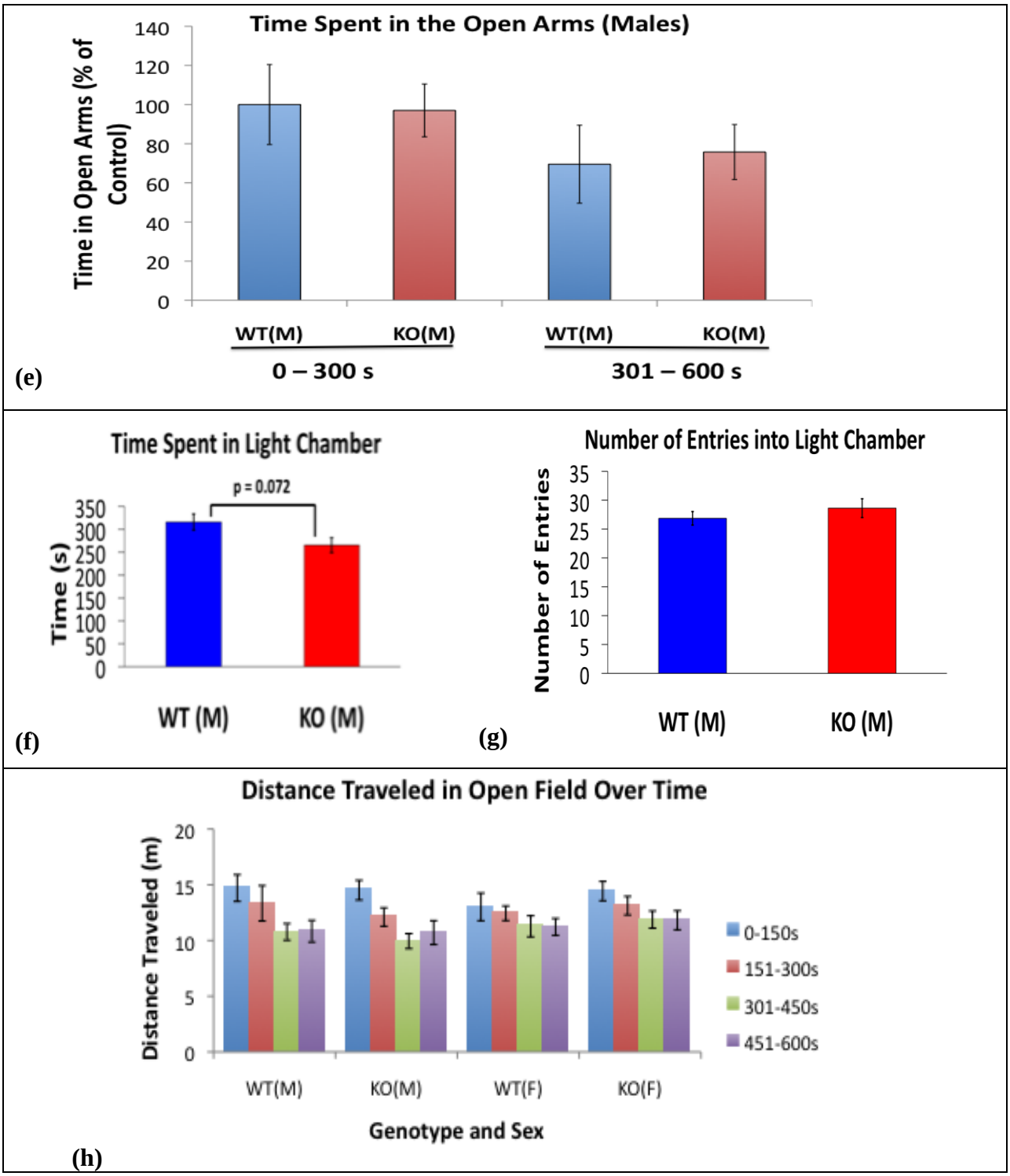

Time Spent in the Open Arms (Males)
Time in Open Arms (% of Control)
140
120
100
80
60
40
20
0
WT(M)
KO(M)
WT(M)
KO(M)
0 – 300 s
301 – 600 s
(e)
Time Spent in Light Chamber
p = 0.072
Time (s)
350
300
250
200
150
100
50
0
WT (M)
KO (M)
(f)
Number of Entries into Light Chamber
Number of Entries
35
30
25
20
15
10
5
0
WT (M)
KO (M)
(g)
Distance Traveled in Open Field Over Time
Distance Traveled (m)
20
15
10
5
0
0-150s
151-300s
301-450s
451-600s
WT(M)
KO(M)
WT(F)
KO(F)
Genotype and Sex
(h)

Figure 3-9: Heightened anxiety displayed by female 5-HT1A-R(-/-) (KO) mice in Elevated-Plus Maze (EPM) and Light-Dark Chamber tests is corrected after neonatal DCP-LA treatment.

Number of mice used EPM: Males: Wild type (WT) 8 and KO 11; Females: WT 9, KO (carrier) 9, and KO (DCP-LA) 7. Light-Dark test: Males: WT 6, KO 9; Females: WT 5, KO (carrier) 5, KO (DCP-LA) 7. In EPM, the KO female mice showed hyper-anxiety in the second half of the test ($p=0.013$ and $p=0.033$), but the KO males were not distinguishable from the wild type. The KO females showed a robust increase in anxiety in the Light-Dark Chamber test ($p=0.0003$ and $p=0.0003$), whereas the KO males were not significantly different from the wild type males. In both tests, DCP-LA rescued the anxiety-like phenotype, EPM ($p= 0.05$ and $p= 0.034$) and Light/Dark Test ($p= 0.0095$ and $p=0.007$). The KO mice of both genders displayed no difference from WT in locomotion (Fig 3.12h), thereby demonstrating that the tests for anxiety in Fig. 3.12 were not influenced by motor abnormalities.

(Samaddar, S., Schroder, R., Alexandra Marsillo, Debata, P.R., Ranasinghe, B., Purkayastha, S., Diallo, S., Kerr, D., Chanthrakumar, P., Tantry, S.J., and Banerjee, P. *Gender-specific Effect of Aberrant Serotonergic Signaling on Neonatal Hippocampal Development Makes Females Prone to Later-life Mood Disorders*. Manuscript in preparation).

An overview of neonatal 5-HT_{1A}-R signaling in mouse hippocampus

- Stimulation of the 5-HT_{1A}-R leads to activation of P-ERK.
- Stimulation of 5-HT_{1A}-R leads to activation of P-ERK via PKC epsilon activation (5-HT_{1A}-R ➔➔PKCε ➔➔ERK).
- 5-HT_{1A}-R mediated signaling causes increase in neuroblast proliferation in the SGZ of the Dentate Gyrus (DG) at P6.
- 5-HT_{1A}-R mediated signaling causes increase in neurogenesis in the DG.
- 8-OH-DPAT is neurogenic rather than neuroprotective.
- 5-HT_{1A}-R deficient mice have significantly reduced neuroblast proliferation when compared to WT.
- 5-HT_{1A}-R deficient mice have no significant difference in neurogenesis when compared to WT.
- Selective PKCε activator, DCP-LA partially or fully rescues neuroblast proliferation in female 5-HT_{1A}-R deficient mice at P6 under both intra-hippocampal (localized) and intra-peritoneal (systemic) injections.
- DCP-LA rescues the aberrant increase in Mossy fiber collaterals in female 5-HT_{1A}-R deficient mice almost to the levels of WT mice.
- Female but not male 5-HT_{1A}-R deficient mice displayed anxiety in elevated Plus Maze (EPM) and Light-Dark chamber tests.
- DCP-LA corrects the elevated anxiety in the female 5-HT_{1A}-R deficient mice in both in EPM and Light-Dark chamber tests close to WT levels.
- No locomotory aberrancies were observed between male and female 5-HT_{1A}-R deficient mice and WT controls in the Open Field Test (OFT).

4. Chapter 4

The role of Vascular Endothelial Growth Factor (VEGF) in Serotonin 1A Receptor mediated Neonatal Hippocampal Development

Vascular Endothelial Growth Factor (VEGF):

Vascular Endothelial Growth Factor (VEGF), is a signal protein produced by cells that is involved in vasculogenesis during embryonic development or in case of injury and also in angiogenesis, ie. formation of new blood vessels from pre-existing vasculature. It is a sub-family of growth factors, of which the most important member is VEGF-A. It is also called the 'founding member' of a family of homodimeric disulfide-bound glycoproteins. Other members are Placenta Growth Factor (PGF), VEGF-B, VEGF-C, VEGF-D. Before the discovery of other isoforms, VEGF-A referred to as VEGF. All the members of the VEGF family bind to tyrosine kinase receptors (VEGFRs) on the cell surface causing them to dimerize and be activated through transphosphorylation. There are four subtypes of VEGF receptors (VEGFRs): VEGFR-1, VEGFR-2, VEGFR-3 and the neuropilin receptor. VEGF-A binds to VEGFR-1 (Flt1) and VEGFR-2 (KDR/Flk-1). They also bind to non-tyrosine kinase receptors. Apart from being an angiogenic factor VEGF has been shown to have neurogenic effects. The major VEGFR involved in angiogenesis and neuroprotection is VEGFR-2. It has been considered to have multifunctional role in neurodevelopment and neurological disorders. It can directly effect neurons and modulate various neurodevelopmental processes like neuronal survival, neuronal migration, axonal growth, and axonal guidance. The roles of the different members of the sub-family in brain development are summarized in Table 1.

Table 1: VEGF ligands and their functions in the nervous system

Ligand	Function
VEGF	Neurogenesis, neuronal survival and proliferation, neuronal migration, axon growth and guidance, glia survival, glia migration, oligodendrocyte migration
VEGF-B	Neurogenesis, neuronal survival
VEGF-C	Neurogenesis, oligodendrocyte precursor proliferation
VEGF-D	Dendrite arborization
PGF-2	Neurite outgrowth

Table adapted from P. Carmeliet, C. R. Almodovar, Cellular and Molecular Life Sciences, 2013.

