AN IN VITRO CHARACTERIZATION OF THE RAPHE NUCLEUS AND THE EFFECTS OF SSRIS ON SYNAPTIC NEUROTRANSMISSION

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Dedicated to my parents,
Funke and Bashir Ashimi,
my sister Laide and brother Idris,
future husband Taofeek,
and the rest of my family and friends
for all their unconditional
love and continued support.

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AN *IN VITRO* CHARACTERIZATION OF THE RAPHE NUCLEUS AND THE EFFECTS OF SSRIS ON SYNAPTIC FUNCTION

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Antidepressants are traditionally used in the treatment of depression. While these drugs rapidly inhibit the reuptake of neurotransmitters in the synaptic terminal by blocking the serotonin and norepinephrine transporters, clinical efficacy can take several weeks. This phenomenon implies that antidepressants not only target neurotransmitter transporters, but have secondary down-stream effects that are important in the treatment of depression. Interestingly, depressed patients respond differently to similar antidepressant therapies, suggesting that although depression is due to a distrubance in the serotonergic neural system, alleviating depression involves more than enhancing

synaptic sertonin concentration. Attention must be given to postsynaptic targets and how elevated serotonin modulates these pathways. Some studies have sought to investigate the mechanism of this delay in antidepressant responses,however, few have investigated the influence antidepressants have on synaptic neurotransmission. Dysfunction in synaptic neurotransmission can have profound affects on the functionality of the nervous system. The goal of this thesis is to evaluate the influence of antidepressants on synaptic neurotransmission. To study this question from a mechanistic standpoint, a primary neuronal co-culture system of serotonergic raphe and hippocampal neurons, was developed. The use of the co-culture neuronal system was devised to recapitulate the raphe/hippocampal pathway, an important serotonergic pathway implicated in the pathophysiology of depression, and provide a foundation in which to study how antidepressants alter synaptic function. The most widely prescribed antidepressants are those that target sertonergic systems, and so selective serotonin reuptake inhibitors were used, with the intention that alterations in synpatic function would elucidate the pathomechanism of depression treatment. The use of raphe and hippocampal neurons provide a unique oppurtunity to manipilate the presynaptic selective serotonin reuptake inhibitor target; the serotonin transporters, have a postysnptic receptors to activate, as well as have an endogenous supply of serotonin. Employing electrophysiological techniques, we found that selective serotonin reuptake inhibitors effect synaptic function by modulation of N-methyl-D-aspartic acid receptors through serotonin 1 A heteroreceptors. Understanding how selective serotonin reuptake inhibitors effect synaptic function will offer a more in depth knowledge base regarding the pathophysiology of the delayed onset of antidepressant action in clinical depression.

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LIST OF ABBREVIATIONS

5-HT- 5-Hydroxytrptamine or Serotonin

8-OHDPAT- 8-hydroxy-2(di-*n*-propylamino) tetralin

AD- antidpressant

AMPA- alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic- acid

AP-5- D-2-amino-5-phosphonopentanoic acid

BDNF- brain derived neurotrophic factor

CIT- Citalopram

CNQX- 6-cyano-7-nitroquinoxaline-2,3-dione

CNS- central nervous system

CREB- cyclic adenosine monophosphate response element binding protein

DA- Dopamine

DAPI- 4',6-diamidino-2-phenylindole

DAT- dopamine transporters

DG- dentate gyrus

DIV- days *in vitro*

DRN- dorsal raphe nucleus

DSM IV- Diagnostic and Statistical Manuel of Mental Disorders

ECT- electroconvulsive therapy

eEF2- eukaryotic elongation factor 2

EPSC- excitatory postsynaptic currents

FLX- Fluoxetine

GABA- gamma (γ) - aminobutyric-acid

GFP- Green fluorescent protein

GPCR- G-protein coupled receptors

FST- forced swim test

HAM-D-Hamilton depression ratio scale

HEK-293- human embryonic kidney 293 cells

HELA- Henrietta Lacks immortalized cervical cancer stable cell line

HPLC- high performance liquid chromatography

IPSC- inhibitory postsynaptic currents

IR- immunoreactivity

LTP- long term potentiation

MAOI- monoamine oxidase inhibitors

MAP2- Microtubule associated protein 2

MDD- Major Depressive Disorder

mEPSC- miniature excitatory postsynaptic currents

mIPSC- miniature inhibitory postsynaptic currents

MK-801- dizocilpine

NE- Norepinephrine

NERI- norepinephrine reuptake inhibitor

NMDA- N-methyl-D-aspartic acid

LDCV- large dense core vesicles

PFC- prefrontal cortex

PSD-95- Postsynaptic Density- 95

PTX- Picrotoxin

sEPSC- spontaneous excitatory postsynaptic current

SERT- Serotonin Transporter

sIPSC- spontaneous inhibitory postsynaptic current

SSRI-selective serotonin reuptake inhibitor

TRP- tryptophan hydroxylase

TTX- Tetrototoxin

VGLUT3- Vesicular Glutamate Transporter 3

VMAT2- Vesicular Monoamine Transporters 2

WAY100635- *N*-{2-{4-(2-methoxyphenyl)-1-piperazinyl}ethyl-*N*-(2-pyridinyl)cyclohexanecarboxamide

CHAPTER 1

INTRODUCTION

Major depressive disorder (MDD) is one of the leading causes of disability in the United States and third worldwide. This disorder affects approximately 37 million people or 17% of the American population 18 years of age or older, and shows a prevalence toward women over men (Jorm, 2001; Kessler et al., 1994; Nestler et al., 2002; Wittchen et al., 1994; Zhao and Sun, 2008). Symptoms of depression have been recognized as early as 400 B.C. when Hippocrates first termed depression as melancholia or "black bile" (Akiskal, 2000). Today MDD is clinically characterized by a set criteria of symptoms put forth by the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) as pervasive low mood, loss of interest or pleasure in everyday activities, excessive weight loss or gain, insomnia or hypersomnia, psychomotor agitation, fatigue, feeling worthless or excessive inappropriate guilt, diminished ability to concentrate or indecisiveness, and recurrent thoughts of suicide. Diagnosis of depression requires occurrence of one core symptom and four or more additional symptoms simultaneously and continuously for more than two weeks (Skolnick et al., 2009).

Based on epidemiological studies approximately half of the risk of depression can be attributed to genetics, however no specific gene has been identified as the "depression- causing gene" to date (Nestler et al., 2002).

Difficulty in narrowing down a depression "vulnerability gene" remains a complex undertaking, because many genes may be involved in the pathophysiology of this complex disorder, thus any single gene identified in a genetic study could produce a miniscule effect that has sub-threshold effects. In addition to the genetic aspect, there is an environmental confound in the neurobiology of depression. Quality of life, stress, traumatic events, and even viral infections have all been associated with the precipitation of MDD (Fava and Kendler, 2000). Stress, for instance, is often described in conjunction with depression. However, not all stressful life events result in depression in an average person. On the other hand depressed individuals find it more problematic to handle moderate stress that is easily managed by most people. These phenomena reinforce the idea that depression is a combination of genetic predisposition and environmental factors.

The first clue about the neurobiology of depression arose during a hypertensive clinical study when physicians discovered that the hypertensive drug reserpine depleted neurons of monoamine neurotransmitters at CNS synapses resulting in depressive- like symptoms in patients (McKenney, 1974; Sandifer, 1978). Physicians hypothesized that clinical presentation of depression may be caused by a decrement in synaptic concentrations of monoamine neurotransmitters. To this end, if a reduction in synaptic monoamine neurotransmitter concentration underlies depressive-like symptoms, agents that

combat this decline by enhancing 5-HT and/or NE at the synapse should alleviate the symptoms of depression. This revelation spawned the creation of the monoamine hypothesis of depression which proposes that depression is a result of a deficiency in biogenic amines, serotonin, norepinephrine and dopamine, and that antidepressants function by restoring these amines and modifying neurotransmission. Formulated based on this hypothesis, the first generations of antidepressants (AD) were synthesized; tricyclics (TCA), which elevate synaptic levels of monoamines and monoamine oxidase inhibitors (MAOI), which inhibit the function of major catabolic enzymes of monoamine neurotransmitters. The multitude of adverse side effects experienced by patients from the use of these agents led to the development of a new generation of drugs.

Today, the most popular antidepressants are selective serotonin reuptake inhibitors (SSRI) and norepinephrine reuptake inhibitors (NERI). While studies have indicated that these newer drugs are only marginally more effective than the older TCAs and MAOIs, the report of fewer adverse side effects makes SSRIs and NERIs more clinically preferable (Taylor et al., 2005). Similar to tricyclics antidepressant SSRIs and NERIs increase levels of serotonin and norepinephrine at the synapse in the central nervous system by competing for binding of 5-HT and NE to their transporters. These reuptake inhibitor drugs bind to serotonin and norepinephrine transporters presynaptically expressed in raphe and locus coeruleus, respectively; resulting in acute and rapid increases in neurotransmitter

levels at these synapses. Unfortunately, SSRIs and NERIs appear to have several non-specific and downstream effects that are not completely understood, similar to the previous generation of antidepressants. Although SSRIs are a standard in antidepressant therapy, clinical efficacy takes several weeks and even months in some cases. These data imply that simply amplifying synaptic concentrations of neurotransmitter is not sufficient for the mollification of depression, and that treatment requires downstream changes in neuronal function, such as synaptic plasticity or gene expression (Gould, 1999; Kobayashi et al., 2008; Manji et al., 2000; Stahl, 1998b; Zhao and Sun, 2008). In the search for an explanation for the delayed onset of SSRI action, the monoamine receptor hypothesis proposes that drug-induced increases in monoaminergic neurotransmission initiates the sensitization state of monoaminergic receptors, which might explain the delayed induction of antidepressant action with use of these drugs (Blier and de Montigny, 1994; Stahl, 1998a). Patients enduring antidepressant therapy often discontinue the treatment before clinical efficacy is reached because of the delayed antidepressant effect. This problem emphasizes the importance of understanding the mechanism by which antidepressants exert their clinical effects.

Animal models have proven to be another difficult feat. Currently there exists no animal model of depression, in part because a depression vulnerability gene has not been identified, thus replication of depression in a genetic knockout mouse model has not been successful. Scientists have used other animal models

in the study of depression to understand the action of known antidepressant and stress responses. For example the force swim test (FST) is readily used as a preclinical indicator of antidepressant efficacy. This test is a measure of despair, in which animals are placed in a beaker of water for a period of time and their movement observed. An increase in the latency to immobility or decrease in total immobility after antidepressant administration is interpreted as a reversal of a depressive- like phenotype. When wild type animals are administered chronic antidepressants in this paradigm, significant decreases in these parameters are observed. While this test might seem like a trivial manner in which to study depression, in order to be classified as an antidepressant and progress through clinical trials the drug under investigation must first go through this preclinical test. Behavior tests such as tail suspension and learned helplessness have also been utilized in animal studies to measure drug efficacy. Social defeat stress, immobilization stress, and chronic unpredictable stress have been used to model depression in rodents. Unfortunately these animal models test wild type or normal animals, whereas depression in humans most likely requires some kind of genetic component. Thus a bona fide animal model of depression cannot be created until researchers discover a “depression gene”, on the other hand we as researchers cannot study depression and actions of antidepressants without the assistance of animal models. While an animal model of depression is not yet available, the

modern animal models provide a foundation in which to explore the neurobiology and develop hypothesis about the etiology of depression.

By way of animal models of depression, researchers have begun to study the impact signaling pathways might have on regulation of neuroplasticity and neurodegeneration in the etiology of mood disorders. A signaling pathway of interest is the neurotrophin pathway. Neurotrophic factors are proteins that regulate neural growth and differentiation during development as well as modulate plasticity and survival of adult neurons and glia. This neurotrophic hypothesis of depression states that a deficiency in neurotrophins support might contribute to hippocampal pathology during the development of depression, so reversing this deficiency through antidepressant therapy could be essential in treatment of MDD (Altar, 1999; Duman et al., 1997; Nestler et al., 2002). Most of the discussion of the neurotrophic hypothesis of depression has been focused on brain derived neurotrophic factor, one of the most prevalent neurotrophins in the adult brain. Stress decreases levels of BDNF protein expression in the dentate gyrus and pyramidal cells of hippocampus in rodents (Smith et al., 1995), and appears to be partially mediated through stress-induced increases in circulating glucocorticoids and partially mediated via stress induced decreases in 5-HT neurotransmission (Vaidya et al., 1997). Chronic antidepressants facilitate amplification in BDNF expression in the hippocampus and reverse stress induced BDNF decreases. Paralleling the animal studies, similarly depressed patients

display lower levels of serum BDNF and postmortem studies illustrate that AD treatment increases hippocampal levels of BDNF (Chen et al., 2001). While the mechanism behind BDNF and its effect in depression are unknown, Shirayama *et al*. showed that administration of BDNF into the DG of hippocampus caused antidepressant effect in the FST (Shirayama et al., 2002). One limitation in further investigation of the BDNF hypothesis is the absence of a BDNF genetic knockout mouse, these mice die shortly after birth. However Adachi *et al.* developed a viral mediated Cre/loxP site specific inducible BDNF knockout selectively in the CA1 or DG of the mice hippocampus. Analysis of a thorough battery of animal behavioral paradigms showed that specific deletion of BDNF in the DG only, attenuated the actions of SSRI antidepressants citalopram and desipramine in FST suggesting that although overall loss of hippocampal BDNF was not enough to manifest in depressive-like symptoms in mice, dentate gyrus BDNF could be important in antidepressant efficacy (Adachi et al., 2008). This study addressed some crucial points in the understanding of the putative association of BDNF and depression; first that DG in hippocampus is critically important in the neurobiology of depression, and that specific proteins such as BDNF underlie the therapeutic effects in antidepressant treatment. In addition to the many modulatory functions of BDNF in the hippocampus, it has also been shown that BDNF enhances LTP and other forms of hippocampal plasticity, and that antidepressant driven enhancement of BDNF can promote hippocampal

function. While the neurotrophic hypothesis of depression is not the primary focus of this thesis document, the idea of BDNF induced antidepressant effects will be further discussed in chapter 5.

Despite the long standing history of depression and appreciation of the fact that neurotransmitter deficiency underlies the manifestation of the disorder, treatment has still proven to be inadequate because of the failure to fully understand how acute enhancements in synaptic monoamines lead to long-term adaptations that ultimately shepherd clinical efficacy in depressed patients. As these early depression findings suggest, impairments in the serotonin system contributes to the development of depression, thus in this thesis document the serotonergic system will be studied to further elucidate the mechanisms of antidepressants and how they modulate synaptic functions.

The Serotonergic System

Serotonin or 5-Hydroxytrptamine (5-HT) is a monoamine neurotransmitter found in neurons, platelets, mast cells, and enterochromaffin. Because 5-HT cannot cross the blood-brain-barrier, the brain synthesizes its own 5-HT which accounts for 1-2% of the body's entire serotonin supply. Tryptophan; the primary substrate for serotonin synthesis, is transported across the blood- brain- barrier where it is hydroxylated in cells by tryptophan hydroxylase. This rate limiting step of the serotonin synthetic pathway requires molecular oxygen and other

cofactors. Upon hydroxylation 5-HT is rapidly decarboxylated by a common neuropeptide enzyme called aromatic amino acid decarboxylase (AADC) or DOPA-decarboxylase. Serotonin can also be continuously metabolized by deamination mechanisms. Monoamine oxidase (MAO) convert 5-HT into 5-hydroxyindoleacetaldehyde which can later be reduced to 5-hydroxytryptophol or oxidized into 5-hydroxyindoleacetic acid (5-HIAA) (Cooper, 1996; Nestler, 2001). Serotonin is exclusively expressed in the dorsal and medial raphe of the rostral brain, a heterogeneous region located between the periaquaduct and fourth ventricle of the midbrain (Smith et al., 2000) (Figure 1-1). Approximately 70% of neurons within the raphe are 5- HT containing; dopamine (DA), GABA, glutamate, and other peptide transmitters make up the remainder of the neurons within the raphe (Michelsen et al., 2007). Early electrophysiological experiments divided the DRN neurons into two distinct types: typical neurons demonstrate a rhythmic firing-pattern and atypical neurons fire irregularly (Nakahama et al., 1981). Serotonergic neurons exhibit a steady tonic state of firing that can be enhanced by stress, behavioral arousal and activity (Morilak and Frazer, 2004). Activity of serotonin is tightly regulated by a family of Na^+ and Cl^- dependent high affinity plasma membrane transporter proteins on the presynaptic terminal of raphe

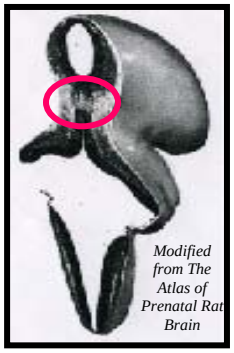


Figure 1-1 Coronal Section of the Raphe at Embryonic Day 15. Dissociated raphe cultures are dissected from the highlighted region

neurons. Serotonin transporters (SERTs) are 630 amino acid, 12 membrane-spanning domain proteins, with two putative extracellular N-linked glycosylation sites (Dahlin et al., 2007). In situ hybridization and histochemistry in rodents utilizing a synthetic oligonucleotide probe illustrated that dorsal and median raphe nuclei showed intense expression of SERTs, while the caudal linear nucleus, raphe magnus nucleus, raphe pontis nucleus, raphe pallidus nucleus and the raphe obscurus nucleus have weak expression patterns of the transporter (Fujita et al., 1993; Zhou et al., 2000). Electron microscopy experiments studying the subcellular distribution of immunogold- silver labeled probes in tissue from rat limbic brain regions identified SERT immunoreactivity (ir) in plasma membranes of raphe axons and axon varicosities, many of which formed synapses on dendritic spines. The authors found SERT ir to a lesser degree in dendrites and in some cases glia (Miner et al., 2000). Comprehensively, raphe axonal SERT staining accounted for an overwhelmingly large portion of immunoreactivity in electron micrographs. Raphe incorporates several afferent and efferent projections to various brain regions including the spinal cord, hippocampus, hypothalamus, and frontal cortex to name a few (Michelsen et al., 2007; Morilak and Frazer, 2004) (Figure 1-2). While absence of

Adapted from Nestler et al 2002

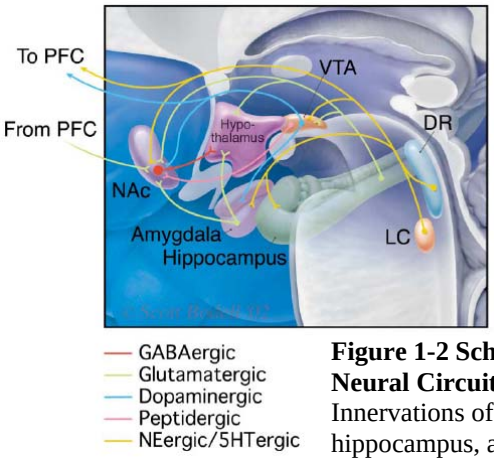


Figure 1-2 Schematic of Neural Circuitry. Innervations of raphe to hippocampus, amygdala, PFC, hypothalamus and other brain regions.

SERT ir in raphe afferents was immunocytochemically confirmed, intense and positive 5-HT staining was visualized in rat tissue using an engineered antibody against 5-HT. Therefore the serotonergic system innervates many brain regions where it modulates synaptic activity (Zhou et al., 2000). This widespread serotonin-guided synaptic modulation alters neuronal function which potentially underlies complex behaviors.

Actions of Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) work by binding to presynaptic serotonin transporters (SERTs) located exclusively on raphe neurons and inhibiting the ability of SERTs to reuptake serotonin into the presynaptic terminal (Figure 1-3). This in turn presumably increases the levels of serotonin (5-HT) available to diffuse to the postsynaptic terminal, bind to 5-HT receptors and modulate postsynaptic receptors including GABA, NMDA, and AMPA. The canonical view describes neurotransmitter transporters such as SERTs as remarkably exclusive proteins that remove these chemicals, such as

Modified from www.Mental1.wordpress.com

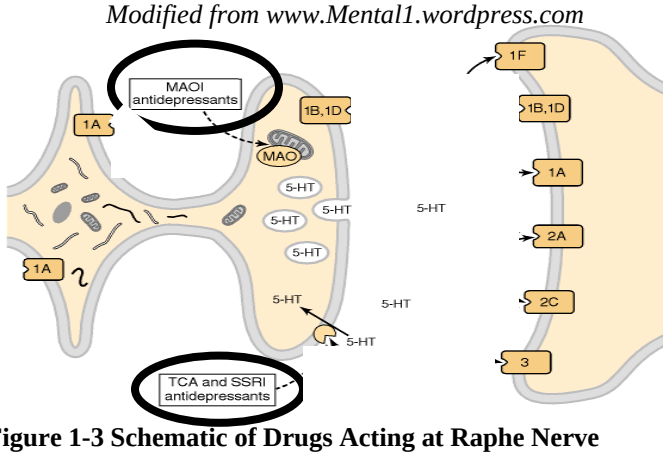


Figure 1-3 Schematic of Drugs Acting at Raphe Nerve Terminals and Receptors. Traditional antidepressants target presynaptic serotonin transporters and targets in a synaptic terminal: outlined.

serotonin, norepinephrine, dopamine, and GABA, from the synaptic cleft, and terminate activity following a sufficient depolarizing event (Bunin and Wightman, 1998; Shen et al., 2004; Zhou et al., 2005). SERTs take up 5-HT while norepinephrine and dopamine are taken up by norepinephrine transporters (NET), or dopamine transporters (DAT) respectively. Contrarily, a report from Zhou *et al*., demonstrated that in the striatal brain slices, fluoxetine increased extracellular concentrations of 5-HT, which was taken up by dopamine transporters (DATs) in striatal terminals in order to maintain the temporal and spatial relationship between DA and 5-HT signaling in the striatum, and as a consequence, 5-HT levels did not remain elevated as expected (Zhou et al., 2005). Clearly, this study raises questions about the specificity of these neurotransmitter transporters, further obscuring our understanding of how these transporters and subsequently reuptake inhibitors work within the brain. In humans the SERT gene originates from a single genomic locus found on chromosome 17 that contains several introns, and express splice variants. In an elegant cloning study Blakely *et al.* was able to discriminate discrete disparities between rat and human SERT proteins through transiently transfected HeLa cells. They reported that TCAs have a higher affinity for human SERTs over rats, conversely amphetamines preferentially bind to rat SERTs. They went on to show that there was no significant species difference between drugs like fluoxetine, cocaine and the substrate serotonin. This group also identified a putative binding site near the transmembrane domain

12 of SERT that could confer substrate and antagonist recognition. These species specific isoforms further complicate the study of ligand binding to transporters which as mentioned are the main target of antidepressant therapeutic drugs such as SSRIs (Barker et al., 1994; Blakely et al., 1994). While the mechanisms of action of SSRI antidepressant drugs are not well understood, the delayed onset of therapeutic effects suggests that along with SERT inhibition of 5-HT reuptake, long-term modifications underlie the beneficial effects of antidepressants, shifting the focus of the field to synaptic alterations that facilitate long-term gene and protein changes.

