The Role of Serotonin 1A Receptor-Mediated Signaling in PSD95 Induction and Synaptogenesis during Neonatal Hippocampal Development

By

Amit Mogha

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

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Abstract

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By

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Previous studies have shown that the 5-HT_{1A}-R is essential for neonatal front brain development. A polymorphism in 5-HT_{1A}-R gene leading to its aberrant expression has been reported to result in severe depression in humans, while its absence up to postnatal day 15 (P15) results in anxiety like behavior in mice. How this receptor functions to regulate the behavior of an animal is still poorly understood. Our prior studies have shown that 5-HT_{1A}-R mediated signaling pathway leads to stimulation of a neuro-protective MAPK➔PKCα pathway in differentiated hippocampal neuron-derived HN2-5 cells. Further studies showed that this pathway is also functional in cultured P15 hippocampal slices and that it causes an induction in field EPSP (fEPSP) in the P15 slices. To further understand the mechanistic role of 5-HT_{1A}-R in synaptogenesis we used organotypic hippocampal slice cultures from C57BL6 mice at P15 to show that the activation of 5-HT_{1A}-R mediated signaling pathway in the hippocampus leads to a massive increase in PSD95 expression and dendritic spine as well as synapse number. The results presented in our study demonstrate that 5-HT_{1A}-R mediated signaling pathway acts via sequential activation of ERK and PKC. *In vivo* studies by intrahippocampally infusing 5-HT_{1A}-R agonist and different signaling inhibitors revealed that the same pathway is involved in the induction of PSD95 and synaptogenesis *in vivo* via activation of PKCα in C57 as well as Swiss Webster mice. We further demonstrated that intra-hippocampal infusion of antidepressant drug like Fluoxetine, which is

Selective Serotonin Reuptake Inhibitor (SSRI), leads to the induction of PSD95 and synaptogenesis through the same pathway. Equipped with this knowledge, we activated the 5-HT_{1A}-R mediated signaling pathway in 5-HT_{1A}-R (-/-) mice by activating the downstream effector PKCα by intrahippocampally infusing its activator Bryostatin, which is also an Alzheimer's drug, to boost PSD95 expression and synaptogenesis. We also observed that the 5-HT_{1A}-R (-/-) mice have less number of synapses in the hippocampus. We, therefore for the first time elucidate the signaling pathway which explains how 5-HT_{1A}-R regulates hippocampal sculpting and function, which may determine the affective normalcy of an adult.

This thesis is dedicated to my loving family:

My parents for their blessings and unconditional support ,

My loving wife Jyotsana, who stood by me in every situation,

My Brothers and loving sister who encouraged me always.

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ABBREVIATIONS

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

PDK-1	Phosphoinositide-dependent kinase-1
PI-3K	Phosphoinositide *tris*- phosphate kinase
PIP2	Phosphoinositide *bis*- phosphate
PKC	Protein kinase C
PL-C β	Phospholipase C β
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphotidylserine
PSD	Post Synaptic Density
PSD95	Post-synaptic density protein of 95 kda
RIPA Buffer	Radioimmune precipitation buffer
5-HTT	5-HT transporter, SERT
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TPH	Tryptophan hydroxylase

CHAPTER 1

INTRODUCTION

Serotonin

Serotonin or 5-hydroxy tryptamine (5-HT) is a monoamine neurotransmitter. Serotonin is well known to regulate various central nervous system functions and has been implicated in a wide array of physiological, behavioral and pharmacological effects including regulation of mood, appetite, sleep, as well as muscle contraction. It is biochemically derived from the amino acid tryptophan and is primarily found in the gastrointestinal tract, platelets and in the central nervous system of mammals including humans.

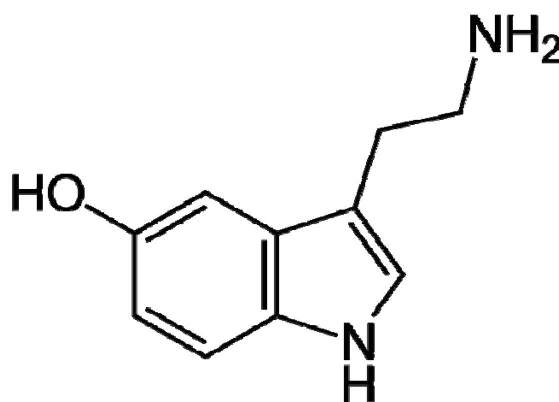


Figure 1.1 Structure of Serotonin

About 80 percent of the total serotonin is located in the enterochromaffin cells in the gut (Berger, Gray et al. 2009) . Because serotonin cannot cross the blood brain barrier, it is synthesized inside the brain itself for the neurotransmitter functions. In the central nervous

system serotonin biosynthesis takes place in the serotonergic neurons of the raphé nuclei in the brain stem.

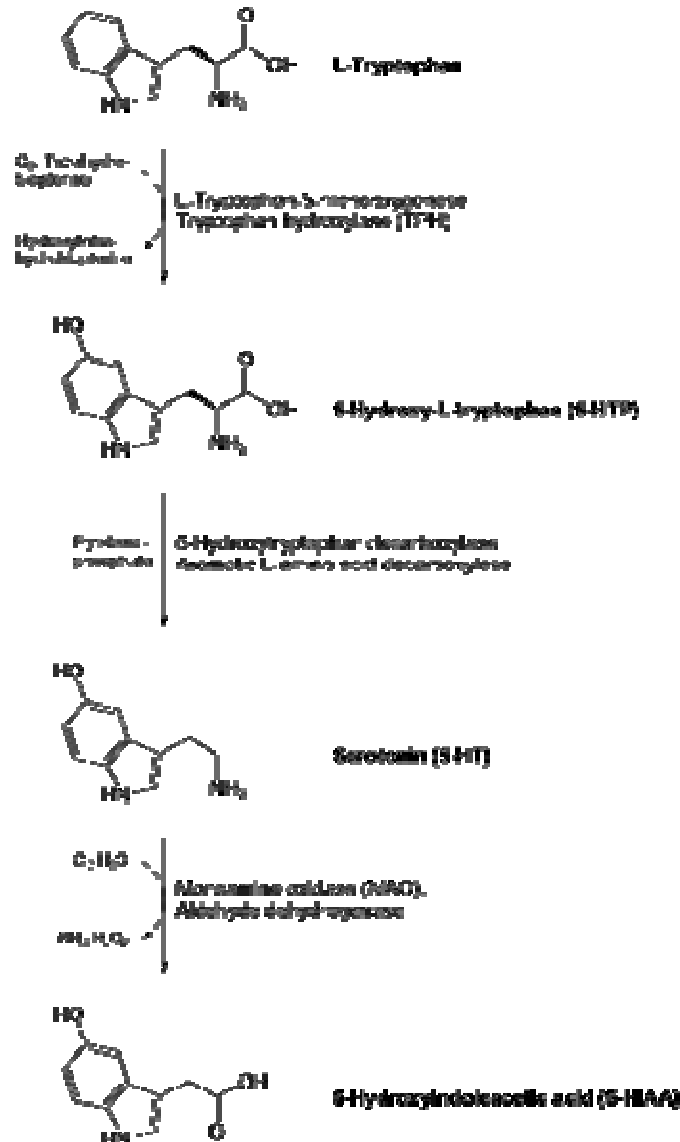


Figure 1.2 Biosynthesis of Serotonin

Due to the cell type specific expression of the enzyme tryptophan hydroxylase (TPH), which is the rate limiting enzyme in the biosynthesis of serotonin, neurons of the raphé nuclei

specifically synthesize serotonin (Wood 2001). Serotonin biosynthesis is a two-step process. In the first rate limiting step, in presence of TPH, tryptophan is converted to 5-hydroxytryptophan (5-HTP), which is further converted to 5-hydroxytryptamine (5-HT) or serotonin by the enzyme 5-HTP decarboxylase (Wood 2001). A negative feedback mechanism via activation of 5-HT_1 autoreceptors causes inhibition in the neuronal firing and suppression of the serotonin release from the serotonergic neurons, thus providing a control over the serotonin levels in the brain (Wood 2001).

Specific transporter protein (Serotonin Transporter or SERT) for the serotonin molecules is located on the serotonergic neurons at the levels of soma, dendrites and axons, providing a mode of regulation upon the concentration of serotonin at the synapse (Cour 2001). Binding of serotonin molecule to the SERT molecule terminates its synaptic effect.

Distribution of serotonin in different brain regions

Serotonergic cell bodies, which are present only in discrete group of cells in the raphé nuclei, send axonal projections to almost every area of the CNS. The serotonin system is divided into two subsystems, rostral and caudal (Fig.1.3). The rostral subdivision has the cell bodies located in the midbrain and rostral pons, which provide axonal projections to the forebrain. The caudal subdivision is primarily located in the medulla oblongata and it provides descending axonal projections to spinal cord and brain stem nuclei (Dahlstrom 1964; Fuxe 1965).

The discrete clusters of serotonergic neurons in the raphé nuclei are made up of heterogeneous populations (Jacobs and Azmitia 1992). 5-HT cells are clustered into nine groups ($B_{1–9}$) (Fig. 1.3), B_1 being the most caudal group of cells (Dahlstrom 1964). The largest group of cells is B_7 which is continuous with B_6. B_6 and B_7 are often considered together as dorsal raphé

nucleus, B_6 being the caudal extension. B_8 is considered as median raphé nucleus. B_9 is a part of ventrolateral tegmentum of pons and midbrain and forms a lateral extension of the median raphé. Serotonergic projections innervating the cerebral cortex and the other regions of the forebrain arise from dorsal raphé, median raphé and B_9 cell groups. Axons from the median raphé neurons heavily innervate hippocampus, hypothalamus and septum, whereas the axons from dorsal raphé project mainly to striatum and frontal cortex (Piñeyro and Blier 1999; Ciranna 2006). The other raphé nuclei, B_1-B_4, are situated more caudally in the midpons to caudal medulla and contain a smaller number of serotonergic cells providing axonal projections to caudal medulla and spinal cord (Ciranna 2006). The clusters B_4-B_9 are now commonly referred to as rostral group, which projects mainly to the forebrain and the cells in B_1-B_3, the caudal group, innervate the spinal cord (TÖRk 1990).

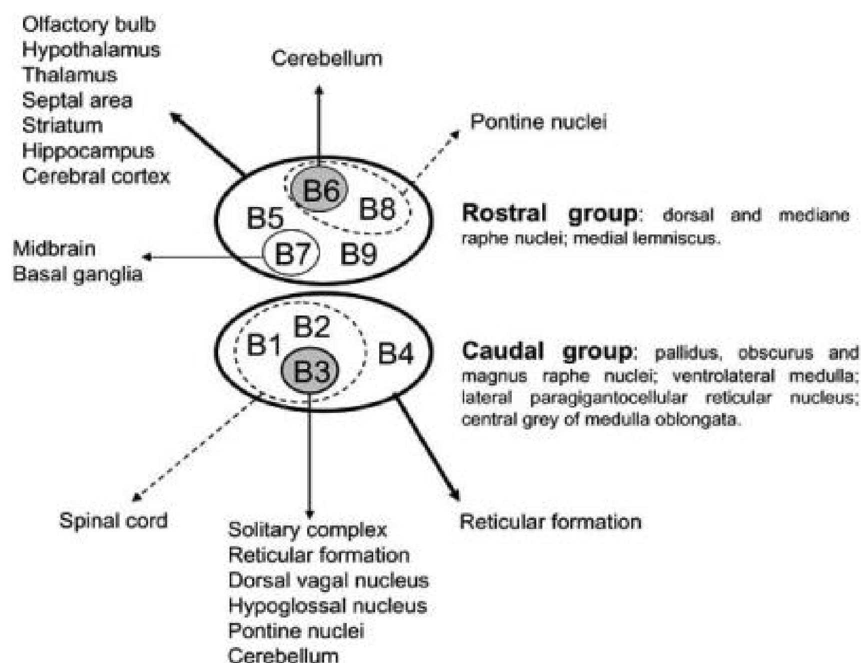


Figure 1.3: Serotonin system. Principal groups of serotonergic neurons in the CNS and their projection sites (Ciranna 2006)

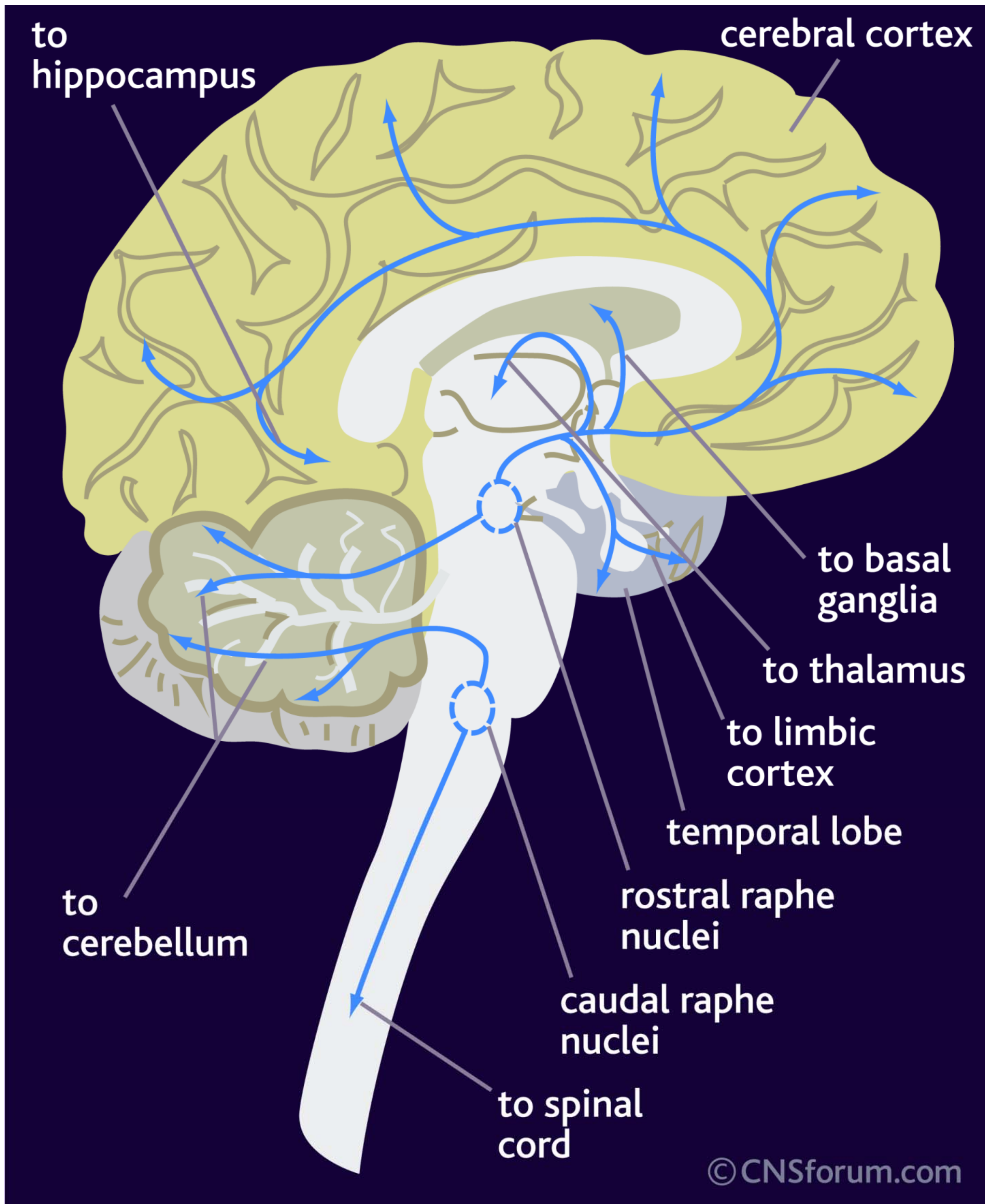


Figure 1.4: Distribution of serotonergic cell groups and their major projections

Serotonin Receptors

The effects of serotonin are subserved by 13 heptahelical G-protein coupled receptors (GPCRs) and a ligand gated ion channel family (5-HT_3). These receptors are divided into seven distinct classes (5-HT_1 to 5-HT_7) largely on the basis of their structural, transductional and operational characteristics (Barnes and Sharp 1999; Hannon 2002). Except for the 5-HT_3-R all the other 13 GPCRs exert their action on a variety of intracellular functional proteins such as adenylyl cyclase, PLC, voltage gated N-type Ca^{2+} channels, hyperpolarizing potassium channels, etc. Based on their effect on adenylyl cyclase these receptors can be classified into two distinct groups. The first group consists of the 5-HT_1 type receptors that couple to adenylyl cyclase inhibiting G_i protein. The second group, on the other hand is comprised of the receptors coupled to adenylyl cyclase stimulating G_s proteins: 5-HT_4, 5-HT_6 and 5-HT_7 (Hannon 2002; Adayev 2005).

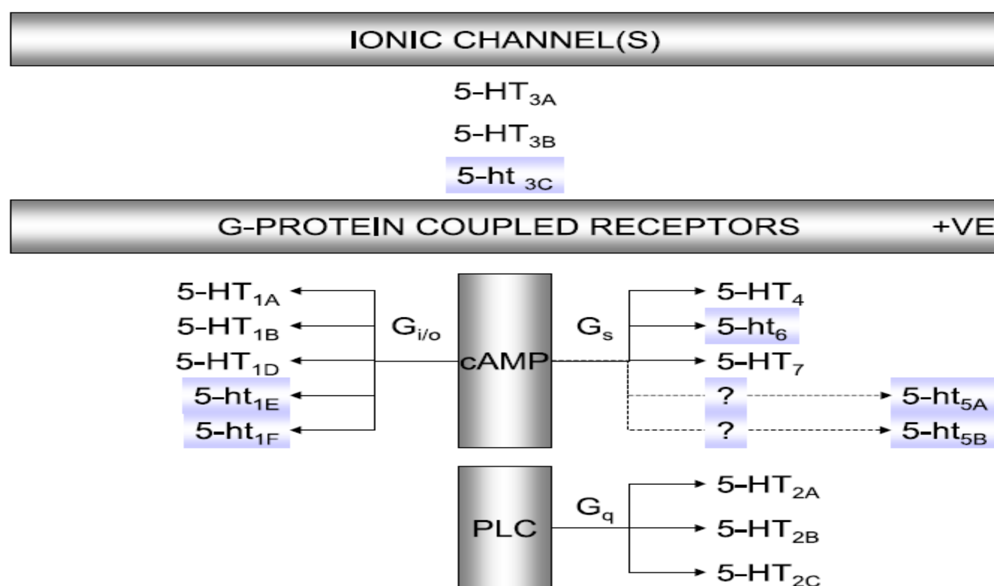


Figure 1.5: Current classification of 5-HT receptors (Hannon 2002)

A typical GPCR is composed of seven transmembrane α-helical domains with an intracellular C- terminus and an extracellular N-terminus. The α-helices alternately run in and out of the plasma membrane and are interspersed by three extracellular and three cytoplasmic loops. Interaction of the receptors with their cognate G-proteins occurs mainly at the second and third cytoplasmic loops (Adayev 2005).

Each of the G-proteins is a receptor-associated heterotrimer of three polypeptide subunits termed α, β, and, γ. The α subunit is responsible for GTP and GDP binding and for GTP hydrolysis, whereas the β and γ subunits are associated in a tightly linked βγ complex (Gilman 1987; Pierce, Premont et al. 2002). Both the α-subunit and the βγ dimer signal through the activation, or inhibition, of effector proteins. Agonist activation of the receptors induces conformational changes which are re-arrangements of membrane helices 6 and 3. This 'activated receptor' can interact with the heterotrimeric G protein, and serves as a GEF (Guanine Nucleotide Exchange Factor) to promote GDP dissociation, and GTP binding and activation. The activated heterotrimer dissociates into an α subunit and a βγ dimer (Gilman 1987; Pierce, Premont et al. 2002). Both of the α-subunit and the βγ dimer can activate several effectors. Hydrolysis of GTP to GDP — a process that is now known to be regulated by RGS (regulator of G-protein signaling) proteins — leads to reassociation of the heterotrimer and termination of the activation cycle.

GPCR signaling is regulated by several mechanisms, among which desensitization has been studied most thoroughly. Desensitization operates at both the level of receptor as well as downstream levels (Ferguson 2001; Pierce, Premont et al. 2002). Desensitization is usually controlled by receptor phosphorylation which is generally mediated by second messenger

kinases (e.g. Protein Kinase A, Protein Kinase C) or a distinct family of G-protein coupled receptor kinases (GRKs) (Pitcher, Freedman et al. 1998).

