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Using A Novel Optogenetic Approach To Directly Assess 5-Ht1a Somatodendritic Autoreceptor Function In Response To Chronic Selective Serotonin Reuptake Inhibitor Treatment

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USING A NOVEL OPTOGENETIC APPROACH TO DIRECTLY ASSESS 5-HT$_{1A}$ SOMATODENDRITIC AUTORECEPTOR FUNCTION IN RESPONSE TO CHRONIC SELECTIVE SEROTONIN REUPTAKE INHIBITOR TREATMENT

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DEDICATION

I would like to dedicate this work to all those individuals who are affected, directly or indirectly, by depression and/or any other mental disorder. I truly hope that adequate treatment will soon be available.

ACKNOWLEDGMENTS

There are many people that deserve credit for my success in graduate school. First and foremost, I would like to thank my mentor, Dr. Rodrigo Andrade, for his guidance and support over the past four years. I would also like to thank each of my committee members: Dr. Karen List, Dr. Paul Walker, Dr. Gregory Kapatos, Dr. Michael Bannon and Dr. Sokol Todi. I am grateful for your thoughts and input into my project, and for your dedication to my success. I would also like to acknowledge my previous mentor, Dr. Keith Lookingland, for believing in me and always putting a smile on my face. And to Dr. Stanley Terlecky, for his continued enthusiasm and kindness. I would like to thank the current and previous members of the Andrade lab, especially Peter Tanaka, Alicia Aleardi, Daniel Huereca, and our 'lab mom', Elaine Andrade.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

Depression and Antidepressants

Depression is a serious medical illness that is characterized by deep, persistent feelings of sadness, worthlessness and general loss of interest and pleasure in everyday activities (anhedonia). It manifests itself throughout many aspects of life, often causing sleep disturbances, concentration deficits, chronic fatigue, and changes in appetite and weight. Depression is also a very common condition, affecting approximately 8% of teenagers and adults in the United States (Pratt and Brody, 2014). It also represents a substantial financial burden. According to Greenberg et al. (2003), this illness cost the United States an estimated 83.1 billion dollars in 2000, which is a significant increase from the 43.7 billion estimated to have been spent in 1990. Unfortunately, depression is often accompanied by suicidal ideation, making suicide the 10th leading cause of death in the United States in 2013 (Kochanek et al., 2014).

Fortunately, there are a number of effective antidepressant medications. Whereas the increasing prevalence of depression remains a matter of debate, the ever enhancing popularity of antidepressants is undeniable. According to the National Center for Health (2014), these medications are used by an estimated 11% of American adults, which is more than a four-fold increase from 1988-1994 to 2007-2010. Antidepressants are the third most common class of prescription drug used by adolescents (12-19 years of age) and the most common class used by adults (20-59 years of age) in the United States (Gu et al., 2010). This is not an insignificant feat, and warrants further elaboration.

History of Antidepressant Treatments- TCAs and MAOIs

While mood enhancing therapies can likely be traced back to the beginning of human existence, the history of modern depression pharmacotherapies began in the late 19th century with two classes of agents, the Tricyclic Antidepressants (TCAs) and the Monoamine Oxidase Inhibitors (MAOIs), whose antidepressant properties were discovered simply by chance.

TCAs are iminobenzylic derivatives. The first phenothiazine (which would serve as the basis for subsequent synthesis of iminodibenzyl in 1899) was created in 1883 by the German scientist Heinrich Bernthsen (reviewed in Lopez-Munoz, 2009). At the time, he was experimenting with dyes for use in the textile industry. However, a larger pharmacological role was not recognized until the 1940's when phenothiazine-based antihistamines were discovered to cause pronounced sedative effects (reviewed in Pletscher, 1991). This led scientists at the Swiss company J.R. Geigy AG to begin work synthesizing derivatives of iminodibenzyl in an attempt to create a compound with sedative and/or hypnotic effects that would be useful in 'calming' the symptoms of psychiatric patients (reviewed in Lopez-Munoz and Alamo, 2009) At least in rodent models, a number of these derivatives showed promise. One of these agents, G-22150, was sent to clinician Roland Kuhn to investigate its potential use as a hypnotic. Kuhn found the compound to be unreliable for inducing sleep, but observed a positive effect on the mood of some patients (reviewed in Kuhn, 1958). It was forgotten about until 1952, when a related phenothiazine, chlorpromazine, was found to have antipsychotic effects (reviewed in Pletscher, 1991). Due to issues with tolerance of agent G-22150, Kuhn was sent a different compound, agent G-22355, to test its efficacy for treating psychosis. Although it actually made many of the schizophrenic patients worse, Kuhn noticed that it improved the mood of three patients that were also diagnosed with depression. Another 37 depressed patients were given agent G-22355, and its antidepressant properties were confirmed (reviewed in Kuhn, 1958). Although these findings were initially perceived with skepticism, agent G-22355 (imipramine) was put on the Swiss market in 1957 as Tofranil and entered the U.S. market in 1959 where it quickly became the drug of choice for depression (reviewed in Pletscher, 1991).

MAOIs represent the second class of antidepressant medication to enter the scene. These hydrazine compounds initially became of interest to clinicians in 1951 when they were discovered to have antitubercular properties by two independent American groups, led by Herbert Fox (Hoffmann-La Roche Laboratories) and Harry Yale (Squibb Institute for Medical Research)

(reviewed in Lopez-Munoz and Alamo, 2009). As such a treatment was in high demand, clinical trials for isoniazid and iproniazid were carried out at New York State Hospitals almost immediately. While both drugs were shown to be efficacious (so much so that isoniazid remains a standard treatment of tuberculosis), the safety profile of isoniazid was significantly better than that of iproniazid. Therefore, iproniazid was largely abandoned for its application for tuberculosis. However, it was not discarded just yet. Physicians carrying out the trials at Sea View Hospital noted that, compared to patients treated with isoniazid, those given iproniazid exhibited CNS stimulating psychological effects including significantly greater vitality and enhanced social activity (Crane, 1956). These observations led Nathan Kline to begin clinical trials assessing the mood-enhancing ability of iproniazid in patients without tuberculosis. In the first trial, iproniazid was reported to significantly improve the mood of approximately 70% of the subjects (reviewed in Kline, 1958). With such positive results, the interest and investigation of iproniazid as an antidepressant grew. Just one year following the conclusion of this trial, iproniazid (marketed only for the treatment of tuberculosis under the trade name Marsilid) had already become widely utilized for its antidepressant properties (reviewed in Lopez-Munoz and Alamo, 2009; Pletscher, 1991).

The MAOIs and TCAs were efficacious for a majority of depressed patients. However, they had a number of serious side effects. For MAOIs these included the risk of jaundice, nephrotoxicity, and hypertensive crisis. In fact, a number of MAOIs had to be withdrawn from the market in the 1960's for this reason (reviewed in Pletscher, 1991). TCAs also suffered from safety and tolerability issues due to their nonselective actions on a variety of neurotransmitter systems and low therapeutic index (reviewed in Ferguson, 2001). This fueled the search for the mechanism of action of antidepressants in order to create drugs with safer and more tolerable profiles.

Search for the Mechanism of Action of Antidepressants

It did not take long before the direct target of MAOIs and TCAs were discovered. In 1952 Zeller and Barsky demonstrated *in vivo* that iproniazid inhibited an enzyme, monoamine oxidase (MAO), which was found to be responsible for breaking down central norepinephrine (NE), dopamine (DA) and serotonin (5-HT) (Shore et al., 1957a; Blaschko, 1952). This suggested that MAOIs acted to increase the levels of these chemicals in brain tissue (Shore and Brodie, 1958). TCAs were found to inhibit NE, DA and 5-HT reuptake in central neurons (Glowinski and Axelrod 1964; Carlsson et al., 1968). It was thought that inhibiting reuptake increased the 'free' forms of the chemicals to enhance each of the neuromodulatory systems. Since all three compounds were known to have psychological roles, modulation by TCAs or MAOIs could theoretically underlie the clinical efficacy of these drugs. Zoning in on one of them, if singularly responsible, could potentially pave the way for development of a medication that retained the antidepressant properties but had a reduced risk of off-target side effects.

Research at the time was largely centered on the reserpine model of depression. Reserpine was discovered to induce sedation and anhedonia (in addition to other peripheral effects) in laboratory animals (Pletscher et al., 1955), and to cause depression-like symptoms in some 'at risk' patients who were taking it for hypertension (reviewed in Bunney and Davis, 1965). Because these behavioral effects in animals were reported to be blocked by all effective MAOIs and TCAs, the reserpine model became a prime tool for screening new potential antidepressants and for investigating the mechanism of action of these medications (reviewed in Bunney and Davis, 1965).

The next step was to determine the neuromodulator responsible for blocking reserpine's effects. All three chemicals were depleted by acute reserpine administration (Shore et al., 1957b; Glowinski et al., 1966). Moreover, the return of NE, DA, and 5-HT to typical brain tissue levels coincided with the recovery from 'depression' (Shore et al., 1957b; Glowinski et al., 1966). Administration of MAOIs and TCAs prior to reserpine, in addition to blocking the behavioral

effects, also prevented or attenuated central NE, DA and 5-HT depletion (Shore et al., 1957a; Brodie and Shore, 1957). However, there were a number of observations that pointed to NE and DA as key components of the reserpine model of depression. In laboratory animals for example, administration of α-methyl-tyrosine (to block NE and DA synthesis) was reported to cause reserpine-like sedation (Spector et al., 1965). Similarly, administration of the DA and NE precursor 3,4-dihydroxyphenylalanine, but not the 5-HT precursor 5-hydroxytryptophan, was observed to transiently reverse the behavioral and physical 'depression' induced by reserpine (Carlsson et al., 1957). This contributed to the catecholamine hypothesis of depression (Schildkraut, 1965; Bunney and Davis, 1965), which posited that depression was associated with deficiency of catecholamines (particularly NE) whereas "elation" was associated with an excess. Similarly, it was thought that drugs that depleted or inactivated central NE caused depression, while medications that increased or potentiated NE produced behavioral stimulation and were clinically effective antidepressants (Schildkraut, 1965). In light of these reports, pharmaceutical companies focused on developing NE-potentiating compounds as prospective antidepressant agents.

Development of SSRIs

However, this rationale did not last for long. In the 1960's physician Paul Kielholz noted that TCAs, while structurally similar and generally thought to be functionally interchangeable, seemed to have slightly different effects on a patient's motivation, mood, and cognition, which he thought should be factors that should be considered for treating individual patients (reviewed in Healy, 2000). Carlsson was the first to link Kielholz's observations to alterations in discrete neurotransmitter systems, and suggested that selectively enhancing the 5-HT system may retain the mood-enhancing therapeutic benefits of antidepressants while avoiding some of the undesirable side effects. This led Carlsson, Corrodi, and Berndtsson at Astra pharmaceuticals to begin work on developing such a drug. They used an antihistamine to synthesize zimelidine which was patented in 1972, put through clinical trials by 1980, and reached the European market by 1982 (reviewed by Healy, 2006). However, soon after it was reported to have caused a serious

neurological disorder in a few patients and was removed almost immediately. Nevertheless, the rationale, proven efficacy, and decreased risk of the 'typical' TCA and MAOI-associated side effects made this an attractive new route for investigation.

The scene shifted as pharmaceutical companies began actively seeking compounds that selectively targeted the 5-HT system. This is precisely how fluoxetine came to be (reviewed by Healy, 2006). In the early 1970's Bryan Molloy of Eli Lilly was in the process of synthesizing new NE-targeting potential antidepressants using an antihistamine as a starting point. The derivatives were then screened for their ability to inhibit reuptake of NE using rat brain synaptosomes. Although the compounds that failed to display selective NE properties were not of particular interest to Eli Lilly, it was proper practice that they be investigated further. Of note, one of these compounds, LY-110140, was found to have remarkable selectivity for 5-HT reuptake (Wong et al., 1975). At this same time the search for 5-HT-targeted antidepressants began, suddenly making LY-110140 a very attractive potential agent. Although LY-110140 failed the reserpine 'gold standard' test for antidepressant activity, additional testing was continued. In 1975 it was named fluoxetine, and after demonstrations of its antidepressant efficacy in clinical populations it was approved by the FDA and marketed as Prozac (reviewed in Healy, 2006). Other 5-HT-targeted antidepressants entered the market in the next few years. These included citalopram (Celexa), escitalopram (Lexapro), sertraline (Zoloft), and paroxetine (Paxil). Notably, it was not until 1992 that the term 'Selective serotonin reuptake inhibitor' (SSRI), which was created to promote sales of Paxil, became used to classify this new group of drugs (reviewed in Healy, 2006). SSRIs quickly became the class of antidepressant medications that was most widely prescribed by physicians (Lieberman, 2003).

Serotonin Hypothesis of Depression

The success of SSRIs clearly suggested that the 5-HT system, despite lacking a role in the reserpine-induced sedation, could be targeted to resolve depression in some individuals. Of course, with time it became more apparent that the reserpine model, whose behavioral effects

were based on NE and DA depletion, was not an entirely accurate portrayal of depression in humans. Some drugs that were predicted to be antidepressants were discovered to have no therapeutic benefits and other clinically effective drugs, such as fluoxetine, did not pass the reserpine-based test.

It is important to note that a 5-HT hypothesis of depression was not a new idea, but had been proposed back in 1967 by Alec Coppen and in 1969 by Lapin and Oxenkrug. In addition to the demonstrated alterations in the 5-HT system by antidepressants, additional support for this theory came from reports that linked low central 5-HT or a hypofunctioning of the 5-HT system to depression. However, it is notable that there was skepticism for this idea relatively early on. Instead, it was generally accepted that just because increasing 5-HT relieves depressive symptoms, it does not necessarily mean that there was low 5-HT to begin with, or that low 5-HT caused the depression (de Montigny, 1981). Nevertheless, the importance of 5-HT in the efficacy of antidepressant treatments was becoming ever more apparent, with three major findings that highlighted the role of the 5-HT system in the clinical benefits of these medications.

Involvement of the Serotonin System in the Therapeutic Effects of Antidepressants

The first of these came from a group led by Coppen. It had previously been demonstrated in rodents that administration of an MAOI along with the 5-HT precursor L-tryptophan caused elevations in brain 5-HT more than the MAOI alone (Hess and Doepfner, 1961). Building off this, Coppen and colleagues found that L-tryptophan significantly potentiated the efficacy of the MAOI tranylcypromine in a clinically depressed population (Coppen et al., 1963).