VEGF and Neurogenesis:

It is well known that neurogenesis in the adults takes place in two distinct regions, the SGZ (Sub-granular Zone) of the hippocampus and the SVZ (Sub-ventricular zone), where the NSCs (Neural stem cells) grow in close apposition to growing capillaries. VEGF thus exerts direct or indirect effect on the NSCs. There have been numerous studies showing that VEGF induces neurogenesis in adults. Testosterone induces the up-regulation of VEGF, which in turn stimulates angiogenesis in the vocal cords of the adult song birds (HVC, Higher Vocal Center). In response to VEGF, the endothelial cells secrete brain-derived neurotrophic factor (BDNF) that causes neural cell proliferation (Carmeliet and Almodovar; Louissaint Jr, Rao et al. 2002). Expression of VEGF in the hippocampus is increased by enriched environment and spatial learning acting through KDR/Flk1 (VEGFR-2). Viral inhibition of VEGF, completely blocks environment induced neurogenesis (Cao Lei 2004). The close association of the vasculature with the neuroprogenitor cells in the SGZ of the hippocampus has been well described (Palmer, Willhoite et al. 2000). VEGF has been defined to be a potential target of antidepressants (Warner-Schmidt and Duman

2008). Multiple classes of antidepressants induce VEGF, which triggers VEGF-Flk1 signaling thereby boosting cell proliferation and producing the necessary therapeutic effects (Warner-Schmidt and Duman 2007). Dentate Gyrus (DG) specific knockdown of the VEGF at a time period of cAMP activation have shown that expression of VEGF is essential during the time window of cAMP-CREB signaling for the action of the antidepressants (Lee Jeong-Sik 2009).

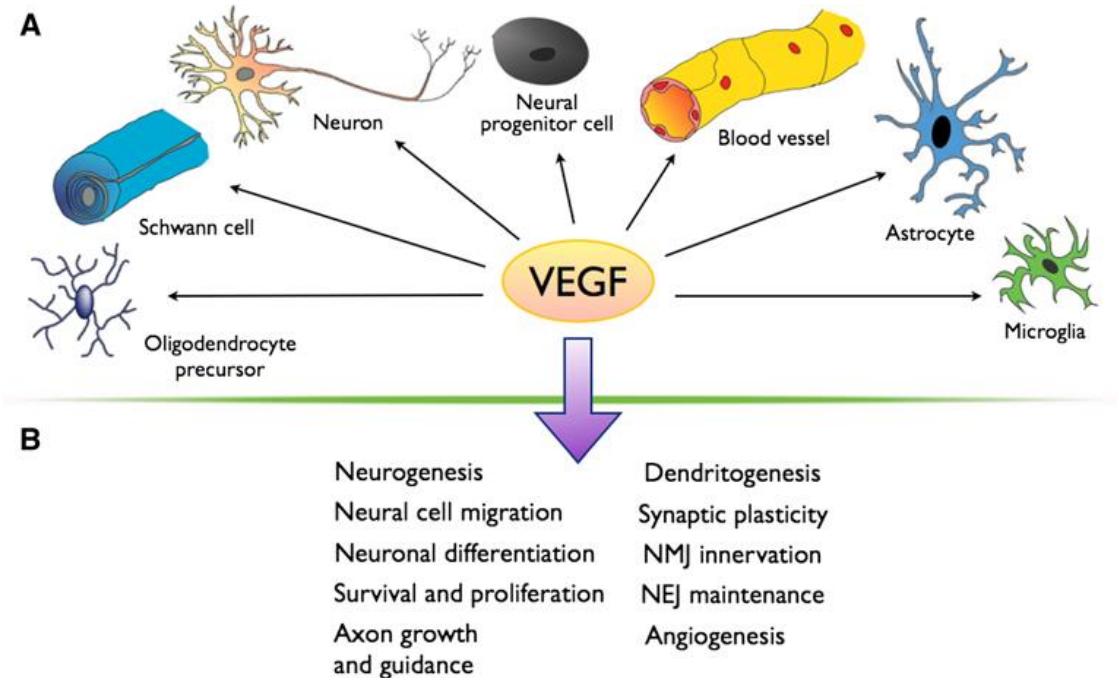


Figure 4-1: VEGF and its effects on different types of cell.

P. Carmeliet, C. R. Almodovar, Cellular and Molecular Life Sciences, 2013.

VEGF and Serotonin 1A Receptor:

Several studies has elucidated that VEGF plays a very important role in the action of antidepressants. One such antidepressant that is widely prescribed is Fluoxetine (Prozac), which is actually a Selective Serotonin Reuptake Inhibitor (SSRI). Pharmacological inhibition of VEGF receptor signaling is sufficient to block behavioral actions of Fluoxetine (Greene 2009). This

finding was crucial since it was suggestive of an involvement of the Serotonin 1A Receptor (5-HT_{1A}-R) in the effects of VEGF in depression. Activation of 5-HT_{1A}-R induces VEGF and blocking of this receptor inhibits VEGF expression as well as behavioral effects of Fluoxetine, indicating that 5-HT_{1A}-R located on the neurons might be important for VEGF mediated antidepressant action of Fluoxetine (Greene 2009). Blocking the VEGFR-2 with the antagonist SU5416 has indicated that VEGF is involved in neurogenesis after traumatic brain injury, and that this process involves the Raf/MEK/ERK cascade (Lu Kwok-Tung 2011).

All these studies have illustrated the crucial role of VEGF in neuroproliferation and neurogenesis. It has also shown VEGFR-2 (Flk1/KDR) as the receptor involved in neurodevelopment and that the Raf/MEK/ERK cascade mediates this developmental outcome. Moreover the link between VEGF and 5-HT_{1A}-R has also been elucidated especially with the studies involving various antidepressants, which are mostly SSRIs. Taking into account all these studies, it can be said beyond any doubt that VEGF plays an important role in neurodevelopment. Nevertheless, all these studies have been done with adult brains, and nothing much is known about the role of VEGF during the neonatal stages. The current study attempts to understand the importance of VEGF in 5-HT_{1A}-Receptor mediated neonatal hippocampal development.

Materials and Method:

Animals: As discussed in detail in Chapter 2.

Materials:

The VEGF antibody was obtained from Millipore (Temecula, CA, USA). The remaining details have been included in Chapter 2. Mice were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee.

Intra-hippocampal drug injection:

VEGFR1/2 antagonist (a potent and selective inhibitor of Flk1/KDR) Semaxanib, SU5416/S8442 (final conc: 10 μM). The remaining details have been included in Chapter 2.

Processing of the brains:

For neuroblast proliferation and VEGF expression studies, following injection, the mouse pups were allowed to recover from anaesthesia and returned to their mother. After 24 hours the pups were perfused with 4% paraformaldehyde, and the brains isolated. After at least 24 hours the brains were suspended in 30% sucrose solution, cryosectioned making coronal sections 30-μm thick, and then subjected to immunohistochemical staining.

Drug Concentrations:

Drug concentrations were made for the observed hippocampal volume of 5 μl of a P6 hippocampus. Drugs were administered in 0.5 μl PBS and sham (0.1M PBS).

SU5416: 200 μM stock solution in DMSO was diluted with PBS (total volume 20 μl) and from there 0.5 μl was injected to achieve a final concentration of 10 μM (Mologni, Sala et al. 2006) in the hippocampus. Appropriate carrier (PBS plus 0.5 % DMSO) was used as control. Further details have been described in Chapter 2.

Cryosectioning and Immunostaining of slices: As described in Chapter 2. The VEGF antibody (Millipore, Temecula, CA) was used at a 1:300 dilution.

Confocal Microscopy of Immunostained slices and Quantification:

For neuroblast proliferation experiment (DPAT-evoked in absence and presence of SU5416), we looked for BrdU (proliferation marker) and Doublecortin (DCX) (neuroblast marker) double labeled cells. For VEGF expression studies, the intensity and distribution (in the dentate gyrus) of VEGF and DCX was measured. At least 3 slices were imaged and analyzed for each treatment and at least 3 such sets of experiments were performed for proper statistical analysis. Sequential single images with all required wavelengths were taken 6-7 per slice and also z-stacks were acquired. Volume rendered images were created from the Z-stacks, which were used for analysis. 3D- animation videos were created to exactly view the positions of the cells proliferating and also the mature neurons.

Statistical Analysis:

BrdU and DCX double labeled cells were counted with ImageJ point selection tool, and then plotted in GraphPad Prism for bar graph construction and statistical analysis. Paired T-test revealed a significant reduction of the proliferating neuroblasts in the presence of SU5416. The intensity of VEGF and DCX was measured by ImageJ analysis tool and then the values plotted as percent carrier. Anova was performed to determine the statistical significance.

Results:

VEGF has been previously shown to have not just angiogenic properties but also neurogenic effects on neurons. We wanted to find out whether this effect was present even in the neonatal stages and whether VEGF was indeed one of the key signaling molecules in the 5-HT_{1A}-R mediated process of neuroproliferation.

In vivo stimulation of the 5-HT1A-R in the hippocampus in the P6 mouse causes induction of VEGF and DCX in the dentate gyrus:

To study if 5-HT_{1A}-R signaling induces VEGF in the Sub-granular Zone of the P6 hippocampus, we stimulated the receptor with 8-OH-DPAT in the absence and presence of 5-HT_{1A}-R antagonist WAY and the PKC epsilon inhibitor, Myr-εV1-2. After 24 hours, we checked for the expression of VEGF and the neuroblast marker Doublecortin (DCX) in the Sub-Granular Zone (SGZ) of the dentate gyrus. Double staining with the neuroblast marker (DCX) (green), and VEGF (blue) clearly shows the expression of the signal protein in the SGZ. The 8-OH-DPAT-evoked induction of VEGF was blocked in the presence of Myr-εV1-2 ($p< 0.0001$). Additionally, since VEGF induction was blocked in the presence of PKC epsilon inhibitor, VEGF was located downstream of PKC epsilon in this 5-HT_{1A}-R-mediated signaling cascade.