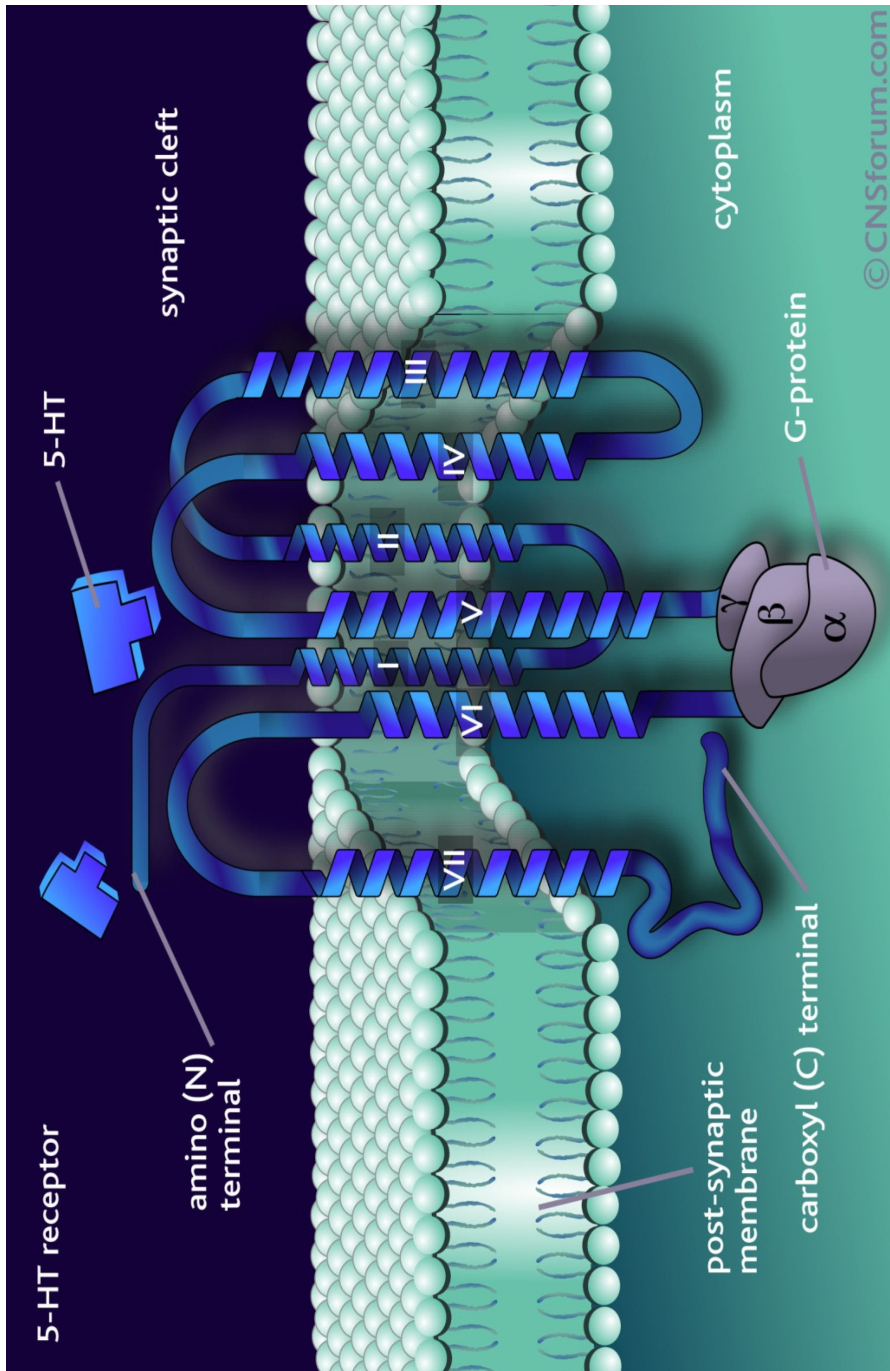


Figure 1.6: Structure of a G-protein coupled 5-HT receptor.

5-HT_{1A}-Receptor

Serotonin 1A (5-HT_{1A}) receptor is a heptahelical transmembrane GPCR, expressed all over the front brain and highly concentrated in the hippocampus. It is coupled to various hetero-trimeric G-proteins including G_i and G_o. $G_{\alpha q}$ and $G_{\alpha o}$ upon activation stimulate PLCγ while the ligand binding to the receptor leads to the activation of the G-proteins and results in the release of the α-subunit and βγ complex. The $G_{\beta\gamma}$ complex on the other hand, activates PLCß and other signaling pathways including ERK/MAPK and PI-3K pathway. Among the pathways activated by agonist bound 5-HT_{1A}-R, ERK1/2 pathways are reported to be important for neuronal proliferation and neuronal survival (Adayev 2003). Studies report that 5-HT_{1A}-R may also mediate opening of inwardly rectifying K^+ channels in many neurons leading to hyperpolarization of the cells (Katayama 1997; Bonasera 2000). Agonist mediated acceleration of Na^+/H^+ exchange has also been reported (Garnovskaya 1997). Thus depending on the type of internal machinery of the effector molecules in a cell the final effect of the receptor activation may be different. This receptor has often been reported to exert inhibitory effect, though the same receptor can stimulate a number of effector molecules causing calcium mobilization and cell proliferation in different cell types (Albert 1998).

Studies have established the pivotal role of 5-HT_{1A}-R in brain development. In mice, absence of the serotonin 1A (5-HT_{1A}) receptor during early postnatal period up to postnatal day 21 (P21) in the front brain results in elevated levels of anxiety (Gross 2002). Furthermore, a mutation in the promoter of the *5-HT_{1A} receptor* gene, which leads to overexpression of the 5-HT_{1A} receptor (5-HT_{1A}-R) especially in the serotonergic raphé neurons has been correlated to depression and suicide (Lemonde 2003). Corroborative research has shown that overexpression

of this receptor in the presynaptic serotonergic neurons in neonatal mouse brain does elicit heightened vulnerability to stress and depression-like symptoms in adult mice (Richardson-Jones, Craige et al. 2010). Elevated expression of the somatodendritic (auto) 5-HT_{1A}-R in the 5-HT-synthesizing raphé neurons results in increased inhibitory signaling through a feedback mechanism, attenuating 5-HT firing at synapses with front-brain neurons that express 5-HT_{1A}-R but do not synthesize 5-HT (Banerjee 2007). Thus, elevated expression of 5-HT_{1A}-R in the presynaptic raphé neurons would eventually lead to a decrease in 5-HT_{1A}-R-mediated signaling in the postsynaptic neurons in front brain regions, such as hippocampus and the prefrontal cortex. Synaptic strengthening in the acutely isolated mouse brain slices has been reported when treated with 5-HT_{1A}-R agonist 8-OH-DPAT (Mehta, Ahmed et al. 2007) (Fig.1.7). Based on this concept, the three studies discussed above collectively indicate that the post-synaptic 5-HT_{1A}-R plays a direct role in neonatal front brain development, which critically determines the emotional normalcy of an individual. However, what intracellular signaling is initiated at the postsynaptic 5-HT_{1A}-R and what part of front brain development is promoted by this signaling?

The 5-HT_{1A}-R is known to be linked directly to only a handful of signaling molecules, some of which could in turn initiate complete biochemical pathways linked to events such as neuroprogenitor proliferation or differentiation and synaptogenesis. Some attempts have been made to identify individual signaling molecules that could regulate these two neonatal events, still a complete biochemical link between the 5-HT_{1A}-R and the events remains to be identified. Our preliminary experiments in mouse brain slices demonstrated a 5-HT_{1A}-R-evoked increase in synaptic transmission in the CA1 region of the hippocampus at P15 (Mehta, Ahmed et al. 2007). This study also showed that a 5-HT_{1A}-R-linked mitogen-activated protein (MAP) kinase pathway, linked to protein kinase C (PKC), was involved in the boosted synaptic activity. A few

studies have also demonstrated a possible role of 5-HT_{1A}-R mediated signaling in neurite outgrowth in neuro 2A cells (Fricker, Rios et al. 2005) as well as maintenance of the synapse number in the fascia dentata of adult rats (Faber and Haring 1999). While these reports address a possible involvement of 5-HT_{1A}-R in synaptogenesis, a straight forward study delineating a signaling pathway leading to synaptogenesis has long been awaited but seldom addressed.

The current study addresses the possibility that this pathway is operant *in vivo* in the P15 hippocampus and it regulates synaptogenesis, which is known to be at its peak in the cortex at P15 (Benitez-Diaz 2003).

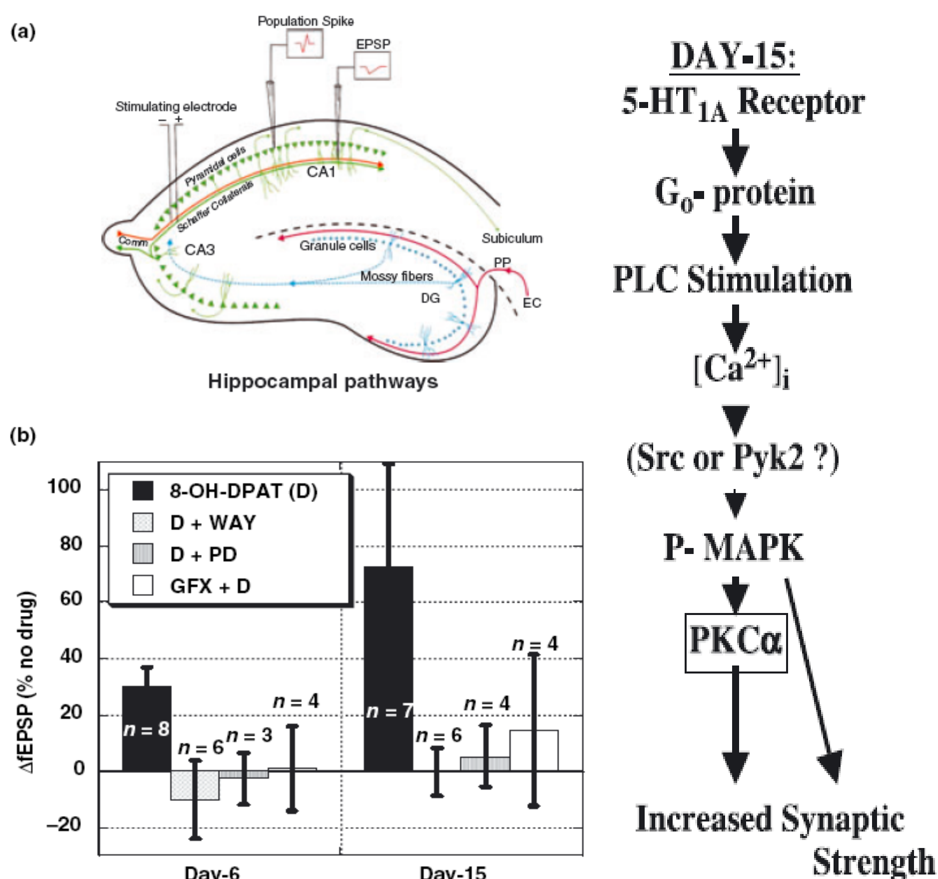


Figure 1.7: 5-HT_{1A}-R mediated signaling pathway leads to synaptic strengthening in hippocampus at P15 (Mehta, Ahmed et al. 2007)

PSD95

The Post Synaptic Density (PSD) Protein of 95 KDa, PSD95- a postsynaptic membrane-associated guanylate kinase- is an established postsynaptic marker of spine and synapse maturation (El-Husseini 2000; Ehrlich and Malinow 2004; Ehrlich 2007). It is also a member of large family of proteins containing the PDZ (PSD95/Disc Large/Zona occludense-1) domains, which provide scaffolding ability to PSD-95 and mediate interactions with other PDZ proteins, intracellular signaling molecules, cell surface adhesion molecules, ion channels and receptors (Kim 2004). These proteins are characterized by three PDZ domains, a Src Homology 3 (SH3) domain, and a Guanylate Kinase-Like (GK) domain. The SH3 and GK domains interact in an intramolecular fashion, but the functional significance of this interaction is unclear (McGee, Dakoji et al. 2001; Tavares, Panepucci et al. 2001). PSD-95 forms multimers, and this process seems to be mediated by amino (N)-terminal 'head-to-head' interactions (Hsueh and Sheng 1999; Christopherson, Sweeney et al. 2003). Self-association is a common feature of many PDZ scaffold proteins, and is sometimes mediated by direct interactions between PDZ domains. Multimerization of PDZ scaffolds might enhance the clustering of partner proteins in large multimolecular assemblies at specific sites, such as the post synaptic density (PSD) (Fig. 1.8). PSD-95 is located close to the postsynaptic membrane (at a mean distance of 12 nm from the postsynaptic membrane), and that it can be labeled by antibodies from both the extracellular and cytoplasmic faces of purified PSDs (Chen, Rojas-Soto et al. 1998; Valtschanoff and Weinberg 2001). It is therefore in a good position to interact with postsynaptic membrane proteins such as receptors, ion channels and cell adhesion molecules, as well as with cytoplasmic proteins. Such interactions are proposed to be important for the localization and clustering of these proteins at the postsynaptic membrane. In neuronal signaling, it is reported to exert a major influence on

synaptic strength and plasticity by recruiting AMPA receptors to the postsynaptic membrane (El-Husseini 2000; Ehrlich and Malinow 2004). Synaptic potentiation induced by the overexpression of PSD-95 seems to mimic LTP, in that it converts silent synapses into functional synapses, drives GluR1 into synapses, occludes LTP and enhances Long-Term Depression (LTD) (Béïque and Andrade 2003; Stein, House et al. 2003; Ehrlich and Malinow 2004).

Intriguingly, the ERK/MAPK pathway mediates induced expression of PSD95, thus exerting a major influence on synaptogenesis (Elkobi 2008). Based on this information, we used PSD95 expression and electron microscopy to demonstrate 5-HT_{1A}-R-mediated synaptogenesis in the P15 hippocampus and determine the signaling cascade involved. To investigate the pathway followed by serotonin (5-HT) released from the 5-HT neurons, we conducted hippocampal administration of a selective serotonin reuptake inhibitor (SSRI) (fluoxetine), which augments serotonin release from the 5-HT terminals, to trigger synaptogenesis in the P15 mice in an Erk and protein kinase C alpha (PKCα)-dependent manner. Based on our finding, we then used bryostatin, a memory-enhancing therapeutic agent for Alzheimer disease, as a means to promote synaptogenesis in 5-HT_{1A}-R(-/-) mice by stimulating PKCα directly at P15.

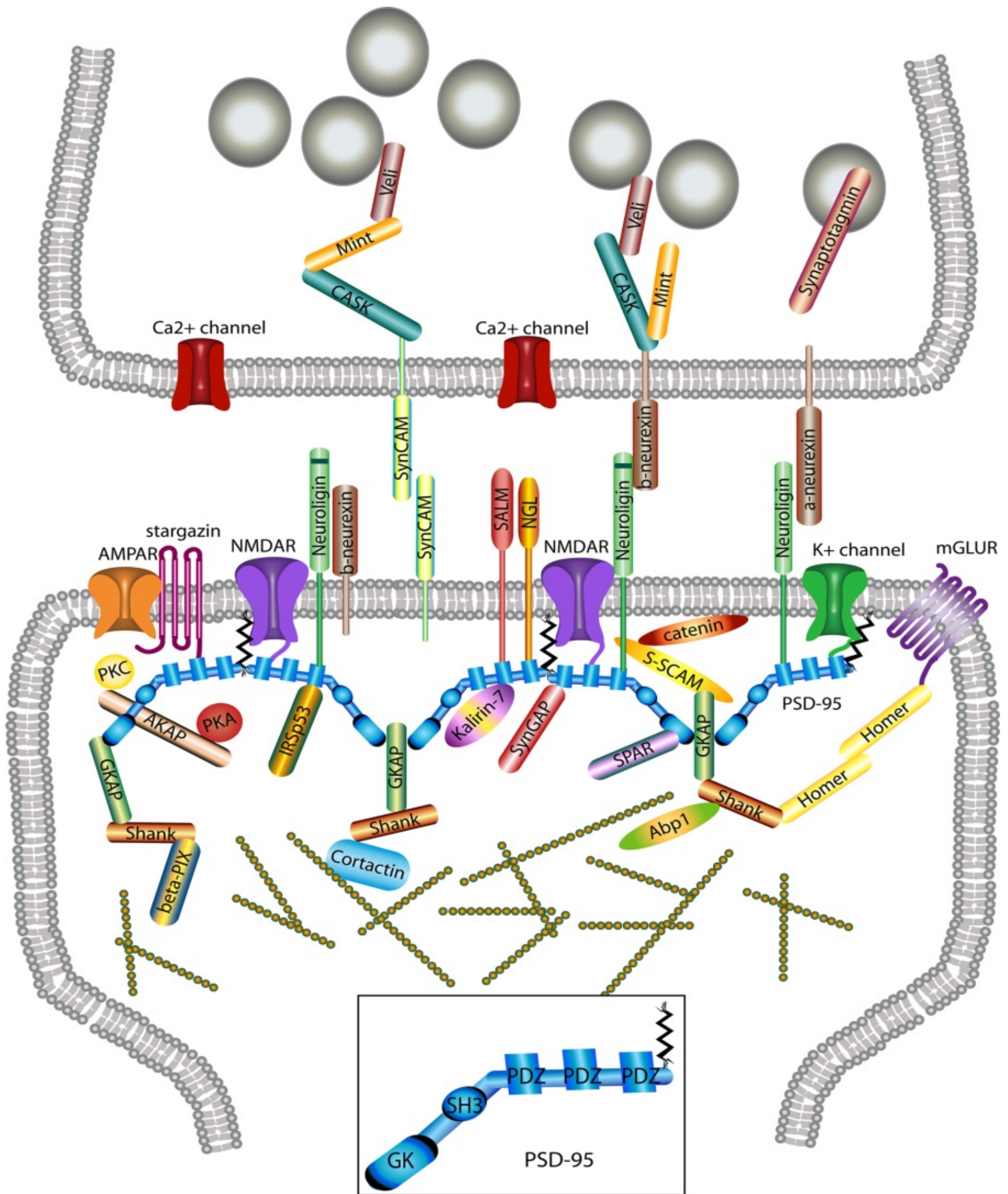


Figure 1.8: Proposed structure of PSD95 and its possible role at post synaptic density

Objective of This Study

The serotonin-1A receptor is required for normal neonatal development of the mouse brain. Based on earlier studies performed in our laboratory as well as other groups, we have postulated that 5-HT1A-R →MAPK signaling plays an important role to cause increased synaptogenesis via heightened expression of PSD95 in the neonatal hippocampus.

To achieve this, we have monitored induction of PSD95 through activation of MAPK isozymes ERK1/2 and PKCα via 5-HT1A-R signaling and its effect on synaptogenesis on postnatal day 15 (P15). We have monitored the involvement of 5-HT_{1A}-R →ERK → PKCα pathway in SSRI mediated augmentation of PSD95 and synaptogenesis. We have confirmed the involvement of 5-HT_{1A}-R mediated signaling pathway by using the 5-HT_{1A}-R (-/-) mice.

Thus, the major purpose of this project is to elucidate the role of 5-HT1A-R mediated activation/ inhibition of key signaling molecules in brain development which will have therapeutic importance in developmental disorders and may shed new light on the etiology of multiple brain disorders. Earlier studies have shown that ERK-mediated signaling causes an induction of PSD95 in the gustatory cortex and taste learning is dependent on this boost in PSD95 expression (Elkobi 2008). Thus it appears that a signaling pathway involving Erk mediated PSD95 expression plays an important role the formation of memory forming synapses in this brain region. Occurrence of this signaling cascade in the limbic region, which determines the emotional state of an individual, has yet to be determined. Our overall objective was also to elucidate for the first time the framework of a synaptogenic 5-HT_{1A}-R-mediated signaling cascade starting from an extra cellular event to the final outcome in the form of synaptogenesis in the hippocampus.

CHAPTER 2

MATERIALS AND METHODS

Animals

Following mice at age postnatal day 15(P15) were used for the experiments:

C57 BL6: 5-HT_{1A}-R (+/+) and 5-HT_{1A}-R (-/-) (Jackson Laboratory, CA)

SW: 5-HT_{1A}-R (+/+) and 5-HT_{1A}-R (-/-) (Jackson Laboratory, CA)

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. All the animals were kept in a 12 hour light/ dark cycle with libitum access to food and water.

Materials

The antibodies to P-Erk and P-PKCα were obtained from Cell Signaling (Beverly, MA, USA). PSD95 and Erk1/2 and the horse radish peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-ß-actin antibody, Bryostatin, 8-OH-DPAT, Bryostatin, and WAY100635 were obtained from Sigma Chemicals (St. Louis, MO, USA). Bisindolylmaleimide or GF109203X (GFX) and U0126 were purchased from Calbiochem (La Jolla, CA, USA). The NeuN antibody was procured from Millipore (Billerica, MA, USA). The Alexafluor- labeled fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA).

Methodological Approaches

Hippocampal Slice Culture:

Mouse pups at P15 were anesthetized using the mixture of Ketamine (100mg/Kg) +Xylazine (10mg/Kg) (in the ratio recommended by the IACUC protocol) and decapitated. Under sterile conditions, the brains were isolated and then cut at 60° from the longitudinal fissure at the top using a hippocampus- dissecting tool to expose the hippocampus. The hemispheres containing the hippocampi were then placed in modified Gey's balanced salt solution (mGBSS) at 4^oC for 30-40 minutes, while bubbling a mixture of 95% O_2 and 5% CO_2. Individual hippocampi were isolated using dissection tool and then 400 μM thick transverse slices were prepared using a tissue chopper (Stoelting, Wood Dale, IL, USA). The slices were placed in ice cold mGBSS and inspected using a dissection microscope for the presence of uninterrupted bright transparent neuronal layers characteristic of the hippocampal structure. Only such slices were placed on Millicell CM filters (Millipore, Bedford, MA, USA). The filters were placed in a six well dish with 1ml of medium in each well. The slices were kept on high K^+ culture medium (25% horse serum, 50% Basal Essential Media- Eagles, 25% Eagle's Balanced Salt Solution (EBSS), 25 mM Na-HEPES, 1mM Glutamine, 28 mM Glucose, pH 7.2) for the first two days. After incubation at 32^oC in a 5% CO_2 atmosphere, the culture medium was changed to physiological K^+ slice culture medium (20% dialyzed fetal bovine serum, 5% Basal Essential Media- Eagles, and EBSS modified to adjust the K^+ concentration to 2.66 mM). After 20% dialyzed serum treatment for two days and the slices were placed in 5% serum medium (same medium as above but with 5% serum) for two days. This was followed by treatment with inhibitors and antagonists for 30 minutes (overnight for WAY) followed by treatment with agonist for the specific time periods (16 to 24 hours for expression regulation and 15' to 60' for

phosphorylation regulation experiments). The slices were placed in serum free medium for 1 hour before the drug treatment in case of short time treatment (up to 1 hour) with agonist but not with long time treatment as the slices will die. After drug treatment the slices were either fixed for the immunohistochemistry or were lysed for western blot analysis.

mGBSS composition (in mM) : $CaCl_2$ (1.5), KCl (4.9), KH_2PO_4 (.02), $MgCl_2$ (11.0), NaCl (138), $NaHCO_3$ (2.7), Na_2HPO_4 (0.8), Na-HEPES (25), glucose 6% (w/v), pH 7.2. The slices were routinely treated with drugs on the sixth day of culture.