The second important discovery was made in the mid 1970's by a group consisting of Shopsin, Gershon, Goldstein, and Friedman. They found that depressed patients currently in remission with either a TCA (imipramine) or MAOI (tranylcypromine) relapsed if they were given the 5-HT synthesis inhibitor *p*-chlorophenylalanine, but not if they were given the NE and DA synthesis inhibitor α-methylparatyrosine (Shopsin et al., 1975; Shopsin et al., 1976). The same group also found that *p*-chlorophenylalanine (but not α-methylparatyrosine), by depleting brain 5-

HT, prevented the effects of imipramine and tranylcypromine on the 5-HT system in rodents (Friedman et al., 1974; Friedman et al., 1976).

The third important finding was reported by Delgado and colleagues. It had been previously shown that dietary restriction of the essential amino acid (and 5-HT precursor), L-tryptophan, or ingestion of a tryptophan-free amino acid drink caused depletion of plasma tryptophan, CSF tryptophan, and reductions in brain tryptophan and 5-HT in laboratory animals (Young et al., 1989; Moja et al., 1989). Based on the demonstration that these manipulations also resulted in reductions in plasma tryptophan in humans (Delgado et al., 1989; Young et al., 1985), the group investigated whether tryptophan depletion in MAOI, TCA and SSRI-remitted patients would cause relapse. Using a double-blind, placebo controlled, crossover design they found that a majority of patients exhibited depressive symptoms, the severity of which were correlated with the extent of plasma free tryptophan depletion (Delgado et al., 1990). The depression subsided once the patients returned to their normal diets (and continued taking their antidepressant medication). Together, these studies suggested that an enhancement of 5-HT neurotransmission was likely an important part of the mechanism of action and maintenance of the therapeutic effect of these drugs.

Notably, in addition to the traditional classes of antidepressants described above, there have been reports that support an essential role for the 5-HT system in a variety of other antidepressant-promoting drugs and treatments. These include the combined serotonin-norepinephrine reuptake inhibitors (SNRIs), ketamine (Gigliucci et al., 2013), deep brain stimulation (Hamani et al., 2010), and even electroconvulsive therapy (reviewed in de Montigny, 1981). From these observations it is thought that a vast majority of antidepressant treatments exert their clinical effects directly or indirectly by enhancing 5-HT neurotransmission.

Mechanism of Action of Antidepressants, Revisited

There are a number of important points about the current status of the available antidepressants. First, despite the name, SSRIs do not have a ‘clean’ profile. Whereas they are

generally better tolerated and thus provide enhanced compliance compared to the original TCAs and MAOIs (reviewed in Goldstein and Goodnick, 1998), they put patients at risk for a number of side effects that include (but are not limited to) nausea, sexual dysfunction, sleep dysfunction, changes in appetite and weight gain, headache, agitation, and drowsiness (Ferguson, 2001). While the benefits outweigh the risks for many patients, this may not always be the case. Secondly, despite their more selective action on the 5-HT system, SSRIs are likely no more efficacious than the other available therapeutics for the treatment of depression (Rickels and Schweizer, 1990). Collectively, an estimated 30% of patients remain unresponsive to all currently available pharmacotherapies (Little, 2009). Finally, it is puzzling as to why the clinical benefits of antidepressants such as SSRIs take weeks to emerge, since the biochemical effects (reuptake blockade) occur almost immediately (Wong et al., 1975). Taken together, these gaps reflect the fact that we still lack a clear understanding of both the etiology of depression and how antidepressant medications exert their therapeutic effects. Nevertheless, since these drugs are clearly efficacious for a number of individuals, they offer a starting point for which to continue the investigation into this disease.

In the current work we chose to use the classic SSRI fluoxetine. Because of its direct actions on SERT, it makes sense to study the mechanism of action of fluoxetine and other SSRIs in the region of the brain with the highest density of SERT, the dorsal raphe nucleus (DRN) (Hrdina and Vu, 1993). The DRN is also responsible for providing the majority of 5-HT to the forebrain (Jacobs and Azmitia, 1992), thereby making it a prime target for SSRI research.

Autoreceptor Desensitization Hypothesis

So what is responsible for the therapeutic effects of antidepressants? Regarding SSRIs, one prominent theory emerged in the late 1980's (Blier et al., 1987). This "autoreceptor desensitization theory" posits that in order for SSRIs to increase 5-HT neurotransmission and thus enhance mood, 5-HT_{1A} somatodendritic autoreceptors must first become desensitized. The delay

in the clinical effects of SSRIs was thereby attributed to the time it took for a reduction in the 5-HT_{1A} autoreceptor-mediated signaling to occur.

This theory was founded on a property of 5-HT neurons termed autoinhibition, or the ability of cells to negatively regulate their activity by responding to their own neurotransmitter (Carlsson, 1975). Evidence for autoinhibition of serotonergic cells began with *in vivo* studies. It was discovered that administration of agents that increased synaptic availability of endogenous 5-HT (such as TCAs, MAOIs or L-tryptophan) caused suppression of the characteristic pacemaker-like firing of putative 5-HT neurons (Aghajanian, 1972; Aghajanian et al., 1970; Sheard et al., 1972). Because this effect could be induced by localized microiontophoretic application of 5-HT receptor agonists into the raphe nuclei, the 'autoreceptors' mediating the response were also likely to be located in the nuclei (Haigler and Aghajanian, 1974). Finally, electrical stimulation of the raphe in *in vitro* slices to elicit spikes (Pan and Williams, 1989) or stimulation of the ascending 5-HT fibers *in vivo* to cause antidromic spikes in the raphe (Wang and Aghajanian, 1977) both caused transient inhibitory responses of presumed serotonergic neurons. Together, these findings led to the general view that 5-HT autoreceptors located in the raphe were activated under physiological conditions by endogenously released 5-HT and were responsible for negatively regulating the firing rate of serotonergic neurons.

With advancements in pharmacological tools it was discovered that these autoreceptors were G protein-coupled receptors of the 5-HT1 family (Willliams et al., 1988) (later subclassified as the 1A subtype) that signal through their associated G protein of the G_i/G_o class. Notably, because they were found to be localized to the soma and dendrites of 5-HT neurons they are generally referred to as 5-HT_{1A} somatodendritic autoreceptors (Riad et al., 2000). Once activated, it was demonstrated that the βϒ subunits of the G protein opened potassium channels (of the Kir3/GIRK subfamily) (Kofuji et al., 1995), thereby causing a transient hyperpolarization by allowing potassium to flow out of the cell (Williams et al., 1988; Penington et al., 1993). This

provided an explanation as to how localized endogenous release of 5-HT could cause a transitory suppression of 5-HT cell firing.

With this in mind, according to the autoreceptor desensitization theory (Fig. 1) acute exposure to SSRIs would produce a buildup of extracellular 5-HT in the DRN, leading to the activation of these autoreceptors and a suppression of the pacemaker-like firing of 5-HT neurons. This would result in a reduction of 5-HT output, thereby preventing the enhancement of 5-HT signaling deemed essential for the antidepressant effects of SSRIs. With prolonged administration in rodents there was observed to be a progressive recovery in the firing rate which returned to baseline levels by 14 days (Blier and de Montigny, 1983). It was thereby proposed that a desensitization of the somatodendritic 5-HT_{1A} autoreceptors was an essential prerequisite for SSRIs to enhance 5-HT neurotransmission and elicit their therapeutic effect (Blier et al., 1987).

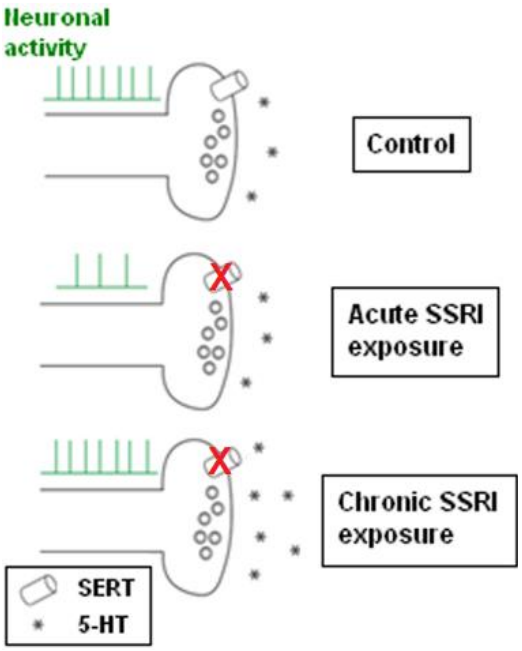

Figure 1. Effects of SSRI administration on the activity of 5-HT neurons in the DRN according to the autoreceptor desensitization theory. Under control conditions (top), 5-HT neurons are thought to exhibit a pacemaker-like firing pattern. 5-HT released from a spike can be taken back up into the presynaptic cleft by the serotonin transporter, SERT. Acute SSRI exposure (middle) prevents the reuptake of 5-HT. It was hypothesized that elevated extracellular 5-HT in the DRN activates 5-HT_{1A} somatodendritic autoreceptors (not shown) to cause a reduction in firing. According to the autoreceptor desensitization theory, with prolonged SSRI treatment these autoreceptors exert less inhibitory control, thus allowing a resumption of firing. Combined with continued reuptake blockade, this results in enhancement of 5-HT neurotransmission (bottom).

Goal of the Current Work

Despite its broad acceptance, there has been inconsistent support for this autoreceptor desensitization hypothesis over the years (Le Poul et al., 1995; Hervas et al., 2001; O'Conner

and Kruk, 1994; Davidson and Stamford, 1998; reviewed in Hjorth et al., 2000). A clear understanding of this phenomenon has largely been prevented due to a lack of available tools that permit direct assessment of the functional state of the 5-HT_{1A} autoreceptor following chronic SSRI treatment. Fortunately, recent expansion of our optical sensor toolbox and advancements in genetic targeting strategies have provided the means to fill in the missing gaps. Therefore, the goal of the present work was to re-examine the theory that chronic SSRI treatment causes desensitization of 5-HT_{1A} somatodendritic autoreceptors.

The key to our experiments was the use of genetically modified mice that express the light-sensitive protein Channelrhodopsin (ChR) selectively in 5-HT neurons in our slice preparation. This optogenetic approach permits unambiguous identification of 5-HT neurons, allows precise temporal control of serotonergic cell activity, and facilitates the release of 5-HT and subsequent activation of somatodendritic autoreceptors. Thus the 5-HT_{1A}R I_{light} signal is a direct readout of autoreceptor function. Three mice models were tested for this purpose and found to be potentially useful. However, one model ("SERT-Cre X Ai32" mice) was found to have particularly robust ChR expression and was therefore utilized in the current work. These mice were treated for two weeks with fluoxetine (Prozac) (10mg/kg/day) or a 5% dextrose solution via a subcutaneously-implanted osmotic minipump. Electrophysiological whole-cell recordings were then carried out on DRN 5-HT neurons maintained in brainstem slices. Collectively, this novel application of optogenetics provided a way to directly assess autoreceptor function in order to lay to rest the controversy of whether or not there is a functional reduction in the 5-HT autoinhibitory signal following chronic SSRI treatment.

CHAPTER 2 RESULTS

Use of Optogenetics to Selectively Evoke 5-HT_{1A} Autoreceptor-mediated Autoinhibition

Despite their demonstrated efficacy and widespread use, we still lack a clear understanding of how antidepressants such as the SSRIs exert their therapeutic effect. One prominent theory holds that a gradual, adaptive reduction in the sensitivity of somatodendritic 5-HT_{1A} autoreceptors is a critical component in the mechanism of action of SSRIs (Blier et al., 1987). The characteristic delay in the onset of the antidepressant effects was thus attributed to the time needed for these autoreceptors to desensitize (Blier et al., 1987). This autoreceptor desensitization hypothesis quickly became a platform for rational drug design. However, there has been inconsistent support for this idea over the years, thereby calling for reexamination of the functional status of the autoreceptor following prolonged SSRI treatment. Methodological limitations have made testing of autoreceptor function difficult by electrophysiological means, and are likely responsible for the inconsistent reports.

There are two main challenges faced by electrophysiologists in this regard. The first involves the identification of 5-HT neurons. Due to the financial and temporal burden of post hoc analysis, electrophysiologists generally relied on the 'classical features' of serotonergic cells that include biphasic action potentials, responsiveness to 5-HT and LSD, and slow, regular pattern of discharge (Aghajanian and Vandermaelen, 1982; Vandermaelen and Aghajanian, 1983). However, there is evidence that these criteria are not sufficient for distinguishing between 5-HT neurons and other cells present in serotonergic nuclei such as the DRN (Kirby et al., 2003; Beck et al., 2004; Schweimer and Ungless, 2010). A second challenge involves the manipulation of 5-HT_{1A} somatodendritic autoreceptors. The currently available 5-HT_{1A} receptor-targeted drugs cannot selectively distinguish between 5-HT_{1A} presynaptic autoreceptors and postsynaptic heteroreceptors or between 5-HT_{1A} receptors present on serotonergic versus non-serotonergic cells within the raphe (Beck et al., 2004), which makes examination of somatodendritic autoreceptor signaling in the absence of perturbations by local or distal feedback circuitry

problematic. It is also difficult to achieve pharmacologically-induced receptor-mediated responses on a timescale similar to that occurring under physiological conditions. Alternatively, electrical field stimulation can be used to activate the autoreceptors by endogenously released 5-HT, thereby preserving more of the spatial and temporal aspects of physiological signaling (Yoshimura and Higashi, 1985; Williams et al., 1988; Pan et al., 1989). However, the elaborate standardization required to adequately compare autoreceptor-mediated responses of individual cells from electrical stimulation has largely prevented its application for electrophysiological examination of 5-HT_{1A}R signaling following chronic SSRI treatment.

Importantly, recent advancements in genetic targeting strategies and optical stimulation techniques have provided the tools necessary to overcome many of these previous challenges and potentially allow us to gain unmatched insight into autoreceptor function in response to prolonged SSRI administration. To this end, we took advantage of the light-sensitive ion channel, Channelrhodopsin (ChR) (Boyden et al., 2005). By selectively targeting ChR to 5-HT neurons, we reasoned it would be possible to combine the temporal control afforded by light stimulation with the real time cellular level insight provided by whole-cell electrophysiological recordings to achieve a direct readout of autoreceptor function.