Synaptic Neurotransmission and Antidepressant Therapy

The role of synaptic neurotransmission in antidepressant treatment has recently drawn some attention, diverting the field in a direction that could solve the age old riddle of the missing link between acute monoamine elevation after SSRI exposure and the delayed therapeutic effects. As previously discussed, conventional knowledge suggests that antidepressants such as fluoxetine, a SSRI, inhibit the serotonin transporter function and elevate 5-HT levels at synaptic terminals. It is not known how these changes may alter synaptic transmission in the long-term, however the monoamine theory of depression suggests that modifications in monoaminergic neurotransmission is instrumental in the pathophysiology and treatment of depression (Millan, 2004; Pittaluga et al.,

2007). Mahgoub *et al.* demonstrated that in active dissociated hippocampal neuronal cultures exogenous administration of serotonin decreases CREB phosphorylation and gene expression while decreasing the firing rates of these neurons; however, with the addition of TTX, 5-HT causes the reciprocal action, suggesting serotonin fosters a tight balance between protein kinases and phosphatases (Mahgoub et al., 2006). The authors were able to demonstrate that neuronal activity within the serotonergic system could drive downstream gene expression in an activity dependent manner through the activation of 5-HT receptors. Another study conducted by Airan *et al.*, utilized voltage sensitive dye imaging to allow millisecond- timescale analysis of disease related neural activity and induced depressive like symptoms in rodents through a chronic mild stress behavioral paradigm and administered chronic fluoxetine or imipramine. In ventral hippocampal evoked slice recordings, they found that there was a reduction in DG activity in CMS-treated animals compared to antidepressant treated animals. The direct opposite results were observed in the CA1 region of the same animals, stating that CA1 contribution is compatible with enhanced hippocampal output while DG contribution is correlated with reduced activity in depression. In antidepressant treated animals the group reported opposite findings from the CMS-treated animals, elevation of DG activity, combined with a decrease in CA1 activity. They hypothesize that deficiencies in the input/output relationship between the DG and CA1 of the hippocampus might be the

underlying mechanism in depression and its treatment (Airan et al., 2007). These studies provide convincing evidence that increases in 5-HT and hippocampal synaptic activity modifications are not directly responsible for the clinical actions of monoamine based antidepressants; but rather, are the avenues in which gradual neuro-adaptations follow in response to this enhanced neurotransmission and mediate therapeutic effects. Notwithstanding the interesting findings of these studies, there still remains a void in the clear understanding of the mechanism behind chronic selective serotonin reuptake inhibitors on long-term synaptic transmission and the down stream modulations that ensue following antidepressant therapy. More recently it has been hypothesized that the delayed onset of antidepressant effects might be a consequence of antidepressant induced restoration of a dysfunction in glutamatergic synaptic transmission in many brain regions including hippocampus (Bleakman et al., 2007; Charney and Manji, 2004; Krishnan and Nestler, 2008; Pittenger et al., 2007). Thus antidepressant-induced changes in hippocampal glutamatergic activity activate intracellular cascades that increase expression of proteins such as CREB, nuclear PKA, adenylate cyclase, as well as BDNF (Marchetti et al., 2010). Requirement of induction of these intracellular pathways might mediate the delayed onset of antidepressant action.

The dorsal and medial raphe innervates different regions of the brain, including the hippocampus. Several studies have shown that some aspects of depression and other mood disorders can be traced to dysfunction in hippocampal

sub-regions synaptic activity (Jorm, 2001; Malberg et al., 2000; Nestler et al., 2002; Zhong et al., 2008) (Figure 1-2). The literature also suggests that modulations in 5-HT concentration affect the synaptic transmission along the serotonergic pathway to the hippocampus. (The raphe and hippocampal serotonergic pathway will be further explored in Chapter 3.) As previously mentioned, SERTS are responsible for taking up as much as 80% of 5-HT released into the presynaptic terminal, thus SSRI application leads to a rapid suppression of 5-HT firing activity by inhibiting the activity of serotonin transporters (Amara and Arriza, 1993; Amara and Kuhar, 1993; Clark and Amara, 1993; Montanez et al., 2003). Although there is a transient increase in serotonin, the activation of 5-HT_{1A} autoreceptors results in inadequate enhancement of serotonin in postsynaptic structures such as the hippocampus thereby blunting any resulting postsynaptic effects (Blier et al., 1998; El Mansari and Blier, 2008; Haddjeri et al., 1998; Kobayashi et al., 2008; Pineyro and Blier, 1999; Zhong and Yan, 2004). Overtime, chronic use of SSRIs desensitizes 5-HT_{1A} autoreceptors on raphe neurons and permits the elevated synaptic serotonin to modulate postsynaptic receptors. Electrophysiological studies have shown that the high density of 5-HT receptors in limbic regions such as the hippocampus can affect membrane excitability depending on the cell and receptor type (Andrade, 1998; Beique et al., 2004a; Beique et al., 2004b). The CA1 region of the hippocampus, for instance, has ten different 5-HT receptor subtypes. Physiological studies have

ascribed functional roles to 5 receptors types. The 2 cell types in CA1, pyramidal cells and GABA interneurons differentially express these 5-HT receptors subtypes, which are consistent with the functional responsibilities displayed in hippocampus. This differential expression in CA1 allows for precise and specific functional regulation of hippocampus neuronal networks. Antidepressants also induce changes in long term potentiation of hippocampal glutamatergic synapses that can be influenced by activation of monoaminergic pathways projecting to the hippocampus, underscoring the intricate relationship between serotonergic and glutamatergic synaptic systems. Modulation in one system can have profound effects on the other. For example, serotonin inhibits glutamate release at presynaptic α_2-adrenergic. Conversely glutamate enhances 5-HT release by acting at presynaptic NMDA and AMPA receptors creating a feedback loop (Gartside et al., 2007; Pallotta et al., 1998; Pittaluga et al., 2007; Tao and Auerbach, 1996). Interestingly, antidepressants working through these pathways change NMDA receptor levels and Cam Kinase II via BDNF changes, which has been shown to mediate synaptic plasticity and its molecular mediators (Popoli et al., 2002). With all of the different studies on the effects of 5-HT on hippocampal synaptic function it is rather daunting to elucidate the dysfunction in the finely tuned network amidst raphe and hippocampus in a depressed state; moreover, how chronic SSRI treatment alleviates this dysfunction remains unclear, with

hypothesis of synaptic augmentation in depression treatment ranging from increases in gene expression to restructuring of intracellular machinery.

One caveat of the aforementioned studies is the experimental system. Mahgoub *et al.* carried out experiments in cultured dissociated hippocampal neurons. In using this system they can only study one angle of this story; how 5-HT enhancement affects postsynaptic hippocampal receptors and consequently synaptic function. As elevated synaptic serotonin concentration is the end result of SSRI administration, this paradigm overlooks the influence of serotonin transporters only indirectly assessing serotonergic effects on hippocampal synaptic activity and gene expression in relation to antidepressant function. Additionally, exogenous application of 5-HT into the hippocampal cultures introduce another limitation to the Mahgoub *et al.* study. As previously mentioned 5-HT is readily oxidized by monoamine oxidases, and this intrinsic property of serotonin makes it nearly impossible to accurately determine the final concentration of 5-HT necessary to act at hippocampal synapses that would resemble a physiological condition. The Airan *et al.* study, induced stress in rats, administered chronic fluoxetine, and performed evoked recording in ventral hippocampal slices. While fluoxetine blocks raphe SERTs, the resulting increase in 5-HT affects all areas of the brain that receive serotonergic innervations, including the hippocampus. Hence, the interaction between all of these regions as a result of 5-HT increase might also contribute to hippocampal function in terms

of depression treatment. While this approach might be more physiological in terms of studying a complete neuronal circuit, it does not allow for a direct mechanistic identification of how SSRIs mediate antidepressant effects by way of the synaptic pathway between raphe and hippocampus, which has implicated in depression pathophysiology. So in a sense, the Airan *et al.* study's broad approach adds complexity to the understanding of an already multifaceted question while the Mahgoub *et al.* study does not entirely address the full scope of the serotonergic circuitry in the neurobiology of depression. Studies of this sort inherently and unintentionally introduce artifacts that make it more challenging to understand the physiology of depression and how to most adequately develop remedies for the symptoms. Even with the findings in these elegant studies, the question of why classical antidepressants have a delayed therapeutic effect still remains unanswered.

Goals of the Thesis

The goal of this thesis document is to better understand the delayed therapeutic effects in antidepressant regimens, by investigating the effect of chronic selective serotonin reuptake inhibitors on synaptic function in a neuronal raphe/hippocampal *in vitro* co-culture system. Recapitulation of the serotonergic pathway between raphe and hippocampus will provide the skeleton for this study. Using this experimental approach has many advantages that other studies lack.

First, the co-cultures contain an endogenous supply of serotonin from the raphe neurons in culture, thus eliminating the requirement for an exogenous application of 5-HT and providing a continuous supply of this monoamine. Secondly, in using raphe neurons, I have the added bonus of the availability of serotonin transporters, which are the primary target of selective serotonin reuptake inhibitors. Which leads to my third point: because this co-culture system has SERTs, SSRIs can be applied directly to the cultures, hopefully providing some insight into their mechanism of action in antidepressant therapy at a synaptic level. Next, replicating the circuitry between raphe and hippocampus *in vitro* allows me to mechanistically address the question of how SSRIs act in this pathway and mediate synaptic effects by using whole-cell voltage-clamped electrophysiological experiments. This system presents the opportunity to evaluate the relationship between 5-HT heteroreceptors, glutamatergic and GABAergic receptors on hippocampal neurons and how they behave in response to SSRI-induced 5-HT enrichment. To accomplish this, I had to first fully characterize the dissociated raphe neuronal culture system that would eventually be incorporated into the co-culture, as extensive work has already been performed using dissociated neuronal hippocampal cultures. Raphe cultures will be surveyed by employing electrophysiological, immunocytochemical, and biochemical techniques with the intention of understanding how the raphe behaves in culture, so as to better comprehend the resulting findings of the

raphe/hippocampal co-culture experiments. A thorough examination of electrophysiological parameters, pharmacological manipulations and immunocytochemistry will also be assessed in dissociated neuronal co-cultures to address the conundrum of how SSRI antidepressants influence synaptic neurotransmission. Results produced from these studies will provide some insight into the pathophysiology of the delayed therapeutic effect of antidepressant treatment.

CHAPTER 2

DEVELOPMENT AND CHARACTERIZATION OF THE *IN VITRO* MIDBRAIN RAPHE NUCLEUS CULTURE SYSTEM

Background

Establishing a working dissociated raphe neuronal *in vitro* culture system was central to the goals of this study. As previously mentioned, the ultimate objective of this thesis is to evaluate whether the cause of delayed antidepressant actions can be attributed to dysfunctions in the raphe to hippocampus synaptic circuitry. Development of a dissociated raphe/hippocampal neuronal co-culture allows a analytical investigation of both electrophysiological and biochemical properties to gain a mechanistic understanding of the actions of SSRIs in the treatment of depression. Therefore, it was necessary to properly understand how raphe neurons work alone in culture as to better comprehend the subsequent co-culture results. The location of raphe neurons in the midbrain presents a challenging task when attempting to precisely dissect out this region. Surprisingly, few studies have ventured into *in vitro* dissociated culture approaches as a means in which to studying raphe neurons, in part because of the location of the raphe nucleus. As described in Chapter 1, the raphe is found between the fourth ventricle and periaquaduct of the rat midbrain and is the sole source of serotonin in the brain. Brain regions such as the noradrenergic locus coeruleus neurons, have also been

implicated in the pathophysiology of depression and are located in close proximity to the raphe nuclei. Hence, accurate and precise removal was imperative as not to contaminate the raphe nuclei with neurons from other brain regions. Despite the laboriousness in dissecting the raphe nucleus, two groups in particular conducted an *in vitro* study of the 5-HT pathway in raphe neurons. Ahead of their time, Johnson *et al.* cultured dissociated raphe neurons from prenatal (P0-P3) rats and plated on a background of cortical, hippocampal and mesopontine glial cells. They performed evoked electrophysiological recordings and immunocytochemical experiments from cultures incubated for 10 weeks. The authors showed that dissociated raphe cultures formed functional synapses which responded to glutamatergic and GABAergic ligands, suggesting raphe has functional ligand gated excitatory and inhibitory receptors (Johnson, 1994; Johnson and Yee, 1995). In a similar study, Gartside *et al.* used raphe slice recordings to examine the glutamatergic receptor regulation of the serotonergic system. By analyzing field recordings, this group concluded that an acute application of AMPA and NMDA activate raphe neurons and increase their firing rates. Interestingly, they found that NMDA activates raphe GABAergic interneurons, which inhibit raphe firing via $GABA_{A/B}$ receptor activation, creating a feedback loop to tightly regulate raphe synaptic activity (Gartside et al., 2007). These studies provided important information about raphe synaptic transmission; however these culture techniques were not suitable for the objectives of this

study. First a slice preparation of raphe does not include the afferent hippocampal subregions, thus studying the actions of antidepressant in raphe/hippocampal neuronal circuitry would be unattainable. Typically slice recording are acute preparations, so the effects of chronic antidepressants could not be carried out in such a system. The Johnson group incubated their raphe cultures for 10 weeks before performing experiments. While this would be ideal to study chronic antidepressants, dissociated neuronal cultures typically do not last past 3 weeks in culture without having to introduce other cell types such as glia and cortical neurons in order to support the microenvironment conditions. This could introduce artifacts that could confound the results collected from the experiments and again not directly answer questions about the raphe to hippocampal synaptic pathway.

To this end, a dissociated raphe neuronal *in vitro* culture system was developed in order to gain a thorough comprehension of the serotonin system, in order to better study how SSRIs and more importantly serotonin play a role in synaptic function.

Results

Culture Development and Immunocytochemical Characterization

It was important to first determine the optimal developmental time point in which to extract the raphe nucleus in order to obtain the highest yield of serotonergic neurons. Previous studies cultured neurons from rats ranging from embryonic day 13-18 (Lautenschlager et al., 2000). Careful examination of the embryonic rat atlas showed that raphe nuclei were not visible until embryonic day 15 and continued to expand through day 16. Based on this information cultures were prepared from embryos ranging from embryonic day 14-16 (E14-16) to determine which age would be suitable for the study. At 14 DIV, the cultures were subjected to immunocytochemistry to confirm the successful removal and survival of cultures raphe neurons. Presence of raphe neurons was confirmed using an antibody against the serotonin transporter α-SERT located specifically on serotonergic neurons (Fujita et al., 1993; Miner et al., 2000). Preliminary immunocytochemistry experiments showed that cultures prepared from the raphe neurons of E15 animals had strong and specific immunostaining for α-SERT (Figure 2-1 middle) compared to cultures made from E14 or E16 that showed no positive staining when visualized under fluorescent microcopy (Figure 2-1 left and right respectively). The lack of positive staining at E14 is probably a result of the raphe nucleus not being fully developed at that age. At E16 more structures have developed in addition to the raphe nucleus making it more difficult to

successfully culture just raphe neurons. Embryonic day 15 cultures were used for all future studies based on data collected from the preliminary time course experiments. Once it was determined to culture E15 raphe nuclei, immunocytochemistry was repeated in dissociated raphe cultures, immunostaining with α-SERT and co-staining with microtubule associated protein (α-MAP-2) a neuronal marker antibody used to confirm neuronal cells in cultures at 14 DIV and visualized using a confocal microscope to allow examination of neuronal processes. Robust and positive immunostaining of the α-SERT and α -MAP-2 antibody was observed throughout the axonal and dendritic processes of E15 raphe neurons, suggesting the presence of serotonergic raphe neurons in the culture (Figure 2-2A) (Miner et al., 2000; Zhou et al., 2000). As a negative control, mature dissociated hippocampal neurons were immunostained in the same manner as the raphe cultures. There was no visible staining of the α-SERT antibody visible in the processes indicating raphe cultures successfully contain serotonergic neurons and that the α-SERT antibody is specific to the transporter protein found on serotonergic neurons and that (Figure. 2-2B). After confirmation of SERT expressing raphe neurons, the next logical step was conferral of serotonin in raphe neurons.

Dissociated Raphe Cultures Produce and Metabolize Serotonin.

Thus far, the immunocytochemical data suggests that the dissociated cultures are serotonin transporter expressing raphe neurons. But it is still necessary to confirm that this raphe neuronal culture system has endogenous serotonin. Without this validation, it will be impossible to directly investigate the effects antidepressants and more importantly serotonin exert on synaptic neurotransmission. In collaboration with Dr. Anne Andrews at The University of California, Los Angeles dissociated raphe cultures were put through high-performance liquid chromatography (HPLC) experiments to detect serotonin concentrations in mature culture systems. The literature suggests serotonin can be detected as early as 7 days *in vitro*, increasing in concetration with maturity, and that the exocytotic mechanism of 5-HT release from raphe is fully developed (Birthelmer et al., 2007). Therefore it is fully expected to detect maximal levels of serotonin in cultures at 14 days *in vitro*. HPLC allows for analysis of serotonin and its metabolite 5-Hydroxyindoleacetic Acid (5-HIAA) concentrations in both cell and media samples. Serotonin is a rapidly oxidized monoamine, so when measuring serotonin it is also important to look at the metabolite to provide a complete analysis of the 5-HT profile. This experiment is two-fold in purpose in that serotonin can be measured in culture and media while confirming that the SSRIs, fluoxetine and citalopram, being used in this study do increase serotonin levels. The doses used in these as well as other experiments were based on

previous studies that observed antidepressant effects with administration of both fluoxetine and citalopram at these concentrations (methods) (Deak et al., 2000; Kelly et al., 1989; Orsulak et al., 1988; Pato et al., 1991; Tao and Auerbach, 1996). SSRI treated dissociated raphe cultures showed a strong trend toward an increase in 5-HT concentration in cell samples [flx: 72.38 nM, cit 66.96 nM] as compared to vehicle treated [42.03 nM] (Figure 2-3A left). Cell fraction concentrations of the metabolite 5-HIAA appeared to be unaltered with the addition of SSRIs (Figure 2-3A right). Analysis of media samples revealed a significant increase with fluoxetine treatment [65.66 nM $p= 0.045$] and an even more significant increase with citalopram exposure [121.1 nM $p= 0.004$] in comparison to vehicle treated samples [20.02 nM] (Figure 2-3B left). Similar to cell samples, SSRI administration did not effect serotonin metabolite concentrations in media samples (Figure 2-3B right). Collectively, the HPLC data shows that neurons cultured from E15 embryos produce serotonin as well as the metabolite in an *in vitro* dissociated culture system. Because such high concentrations of 5-HT were detected in medium samples, it can be inferred that raphe neurons are also releasing serotonin into the media. With proof of 5-HT production and release in raphe cultures, electrophysiological parameters can be evaluated to further enrich the knowledge base of this raphe culture system.

Electrophysiological Survey of Dissociated Raphe Neurons

The raphe nucleus and serotonin shape the pathways of various regions in the brain, thus it is vital to understand the interplay between raphe and serotonin independently in order to fully appreciate how 5-HT behaves in afferent terminals. Executing electrophysiological experiments in the dissociated raphe neuronal culture offers information about the functionality of raphe neurons and its receptors. Previously, Johnson *et al.* showed functional glutamate and GABA receptors on raphe neurons in slice preparation field recordings (Gartside et al., 2007; Johnson, 1994; Johnson and Yee, 1995). However, a dissociated *in vitro* culture system might behave differently than the more physiological slice preparations because of artificial culture conditions used, as well as the rewiring of synaptic circuitry in culture. The combination of glutamatergic and GABAergic synaptic responses are examined by monitoring spontaneous network activity. Network activity recordings can be further divided into excitatory responses (sEPSC), by adding picrotoxin (PTX) and inhibitory (sIPSC) by adding CNQX and AP-5. Comparisons between recordings performed under these various conditions provide information about the overall synaptic activity of dissociated raphe neuronal cultures (Figure 2-4A). While there was a significant increase in the frequency of glutamatergic activity as compared to both network and inhibitory responses [50 Hz p= .007; p=6.4 e-6], GABAergic activity frequency was significantly lower compared to network and glutamatergic

activity [1.2 Hz p= 1.5 e-7] (Figure 2-4B). There were no significant differences between the amplitude of responses from network, glutamatergic, or GABAergic recordings (Figure 2-4C). This suggests that raphe network activity is overwhelmingly glutamatergic in nature and facilitated through excitatory receptors. Synaptic responses meditated through inhibitory receptors appear to have a lesser contribution to the overall network activity in dissociated raphe neuronal cultures. Cumulatively, the electrophysiological experiments demonstrate that the dissociated raphe cultures express functional excitatory and inhibitory receptors.

To study the acute effects of serotonin on raphe spontaneous network activity, whole-cell voltage-clamped recordings were performed on mature dissociated raphe cultures without isolating glutamatergic or GABAergic activity (Figure 2-5A). Acute perfusions of 5-HT during the recording led to a non-significant increase in network activity frequency, which returned to baseline (4K) after washing serotonin out [4K: 70.7 Hz, 5-HT: 84.6 Hz, after 5-HT: 65.7 Hz] (Figure 2-5B). Acute 5-HT perfusions had little to no effect on the amplitude responses of sPSCs in the raphe cultures, although the amplitude was slightly enhanced after the removal of 5-HT from the external solution [4K: 11.9 pA, 5-HT: 13.8 pA, after 5-HT: 16.5 pA p=.078] (Figure 2-5C). It appears that serotonin has a mild and subtle effect on spontaneous network activity.

After studying the electrophysiological properties of dissociated raphe cultures, immunocytochemical experiments were performed to investigate the types of synaptic boutons responsible for the previously studied synaptic activity at raphe nerve terminals. Antibodies against SERT and the vesicular monoamine transporter 2 (VMAT-2) (Figure 2-6A), as well as the vesicular glutamate transporter 3 (VGLUT-3) (Figure 2-6B) were utilized to accomplish this task. Co-staining with these antibodies not only provides information about the transporters present at raphe nerve terminals, but also helps to validate that the whole-cell voltage-clamped experiments previously carried out were indeed a result of synaptic release from neurotransmitter filled vesicles. Examination of images captured by confocal microscopy indicated that roughly one-third of raphe neuronal synaptic vesicles that stained positive for α-SERT co-stained with α-VMAT-2 (Figure 2-6C) suggesting the presence of monoaminergic presumably serotonergic vesicles. Approximately 10% of α-SERT positive raphe synaptic boutons co-stained with α-VGLUT-3 indicating that these vesicles contained glutamate (Figure 2-6C). Other vesicular glutamate receptors (VGLUT-1 and VGLUT-2) are not highly expressed in raphe neurons (Herzog et al., 2004), and were not visible with immunocytochemical staining.