In vivo stimulation of the 5-HT_{1A}-R in the hippocampus in the P6 mouse causes induction of VEGF and DCX in the dentate gyrus:

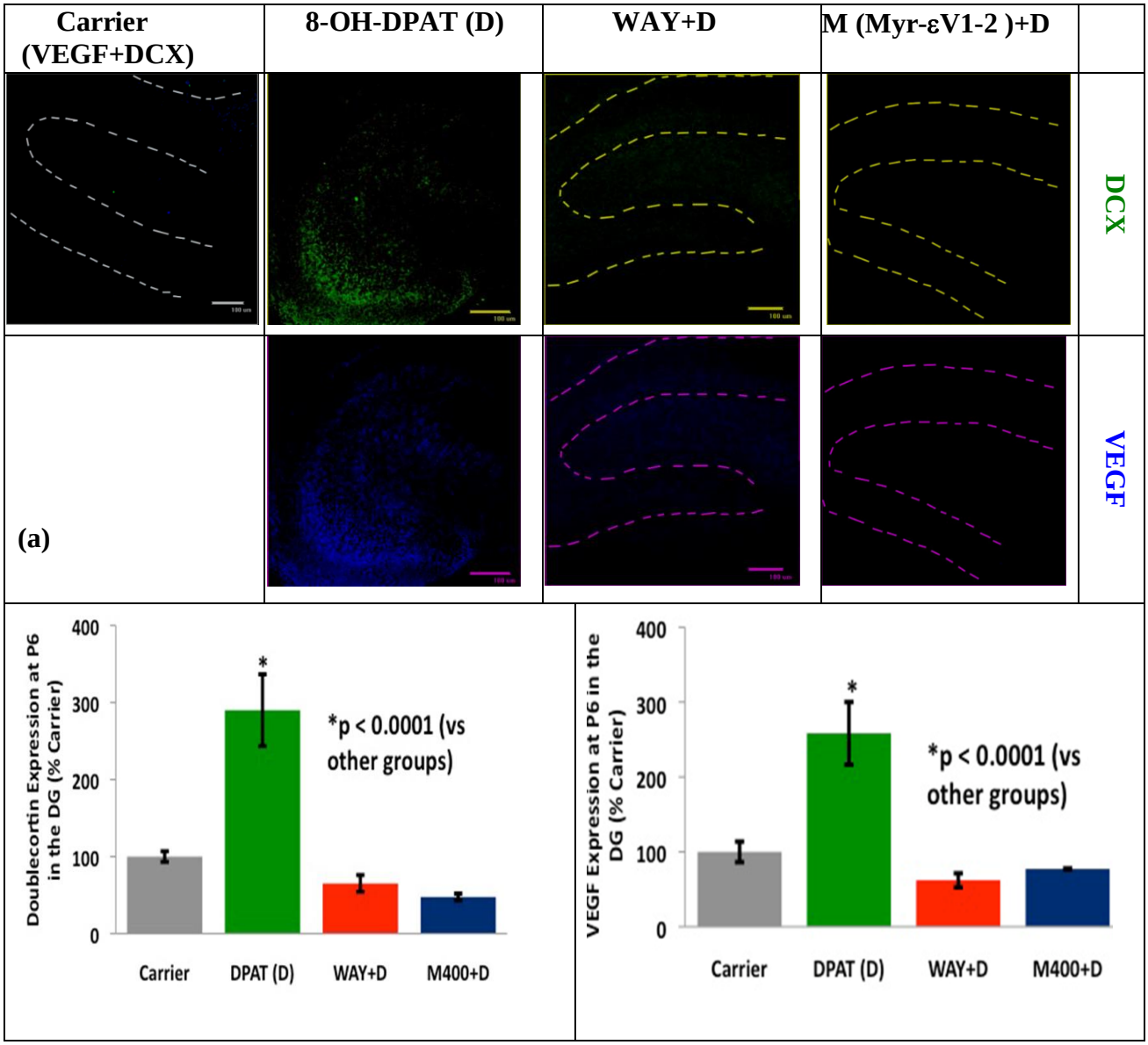


Figure 4-2: Serotonin 1A Receptor mediated induction of VEGF.

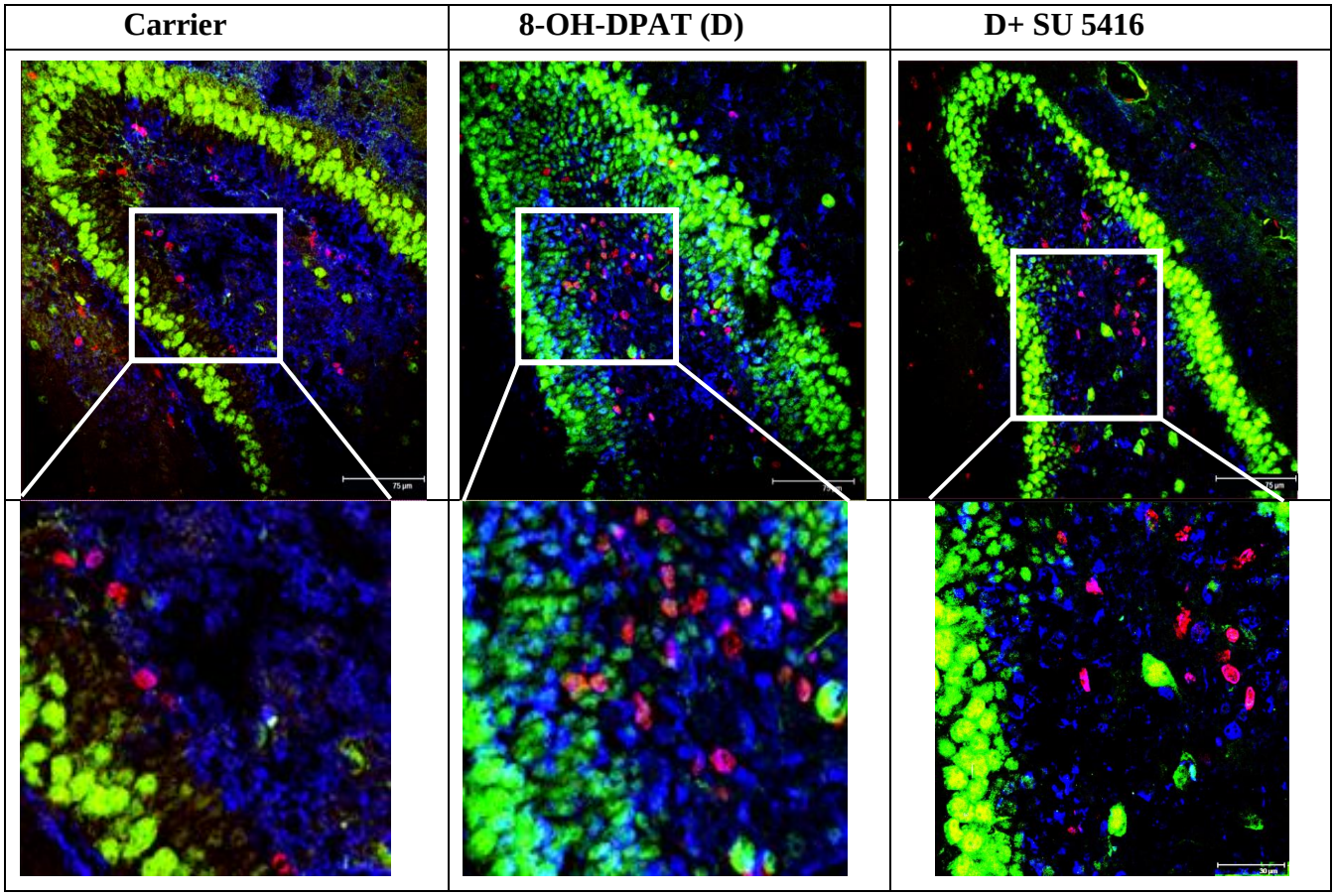
(a) Expression of VEGF in the Sub-granular Zone of the Dentate Gyrus in P7 mouse pup after 24-h stimulation with 8-OH-DPAT (D) n the absence and presence of antagonist WAY, and

PKC epsilon inhibitor Myr-εV1-2 (M). The upper panel shows the staining with DCX, and the lower panel with VEGF in all the treatment groups except for the Carrier for which a merged image has been shown with both the colors. The contour in the images are made to locate the dentate gyrus especially in case of very low expression in WAY-treated and Myr-εV1-2-treated brains. (b) Quantification and statistical analysis for both DCX and VEGF at P7.