Three genetically modified mice models were tested for this purpose (Fig. 2). The first was a BAC transgenic mouse that expressed ChR under the control of the tryptophan hydroxylase 2 (TPH2) promoter elements ("TpH2-ChR") (Zhao et al., 2011). Previous characterization revealed moderate ChR2-EYFP expression in the DRN that was restricted to cells that were TPH2 positive, suggesting no ectopic expression of ChR-EYFP (Zhao et al., 2011). The second and third used the serotonin transporter (SERT) or the Pet1 transcription factor promoter/enhancer to drive expression of Cre recombinase (Gong et al., 2007, and Scott et al., 2005, respectively). In order to express ChR in 5-HT neurons these transgenic mice were crossed with a conditional ChR knock-in mouse (containing a *loxP*-STOP-*loxP*-ChR-EYFP expression cassette) (Madisen et al.,

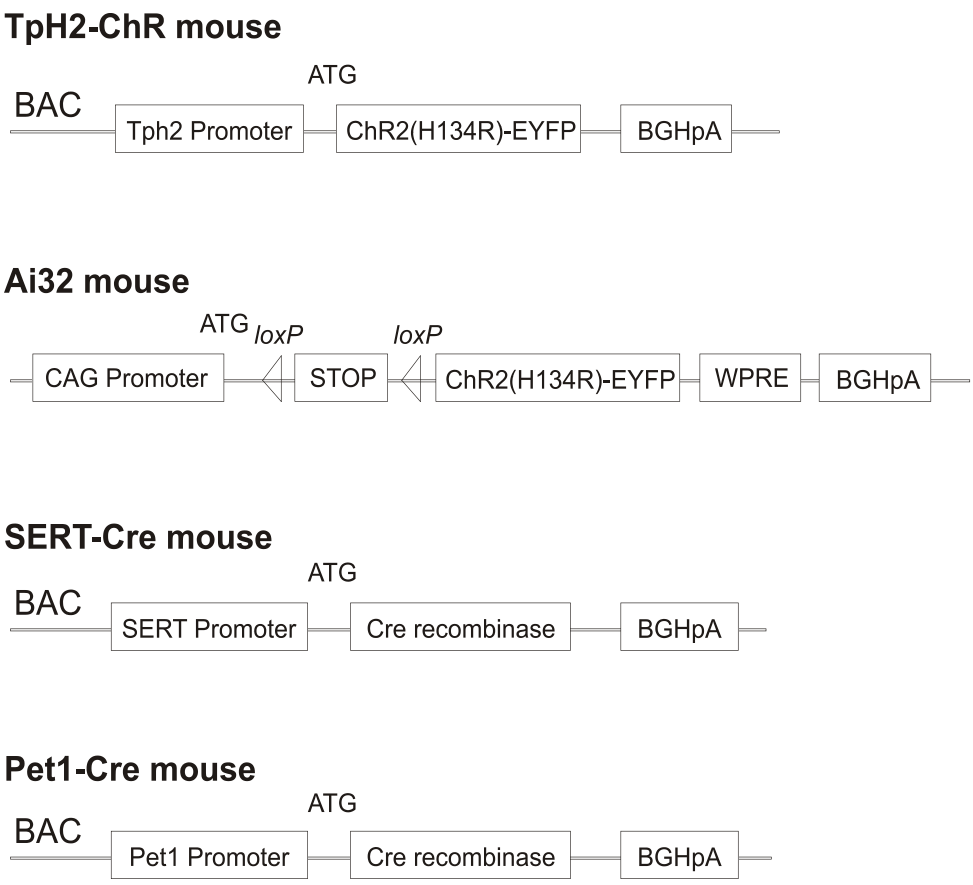

Figure 2. Genetically modified mice models for the selective targeting of the light-gated ion channel, ChR, to serotonergic neurons in our preparation. The "TpH2-ChR" transgenic mouse (top) utilized the tryptophan hydroxylase 2 promoter elements to direct expression of the ChR2-EYFP fusion construct to 5-HT cells. Alternatively, Cre recombinase driver mice (SERT-Cre or Pet1-Cre; bottom) were crossed with a conditional ChR knock-in mouse (Ai32; middle) to allow excision of the *loxP*-flanked STOP cassette and expression of ChR2-EYFP in 5-HT neurons. (BAC: bacterial artificial chromosome; ChR2(H134R): ChR2 variant; BGHpA: bovine grown hormone polyadenylation signal; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element.) (Refer to methods for additional mouse information.)

2012) to produce "SERT-Cre X Ai32" and "Pet1-Cre X Ai32" offspring. Selective targeting of Cre recombinase to serotonin neurons using Pet1 promoter/enhancer regions has previously been demonstrated (Scott et al., 2005). To test whether the serotonin transporter promoter would also be useful in this regard, we crossed a SERT-Cre mouse with an "Ai3" reporter mouse (Madisen et al., 2010). As shown in figure 3, qualitative assessment of brainstem slices revealed extensive

co-localization of Cre recombinase activity with SERT and TPH2, without any apparent ectopic expression. Therefore, all three mice models provided a means to selectively target ChR to 5-HT neurons in our preparation.

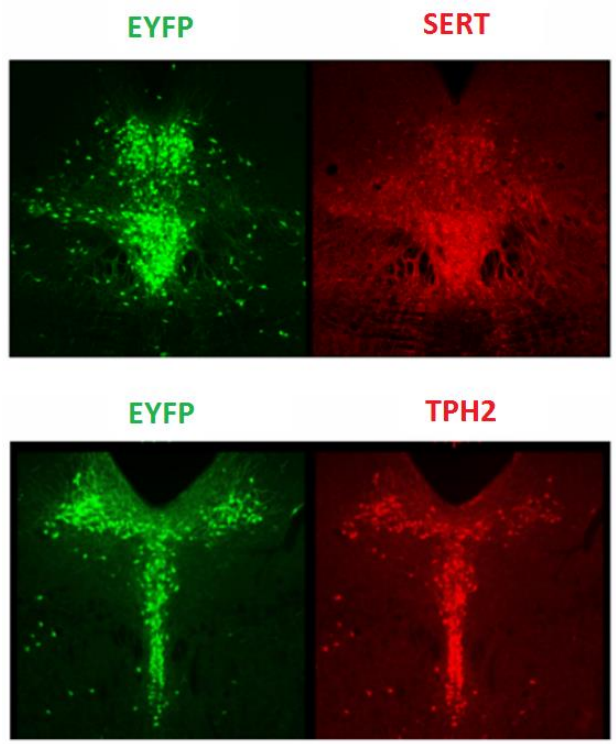

Figure 3. Use of the SERT promoter to target Cre recombinase to 5-HT neurons in brainstem slices. Coronal brainstem slices from a SERT-Cre X Ai3 reporter mouse reveal co-localization of the serotonin transporter (SERT; Alexa Fluor® 568) or tryptophan hydroxylase 2 (TPH2; Cy™3) with Cre recombinase activity (as detected by EYFP expression; DyLight™488) in the DRN.

To investigate the utility of each targeting strategy for our current purpose, we conducted recordings of 5-HT neurons from the ventromedial region of the DRN (vmDRN) in brainstem slices from each of the mice. In voltage clamp, brief flashes (5 ms) of blue light (455-490 nm) produced robust ChR-mediated currents (Fig. 4A). Importantly, the presence of ChR-mediated currents thereby provided the means to unambiguously confirm the serotonergic identity of a patched cell, and do so in a relatively simple manner.

To determine which of the three models would provide the greatest optical control of 5-HT neurons we compared the amplitude of the ChR-mediated currents between the groups. These currents were largest in the SERT-Cre X Ai32 (521.3 ± 22.7 pA) mice compared to the Pet1-Cre X Ai32 (485.2 ± 45.3 pA) or TpH2-ChR (186.9 ± 109.9 pA) mice (in agreement with earlier reports (Zhao et al., 2011)) (Fig. 4B). In light of these findings, we chose to proceed using just the SERT-

Cre X Ai32 mouse. Further characterization revealed that under current clamp conditions, the light-stimulated ChR-mediated depolarization was sufficient to produce a spike, and do so in a consistent manner (Fig. 4C). In summary, selective and robust expression of ChR in 5-HT neurons in the SERT-Cre X Ai32 mouse allowed identification of 5-HT neurons and permitted reliable control of serotonergic cell activity.

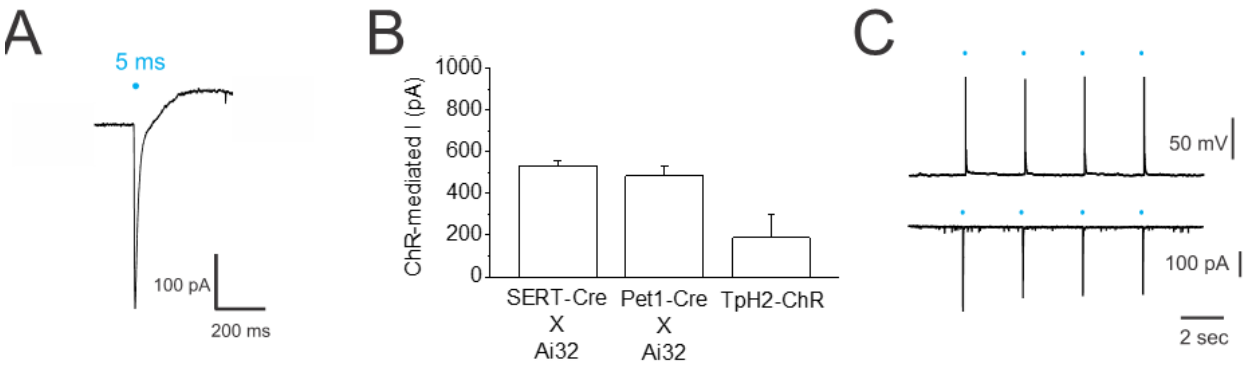

Figure 4. Characterization of ChR in 5-HT cells of SERT-Cre X Ai32, Pet1-Cre X Ai32 and TpH2-ChR mice models. A. A brief (5 ms) flash of blue light (~5 milliWatts (mW) full field; indicated by a blue circle) caused the appearance of an inward ChR-mediated current in a ChR-expressing 5-HT cell from a SERT-Cre X Ai32 mouse. **B.** Light-evoked ChR-mediated currents were largest in SERT-Cre X Ai32 mice (531.3 ± 22.7 pA; 82 cells, 19 mice) compared to Pet1-Cre X Ai32 (485.2 ± 45.3 pA; 13 cells, 5 mice) or TpH2-ChR (186.9 ± 109.9 pA; 8 cells, 2 mice) animals. (Data are presented as mean ± SEM). **C.** In a ChR-expressing serotonergic neuron from a SERT-Cre X Ai32 mouse, repeated light stimulation triggered ChR-mediated currents (bottom) that provided reliable control over 5-HT neuronal activity (top).

We next studied whether optical stimulation of ChR-expressing serotonergic neurons in SERT-Cre X Ai32 mice could evoke the phenomenon of autoinhibition. In voltage clamp, light flashes often resulted in a transient outward current (Fig. 5A). In current clamp, this was observed as a pronounced inhibitory afterpotential (Fig. 5B) capable of causing temporary silencing of neuronal activity. Application of the selective 5-HT_{1A} receptor antagonist WAY100635 (1 µM) resulted in complete suppression of this light-evoked current (Fig. 5A, 7/7 cells tested), indicating that the 5-HT_{1A} autoreceptor was mediating the response. Across cells, slices and mice, this 5-HT_{1A} autoreceptor-mediated current ("5-HT_{1A}R I_{light}") was found to be maximally activated with 3 flashes of light (5 ms, 50 ms apart; Fig. 5C). Importantly, this optogenetic approach therefore

facilitated direct cell-to-cell comparison of the response without the need for additional standardization methodologies. The autoreceptor-mediated current response was observed to remain stable for 30 minutes or more (Fig. 5D, n = 4 cells), thereby potentially providing a within-cell comparison of drug-induced alterations in the autoinhibitory signal. Having demonstrated the utility of this optogenetic strategy to provide dependable control over the phenomenon of autoinhibition, we next sought to apply this approach to directly assess changes in autoreceptor sensitivity following prolonged SSRI administration.

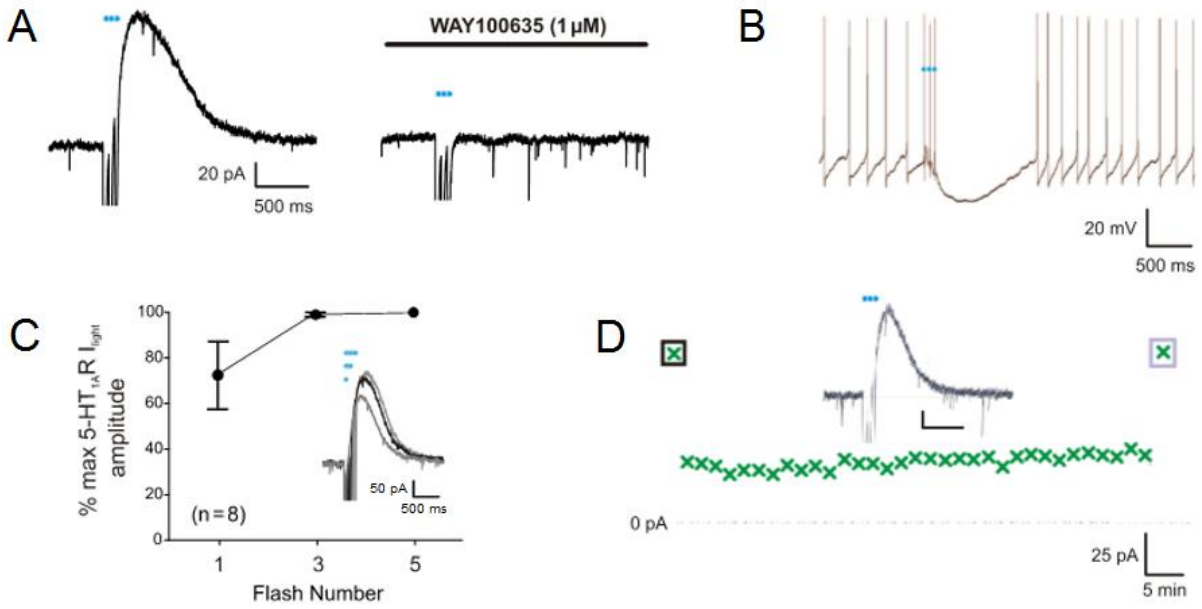

Figure 5. Genetically modified SERT-Cre X Ai32 mice permit optical stimulation-mediated control over the phenomenon of autoinhibition. A. Brief flashes of light evoked the appearance of ChR-mediated inward currents followed by a transient outward current (left). In the same cell, blockade of this response with bath application of the selective 5-HT_{1A}R antagonist WAY100635 (1 µM) indicates that the current is mediated by 5-HT_{1A} autoreceptors (right). **B.** In current clamp, light stimulation caused a 5-HT_{1A}R-mediated hyperpolarization that was sufficient to cause temporary silencing of neuronal activity (induced by a 50 pA current injection). **C.** Experimentation with stimulation parameters revealed that three flashes of light (50 ms apart at ~5 mW full field) was sufficient to achieve 99 ± 0.97% of the maximal 5-HT_{1A}R I_{light} response. The responses of a single cell to 1, 3 and 5 flashes of light are shown in the inset (black trace is from 3 flashes). **D.** The 5-HT_{1A}R I_{light} response of a 5-HT neuron remained stable over time, as illustrated by the amplitude of the response (crosses) evoked every 2 minutes from 3 flashes of light given 50 ms apart (~1.3 mW full field). Overlaid traces are from the first (black trace) and last (gray trace) response of the cell in the experiment (scale bar is 25 pA by 1 sec). Note: for illustration purposes ChR-mediated currents were truncated.