Western Blotting:

The drug treated slices were lysed in 1ml RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4 plus freshly added protease inhibitor cocktail; Roche Diagnostic GmbH, Mannheim, Germany), the lysates were resolved on a 8% acrylamide gel, protein bands transferred to nitrocellulose membrane, blocked in 5% solution of dry milk dissolved in 0.1% Tween in TBS (20 mM Tris- HCL, pH 7.4, 0.8% NaCl). The membranes were then probed with PSD-95 (1:1000) antibody followed by treatment with HRP linked goat anti-mouse IgG (1:40000). The immune-reactive bands were visualized using the supersignal luminol kit (Pierce) and incubation with an X-ray film. The β-actin bands were used to normalize the proteins.

Immunohistochemistry of cultured slices:

The cultured and drug treated slices were washed quickly with chilled 10mM Phosphate buffer (PB) and then fixed overnight at 4^oC in 4% PFA. The sections were then removed from the membrane with the brush and placed in a 48 well plate in PBS. This was followed by 2-3

washes of 15 minutes each with 1X PBS. For immune-fluorescence staining, free floating sections were first incubated in 10% Triton X-100 for 30 minutes at room temperature and then rinsed with 1X PBS (0.2M) 3 times. Sections were then blocked with blocking buffer (0.1% Triton X-100- 10% serum from the animal used to raise the 2° antibody-in 1X PBS) for overnight at 4^oC. This was followed by treatment with primary antibody for PSD95 (1:200) in antibody solution (0.1% -Triton X-100- 2% serum-in 1X PBS) for 48 hours at 4^oC with gentle rocking. The samples were washed 3 times for 30 minutes each in 1X PBS and then treated with goat anti mouse fluorescent 2° antibody covalently linked to Alexa Fluor 568 (Red). After 24 hours of secondary antibody treatment at 4^oC the sections were washed in 1X PBS and then the slices were blocked again for 24 hours. Second primary antibody treatment was done for NeuN (1:150) for 48 hours followed by washing 3X with PBS. This was followed by second secondary antibody treatment (goat anti mouse linked with Alexa Fluor 488) for 24 hours. Slices were washed three times for 30 minutes each and then were mounted on slides with ProLong DAPI antifade reagent (Molecular Probes, Eugene, OR, USA) for visualization and photography using a laser confocal microscope. In case of cryo-sectioned slices, slices were washed with PBS three times for 30 minutes each before blocking over-night and then were treated in the way explained above.

Intra-cranial Brain Injections:

Mouse pups at P15 were anesthetized using the mixture of Ketamine (100mg/Kg) +Xylazine (10mg/Kg) in the ratio recommended by the IACUC protocol. Before the injection, the mice were anesthetized using xylazine (10 mg/ Kg) and ketamine (100 mg/ Kg). The heads were shaved, cleaned with 70% ethanol and then, using a sterile scalpel, a midline incision was made and subcutaneous muscle and fascia were retracted to expose the skull. Injections were

made at stereotaxic coordinates corresponding to Bregma: anterioposterior (AP) = −1.8 mm, mediolateral (ML) = −1.5 mm, dorsoventral (DV) = −1.8 mm. This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure (Le Duigou, Wittner et al. 2005) (Fig. 2.1). Small holes were made in the skull on both sides in the pups to be used for behavioral studies and on the right side in pups used for immunohistochemistry or electron microscopy. Holes were carefully made by 27 gauge needle as the mouse pups were too small for a dental drill. After making holes 10µl Hamilton syringe was used to deliver the drugs into the hippocampi of the pups (Paxinos 2001) at the rate of 1 µl per minute using a stereotaxic set-up (KDS Model 310 plus infusion-withdrawal syringe pump). Before injection it was ensured that the mouse is in deep anesthesia by checking for regular, relaxed respiration and the lack of response to tail/toe pinch. After the injection, the holes in the skull were sealed with sterile bone wax and the skin and muscle were placed back in place with appropriate 7-mm stainless steel clips (Reflex Skin closure system; Cellpoint Scientific). For wound healing, a topical antibiotic (Triple Antibiotic, containing Polymyxin B, Bacitracin, and Neomycin) were applied and for post-operative care the animal were kept in a warm blanket at 35-37 °C taking precautions to avoid thermal injury. After injections, pups were kept under constant supervision until they came back from anesthesia. The animals were fed on Clear H_2O Diet Gel after surgery till the dissection.

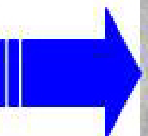


Figure 2.1: Intracranial hippocampal injections for *in vivo* study

Drug Concentrations:

Drug concentrations were made for the observed hippocampal volume of 10µl a P15. 1µl injections were given for all the drugs and sham (0.1M PBS). Stock solutions were made as follows:

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

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

U0126: 100µM stock solution in DMSO for the final concentration of 10µM in the hippocampus.

Ro 31-8220: 100nM stock solution for 10nM final concentration in the hippocampus.

Bryostatin: 200nM stock solution for 20nM final concentration in hippocampus.

Fluoxetine: 18 mg/Kg as described earlier (David, Samuels et al. 2009). Since the density of brain tissue is 1.05 g/ml, which is quite close to that of water, we approximated this value to 18 mg/L, which was about 52 µM. A 520-µM solution in PBS (1 µl) was injected per P15 hippocampus to achieve the final concentration of 52 µM in the whole hippocampus.

Animal perfusion for immunohistochemistry and electron microscopy:

After 16-24 hours post intracranial injections mouse pups were subject to intra-cardial perfusion. Before dissection, animals were anesthetized with the Ketamine+Xylazine mixture according to the dosage, described above and were perfused intra-cardially after they were

completely unconscious with 0.1M PBS solution. This was followed by perfusion with 5% Glutaraldehyde + 2% paraformaldehyde (EM) or 4% paraformaldehyde (IHC) for 15 minutes. Brains were removed and were post fixed in the same perfusion solution overnight. Post fixed brains were transferred to either 30% sucrose solution or 0.1M PBS for cryo-sectioning and EM respectively.

Cryo-sectioning of brain samples and immunohistochemistry of cryo-sectioned slices:

Perfused brains were cryo-sectioned at -20°C. Cerebellum was removed prior to sectioning. Prefrontal part was also removed and from the remaining part of the brains 30µ thick sections were made from the front till the dorsal hippocampus was visible in the slices. This was confirmed by observing the brain slices under a light microscope. The slices were put in 0.1M PBS in a 24 well plate with 4-6 slices per well. Brains were consistently injected with the calculated amount of different pharmacological drugs on the right hippocampi. Only ipsilateral hippocampi from different animals were compared, as no effect of drug infusion was observed on the contralateral hippocampi.

Confocal Microscopy of the immunostained slices, fluorescence quantification, counting of spine numbers and statistical analysis:

The cultured and cryo-sectioned slices were viewed under Nikon C1-LU3 laser scanning confocal system (Melville, NY, USA). We used 488nm exciting wavelength for NeuN and 568nm for PSD95; the slices were viewed at different magnification with objective lenses 20x and 40x magnification. All the pictures were taken at 1024x1024 resolutions with the frame average of 4. Z-stacks were also acquired at 40x magnification, which were used to create maximum volume renders. The Nikon EZ-C1-system software was used to determine the total

thickness of each slice after adjusting channels to obtain pictures from each exciting wavelength separately while blocking the laser beam of the other, exciting wavelength. Subsequently, the superimposed images were created from the individual colored images. Quantification was done by recording the fluorescence of PSD95 in the pictures by the “ImageJ” software. Same magnification pictures were compared. In case of the spine number and fluorescent intensity, spines were counted manually and the number in per unit of the dendritic length was calculated. Puncta intensity was obtained by drawing contours around the puncta carefully and then measuring the fluorescent intensity in the contour. All the values were then converted to percent values of the Carrier treated samples. Statistical analysis was carried out using ANOVA with Bonferroni post hoc test.

Genotyping to Screen 5-HT$_{1A}$-R (-/-) Mice

Having been anesthetized with ketamine/xylazine mixture, 2 mm of tail was removed from each mouse and placed into a 1.5 ml Eppendorf tube. To each tube, 500 µl of 20 mM Tris-cl (pH 8), 5mM EDTA (pH8), 400 mM Nacl, 1% SDS and 0.4mg/ml Proteinase K (Invitrogen, Cat. # 25530-0-15) was added and then incubated at 55° C overnight. The next day, 500 µl of phenol, chloroform and isoamyl alcohol (25:24:1) was added and inverted for 30 minutes at room temperature. Phase separation was achieved after 5 minutes in a micro-centrifuge (Eppendorf) at 14 thousand rpm after which the upper, aqueous was removed from the organic layer and placed in a fresh 1.5 ml tube. The DNA was precipitated by adding 500 µl of isopropanol and centrifugation at 13,200g for 15 minutes at 4°C. The DNA pellet was washed with twice 70% ethanol and dissolved in 60µl of TE.

For the PCR reaction 50ng of DNA was used. To each 0.2ml tube (USA Scientific, 1402-8100) 0.1µl of Platinum Taq Polymerase (Invitrogen, 10966-034) was added along with the Invitrogen PCR Buffer, 2mM $MgSO_4$, 0.25 mM dNTPs, 500 nM of both forward and reverse primers, and the DNA. The volume was brought to 20µl with H_2O. The thermo cycler used was from 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 : CACCTTGCTCCTGCCGAGAAA

NeoH: AGAAGGCGATAGAAGGCGATG

The product size was 464 bp.

PCR Conditions:

1. 94°C 3 minutes.

2. 94° C 30 seconds

3. 65°C 30 seconds

4. 68°C seconds

5. Go to step (2) 29 more times (total 30X).

6. 68° C 7 minutes.

7. 4° C hold.

The PCR product was run for 1 hour at 100 volts on a 1.5% agarose, TBE gel stained with ethidium bromide and visualized under UV light.

Electron microscopic studies:

Hippocampi were removed and 1mm thick sections were cut. After washing with 0.1M PBS for 30 minutes (3X) brain sections were post fixed in 1% osmium tetra-oxide for 90 minutes. Sections were rinsed again with 0.1M PBS for 30 minutes and dehydrated with graded ethanol (50%, 70%, 85%, 95%, 2X 100%) for 10 minutes each step, infiltrated with propylene oxide and plastic Spurr medium, and finally embedded with pure Spurr medium (Spurr 1969) in BEEM capsules and polymerized in oven at 70°C overnight. Ultrathin sections (70-100 nm) were placed on uncoated 200 mesh copper grids, stained with saturated uranyle acetate (UA) in 50% ethanol for 2 minutes, rinsed with 0.22µm Millipore- filtered distilled water for 2 minutes to obtain clean section without any trace of UA residue, stained with Reynolds's lead citrate for 90 seconds, and rinsed with Millipore- filtered distilled water for 90 seconds (Wen, Yang et al. 2002). Sections were examined and photographed with Hitachi 7500 EM operated at 80kV.

Statistical analysis

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

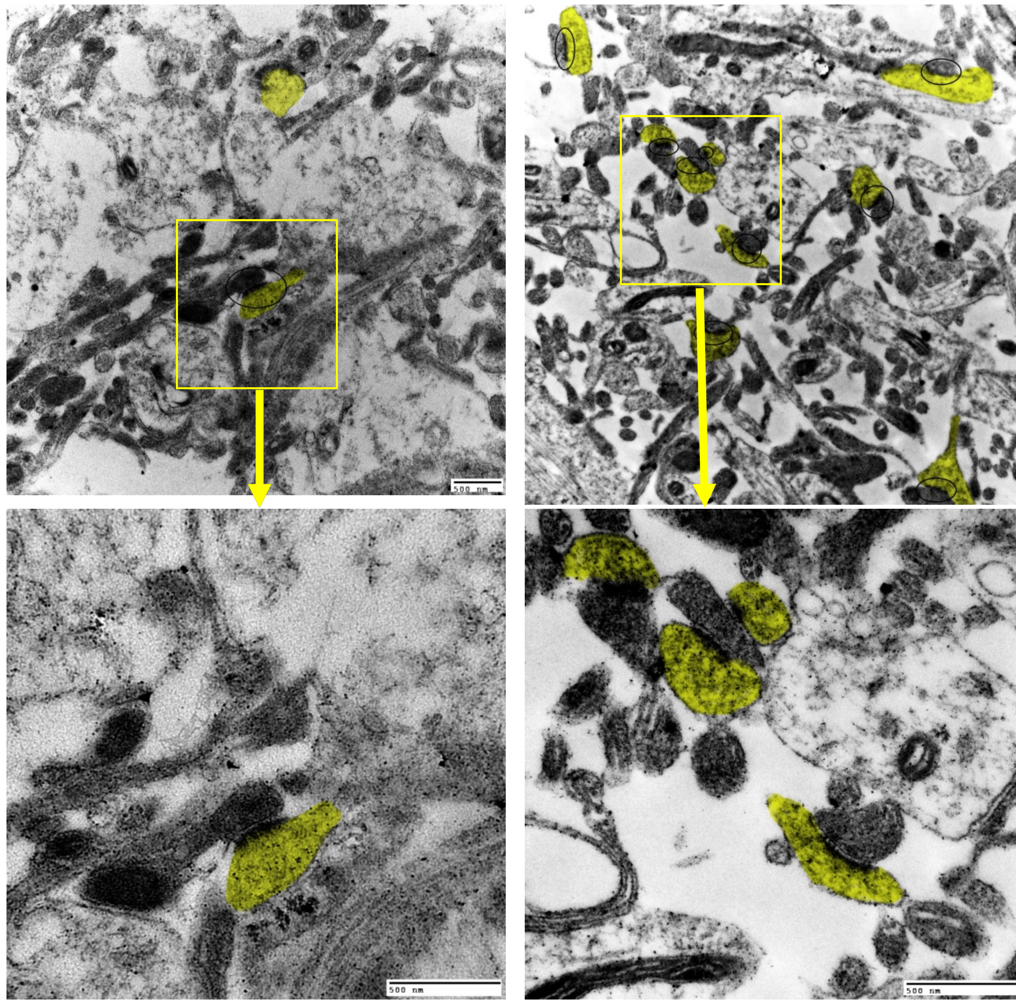
CHAPTER 3

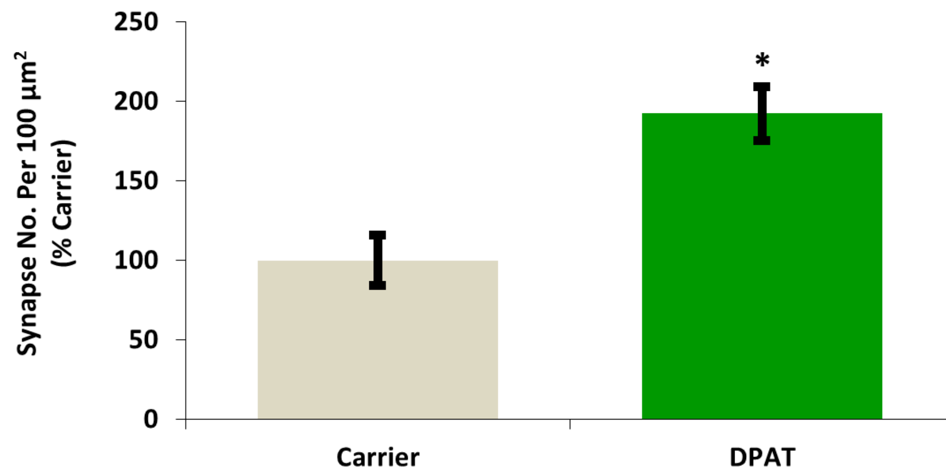
EXPERIMENTAL RESULTS

Stimulation of the 5-HT$_{1A}$-R in organotypic cultures of P15 hippocampal slices augments synaptogenesis:

We first monitored the influence of 5-HT$_{1A}$-R signaling on synaptogenesis in organotypic cultures of hippocampal slices by using electron microscopy. The cultured P15 hippocampal slices at 6DIV were treated with the 5-HT$_{1A}$-R agonist 8-OH-DPAT (DPAT) at 100nM concentration or carrier to show that the DPAT-treated slices harbored a significantly higher number of synapses ($p<0.0001$) (Fig. 3.1). There was significant artifact in the tissue because the slices were cultured *in vitro* for 6 days and lots of cells are lost during these 6 days. But all the slices were treated in the similar way and were comparable.

Fig. 3.1: Treatment of cultured hippocampal slices from P15 mice with a 5-HT$_{1A}$-R agonist causes increased synaptogenesis and PSD95 expression. Organotypic cultures of P15 mouse hippocampal slices at 6DIV were treated with carrier or 100 nM DPAT for 16 h, following which the slices were fixed and subjected to electron microscopy. **(a)** Synapses with post-synaptic density were counted using three stained sections and four fields of view per section (the post-synaptic terminal shown in yellow) (Scale bar: 500 nm). **(b)** Quantification showed a significant increase in the number of synapses following DPAT treatment (*$p < 0.001$ DPAT vs Carrier).


500 nm
500 nm
500 nm
Carrier
DPAT


Synapse No. Per 100 µm²
(% Carrier)
250
200
150
100
50
0
*
Carrier
DPAT

Stimulation of the 5-HT$_{1A}$-R in organotypic cultures of P15 hippocampal slices augments PSD95 expression:

We used Western blotting to monitor the influence of 5-HT$_{1A}$-R signaling on PSD-95 expression in cultured hippocampal slices from P15 mice. We observed a significant increase ($p \leq 0.004$) in PSD95 expression in the presence of DPAT (100 nM). Time course experiment confirmed that PSD95 levels reach a peak after 16 hours of DPAT treatment. The induction was abrogated in the presence of the 5-HT$_{1A}$-R antagonist WAY100635 (4 μM), thus confirming the involvement of the 5-HT$_{1A}$-R in the induction of PSD95 (Fig. 3.2).