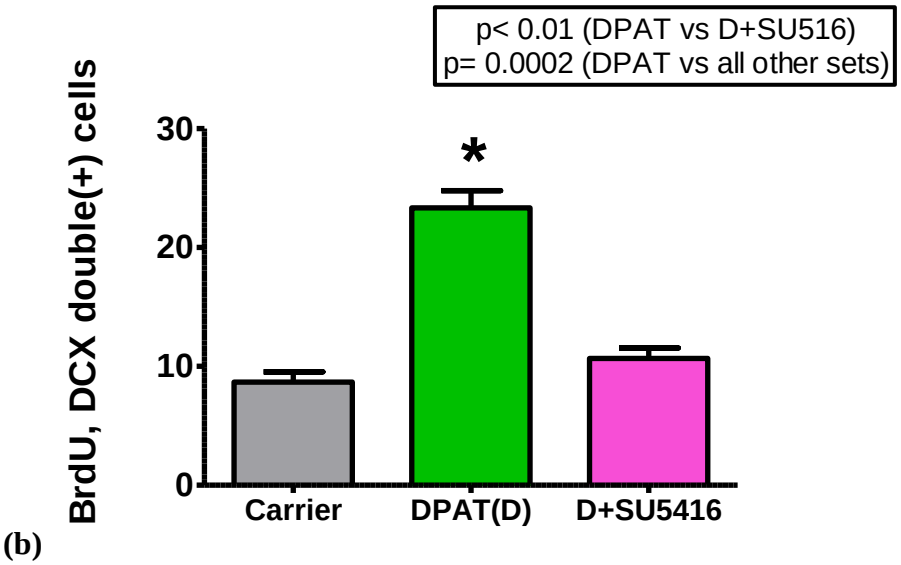
VEGFR1/2 antagonist SU5416, blocks 5-HT1A-R mediated increase in neuroblast proliferation in P6 mouse hippocampus:

Studies in adult mice have illustrated that VEGF stimulates neurogenesis and the VEGFR2/ Flk1 is the receptor that is involved in the neurogenic effect of VEGF. We wanted to confirm whether the same Flk1 receptor was also involved in the 5-HT_{1A}-R-mediated neuroproliferation in the SGZ during early brain development at P6. We stimulated the receptor with 8-OH-DPAT in the absence and presence of VEGFR 1/2 antagonist SU5416 and analyzed the number of proliferating neuroblasts. The 8-OH-DPAT-evoked increase BrdU (red) and DCX (blue) double positive (purple) cells in the SGZ was blocked in the presence of D+SU5416 ($p<0.01$). This result not only shows that VEGFR 1/2 is involved in neuroproliferation in the developmental stages but also tells us that the 5-HT_{1A}-R-mediated neuroproliferative effect at P6 could be further amplified by VEGF signaling.

VEGFR1/2 antagonist SU5416, blocks 5-HT_{1A}-R mediated increase in neuroblast proliferation in P6 mouse hippocampus



(a)



(b)

Figure 4-3: A VEGFR1/2 antagonist blocks 5-HT1A-R-mediated increase in neuroblast proliferation.

(a) Analysis of the number of neuroprogenitor cells proliferating in a P6 mouse hippocampus, Sub-granular Zone after stimulation of the 5-HT_{1A}-R, in the absence and presence of VEGFR1/2 antagonist, SU5416. The 8-OH-DPAT (D)-evoked increase in number of BrdU (red) and DCX (blue), double positive (purple) cells was blocked in the presence of SU5416 ($p<0.01$). The top panel consists of representative images from the different treatment groups with relevant areas magnified to show the distribution of the double positive cells in the bottom panel. (b) The graph represents the statistics of the number of double positive cells across the different treatment groups.

As discussed previously, apart from being vasculogenic and angiogenic, VEGF is also neurogenic and neuroprotective. But all these studies had been performed in adult animals and the role of this signal protein in shaping the central nervous system during the developmental stages was not well understood. Our studies strongly suggest a crucial role of VEGF in the development of the brain during the early postnatal stages. Increased expression of VEGF in response to stimulation of the Serotonin 1A Receptor with 8-OH-DPAT, in the SGZ indicates that VEGF might have an important role to play in the dentate gyrus. Moreover, blockade of this induction by the PKC epsilon inhibitor, shows that the release of this neurogenic factor is controlled by the receptor via the epsilon isoform of the kinase. Co-localization with DCX, which labels neuroprogenitor cells throws light to its close association with these future neurons and to the fact that it might be neurotrophic in these cells.

Blocking of the Flk1/KDR receptor, with the selective and potent antagonist SU5416, in the presence of the 5-HT_{1A}-R agonist 8-OH-DPAT, further confirms the involvement of VEGF in 5-HT_{1A}-R-mediated neuroblast proliferation. Moreover, the reduced neuroblast proliferation reinstates the importance of the VEGFR1/2 in shaping the central nervous system, with VEGF-A as the principal subtype of the protein that is regulating neuroproliferation in early stages of hippocampal development.

VEGF seems to play a more pleiotropic role in developing the central nervous system. Application of VEGF causes a dose-dependant increase in the neuronal microtubule markers TUJ1 and MAP2 and neuronal cell body diameter in primary cortical neurons (Rosenstein, Mani et al. 2003). VEGF acts on the neuronal microtubular content which is involved in growth, stability and maturation of the neuronal processes by the Flk1 receptor, since aberrance in the receptor causes inhibited neurite growth and also the number of emerging dendrites (Rosenstein,

Mani et al. 2003). A more convincing evidence of the role of VEGF in synapse formation comes from the studies with soluble VEGF receptor protein sequestered VEGF-A and decreased the number of dendritic spines in newly born granule cells in the olfactory bulb (Licht, Eavri et al.), although it increases the spine density in mature granule cells of the same region, indicating to a more regulatory role of VEGF, whereby it makes and breaks connections at different stages of development according to the requirements (Licht, Eavri et al.).

VEGF also modulates synaptic plasticity (Tillo Miguel 2012). Overexpression of VEGF increased Long Term Potentiation (LTP) and blockade of endogenous VEGF completely eliminated LTP (Licht, Goshen et al.). VEGF released from the neuronal cells had prominent effects on the activation calcium/calmodulin protein kinase II and cAMP responsive element binding protein as well as on ERK, all in a VEGFR2 dependent manner (Kim, Choi et al. 2008).

5. Chapter 5

Discussion

The hippocampus with its intra and inter connections forms the basis of understanding of mood disorders. Our studies have once again pointed out the relevance of serotonergic signaling in brain development. Previous studies have already illustrated the role of the 5-HT_{1A}-R heteroreceptors in the development of anxiety in adulthood and have provided evidence for a 'critical time window' when the receptor signaling is most essential (Gross 2002). Our studies have shown that indeed the early postnatal week is crucial for neuroblast proliferation. Antidepressants which are selective serotonin reuptake inhibitors (SSRIs) have been shown to exert their effects accompanied by elevated neurogenesis (Santarelli 2003). Our studies have shown that stimulation of the 5-HT_{1A}-R by a pharmacological agent like 8-OH-DPAT, or by a frequently used antidepressant Fluoxetine, causes elevated neurogenesis confirming the role of the receptor in early postnatal development.

Earlier studies have shown elevated serotonin levels in the females when compared to the males (Arango, Underwood et al. 1995; Boldrini, Underwood et al. 2008). This might mean existence of some aberrancy in the female hippocampal development. Our studies explicitly point out this difference in development as observed by the markedly reduced neuroproliferation and also the excessively extended mossy fiber arborization (at P18) in the *stratum oriens* in female 5-HT_{1A}-R deficient mice (KO) mouse when compared to WT controls. These morphological differences in the hippocampus were corrected to almost WT levels when the downstream molecule PKC epsilon was selectively activated with DCP-LA, once again confirming the involvement of PKC epsilon in 5-HT_{1A}-R mediated early hippocampal

development from our previous studies (Adayev 1999; Mehta 2007; Purkayastha, Fernando et al. 2009).

As previously mentioned, studies with depressed patients and suicide victims have shown that the incidence of anxiety-related disorders and post-traumatic stress disorder (PTSD) is higher in females than in males (Arango, Underwood et al. 1995; Victoria Arango 2001; Boldrini, Underwood et al. 2008). This sex-dependant anxiety-related behavior can be partially explained in the light of the differential hormonal control in males and females. The three primary types of the female sex hormone are estrone, estriol and 17β estradiol. Apart from ovary, 17β estradiol is also synthesized in the brain. Estradiol have been shown to exert its actions on the hippocampus. Estrogen receptors (ERα and ERβ) are located in diverse areas within the hippocampus like dentate gyrus, CA3 and even along the mossy fibers. 17β estradiol enhances mossy fiber sprouting *via* the action of BDNF. It has been suggested that 17β estradiol inhibits GABAergic interneurons, causing disinhibition of the principal excitatory cells in the hippocampus. Circulating androgens in the adult male rats suppress mossy fiber plasticity by reducing BDNF protein levels (Scharfman and MacLusky 2013).

The CA3 region axon collaterals undergo simultaneous repetitive firing, as a result of which they are autoassociative, following Hebb's cell assembly theory 'Cells that fire together, wire together'. They have been shown to be involved in pattern completion. Encoding and retrieval are the two most important components of this process. Thus if these cells are presented with only a partial previous pattern, they are able to retrieve the complete pattern (Kessner 2007). Encoding requires synaptic plasticity and the mossy fibers increases the pyramidal cell depolarization at the same time with the CA3 pyramidal neurons to increase the long-term memory. In the case of females, the increased mossy fiber sprouting may cause greater retrieval

of memory, which can be related to pattern completion sometimes of certain stressful or fearful memories, causing hyperexcitability leading to anxiety. In males, the reduced mossy fiber sprouting, and consequently lower excitability might lead to lesser anxiety-related disorders (Scharfman and MacLusky 2013).