Effects of Chronic Fluoxetine Treatment on Autoreceptor-mediated Currents

SERT-Cre X Ai32 mice ~5 weeks of age were treated with the classic SSRI fluoxetine (10 mg/kg/day) or a 5% dextrose solution (sham) for 14 days via subcutaneously implanted osmotic pumps. This treatment regimen was chosen because it has been shown to alleviate depression-like symptoms in rodent models of anhedonia (Machado et al., 2012) and induce suppression of serotonin cell firing that recovered by 14 days (Czachura and Rasmussen, 2000). In the current work we found that it provided blood levels of fluoxetine and its major active metabolite norfluoxetine of 355.9 ± 100.25 ng/mL (mean ± SD, 5 mice) that were comparable to human therapeutic levels (50-500 ng/mL). Based on reported values for SERT binding affinity and 5-HT uptake inhibition (~1 nM and ~10 nM, respectively; Owens et al., 2001), the estimated 1 µM fluoxetine achieved with this treatment should be sufficient to near maximally block 5-HT reuptake in the current experiments. At the conclusion of treatment, the mice were sacrificed and whole-cell electrophysiological recordings were performed on identified 5-HT neurons of the vmDRN.

Four parameters of the autoreceptor signal were quantified to facilitate comparison across treatment groups: the amplitude (pA), rise time (ms), half-decay (ms) and charge transfer (pAxs) (see methods). Non-treated and sham treated littermates were not significantly different from one another in terms of the ChR-mediated currents (521.3 ± 22.7 pA (82 cells, 19 mice) and 527.6 ± 19.4 pA (54 cells, 9 mice), p = 0.91; student's t-test) or any of the autoreceptor-mediated responses (Fig. 6; 5-HT_{1A}R I_{light} amplitude (p = 0.28), rise time (p = 0.70), half-decay (p = 0.31), and charge transfer (p = 0.64); student's t-test). Therefore, data from the two groups were pooled to enhance statistical power.

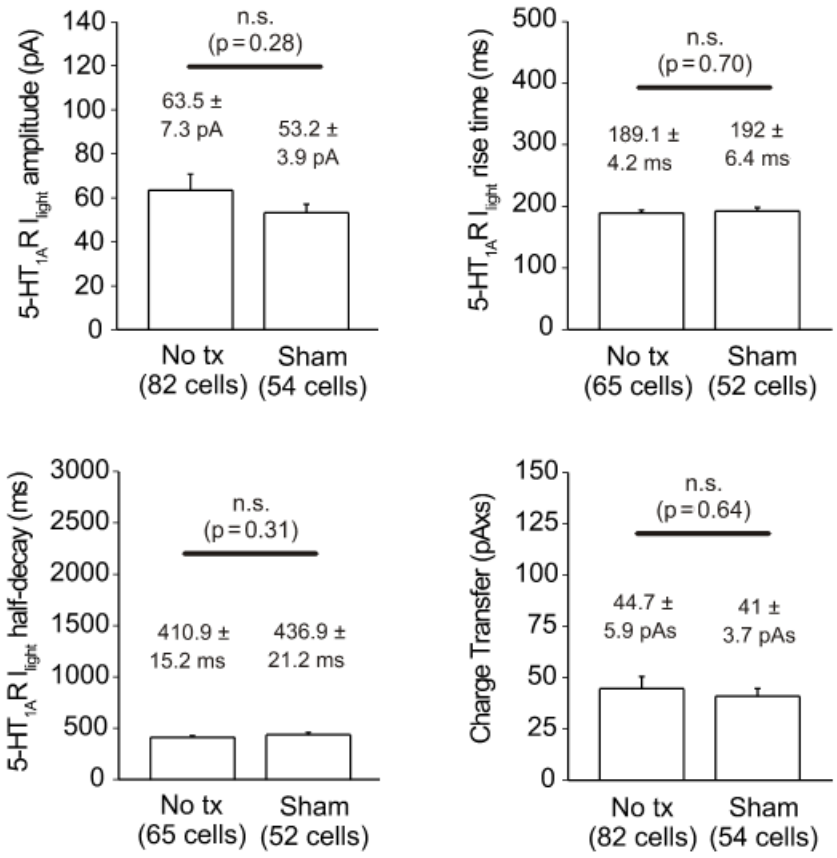

Figure 6. The 5-HT$_{1A}$R I_{light} response characteristics of 5-HT neurons from non-treated and sham treated SERT-Cre X Ai32 mice were not significantly different from one another. Data are presented as mean ± SEM. Student's t-test was used for statistical analysis. Data were obtained from a total of 9 sham treated mice and 19 non-treated mice.

As illustrated in figure 7, chronic fluoxetine treatment resulted in drastic alterations of 5-HT$_{1A}$R I_{light} characteristics. Compared to controls, the amplitude of the autoreceptor signal was significantly decreased (59.4 ± 4.7 pA to 30.5 ± 2.5 pA, p = 1.77E-6; student's t-test). There was also a prolongation of the response, as indicated by a significant increase in the rise time (190.4 ± 3.7 ms to 288.1 ± 9.5 ms, p = 6.27E-22; student's t-test) and half-decay (422.4 ± 12.7 ms to 1167.7 ± 52.2 ms, p = 2.34E-38; student's t-test). Despite the smaller amplitude, the longer timecourse resulted in an overall enhancement of total charge transfer (43.2 ± 3.8 pAxs to 58.2 ± 5.6 pAxs, p = 0.022; student's t-test). As illustrated in figure 7C, the strength of the relationship between 5-HT$_{1A}$R I_{light} amplitude and charge transfer was found to be significantly influenced by

chronic treatment ($p < 0.001$, moderated regression analysis). To summarize, autoreceptor-mediated currents of chronic fluoxetine treated mice were smaller in amplitude but longer in duration, thereby resulting in an overall increase in the total charge transfer. Because the net response was not significantly reduced, but actually increased from prolonged fluoxetine exposure, the data are therefore not consistent with the autoreceptor desensitization hypothesis.

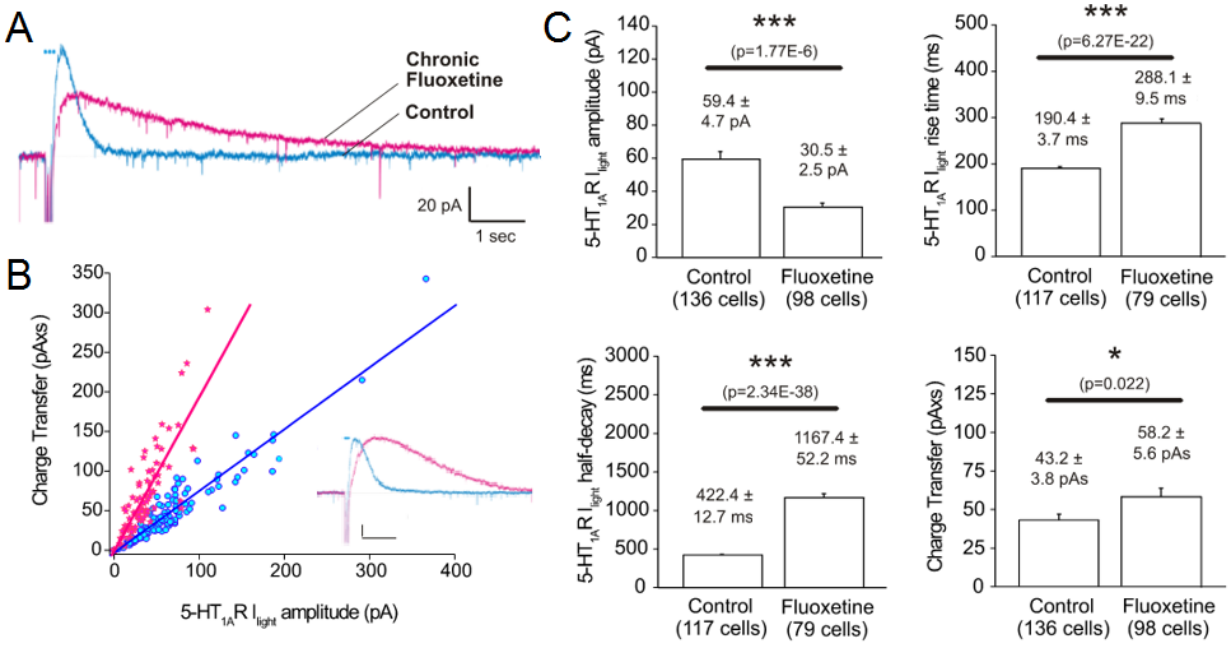

Figure 7. Chronic fluoxetine treatment induces alterations in the 5-HT$_{1A}$ autoreceptor-mediated response. **A.** Representative traces from cells of control and chronic fluoxetine-treated mice. **B.** Quantitative analysis of the autoreceptor-mediated response by comparison of amplitude, rise time, half-decay and charge transfer. Data are presented as mean ± SEM. Statistical analysis was performed with student's t-test. * = $p < 0.05$, *** = $p < 0.01$. **C.** Scatter plot of the total charge transfer (pAxs) versus the 5-HT$_{1A}$R I_{light} amplitude (pA) of individual cells from chronically treated mice (stars; 98 cells) and control mice (circles; 136 cells). Lines of best fit were included to emphasize the strong relationship between the two variables for control ($R^2 = 0.95$, $p < 0.001$) and chronic fluoxetine treatment ($R^2 = 0.86$, $p < 0.001$). Moderated regression analysis revealed a highly significant ($p < 0.001$) effect of treatment on the strength of this relationship. **C, inset.** To better illustrate the impact of the prolonged timecourse and enhanced charge transfer on the autoreceptor-mediated response, traces from cells of control and chronic treated mice with similar amplitude are overlaid (scale bar is 25 pA by 1 sec). Data were obtained from a total of 28 control and 16 chronic fluoxetine treated mice.

Although the net effect from chronic treatment was an enhancement of the autoreceptor response, because of the reduction in peak amplitude it was plausible that there was mild desensitization of the autoreceptor. In order to further investigate this possibility we turned to a different experimental approach.

5-HT Dose Response Analysis

To this end, we tested for autoreceptor sensitivity changes in chronic fluoxetine treated mice by constructing 5-HT dose response curves. To account for any changes in 5-HT reuptake (due to residual fluoxetine or adaptive changes in SERT expression or function) the experiments were performed in the presence of fluoxetine (1 µM) (see methods). Our laboratory has found this approach capable of providing reproducible responses within a cell over repeated 5-HT applications (Fig. 8A) and moreover, in our testing conditions, allowing for selective assessment of 5-HT_{1A}R function (Fig. 8B). Data were fit to the Hill equation and cells across treatment groups were compared on the basis of the maximal current response (I_{max}) and the concentration of 5-HT (µM) necessary to produce a half-maximal response (EC_{50}). If there was desensitization of the 5-HT_{1A}R, one would expect to see a significant decrease in the I_{max} and increase in the EC_{50}. However, as shown in figure 8C-F, the chronic treatment group (I_{max} = 52 ± 6.2 pA; EC_{50} = 2.1 ± 0.4 µM) did not differ from the control group in either parameter (I_{max} = 57.3 ± 6 pA ($p = 0.56$); EC_{50} = 2.8 ± 0.8 µM ($p = 0.53$) (student's t-test)). Therefore, in agreement with the optical stimulation experiments, pharmacological assessment revealed no evidence of autoreceptor desensitization from chronic treatment. Collectively, our data provide support for the idea that the 5-HT_{1A} somatodendritic autoreceptor-mediated signal, despite being altered, is actually preserved following two weeks of fluoxetine administration.