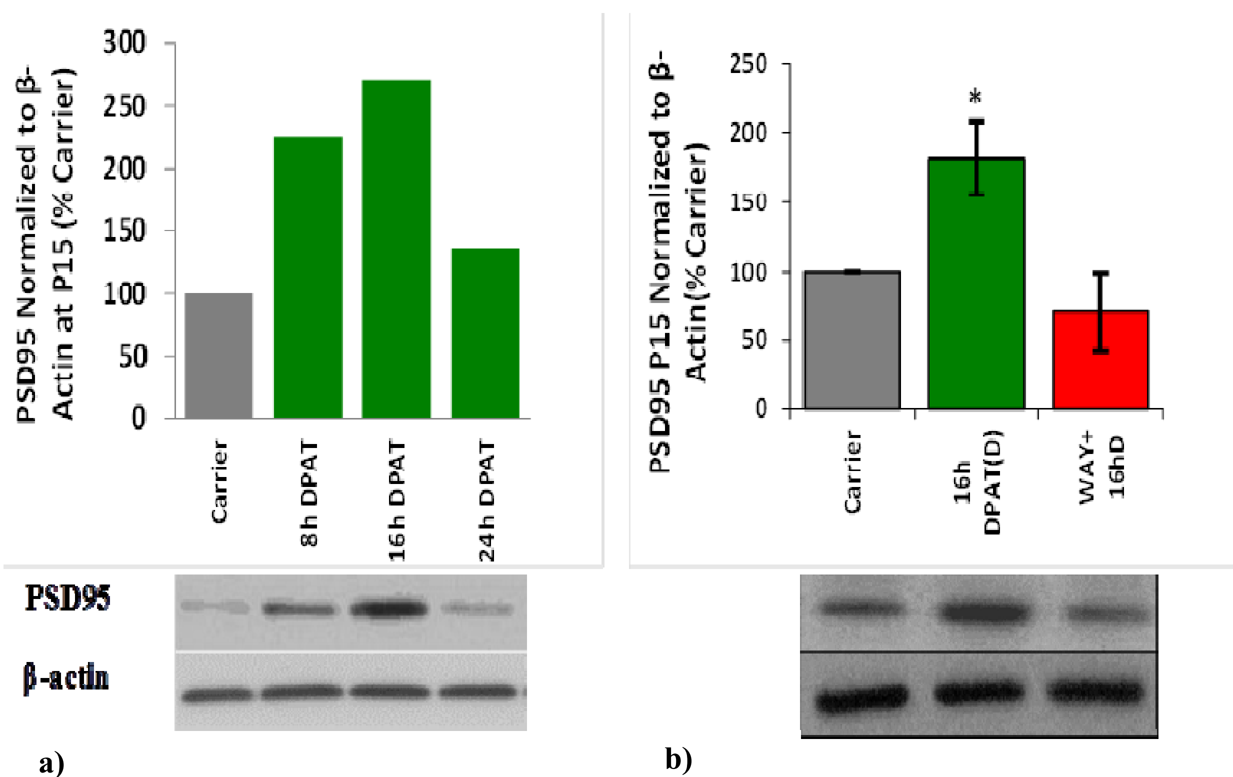
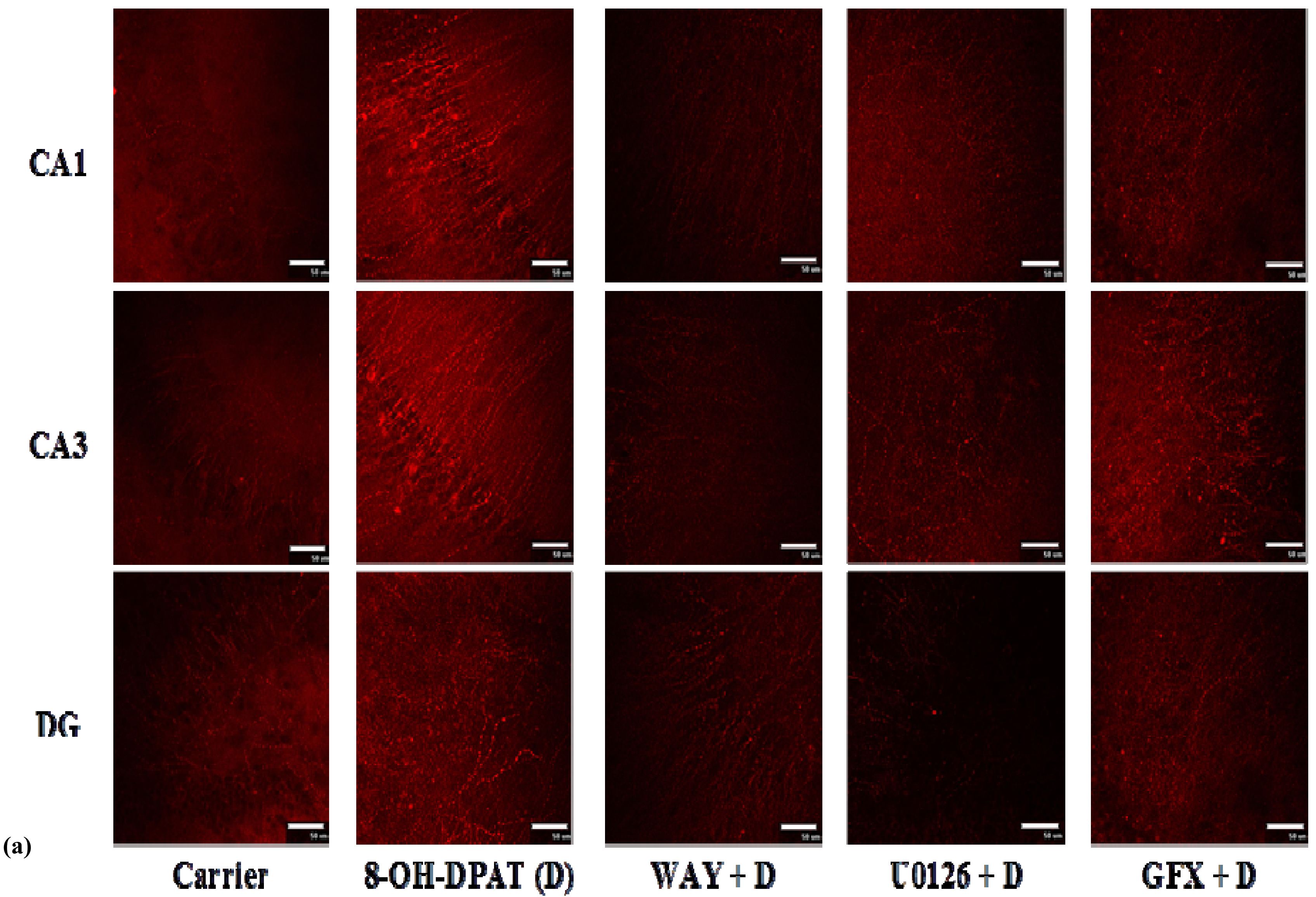


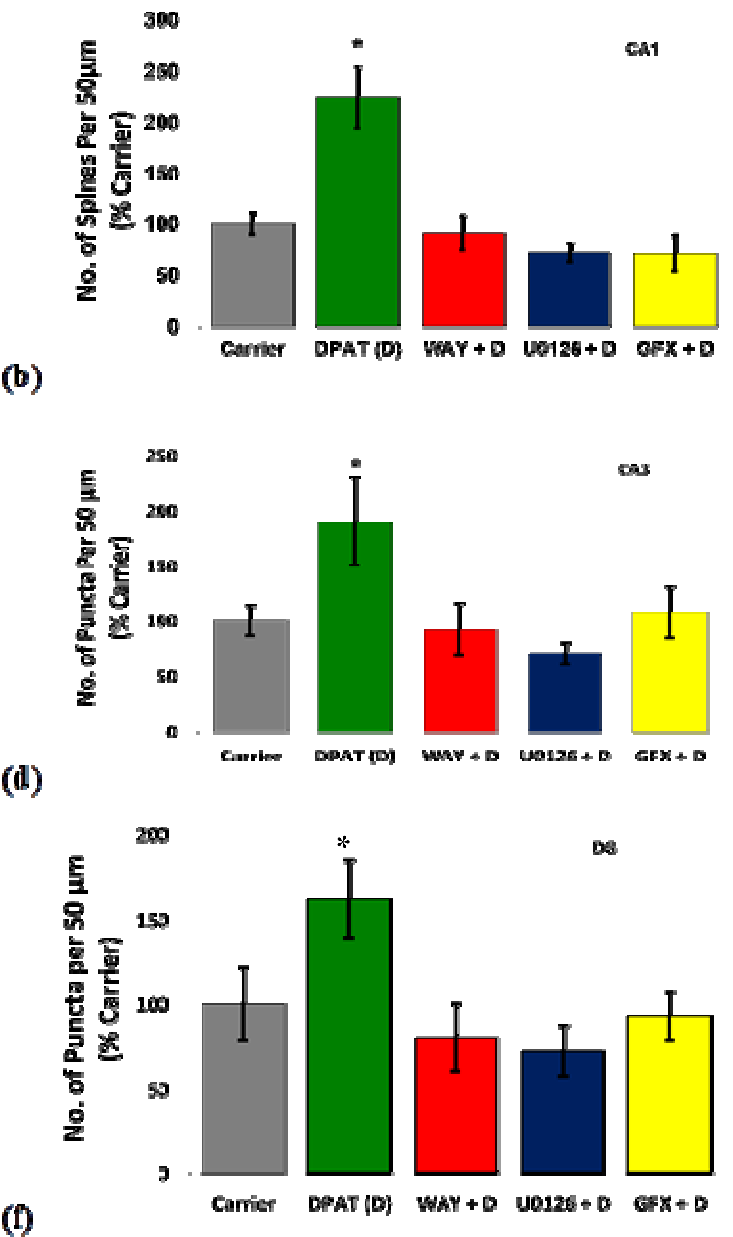
Figure 3.2: Treatment of cultured hippocampal slices from P15 mice with a 5-HT$_{1A}$-R agonist causes increased PSD95 expression. Organotypic cultures of P15 mouse hippocampal slices at 6DIV were treated with carrier or 100 nM DPAT for 16 h, following which the slices were lysed and subjected to western blotting. **(a)** Western blot analysis of the treated slices showed a significant increase PSD95 expression following 100-nM DPAT treatment, which was eliminated in the presence of WAY100635 (4 µM) (*$p \leq 0.004$ for DPAT vs other sets, obtained from three discrete experiments).

Increased PSD95 in dendritic spines upon 5-HT_{1A}-R activation in cultured hippocampal slices: involvement of ERK1/2 and PKC

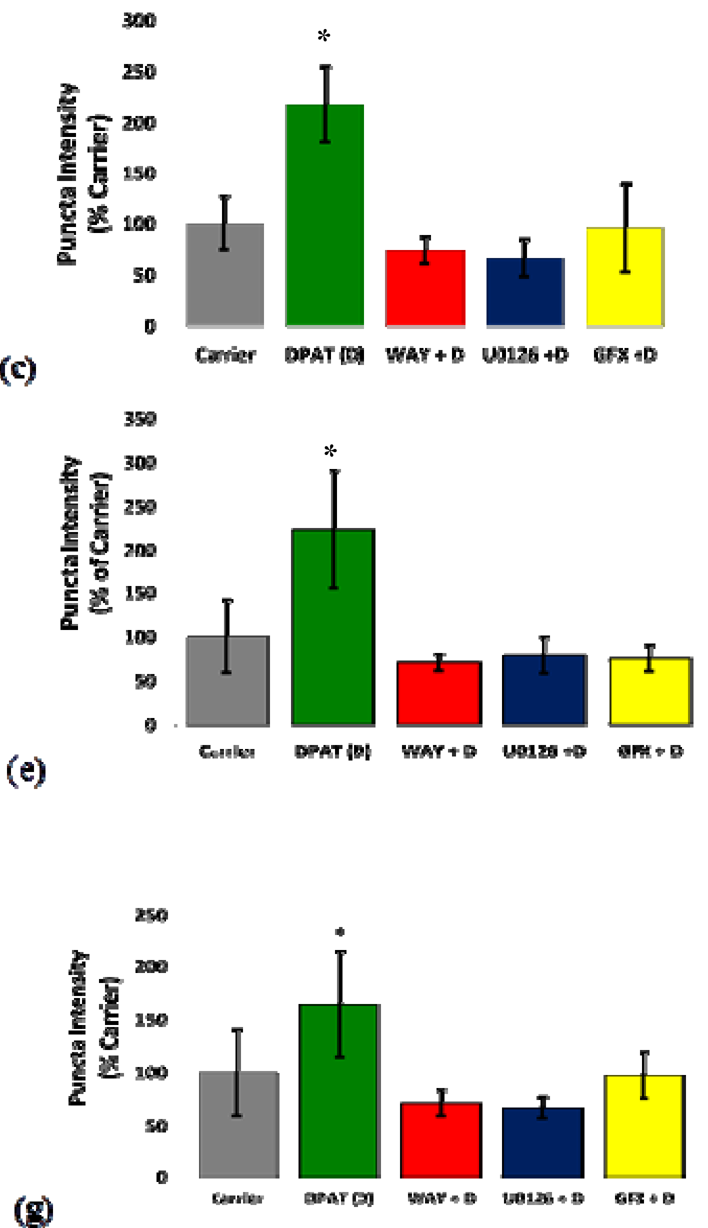
5-HT_{1A}-R-evoked increase of PSD95 expression was also evident from immunohistochemistry (Fig. 3.3a) and it was inhibited in presence of WAY100635 and the pharmacological inhibitors of MEK (U0126, 10 μM) and PKC (GFX, 2 μM), respectively (Fig. 3.3 b-g). PSD95 was shown as punctuate staining in the spines. The number of puncta per unit length of the dendrites and puncta intensity representing the number of PSD95 molecules per spine showed a striking increase in presence of DPAT (D). More number of spines represents higher number of synaptic connection while higher puncta intensity represents more number of PSD95 molecules recruited to the post-synaptic densities. This DPAT-evoked increase in spine formation was eliminated in presence of WAY, U0126 and GFX, thereby confirming the involvement of MEK➔Erk1/2 signaling and PKC in the 5-HT_{1A}-R-mediated induction of PSD95.

Fig. 3.3: Increased PSD95 in dendritic spines upon 5-HT_{1A}-R activation in cultured hippocampal slices: involvement of ERK1/2 and PKC. Organotypic cultures of P15 hippocampal slices at 6DIV were treated with DPAT (100 nM) in the absence and presence of WAY100635 (4 μM), U0126 (10 μM), or GFX (2 μM) for 16 h (Scale bar: 50 μm). The slices were fixed and immunostained to detect PSD95 expression in dendritic spines. DPAT treatment caused a significant increase in both PSD95 expression (puncta intensity, c, e, and g) as well as spine number per unit length (b, d, and f). This increase in PSD95 and spine number was eliminated in the presence of WAY100635, U0126, and GFX (a-g). Quantification from three triplicate experiments revealed significance (b, d, and f: $*p < 0.001$ DPAT versus all other groups); (c, e, and g: $*p < 0.001$ DPAT versus all other groups).


CA1
CA3
DG
(a)
Carrier
8-OH-DPAT (D)
WAY + D
U0126 + D
GFX + D



(b)
No. of Spines Per 50µm (% Carrier)
CA1
Carrier
DPAT (D)
WAY + D
U0126 + D
GFX + D
(d)
No. of Puncta Per 50 µm (% Carrier)
Carrier
DPAT (D)
WAY + D
U0126 + D
GFX + D
(f)
No. of Puncta per 50 µm (% Carrier)
DG
Carrier
DPAT (D)
WAY + D
U0126 + D
GFX + D



(c)
Puncta Intensity (% Carrier)
Carrier
WAY + D
U0126 +D
GFX +D
(e)
Puncta Intensity (% of Carrier)
DPAT (D)
WAY + D
(g)
Puncta Intensity (% Carrier)

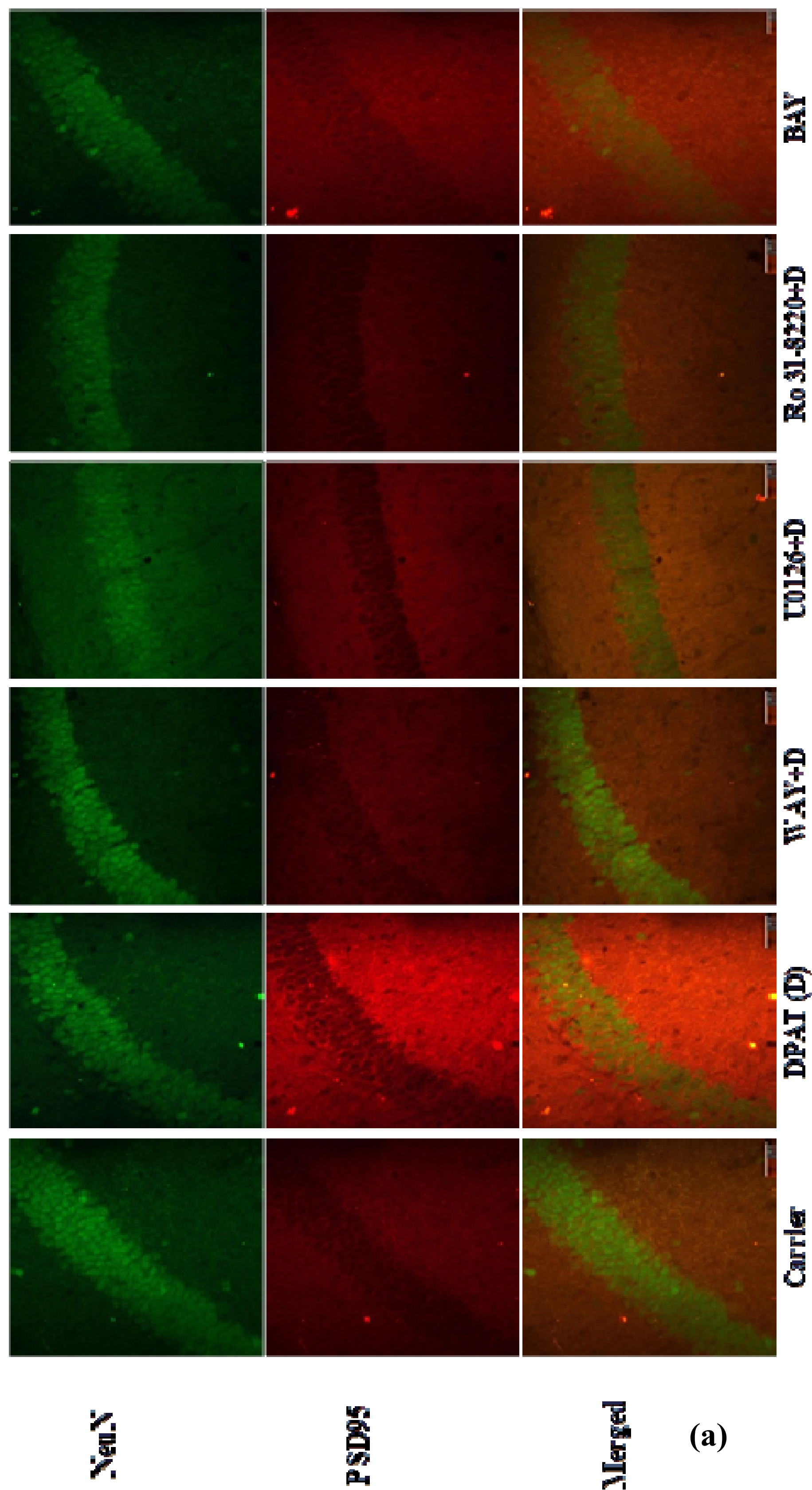
Intra-hippocampal injections and *in vivo* analysis of the synaptogenic 5-HT$_{1A}$-R signaling pathway

Our earlier studies in the hippocampal HN2-5 cells and cultured hippocampal slices indicated that a 5-HT$_{1A}$-R→→ERK→PKCα pathway played an important role in neuroprotection as well as increased synaptic activity (Adayev 2003; Mehta, Ahmed et al. 2007). In order to confirm that the same 5-HT$_{1A}$-R→→ERK→PKCα pathway also functions *in vivo,* we performed intra-hippocampal injections (Ohno, Yamamoto et al. 1992) of DPAT with or without the pharmacological inhibitors.

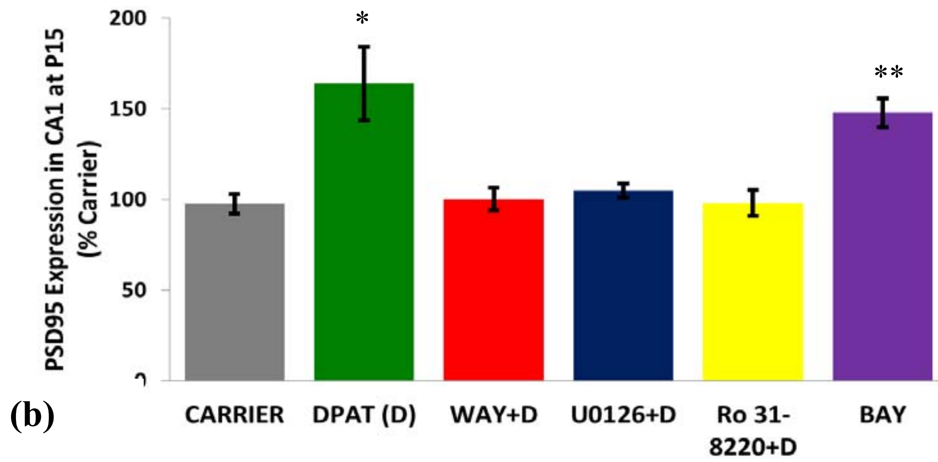
Immunohistochemistry confirms PSD95 induction in the hippocampus:

After 24 h, frozen sections were made from the brains after intracardiac perfusion of paraformaldehyde and the sections were processed for immunohistochemistry. The red staining for PSD95 was markedly higher in sections obtained from the DPAT-injected brains than in sections from carrier-treated brains ($p<0.0001$). This increase in the PSD95 staining was absent in the slices from hippocampi that received WAY100635, U0126, or Ro 31-8220 (a selective PKCα inhibitor ≤10 nM; K_D= 5 nM) in addition to DPAT (Hongpaisan and Alkon 2007). We also activated 5-HT$_{1A}$-R alternatively with BAY, another agonist of 5-HT$_{1A}$-R, which also boosted the PSD95 expression significantly above the Carrier treated slices. There was no significant difference observed between the staining in 8-OH-DPAT treated slices versus BAY treated slices ($p=1$). This confirmed that the same 5-HT$_{1A}$-R mediated signaling pathway as discussed above boosts the expression of PSD95 *in vivo* (Fig. 3.4).

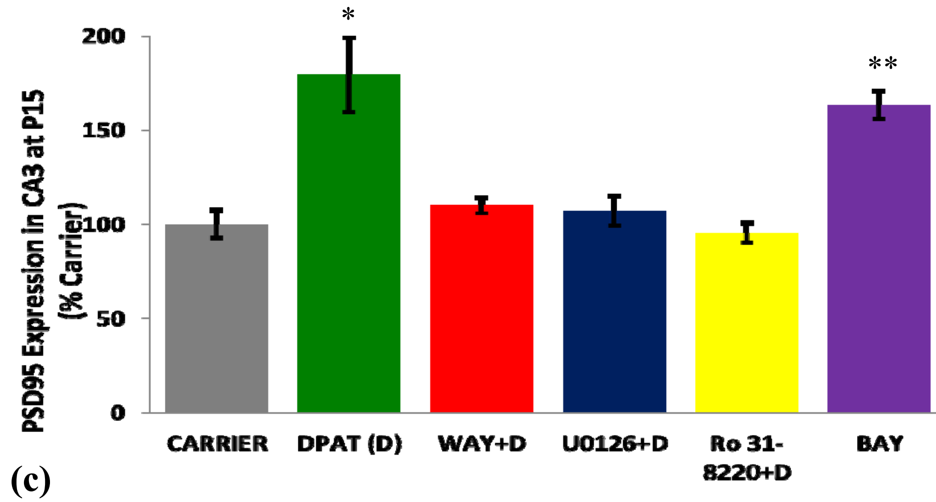
Fig. 3.4: The 5-HT$_{1A}$-R mediated signaling pathway is involved *in vivo* in heightened expression of PSD95 in the P15 hippocampus. (a) A DPAT (100 nM)-evoked induction in PSD95 expression in 24 h is eliminated in the presence of WAY100635 (4 μM), U0126 (10 μM), and the selective PKCα inhibitor Ro 31-8220 at 10 nM. A second 5-HT$_{1A}$-R agonist BAY3702 (100 nM) also causes induced PSD95 expression (Scale bar: 50 μm). **(b-d)** Densitometric quantification from three experiments shows a significant induction of PSD95 in CA1, CA3 and DG respectively following infusion of DPAT or BAY3702 (BAY) (*$p < 0.001$ DPAT versus all other groups except BAY3702; **$p = 0.1345$ DPAT versus BAY3702). **(e)** Only ipsilateral hippocampi had an effect of the infused drug. No effect was observed on the contralateral hippocampi. For all the experiments only ipsilateral hippocampi were compared.