Traditionally, mossy fiber pathway was thought to be a part of the tri-synaptic circuit of the hippocampus, in which the axonal projects of the granule cells of the dentate gyrus form excitatory connections with the pyramidal neurons of the CA3 region. However, this pathway is far more complex than what it was perceived to be initially. The mossy fibers on its way towards the CA3 pyramidal cells gives rise to *en passant* swellings along the axon in the *stratum lucidum* which travel parallel to the CA3 layer. These swellings gave rise to the giant mossy fiber boutons. These boutons contain clear vesicles containing the neurotransmitter glutamate. They have also been shown to be positive for GAD 67 (Glutamic acid decarboxylase) and GABA. Each of these giant boutons synapses with the proximal dendrites of the CA3 pyramidal neurons called thorny excrescences. These giant mossy fiber boutons also have filamentous extensions called filopodia which synapse in the inhibitory interneurons. Acśady and coworkers showed that the number of synapses that the filopodial extensions make with the CA3 interneurons is about 4-5 times higher than the number of synapses that the giant boutons make with CA3 pyramidal neurons thorny excrescences. Each of these interneurons connects to several hundred principal target CA3 neurons. The net effect is a feed forward inhibition of the CA3 pyramidal neurons. Additionally, the mossy fibers branches into several collaterals before entering the CA3 region, where it synapses with the cells of the hilus. The collaterals make excitatory connections with the mossy cells of the hilus and the basket cells (inhibitory), which in turn connect with other granule cells ultimately inhibiting them. Thus the net effect of the mossy fiber connection on the

pyramidal cells is more inhibitory than excitatory (L. Acsady 1998). Interestingly, at the CA3 interneuron – CA3 pyramidal cell synapse, the probability of neurotransmitter release is of a higher magnitude than at the mossy fiber – CA3 pyramidal cell synapse. Furthermore, the quantal amplitude of release is also about three fold larger at the synapses of the interneuron with CA3 neurons than at the MF synapses with the CA3 cells (C. E. Ribak 1983; D. A. Henze 2000; D. A.. Henze 2000; McBain 2003; McBain 2006). Excitation of both inhibitory interneurons and the excitatory target cells creates a self-regulatory mechanism where weak excitatory signals are eliminated and only the strong inputs are transmitted to specific target cells (Michael Frotscher 2006). However, the immature granule cells are not susceptible to GABAergic inhibition, and causes excitatory effects on the CA3 pyramidal neurons. Our findings of marked reduction of the neuroblast proliferation in the female KO mice could therefore lead to a net decrease in the feed forward inhibition in the mossy fiber pathway, thereby causing a net excitation in CA3 and a consequent increase in activity at the mossy fiber synaptic connections with CA3 neurons (Fig. 5.1). This lack of inhibition and auto-regulation is probably responsible for the observed increase in the Timm-stained collaterals in the *stratum oriens* of the CA3 region. DCP-LA prunes the unnecessary excessive connections and restores normal levels of synapses. Stunted neuroproliferation in the female (KO) mice with no significant difference in neurogenesis in adulthood, might be due to aberrant hippocampal development and unfocused synaptic connections. This increased synaptic connectivity leads to hyper-excitation in the female KO mice causing anxiety-like phenotype. Nevertheless, our studies did not show any marked difference in the level of neurogenesis in the KO mice when compared to controls. This might be due to compensatory effects of the catecholaminergic system that develops right after the serotonergic system and might augment neurogenesis in KO mice. These findings fit very well

with the fact that there is no difference in the size of the hippocampus between the KO and wild type mice. However, in spite of compensatory effects of the catecholaminergic system in restoring neurogenesis, the actual aberrancies lie in the hippocampal connections that are formed during those early formative days (critical postnatal time window) which shape the neuropsychiatric makeup for later-life behavioral anomalies.

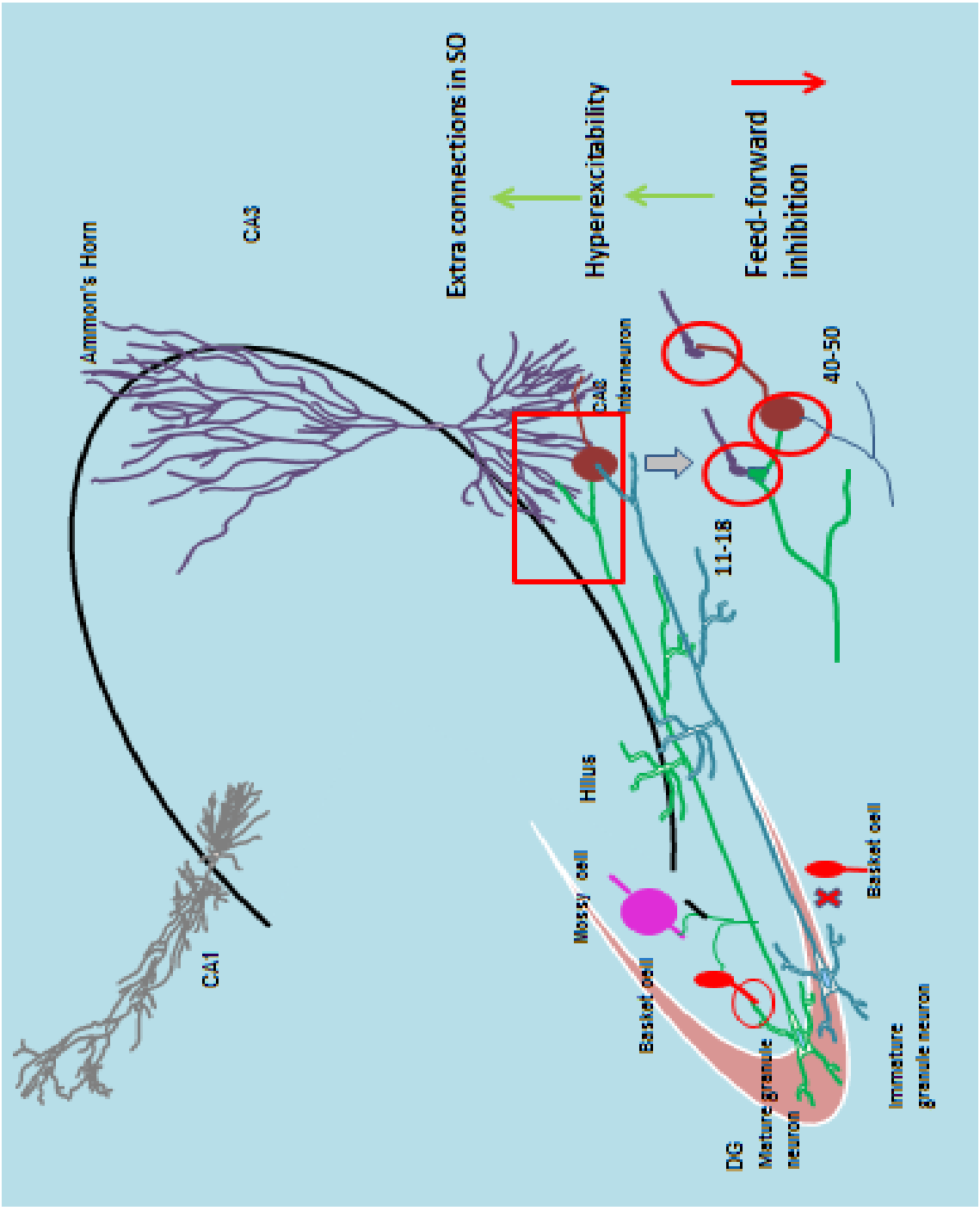


Figure 5-1: Diagrammatic representation of the mossy fiber connectivity in mature and immature granule neurons.

Inhibitory basket cells in the hilus inhibit some of the granule cells in the dentate gyrus. The ones that are not inhibited by these basket cells, forms synaptic connections with CA3 pyramidal cells and CA3 interneurons. CA3 interneurons in turn inhibit CA3 pyramidal neurons. The number of mossy fiber-CA3 interneuron synapse is four to five hold higher than number of mossy fiber-CA3 pyramidal cell synapse. The CA3 interneurons in turn inhibit the CA3 pyramidal cells. Moreover the quantal release of neurotransmitter is also higher in the CA3 interneuron-CA3 pyramidal cell synapse than the mossy fiber-CA3 pyramidal cell synapse causing an overall feed-forward inhibition. Conversely newly formed granule cells are not inhibited by the hilar basket cells though they synapse similarly like mature granule cells with the CA3 pyramidal cells and the CA3 interneurons adding to the net inhibitory effect. In the absence of the 5-HT_{1A}-R, a sharp decrease of newly formed granule cells decreases the overall feed-forward inhibition, leading to over-excitation and ultimately formation of extra mossy fiber arborization and extra connections in the *Stratum oriens*

Several studies throw light on the multifaceted role of VEGF in shaping the central nervous system (see Chapter 4). In the context of our studies illustrating the role of VEGF signaling *via* the VEGFR1/2 in neuroproliferation, together with the understanding of development of the mossy fiber connections, it can be argued that VEGF, one of the downstream targets of the serotonin 1A receptor cascade, is not only neurotrophic, but also plays a key role in maintaining these connections, by acting on the microtubular proteins, increasing and decreasing spine density allowing an intrinsic regulatory mechanism to control hippocampal development neuropsychiatric development (Fig. 5.2).

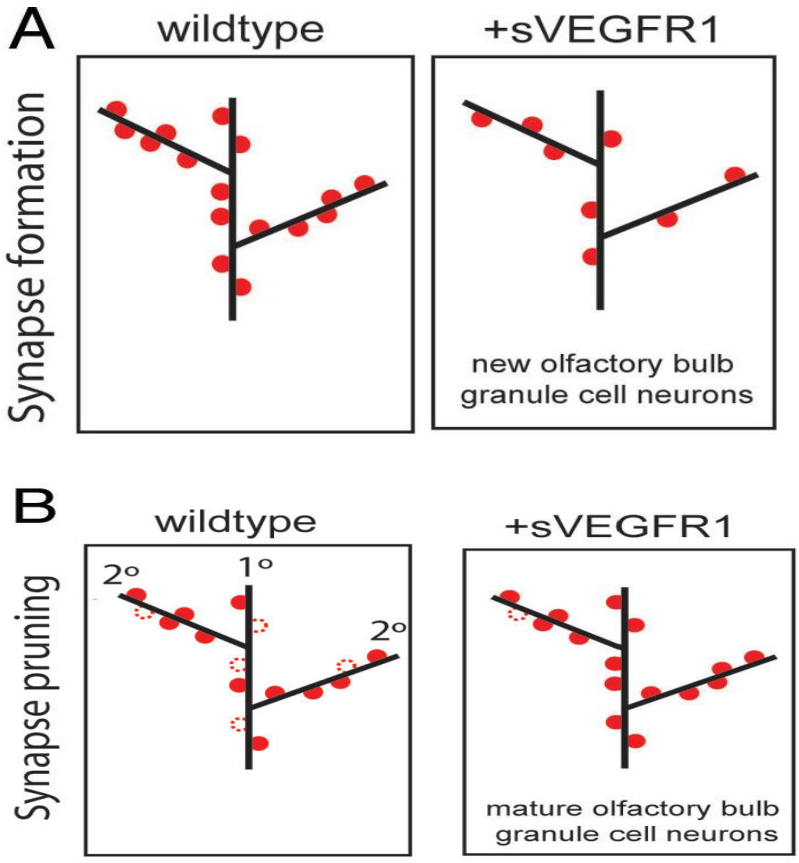


Figure 5-2: Role of VEGF in synapse formation.