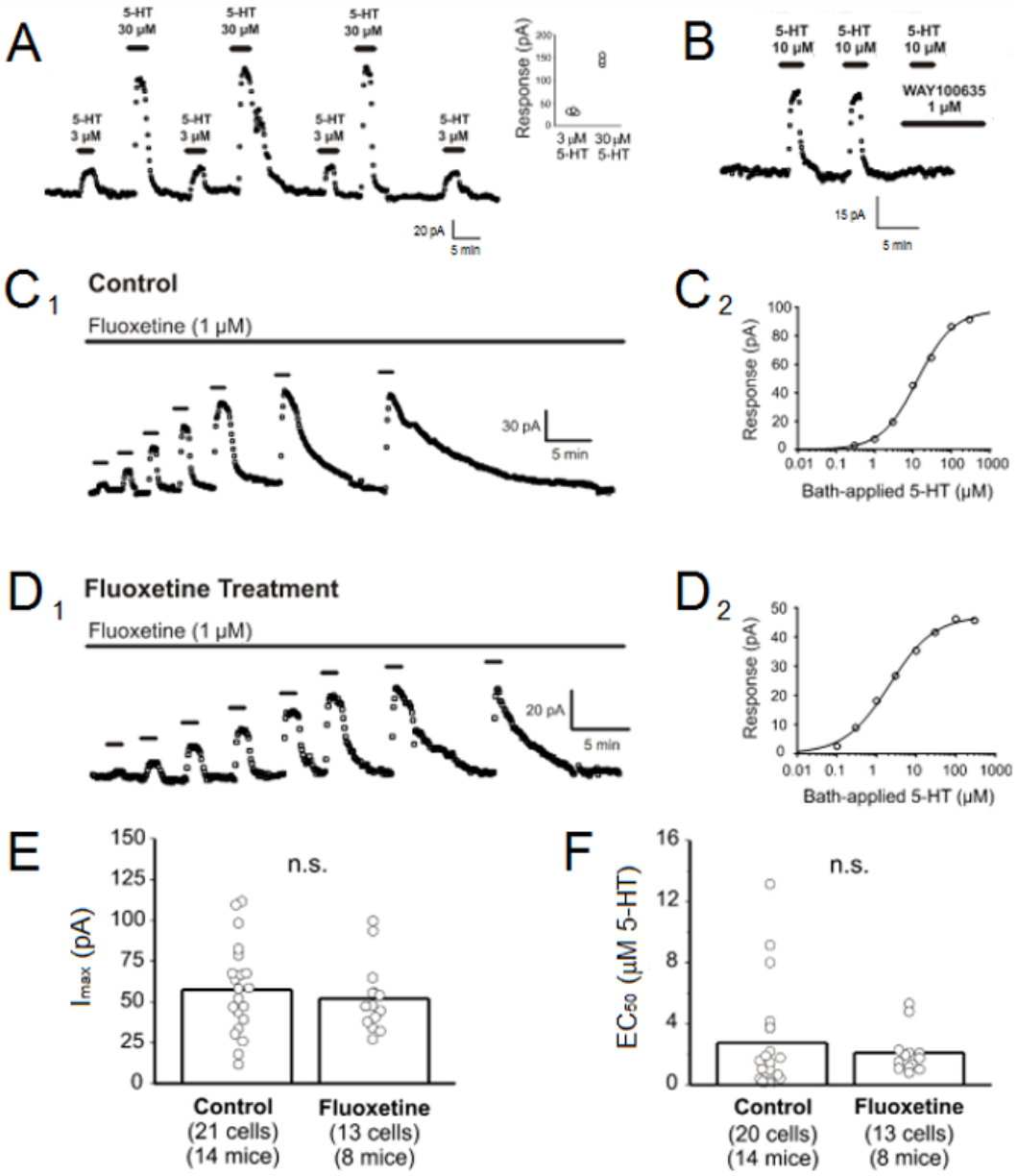

Figure 8. 5-HT concentration response curves conducted in the presence of bath fluoxetine (1 µM) do not indicate a change in 5-HT$_{1A}$ autoreceptor sensitivity following chronic fluoxetine treatment. Repeated bath application of 5-HT (3 µM and 30 µM) produced consistent responses (**A**) that were mediated through the 5-HT$_{1A}$ receptor, as demonstrated by blockade of the response by application of the selective 5-HT$_{1A}$ receptor antagonist, WAY100635 (1 µM) (**B**). Example 5-HT dose response data from a control cell (**C$_1$** and **C$_2$**) and a chronic fluoxetine treated cell (**D$_1$** and **D$_2$**) obtained in the presence of bath fluoxetine (1 µM). Data were fit to the Hill equation and the I_{max} and EC_{50} results were compared between the two groups. The I_{max} (maximal 5-HT$_{1A}$R-mediated response amplitude) (**E**) and EC_{50} (concentration of 5-HT in µM needed to obtain 50% of the maximal response) (**F**) of 5-HT neurons from chronic fluoxetine treated mice were not significantly different from those of controls (p = 0.56 and p = 0.53, respectively; student's t-test).

So if a desensitization of the autoreceptor was not causing the reduction in 5-HT_{1A}R I_{light}, what else could be responsible? Interestingly, a previous study utilizing whole-cell recordings coupled with electrical stimulation to evoke 5-HT release noticed that acute bath-applied fluoxetine caused a reduction of the autoreceptor-mediated response amplitude and prolongation of the signal (Pan et al., 1989). The similarity between the previously observed effects of acute exposure (Pan et al., 1989) and those from chronic treatment in the current work (see Fig. 7) raised the possibility that simply impairing 5-HT clearance could account for the chronic treatment-like responses. Therefore we next tested whether acute fluoxetine would cause similar effects on 5-HT_{1A}R I_{light} in treatment naïve mice in our experimental conditions.

Effects of Acute Fluoxetine

To this end, fluoxetine (1 µM) was bath-applied to slices derived from non-treated littermate controls. A minimum of 20 minutes of exposure was utilized to ensure the drug had fully penetrated the slice and the response had reached steady state (unreported observation). As shown in figure 9, the amplitude (28 ± 5.7 pA), rise time (326.8 ± 23.3 ms), half-decay (1223.7 ± 195.2 ms) and charge transfer (60.7 ± 13.1 pAxs) from acute exposure were not significantly different from the corresponding values of chronic treatment ($p = 0.97$, $p = 0.08$, $p = 0.84$, $p = 0.98$, respectively; one-way ANOVA with post hoc Tukey). Moreover, all measurements trended towards and/or reached significance in regards to controls (5-HT_{1A}R I_{light} amplitude ($p = 0.006$), rise time ($p < 0.001$), half-decay ($p < 0.001$), charge transfer ($p = 0.30$); one-way ANOVA with post hoc Tukey). As shown in figure 9B, the strength of the relationship between 5-HT_{1A}R I_{light} amplitude and charge transfer of individual cells acutely exposed to fluoxetine closely resembled that of chronic treatment ($p = 0.22$, moderated regression analysis) and was significantly different from that of control cells ($p < 0.001$, moderated regression analysis). Therefore, in agreement with earlier reports (Pan et al., 1989), acute fluoxetine exposure to treatment naïve mice resulted in an autoreceptor-mediated signal with a smaller peak amplitude but longer timecourse. Importantly, these alterations from acute exposure resembled those of chronic treatment.

Together, the data suggest that simply a reduction in 5-HT reuptake, instead of a chronic adaptation in autoreceptor sensitivity, could potentially account for the chronic treatment-like effects on the autoreceptor-mediated response.

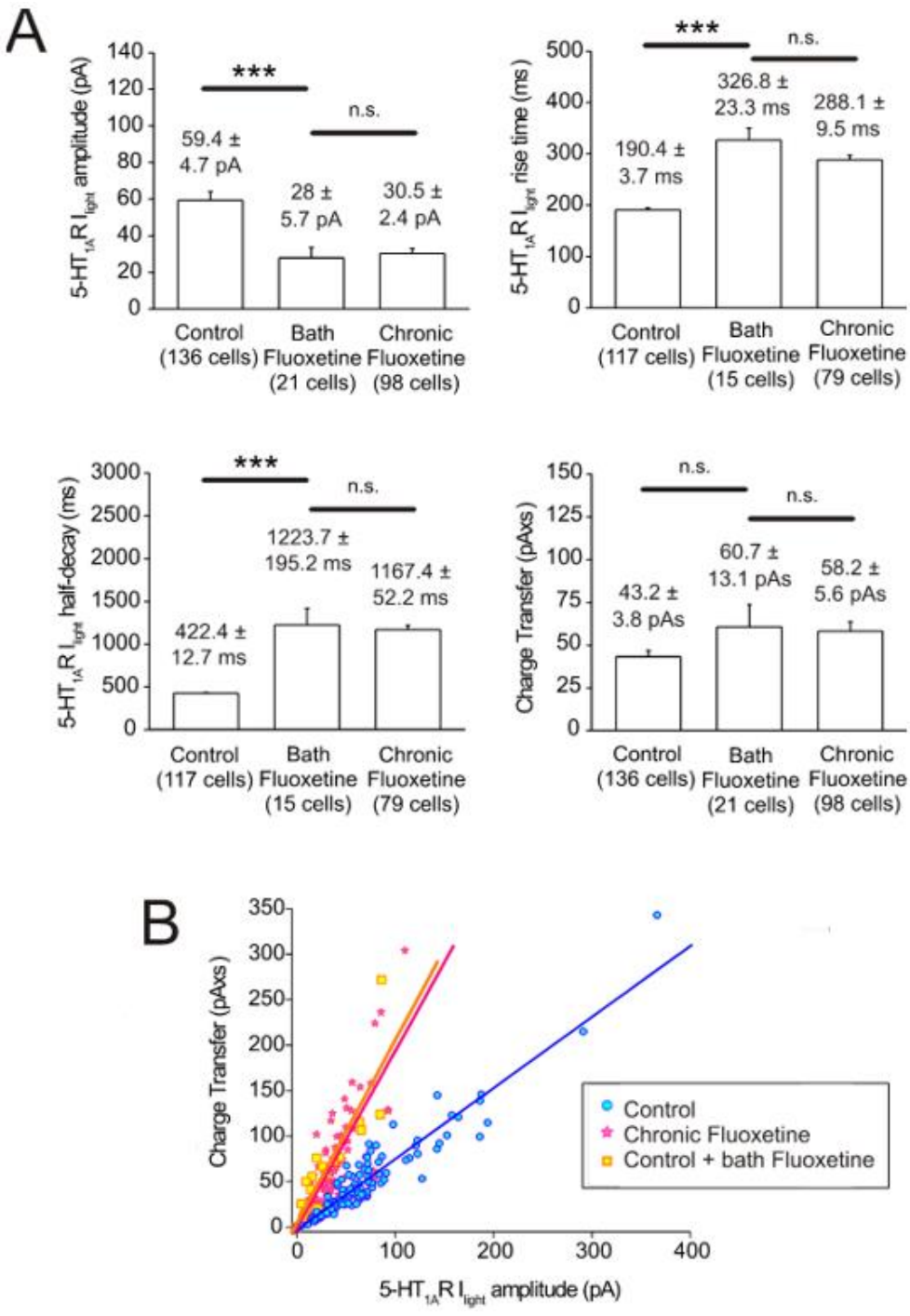

Figure 9. *(legend on next page)*

Figure 9. Bath application of fluoxetine (1 μM) produces 5-HT$_{1A}$R I$_{light}$ characteristics in control cells that are similar to those of chronic fluoxetine treatment. A. The 5-HT$_{1A}$R I$_{light}$ amplitude from acute fluoxetine was not different from chronic treatment ($p = 0.97$) but was significantly smaller than control conditions ($p = 0.006$). The rise time and half-decay were not different from their corresponding chronic treatment values ($p = 0.08$ and $p = 0.84$, respectively) but were significantly different from those of controls ($p < 0.001$ and $p < 0.001$). The charge transfer did not differ from chronic treatment ($p = 0.98$) or control ($p = 0.30$) values. Data are presented as mean ± SEM. Significance was assessed with one-way ANOVA and post hoc Tukey. * = $p < 0.05$, *** = $p < 0.01$. **B.** Scatter plot of the charge transfer (pAxs) versus the amplitude (pA) of the autoreceptor-mediated response for individual cells acutely exposed to fluoxetine (squares; 21 cells) revealed a strong correlation ($R^2 = 0.87$, $p < 0.001$) between the two variables. The line of best fit is included to illustrate this point. The strength of this relationship for acute fluoxetine was not significantly different from that of chronic fluoxetine treatment ($p = 0.22$) but was significantly different from that of control conditions ($p < 0.001$, moderated regression analysis).

However, it is notable that bath-applied fluoxetine does not fully recapitulate the conditions of chronic treatment. In order to more accurately test whether the 5-HT$_{1A}$R I$_{light}$ alterations observed following 14 days of fluoxetine were due to a reduction in 5-HT clearance, we next explored whether subchronic fluoxetine administered via osmotic pump would produce similar results to those of bath fluoxetine and chronic treatment.

Effects of Subchronic Fluoxetine Administration

Mice were treated with fluoxetine for ~72 hours (71.3 ± 14 hours (mean ± SD), 5 mice) in a manner identical to that of chronic treatment. This timepoint was chosen because it has been shown to be premature for the emergence of physical adaptations previously associated with autoreceptor desensitization, such as the recovery of serotonin cell firing (Czachura and Rasmussen, 2000) or onset of the antidepressant effects in rodents (Dulawa et al., 2004), and therefore should provide insight as to whether simply blocking 5-HT reuptake produces chronic treatment-like effects on the 5-HT$_{1A}$R I$_{light}$ signal. At the time of sacrifice, the blood levels of fluoxetine and its major active metabolite norfluoxetine were approximately half of those obtained under chronic conditions (173.1 ± 116.7 ng/mL (mean ± SD), 5 mice). Nevertheless, there were noticeable effects on the 5-HT$_{1A}$R I$_{light}$ characteristics. As illustrated in figure 10A, the 5-HT$_{1A}$R I$_{light}$

amplitude (39.3 ± 5.3 pA), rise time (272.1 ± 13.2 ms), half-decay (873 ± 69.5 ms) and charge transfer (54.4 ± 7.7 pAxs) trended towards their corresponding chronic treatment values ($p = 0.50$, $p = 0.43$, $p < 0.001$, $p = 0.91$, respectively; one-way ANOVA with post hoc Tukey). They were significantly different from their respective controls (amplitude ($p = 0.02$), rise time ($p < 0.001$), half-decay ($p < 0.001$)) with the exception of total charge transfer ($p = 0.39$) (one-way ANOVA with post hoc Tukey). Notably, the strength of the relationship between response amplitude and charge transfer of 5-HT cells from subchronic treated mice was intermediate between that of control and chronic fluoxetine treated mice (Fig. 10B, $p < 0.001$ compared to both control and chronic conditions; moderated regression analysis). As the drug blood levels were approximately half that of chronic treated mice, this suggests that the relationship may be mediated by the extent of 5-HT reuptake blockade. Consistent with the effects of acute fluoxetine, the effects of subchronic treatment on the autoreceptor-mediated response provide support for the idea that prolonging the extracellular lifetime of 5-HT may be sufficient to cause alterations in $5\text{-HT}_{1A}\text{R}$ I_{light} that are similar to those observed after 14 days of fluoxetine administration. Together, these data suggest that the reduction in the autoreceptor-mediated signal amplitude seen following prolonged SSRI treatment is not the result of a chronic adaption in the sensitivity of the 5-HT_{1A} autoreceptor. Instead, the altered amplitude and kinetics of the response are likely due to a reduction in 5-HT clearance.