Recycling Properties of Chronically Treated SSRI Raphe Neurons

The immunocytochemical study offered insight into some presynaptic properties of the dissociated raphe neuronal culture system. Synaptic vesicle recycling is a presynaptic measure that provides information about the internal machinery that subsequently drives neurotransmission at a synaptic terminal. Neurotransmission is initiated by fusion of synaptic vesicles with the plasma membrane which allows neurotransmitters in the vesicle to be released into the synaptic cleft. Upon release, vesicles are endocytosed, and recycled back to a pool that can be refilled and ready for release in the event of consequent stimulation. Synaptophysin-pHluorin is a genetically engineered optical indicator of vesicle release and recycling. It is comprised of a pH- sensitive form of green fluorescent protein (GFP) fused to the lumenal side of a vesicle associated membrane protein and can be utilized to manipulate differences in pH. At rest, the acidic intracellular compartment of synaptic vesicles (pH 5.5) prohibits synaptophysin-pHluorin fluorescence. Upon stimulation vesicles fuse with the plasma membrane allowing the pHluorin to be exposed to the neutral cytoplasmic space (pH 7.4) which results in fluorescence at the presynaptic terminal (Tian and Looger, 2008). During endocytosis rapid reacidification of vesicle occurs and fluorescence is quenched thereby decreasing in intensity. Optically, the increase in fluorescence is a combination of simultaneous vesicle endocytosis and exocytosis, therefore when the data is graphed the increase in fluorescence intensity is depicted as a

more slanted slope shape (Figure-2-7A). Folimycin is a highly sensitive and specific inhibitor of the H^+-ATPase. When applied to the bath solution during stimulation it inhibits acidification of vesicles thus removing the endocytosis component of the first response illustrated graphically by a sharper slope appearance. Following this initial rapid increase in the fluorescence of exocytosed vesicles, the decay is abolished and fluorescence is maintained. Analyzing imaging experiments yields a wealth of information including, the rate of recycling, the size of the recycling vesicle pool, as well as how well these vesicles recycle. These imaging techniques were employed to investigate the recycling properties of dissociated raphe neurons in culture. I prepared dissociated raphe cultures as before and infected with synaptophysin-pHluorin lentivirus at 3 days *in vitro*. At 7 DIV, I began treating the cultures with either fluoxetine or citalopram for seven days and imaged at 14 DIV (Jesica Raingo). SSRI treatment did not appear to have any significant affect on raphe vesicle recycling (Figure 2-8A-C). For a more quantitative assessment of raphe recycling alkalization of the vesicles can serve to normalize. At the end of the experiment, NH_4 is applied to the bath solution to allow all vesicles that took up the pHluorin-tagged virus to fluoresce. Delta F with ammonium is taken as 100% fluorescence. The initial measurement which is the response before folimycin can then be interpreted as a percent of recycling vesicles and divided by the total pool to derive a percentage of the recycling pool. SSRI exposure resulted in a slight but non-significant

increase in the size of the raphe recycling pool as compared to vehicle treated raphe cultures [Veh: 12%, Flx: 18%. Cit: 17%] (Figure 2-8D). Upon further evaluation of the images collected it was apparent that dissociated raphe neuronal vesicles were characteristic of small clear vesicles opposed to dense core vesicles (Figure 2-8E). Typically neurons have two typed of secretory vesicle that are involved in regulated exocytosis. Large dense core vesicles (LDCVs) store neural peptides whereas small clear synaptic vesicles store classical neurotransmitters such as glutamate, GABA, acetylcholine, and glycine. Conversely, monoamines differ from other classical transmitters and have been reported to appear in both LDCVs and smaller vesicles (Liu et al., 1994). Moreover, the appearance of small vesicles in monoaminergic dissociated neurons was intriguing and prompted an investigation to determine the types of vesicles that are responsible for raphe vesicle recycling at nerve terminals. Synaptophysin-pHluorin infected dissociated raphe neurons were immunostained with antibodies against GFP to localize boutons that took up the GFP- fused lentivirus and co-stained with α-VGLUT-3, α -VMAT-2, or α -SERT (Figure 2-9A-C). Confocal imaging revealed that the majority of the recycling vesicles at raphe synaptic boutons appeared to be glutamatergic as 30% of the α -GFP positive puncta colocalized with α -VGLUT-3. Colocalization of α-GFP and α-VMAT-2 indicated that 10% of the boutons packaged monoaminergic transmitters, further staining with α -SERT showed that 13% co-stained with α-GFP implying that the VMAT-2 positive puncta could be

serotonergic (Figure 2-9D). Analysis of the raphe electrophysiological and immunocytochemical data suggest that although only 10% of α -VGLUT-3 positive puncta colocalized with α -SERT in dissociated raphe cultures, these glutamatergic axonal vesicles are the predominating driving force of raphe synaptic transmission.

Discussion

The focus of this chapter was to develop and fully characterize the raphe neuronal culture system. Immunocytochemical analysis of the raphe dissociated cultures validated the specificity of the α-SERT antibody which was used as a serotonergic neuronal marker illustrated by positive and specific staining in raphe cultures that was not observed in dissociated hippocampal cultures. These experiments also designated that the neurons dissected from the midbrain of E15 rat embryos were indeed serotonergic raphe neurons that expressed native 5-HT transporters. While this might seem like a trivial task, the inability to master this dissociated raphe culture system would impede the progress of the study.

Confirming the presence of serotonin in the dissociated raphe cultures by high performance liquid chromatography analysis provided information that was vital to the legitimacy of the study. It is important to note that there are significantly higher concentrations the 5-HT metabolite 5-HIAA in both cell and media samples of raphe cultures. While this might seem like a minute fact, it illustrates that the dissociated raphe neurons behave in a similar manner as they should in an intact CNS. As previously mentioned, serotonin oxidizes quickly into its metabolite form, thus it would be expected to circulate at high levels. Measuring the concentration of 5-HIAA can also be an indicator of the initial amounts of 5-HT present before oxidation occurred. It could be hypothesized that the higher the concentration of 5-HIAA, the more serotonin that was produced or

released from raphe neurons. It is worth mentioning that there were no noticeable differences between fluoxetine and citalopram treated raphe cultures in cell samples, implying that these SSRI antidepressants do not significantly affect the ability of raphe neurons to synthesize serotonin. Although citalopram significantly increased 5-HT concentrations in raphe culture media, fluoxetine also increased 5-HT to a lesser degree. This suggests citalopram might be more effective than fluoxetine in binding to SERT and inhibiting the reuptake of serotonin from the synapse. It has been reported that fluoxetine may not be as selective for the serotonin transporter as was once thought, due to its capacity to have secondary binding properties. Previous studies reported that fluoxetine has effects in dopamine reuptake inhibition, muscarinic cholinergic antagonism, noradrenaline reuptake inhibition, nitric oxide synthase inhibition, among others. These non-selective interactions have led to their application outside of the realm of antidepressant treatment such as in pain management therapy (Chen et al., 2005; Schreiber and Pick, 2006; Stahl, 1998c, d). The HPLC experiments provided the first indication that the SSRIs fluoxetine and citalopram were working properly by increasing the synaptic concentration of 5-HT in the media of dissociated raphe culture without exerting any substantial negative effects on presynaptic terminal sites.

Network activity is a combination of glutamatergic and GABAergic synaptic activity, however; it should not be mistaken for a summation of the two responses. For instance, subtracting the sEPSC response from spontaneous network activity is not equivalent to sIPSC responses. Observation of spontaneous activity is a depiction of glutamatergic and GABAergic receptors working in concert to finely modulate each other and regulate synaptic neurotransmission. Use of channel blockers allows isolation of excitatory or inhibitory synaptic responses independent of the other but with the influence of action potentials. Electrophysiological examination of raphe synaptic responses established that glutamatergic responses dominate raphe synaptic neurotransmission, but GABAergic response might function to balance the overwhelming excitatory presence in raphe spontaneous network activity. Acute serotonin did not significantly affect raphe synaptic activity in dissociated cultures as depicted in Figure 2-5. The slight increase observed with 5-HT perfusion returned to baseline upon removal of serotonin from the bath solution. It might be expected that serotonin would have a robust effect because there are 15 different serotonin receptor subtypes which can be activated. However, many 5-HT receptors function in counteracting pathways which might help to explain a modest change following serotonin administration. For instance, 5-HT_{1A} is a G_i/G_o coupled receptor that decreases cellular levels of cAMP upon activation, conversely 5-$HT_{7;}$ a G_s coupled receptor, increases cAMP when activated. In the presence of

serotonin, all 5-HT receptors including these two are activated and in turn activate these opposing intracellular pathways. The following occurrence might not yield the same sizable change that would take place if only one receptor were activated. Of course in research, it is common practice to pharmacologically isolate specific receptors in order to study there effects on a system, therefore uncovering large-scale effects. However, in a native environment where there are no perturbations on the system in question, it is important to consider all components. In this case, all 5-HT receptors expressed on raphe neurons will contribute in some fashion to a 5-HT response, thus the effect of one receptor might be masked by the activation of another. From the electrophysiological experiments two main points can be concluded 1) dissociated raphe neurons *in vitro* have functional excitatory, inhibitory, and 5-HT receptors and that 2) serotonin modulates the responses mediated through these receptors.

Investigation of axonal synaptic vesicles in dissociated raphe neuronal cultures further validated the electrophysiology experiments in the previous section. Co-staining cultures with specific antibodies against vesicular transporter proteins indicated that dissociated raphe neurons have synaptic boutons expressing both glutamatergic and monoaminergic transporters, which presumably are filled with glutamate and serotonin respectively. These data suggest a correlation with the electrophysiology results, showing that the predominating glutamatergic response observed in spontaneous network activity

could be a result of glutamate filled vesicles, and that serotonin might modulate raphe neurotransmission.

The last set of experiments helped in understanding the presynaptic terminal machinery in dissociated raphe neuronal cultures in more depth. Manipulating synaptophysin-pHluorin, I was able to gain a better understanding of how SSRIs fluoxetine and citalopram affect presynaptic vesicle recycling. There were no significant changes in the percent of the recycling pool following treatment with SSRIs, suggesting that antidepressants to not negatively affect raphe vesicle recycling, which further confirms that these drugs function within their capacity to presynaptically hinder reuptake of serotonin and enhance concentration and are not affecting the presynaptic intracellular machinery in raphe neurons. Immunocytochemical techniques further demonstrated that only a portion of dissociated raphe synaptic boutons were infected by pHluorin-tagged lentivirus. Immuno-labeling α-GFP with either antibodies against vesicular transporter proteins or α-SERT suggested that the majority of the axonal vesicles participating in recycling were glutamate filled boutons. Monoaminergic containing vesicles appeared to participate in transmission in a smaller capacity than glutamatergic. These data only account for approximately 50% of synaptic boutons hence the remainder of the vesicles not represented in these immunocytochemical studies could be associated with GABAergic transporters

which would support the electrophysiological data, or are not part of the recycling synaptic vesicles (Tao et al., 1996).

Collectively this battery of experiments examined the functional characteristics of this dissociated raphe neuronal *in vitro* culture system. Data compiled indicated that this system could adequately provide the framework in which to continue to investigate the functional impact of antidepressants on synaptic functions. As previously stated, interpretation of data acquired from any future antidepressant study would prove irrelevant without a methodical evaluation of the dissociated raphe system. Revelations such as serotonin release, functional receptors, and proper transporter expression will serve to strengthen data gathered in the subsequent studies.

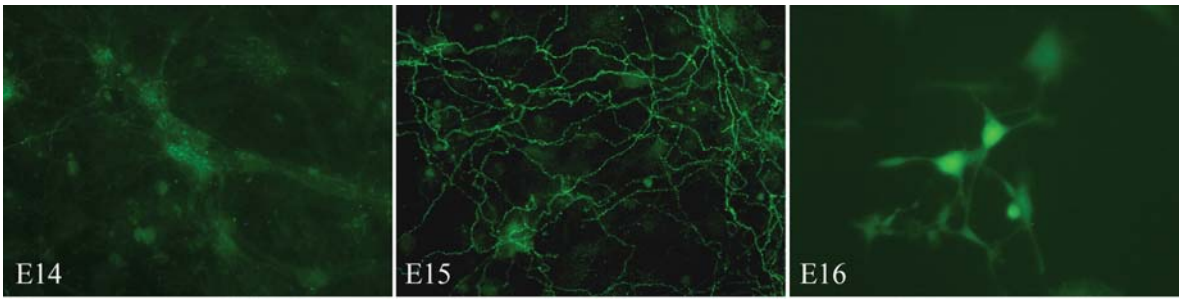


Figure 2-1 Immuno-labeling of α-SERT on Embryonic Raphe Rat Neurons: Left E14 dissociated neurons do not show any specific immunostaining; Middle dissection at E15 illustrates strong labeling for 5-HT transporter; Right E16 dissociated neurons do not show specific SERT staining. Images collected by fluorescent microscopy.

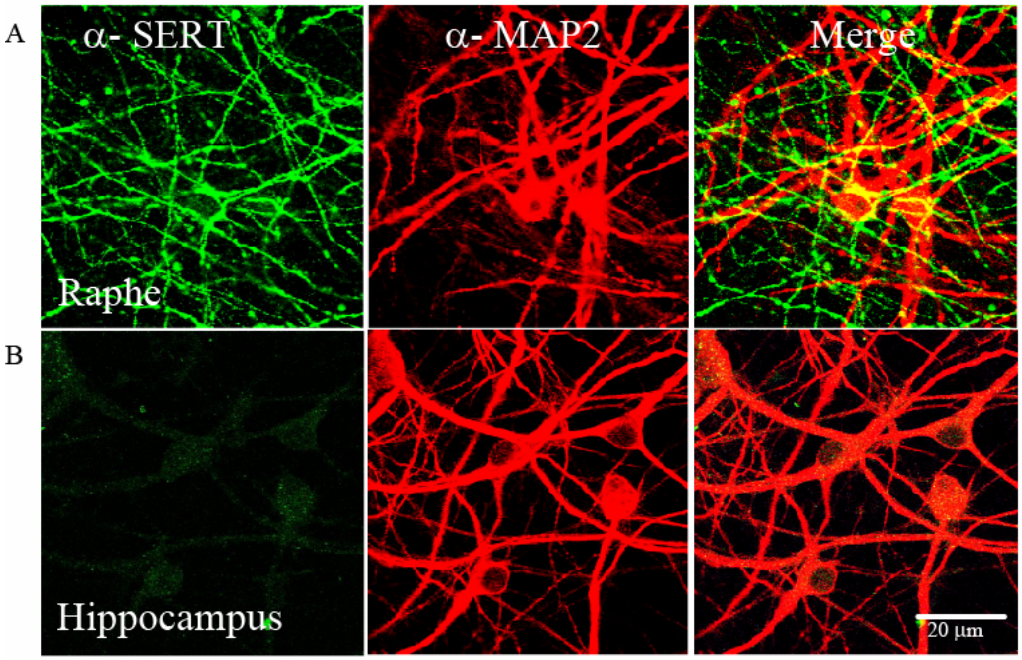


Figure 2-2 Neuronal Confirmation: To confirm the presence of serotonergic neurons in the raphe cultures, immunocytochemistry was conducted utilizing the α-SERT abs (green) to confirm raphe neurons and α-MAP2 abs (red) to confirm neuronal cell-types (A) Dissociated raphe primary neuronal cultures, (B) Dissociated hippocampal primary neuronal culture

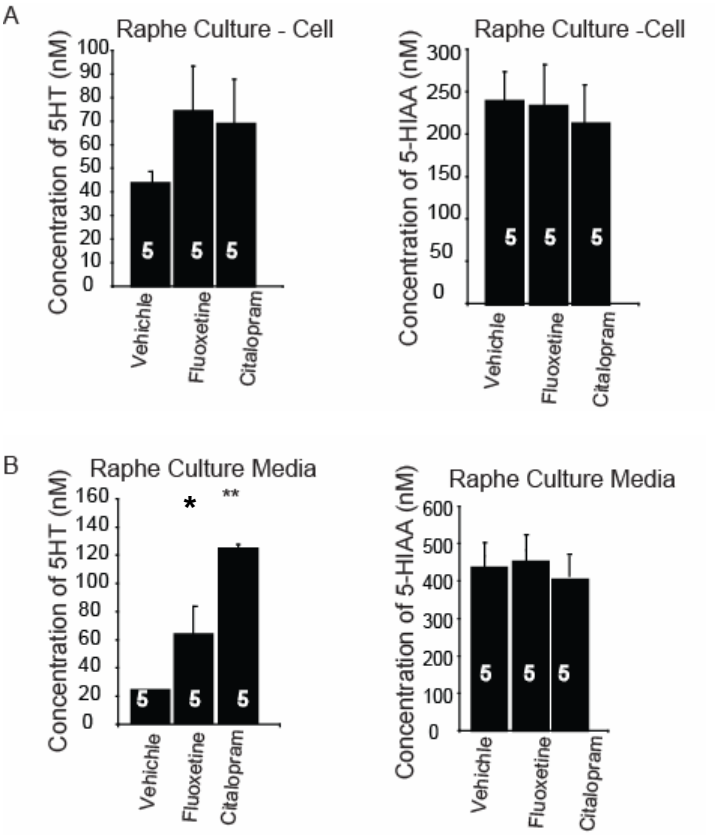


Figure 2-3 Dissociated Raphe Neurons Produce and Metabolize Serotonin in culture: High Performance Liquid Chromatography (HPLC) was conducted to determine the concentration of 5-HT and its metabolite 5-HIAA in mature raphe neuronal culture. Raphe cultures were treated with Fluoxetine (0.3μM), Citalopram (1μM), or Vehicle for 7 consecutive days. Samples were tested in duplicates. **(A)** Bar graphs show quantification of 5-HT (left) and 5-HIAA (right) in raphe cells **(B)** In raphe culture media, the 5-HT concentration is significantly increased with fluoxetine and citalopram treated raphe neurons compared to vehicle treated raphe cultures.

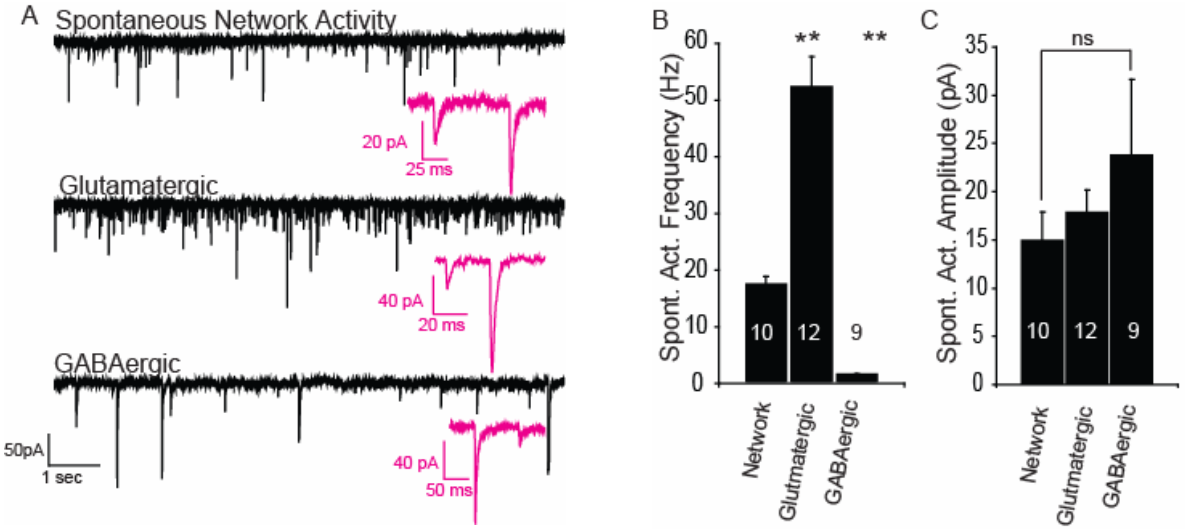


Figure 2-4 Electrophysiological Properties of Raphe Neurons: Whole-Cell Voltage-Clamped Recordings in Raphe Primary Neuronal Cultures **(A)**Sample traces of spontaneous network activity recorded in Tyrode's external solution (top), spontaneous glutamatergic activity recorded in Tyrode's solution + PTX (middle), and spontaneous GABAergic activity recorded in Tyrode's solution CNQX and AP-5. **(B)** Bar graph depicts a significant increase in spontaneous activity frequency in glutamatergic responses in raphe neuronal cultures in comparison to network or GABAergic transmission. (** $p < 0.01$; *$p< 0.05$) The numbers on the bars indicate the numbers of experiments **(C)** Bar graph shows no significant change in amplitudes from network, glutamatergic, or GABAergic.

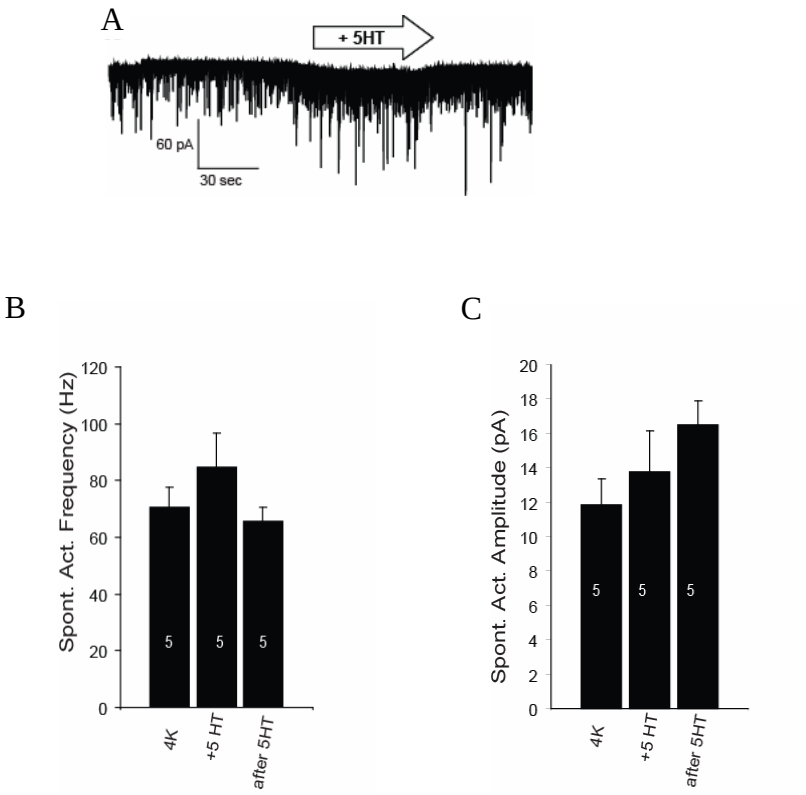


Figure 2-5 Effects of Serotonin on Raphe Network Activity (A) Representative trace of spontaneous network activity in raphe cultures perfused with serotonin (5-HT). **(B)** Bar graph depicts a slight but non-significant increase in spontaneous activity frequency in raphe cultures once 5-HT is added, frequency returns to baseline upon 5-HT washout.