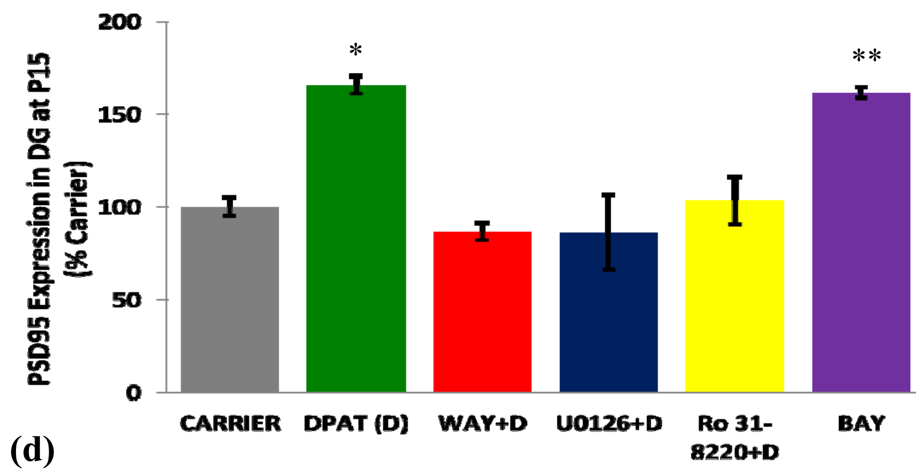
(a)



(b)

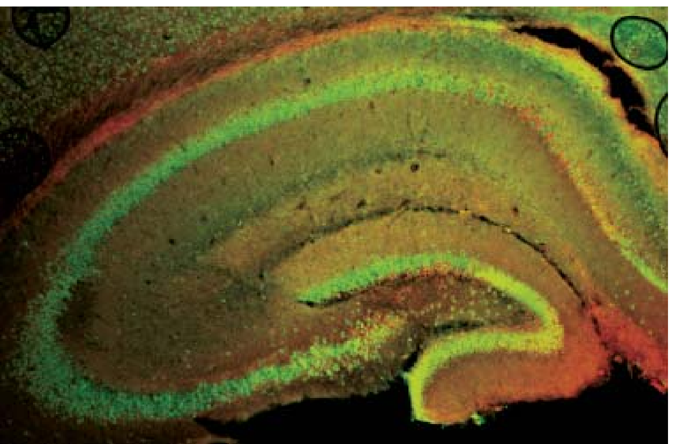


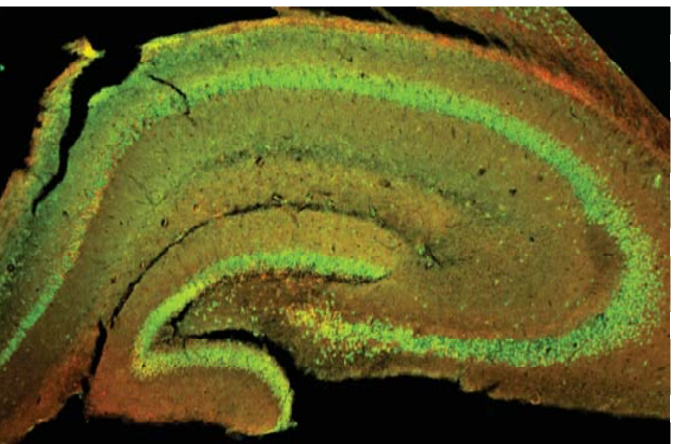
(c)



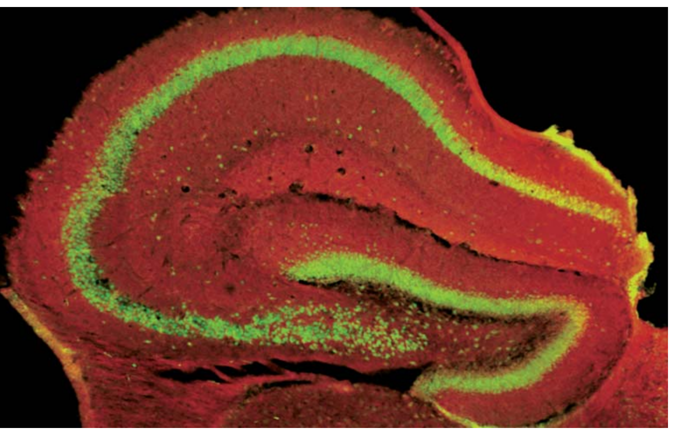
(d)

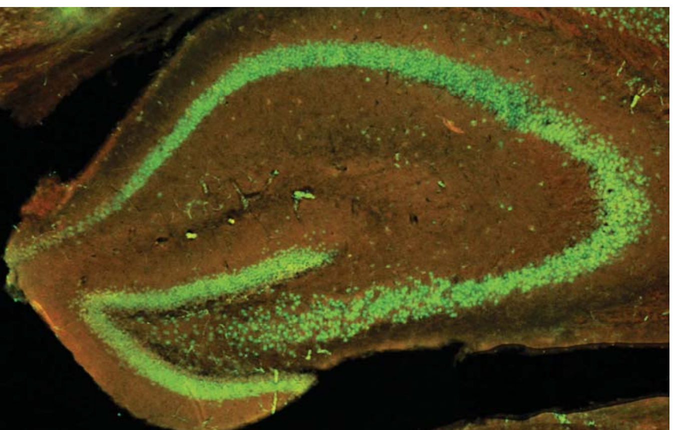
Carrier





DPAT



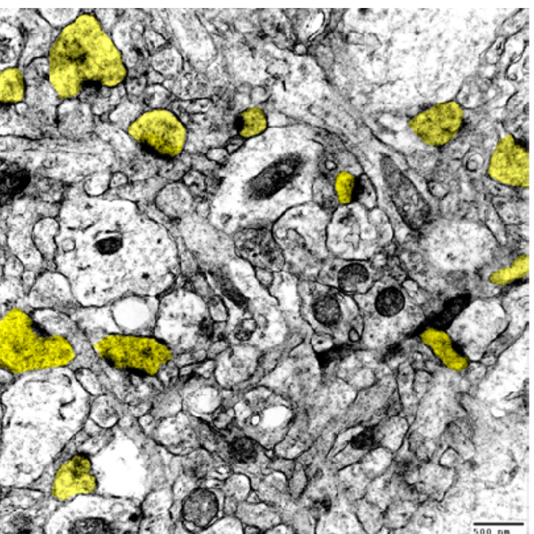


(e) Ipsilateral Contralateral

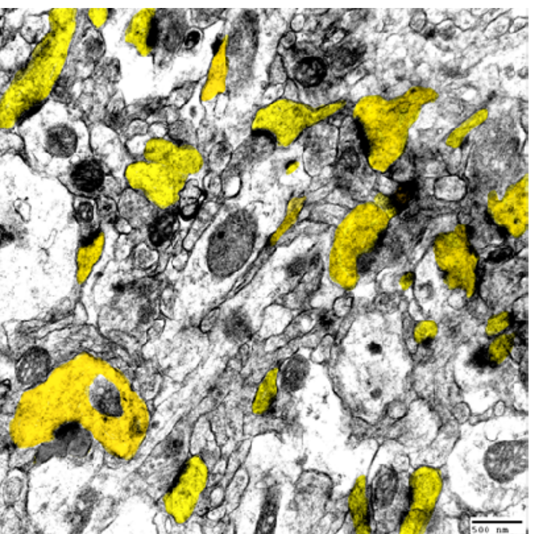
Electron Microscopy confirmed increased synaptogenesis *via* PKCα downstream of 5-HT_{1A}-R in C57BL6 mouse hippocampus:

In order to determine the role of 5-HT_{1A}-R mediated pathway in synaptogenesis *in vivo* we performed electron microscopic analysis on hippocampi obtained from brains that were infused intra-hippocampally with DPAT (final intrahippocampal concentration of 100 nM) in the absence or presence of different pharmacological inhibitors. We observed that DPAT treatment caused a significant increase in the number of hippocampal synapses in the CA1 region. This increase in synaptogenesis was abolished in presence of any of the inhibitors: U0126 (MEK inhibitor); Ro 31-8220 (selective PKCα inhibitor at 10 nM) and WAY (5-HT_{1A}-R antagonist). This confirms that 5-HT_{1A}-R mediated signaling *via* Erk and PKCα causes induced synaptogenesis *in vivo* in P15 mouse hippocampus (Fig. 3.5).

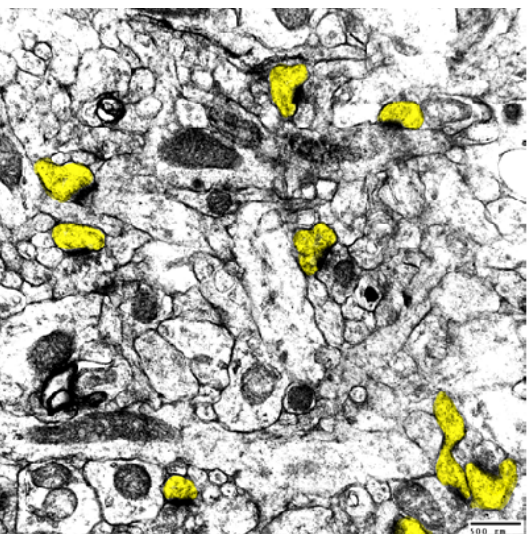
Fig. 3.5: Serotonin 1A receptor-mediated signaling induces synaptogenesis in the P15 hippocampus *in vivo*. (a) Electron microscopy revealed a significant increase in synapse number in the hippocampal CA1 region of the DPAT-infused mice. This DPAT-stimulation of synaptogenesis was eliminated in the presence of WAY100635 (4 μM), U0126 (10 μM), and Ro 31-8820 (Scale bar: 500 nm). **(b)** Quantification from three experiments revealed a significant effect of 5-HT_{1A}-R signaling, which was eliminated in the presence of U0126 and Ro 31-8820 (*$p < 0.001$ DPAT versus all other groups).



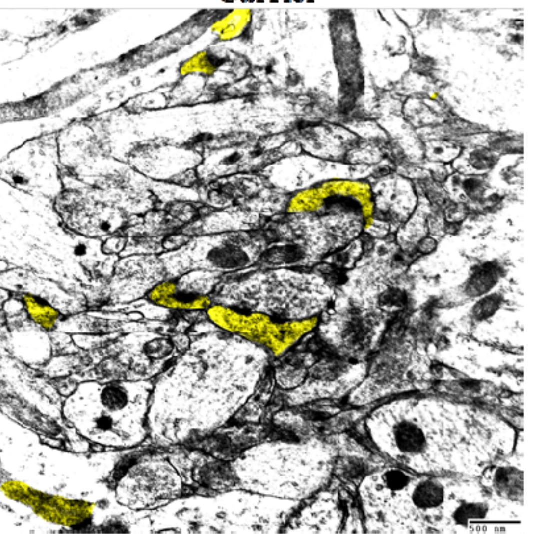
Carrier



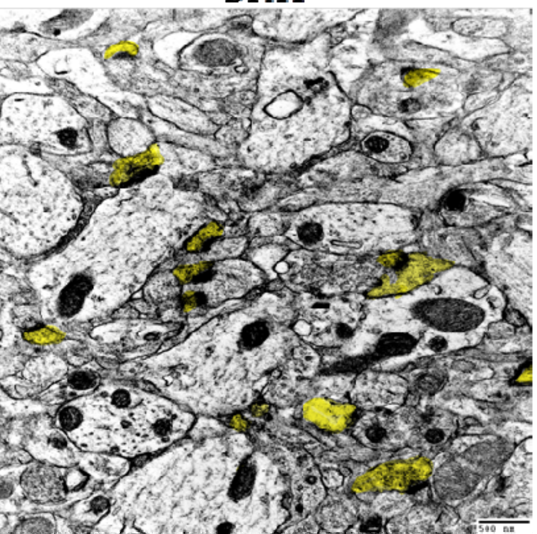
DPAT



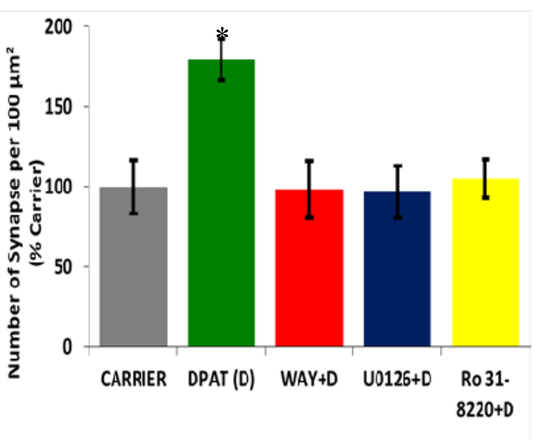
WAY + DPAT



(a) U0126 + DPAT



Ro 31-8220 + DPAT

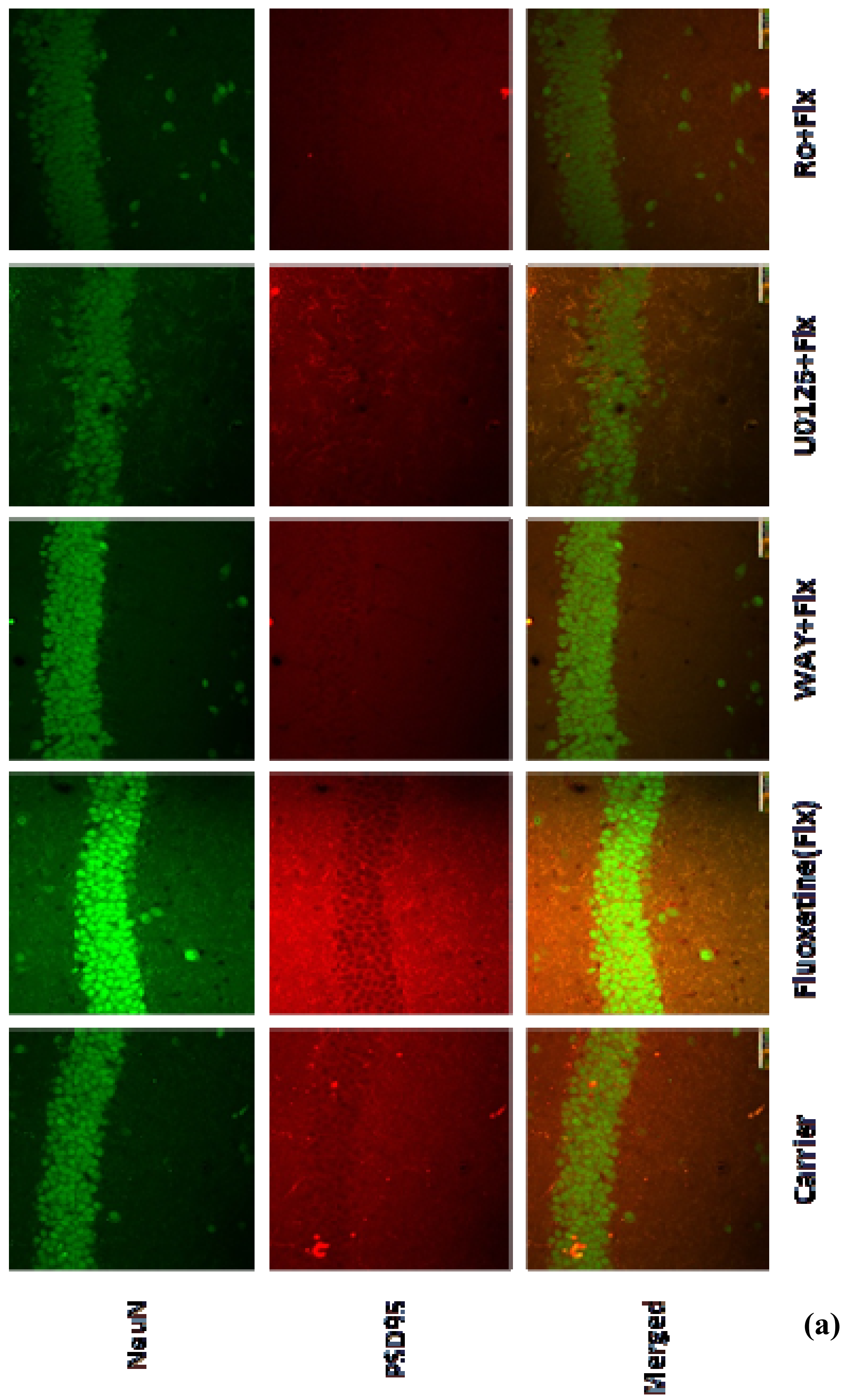


(b)

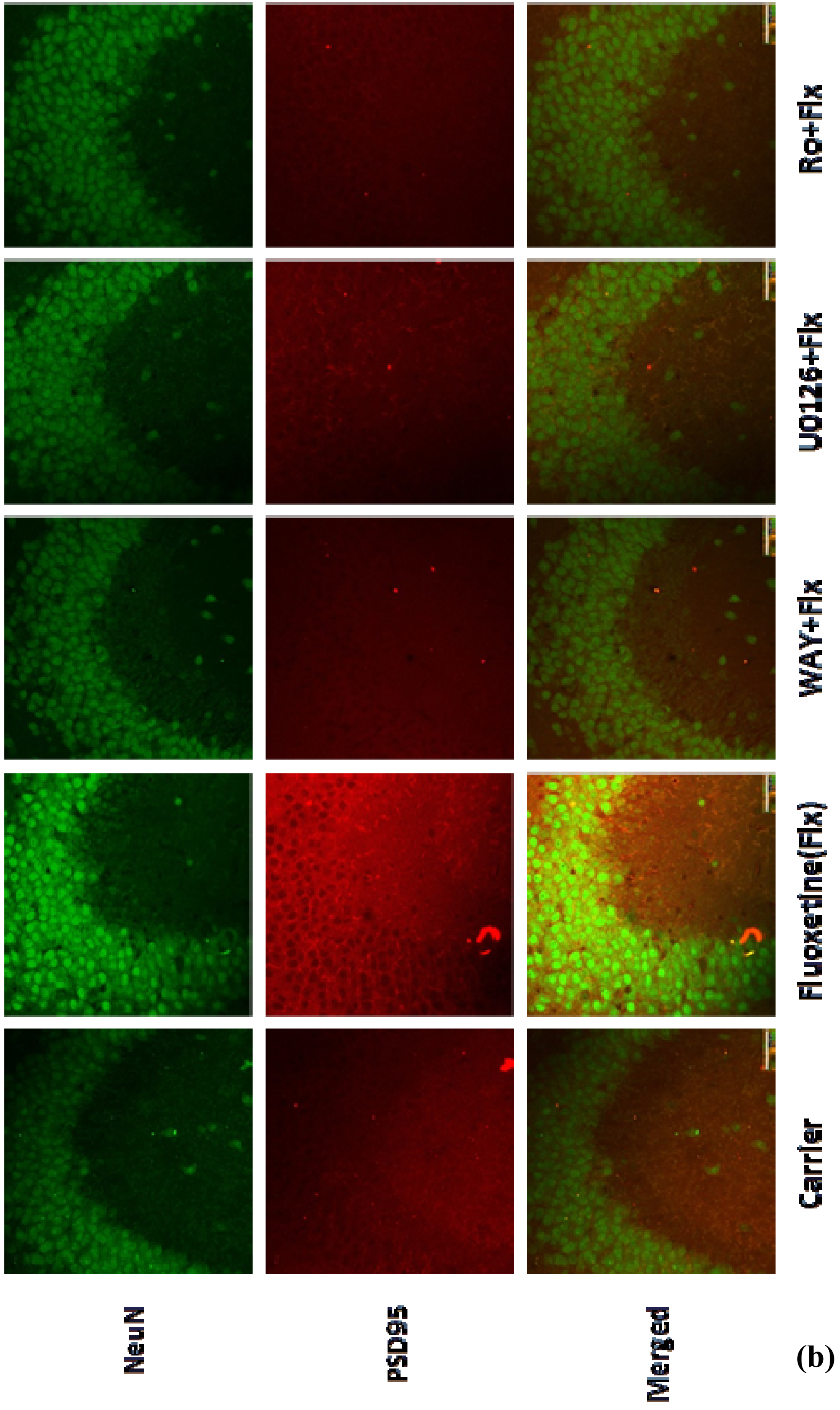
Intrahippocampal infusion of fluoxetine causes 5-HT$_{1A}$-R-, Erk1/2-, and PKCα-dependent induction of PSD95:

Although systemic infusion causes the same effect (He 2001; Malagie 2002), direct intracortical infusion of an inhibitor of the serotonin reuptake protein (SERT) causes a 4-5-fold induction of serotonin release within 60 min (de Groote 2001). We investigated if this augmented serotonin release at the hippocampal, serotonergic synapses elicit the same 5-HT$_{1A}$-R, Erk, and PKCα mediated synaptogenic signaling in P15 mice. As shown in Fig. 3.6, intrahippocampal infusion of fluoxetine into P15 mice caused a significant increase in PSD95 expression and this effect was eliminated upon inhibition of the Erk activator MEK with U0126 and PKCα with Ro 31-8220.