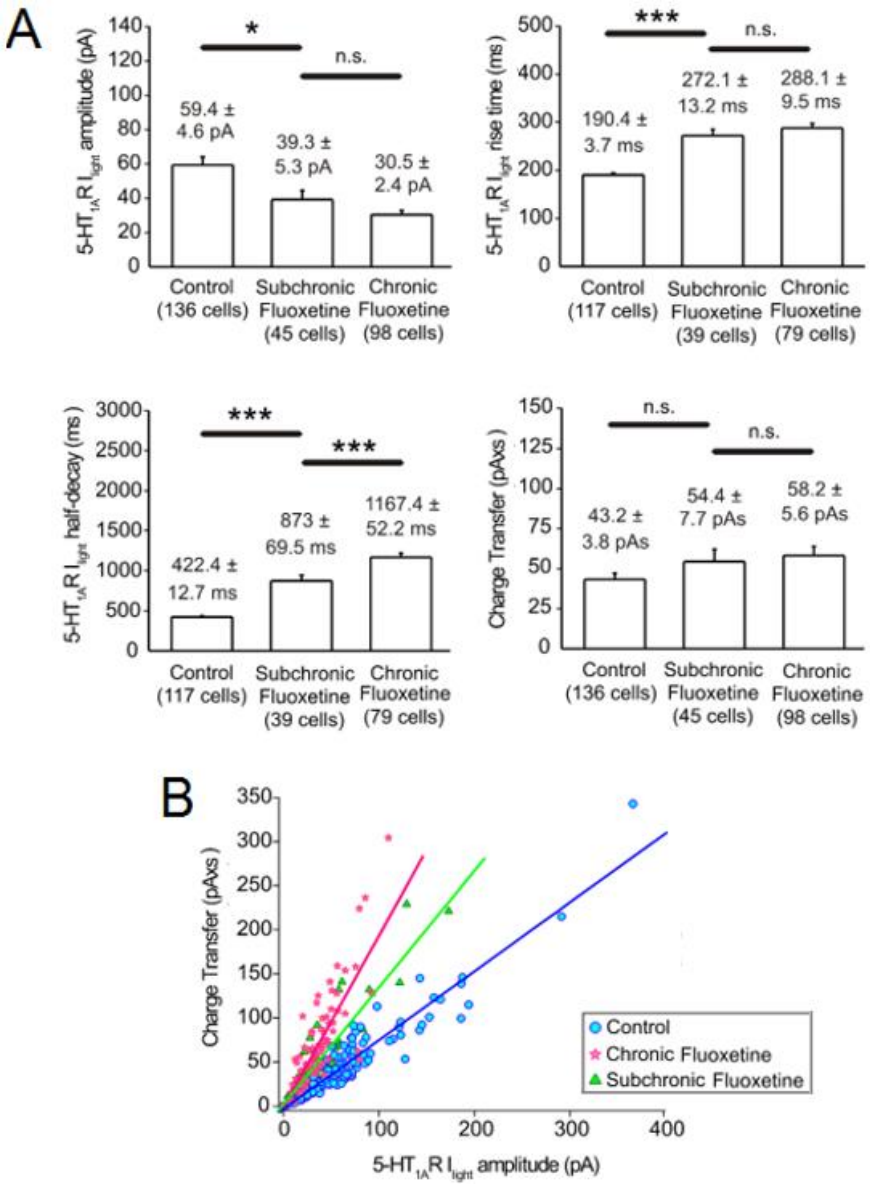

Figure 10. Effects of subchronic fluoxetine treatment on the 5-HT$_{1A}$ autoreceptor-mediated signal. A. Comparison of the 5-HT$_{1A}$R I_{light} characteristics between control and ~72 hours fluoxetine treatment (5 mice) revealed alterations in the amplitude ($p = 0.02$), rise time ($p < 0.001$), half-decay ($p < 0.001$) and charge transfer ($p = 0.39$) that trended towards their corresponding chronic treatment values. With the exception of half-decay ($p < 0.001$), subchronic treatment was not significantly different from chronic treatment in amplitude ($p = 0.50$), rise time ($p = 0.43$) or charge transfer ($p = 0.91$). Data is presented as mean ± SEM. One-way ANOVA with post hoc Tukey was used for statistical analysis. **B.** A scatterplot of charge transfer (pAxs) versus amplitude (pA) for the individual subchronic treated cells (triangles; 45 cells) revealed a relationship between the two variables ($R^2 = 0.92$, $p < 0.001$). The strength of this relationship was significantly influenced by subchronic treatment ($p < 0.001$ compared to control) but, as expected from the sub-maximal drug blood levels, remained significantly different from that of chronic treatment ($p < 0.001$, moderated regression analysis).

Despite their widespread, ever-growing use, the mechanism of action of SSRI antidepressants remains only partially understood. One prominent theory posits that in order for these medications to elicit their therapeutic effect, 5-HT_{1A} somatodendritic autoreceptors must become desensitized (Blier et al., 1987). Despite its general acceptance, there has been inconsistent support for the theory over the years. However, until now, direct assessment of autoreceptor sensitivity following prolonged SSRI exposure has been prevented due to methodological limitations. With advancements in genetic targeting strategies and expansion of our optical toolbox, the goal of the current work was to reinvestigate this theory. Using a novel application of optogenetics to directly assess vmDRN 5-HT_{1A} somatodendritic autoreceptor function, we found, in contrast to the prevailing theory, that the autoreceptor-mediated signal is actually preserved following chronic fluoxetine treatment.

Benefits of Optogenetics for Assessment of Autoinhibition

The use of optogenetics, the targeting of light-sensitive proteins to cells to control their activity, has quickly become a popular neuroscience tool. It has been utilized to study neuronal circuitry (Pollak Dorocic et al., 2014), investigate signaling pathways (reviewed in McGregor et al., 2015), and deduce the role of selective neuronal populations on behavior (McDevitt et al., 2014). It is the only currently available method of controlling a specific neuronal cell type within a heterogeneous region of the brain and/or brain tissue. It also allows temporally precise, synchronous control of a neuronal cell type in a population-wide manner. In the current work, we utilized genetically-modified mice (SERT-Cre X Ai32) that directed expression of the light-sensitive cation channel, Channelrhodopsin (ChR) (Boyden et al., 2005) selectively to serotonergic neurons in our slice preparation using the serotonin transporter promoter. A variant of ChR (ChR2(H134R)) was chosen for this purpose as it has been shown to exhibit enhanced light sensitivity and thus provide larger photocurrents compared to the classic protein (Lin, 2011). Unlike some other light-sensitive constructs, additional compounds are not required for its activity, since its cofactor, all-*trans* retinal, is known to be present in the invertebrate brain at levels that

are sufficient for light transduction (Boyden et al., 2005). Light stimulation at the relatively low levels and frequency utilized in our experiments did not cause any noticeable harm to cells. Notably, this study represents the first application of optogenetics to examine autoinhibition of serotonergic neurons. This approach allowed us to overcome a number of limitations that have previously prevented direct assessment of 5-HT autoinhibition, and in particular, examination of 5-HT_{1A} autoreceptor sensitivity following chronic SSRI treatment.

One of the major obstacles for the study of serotoninergic neurons is their identification. It has been known for many years that the dorsal raphe nucleus is a heterogeneous nucleus, containing not only 5-HT cells, but also glutamatergic, GABAergic, and dopaminergic cells (Jacobs and Azmitia, 1992; Kohler and Steinbusch, 1982). Therefore, electrophysiological criteria to facilitate the identification of 5-HT neurons were described relatively early on (Vandermaelen and Aghajanian, 1983; Aghajanian and Vandermaelen, 1982). These 'classic' serotonergic characteristics included: biphasic action potentials, responsiveness to 5-HT and LSD, and slow (0.5 to 2 spikes/sec), regular pattern of discharge. Although these criteria have been frequently utilized throughout the years, there has been mounting immunohistochemical evidence that they do not accurately discriminate between serotonergic and non-serotonergic neurons (Kirby et al., 2003; Beck et al., 2004). Not only do many non-5-HT containing cells meet these criteria (Kirby et al., 2003), but because the DRN 5-HT population is much more heterogeneous than previously imagined, some 5-HT neurons do not meet the criteria (Schweimer and Ungless, 2010; reviewed in Andrade and Haj-Dahmane 2013). The genetic targeting strategy utilized in the current work allowed us to overcome this limitation and easily confirm the identity of a patched neuron as serotonergic by simply flashing light and looking for the presence of ChR-mediated currents (Fig. 4).

Another obstacle that has limited our insight into 5-HT_{1A}R-mediated signaling and its potential alteration from SSRI treatment by electrophysiological means involves stimulation techniques. A common method of activating 5-HT_{1A} autoreceptors *in vivo* has been systemic

administration of a 5-HT_{1A}R agonist to an anesthetized rodent. However, due to the fact that no available pharmacological agent selectively distinguishes between the 5-HT_{1A} somatodendritic and heteroreceptors, systemic administration activates not only the 5-HT_{1A} somatodendritic autoreceptors but also 5-HT_{1A}R's located on GABAergic cells in the DRN (Beck et al., 2004) and 5-HT_{1A}R's on postsynaptic forebrain targets, both of which directly or indirectly feed back to affect the activity of the recorded neuron (Challis et al., 2013; reviewed by Altieri et al., 2013). A common method for pharmacologically manipulating the autoreceptor in brain slices is through bath application. As is the case with systemic administration however, this also lacks the temporal precision necessary to stimulate the receptors on a physiologically relevant timescale, thereby preventing a complete understanding of the physiological response. Another notable *in vivo* and *in vitro* option is microiontophoresis, which allows brief (sub-second) application of an exogenous agonist to a localized brain region. Interestingly, although it has been adopted for the examination of autoreceptor sensitivity from chronic MAOI, TCA, and some SSRI treatments, to the best of our knowledge it has never been used to study DRN 5-HT_{1A} somatodendritic autoreceptor sensitivity following chronic fluoxetine exposure.

Until now, the only available stimulation technique that would cause release of endogenous 5-HT within the DRN, and do so on a subsecond timescale was electrical field stimulation in brain slices (Yoshimura and Higashi, 1985; Williams et al., 1988; Pan and Williams, 1989; Pan et al., 1989). However, this technique had a number of limitations when it came to the study of SSRI effects on 5-HT_{1A}R signaling. First, nonselective stimulation of the DRN caused release of not only 5-HT, but also GABA and glutamate, which have been shown to impact the readout of the 5-HT_{1A}R-mediated signal (Pan et al., 1989). However, this could be overcome by addition of GABA and glutamate receptor antagonists to the extracellular solution. A more important limitation was the complex standardization required to adequately compare responses between cells both within slices and across slices. Because standardizing between cells of control and treated rodents was even more problematic, this issue largely prevented the application of

electrical stimulation for electrophysiological assessment of autoinhibitory signaling in response to chronic 5-HT reuptake blockade.

The optogenetic methodologies implemented in the current work overcame these previous limitations. The use of light to selectively stimulate 5-HT cells in brain slices provides precise temporal, synchronous control of endogenous 5-HT release and selective activation of 5-HT_{1A} autoreceptors (Fig. 5A). By its nature, light stimulation bypasses the need for standardization in our preparation since light penetrates the slice almost instantaneously to induce simultaneous release of 5-HT from cells irrespective of cell depth or distance from the light source. Coupled with electrophysiology this technique provided real time, cellular level insight into autoreceptor signaling. Moreover, with this approach the autoreceptor-mediated response from one cell could be continuously sampled over time (Fig. 5D). It also allowed multiple cells to be sampled from each mouse, thereby providing a more complete picture of the effects of SSRI treatment on the heterogeneous DRN 5-HT cell population (reviewed in Andrade and Haj-Dahmane, 2013). Collectively, the current optogenetic approach permitted, for the first time, a direct readout of 5-HT_{1A} autoreceptor signaling and allowed comparison of the signal between control and chronic fluoxetine treated mice.

Major Findings

The major finding of the current work is that the autoinhibitory signal mediated by somatodendritic 5-HT_{1A} autoreceptors was preserved following prolonged 5-HT reuptake blockade. Support for this came from experiments utilizing genetically modified SERT-Cre X Ai32 mice that targeted ChR to 5-HT neurons in our brainstem slice preparation (Fig. 3). Brief (5 ms) flashes of light was sufficient to selectively and reliably control the activity of serotonergic neurons (Fig. 4) and provide a direct readout of the 5-HT_{1A} autoreceptor-mediated signal (Fig. 5). As shown in figure 7, compared to controls, chronic treatment with fluoxetine resulted in a reduction in the 5-HT_{1A}R I_{light} amplitude but a dramatic increase in the duration, thereby causing a net enhancement in the autoreceptor-mediated signal as assessed by total charge transfer. In order

to clarify whether the reduction in amplitude was due to a chronic adaptation in the sensitivity of the autoreceptor, we conducted 5-HT dose response analysis on identified 5-HT cells from control and chronic treated mice. Importantly, the experiments were performed in the presence of bath fluoxetine to account for potential reductions in 5-HT reuptake due to residual fluoxetine/norfluoxetine or alterations in SERT function or expression. Importantly, neither the I_{max} or EC_{50} were different between the groups (Fig. 8). Collectively, the data suggested that the sensitivity of the 5-HT_{1A} autoreceptor was not attenuated following chronic SSRI administration.

Since it was previously reported that acute fluoxetine induced alterations in the autoreceptor-mediated signal that were similar to the observed changes from chronic fluoxetine in the current work, we next sought to explore whether simply a reduction in 5-HT reuptake—instead of a chronic attenuation of autoreceptor function—could account for these 'chronic' effects. Exposure to bath-applied fluoxetine (Fig. 9) or subchronic treatment (Fig. 10) produced 5-HT_{1A}R I_{light} signals that were significantly smaller in amplitude and exhibited a significant prolongation of their timecourse compared to controls. Because of the similarity to chronic treatment responses, the results indicate that the effects from chronic treatment are unlikely to be the result of chronic adaptations, but appear to be due simply to a reduction in 5-HT clearance.