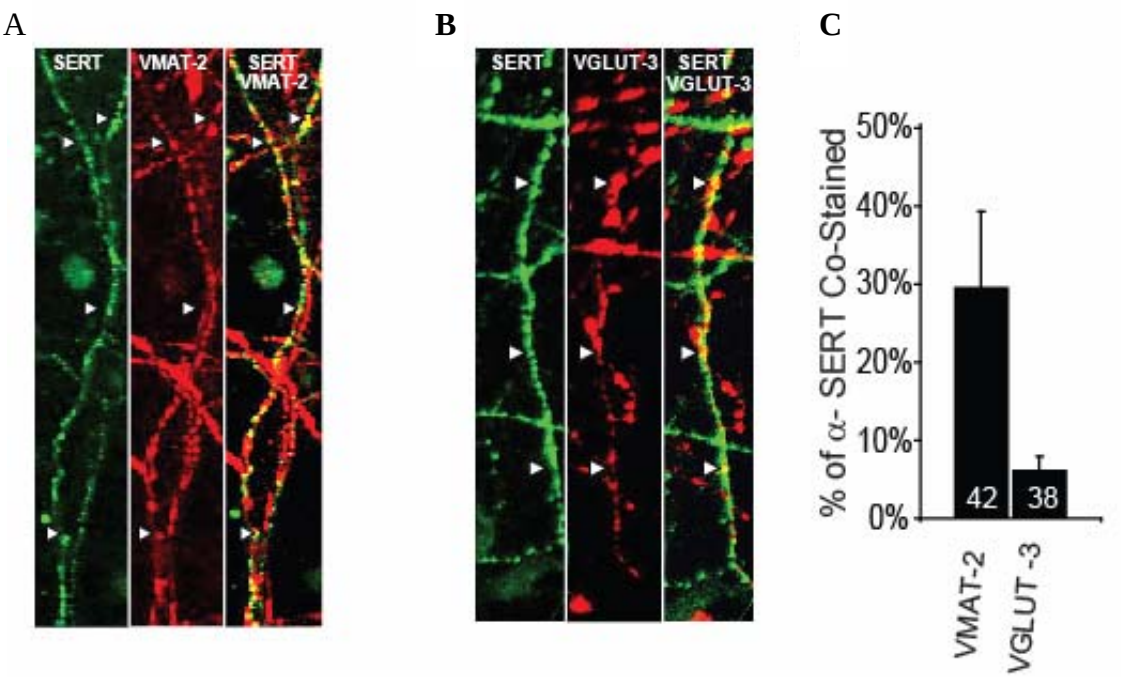


Figure 2-6 Raphe Synaptic Boutons (A) Dissociated raphe neurons were immunostained with antibodies to SERT (green) and VMAT-2 (red) to determine the amount of monoaminergic synaptic vesicles in raphe neurons. **(B)** Dissociated raphe neurons were immunostained with antibodies to SERT (green) and VGluT-3 (red) to determine the amount of glutamatergic synaptic vesicles in raphe neurons. **(C)** The bar graph illustrates both monoaminergic and glutamatergic vesicles are present in raphe nerve terminals when co-stained with the SERT antibody. All immunostaining was done at 14 DIV and visualized via confocal microscopy. Colocalized puncta was quantified in 50 nm sections per coverslip. White arrows indicated colocalized puncta

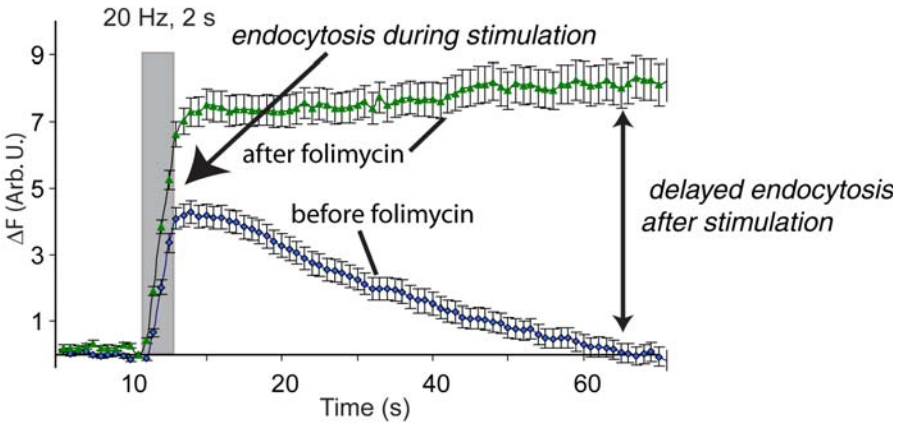
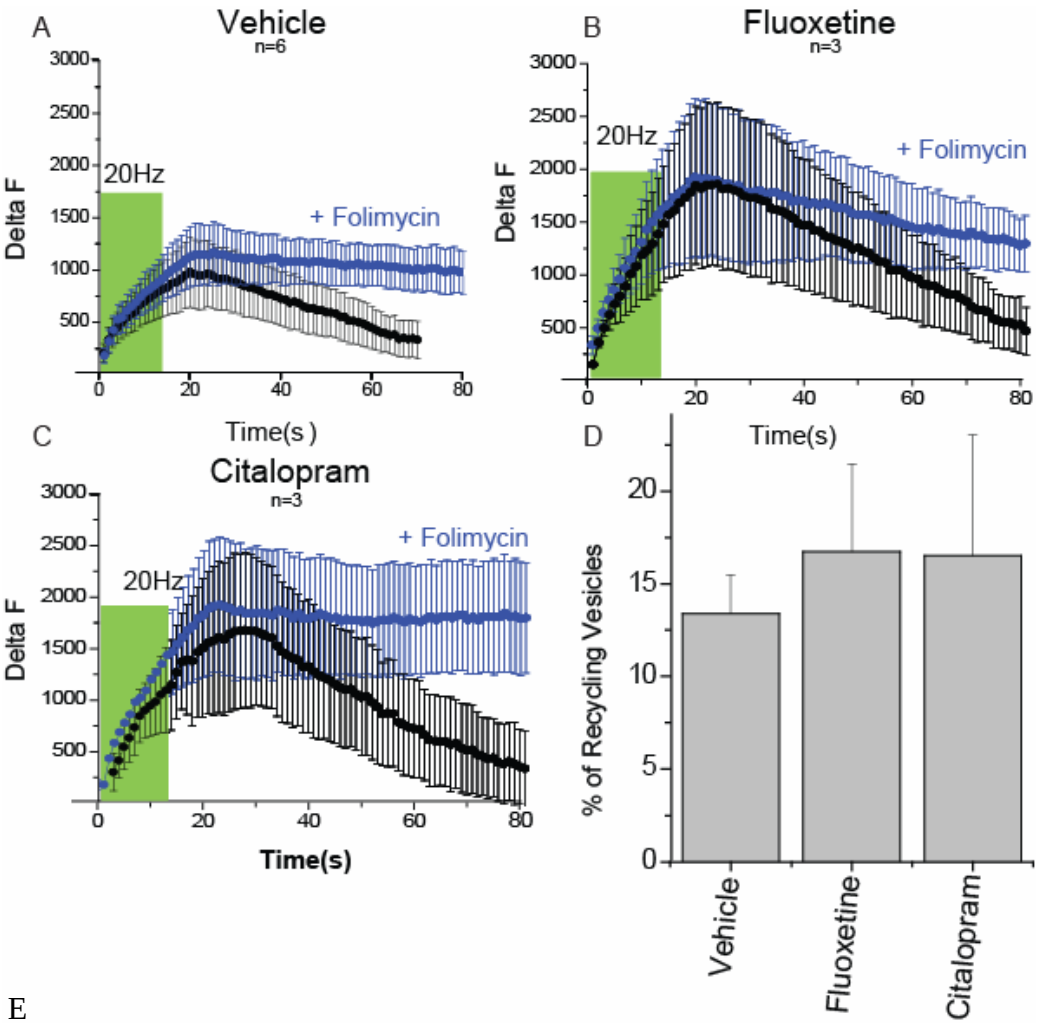


Figure 2-7 Schematic Representative Trace of Synaptophysin-pHluorin Imaging Experiments: Blue Trace is response after 20 Hz stimulation without folimycin. Response is a mixture of endo- and exocytosis. Green trace is same stimulation after folimycin, increase is more rapid and there is no decline in fluorescence; representative of exocytosis axonal boutons only.


A
Vehicle
n=6
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+ Folimycin
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B
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+ Folimycin
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% of Recycling Vesicles
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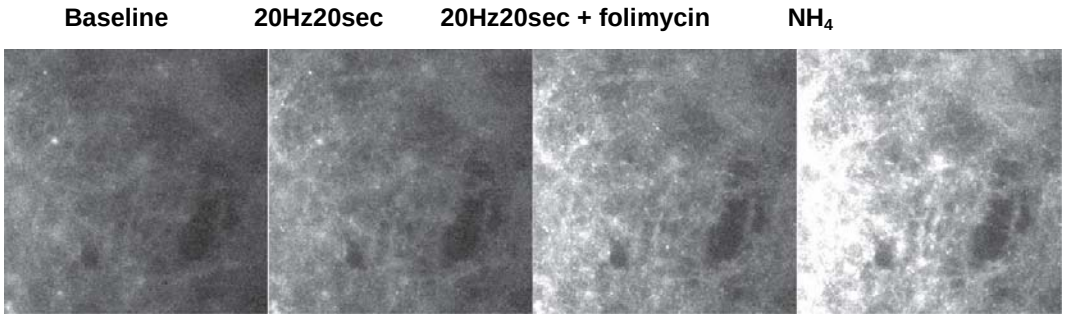

Baseline
20Hz20sec
20Hz20sec + folimycin
NH4

Figure 2-8 Recycling Properties of SSRI Treated Raphe Neurons: Imaging experiments were performed in mature dissociated raphe neurons treated with Vehicle, 0.3 μM Fluoxetine, or 1μM Citalopram. Representative traces of fluorescence bleaching at 20 Hz stimulation (black) and after bath application of folimycin (blue). **(A)** Vehicle treated raphe neurons. **(B)** Fluoxetine treated, **(C)** Citalopram. **(D)** Bar graph shows a slight but non-significant increase in the percentage of recycling synaptic vesicles in SSRI treated raphe cultures as compared to vehicle treated. **(E)** Representative fluorescent image of synaptophysin-pHluorin infected dissociated raphe cultures during imaging experiments.

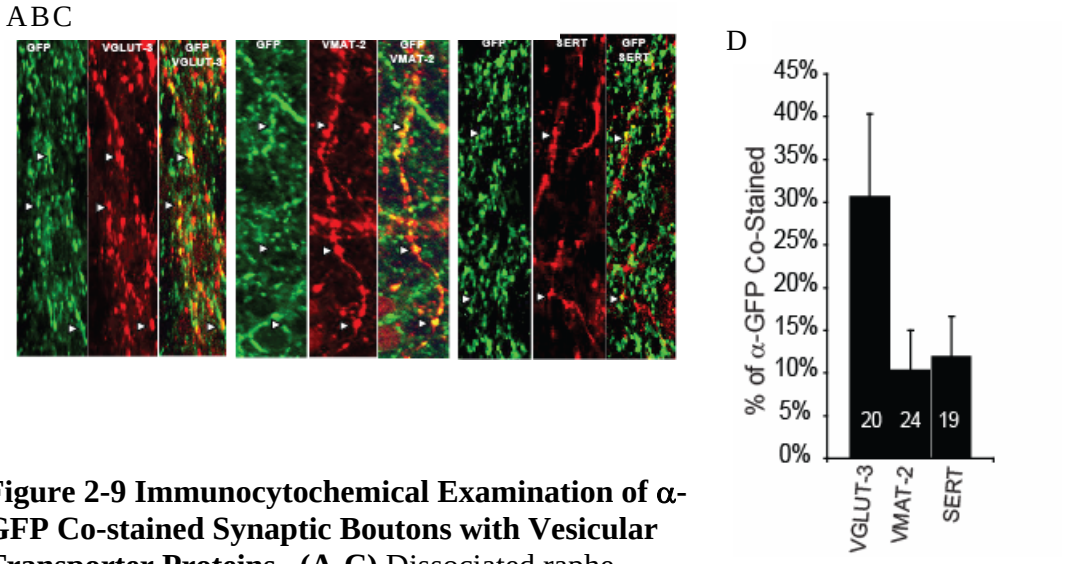


Figure 2-9 Immunocytochemical Examination of α-GFP Co-stained Synaptic Boutons with Vesicular Transporter Proteins. (A-C) Dissociated raphe neurons were infected with synaptophysin pHluorin lentivirus at 3 DIV, incubated and immunostained with antibodies to GFP (green) and a vesicular transporter once matured, (14 DIV). Coverslips were visualized by confocal microscopy. Colocalized puncta was quantified in 50 nm sections per coverslip. White arrows indicated colocalized puncta **(A)** GFP and VGLUT- 3, **(B)** GFP and VMAT-2, **(C)** GFP and SERT. **(D)** Bar graph shows a majority of recycling vesicles that took up the virus were glutamatergic vesicles.

CHAPTER 3
DEVELOPMENT AND CHARACTERIZATION OF THE *IN VITRO* RAPHE/HIPPOCAMPAL CO-CULTURE SYSTEM

Background

Several limbic brain regions including the hippocampus have been implicated in depressive symptoms. The hippocampus is a region of the brain that is responsible for numerous cognitive and behavioral functions. Lesions in the hippocampus result in learning and memory deficits as well as behavioral abnormalities (Fossati et al., 2002). The hippocampus is a unique region in which to study depression because the serotonin, norepinephrine, and glutamate systems, which are all involved in depression, interact and thus provide an ideal arena to carry out this study (Millan, 2004; Pittaluga et al., 2007; Yuen et al., 2005). Previous postmortem studies demonstrated that depressed individuals had smaller hippocampal volume than both control individuals and patients that had been through antidepressant therapy suggesting that dysfunction in the hippocampus can be directly attributed to the pathophysiology of depression. Furthermore, it was also been reported that chronic SSRI treatment modulates precursor cells in the dentate gyrus, a subregion of the hippocampus (Airan et al., 2007; Kobayashi et al., 2008; Malberg et al., 2000; Santarelli et al., 2003) and leads to increases in spine density in the hippocampus (Hajszan et al., 2005). Additionally, 5-HT has been shown to regulate synaptic neurotransmission in the hippocampus

(Kobayashi et al., 2008; Roychowdhury et al., 1994; Segal, 1981). Information gathered from these studies indicates that the serotonergic synaptic pathway between the raphe and hippocampus is extremely important in the neurobiology of depression. Therefore, it was imperative to this study to comprehensively understand the synaptic relationship between the hippocampus specifically the dentate gyrus (DG) and the raphe nuclei (Zhong et al., 2008). The objective of this study was to evaluate the effect of antidepressants, specifically SSRIs, on synaptic neurotransmission. To completely appreciate the impacts of SSRIs on the raphe/hippocampal pathway it was important to engineer a system that could supply endogenous serotonin, serotonin transporters, raphe afferents, as well as serotonergic auto and heteroreceptors. To this end, an *in vitro* neuronal raphe/hippocampal co-culture was developed and electrophysiological experiments were performed.

Results

Immunocytochemical Characterization

Development of the raphe/hippocampal co-culture was challenging and required meticulous planning. Because there was no working protocol for an *in vitro* co-culture system, it took several trials to create the perfect blend of hippocampal and raphe neurons in culture. Rats at postnatal day 0-2 (P0-2) were cultured for the dissociated hippocampal neurons. Four days later, embryonic 15 (E15) raphe neurons were cultured and plated directly on top of hippocampal neurons and allowed to mature in incubation until 14 DIV for acute and 21 DIV for chronic experiments (Figure 3-1). Surprisingly, the adhesion matrix used for coating the coverslips presented a unique opportunity that distinguishes between the two neuronal populations in culture without the use of a fluorescent tag. Hippocampal cultures are typically plated on matrigel coated coverslips. Unlike the dissociated raphe cultures which are plated on poly-lysine, the co-cultures appear to thrive on matrigel similar to the hippocampal cultures as confirmed by immunocytochemical α-MAP-2, α -SERT, and DAPI staining (data not shown). In the dissociated co-culture system, raphe neurons form cluster-like structures, completely separate from the dispersed hippocampal neurons (confirmed by immunocytochemistry- data not shown), making it difficult to patch during recordings, but advantageous in distinguishing between raphe and hippocampal, (Figure 3-2). To that end, during whole-cell voltage -clamped recordings neurons

that were distinctly separate from the raphe clusters were patched. For raphe neuronal culture recordings, neurons are plated on poly-lysine coated coverslips in which neurons can mature in a more disperse uniform pattern throughout the culture and allow for patching during electrophysiological recordings (Chapter 2). To validate the presence of both neuronal types in culture, dissociated co-cultures were immunostained with the antibodies against SERT and MAP-2 similar to raphe cultures in chapter 2. Observation of the co-immunostained coverslips illustrated specific and positive labeling with both antibodies, distinctly different from the images of the immunostained raphe and hippocampus alone (Fig 3-3). This indicates that dissociated co-cultures have both raphe and hippocampal neurons maturing in concert. Quantification of proportions of each neuronal type indicated that from the 1 rat embryo and 2 rat pups per plate the ratio of raphe to hippocampal neurons is approximately 2.5 to 1.

Dissociated Raphe Neurons Produce and Metabolize Serotonin in Raphe/Hippocampal Co-cultures

Similar to dissociated raphe cultures, it was necessary to verify that serotonin was still being produced and released once dissociated raphe neurons were co-cultured with hippocampal neurons. HPLC experiments were carried out in dissociated co-cultures to measure serotonin and 5-HIAA concentrations in both cell and media samples. Upon analysis of the HPLC data from co-cultures there were no observable changes in the serotonin or 5-HIAA concentration in

cell samples [Veh: 146.6 nM, Flx: 150 nM, Cit: 154.3 nM] (Figure 3-4A). Analysis of co-culture media samples showed identical trends as the raphe media samples, an increase in SSRI treatments and to a higher extent with citalopram [Veh: 88.1 nM, Flx: 129.8 nM, Cit:226.9 nM] (Figure 3-4C). While the 5-HIAA concentrations were unchanged by SSRI exposure, the metabolite was much higher then 5-HT as it was observed in dissociates raphe cultures (Figure 3-4 B & D). The data collected from the HPLC experiments confirm that the dissociated raphe neuronal cultures and co-cultures produce and presumably release serotonin. The data also suggest that SSRIs are working to increase 5-HT at the synapse and that citalopram might be more effective in binding to SERTs. Again these results indicated that the co-cultures are capable of producing and releasing 5-HT in culture media, which allows me to study how serotonin might modulate hippocampal synaptic function following SSRI exposure.

Electrophysiological Study of the Co-culture

Following electrophysiological characterization of the dissociated raphe culture, I wanted to further delineate the differences between raphe and hippocampal neurons in culture. As previously mentioned only raphe neurons stained positive for α-SERT (Figure 2-2), and when plated on the adhesion matrix matrigel they mature and disperse differently than hippocampal neurons. I wanted to also show a difference in the spontaneous network activity between the

two neuronal populations (Figure 3-5A). Spontaneous network activity frequency recorded from either hippocampal neurons alone or co-cultures were significantly reduced in comparison to raphe spontaneous network frequency. There were no distinguishable differences between the sPSCs frequency of the hippocampal and co-culture suggesting that the responses are both recorded from hippocampal neurons [Hippo: 6.5 Hz, Co-culture: 5.7 Hz] (Fig 3-5B). This piece of evidence further validates that the co-culture recordings are from patched hippocampal neurons and not raphe. When amplitudes of spontaneous activity were examined there was a significant increase in hippocampal neurons as compared to raphe and co-cultures [Hippo: 27.5 pA, Co-culture: 9.6 pA p=0.0004] (Figure 3-5C). It is possible that raphe neurons attenuate the amplitude responses of hippocampal neurons in the co-culture system. This concept will be revisited later in the discussion.

Effects of SSRIs on Spontaneous Miniature Neurotransmission in a Co-culture Neuronal System

Miniature postsynaptic currents (mPSCs) are a spontaneous form of transmitter release that is independent of an action potential, depolarization, or calcium influx from voltage gated calcium channels (VGCCs). The mPSCs can be pharmacologically isolated into excitatory (mEPSCs) and inhibitory (mIPSCs), transmission which serve to maintain the balance in the central nervous system.

Miniature EPSCs or IPSCs can be useful in studying the activity of an individual synapse at the site of interaction between a presynaptic and postsynaptic terminal. Excitatory transmission is controlled predominantly by glutamatergic receptors, such as AMPA receptors, and inhibitory transmission by GABA receptors. In relation to this experiment these electrophysiological techniques were incorporated to study how SSRIs affect neurotransmission homeostasis. Because spontaneous miniature synaptic transmission provides insight into the basic properties of synapses; such as quantal size, it also allows for the understanding of what type of effects SSRIs and more directly 5-HT can have on synaptic properties in the neuronal co-culture system. An important hallmark of miniature neurotransmission is the spontaneous aspect of these types of recordings. This is achieved by addition of tetrodotoxin (TTX) to a Tyrode's bath solution to block action potentials and study spontaneous activity exclusively. Further delineation between excitatory and inhibitory response can be accomplished by the addition of either AP-5 and PTX to block NMDA and GABA receptors respectively and seclude excitatory AMPA receptors, or AP5 and CNQX to inhibit AMPA receptors and selectively examine inhibitory GABA receptors. Information regarding the frequency and amplitude of spontaneous synaptic currents can be collected from surveying mEPSCs and mIPSCs recordings. Changes in mPSC frequencies are indicative of changes in presynaptic function while changes in amplitude are reflective of postsynaptic changes. While these are commonly

accepted dogmas in electrophysiology, future experiments will further parse out any presynaptic or postsynaptic component.