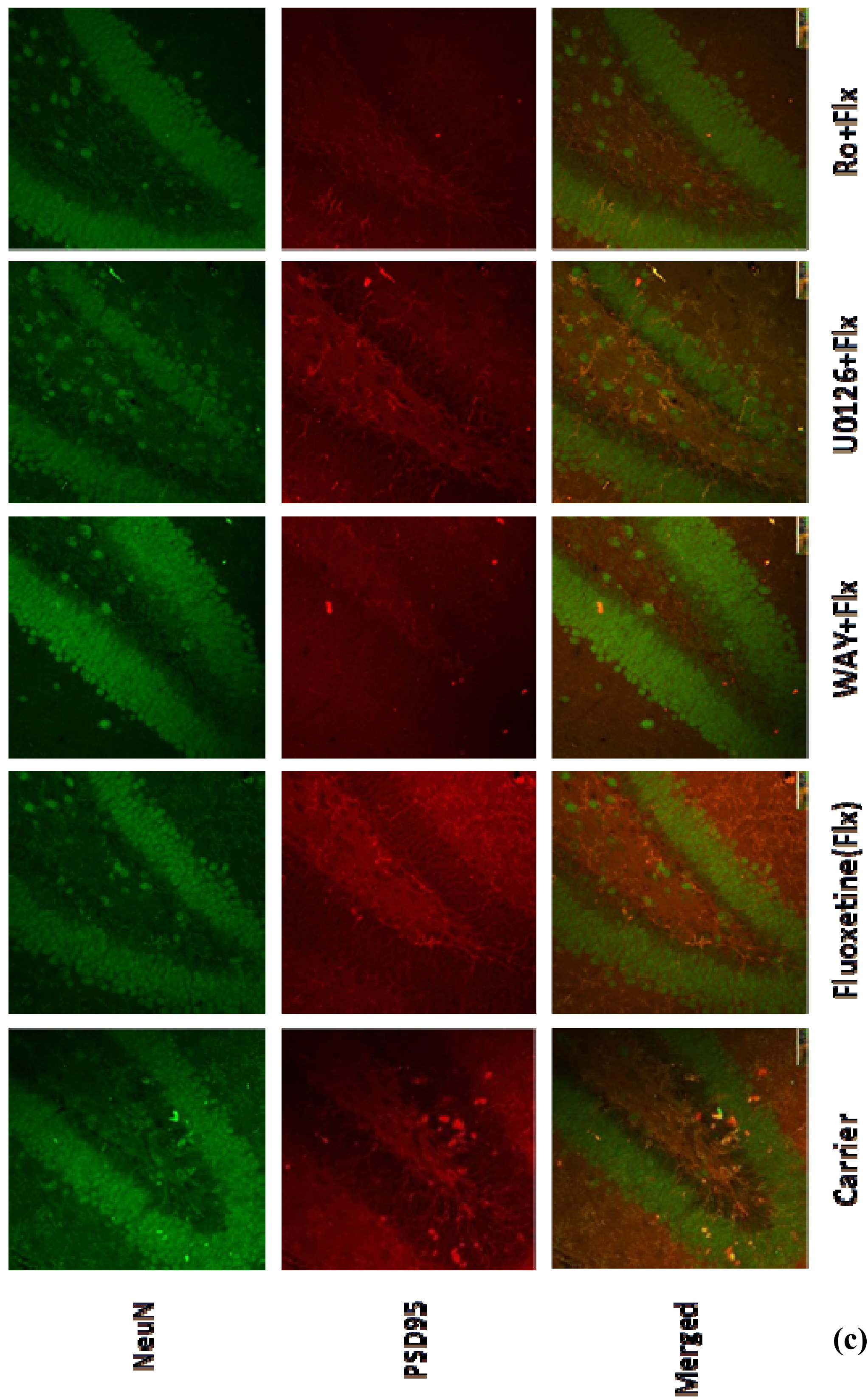
Figure 3.6: Intra-hippocampal infusion of fluoxetine into P15 hippocampus causes induction of PSD95 through 5-HT$_{1A}$-R signaling involving Erk and PKCα. (a-c) Intrahippocampal infusion of fluoxetine (Flx) caused a significant increase in PSD95 expression in all regions of the hippocampus, i.e. CA1, CA3, and DG respectively (Scale bar: 50 μm). **(d-f)** Quantification from three experiments each performed with quadruplicate sections showed a significant increase in PSD95 expression in the presence of Flx, which was eliminated in the presence of WAY100635, U0126, and Ro 31-8820 (*$p < 0.0001$ Flx versus all other groups for each hippocampal region).



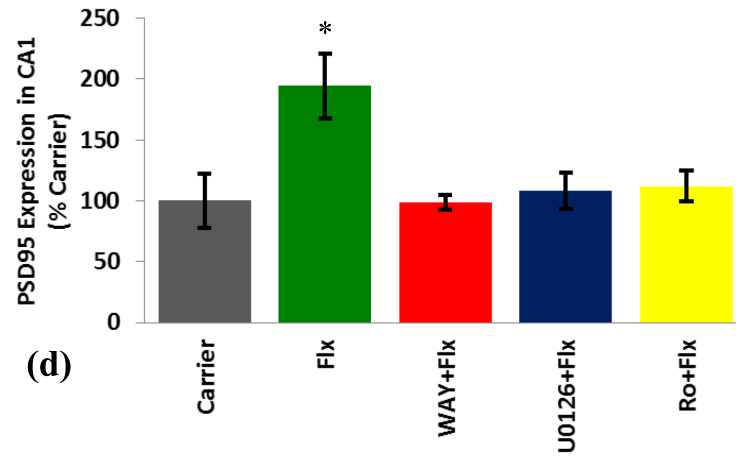
(a)

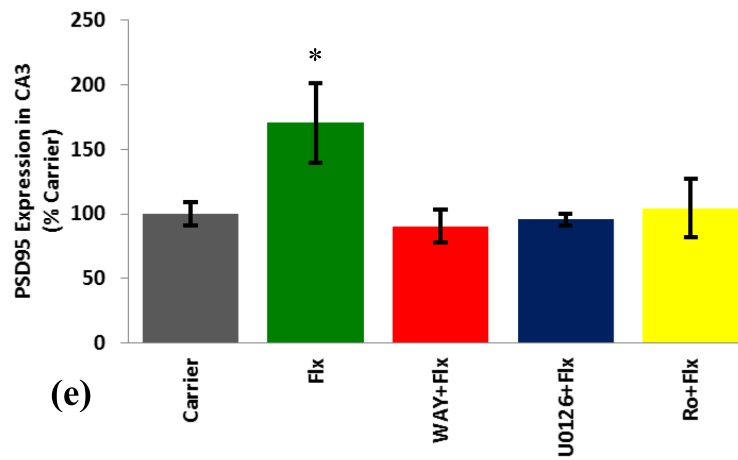


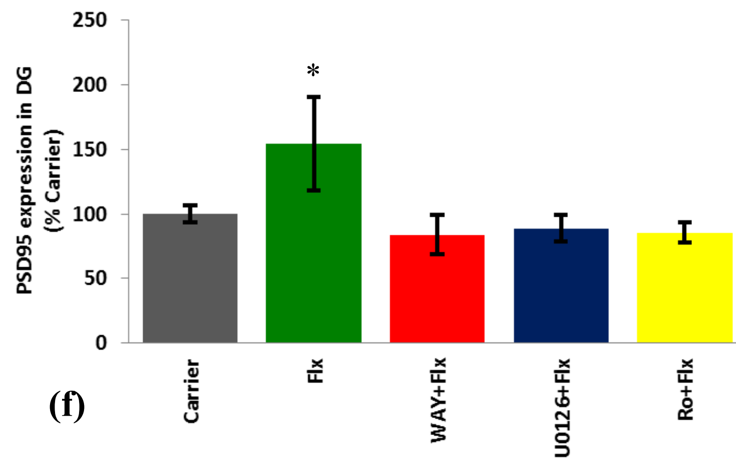
(b)



(c)


PSD95 Expression in CA1
(% Carrier)
250
200
150
100
50
0
*
Carrier
Flx
WAY+Flx
U0126+Flx
Ro+Flx
(d)

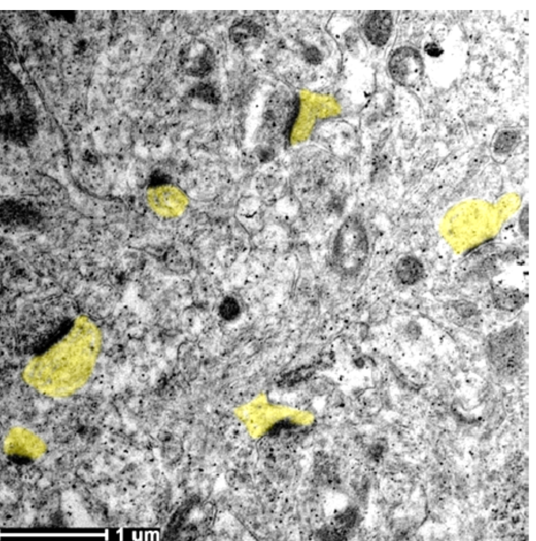

PSD95 Expression in CA3
(% Carrier)
250
200
150
100
50
0
*
Carrier
Flx
WAY+Flx
U0126+Flx
Ro+Flx
(e)


PSD95 expression in DG
(% Carrier)
250
200
150
100
50
0
*
Carrier
Flx
WAY+Flx
U0126+Flx
Ro+Flx
(f)

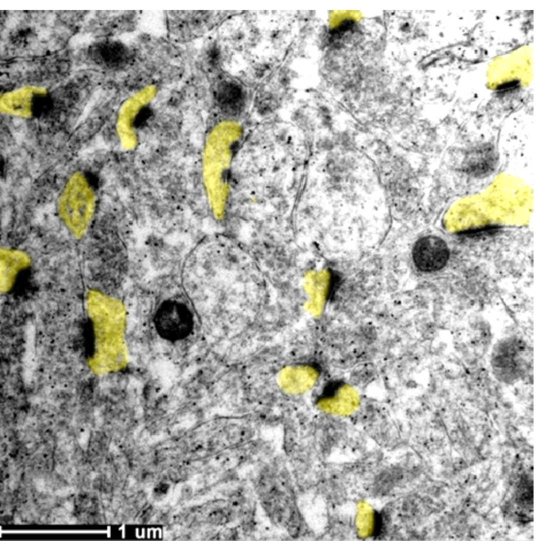
Intrahippocampal infusion of fluoxetine causes 5-HT$_{1A}$-R-, Erk1/2-, and PKCα-dependent induction of Synaptogenesis:

We investigated if the augmented serotonin release, after fluoxetine infusion at the hippocampal serotonergic synapses, elicits the same 5-HT$_{1A}$-R, Erk, and PKCα mediated synaptogenic signaling in P15 mice. As shown in Fig. 3.7, intrahippocampal infusion of fluoxetine into P15 mice caused a significant increase in the number of synapses and this effect was eliminated upon inhibition of the Erk activator MEK with U0126 and PKCα with Ro 31-8220. This effect is in accordance with an increased expression of PSD95.

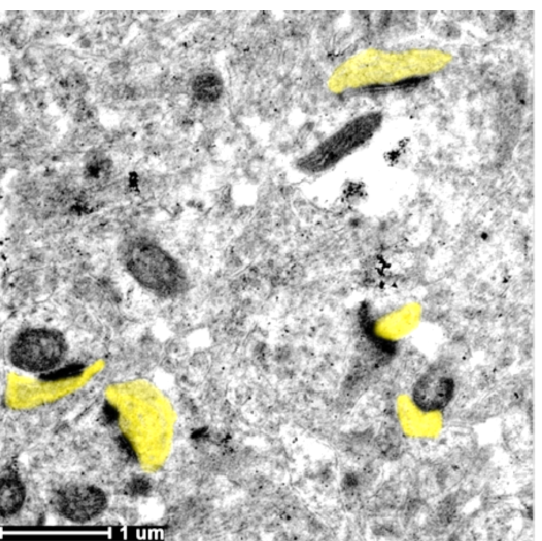
Figure 3.7: Intra-hippocampal infusion of fluoxetine into P15 hippocampus causes increased number of synapses through 5-HT_{1A}-R signaling involving Erk and PKCα. (a) Intrahippocampal infusion of fluoxetine (Flx) caused a significant increase in the number of synapses in all CA1 region of the hippocampus (Scale bar: 1 μm). **(b)** Quantification by counting the number of synapses manually with quadruplicate sections showed a significant increase in number of synapses in the presence of Flx, which was eliminated in the presence of WAY100635, U0126, and Ro 31-8820 (*$p< 0.0001$ Flx versus all other groups).



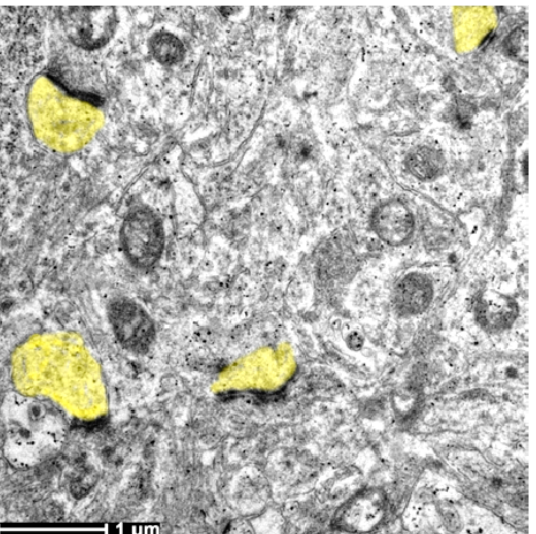
Carrier



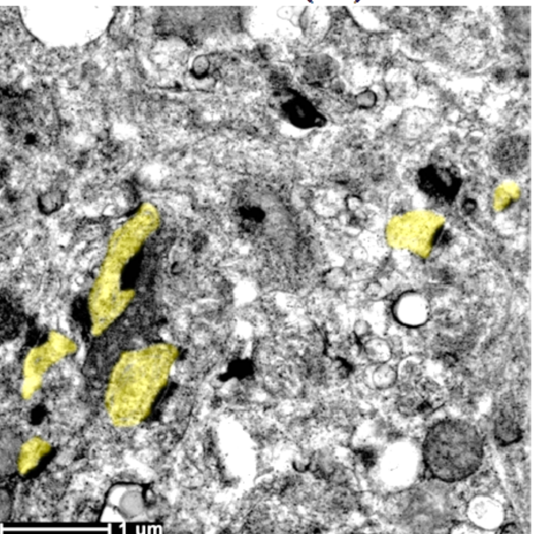
Fluoxetine (Flx)



WAY+Flx

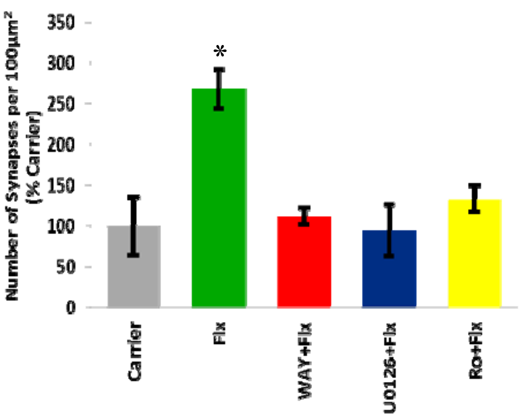


U0126+Flx



Ro 31-8220+Flx

(a)



(b)

The hierarchy of signaling molecules in the 5-HT$_{1A}$-R➔➔Erk1/2➔PKCα pathway in C57BL6 mouse hippocampus:

The widespread induction of PSD95 over the entire hippocampus enabled us to perform Western blot analysis on whole hippocampal lysates to study the possible link among 5-HT$_{1A}$-R, Erk, and PKCα. We used T^{638} phosphorylation of PKCα and dual phosphorylation of T^{202} and Y^{204} on Erk1/2 as a measure of stimulation of these kinases. As shown in Fig. 6a and b, short-term stimulation of the hippocampal 5-HT$_{1A}$-R by intrahippocampal infusion of 8-OH-DPAT (final concentration of 100 nM) in the absence and presence of inhibitors followed by sacrifice of the treated mice after 60 minutes, removal of the hippocampi and lysis in protease-containing buffers yielded protein samples that could be analyzed by Western blotting for P-PKCα and P-Erk. We observed a significant induction of P-PKCα in the DPAT-infused hippocampi, which was eliminated in the presence of WAY100635, U0126, and Ro 31-8220 (Fig. 3.8a). In contrast, 5-HT$_{1A}$-R-mediated induction of P-Erk was eliminated in the presence of WAY100635 and U0126 but not Ro 31-8220 (Fig. 3.8b). These two sets of data place Erk above PKCα in a 5-HT$_{1A}$-R signaling pathway, which is most probably involved in 5-HT$_{1A}$-R, Erk, and PKCα-mediated signaling the long-term effect of which would be increased synaptogenesis (Fig. 3.8).

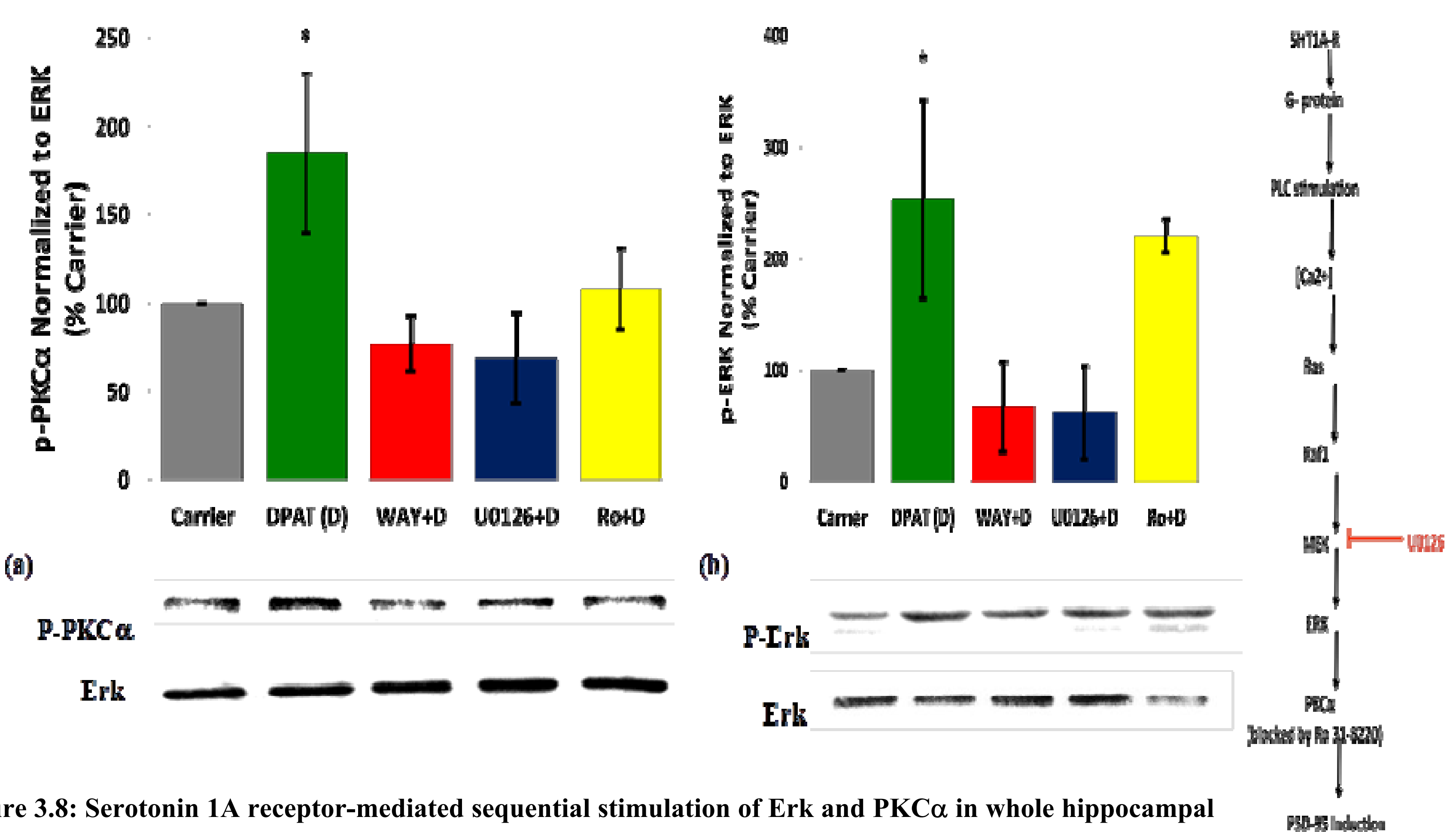


Figure 3.8: Serotonin 1A receptor-mediated sequential stimulation of Erk and PKCα in whole hippocampal tissue from treated mice. **(a)** and **(b)**. The PKCα stimulation is blocked in the presence of both U0126 (inhibits MEK) as well as Ro 31-8220 (Ro) (inhibits only PKCα ≤10 nM), but the activation of Erk is not blocked by Ro. **(c)** Data presented in (a) and (b) place Erk above PKCα in a signaling pathway, as elucidated in our earlier reports (Adayev 2003; Mehta, Ahmed et al. 2007). (a, *$p < 0.0001$ DPAT versus all other groups); (b, $p<0.005$ DPAT versus Carrier; $p < 0.001$ DPAT versus WAY100635 and U0126; $p=1$ DPAT versus Ro31-8220).