Potential Limitations of the Current Approach

There is a notable potential limitation with this approach. The ChR-mediated currents were significantly smaller in the chronic (450.99 ± 15.57 pA, 98 cells) and subchronic (408.53 ± 27.27 pA, 45 cells) fluoxetine treatment groups compared to controls (529.80 ± 15.64 pA, 136 cells; $p = 0.007$ and $p = 0.001$, one-way ANOVA with post hoc Tukey). The chronic and subchronic treatment ChR-mediated amplitudes were not significantly different from one another ($p = 0.668$, one-way ANOVA with post hoc Tukey). It is currently unclear as to why the currents were reduced. One explanation could be that there is a decrease in ChR expression. At the level of transcription, the general consensus is that fluoxetine does not result in an attenuation of SERT expression (and thus would not alter transcription of Cre recombinase) (Hrdina and Vu, 1993; Koed and

Linnet, 1997; Neumaier et al., 1996; Le Poul et al., 2000). It would also be unlikely that fluoxetine treatment would influence the accessibility of the *Rosa26* locus that the conditional ChR expression cassette was inserted. Moreover, the time for changes in transcription to have an impact on protein levels makes it unlikely that an alteration in transcription could underlie the observed changes for the subchronic conditions. Alternatively, Cre recombinase expression (driven by the SERT promoter) may be reduced by fluoxetine's reported indirect actions on SERT translation, as mediated by microRNAs (Baudry et al., 2010). However, if there was a significant decrease in Cre recombinase transcription or translation, this would likely result in an all-or-nothing pattern of ChR expression instead of the modest decrease in ChR-mediated currents that were observed. To resolve this in a simple manner, future studies could utilize techniques such as *in situ* hybridization and immunohistochemistry to test for changes in ChR mRNA or protein levels.

Instead of an alteration in ChR expression, it may be possible that fluoxetine produced modifications in the properties of 5-HT neurons that could reduce the impact of light stimulation on the amplitude of the ChR currents. However, we did not find any difference in the resting membrane potential between control (-77.32 ± 0.64 mV, 136 cells) and chronic (-76.28 ± 0.77 mV, 98 cells; $p = 0.835$, one-way ANOVA post hoc Tukey) or subchronic treatment conditions (-78.96 ± 1.15 mV, 45 cells; $p = 0.711$, one-way ANOVA post hoc Tukey). Although speculative, fluoxetine may influence the internalization or recycling of ChR from the membrane. Detailed characterization of the ChR-mediated current amplitude from repeated stimulation between treatment and control groups may provide clarification. Alternatively, immunoelectron microscopy of immunogold labeled ChR could also be useful in this regard.

Despite the significant reduction in amplitude, it is important to note that these ChR-mediated currents were still well above the estimated threshold that would be necessary to induce a spike. Nevertheless, even if the 5-HT_{1A}R I_{light} readout from fluoxetine administration was partially hindered by the efficacy of optical stimulation, it would only strengthen our conclusion that

prolonged fluoxetine treatment does not cause autoreceptor desensitization, since the 5-HT_{1A} autoreceptor-mediated signal in the chronic treatment group reported in the current work would therefore be an underestimate of the 'actual' autoreceptor-mediated response.

Previous Reports that Cast Doubt on the Autoreceptor Desensitization Hypothesis

In agreement with the current work, there have been other reports that cast doubt on the hypothesis that chronic SSRI exposure causes a reduction in 5-HT_{1A} somatodendritic autoreceptor-mediated signaling. For example, following prolonged fluoxetine treatment, biochemical studies have failed to find reductions in 5-HT_{1A} R mRNA in the DRN (Hervas et al., 2001), alterations in the density of binding sites, modifications of the binding parameters, or changes in the proportion of the autoreceptor present in its high affinity, G protein-coupled state (Castro et al., 2003; Le Poul et al., 2000; Le Poul et al., 1995; Hervas et al, 2001; Hensler, 2002). Studies employing *in vivo* microdialysis or fast scanning cyclic voltammetry to assess the functional status of the autoreceptor have also failed to consistently demonstrate an attenuation of autoreceptor-mediated control over 5-HT release following chronic SSRI treatment (O'Connor and Kruk, 1994; Davidson and Stamford, 1998; reviewed by Hjorth et al., 2000). Together, these reports provide sufficient support to call into question the validity of the autoreceptor desensitization hypothesis.

Previous Reports that Chronic Fluoxetine Treatment Causes 5-HT_{1A} Autoreceptor Desensitization

Although the current approach is the first to allow direct assessment of the autoreceptor-mediated signal and thus permit unmatched insight into its potential regulation by SSRI treatment, it was initially puzzling that the conclusions from a majority of other electrophysiological studies were in opposition to our current findings. However, these disparities can be largely reconciled after consideration of a number of important factors. In addition to those discussed above (i.e. potential misidentification of '5-HT' cells), there are a few additional points that deserve mention.

Evidence for desensitization stemmed from two lines of electrophysiological observations. First, acute SSRI exposure caused a suppression of 5-HT cell firing, which recovered slowly over a two week period (de Montigny et al., 1984). Because it was thought that 5-HT_{1A} somatodendritic autoreceptors normally act to regulate the basal firing rate of 5-HT neurons, it was logical that a reduction in their inhibitory signaling was critical for the resumption of neuronal activity (reviewed in Blier, 2001). However, as recently reviewed by our group (Andrade et al., 2015), the evidence to support the idea that 5-HT_{1A} autoreceptors act to homeostatically regulate the pacemaker-like firing of serotonergic neurons is inconsistent and possibly insufficient. There are a number of alternative roles for 5-HT_{1A} autoreceptors, including synchronizing neuronal networks (Andrade et al., 2015) or functioning as sensors to prevent excessive local 5-HT elevations (Adell et al., 2002). With this view, many alternate factors could account for the recovery in firing observed to occur following prolonged 5-HT reuptake blockade. For instance, since glutamatergic and noradrenergic inputs are known to provide excitatory drive to 5-HT cells (Baraban and Aghajanian, 1980; reviewed in Altieri et al., 2013), it is conceivable that they exert an enhanced influence over the course of treatment. It is also possible that there are adaptations in the intrinsic properties of serotonergic cells, or changes in various components of intracellular signaling cascades and/or in neurotrophic factor signaling from chronic SSRI treatment (reviewed in Duman et al., 1997) that contribute to the recovery of firing. Collectively, in our view the use of 5-HT cell firing as a direct indicator of 5-HT_{1A} autoreceptor function may not be entirely justified.

The second line of electrophysiological support for the hypothesis originated from studies examining the functional status of the autoreceptor using pharmacological agents. For example, following two weeks of SSRI treatment, it was reported that agonist administration had less of an effect on suppressing the firing of presumed 5-HT cells compared to cells of treatment-naïve rodents (Blier et al., 1998). This was therefore attributed to a desensitization of the autoreceptor. But once again, firing is by no means a direct readout of the functional state of the autoreceptor. As described above, a number of factors that influence serotonergic firing—especially potentially

following chronic fluoxetine treatment—may override the contribution of the 5-HT_{1A} R. If this were true, then the observed dampened ability of 5-HT_{1A} R agonists to reduce the firing of putative 5-HT cells following chronic treatment may in fact occur, but do so independently of any actual change in autoreceptor sensitivity. To summarize, the almost exclusive reliance on firing rate as a readout of autoreceptor function by electrophysiologists may have led to the possibly unfounded conclusion that chronic SSRI treatment resulted in a desensitization of 5-HT somatodendritic autoreceptors.

Previous Reports of the Effects of Acute and Subchronic Fluoxetine on the 5-HT_{1A} Autoreceptor-mediated Signal

So if others observed 'autoreceptor desensitization' from chronic treatment of fluoxetine, then why did they not see a similar 'desensitization' from acute or subchronic fluoxetine administration? Concerning acute exposure, Pan and colleagues (1989) (using electrical field stimulation to induce endogenous 5-HT release on a timescale comparable to that of physiologic conditions) did report the amplitude reduction, they just did not call it desensitization. Interestingly, to the best of our knowledge, no other electrophysiological studies used acute fluoxetine when comparing their control group to chronic fluoxetine treatment to assess autoreceptor sensitivity. It would have been interesting to see what they would have concluded had they done so.

In terms of subchronic treatment, *in vivo* electrophysiological studies (with one exception) did not test for alterations in autoreceptor sensitivity at earlier (<14 days) timepoints, presumably because there were so few active cells and their only readout of autoinhibition was firing suppression. Notably, there was one group that did assess autoreceptor sensitivity from fluoxetine treatment, *in vivo*, at subchronic timepoints (Czachura and Rasmussen, 2000). Given the thoroughness of their work, it is puzzling why they performed statistical analysis using the treatment-naïve data as the control, as one of the subchronic timepoints would have been more appropriate. It would be interesting to know whether analysis of 14 days of treatment compared to 3 days of treatment would have led them to the same conclusion that there was significant 5-

$HT_{1A}R$ desensitization solely following chronic administration. There is one more point that deserves mention. Being the only study to examine both the progressive recovery of firing and 'autoreceptor sensitivity' (albeit indirectly from firing suppression) throughout the early stages of SSRI administration, it unintentionally provided evidence against the rationale that firing recovery is due to a reduction in autoreceptor function. Specifically, it was demonstrated that the basal firing rates of putative 5-HT cells had fully recovered by day 14 of fluoxetine treatment. However, the 'blunted' ability of an agonist to suppress firing at 14 days appears to be only half-maximal compared to the effect at 21 days of treatment. These inconsistencies therefore work against the argument of autoreceptor desensitization.

In addition to the *in vivo* experiments described above, there was a second study that investigated the effects of subchronic SSRI treatment, this time conducted in *in vitro* brain slices (Le Poul et al., 1995). Consistent with the majority of chronic treatment reports, Le Poul and colleagues observed a reduced ability of a 5-HT_{1A} receptor agonist to suppress the firing of presumed 5-HT neurons following 21 days of fluoxetine treatment. However, this only occurred in ~60% of the sampled cells, whereas there were no obvious effects on the other ~40%. Moreover, when they compared the extent of this blunted response between 'less responsive' cells at 21 days of treatment to those after 3 days of treatment, they found no difference. Instead, they observed the percentage of sampled cells exhibiting a blunted response to slightly increase with the duration of treatment, from ~43% at day 3 to ~60% at day 21. Such a small increase could potentially be explained if, consistent with the current work, the drug had failed to reach steady state levels by 3 days of treatment (although there were no reports of drug blood levels by Le Poul and colleagues). Collectively, the few times when the effects of acute or subchronic fluoxetine exposure were examined in the past, the results from such studies have not provided strong evidence to support the autoreceptor desensitization theory.

Potential Factors Contributing to the Alterations in 5-HT_{1A}R I_{light}

We found that acute, subchronic, and chronic fluoxetine exposure caused a prolongation of the autoreceptor-mediated signal. This is consistent with other reports of the effect of acute fluoxetine on the lifetime of electrically-evoked 5-HT in the extracellular space (Roberts et al., 2005; Bunin et al., 1998) and its effect on the 5-HT_{1A}R-mediated response (Pan et al., 1989). As a competitive inhibitor of SERT, the enhanced 5-HT_{1A}R signal rise time and half-decay have been attributed to the increased lifetime of 5-HT in the synaptic cleft, which potentially allows it to repeatedly activate receptors for a longer period of time and possibly travel further to act on more distally located receptors. Therefore, a reduction of 5-HT clearance from the extracellular space was likely responsible for the alterations in 5-HT_{1A}R I_{light} observed in the current work.

Of note, in terms of chronic treatment, this could potentially be due to an adaptation in SERT expression or function, or from the presence of residual fluoxetine and/or its active metabolite, norfluoxetine. As described above, the general consensus is that prolonged administration of fluoxetine does not reduce SERT expression, as assessed by *in situ* hybridization, Northern blot, competitive RT-PCR and autoradiography (Hrdina and Vu, 1993; Koed and Linnet, 1997; Neumaier et al., 1996; Gobbi et al., 1997; Le Poul et al., 2000). Reports on the functional status of SERT following chronic fluoxetine treatment are less clear. Using electrical stimulation to evoke 5-HT release in brain slices, O'Connor and Kruk (1994) found no change in SERT function as determined by the absence of a prolongation of the extracellular 5-HT signal in chronic treated rodents compared to controls. However, it should be noted that fast scanning cyclic voltammetry is limited to sampling bulk extrasynaptic overflow of 5-HT, and therefore may be restricted as to the data it can provide. Gobbi et al. (1997) also found the ability of SERT to take up [^{3}H]5-HT into rat brain synaptosomes to be unaltered following 21 days of treatment with fluoxetine, supporting the idea that SERT function is not impacted under these conditions. On the other hand, Descarries and Riad (2012) observed an attenuation of SERT expression and function as assessed by electron microscopy of immunogold-labeled SERT. The

authors reported that acute fluoxetine had no effect on SERT internalization in control rats, but caused a 48% decrease in plasma membrane levels with a concurrent increase in the cytosolic levels of SERT in chronic fluoxetine treated rats. Because they also observed a reduction in the overall level of SERT, they suggested that chronic exposure resulted in internalization and subsequent degradation of the protein. Collectively, while it seems unlikely that there is a reduction in SERT expression, determination of the functional status of SERT warrants additional investigation.

An alternative possible explanation for the prolongation of 5-HT_{1A}R I_{light} is that residual fluoxetine and norfluoxetine are present during experiments. Whereas it is well known that norfluoxetine levels in the brain and plasma in rodents require approximately 7 days for washout *in vivo* (Homberg et al., 2011; Gardier et al., 1994), less is known about its lifetime in slices. Although the acute and subchronic exposure experiments in the current work are consistent with the idea that residual drug is responsible for the alterations in 5-HT_{1A}R I_{light}, additional possibilities cannot be ruled out. For example, although it is speculative, dysregulation of the intracellular signaling components such as an impairment of RGS (regulator of G protein signaling) proteins could potentially play a role. In summary, while it is not currently clear whether chronic alterations in SERT or continued presence of fluoxetine and norfluoxetine were responsible, it is likely that the consequential attenuation of 5-HT clearance caused the increased timecourse of 5-HT_{1A}R I_{light} in 5-HT neurons exposed acutely, subchronically or chronically to fluoxetine.