Adapted from Miguel Tillo, Christiana Ruhrberg and Francesca Mackenzi, 2012, Cell Adhesion and Migration (Tillo Miguel 2012), (Licht, Eavri et al.).

VEGF signaling leading to increased neuroproliferation can take place through two different mechanisms. VEGF can activate ERK, amplifying the signal in a cell-autonomous fashion leading to increased cell proliferation. The other possibility is that VEGF released from a mature granule cell binds to a neuroblast expressing the VEGF receptor resulting in the division of the neuroblast where VEGF acts as a neurogenic factor in an indirect fashion.

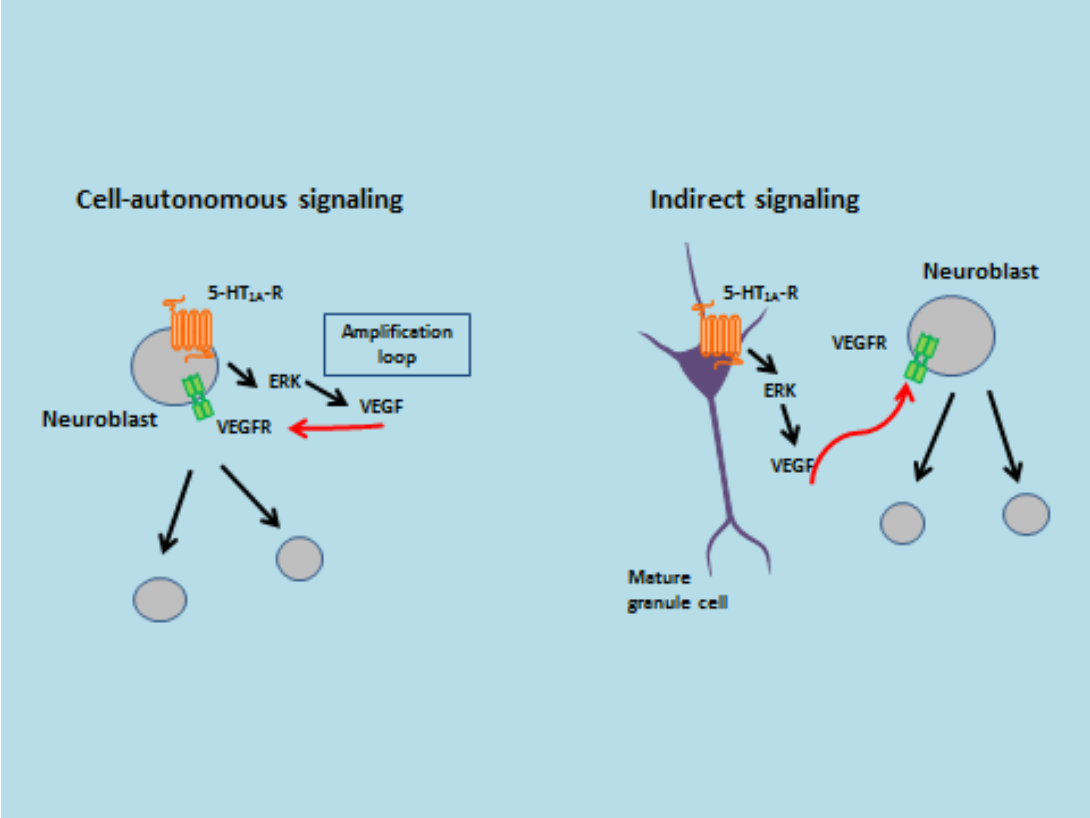


Figure 5-3: Role of VEGF in cell-autonomous and indirect signaling.

ERK after phosphorylation activates both cytoplasmic as well as nuclear targets. Nuclear translocation of ERK1 and ERK2 is critical for gene expression. ERK is known to activate targets like RSK-related kinases, Mitogen- and Stress- activated Protein Kinases (MSKs). MSKs phosphorylate and activate the Activator Protein 1(AP-1), which is again important for CREB phosphorylation. The human VEGF gene promoter sequence revealed several consensus transcriptional response elements (TREs) like AP-1, AP-2, GATA-6, IL-6RE and others (Mueller, Vigne et al. 2000). The existence of these TREs like AP-1, might indicate an ERK regulated VEGF gene expression which in turn might be responsible for proliferation of the neuroblasts.

We have targeted the 5-HT_{1A}-R and its downstream signaling molecules by various well-established pharmacological reagents which have been frequently used for studies reported in

many high-impact publications. The use of 5-HT_{1A}-R agonist 8-OH-DPAT (Boutrel, Monaca et al. 2002; Gross 2002; Santarelli 2003; Lo Iacono 2008), antidepressant Fluoxetine (Lemonde, Du et al. 2004; Greene 2009; Richardson-Jones 2011), 5-HT_{1A}-R antagonist WAY100635 (Gross 2002; Lo Iacono 2008; Richardson-Jones 2011), PKC epsilon inhibitor Myr-εV1-2 (Johnson, Gray et al. 1996; Chen, Hahn et al. 2001; Yamamoto, Kawamata et al. 2006) which is a myristoylated peptide εV1–2 (EAVSLKPT), specific MEK inhibitor U0126 (Crews, Alessandrini et al. 1992; Cowley, Paterson et al. 1994; Rosen, Ginty et al. 1994), PKCε selective activator DCP-LA (Tanaka and Nishizaki 2003; Yamamoto, Kanno et al. 2005; Hongpaisan, Sun et al. 2011), VEGFR1/2 antagonist SU5416 (Takamoto T 2001; Greene 2009) have been well established as selective pharmacological reagents. We have also selected reagents with are specific in their actions like replacing PD98059 with U0126, since the latter is capable of inhibiting both MEK1 and MEK2 while PD98059 inhibits MEK1 more potently than MEK2. Furthermore, we have directly implanted these reagents into the hippocampus to avoid any indirect effect through the raphé 5-HT_{1A} receptor. While a genetic knock-out is a way of specifically blocking the action of a protein it also brings about activation of other genes. Therefore, highly selective agents have been used to answer our questions. In this study, we used such pharmacological inhibitors to block the supra-basal level of proliferation and ERK activation. In future studies we will test the effect of these inhibitors on the basal level of neuroproliferation and ERK activation.

Our studies have successfully delineated a signaling pathway mediated by the serotonin 1A receptor involved in early hippocampal development and circuitry. We have also identified a potential therapeutic target, PKCε, which can be regulated to combat these mood disorders.

6. Chapter 6

Future Studies

Studies so far have established a 5-HT_{1A}-R mediated signaling pathway operating at P6 boosts neuroblast proliferation and subsequent neurogenesis. The presence of 5-HT_{1A}-R signaling is particularly important for the development of proper mossy fiber connectivity and also normal anxiety-like behavior in adulthood. There also exists a female-specific suppression of neuroblast proliferation, increase in mossy fiber connectivity, and escalation of anxiety phenotype in the 5-HT_{1A}-R(-/-) mice. We have successfully illustrated this correlation but the bridging molecules are yet to be studied.

Moreover as discussed earlier, our studies have targeted the 5-HT_{1A}-R and its signaling molecules by various well-established and widely-used pharmacological reagents. In the future, we intend to use genetic knockdown and constitutively active systems to manipulate the pathway at various stages to study its effects. Thus our future studies will consist broadly of two distinct aims.

- **Genetically manipulate 5-HT_{1A}-R and downstream target PKC epsilon both in dividing and non-dividing cells to study neuroproliferation, mossy fiber connectivity and later-life behavior.**
- **Study the interdependence of sex hormones and brain development in relation to the 5-HT_{1A}-R mediated signaling cascade to understand the mechanistic underpinnings of this sex-based effect.**

We have developed some strategies to conduct the future studies. Since it is not known whether the 5-HT_{1A}-R is expressed in the dividing neuroblasts or the non-dividing mature granule neurons (or both) in the dentate gyrus, we have designed experiments to examine only mitotic

cells (by retroviral expression) and both mitotic and post-mitotic cells (by lentiviral expression). We will suppress expression of 5-HT_{1A}-R using targeted shRNAs in P6 WT mouse hippocampus and test its effect on activation of Erk, neuroproliferation and neurogenesis. We have recently standardized this strategy using a 5-HT_{1A} receptor-specific shRNA cloned into a ViraPower™ HiPerform™ Gateway® Expression System (Invitrogen, US). This viral construct also harbors a green fluorescent protein (GFP) cassette and expression of the target protein is marked by co-expression of GFP expression in the same tissue. Expression levels of 5-HT_{1A}-R will be tested by RT-PCR analysis of hippocampal lysates as shown in Fig. 6.2b. Control mice will receive the virus without the shRNA. Knocking down of 5-HT_{1A}-R should cause a sharp drop in neuroproliferation and neurogenesis. Lentiviral transduction occurs in both proliferating as well as post-mitotic cells. Therefore, above strategy will not clarify if the neurogenic 5-HT_{1A}-R➔➔PKCε➔➔Erk1/2 pathway is functional in neuroprogenitor cells or if 5-HT_{1A}-R, expressed only by the post-mitotic cells, signals through this pathway to cause secretion of other molecules such as VEGF, which binds to VEGF receptors expressed by the neuroprogenitor cells in the subgranular zone (SGZ) of the DG to cause proliferation and neurogenesis (Greene 2009; Samaddar 2010; Samaddar 2011). To address this possibility we will express the 5-HT_{1A}-R-targeted shRNA from a retroviral system (pRevTRE from Clontech), which is selective toward dividing cells.