Downstream involvement of PKCα confirmed and utilized to induce PSD95 and synaptogenesis in C57BL6 5-HT_{1A}-R (-/-) mice

To further confirm the role of 5-HT_{1A}-R mediated signaling pathway in the expression of PSD95, we used 5-HT_{1A}-R (-/-) C57BL6 mice. These mice were injected with Carrier, DPAT and Bryostatin. Bryostatin is a well-known memory enhancing drug which activates PKCα (Hongpaisan and Alkon 2007). We observed that PSD95 expression was significantly higher ($P<0.0001$) in Bryostatin treated brains than carrier treated or DPAT treated ones (Fig. 3.9 a-c). This enhancement was seen in CA1 region of the hippocampus (Fig. 3.9) as well as CA3 and DG regions of the hippocampus.

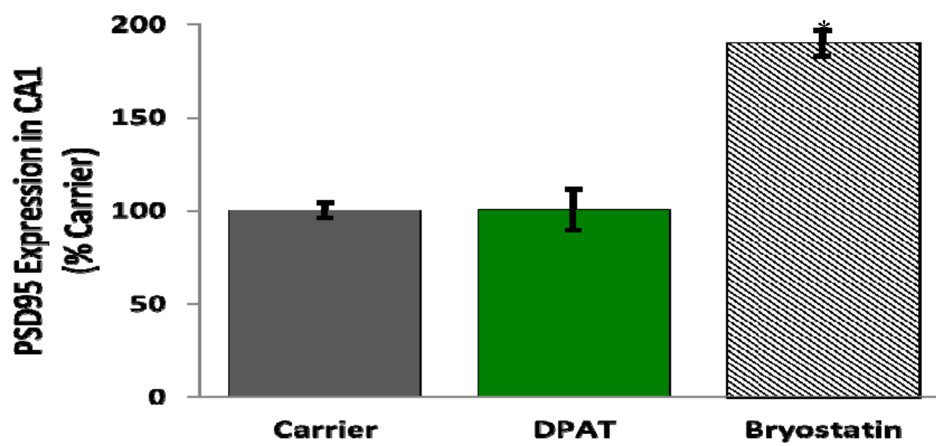
Figure 3.9: Bryostatin treatment of P15 5-HT_{1A}-R (-/-) C57BL6 mice leads to heightened expression of PSD95 and boosted synaptogenesis. (a-c) In the 5-HT_{1A}-R-deficient mice, PSD95 staining is increased in the CA1, CA3 and DG regions in bryostatin- but not DPAT-infused mice (Scale bar 50 µm). **(d)** Electron microscopy revealed a simultaneous increase in the number of synapses in the bryostatin-treated animals (Scale bar: 500 nm) (f, $p < 0.0001$ bryostatin versus all other groups; $n=3$).

PSD95

(b)

Merged

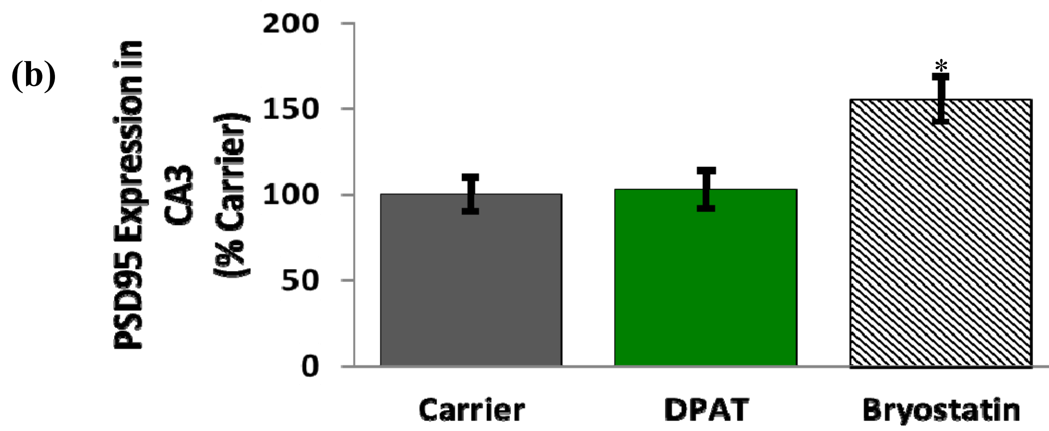
(a) Carrier DPAT Bryostatin



(c)

Carrier DPAT Bryostatin

(b)



NeuN

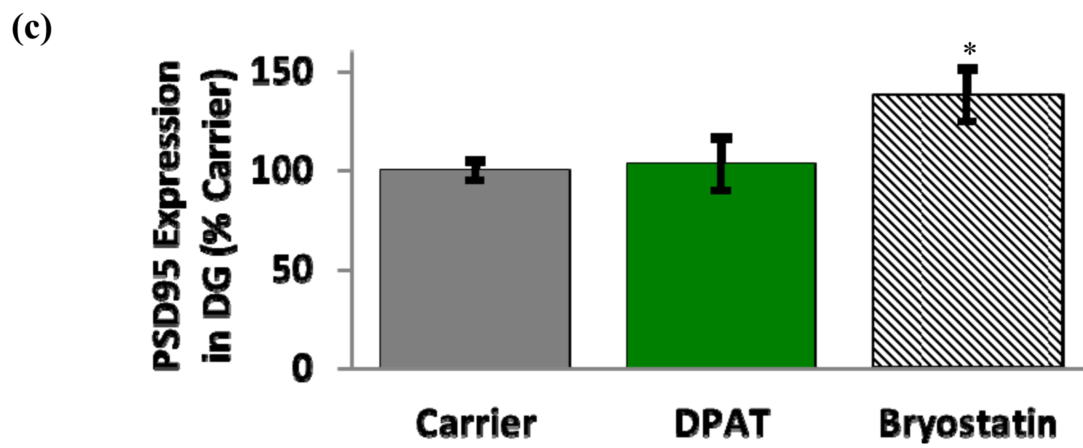
(d)

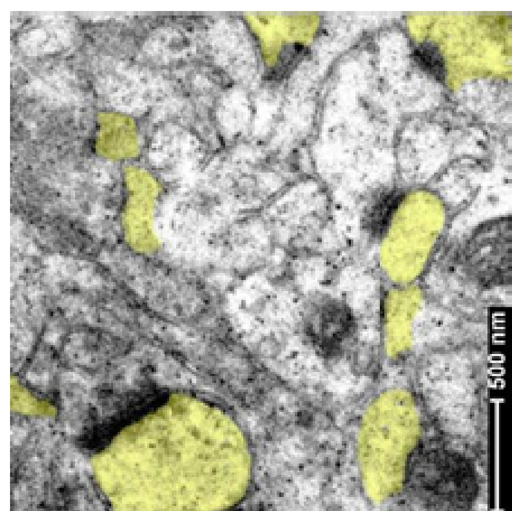
PSD95

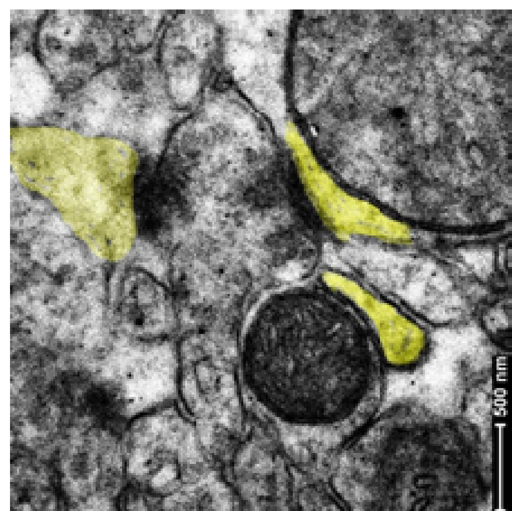
Merged

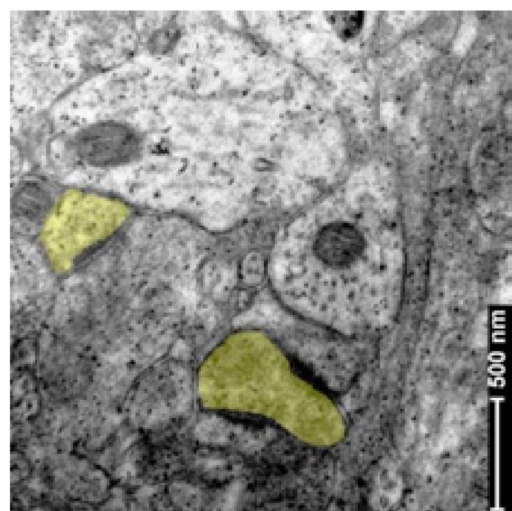
Carrier DPAT Bryostatin

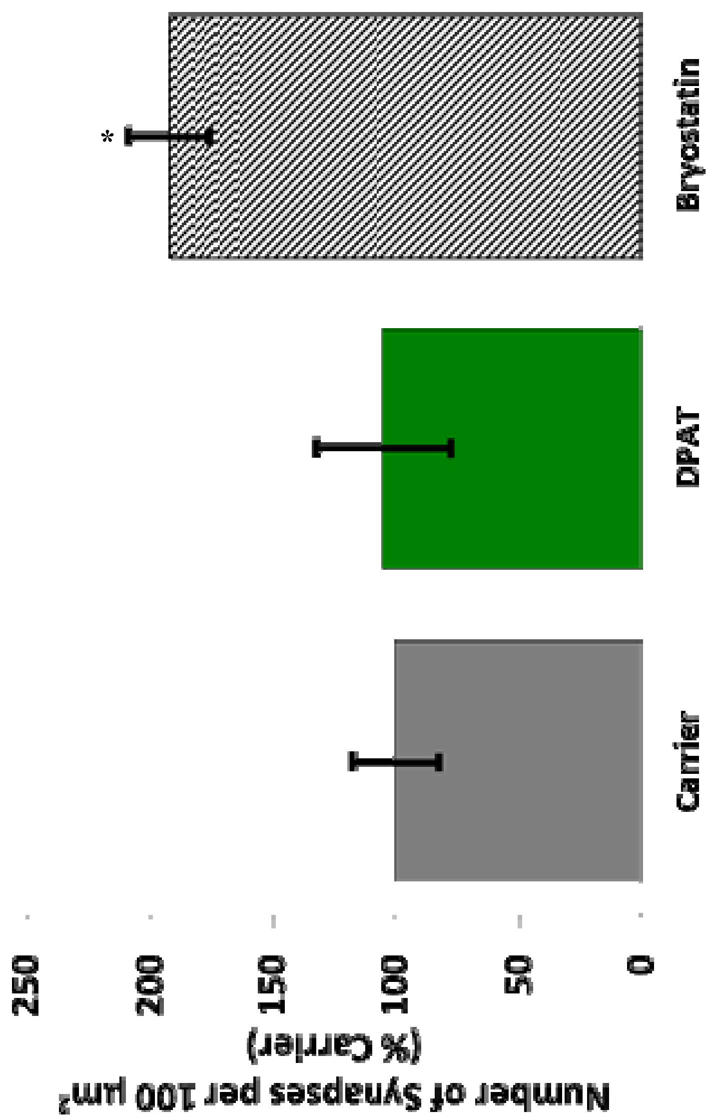
(c)











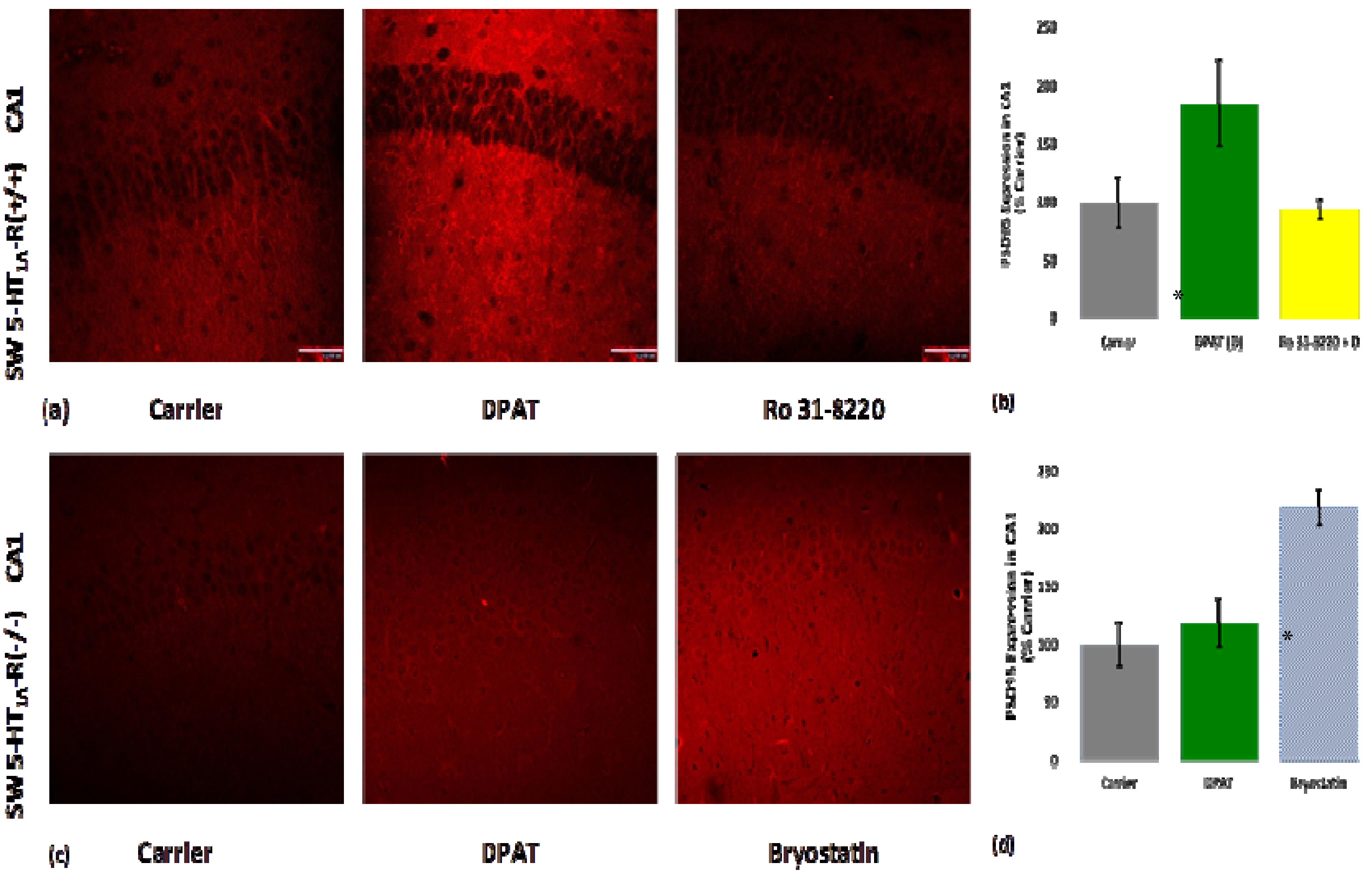
(d)

***In vivo* analysis in Swiss Webster mice:**

Immunohistochemistry in 5-HT_{1A}-R (+/+) mice to show the involvement of PKCα in the induction of PSD95

In order to confirm the involvement of PKCα in the increased expression of PSD95 we performed study with 5-HT_{1A}-R (-/-) mice. In the 5-HT_{1A}-R (-/-) mice while the receptor is altogether absent we stimulated a downstream molecule, PKCα by injecting suggested dosage of Bryostatin (Hongpaisan and Alkon 2007) intra-hippocampally. The results show that in these mice DPAT treated hippocampi have no increase in PSD95 staining compared to the carrier treated ones while the expression levels of PSD95 in Bryostatin treated hippocampi was significantly higher (Fig. 3.10 b). This confirms that the 5-HT_{1A}-R mediated signaling pathway leads to higher expression of PSD95. A parallel experiment with wild type mice with intact 5-HT_{1A}-Rs shows that even if 5-HT_{1A}-R is activated, inhibition of a downstream effector, PKCα, results in the down regulation of PSD95 (Fig. 3.10 a).

Figure 3.10: 5-HT_{1A}-R and PKCα-mediated induction of PSD95 also occurs in SW mice: downstream stimulation of PKC with bryostatin elicits induction of PSD95 in SW 5-HT_{1A}-R(-/-) mice. (a) DPAT-infusion caused an increase in PSD95 expression, which was eliminated in the presence of WAY100635 (Scale bar: 50 μm). **(b)** Quantification of volume-rendered images from three experiments revealed that this induction was significant ($p < 0.0001$ DPAT versus all other groups). **(c, d)** In the 5-HT_{1A}-R deficient mice, bryostatin but not DPAT infusion caused a significant increase in PSD95 expression in the CA1 region ($p < 0.0001$ DPAT versus all other groups) (Scale bar 50 μm).


SW 5-HT1A-R(+/+) CA1
(a)
Carrier
DPAT
Ro 31-8220
250
200
150
100
50
0
*
(b)
SW 5-HT1A-R(-/-) CA1
(c)
Carrier
DPAT
Bryostatin
0
*
(d)

Absence of the 5-HT$_{1A}$-R results in a decreased density of excitatory synapses in the hippocampus of P15 mice:

Based on our observation that 5-HT$_{1A}$-R signaling in the hippocampus was critical in synaptogenesis, an obvious question remains that "Is the hippocampal synaptic density significantly lower in the 5-HT1A-R (-/-) mice?" As shown in Figure 3.11, the 5-HT$_{1A}$-R (-/-) mice harbor a significantly lower number of excitatory synapses in the CA1 region. As explained in the discussion, this could have a major detrimental effect on hippocampal maturation, which in turn could account for the behavioral complications observed in these mice.

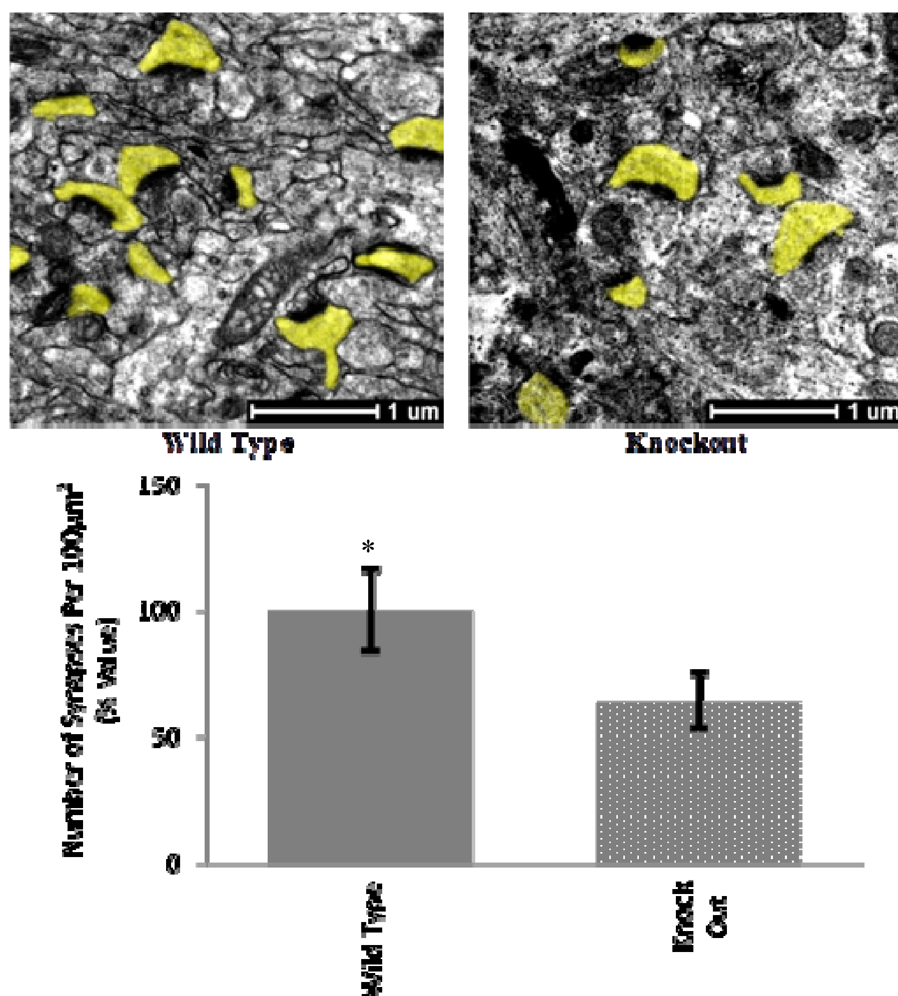


Figure 3.11: The 5-HT_{1A}-R(-/-) mice harbours less hippocampal synapses. Comparison of the electron micrographs of the CA1 area from the Wild Type (top left) and 5-HT_{1A}-R knockout (top right). Absence of 5-HT_{1A}-R leads to a less number of synapses in this area which is evident from the quantification (lower panel).

5-HT_{1A}-R mediated signaling leads to stable increase in synaptogenesis:

Activation of 5-HT_{1A}-R mediated signaling pathway, by injecting DPAT or by increasing the intrinsic serotonin levels, leads to an increase in the number of synapses in the CA1 area of hippocampus. This increase in the synapse number is stable which is evident from significant difference in the numbers of synapses in Carrier treated and agonist treated mice after five days of injections. Figure 3.12 shows increased synapses with the post synaptic areas pseudo colored in yellow.