Lacking a vast body of literature concerning SSRIs and *in vitro* synaptic effects to draw information from it was challenging to predict what might be expected. As a starting point, acute miniature experiments were first performed to see if any synaptic effects could be observed. Current literature suggests that acute SSRI treatment causes an activation of somatodendritic 5-HT_{1A} autoreceptors that occludes the transient increase in serotonin induced as a result of blocking the reuptake of SERTs (Stahl, 1994; Stahl, 1998b). This occurrence reduces raphe firing, however, how this event might affect hippocampal synaptic function is not well understood. To study these properties, dissociated neuronal co-cultures developed in the previous system were treated with fluoxetine at 13 DIV for 18-24 hours and hippocampal neurons in co-cultures were recorded from the following day, 14 DIV (Figure 3-6A & D). Preliminary findings showed a significant increase in mEPSC frequency [Veh: 0.24 Hz, Flx: 0.68 Hz p= 0.013] without altering the frequency of mIPSCs events [Veh: 0.132 Hz, Flx: 0.131 Hz] (Figure 3-6 B & E). Fluoxetine also caused a slight but significant increase in mIPSC amplitude [Veh: 7.6 pA, Flx: 9.9 pA p= 0.023] with no change in mEPSC amplitude [Veh: 10 pA, Flx: 13.4 pA] (Figure 3-6 C & F). To further investigate these findings cultures can be immunostained with synaptic antibodies for pre and post- synaptic markers. Usually a change in miniature amplitudes suggest a

postsynaptic change in receptor number or the responsiveness of the receptors, while an alteration in frequency indicates a presynaptic modification in the release probability, quantal size or presynaptic synapse number. When the acutely treated co-cultures were co-immunostained with antibodies against synapsin, a presynaptic marker, and either α-PSD-95 an excitatory postsynaptic marker or α -gephyrin an inhibitory postsynaptic marker, there was an increase in both colocalized excitatory [Veh: 530.5, Flx: 785.3] and inhibitory puncta [Veh: 244.2, Flx: 431.8 p= 0.048] after fluoxetine treatment however only puncta co-labeled with inhibitory markers were significant (Figure 3-6G), suggesting there might be an augmentation in the number of inhibitory receptors upon acute fluoxetine treatment. These data illustrate that fluoxetine influences inhibitory synaptic function of hippocampal neurons when used acutely, however, as previously mentioned, the neuro-chemical effectiveness of antidepressants are observed after chronic use. Although differences observed in the miniature electrophysiology and immunocytochemical experiments are promising, it only provides a starting point for the study. Therefore, to further delineate the effects of antidepressants, these miniature experiments were repeated under chronic conditions.

As previously stated clinical antidepressant therapy efficacy takes anywhere from several weeks to months. For that reason, a consecutive 10 day SSRI treatment paradigm was followed to better recapitulate this physiological situation. In addition to fluoxetine, citalopram, another SSRI were used, as both

antidepressants are from the SSRI family of drugs, therefore alterations observed with both drugs might point to a mechanistic effect of SSRIs on synaptic neurotransmission. Starting at 12 DIV, dissociated co-cultures were treated with SSRIs and all recordings were conducted between 21-24 days after the plating of the hippocampal neurons (Figure 3-7A), a time at which the antidepressant effect should have taken place (O'Leary et al., 2009). Neither chronic fluoxetine nor citalopram had an effect on mEPSC frequency [Veh: 1.2 Hz, Flx: 1.3 Hz, Cit: 1.2 Hz]. However citalopram produced a significant decrease in mEPSC amplitude and fluoxetine had no effect [Veh: 9.8 pA, Flx: 9.5 pA, Cit: 7.1 pA p= 0.05] (Fig 3-7 B-C). Under spontaneous miniature inhibitory conditions chronic fluoxetine had no affect in either amplitude or frequency (Figure 3-7D). Conversely, chronic citalopram revealed a significant and robust increase in mIPSC frequency and no change in mIPSC amplitudes [Veh: 0.23 Hz; 7.2 pA, Flx: 0.26 Hz; 8.6 pA, Cit: 0.85 Hz p= 0.027; 6.8 pA] (Figure 3-7E-F). Immunocytochemical experiments were performed to investigate whether the decrease in mEPSC amplitude after citalopram administration could be a result of a variance in excitatory synapse number. Quantification of both excitatory and inhibitory colocalized puncta depicted a decrease in excitatory puncta, co-immunostained with synapsin and PSD-95 [Veh: 860.8, Cit: 441.1 p= 0.002] (Figure 3-7G). Evaluation of inhibitory puncta demonstrates a non-significant trend toward an increase in colocalized puncta following citalopram treatments [Veh: 365.9, Cit: 577.9

p=0.056] (Figure 3-7G). As aforementioned, amplitude changes are thought to imply a postsynaptic change, whereas frequency changes point to a presynaptic modification. Since no change was seen in inhibitory synapse number, the observed mIPSC frequency increase with chronic citalopram suggests an alteration in GABAergic vesicle release or presynaptic inhibiting synapse.

Because the goal of this study is to investigate the role of SSRIs in synaptic function, the changes with citalopram, while interesting, did not fully address the purpose of this study. In order to make inferences about the effect of SSRIs on synaptic miniature neurotransmission, changes that occurred with exposure to both fluoxetine and citalopram were pursued. Other basic electrophysiology parameters were examined and discussed in chapter 4.

Effects of Age and Raphe Neurons on Hippocampal Synaptic Function

During the co-culture electrophysiological experiments, it appeared that some basic electrophysiological properties differed from recordings done in hippocampal cultures alone. The first speculation was that the dissimilarity was because of the different ages at which the cultures were studied. Usually hippocampal electrophysiology studies are done at 14 days *in vitro*; alternatively to mimic chronic SSRI antidepressant use, many of the co-culture studies are conducted at 21 DIV. Dissociated co-cultures were prepared identically to previous experiments, half of the coverslips were recorded from at 14 DIV and

the other half were examined at 21 DIV. In a side-by-side comparison of dissociated co-cultures recorded at 14 DIV (acute paradigm) or 21 DIV (chronic paradigm), there were no distinguishable differences in either the events amplitude or frequency of both excitatory and inhibitory miniature synaptic currents in dissociated co-cultures (Figure 3-8A & B white bars). Once age of the cultures was eliminated as a reason for the disparities in miniature synaptic currents, attention was turned to the type of cultures used, hippocampal alone versus the raphe/hippocampal co-culture. Particularly the amplitude of the mEPSCs and mIPSCs were significantly lower in the co-cultures than that of hippocampal neuronal cultures (Figure 3-8 A & B). In concurrence with the spontaneous miniature electrophysiological data, the amplitude of the network activity was also significantly reduced in the co-culture compared to the hippocampal neurons (Figure 3-8C). The frequency did not appear to be altered by the presence of raphe neurons in the cultures. It is not quite understood how the existence of raphe neurons in the hippocampal culture could exert such a significant effect on amplitude events seen in these experiments. As previously mentioned, this raphe/hippocampal co-culture system was devised in order to study SSRI antidepressant effects on synaptic function. While the decreased event amplitudes in raphe/hippocampal co-cultures are still a mystery, hopefully the lack of an antidepressant effect was not masked by these phenomena. It cannot be

unequivocally stated that occurrences observed in this system are physiological and are not an artifact of the culture itself.

Collectively, these specific miniature synaptic transmission experiments provided some insight into how serotonin influences hippocampal neurotransmission, but none of the changes detected could be directly attributed to an effect of selective serotonin reuptake inhibitors. The lack of obvious changes suggest that the modifications exerted from the application of SSRIs are subtle and gradual, possibly so much so, that the electrophysiologically parameters measured were not sensitive enough to detect any of these changes. Most drugs typically work by modest alterations on its targets rather than substantial ones. The results gathered from this set of experiments imply that the depressive state is much more of an understated mechanistic dysfunction, which might be the reason it has been so challenging to understand its exact pathophysiology.

Discussion

Characterizing the serotonergic pathway between raphe and hippocampus in an *in vitro* culture system provided important support for the electrophysiological experiments used to study how the delayed onset of antidepressant efficacy might be a result of SSRI-mediated synaptic modifications. Existence of both neuronal types, raphe and hippocampus, was confirmed by immunocytochemical labeling with α-SERT and α-MAP. Co-staining with these specific antibodies illustrated a symbiotic pairing in the co-culture. I believe this association strengthened the dissociated co-cultures in some aspects. As mentioned many of the studies were conducted at 21-24 DIV, which is quite a mature time point. However, DAPI immunostaining showed that these co-cultures were alive and healthy when compared to raphe or hippocampal cultures alone of the same age (data not shown), making it the ideal system in which to carry out chronic SSRI studies.

HPLC experiments showed that similar to the dissociated raphe cultures, the co-cultures also produce and release serotonin and its metabolite. I did however notice a very slight trend toward a decrease in 5-HIAA concentration in cell samples of both raphe and co-cultures following SSRI exposure. It is probable that re-uptake inhibition at the presynaptic terminal could be modifying internal 5-HT concentration in order to preserve what remains after vesicle release. That is, because SERTs are not taking serotonin back up into the presynaptic terminal due to SSRI function, there might be a reduction in

mitochondrial oxidation of 5-HT, thus a reduction in its metabolite; however this concept was not further investigated.

Spontaneous network activity evaluation of hippocampal neuronal culture and co-cultures strengthen the argument that co-culture electrophysiological recording were from hippocampal and not raphe neurons in the co-culture. Although there was no optically fluorescent means of distinguishing raphe from hippocampal neurons in the co-cultures, the decrease in spontaneous network activity frequency in both hippocampal and co-cultures in comparison to raphe cultures strongly affirm this concept.

Though there was no parallel change in miniature neurotransmission with the use of chronic fluoxetine and citalopram in the co-culture preparation, fundamental information was attained. First the mPSC modulation after acute fluoxetine treatment might have been a result of some of the non-specific binding properties discussed in Chapter 2 (Schreiber and Pick, 2006; Stahl, 1998c, d) that are distinctively separate from antidepressant efficacy effects. After chronic exposure to fluoxetine which better resembles antidepressant use, no alteration was detected. Alternatively, these experiments could imply that these changes might also be a result of the short-term activation and consequent desensitization of 5-HT_{1A} autoreceptors as well as activation of 5-HT_3 and 5-HT_2 heteroreceptors which are consistent with previous data (Ye et al., 2008). In an acute bath application of 30 μM fluoxetine, Ye *et al*. witnessed a potentiation of both mIPSC

frequency and amplitude. Although fluoxetine should theoretically act presynaptically at SERTs, other studies including Ye *et al*. have reported unexplainable synaptic alterations in hippocampal cultures after fluoxetine treatment (Balfour, 1980; Derry et al., 2007; Goren et al., 2007; Robinson et al., 2003; Ropert and Guy, 1991; Tunnicliff et al., 1999). These findings only further complicate the molecular understanding of fluoxetine as an antidepressant.

On the contrary electrophysiological results collected after chronic citalopram administration implies that cit could be a "true SSRI". The observed changes in dissociated co-cultures after chronic citalopram also suggest that there might be a specific reduction in excitatory AMPA receptor number or function as demonstrated in mEPSC events paired with a significant enhancement in inhibitory presynaptic terminal or release probability mediated through GABAergic boutons. It has been reported that serotonin release might be involved in GABAergic synaptic systems. Chronic inhibition of SERTs could lead to a reduction in 5-HT metabolism which could lead to an increase in GABA (Zhong and Yan, 2004). In a postmortem study, a significant reduction in GABA neurons was found in the orbitofrontal cortex of patients suffering from MDD (Rajkowska et al., 1999; Taylor et al., 2005). The increase in mIPSC frequency upon citalopram exposure observed in my experiments could be a manifestation of rectifying the GABAergic neuronal volume at raphe synapses mirroring the observations in the postmortem study. Likewise, the observed decrease in

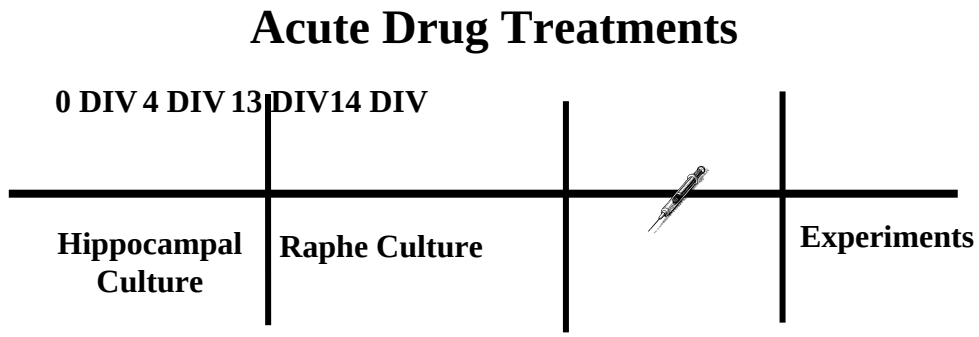
mEPSC amplitude following chronic citalopram is consistent with studies suggesting that SSRIs lead to a reduction in glutamatergic receptors (Pittaluga et al., 2007). This idea of excitatory receptor changes will be further discussed in following chapters.

The most astonishing finding revealed from this set of experiments was the reduction in amplitudes of co-culture events in contrast to hippocampal cultures alone. In every electrophysiological measure done in this study hippocampal amplitude events in co-cultures were significantly smaller than hippocampal amplitude events in hippocampal cultures alone. Activation of different serotonin receptors could be the contributing factor in the reduction in miniature and network event amplitudes (Yuen et al., 2005; Zhong and Yan, 2004). Many of the serotonin receptors are G-protein coupled receptors; therefore, activation of 5-HT heteroreceptors by serotonin might lead to interactions with both excitatory and inhibitory receptors located at hippocampal synapses and affect their ability to respond to neurotransmitter release resulting in diminished amplitude responses. Conversely, auto-activation of the 5-HT autoreceptors may also play a role in decreasing the effects neurotransmitters might have on the postsynaptic glutamate and GABAergic receptors, which could also results in weakened amplitude events in the co-cultures. Activation of these receptors might result in the reduced capacity for the presynaptic terminal to

release neurotransmitters into the synaptic terminal and activate postsynaptic receptors (Tao et al., 1996; Zhong and Yan, 2004).

As a whole, these experiments permitted the continued evaluation of other synaptic parameters on the quest to determine if synaptic dysfunction is behind the delayed onset of antidepressant action in depressed states. The findings indicated that dissociated raphe and hippocampal neurons were making functional synaptic connections and producing and releasing serotonin in the *in vitro* co-culture system.

A



B

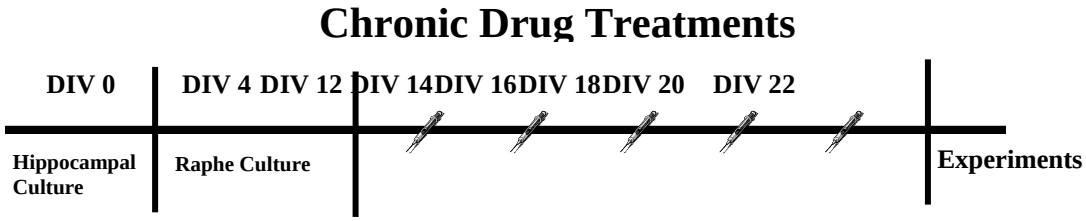


Figure 3-1 Timeline of Raphe/Hippocampal Co-culture Dissection Preparation and Experiments (A) For acute experiments co-cultures were treated once overnight for 18-24 hrs and experiments were done at 14-16 DIV **(B)**For chronic experiments SSRIs were given every other day for 10 consecutive days. All experiments were conduced between 22-25 DIV

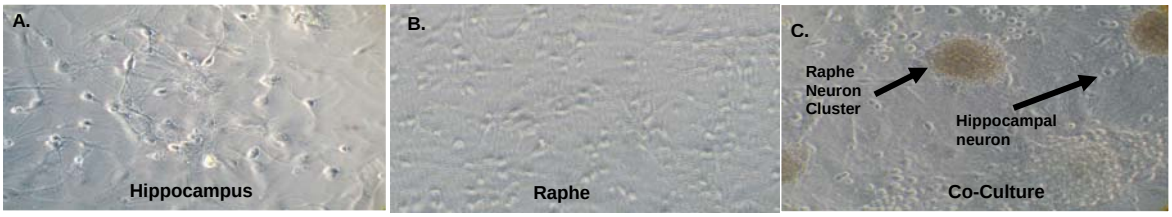


Figure 3-2 Light Microscopy Images of Culture Systems (A) Hippocampal Neuronal Culture plated on matrigel, **(B)** Raphe Neuronal Culture plated on poly-lysine, **(C)** Raphe and Hippocampal Neuronal Co-Culture. Black arrows point to the raphe clusters and hippocampal pyramidal cell formed on matrigel coated cover slips.

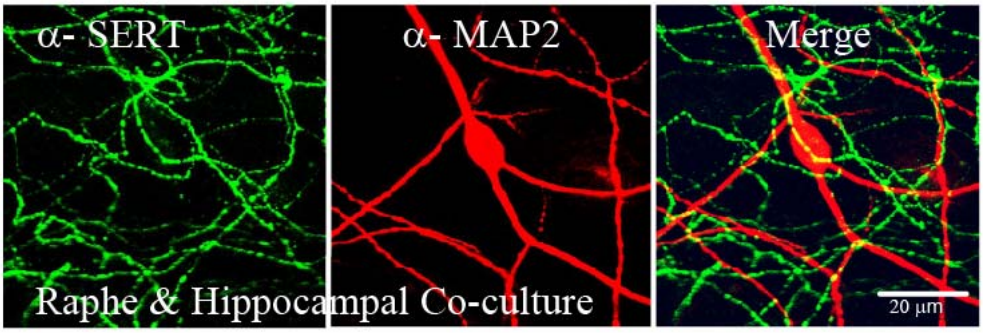


Figure3-3 Neuronal Confirmation: To confirm the presence of serotonergic and hippocampal neurons in the co-cultures, immunocytochemistry was conducted utilizing the α-SERT abs (green) to confirm raphe neurons and α-MAP2 abs (red) to confirm neuronal cell-types in dissociated raphe/hippocampal primary neuronal co-cultures express α -SERT in raphe processes that synapse onto hippocampal dendrites (α-MAP).

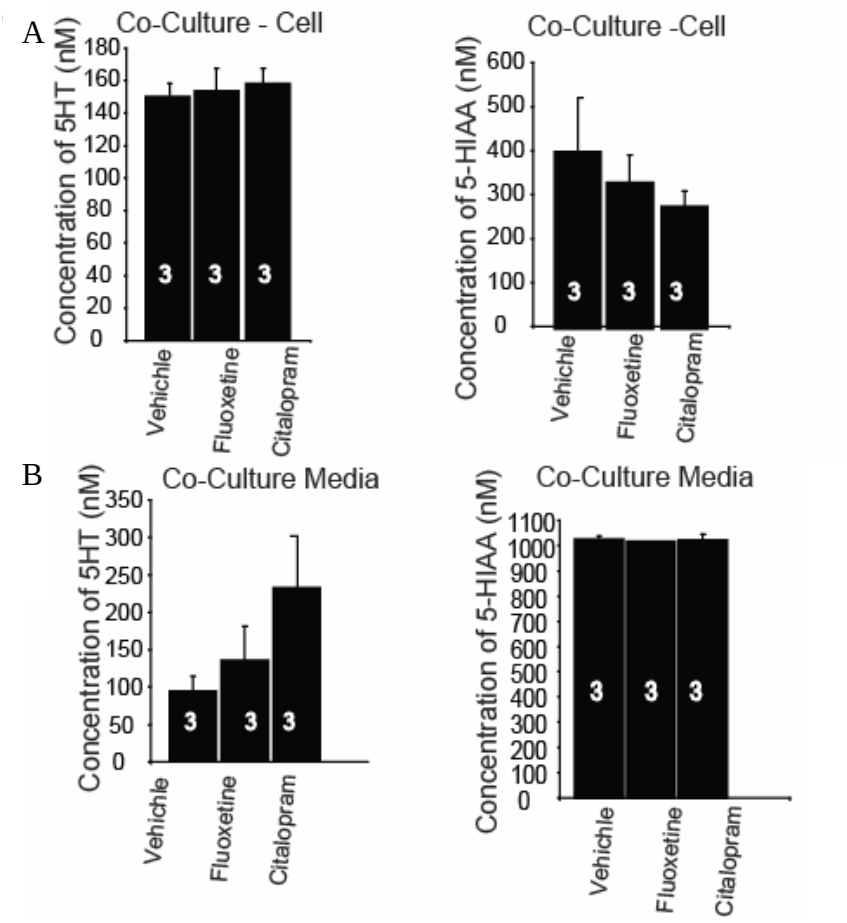


Figure 3-4 Dissociated Raphe/Hippocampal Co-cultures Produce and Metabolize Serotonin in Culture: High Performance Liquid Chromatography (HPLC) was conducted to determine the concentration of 5-HT and its metabolite 5-HIAA in mature raphe/hippocampal neuronal co-culture. Raphe cultures were treated with Fluoxetine (0.3μM), Citalopram (1μM), or Vehicle for 10 consecutive days. Samples were tested in duplicate. **(A)** Bar graphs show quantification of 5-HT (left) and 5-HIAA (right) in co-cultures **(B)** In co-culture media, the 5-HT concentration trends toward an increase with citalopram treated raphe neurons compared to vehicle treated.