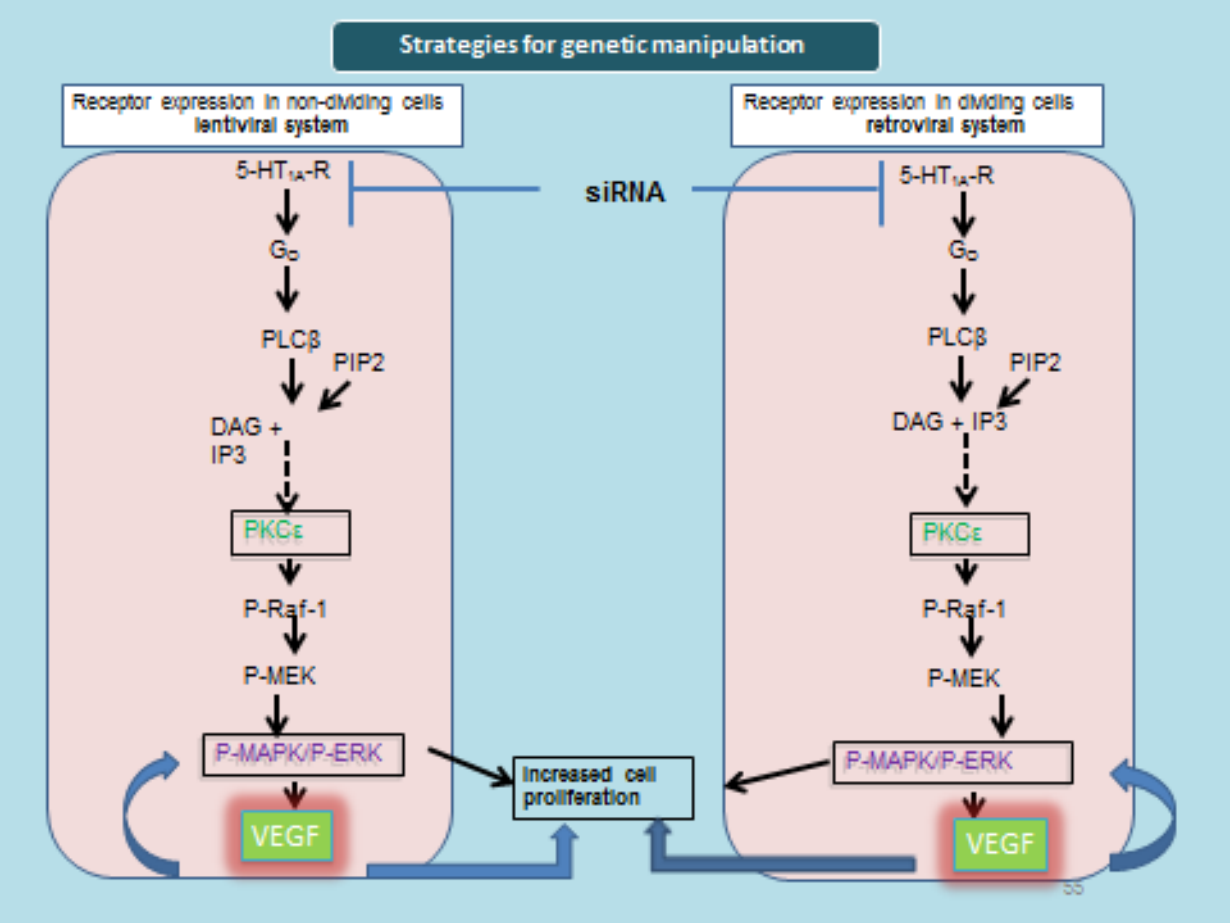


Figure 6-1: Experimental strategy for expressing 5-HT1A-R-targeted shRNA in retroviral and lentiviral system

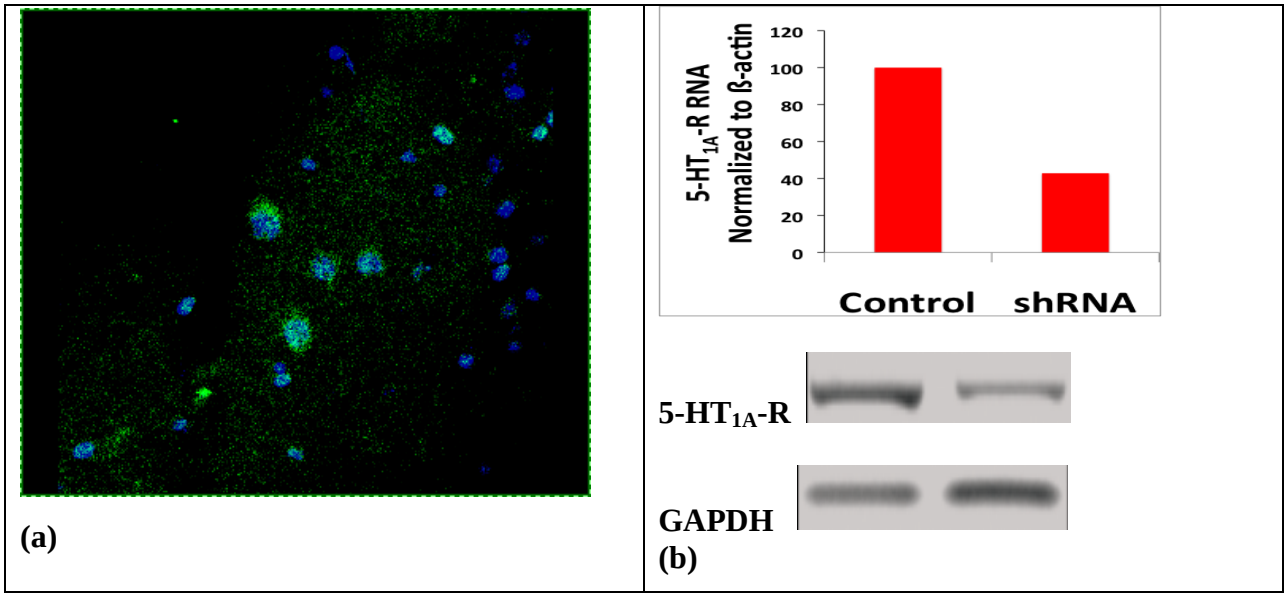
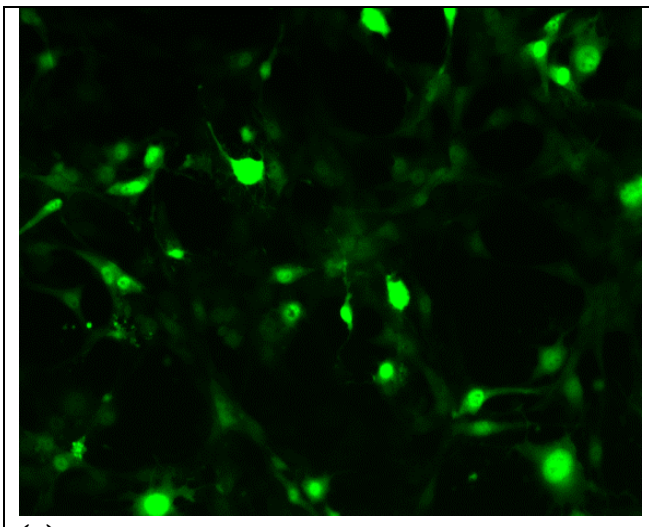


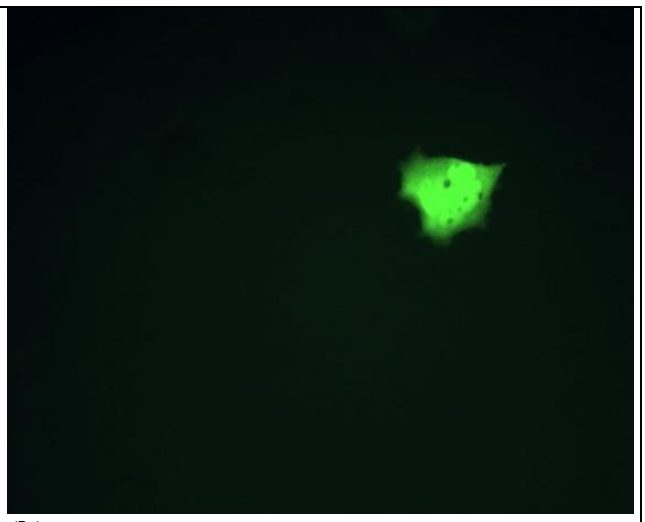
Figure 6-2: Lentivirus-mediated co-expression of GFP and 5-HT1A-R shRNA in mouse hippocampus

5-HT_{1A}-R-targeted shRNA-expressing lentivirus was infused int the hippocampus. **(a)** Confocal images obtained 24 h after intra-hippocampal infusion of lentiviral shRNA against 5-HT_{1A}-R. The nuclei were counter stained with HOECHST33342 (blue). **(b)** RT-PCR analysis using 5-HT_{1A}-R and GAPDH (internal control) primers shows a dramatic decrease in 5-HT_{1A}-R mRNA.