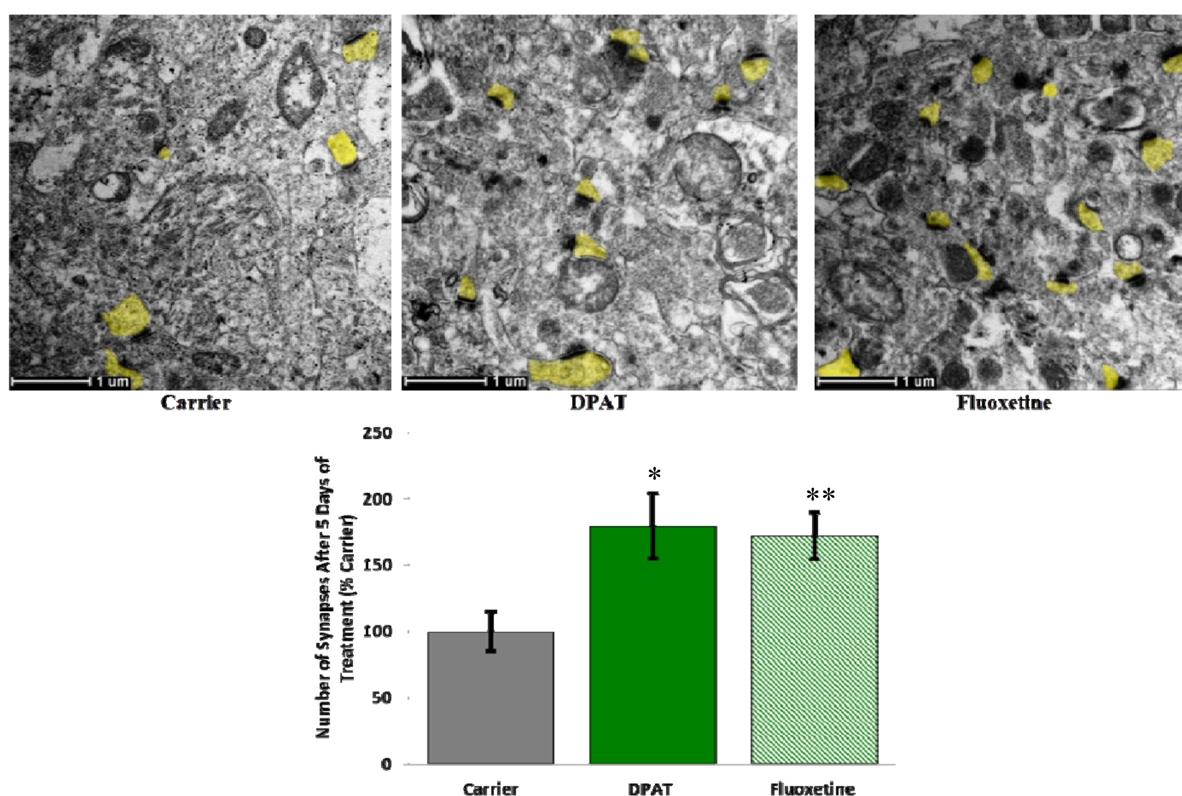


Figure 3.12: 5-HT_{1A}-R mediated synaptogenesis is stable: Higher synaptic density persists in the CA1 region five days after 8-OH-DPAT or Fluoxetine treatment at P15. Significantly higher number synapses were observed five days after the infusion of DPAT or Flx ($n = 3$, $p < 0.001$ for both D and Flx, compared to carrier-treated).

CHAPTER 4

DISCUSSION

Summary of the key findings:

- 5-HT1A-R mediates synaptic strengthening in the hippocampus at P15.
- 5-HT1A-R mediated signaling pathway via MAPK and PKC leads to an increase in the expression of PSD95.
- Activation of 5-HT_{1A}-R mediated signaling in organotypically hippocampal slices leads to increase in number of dendritic spines as well as synapses in the hippocampus.
- *In vivo* experiments including the intra-hippocampal injections confirmed the role of 5-HT_{1A}-R mediated signaling pathway in PSD95 expression.
- Electron microscopy confirmed that the 5-HT1A-R mediated signaling pathway leads to an increase in the number of synapses in hippocampus.
- Increasing the serotonergic firing by SSRI infusion leads to PSD95 induction which is dependent on 5-HT_{1A}-R mediated signaling pathway via MAPK→PKCα
- Absence of 5-HT_{1A}-R can be bypassed by activating a downstream effector protein. Bryostatin treatment leads to an increase in the PSD95 expression in the 5-HT_{1A}-R (-/-) mice and can rescue the synapse deficit in these mice, which confirms the involvement of PKCα in PSD95 induction.

- $5\text{-}HT_{1A}$-R→→MAPK→PKCα pathway is not strain specific: it is functional and leads to PSD95 induction in Swiss Webster mice.

- Absence of the $5\text{-}HT_{1A}$-R leads to a deficit in the number of synapses in CA1 at P15.

- $5\text{-}HT_{1A}$-R mediated synaptogenesis is stable and increased synapses do not diminish even after 5 days of agonist infusion.

Systemic elimination of a gene product, such as the 5-HT$_{1A}$-R affects both 5-HT-synthesizing raphé neurons and also the 5-HT-free postsynaptic front brain neurons, which disallows us to study the effect of selective effect in the hippocampus. Similarly, application of pharmacological agents, such as Ro 31-8220 and bryostatin, would affect the PKCα activity all over the body. The strength of our approach is that we have avoided the ensuing confound in both situations by inhibiting the 5-HT$_{1A}$-R pathway locally, *via* intra-hippocampal infusion of pharmacological agents. After elucidating the 5-HT$_{1A}$-R-linked cascade up to PKCα, we have confirmed the lack of its synaptogenic effect in the 5-HT$_{1A}$-R(-/-) mice and then used the acquired knowledge to trigger synaptogenesis by stimulating a downstream signaling member (PKCα) of the same pathway.

As shown in earlier studies, the presence of the 5-HT$_{1A}$-R in the front brain of neonatal mice is critical for the development of normal anxiety levels in adulthood (Gross 2002). Furthermore, overexpression of the 5-HT$_{1A}$-R in the presynaptic raphé neurons of neonatal mice, which is expected to cause diminished 5-HT firing at synapses with the post-synaptic front-brain targets (Banerjee 2007), results in depression-like symptoms in adulthood (Richardson-Jones, Craige et al. 2010). Although such findings demonstrate the importance of the 5-HT$_{1A}$-R in neonatal front brain development, how exactly this receptor would regulate brain development was unclear. Results presented in our study elucidate the critical role of a 5-HT$_{1A}$-R-mediated signaling cascade *via* Erk and PKCα in the formation of post-synaptic density (PSD)-containing synapses during early postnatal hippocampal development at P15. Our data demonstrate that the effects of the inhibitors of the Erk pathway (U0126) and PKCα (Ro 31-8220) on PSD95 induction and synaptogenesis were mutually occlusive, thus establishing that these two signaling molecules were in the same pathway. Short term 8-OH-DPAT treatment (60 min) followed by

Western blotting revealed that the activation of PKCα was sensitive to U0126, which placed PKCα downstream of Erk. This inference is consistent with our earlier observation that in hippocampal neuron-derived HN2 cells, Erk physically binds to and phosphorylates PKCα at T^{638}, which is crucial for the activation PKCα (Debata 2010).

Electron microscopy demonstrates a significant increase in PSD-containing CA1 synapses. Since the presence of PSD characterizes excitatory synapses (de Bartolomeis 2004), this $5\text{-}HT_{1A}$-R-mediated pathway clearly plays a central role in the sculpting of the excitatory glutamatergic synapses at the CA1 pyramidal neurons. In acutely isolated hippocampal slices from P15 mice, $5\text{-}HT_{1A}$-R signaling causes an increase in field excitatory post-synaptic potential (fEPSP) in the Schaffer Collateral pathway (Mehta, Ahmed et al. 2007). This may occur due to 5-HT firing from the serotonergic raphé neurons at their synaptic contacts with $5\text{-}HT_{1A}$-R- and GABA-expressing interneurons, thereby causing $5\text{-}HT_{1A}$-R-mediated stimulation of hyperpolarizing K^+ channels (Dascal 1993; Okuhara 1994; Jeong, Han et al. 2001), and inhibition of GABA release at synaptic contacts of the interneurons with the glutamatergic neurons of the Schaffer Collateral pathway. Alternatively, the 5-HT raphé neurons that form synapses with the $5\text{-}HT_{1A}$-R-containing glutamatergic neurons in the hippocampus cause postsynaptic $5\text{-}HT_{1A}$-R signaling that directly suppresses $GABA_A$ receptor-mediated inhibitory signaling *via* PKC-mediated phosphorylation of the $GABA_A$ receptors in the glutamatergic neurons of the hippocampus (Leidenheimer, McQuilkin et al. 1992).

Prior studies have shown that multiple subunits of the $GABA_A$ receptor are substrates of the Ca^{2+}-dependent conventional PKCs. The resultant phosphorylation inhibits the $GABA_A$ receptor (Leidenheimer, McQuilkin et al. 1992) and leads to its internalization (Chapell, Bueno

et al. 1998). Consequently the $GABA_A$ receptor-gated chloride current is reduced when PKC is activated, an effect that is abrogated by PKC inhibitors (Leidenheimer and Chapell 1997). Additionally, PKC-mediated phosphorylation of the *β3* subunit of the $GABA_A$ receptor at Ser409 or Ser410 elicits a decrease in $GABA_A$ activity in cortical neurons (Brandon, Delmas et al. 2000). Finally, this PKC-mediated phosphorylation regulates $GABA_A$ receptor activity *in vitro* as well as *in vivo* (Kumar, Khisti et al. 2005). Thus, the activation of PKCα (a conventional Ca^{2+}-dependent PKC) *via* $5\text{-}HT_{1A}$-R mediated signaling is expected to cause an inhibition of $GABA_A$ receptor function in the hippocampus. Such overall reduction of inhibitory signals leads to an increase in the net excitatory signals received by the pyramidal neurons in the CA1 area. This is consistent with our previous finding that fEPSP increases in a dramatic manner in the pyramidal neurons of the CA1 area in acutely isolated hippocampal slices within 30 minutes of 8-OH-DPAT treatment (Mehta, Ahmed et al. 2007).

Augmented electrical activity due to the dis-inhibition mechanisms described above could cause increased synaptic strength, induction in PSD95, and augmented synaptogenesis as observed in the hippocampus after 24-h of *in vivo* 8-OH-DPAT treatment. However, beyond the acute phase of fEPSP (beyond 2 h), alternative and Erk and PKC-dependent mechanisms could also operate to cause such induction. Prior studies by Elkobi and coworkers have demonstrated Erk-dependent PSD95 induction in the gustatory cortex as an essential step in taste learning (Elkobi 2008), however, the link between Erk activity and PSD95 induction is not clear yet. Our results strongly indicate that Erk-dependent induction of PSD95 involves PKCα in the hippocampal neurons. Among molecules that could link PKC to the induction of PSD95, neuroligin-1 (Nrg), which elicits induced expression of PSD95 (Bao 2004), is a likely candidate. The intracellular domain of Nrg is shed in a PKC-dependent manner (Ozaki 2004), following

which this intracellular Nrg fragment complexes with the transcription factor Eos to bind to an enhancer element in the *PSD95* promoter to elicit induced expression of PSD95 (Bao 2004).

The mouse and rat pups first open their eyes between P12 and P16, which is marked by a dramatic increase in PSD95 expression and redistribution into synapses (Yoshii 2003). As mentioned in the introduction, P15 is at the epicenter of a major synaptogenic event (Benitez-Diaz 2003), and a significant part of it is visual activity-driven. By elucidating 5-HT_{1A}-R, Erk, and PKCα-mediated PSD95 induction and synaptogenesis and also demonstrating that the absence of the 5-HT_{1A}-R in the 5-HT_{1A}-R(-/-) mice is associated with a decrease in synapse density in the CA1 region at P15, we have established that the hippocampal 5-HT_{1A}-R plays the essential role of orchestrating synaptogenesis. Our study strongly indicates that sluggish hippocampal development because of impaired 5-HT_{1A}-R, Erk, PKCα signaling could be remedied by the use of the memory-enhancing drug bryostatin. Our results also reveal that administration of fluoxetine (Prozac) also stimulates this signaling pathway, thereby enhancing PSD95 production, which is a reliable marker of synaptogenesis. Normal brain development requires that the early postnatal synaptogenesis be followed by a phase of synaptic pruning for the refinement of synaptic connections. Therefore, the use of Prozac during the period of pruning may impair brain development by triggering unwanted synaptogenesis through the same 5-HT_{1A}-R, Erk, PKCα pathway.

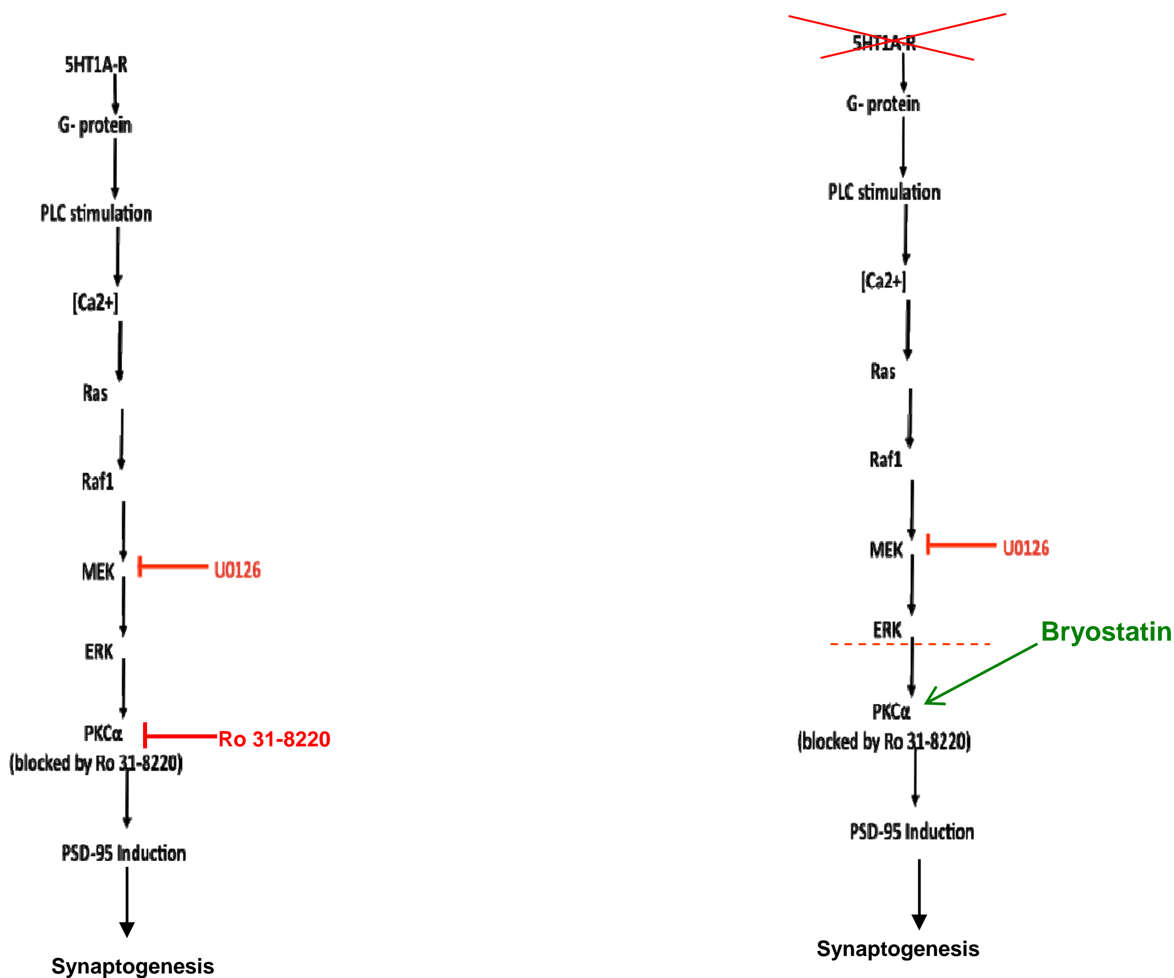


Figure 4.1: Possible mechanism for PSD95 expression and Synaptogenesis via 5-HT$_{1A}$-R mediated signaling pathway: (a) 5-HT$_{1A}$-R mediated signaling in wild type mice leads to PSD95 induction and synaptogenesis involving the sequential activation of ERK and PKCα; **(b)** Activation of a downstream effector PKCα in 5-HT$_{1A}$-R(-/-) mice can rescue the lack of synaptogenesis.

CONCLUSION AND POSSIBLE STRATEGIES FOR FUTURE STUDIE

Present study further opens up several possible directions to investigate. One of the major areas to explore is the behavioral implications of the effects of 5-HT_{1A}-R mediated signaling pathway during developmental phase of the brain. We have investigated that activation of 5-HT_{1A}-R via pharmacological agents or via increasing the serotonin levels, both lead to structural and molecular changes in the hippocampus. These changes are stable and the increased numbers of synapses do not diminish even after 5 days of the agonist treatment. The interesting question here would be that how does these structural changes affect the behavior of the animal. A possible strategy for investigating in this direction is given in figure 4.2. By blocking the 5-HT_{1A}-R signaling in the wild type mice by intrahippocampal stereotaxic injections of inhibitors or genetic knock down by siRNA at P15 while bypassing this pathway by activating PKCα, we can expect to observe behavioral changes during adulthood in these to mice populations. Because either by activating or by blocking the 5-HT_{1A}-R signaling we are either increasing the synaptogenesis or decreasing it from the normal baseline levels, we can expect behavioral deviation in both populations of mice.

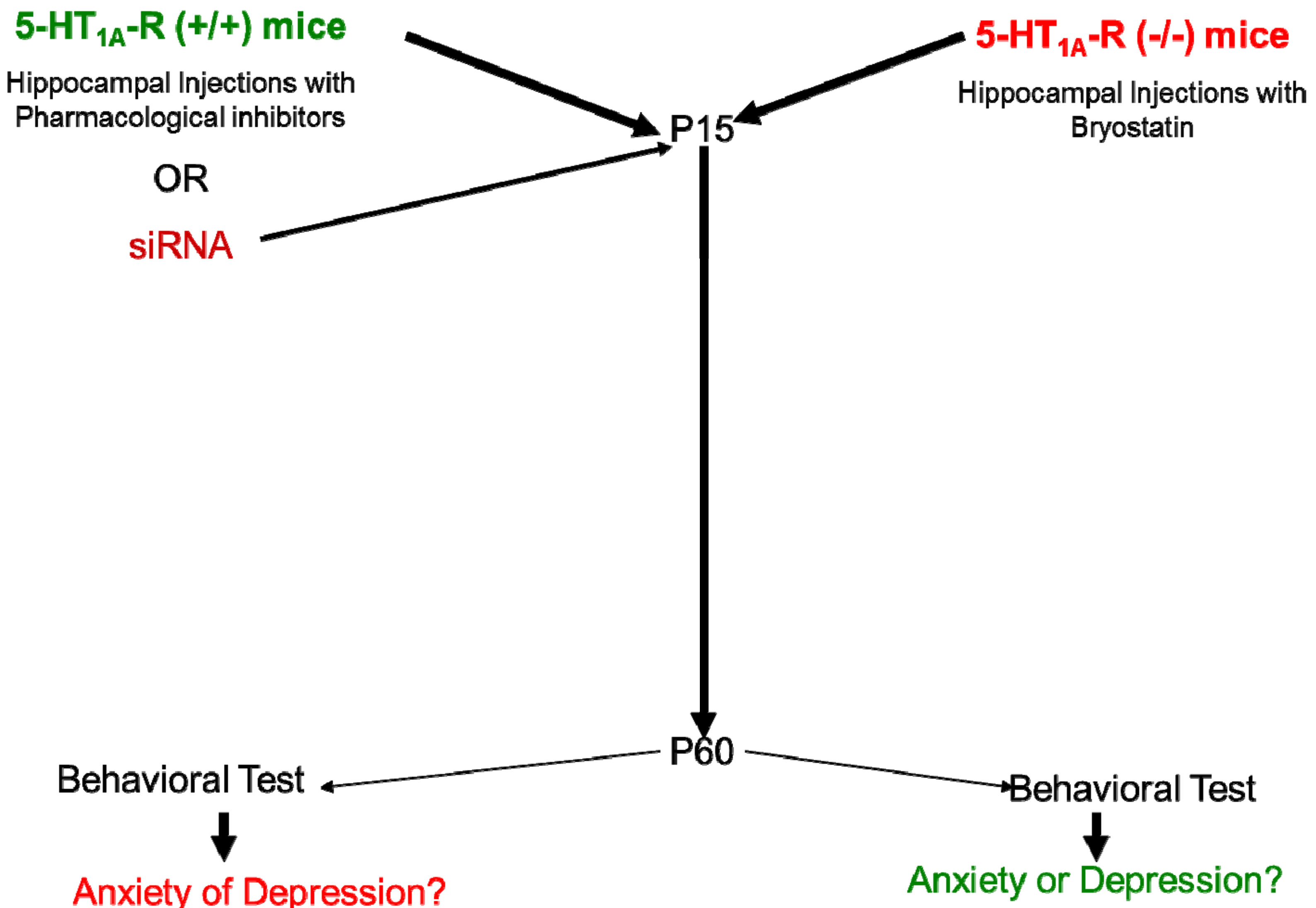


Figure 4.2: A possible strategy for future study: Blocking the 5-HT_{1A}-R mediated signaling in wild type mice and activating the pathway at downstream site in 5-HT_{1A}-R (-/-) mice, and investigating the behavioral changes during adult hood would be intriguing to monitor the role of this receptor in maintain the affective normalcy of the animal

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