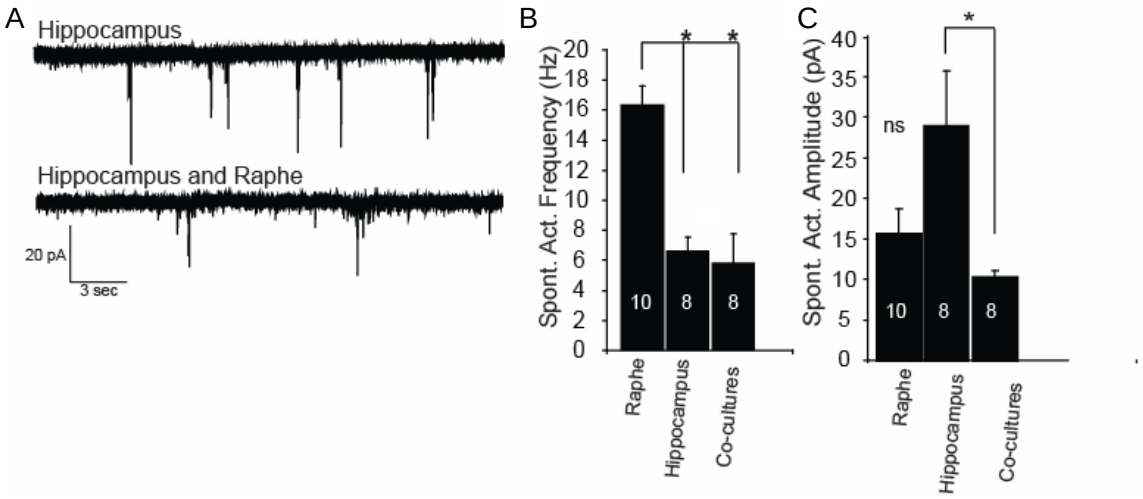
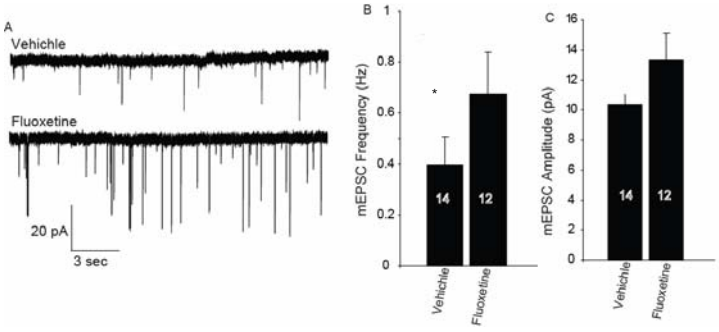


Figure 3-5 Comparison of Network Activity Between Neuronal Culture Types (A) Sample traces of network activity from mature hippocampal cultures and mature raphe & hippocampal co-cultures. Raphe bar included for comparison **(B)** Bar graph depicts a significant increase in spontaneous activity frequency in raphe neurons with no change between hippocampal and co-culture neurons. **(C)** Bar graphs shows an increase in spontaneous activity amplitude in hippocampal neurons compared to co-cultures and no significant change between raphe neurons.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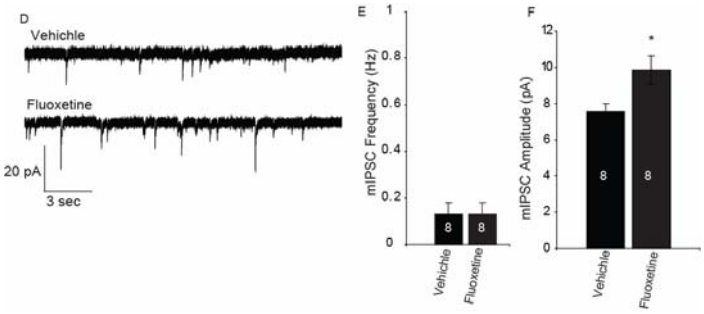
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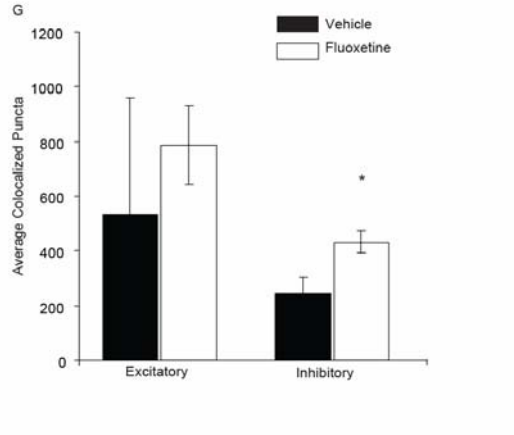
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Figure 3-6 Acute SSRI Treatment has Little Effect on Spontaneous Miniature Neurotransmission in Mature Co-cultures: Whole-cell voltage-clamped recordings of spontaneous miniature synaptic current in co-cultures. **(A)** Sample traces of mEPSCs recorded from 14 day old cultures treated with Vehicle or Fluoxetine (0.3 μM) overnight (18-24 hrs). **(B)** Bar graph shows a significant increase in mEPSC frequency after fluoxetine exposure **(C)** Bar graph shows no differences in mEPSC amplitude among vehicle and Flx treatments. The numbers on the bar graphs indicated the number of experiments. **(D)** Sample traces of mIPSCs recorded from 14 day old cultures treated with Vehicle or Fluoxetine (0.3 μM) overnight (18-24hrs). **(E)** Bar graph shows no change in mIPSC frequency. **(F)** Bar graph shows a significant increase in mIPSC amplitude between vehicle and Flx treatments, (*$p < 0.05$). **(G)** Bar graph represents quantification of co-stained co-culture puncta. Excitatory synapses were co-stained with α -Synapsin (presynaptic) and α-PSD (postsynaptic). No significant changes are observed in excitatory co-localized puncta (left). Inhibitory puncta was co-stained with α -Synapsin and α-Gephyrin (postsynaptic). Fluoxetine significantly increased co-localized inhibitory puncta (right) in acutely treated co-cultures; (* $p = 0.05$).

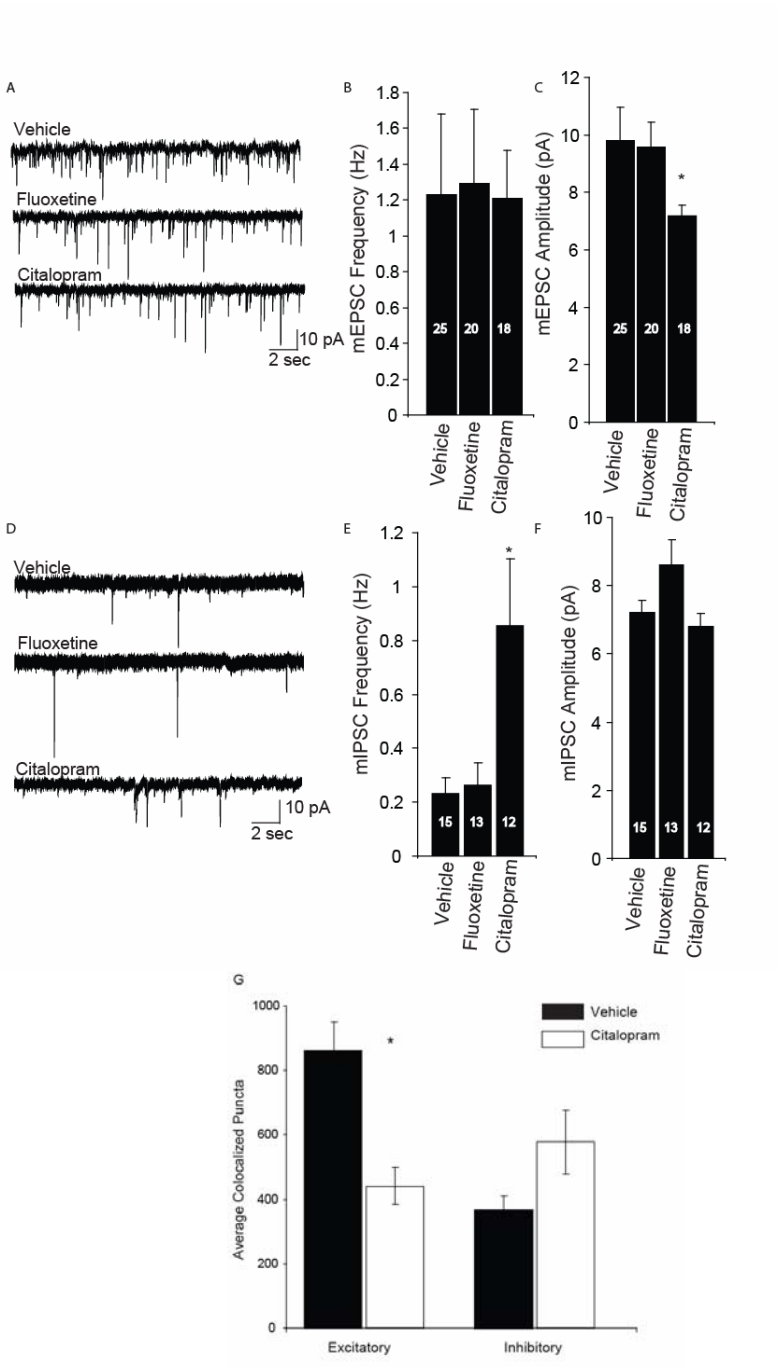
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Figure 3-7 Spontaneous Miniature Neurotransmission is Not Affected in Chronic SSRI Treated Mature Co-cultures: Whole-cell voltage-clamped recordings of spontaneous miniature synaptic current in co-cultures. **(A)** Sample traces of mEPSCs recorded from 21 day old cultures treated with Vehicle, Fluoxetine(0.3 μM), or Citalopram (1μM) 10 consecutive days. **(B, C)** Bar graph shows no differences in mEPSC frequency or amplitude among vehicle, Flx, and a significant decrease in mEPSC event amplitudes after chronic Cit treatments. The numbers on the bar graphs indicated the number of experiments. **(D)** Sample traces of mIPSCs recorded from 21 day old cultures treated with Vehicle, Fluoxetine(0.3 μM), or Citalopram (1μM) 10 consecutive days. **(E)** Bar graph shows a significant increase in mIPSC frequency with Citalopram, (*$p < 0.05$). **(F)** Bar graph shows no differences in mIPSC amplitude among Vehicle, Flx or Cit treatments. **(G)** Bar graph represents quantification of co-stained co-culture puncta. Excitatory synapses were co-stained with α -Synapsin (presynaptic) and α-PSD (postsynaptic). Chronic citalopram significantly decreases excitatory co-localized puncta (left). Inhibitory puncta was co-stained with α -Synapsin and α-Gephyrin (postsynaptic). No change was detected in co-localized inhibitory puncta (right), (* $p = 0.05$).

A B C

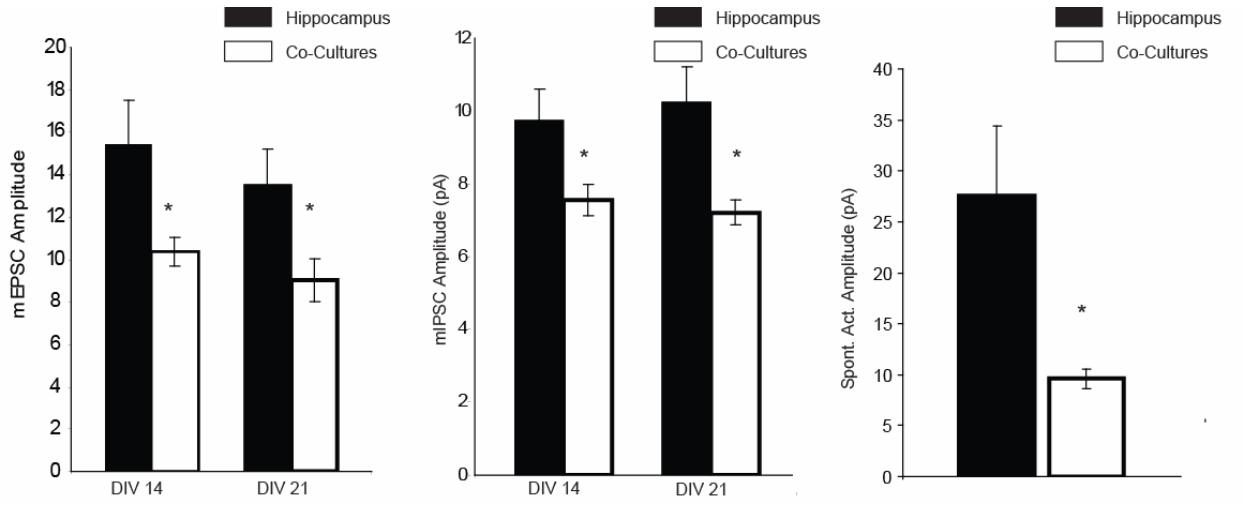

Figure 3-8 Comparison Between Age vs. Neuronal Culture Type. (A) Bar graph shows a significant decrease in mEPSC event amplitudes of co-cultures at 14 and 21 DIV compared to hippocampal cultures at the same time points, **(B)**Bar graph depicts a significant decrease in mIPSC event amplitudes of co-cultures at 14 and 21 DIV compared to hippocampal cultures at the same time points **(C)** Bar graph shows a significant reduction in network activity amplitude of co-cultures.

CHAPTER 4

THE EFFECT OF CHRONIC SSRIS ON NMDA RECEPTOR SYNAPTIC FUNCTION

Background

The monoamine hypothesis of depression has branded serotonin transporters as the premiere target of antidepressant drug therapy. However, the delayed effects of SSRIs have led to an exploration of new drugs that work more efficiently. Drugs that alter glutamatergic synaptic systems have more recently become clinically relevant because of their robust and rapid antidepressant effects in depressed patients. Malfunction in the glutamatergic ligand-gated NMDA ion channel has been implicated in the pathophysiology of numerous mental illnesses including depression, because of its profound effects on neuronal function such as synapse formation, synaptic plasticity, as well as learning and memory (Pittaluga et al., 2007; Zarate et al., 2003). NMDA receptor antagonists as contenders for antidepressant therapy first surfaced from an animal stress study. After one week of low-intensity shock training, LTP in mouse hippocampal slices were studied. The authors concluded that inescapable but not escapable stress disrupted hippocampal long-term potentiation, a phenomenon that is dependent on NMDA receptor function (Shors et al., 1989; Trullas and Skolnick, 1990), inferring that NMDA receptor function and the pathways affected by them could be involved in

mitigating depressive symptoms. Zarate *et al.* reported that glutamatergic transmission was enhanced in depressive states and administration of antidepressants led to a down-regulation of glutamate release (Zarate et al., 2003). Over the past twenty years different NMDA receptor antagonists like use - dependent channel blocker MK-801 or competitive blocker AP-7 have shown an antidepressive effect in conventional behavioral paradigms designed to test despair and depression in rodents. Other studies have shown that antidepressant therapy can depress NMDA receptor function and ligand binding (Pittaluga et al., 2007). These experiments also suggest that if SSRIs and traditional antidepressants work by indirectly inhibiting NMDA receptor function over 2-4 weeks, then NMDA antagonist could circumvent this prolonged pathway and directly mediate effects in a much shorter time frame (Skolnick et al., 2009; Trullas and Skolnick, 1990).

Stemming from the animal studies, physicians have treated depressed patients that were unresponsive to at least two other antidepressants with a one-time low dose of ketamine, a potent non-competitive NMDA receptor antagonist, and observed robust and long-lasting antidepressant effects that persisted for over two week and in some cases reached remission according to the HAM-D score (Berman et al., 2000; Pittaluga et al., 2007; Skolnick et al., 2009; Zarate et al., 2006a; Zarate et al., 2006b). Why ketamine is so effective has not been comprehensively elucidated. In one study, human SERT constructs were

transfected into HEK 293 to study the interactions of antidepressants and IV anesthetics. The authors reported that ketamine inhibited SERT function when given alone, however, fluoxetine exposure attenuated this inhibition such that the effect was no longer distinguishable at clinically relevant doses (Zhao and Sun, 2008). The mechanism of action of ketamine as an antidepressant could be a consequence of its competitive binding to SERTs, thus allowing 5-HT to not be removed from the synapse presynaptically by SERTs, in addition to inhibiting the function of NMDA receptors and propagating intracellular postsynaptic cascades (Martin et al., 1990; Nishimura et al., 1998). Collectively, these studies implicate NMDA receptors as a viable candidate for investigating the mechanism of antidepressant therapy.

Results

Previously, I investigated the effects of antidepressants on AMPA receptor-mediated miniature postsynaptic currents (mEPSCs) by adding TTX, PTX, and AP-5 to the Tyrode's external bath solution. We observed small changes with citalopram (e.g. a decrease in mEPSC amplitude and an increase in mIPSC frequency) in these experiments; however the changes were not consistent with both SSRIs. Given that excitatory neurotransmission is mediated through two different glutamatergic neuronal receptors (AMPA and NMDA); the next logical step was to investigate the role of NMDA receptors in chronic SSRI therapy. To isolate NMDA receptors TTX, PTX, and CNQX were added to a bath solution that contained a low concentration of magnesium to relieve the co-agonist Mg^{2+} block in NMDA receptors. Changes in the charge of event responses were studied. A difference in receptors at the postsynaptic terminal membrane can be measured by looking at changes in the charge under a peak. Dissociated co-cultures were chronically treated with fluoxetine and citalopram as in the previous experiments and recorded on 22 DIV.

SSRIs' effects on NMDA synaptic transmission

Upon whole-cell voltage-clamped electrophysiological examination of spontaneous NMDA miniature events, chronic SSRI (fluoxetine and citalopram) resulted in a significant decrease in the charge of NMDA-mediated currents,

compared to vehicle treated co-cultures [Veh: 17.7 pC, Flx: 13.6 pC p= 0.003, Cit: 14 pC p= 0.016] (Figure 4-1A & B). To ensure that the responses observed were actually mediated through NMDA receptors 50μM of AP-5 a competitive NMDA receptor blocker was applied acutely during recordings. AP-5 abolished the NMDA response, suggesting that the observed effects of chronic SSRIs on spontaneous NMDA transmission were specific to NMDA receptors.

To further explore this possibility, the global NMDA response was studied in the co-culture system by directly perfusing 1mM NMDA during recordings to activate all surface NMDA receptors synaptically and extrasynaptically. If the change observed in the spontaneous NMDA-mediated mEPSC activity has a result of a re-trafficking of receptors, measuring the global response gives a overall evaluation of NMDA receptors. In raphe/hippocampal co-cultures chronic SSRI treatment resulted in diminished NMDA average peak amplitudes as compared to vehicle [Veh: 72.4 pA, Flx: 45.9 pA p= 0.0098, Cit 48.8 pA p= 0.024] (Figure 4-2 A & C black bars). In performing these global NMDA experiments it became apparent that the average peak amplitude was severely reduced compared to hippocampal NMDA peak amplitude responses (Figure 4-3A). Akin to raphe-mediated reductions observed in previous miniature electrophysiological experiments (Chapter 2), this suggested that the presence of raphe neurons in the co-culture might again contribute to the decrease in NMDA receptor-mediated currents.

5-HT_{1A} receptor effects on NMDA receptor activity

Observation of the diminished NMDA receptor-mediated global responses in dissociated co-cultures prompted an investigation into how the raphe neuronal circuitry might play a role in the reduction of specific measures of hippocampal synaptic activity. Previous studies have shown that 5-HT can cause a decrease in NMDA receptor current amplitude by activating synaptic 5-HT_{1A} receptors (Pallotta et al., 1998; Yuen et al., 2008; Yuen et al., 2005; Zhong et al., 2008). Specifically, Yuen *et al*. demonstrated by slice electrophysiology in PFC pyramidal neurons that serotonin reduces NMDA receptor current through activation of 5-HT_{1A} receptors. Literature has also suggested that 5-HT_{1A} receptor activation can induce hyperpolarization of hippocampal neurons through opening inward rectifying potassium channels and reducing calcium currents via inhibition of voltage gated Ca^{2+} channels (Andrade et al., 1986). A decrease in Ca^{2+} flow reduces the activity of NMDA receptors during a depolarizing event. 5-HT_{1A} receptors are found in dendritic compartments and are associated with glutamate receptor rich dendritic spines (Kia HK, 1996). Furthermore it is highly probable that NMDA and 5-HT_{1A} receptors interact to co-modulate hippocampal synaptic activity.

To test whether the activation of 5-HT_{1A} receptor could be the cause of the small NMDA responses in co-cultures, an antagonist WAY 100635 (W) was co-administered with both SSRIs and run through the same NMDA perfusion

experiments (Figure 4-2 B). With the co-application of 5-HT_{1A} receptor antagonist, the global NMDA response significantly increased in each treatment (Veh, Flx, & Cit) compared to control amplitudes [Veh + W: 151.2 pA, Flx + W 80.3 pA, Cit + W: 76.7 pA] (Figure 4-2 C white bars). Interestingly, the same decrease in global NMDA response was observed between SSRI treated and vehicle even with WAY100635 administration (Figure 4-2 C). While the peak amplitude was not restored to levels seen in the hippocampal recordings, the data suggest that 5-HT_{1A} may be partially responsible for the NMDA receptor current effect observed in the co-cultures. Similar to these findings, Gartside *et al.* reported only seeing a 10% increase in NMDA response after 5-HT_{1A} antagonist exposure in raphe midbrain slices (Gartside et al., 2007).

Combined with the previously reported findings presented in chapter 3, there is growing evidence that serotonin might have a negative effect on hippocampal amplitudes electrophysiologically (Deak et al., 2000; Kobayashi et al., 2008; Pittaluga et al., 2007). If so, it is possible that hippocampal neurons in co-cultures already have smaller current amplitudes even before application of SSRIs or the 5-HT_{1A} antagonist. To further probe this finding, dissociated hippocampal cultures were chronically treated with the 5-HT_{1A} agonist 8-OH-DPAT and global NMDA currents were recorded. Chronically treated hippocampal cultures showed a 69% reduction in global NMDA response compared to vehicle treated hippocampal cultures [Veh: 972.4 pA, 8OH-DPAT

302.9 pA p= 1.94 e -8] (Figure 4-3A-B). The peak amplitude of NMDA responses was still greater in agonist treated hippocampal neurons when compared to vehicle treated co-cultures and WAY100635 treated co-cultures, by 76% and 50%, respectively (Figure 4-2 & 3C). These data suggest that (a) 5-HT_{1A} receptors modulate NMDA receptors by attenuating their function, and (b) 5-HT itself can also modulate the NMDA response, which might explain the depressed amplitudes observed in the co-cultures.

To probe more deeply into the mystery of the reduced NMDA receptor response in co-cultures, an immunocytochemical approach was taken, with the hypothesis that 5-HT_{1A} receptors are mediating NMDA receptor trafficking at the membrane. Examining changes in surface NMDA receptors can be accomplished by taking advantage of the ability to conduct these experiments under permeable or impermeable conditions. An antibody against the NR1 subunit of NMDA receptors was co-labeled with α-MAP-2 to elucidate whether 5-HT_{1A} receptor activation can alter NMDA receptor expression in dissociated hippocampal and co-cultures. The NMDA receptor forms a heterotetramer between two NR1 and two NR2 subunits. NR1 is the obligatory subunit in the NMDA complex and so probing with an antibody against this subunit should provide an idea of NMDA localization. Preliminary immunocytochemistry experiments with α -NR1 co-stained with α-MAP2 illustrated that the surface NR1 subunit was reduced in all co-cultures and even more so in those treated with SSRIs (Figure 4-5). In

comparison to hippocampal cultures alone, addition of 8OH-DPAT significantly reduced the α-NR1 immunoreactivity. This experiment suggests that NMDA receptor trafficking might be the explanation for the reduction in NMDA receptors currents observed in the dissociated co-cultures. However, it will be necessary to repeat these experiments to confirm these results.

Discussion

After the primary miniature synaptic event recordings failed to reveal any information directly answering the question of SSRI-mediated synaptic effects NMDA receptor- mediated spontaneous miniature currents were probed. Chronic SSRI exposure resulted in a significant decrease in NMDA mEPSC event charge that was abolished upon an acute AP-5 treatment in the dissociated co-cultures. This finding can be interpreted as a possible alteration in NMDA receptor membrane expression. This was the first indication that NMDA receptors could be involved in the synaptic component of the pathophysiology of depression. To better understand the mechanism by which NMDA receptors might be functioning, global NMDA amplitude responses were recorded under the same chronic SSRI paradigm. Initial observation showed a significant decrease in the global NMDA current amplitudes suggesting that NMDA receptors were reduced synaptically and extrasynaptically. Such a large scale decrease could be a result of receptor trafficking. However after closer evaluation of this response it appeared that the current amplitude responses were substantially reduced in comparison to hippocampal global NMDA current amplitudes. Again, it appeared that the existence of raphe neurons in the co-culture was hindering hippocampal synaptic current responses. Yuen *et al.* demonstrated that in PFC slice electrophysiology serotonin reduced NMDA EPSCs through activation of 5-HT_{1A} receptors (Yuen et al., 2005). Therefore, it was possible that 5-HT_{1A} receptor activation was behind

the reduction in NMDA current after chronic SSRI administration in the co-cultures. Co-application of the 5-HT_{1A} receptor antagonist WAY 100635 and either fluoxetine or citalopram in the co-culture attenuated the decrease in global NMDA current amplitude suggesting that this phenomenon was mediated through 5-HT_{1A} receptors. However peak NMDA amplitudes in co-cultures did not reach the same magnitude as hippocampal NMDA amplitude, further suggesting that serotonin plays an important role in the raphe/hippocampal pathway. To more closely determine if 5-HT_{1A} receptors directly reduce NMDA receptors function, receptor agonist 8OH-DPAT was chronically applied in dissociated hippocampal cultures. 5-HT_{1A} receptor activation in hippocampal cultures resulted in a significant reduction in global NMDA current amplitudes that mimicked, but was not equal, to NMDA-mediated currents observed in the co-cultures. Moreover, immuno-staining for NR1 showed a decrease in surface NMDA receptors after chronic SSRI and 5-HT1A agonist treatment in co-cultures and hippocampal cultures respectively.