If lentiviral transduction blocks D-evoked proliferation but not retroviral transduction, then we would infer that the 5-HT_{1A}-R➔➔PKCε➔➔Erk1/2 pathway operates in the postmitotic hippocampal cells to generate a secreted signal (e.g. VEGF), which binds to the cognate receptors on the SGZ cells to trigger proliferation and neurogenesis. Co-expression of GFP indirectly marks shRNA expression (Fig. 6.1 a). Since the currently available 5-HT_{1A}-R antibodies are not reliable, especially at low receptor expression, this retroviral approach will assess the functional output of the 5-HT_{1A}-R signaling pathway and, thereby, overcome a major technical difficulty. A block of D-evoked neuroproliferation by retroviral transduction will indicate 5-HT_{1A}-R➔➔PKCε➔➔Erk1/2 pathway is a neuroprogenitor-autonomous signal that triggers its own division in the SGZ.



(a)



(b)

Figure 6-3: Expression of pRev-TRE-GFP. (a) **PT67 cells transfected with pRevTRE-GFP.** (b) **An HN2-5 cell infected with pRevTRE-GFP.**

The basal level of proliferation in the KO mouse DG at P6 was significantly lower than in WT, which was rescued by activating the downstream signaling molecule PKCε with a widely-used pharmacological activator DCP-LA ((Tanaka and Nishizaki 2003; Hongpaisan, Sun et al. 2011)). The existence of the signaling pathway also in the 5-HT_{1A}-R (-/-) (KO) mice will be re-demonstrated by using the same lentiviral and retroviral strategy to express constitutively active PKCε in the hippocampus of KO mice. This should boost the rest of the neurogenic pathway, thereby rescuing neuroproliferation at P6 and also connectivity and later-life behavior as observed with DCP-LA.

The experimental design involving the expression of constitutively active PKCε in both mitotic and post-mitotic cells is illustrated in the following figure.

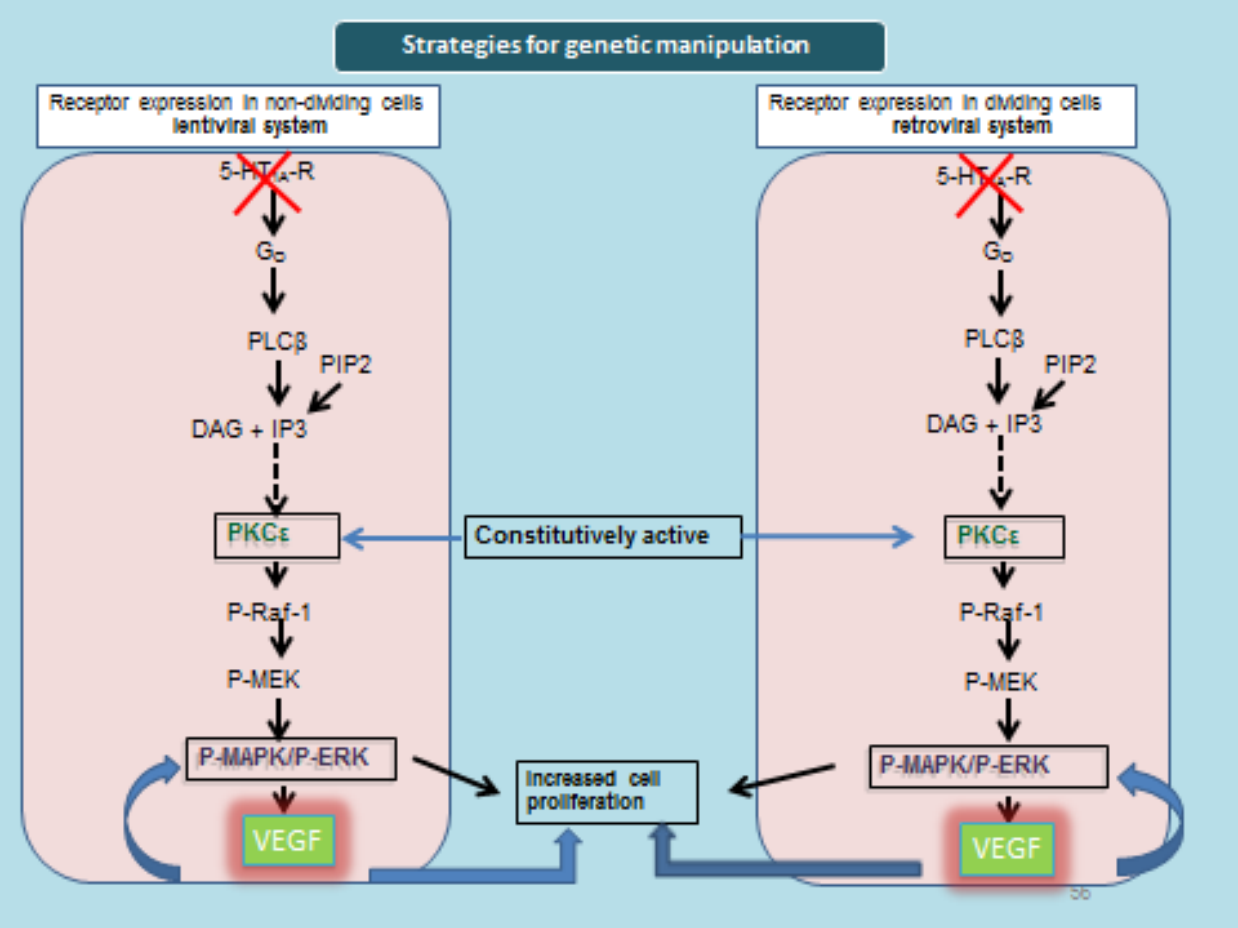


Figure 6-4: Experimental strategy for expressing constitutively active PKCε in the lentiviral and retroviral system in P6 mouse DG.

As already mentioned in the Discussion there are sex-based morphological differences existing during early post-natal brain development. Development of sex differences pertaining to reproductive organs is exclusive of brain development in males and females. The critical gene for sex differentiation is SRY on the Y-chromosome, which determines the development of the male reproductive system from the bi-potential gonad. The default is the female development pathway. Expression of the SRY gene will result in the differentiation of the Wolffian duct

system into male reproductive system and conversely, in the event of development of female reproductive system, the Wolffian duct system will degenerate and the Mullerian duct system will develop into the female reproductive system. Differentiation of the external genitalia and secondary sex characteristics is usually complete during the gestational period. Sex differentiation of the brain occurs usually just before birth and during the early postnatal weeks.

Steroids unlike peptides are synthesized on demand. The two most important sex steroid hormones are testosterone and estrogen (17β estradiol). Estrogen is derived from testosterone by the enzyme p450 aromatase, also called estradiol synthase. The Estrogen Receptors (ERs), are actually members of a superfamily of nuclear transcription factors characterized by the presence of a central DNA-binding domain that targets the receptor to a hormone responsive element (HRE). There are currently two recognized receptors for estradiol, ERα and ERβ. These ERs are highly expressed in the developing brain, especially the hippocampus (McCarthy 2008). Thus estradiol seems play a crucial role in the development of brain during the early stages. Studies with Ishikawa adenocarcinoma cells have shown that estradiol-induced gene transcription is ER dependent and is activated through an Estrogen Responsive Element (ERE) located upstream from the VEGF transcription start site (Mueller, Vigne et al. 2000). However, studies confirming estradiol-induced VEGF expression *in vivo* have not been reported yet.

There is a sensitive period during which estradiol exerts its actions on the developing brain. Though there is no significant difference between the levels of estradiol and estrogen receptors between the two sexes, there is a sex-based difference of cell proliferation in the developing hippocampus. Newborn males have higher rates of cell proliferation than females. This increased rate of cell proliferation was blocked when treated with an aromatase inhibitor or an estradiol receptor antagonist only in the males, but not in the females. Conversely, estradiol

treatment increased cell proliferation in females, but not in males (Bowers 2010). Thus especially in the case of females the cell proliferation appears to be dependent on estradiol. Thus one of the possibilities can be an induction of the aromatase activity by the 5-HT_{1A}-R signaling cascade producing elevated levels of estradiol, which in turn would cause transcription of the VEGF gene, ultimately leading to higher cell proliferation.

The other mechanism of 5-HT_{1A}-R mediated sex-based difference in hippocampal development can take place through an indirect mechanism. A seven-transmembrane domain receptor GPR30 (G-protein coupled estrogen receptor 1) which is an estrogen sensitive G-protein coupled receptor, has recently been isolated. It is responsive to 17β estradiol and is expressed in the mammalian brain, with specific subcellular localizations in the hippocampus. This receptor is yet to be characterized in detail. It is expressed in the post-synaptic terminals and has the potential to associate with other post-synaptic receptors including 5-HT_{1A}-R. Actions of this receptor has been well established in modulating spine plasticity (Akama 2013). There is a possibility that the GPCR30 binds to 5-HT_{1A}-R and then induces a signaling cascade ultimately leading to elevated cell proliferation. In the absence of this 5-HT_{1A}-R, there is no dimerization, which blocks the signaling pathway from being operative. In the event of activating any downstream molecule of the 5-HT_{1A}-R cascade, the estradiol mediated pathway comes into play either by binding to the GPR30 or by binding to the ERs present in the nucleus to transcribe the VEGF gene.

Experiments have yet to be performed to answer above questions and establish a crucial link between the 5-HT_{1A}-R and the sex hormones.

Genetic manipulation of the 5-HT_{1A}-R mediated signaling pathway and understanding the link between the 5-HT_{1A}-R pathway and estradiol receptors will provide us with a better understanding of hippocampal development and will also unravel potential target molecules to overcome several neuropsychiatric and mood disorders, which have their roots in aberrant brain development during this highly sensitive neonatal period.

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