WAY 100635 has been previously shown to significantly enhance NMDA responses, but not AMPA responses in raphe neurons, suggesting that some of the NMDA receptors on raphe neurons are modulated through 5-HT_{1A} autoreceptors (Gartside et al., 2007). Hence, it is entirely possible to assume that hippocampal NMDA receptors are also modulated in part through 5-HT_{1A} heteroreceptors in this dissociated co-culture assay. These findings strongly suggest that chronic

SSRI-mediated serotonin enhancements activate 5-HT_{1A} heteroreceptors that modulate a decrease in NMDA receptor function. The *in vitro* co-culture data collected from the NMDA receptor experiments correlate nicely with clinical ketamine antidepressant observations seen in human subjects. NMDA receptors might be the key to understanding the molecular mechanism by which SSRIs function in antidepressant efficacy.

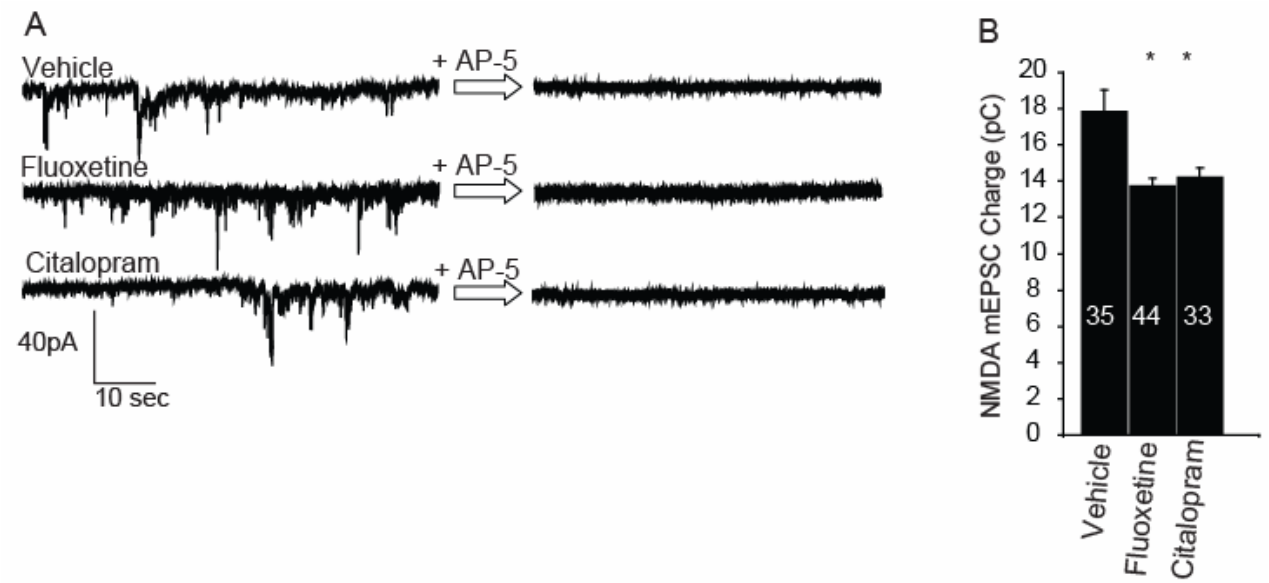

Figure 4-1 Chronic SSRIs Decrease the NMDA Miniature Neurotransmission. (A) Sample traces of whole- cell voltage- clamped mature co-cultures were recorded in Tyrode's solution + TTX, CNQX, and PTX. Cultures were treated with Vehicle, Fluoxetine (0.3μM), or Citalopram (1μM) chronically for (10 days) Application of AP-5 (50μM) abolishes the NMDA mini response. **(B)** The bar graph shows a significant decrease in NMDA mEPSC charge with SSRI treatment compared to vehicle, (*$p < 0.05$). Numbers on bar graphs represent the number of experiments.

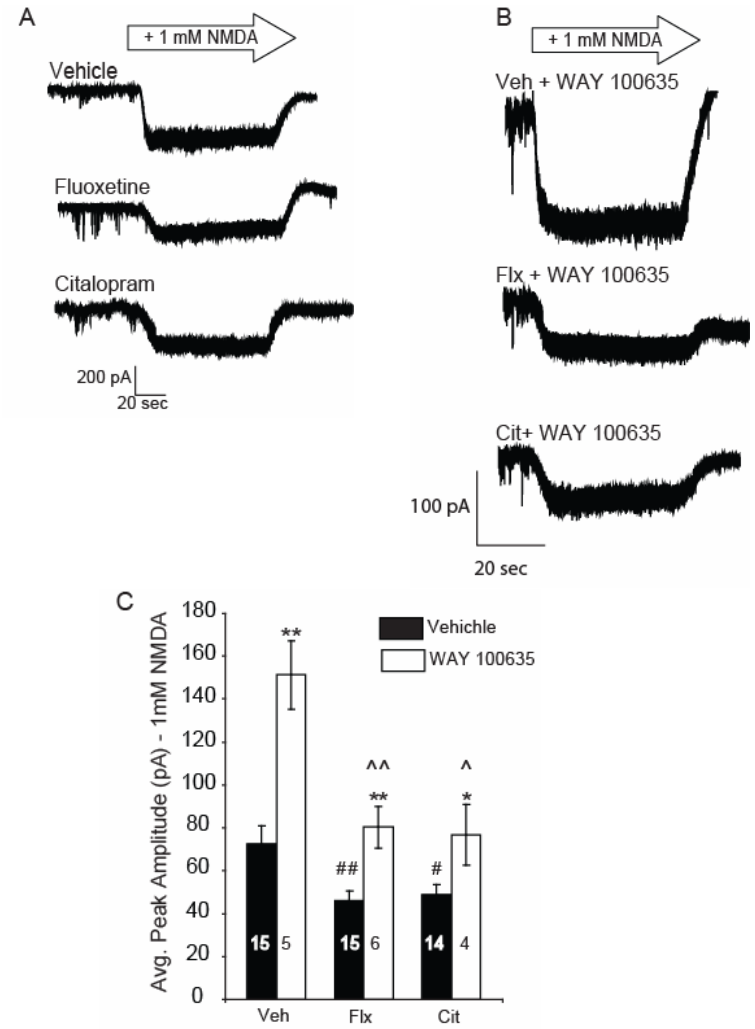

Figure 4-2 5-HT$_{1A}$ Receptor Antagonist Attenuates Reduced Global NMDA Current Response in Co-cultures (A) Representative traces of NMDA (1mM) perfusions in SSRI treated co-cultures. **(B)** Representative traces of co-cultures co-treated with veh, flx, or cit and WAY 100635 **(C)** The bar graph shows a significant decrease in NMDA peak amplitude among Flx and Cit treated cultures compared to Veh, (black bars), ($^{\#}p< 0.05$; $^{\#\#}p< 0.01$). Chronic administration of WAY 100635 results in a significant increase in the peak amplitude among all treatments (white bars) compared to only SSRI treated cultures (black bars), (*p< 0.05; **p< 0.01). Chronic SSRIs administration decreases the peak amplitude upon NMDA application, (^p< 0.05; ^^p< 0.01)

A B

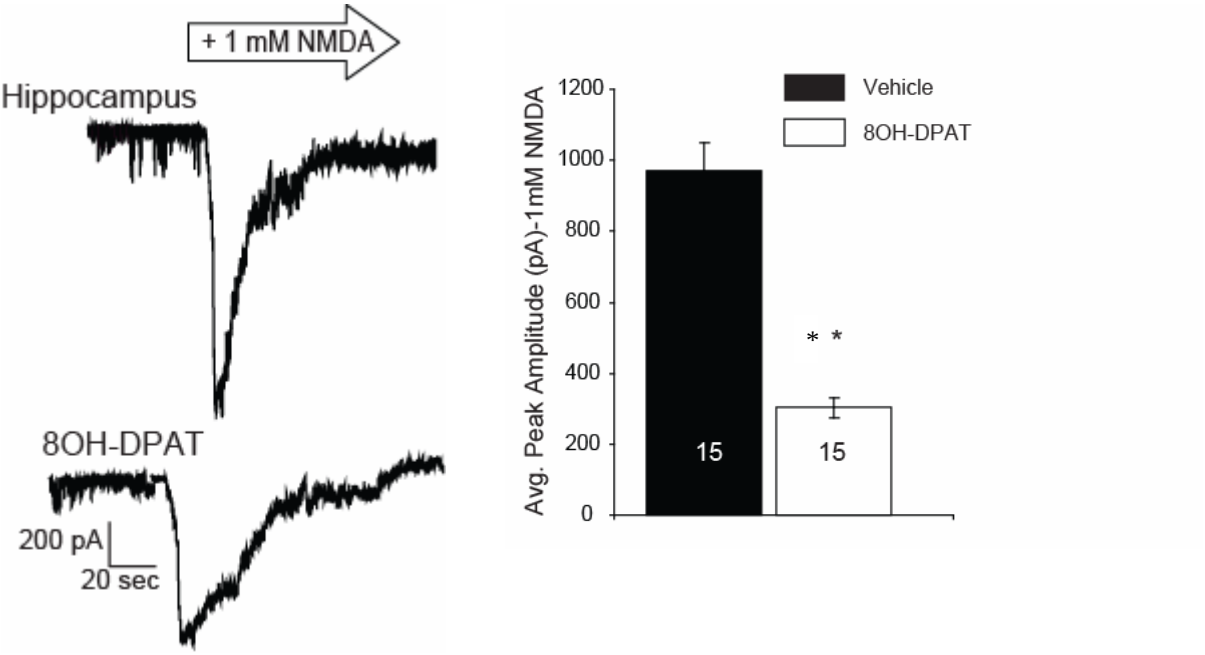

Figure 4-3 5-HT$_{1A}$ Receptor Agonist Attenuates the NMDA Global Response in Hippocampal Neurons. (A) Representative traces show hippocampal response after 1mM NMDA perfusion (top) and hippocampal neurons with 8OH-DPAT chronic exposure **(B)** Bar graph represents the reduction in average NMDA peak amplitude with and without the 5-HT1A agonist, (**p< 0.01).

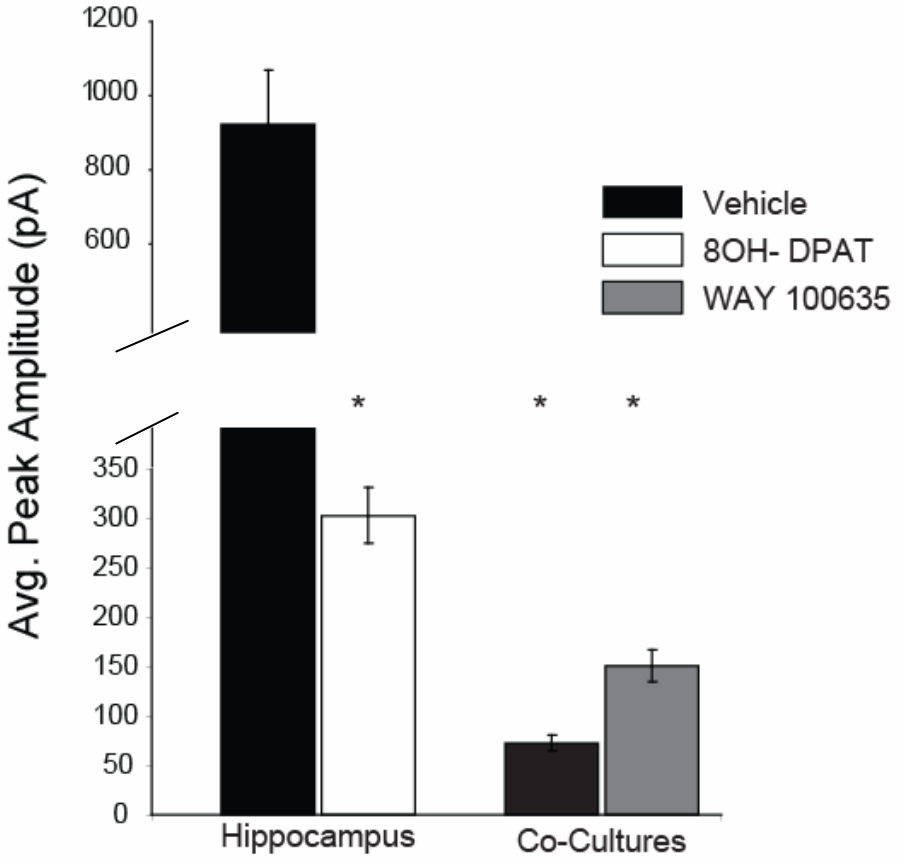

Figure 4-4 Global NMDA Response Comparison. Bar graph shows average amplitudes in response to 1mM NMDA perfusion. Raphe neurons significantly reduce the response, but are partially restored upon WAY100635 treatment. Treating hippocampal cultures with the 5-HT_{1A} agonist mimics the raphe affect in co-cultures, by decreasing the NMDA global response, (*$p<0.01$).

A B

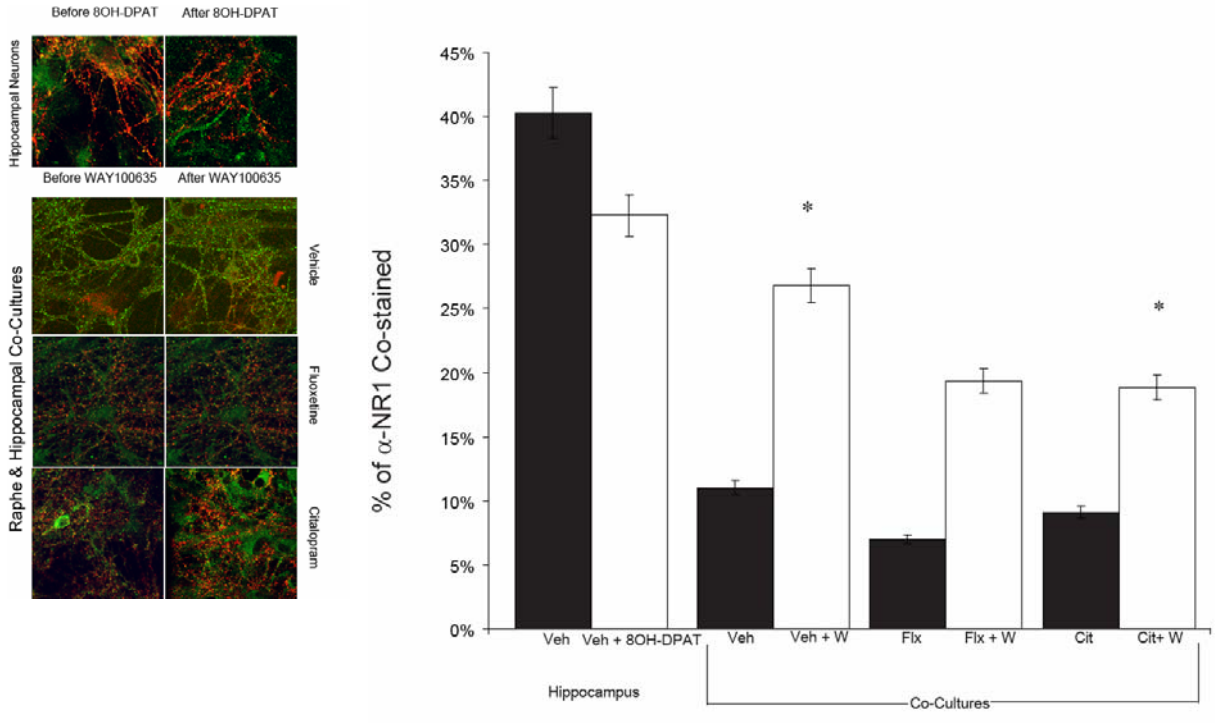

Figure 4-5 NR1 Trafficking of NR1: (A) Confocal images of stained cultures. Hippocampal cultures received 5-HT_{1A} receptor agonist 8OH-DPAT. Co-cultures were co-administered with SSRIs and 5-HT_{1A} receptor antagonist WAY 100635. Both hippocampal and co-culture were co-stained with α-NR1 and α-MAP2 to examine whether 5-HT_{1A} activation mediated NMDA receptor removal from the synaptic membrane. **(B)** Representative bar graph of percent α-MAP2- positive puncta that co-localized with α-NR1

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

A series of immunocytochemical, neurochemcial, electrophysiological, and biochemical experiments were carried out to attempt to answer the intriguing question about the underlying mechanism behind the delayed onset of the clinical antidepressants effect. To accomplish this hefty task a raphe/hippocampal co-culture system was developed to recapitulate the serotonergic pathway implicated in the pathophysiology of depression. Re-creation of this circuitry *in vitro* requires precise and proper dissection of the dentate gyrus of the hippocampus and raphe nuclei. I have demonstrated that antidepressants might modulate their effect through alterations in synaptic neurotransmission. Chronic selective serotonin reuptake inhibitors function by binding to raphe serotonin transporters and increasing synaptic 5-HT which activates 5-HT_{1A} receptors. These heteroreceptors mediate NMDA receptor trafficking from both the synaptic and extra-synaptic membrane in hippocampal neurons resulting in a suppressed NMDA current response. Consequently, intracellular downstream pathways mediated through NMDA receptor function are altered. This gradual cascade of events might be the cause of the delayed onset of antidepressant therapeutic effects. Therefore, chronic SSRI antidepressants modulate therapeutic effects by 5-HT_{1A} receptors mediated NMDA receptor trafficking.

Chapter 2: Development and characterization of the in vitro midbrain raphe nucleus culture system

The first step in this study was to master the dissociated raphe neuronal culture system as it was the framework in which the rest of the study was based. Conditions used in dissociated hippocampal cultures were also used for the raphe cultures; however, one important change to the protocol was the substitution of poly-lysine as an adhesion matrix on the coverslips instead of matrigel. This modification permitted dissociated raphe neurons to mature in a disperse manner and form necessary synaptic connections. After determining that the optimal time to prepare embryonic raphe cultures was at 15 days *in utero* E15 (Figure 2-1), the presence of serotonergic raphe neurons was immunocytochemically confirmed by immuno-labeling with an antibody specific for the serotonin transporter protein α-SERT located exclusively on raphe neurons. Confocal microscopy images illustrated that neurons dissected from E15 rat midbrain expressed SERTs throughout the axonal processes, identifying them as serotonergic raphe neurons (Figure 2-2).

High performance liquid chromatography experiments further validated that these neurons extracted from the embryos were actually serotonergic, as 5-HT and its metabolite 5-HIAA were detected in both cell and media samples. Interestingly, the concentration of 5-HIAA in both samples was several-fold higher than the concentration of 5-HT itself, presumably because of the oxidation

of serotonin. None the less, these neurochemical experiments indicate that the dissociated raphe cultures produce and release serotonin (Figure 2-3). The other piece of critical information gathered from this experiment is the efficiency of the SSRIs, fluoxetine and citalopram, used in this study. SSRIs are supposed to enhance serotonin released in the media. Elevated levels of 5-HT after SSRI exposure suggest that fluoxetine and citalopram are working properly.

Electrophysiological review of spontaneous network activity revealed that dissociated raphe cultures express functional excitatory and inhibitory receptors. Further chemical isolation of glutamatergic or GABAergic synaptic activity indicated that the primary form of neurotransmission in dissociated raphe cultures was modulated through excitatory glutamatergic receptors. It is likely that the inhibitory component of raphe transmission serves as a means to curtail the immense excitatory activity (Figure 2-4). Even though acute application of serotonin did not elicit a significant change in the frequency or amplitude of spontaneous network activity, the slight increase could be attributed to activation of the multiple 5-HT receptors (Figure 2-5). As previously mentioned, some serotonin receptors function in opposing pathways; thus robust changes upon 5-HT exposure might not necessarily be detected. Alterations might be unmasked in a scenario in which 5-HT receptors functioning in similar pathways were pharmacologically isolated and studied. For instance, specific activation of 5-HT $_{4, 6, \& 7}$ receptors, which all increase cellular levels of cAMP might result in a

more pronounced modification in spontaneous network activity currents with the addition of 5-HT. Absence of a profound serotonergic effect in dissociated raphe neurons following an acute application of 5-HT becomes important for experiments conducted in Chapter 3 with the raphe/hippocampal co-cultures. It can be assumed that the responses recorded in the co-culture experiments after SSRI administration and subsequent 5-HT increase are not a direct consequence of raphe 5-HT receptor activation, but more likely a result of serotonin receptor activation on hippocampal neurons. This further allows me to directly study how SSRIs and serotonin affect hippocampal synaptic function.

Discriminating monoaminergic from glutamatergic presynaptic raphe axonal vesicles offered insight into the vesicle composition involved in raphe neurotransmission. Co-immunostaining the dissociated raphe cultures with α-SERT and either α -VMAT-2 or α -VGLUT-3 showed that the majority of the raphe boutons contained vesicles filled with monoamines, and more specifically 5-HT. Although there was no direct stain for serotonin, colocalization of antibodies that are specific to serotonergic neurons in addition to monoaminergic protein transporters suggest that the boutons are loaded with 5-HT. Glutamate filled vesicles only accounted for a small percentage of raphe boutons, suggesting that the dissociated raphe cultures package the canonical excitatory neurotransmitter for release. These immunocytochemical experiments identified approximately 40% of raphe axonal vesicles (Figure 2-6). The remaining boutons

might be categorized upon additional staining with antibodies against the vesicular GABA transporter (VGAT). It is important to note that while α - VMAT-2 should immunostain all synaptic boutons carrying monoamines, I only investigated those that co-stained with the serotonergic marker α-SERT at the presynaptic terminal, therefore axonal vesicles that have boutons filled with other monoamines such as norepinephrine that might not be close enough to the presynaptic terminal to co-stain with α-SERT therefore they were not quantified. In the future immunocytochemical experiments will be necessary to account for the remaining 60% of raphe synaptic boutons.

Synaptophysin-pHluorin lenti-viral infection of the dissociated raphe cultures provided valuable information about the presynaptic machinery of raphe neurons. Employing this type of imaging technique to investigate vesicle recycling has been well studied in dissociated hippocampal neurons (Tian and Looger, 2008). Although there is some information regarding raphe neurons, the challenge of preparing *in vitro* dissociated raphe cultures has resulted in limited exploration of raphe neuronal recycling. Imaging pHluorin-infected raphe cultures suggests that raphe recycling is not significantly altered by a 7 day administration of fluoxetine and citalopram (Figure 2-8). These experiments simply enhance the understanding of SSRIs and what aspects of the synapse are truly affected by their use. Because SSRI antidepressants bind presynaptically to SERTs and increase synaptic 5-HT, the resulting effect should occur at

postsynaptic targets. Examination of the types of synaptic boutons responsible for vesicle recycling in the dissociated raphe neurons illustrated that although only 10% of α -SERT positive puncta co-stained positive for α-VGLUT-3 (Figure 2-6C), that a small proportion is responsible for one-third of raphe synaptic vesicle recycling (Figure 2-9D). Accurate preparation of dissociated raphe neuronal cultures built the foundation for studying *in vitro* effects of selective serotonin reuptake inhibitors on hippocampal synaptic function. Without this seminal step, it would be difficult to directly study a mechanistic view of how synaptic function adaptations might be involved in the delayed onset of antidepressant therapeutic effects.

Chapter 3: Development and characterization of the in vitro raphe/hippocampal co-culture system

After establishing a working dissociated raphe *in vitro* culture system, I proceeded to optimize the conditions for the dissociated raphe/hippocampal co-culture. Dissociated hippocampal neurons were plated on matrigel coated plates first and four days later dissociated raphe neurons were added directly on top of hippocampal neurons. The presence of both hippocampal and raphe neuronal populations was confirmed immunocytochemically via α-SERT and α -MAP2 co-staining. Unlike the hippocampal culture (Figure 2-2B), robust and distinct α -SERT staining was visualized throughout the axonal processes intermingling with

α -MAP2 positive neurons representing cohabitation of dissociated raphe and hippocampal neurons in cultures.

Neurochemical HPLC assessment in dissociated co-cultures showed presence of both 5-HT and its metabolite 5-HIAA in cell samples, and a strong trend toward and increase in media samples following SSRI treatment. Paralleling raphe cultures, 5-HIAA concentration appeared to be significantly higher than the monoamine itself in co-cultures.

Because the co-culture was not prepared using transgenic animals, there were no types of fluorescent identifying factor by which to distinguish raphe from hippocampal neurons in culture. Therefore, I had to rely on my own visual identification during recordings. Apart from the obvious raphe neuronal clusters that are impossible to patch as a result of the matrigel coated coverslips, I selected single pyramidal cells distinctly separate from the raphe clusters (Figure 3-1). Moreover, in the event that I patched onto a raphe neuron that was detached from the clusters, I was unable to maintain a patch or proper giga-seal, however, these obstacles were eliminated when patching on to hippocampal neurons in the co-culture.

In addition to visual identification of hippocampal neurons in co-cultures, spontaneous network activity was studied. While it might seem that measuring spontaneous network activity is too general of a measure, it furnished relevant information assisting with the verification of neuronal cell types in co-cultures

during electrophysiological experiments. In a comparison between recordings from hippocampal neurons alone and hippocampal neurons in co-cultures, there was no observable difference in spontaneous activity frequency (Figure 3-5). However when matched to spontaneous activity frequency in raphe cultures, both cultures had significantly reduced sPSC frequency. Network activity amplitudes in hippocampal neurons were significantly enhanced compared to co-cultures and raphe spontaneous currents. Observing almost identical frequency currents responses in hippocampal neurons alone and co-cultures suggest that the responses surveyed are from hippocampal neurons and not raphe neurons which show more than a two-fold increase in sPSC frequency events.

Studying the effects of SSRIs on spontaneous miniature neurotransmission uncovered little in terms of how antidepressants influence synaptic function. In the acute paradigm of fluoxetine exposure mEPSC frequency and mIPSC amplitude events were enhanced (Figure 3-6). This alteration suggests that SSRIs do indeed have an effect on synaptic function; however, antidepressants do not mediate therapeutic effects until several weeks after starting therapy. The acute antidepressant modulations witnessed in mPSC events could be a result of short-term rapid effects that might be indiscernible after continued use. It is also possible that acute fluoxetine exposure alters gene expression necessary for the long-term subsequent synaptic function to occur after chronic use. However, this idea will not be explored in the scope of this thesis. Future acute SSRI and

protein experiments could assist in answering this question. To better recapitulate antidepressant use in depressed patients, a 12 day chronic model of fluoxetine and citalopram administration was studied in the co-cultures. Many of the changes observed under acute fluoxetine treatment were abolished in the chronic SSRI model. Only mEPSC amplitude and mIPSC frequency were greatly affected by chronic citalopram. Fluoxetine had no detectable effects on any measure of spontaneous miniature neurotransmission after chronic use (Figure 3-7). This occurrence was surprising because fluoxetine and citalopram are both part of the SSRI family of antidepressants. These drugs are assumed to be highly specific to raphe SERTs and thought to behave similarly. However these data suggest that fluoxetine and citalopram do not modulate these specific measures in the same manner. Some studies such as those conducted by Schreiber *et al.* and Stahl *et al.* reported that fluoxetine might not be as selective a SSRI as once thought. Using an animal model of acute pain, they studied the antinociceptive properties of several SSRIs including fluoxetine and citalopram. Citalopram was reaffirmed as a "pure SSRI" (Schreiber and Pick, 2006; Stahl, 1998c), conversely fluoxetine showed several non-SSRI specific properties that have led to their application in other areas such as pain therapy and inhibition of other monoamine reuptake proteins. It is possible that unlike citalopram, fluoxetine could be utilized in other psychiatric disorders other than depression e.g. bipolar disorder or schizophrenia. As the main goal of this study was to examine consistent effects of both SSRIs

with the intention of comprehending how they mediate synaptic function after chronic use, cataloguing citalopram specific modulators while interesting, suggests that these effects are not specific to the antidepressant nature of SSRIs, but possibly to the biochemistry of citalopram. The fact that psychiatrists and pharmaceutical companies have not eradicated fluoxetine as a prescribed SSRI, also suggests that although it might not be as selective as citalopram, it still mediates therapeutic antidepressant effects similar to citalopram and other SSRIs.

Upon evaluation of synaptic activity of hippocampal neurons in dissociated co-cultures, I noticed a reduction in amplitude responses as compared to hippocampal cultures alone (Figure 3-8). The presence of raphe neurons in the co-cultures appeared to attenuate the amplitude response of the hippocampal neurons possibly through activation of the multiple 5-HT receptors expressed on hippocampal neurons. This revelation confirmed that serotonin does modulate receptor function in hippocampal neurons. The exact process by which serotonin heteroreceptors shape glutamatergic and GABAergic receptor activity is not completely understood, however this raphe-mediated suppression of hippocampal receptor function implies that serotonin can have substantial effects in the synaptic properties of hippocampal neurons.

Chapter 4: The effect of chronic SSRIs on NMDA receptor synaptic function

Not identifying congruent changes in dissociated co-cultures with both SSRIs after chronic exposure was puzzling at first; however this presented the opportunity to slightly shift the focus of the study. A more scrutinizing survey of the antidepressant literature unearthed NMDA receptors as a possible candidate in antidepressant therapy. Depressed patients that were unresponsive to other antidepressant therapies were given a one-time low dose of ketamine, a non-competitive NMDA receptor blocker. These patients experienced a rapid and long-lasting antidepressant effect that was not observed with the use of other antidepressant regimens. In addition to the clinical effects of ketamine, malfunction in NMDA receptors has been implicated in the pathophysiology of numerous mental illnesses including MDD because of its profound effects on neuronal function such as synapse formation, synaptic plasticity, as well as learning and memory (Pittaluga et al., 2007; Zarate et al., 2003). Evaluation of NMDA receptor-mediated spontaneous miniature neurotransmission unveiled a significant decrease in NMDA receptors mediated event charge after chronic exposure to fluoxetine and citalopram (Figure 4-1). NMDA mEPSCs were the first measure that demonstrated the same effect with both SSRIs. In pursuing this avenue, examination of global NMDA receptor response in co-cultures illustrated a remarkable decrease in the average peak amplitude of NMDA currents after chronic SSRI administration (Figure 4-2). Not only was the response in co-

cultures diminished amoung SSRI treatments, there was a considerable decrease contrasting to hippocampal neurons (Figure 4-3). These reductions in global NMDA receptors response after SSRI exposure in dissociated co-cultures suggest that trafficking of NMDA receptors from the membrane might contribute to the antidepressant effect.

Previous studies insinuated that the 5-HT_{1A} receptors might modulate NMDA receptor function. These studies have shown that 5-HT can cause a decrease in NMDA receptor current amplitude by activating synaptic 5-HT_{1A} receptors (Pallotta et al., 1998; Yuen et al., 2008; Yuen et al., 2005; Zhong et al., 2008). If 5-HT_{1A} receptor activation reduces NMDA receptor function, then specific inhibition with 5-HT_{1A} receptor antagonists should restore the diminished NMDA global response following chronic SSRIs. Co-administration of WAY 100635 and SSRIs significantly increased the global NMDA response. Targeting 5-HT_{1A} receptors in hippocampal culture with the agonist recapitulate the global NMDA current amplitude reduction observed in co-cultures, implying 5-HT_{1A} receptors directly modulate NMDA receptors function. Manipulating 5-HT_{1A} receptors agonists and antagonist also suggest that both 5-HT_{1A} somatodendritic auto-receptors and heteroreceptors play a role in NMDA receptor-mediated synaptic neurotransmission.

Despite the novel NMDA receptors finding in the dissociated co-culture after chronic SSRI exposures, I cannot be disregard that application of receptor

inhibitors are blocking receptors on both hippocampal and raphe neuronal populations, thus it cannot unequivocally stated that SSRIs have no effect on parameters such as AMPA and inhibitory spontaneous transmission. Inhibiting excitatory and inhibitory receptors in both populations might mask a response that requires raphe receptor activity to facilitate an antidepressant effect. It is possible that I only observed changes in NMDA function because of the sensitive nature of the receptors and how they intrinsically function.

While the exact mechanism by which SSRIs, and more directly serotonin, work to activate 5-HT_{1A} receptors and regulate NMDA receptor function remains unclear, previous NMDAR and $5HT_{lA}$ receptor studies have proposed some intriguing hypotheses that could help make sense of some of the findings of my study. For instance, application of NMDA leads to increases in the firing rate of raphe in a concentration-dependent manner, which in turn results in amplification of serotonin release (Gartside et al., 2007), SSRIs also increase synaptic serotonin in brain regions such as the hippocampus (Stahl, 1998a). Furthermore, the simultaneous enhancements in serotonin and NMDA in my experiments could foster an over-excitation of hippocampal NMDA and 5-HT receptors causing a "scaling" back phenomenon in order to compensate for the bombardment of activated receptors, resulting in a perturbed NMDA response.

Alternatively, both raphe and hippocampal neurons have NMDA receptors that are modulated by NMDA and glutamate (Gartside et al., 2007), so it is possible that the expected large scale response seen in hippocampal recordings may not be revealed in my dissociated co-culture recordings because both hippocampal and raphe NMDA receptors could be competing for NMDA. Hence, in my initial experiments with 100μM of NMDA applied during co-culture recordings, no change was detected (data not shown), moreover, the lack of differences in the NMDA perfusion studies I conducted might have been masked by the activation of NMDA receptors on raphe synapses until application of the higher concentration of NMDA.

The purpose of this thesis document was to provide convincing evidence that synaptic NMDA receptor dysfunction might contribute to the pathophysiology of depression. Pursuing an indirect pathway through the use of chronic SSRI could be the fundamental cause of the delayed onset of therapeutic antidepressant effects. As previously stated SSRIs binds to SERTs on raphe presynaptic terminals and inhibit the re-uptake of 5-HT. Acutely 5-HT_{1A} autoreceptors are activated and blunt the synaptic increase of 5-HT. After chronic use, autoreceptors are desensitized and postsynaptic receptors come into play. Activation of 5-HT_{1A} heteroreceptors along with other 5-HT receptors activates down stream GPCR cascades. 5-HT_{1A} receptor activation in particular somehow changes the NMDA receptor expression at the membrane and reduce NMDA

receptor-mediated transmission which then leads to intracellular alterations that were not studied in the scope of this thesis. There still remains an enormous amount of work that needs to be done to further elucidate the phenomenon of NMDA receptor-mediated antidepressant physiology. For example, it appears that 5-HT_{1A} receptors specifically modulate NR2B-contianing NMDA receptors (Yuen et al., 2005), so NMDA-mediated antidepressant effects might have different efficacies depending on the receptor composition. While robust and rapid effects of ketamine are probably a result of non-selective binding to all NMDA receptors, NR2A and NR2B, ketamine has not gained broad based clinical use because of the dangerous potential in drug abuse. Based on the data discussed in this document, investigating these receptor subtypes might be important in the development of new fast acting antidepressants that work as well as ketamine without the potential hazard for misuse.

The co-culture study I examined provided a sound basis in which to study chronic SSRI effects, but one short-coming of this *in vitro* culture system is that in theory the neurons in culture are not depressed. In fact this might be the reason that I was unable to detect distinct changes in any measure except the NMDA miniature neurotransmission experiments. Therefore, studying SSRI mechanisms in an unaffected model does not completely account for the fact that in depressed patients or animal models of depression there are underlying neural malfunctions that are presumably corrected by antidepressants. Paradoxically, what those

neural malfunctions might be are not clearly understood, hence the need for *in vitro* cultures assays such as the co-cultures system. However, measuring NMDA receptor currents in a depression induced mouse model that subsequently exhibits antidepressant effects in a behavior test for depression such as FST through hippocampal slice physiology might correlate better with the observations in the human diseased state of depression, as well as strengthen the results discussed in this documents using the dissociated raphe/hippocampal co-cultures assay. Also it might be worthwhile to evaluate NMDA receptor-mediated mEPSCs after acute SSRI administration. If NMDA receptor-mediated synaptic function is truly reduced only after chronic SSRIs, then NMDA mEPSC should not be affected after acute use, further suggesting that the delayed onset of AD action results from activation of an indirect pathway to NMDA receptor synaptic neurotransmission.

In addition to extending the electrophysiological studies, it would be intriguing to study if there are any differences in protein expression as a result of chronic SSRI administration. Evaluation of the intracellular downstream pathways that are mediated through NMDA and 5-HT_{1A} receptors could be instrumental in fully comprehending the pathophysiology of depression and why NMDA receptor antagonists bypass this delayed onset pathway of conventional selective serotonin reuptake inhibitors. Much of the antidepressant literature has focused on BDNF as an essential protein in antidepressant efficacy (Adachi et al.,

2008). Altar *et al*. showed that increases in the serotonergic transmission increase BDNF message and protein in the hippocampus and cortex (Altar et al., 2003). It would be worthwhile to examine whether the chronically SSRI treated dissociated co-cultures have any BDNF modifications by western immunoblotting. If there were any changes it might suggest that the reduction in NMDA receptor function upon chronic SSRI induces changes in postsynaptic protein translation. In a perfect scenario, I would predict that BDNF levels would be enhanced in my co-culture system which might then suggest there is a connection between NMDARs and the protein translation machinery. Earlier studies have shown that activation of NMDA receptors increase phosphorylation of eukaryotic elongation factor 2 eEF2 a process known to inhibit protein translation by reducing peptide chain elongation (Scheetz AJ, 2000). Therefore, the decrease in NMDA receptor function seen in my co-culture studies could decrease the Ca^{2+} flow through VGCCs thereby reducing activation of CAMKII and de-phosphorylating eEF2 which would enhance protein translation (Bayer et al., 2001) resulting in an up-regulation of several proteins one of which could be BDNF (Manadas et al., 2009).

The findings discussed in this thesis open the door for a wealth of possibilities in regards to understanding how selective serotonin reuptake inhibitor antidepressants modulate synaptic neurotransmission. In conclusion my data suggest that the delayed onset of antidepressant action might be a result of a

gradual process by which chronic SSRIs increase synaptic concentrations of 5-HT which activate postsynaptic 5-HT_{1A} receptors that then mediate NMDA receptors trafficking from the synaptic membrane and in turn modulates down-stream pathways that eventually mediate the therapeutic effects of antidepressants.

MATERIALS AND METHODS

Cell Culture

Dissociated hippocampal cultures were prepared from the brains of Sprague Dawley rats (Charles River Laboratories) according to previously published protocols (Kavalali et al., 1999). Briefly, whole hippocampi were dissected from the postnatal days 0-2 rats. Dorsal and median raphe was dissected from pregnant Sprague Dawley rats on embryonic day 15. Tissue was trypsinized for 10 min at 37 ^{o} C, mechanically dissociated using siliconized glass pipettes, and plated onto Matrigel -coated coverslips for hippocampal cultures and poly-lysine- coated coverslips for raphe cultures. For hippocampus a concentration of 4nM cytosine arabinoside (Sigma, St. Louis, MO) was added at 1 day *in vitro*, and 2nM cytosine arabinoside for raphe neurons. To prepare co- cultures, hippocampi were plated on matrigel-coated coverslips, and then raphe neurons were added after 3 days *in vitro* (4 DIV). For all experiments except HPLC, neurons were plated on a 24-well plate. All experiments were performed on cultures 14-25 days *in vitro*.

Drug Treatments

18hr – 10 day treatments of all cultures were done with the following drugs: Fluoxetine (Flx) (0.3 μM), Citalopram (Cit) (1μM), *N*-{2-{4-(2-methoxyphenyl)-1-piperazinyl} ethyl-*N*-(2-pyridinyl) cyclohexanecarboxamide (WAY 100635) (20 μM), 8-hydroxy-2(di-*n*-propylamino) tetralin (8OH-DPAT) (10 μM), all drugs were brought up to final concentration in cytosine arabinoside.

Lentiviral Production

HEK 293 cells were transfected using the Fugene 6 transfection system (Roche Molecular Biochemicals) with the expression plasmid, synaptophysin- pHluorin and two helper plasmids, delta 8.9, and vesicular stomatitis virus G protein (VsVg), at 3 μg each of DNA per 75 cm^2 flask (Dittgen et al., 2004). After 48 hrs, lentivirus containing culture media was harvested, filtered at a 0.45- μM pore sized, and immediately used for infection. Raphe cultures were infected at 7 DIV by adding 200 μL of viral suspension to each well and imaging experiments were done at 14-16 DIV.

Immunocytochemistry

For neuronal confirmation and vesicular boutons studies, dissociated hippocampal, raphe, and co-cultures were fixed for 30 minutes with 4% paraformaldehyde, rinsed twice with 1X PBS/Glycine, then blocked in 2% goat serum and 0.4% saponin for 1 hour. The cells were then incubated with primary antibodies, anti-SERT monoclonal (1:500, Chemicon), anti-GFP monoclonal (1:500, Chemicon), anti-MAP2 polyclonal (1:200, Chemicon), anti-VMAT-2 polyclonal (1:200, Synaptic Systems), anti-GFP polyclonal (1:500, Synaptic Systems), anti-VGLUT-3 polyclonal (1:100, Synaptic Systems) overnight at 4^oC. The next day the cells were washed then incubated with fluorescent secondary antibodies, goat-anti- mouse (1:200 Molecular Probes) and goat-anti-rabbit (1:200

Molecular Probes). For the puncta quantification study dissociated raphe and hippocampal co-cultures were fixed for 2 minutes at room temperature in PBS with 2% formaldehyde and 2% sucrose followed by treatment with cold methanol for 10 minutes at -20^{o}C. Then, neurons were blocked in 2% goat serum for 1 hr at room temperature. The cells were then incubated with primary antibodies, anti-Synapsin polyclonal (1:1000, Synaptic Systems), anti-PSD-95 monoclonal (1:200, Affinity Bioreagents), or anti-Gephyrin monoclonal (1:500, Synaptic Systems) added to 0.02% gelatin and 0.5% Triton X-100 in PBS overnight at 4^{o}C. The following day, neurons were washed with PBS and then incubated with fluorescent secondary antibodies, goat-anti- mouse (1:200 Molecular Probes) and goat-anti-rabbit (1:200 Molecular Probes). For receptor trafficking experiments, dissociated co-cultures were fixed for 30 minutes with 4% paraformaldehyde, rinsed twice with 1X PBS/Glycine, then blocked in 2% goat serum for 1 hour. The cells were then incubated with the primary antibody anti-NR1 polyclonal (1:200, Santa Cruz) overnight at 4^{o}C. The next day neurons were washed with PBS/Glycine and re-blocked blocked in 2% goat serum and 0.4% saponin for 1 hour. The cells were then incubated with primary antibodies, anti-MAP2 monoclonal (1:200, Chemicon) overnight at 4^{o}C. The following day the cells were washed then incubated with fluorescent secondary antibodies, goat-anti-mouse (1:200 Molecular Probes) and goat-anti-rabbit (1:200 Molecular Probes).

Coverslips were mounted with Vectashield (Vector Laboratories) and neurons were visualized on a Zeiss Confocal Microscope.

Confocal Microscopy Quantification

In colocalization studies images were quantified by averaging multiple 20 μm sections along a single dendrite in Image J 1.37v NIH.

Electrophysiology

Synaptic activity was recorded from hippocampal pyramidal cells, raphe, or hippocampal pyramidal cells in co-cultures using whole-cell voltage-clamped techniques. Data was acquired using and Axopatch 200B amplifier and Clampex 9 software (Axon Instruments). Recordings were filtered at 1kHz and sampled at 200 μsec. A modified Tyrode solution containing (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2.6H_2O$, 10 Glucose, 10 HEPES, 2 $CaCl_2.2H_2O$, (pH 7.4) was used as external bath solution for all experiments except for NMDA where 0.1 mM $MgCl_2.6H_2O$ was used. The pipette internal solution contained (in mM): 120 K Gluconate, 3 $MgCl_2.6H_2O$, 40 HEPES, 2 NaCl, 2 mg-ATP, 0.3 NaGTP, 10 QX-314, pH 7.2 (300 mOsm). Miniature recordings were conducted in the presence of Tyrode external solution with (in μM): 1tetrodotoxin (TTX), 50 D-2-amino-5-phosphonopentanoate (AP-5), and 50 picrotoxin (PTX) for AMPA mediated mEPSCs, and (in μM): 1 TTX, 50 AP-5, and 10 6-cyano-7 nitroquinoxaline-2, 3-dione, (CNQX) for mIPSCs. For 5-HT perfusion experiments 10 μM 5-HT and 1mM Ascorbic Acid were added to external Tyrode solution. For NMDA

experiments 1mM was perfused on to Tyrode solution with (in μM): 1 TTX, 50 PTX, and 10 CNQX; 50 AP-5 was used to abolish response.

High-Performance Liquid Chromatography

For culture medium 450 μl of conditioned medium was removed and place on ice. 50μl of ice cold 1M $HClO_4$ that has 500nM N-Me-5-HT was added. Samples were vortexed for 30 sec and centrifuged at 7000g at 4^{o}C for 10 min. Supernatant was then transferred to clean tubes. For cells all culture medium was removed. Cells were scraped into tubes. 250 μl of 0.1M $HClO_4$ containing 250 nM N-Me-5-HT was added to each sample. A small gauge needle and 1 ml syringe was used to break up cells by titrating. All homogenates were store at -70^{o}C overnight, packed on dry ice and shipped to Dr. Anne Andrews for analysis.

Statistical Analysis

All error bars represent the standard error of the mean (S.E.M) and all data was tested for statistical significant by means of a two-tailed Student's t-test.

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