In addition to a prolongation of the 5-HT_{1A}R I_{light}, we also observed a reduction in the amplitude of the signal from fluoxetine. This was found by us and others (Pan et al., 1989; Le Poul et al., 1995) to occur at timepoints too premature for the emergence of neuroadaptive changes consistent with an attenuation of 5-HT_{1A}R sensitivity as proposed by the autoreceptor desensitization theory. Therefore, an acute effect of fluoxetine must be responsible. One option is that fluoxetine itself is activating the autoreceptor, thereby partially masking the response of endogenous 5-HT released from light stimulation. However, we find this to be an unlikely

possibility since a reduction in 5-HT_{1A}R I_{light} amplitude was also observed following bath application of the selective 5-HT reuptake inhibitor escitalopram (1 µM) (data not shown). Furthermore, the affinity of fluoxetine for the 5-HT_{1A}R in rodents (~8.3 µM, Owens et al., 1997) suggests that the 1 µM used in our experiments would be unlikely to have a significant direct effect on the autoreceptor.

A second possibility is that fluoxetine, by inhibiting 5-HT reuptake, is causing buildup of extracellular 5-HT in the slice that is sufficient to basally activate the 5-HT_{1A} autoreceptors (Davidson and Stamford, 1995) and cause a reduction in the light-evoked response amplitude (Pan et al., 1989). Consistent with this idea, the onset and extent of 5-HT_{1A}R I_{light} amplitude reduction from acute escitalopram (1 µM) was observed to follow a similar timecourse as the onset and extent of a slowly developing WAY100635 (1 µM)-sensitive sustained outward current (data not shown). Whereas this is a plausible mechanism *in vivo* (since 5-HT neurons are intrinsically active and chronic treatment has been shown to cause ~6 fold increases in the DRN resting 5-HT levels (Rutter et al., 1994)), it is currently unclear whether there was elevated basal 5-HT in our quiescent slice preparation. Therefore we cannot confirm that such 'occlusion' is responsible for the reduced amplitude of 5-HT_{1A}R I_{light} observed from acute, subchronic and chronic fluoxetine exposure in the current work. Future electrophysiological studies may be able to resolve this by comparing the effects of bath-applied WAY100635 on the baseline holding current of 5-HT cells from slices derived from control and fluoxetine treated mice.

Possible Future Antidepressant Treatments

In the present work we have found no evidence to support the theory that prolonged SSRI administration leads to 5-HT_{1A} somatodendritic autoreceptor desensitization. If SSRIs such as fluoxetine do not alter 5-HT_{1A} autoreceptor signaling, then pharmacologically targeting this receptor to limit its initial inhibitory influence over 5-HT neurotransmission with the goal of reducing the onset latency and/or enhancing the therapeutic efficacy would not be advantageous. In line with this idea, although early clinical trials for drugs such as pindolol reported positive

results (Zanardi et al., 1997), the findings have not been consistently replicated (Berman et al., 1999). It was generally concluded that if this strategy provides benefits, they are modest at best. Although there are a few groups who continue on this path, many researchers have turned to new avenues of exploration. The findings of the current work may help with finalizing this transition.

One promising new class of antidepressant agents stemmed from the observation that the anesthetic and 'club drug' ketamine exerted pronounced antidepressant effects when given at subanesthetic doses. Clinical trials confirmed these rumors, with patients (even some that were previously treatment-resistant) exhibiting rapid relief of depressive symptoms that lasted for up to seven days with a single dose (Zarate et al., 2006). Whereas the risk of psychotomimetic effects and potential for abuse makes ketamine far from an ideal solution, investigation of its mechanism of action has been beneficial. Notably, it has fueled the idea that targeting the neurotransmitter glutamate may be a potentially useful strategy for combating depression. This rationale has yielded a number of promising candidates, including Rapastinel (Naurex Inc.), NRX-1074 (Naurex Inc.), Esketamine (Johnson & Johnson) and CERC-301 (Cerecor Inc.). The antidepressant properties of these new agents are generally thought to result from their positive effects on neurotrophic factors and intracellular signaling pathways promoting synaptogenesis (reviewed in Duric and Duman, 2013). Whereas it is possible that ketamine and ketamine-like compounds exert these effects indirectly through the 5-HT system (as suggested by their reported dependence on 5-HT signaling; Gigliucci et al., 2013), there is support for the idea that 'classic' antidepressants may also have a previously overlooked impact on promoting neuronal plasticity (reviewed in Duman et al., 1997). With their fast onset, more tolerable side effect profile and potential for treating previously treatment-refractory cases, these newer ketamine-like drugs may be available in the future as monotherapy or as adjunctive therapy for traditional antidepressants to enhance therapeutic efficacy and provide quicker symptom relief.

Concluding Remarks

In summary, the application of optogenetics to the study of autoinhibition provided unparalleled insight into the effects of chronic SSRI treatment on the 5-HT_{1A} autoinhibitory signal. In contrast to the prominent autoreceptor desensitization theory, we found that prolonged 5-HT reuptake blockade resulted in an enhancement of the autoreceptor response. Therefore, our data suggest that instead of continuing the search for drugs targeting the 5-HT_{1A} autoreceptor in an attempt to decrease the onset latency or enhance the efficacy of antidepressant medications, rational drug design may be more successful by exploring alternative avenues for potential therapeutics.

MATERIALS AND METHODS

Animal Use

All animal care and experimental procedures were approved by the Wayne State University animal investigation committee and are in accordance with the NIH's Office of Laboratory Animal Welfare (OLAW) *Public Health Service Policy on Humane Care and Use of Laboratory Animals.* The Wayne State University Institutional Animal Care and Use Committee (IACUC) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The laboratory animal care and use program conforms to the National Research Council's *Guide for the Care and Use of Laboratory Animals*, 8th Edition.

Experiments were performed on 3 genetically modified mice models. The first was B6;SJL-Tg(Tph2-COP4*H134R/EYFP)5Gfng/J ("TpH2-ChR" mice) (Jackson Laboratories stock number 014555) (Zhao et al., 2011). The second, "SERT-Cre X Ai32" mice, were created by crossing a BAC transgenic mouse expressing Cre recombinase under the control of the serotonin transporter (SERT) promoter (Tg(Slc6a4-cre)ET33Gsat/Mmucd) (MMRRC stock number 017260-UCD) (Gong et al., 2007) with a conditional knock-in mouse (B6;129S-*Gt(ROSA)26Sor*$^{tm32.1(CAG-COP4*H134R/EYFP)Hze}$) (Jackson Laboratory stock number 012569) (Madisen et al., 2012) containing a *loxP*-STOP-*loxP*-ChR(H134R)-EYFP expression cassette downstream of the *CAG* promoter inserted into the *Rosa26* locus. Similarly, B6.Cg-Tg(Fev-cre)1Esd/J mice (Jackson Laboratory stock number 012712) (Scott et al., 2005) were crossed with the Ai32 reporter mice to produce "Pet1-Cre X Ai32" mice. For consistency purposes, only offspring that were heterozygous for the *CAG-COP4*H134R/EYFP* allele were utilized in the study. Mice were housed in the approved animal facility, which was maintained on a 12 hour light-dark cycle. Each cage was enriched with a hut and paper shavings. Food and water were available *ad libitum.*

Treatment

Mice were given 10 mg/kg/day (±)-Fluoxetine HCl (RTI International) or a 5% sterile dextrose solution for two weeks ('chronic' treatment) beginning at ~p35 (postnatal day 35).

Alternatively, mice used for 'acute' experiments began treatment at ~p42. Treatment was administered using an osmotic minipump (ALZET®, model 1002), which was prepared and implanted subcutaneously according to the procedure outlined by the ALZET® company. During pump preparation, expected weight gain was accounted for (1.5 g added to the starting weight of males and 0.75 g to that of females) to ensure the dose remained within the target range (between ~11-9 mg/kg/day) throughout the two weeks. Carprofen (s.c, 5 mg/kg, dissolved in sterile 1x PBS) was given prior to the procedure and again 24 hours later. During surgery, anesthesia was induced and maintained using isofluorane (1-3% via inhalation). Following surgery, mice were housed individually under pre-surgical conditions.

Testing Blood Levels of Fluoxetine/Norfluoxetine

At the time of sacrifice, trunk blood was collected in a blood collection tube (BD Vacutainer® Serum Tube) and shipped overnight to AIT Laboratories (2265 Executive Drive, Indianapolis, IN 48462). To obtain the minimum 1mL required for testing, blood from two mice was typically pooled. AIT laboratories performed UHPLC-MS/MS on the whole-blood sample (test 4150). Of note, pumps were checked at the time of sacrifice to ensure their contents were empty.

Slice preparation

Mice were sacrificed in accordance with the recommendations of the AVMA guidelines on euthanasia using isofluorane followed by decapitation. Slices were prepared essentially as described previously (McGregor et al., 2015). Briefly, the brain was removed and submerged in ice-cold Ringer's solution containing (in mM): 119 ChCl, 2.5 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1 NaH_2PO_4, 10 HEPES, and 22 glucose, bubbled to saturation with 95% O_2/5% CO_2. The posterior half of the brain was mounted onto a stage by application of cyanoacrylate glue and stabilized ventrally by a 2% agarose block. Coronal slices (300 μM thick) of the brainstem were prepared using a vibratome (Vibratome® Series 1000 Sectioning System). Slices were then transferred to a recovery chamber filled with pre-warmed (33°C) Ringer's solution (containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 10 HEPES, and 22 glucose bubbled to saturation

with 95% O_2/5% CO_2) for at least one hour to allow for recovery. This solution was identical to that used for recordings (see below), both of which were supplemented with L-tryptophan (30 μM, Alfa Aesar®) to help preserve 5-HT synthesis and stores in the slice (Evans et al., 2008).

Electrophysiological Recordings

Slices were transferred to a recording chamber on the stage of an upright microscope (BX50WI, Olympus), where they were continuously perfused with Ringer's solution maintained at ~31°C and bubbled to saturation with 95% O_2/5% CO_2. Serotonin neurons of the ventromedial region of the DRN (vmDRN) were initially targeted by their location using differential interference contrast (DIC) imaging. The identity of a patched cell was confirmed by the presence of Channelrhodopsin (ChR)-mediated currents (see below).

Whole-cell recordings were conducted using an EPC10 amplifier (HEKA Instruments) under the control of Patchmaster software (HEKA Instruments). Current and voltage were lowpass filtered at 1-5 kHz and sampled at 2-10 kHz. Recording pipettes were pulled from borosilicate glass (Sutter Instruments) using a horizontal Flaming/Brown micropipette puller (Sutter Instruments, model P-97). The pipettes were filled with a potassium-based internal solution (composition in mM: 120 $KMeSO_4$, 5 KCl, 5 NaCl, 0.02 EGTA, 10 HEPES, 1 $MgCl_2$, 10 myo-inositol, 10 Na_2 phosphocreatine, 4 ATP Mg Salt, 0.3 GTP Na Salt, pH 7.4) and exhibited resistances ranging from 3-4.5 MΩ. Series resistance after breaking into the cell ranged from 5-20 MΩ. Only cells with a resting membrane potential more hyperpolarized than -60 mV (without liquid junction potential (LJP) correction) were included. During recordings, any cells that exhibited unstable baselines or access resistances (R_{access}) that deviated significantly from baseline were discarded.

Characterization of 5-HT Neuron Properties

The resting membrane potential (mV) was determined soon after breaking into a cell, once the voltage had become steady. Series resistance (MΩ) was assessed by injecting a 70 ms-long current pulse (-200 pA) and was subsequently compensated. During voltage clamp recordings,

cells were held at -60 mV and the R_{access} (MΩ, taken as the current deviation from baseline in response to a brief (100 ms) hyperpolarizing pulse (-90 mV)) was continuously monitored.

Light Stimulation and Characterization of the Response Parameters

ChR was excited with brief (5 ms) flashes of blue light (455-490 nm). A high speed shutter (Model T132, UniBlitz®) under experimenter control via Patchmaster was utilized for this purpose. A mercury lamp (USH-102DH, USHIO) served as the light source. To ensure the output was stable, the light power at the microscope objective was checked approximately every 20 hours. The light was delivered through the 40X water-immersion objective to the entire visual field centered on a recorded cell. Full field intensity measured at the 40x microscope objective was ~5 milliWatts (mW).

The first stimulation of each patched cell (3 flashes given 50 ms apart) was used to report the peak amplitude of the ChR-mediated current (pA) and to characterize the 5-HT_{1A}R I_{light} responses, which were based on four parameters. The amplitude of the 5-HT_{1A}R-mediated current (pA) was taken as the mean around the point at which the signal deviated maximally from baseline (mean of the 2 sec segment immediately preceding the light flashes). The rise time (ms) was the time from when the ChR-mediated current crossed baseline until the peak autoreceptor-mediated current amplitude. The half-decay (ms) was the time it took for the autoreceptor-mediated current to return to half of its maximal value (using the same 'peak' location used for rise time). The total charge transfer (pAxs) was the integral, or area under the curve, of the entire current response. Notably, when the amplitude was approximately 5 pA or less it was not possible to accurately determine the rise time or half-decay. In those cases the rise time and half-decay were not included in the analysis. Of note, gender had no apparent effect on the 5-HT_{1A}R I_{light} amplitude, rise time, half-decay, or charge transfer in either control mice ($p = 0.87$, $p = 0.19$, $p = 0.29$, $p = 0.72$, respectively; student's t-test) or chronic fluoxetine treated mice ($p = 0.20$, $p = 0.26$, $p = 0.87$, $p = 0.42$, respectively; student's t-test).

For experiments examining the effect of acute fluoxetine (Fig. 7), fluoxetine (1 µM, LY110140, Eli Lilly Company) was bath-applied for a minimum of 20 minutes before data were obtained. This was done to ensure that the drug had fully penetrated the slice. A concentration of 1 µM should saturate SERT (Ki ~1-2 nM) (Owens et al., 2001; Cheetham et al., 1993) while avoiding off-target effects on receptors present in the brainstem slices, such as the 5-HT_{1A}R (Ki ~8,313 nM) (Owens et al., 1997).

5-HT Dose Response Curves

In voltage clamp, BHI was sampled for ~2 sec every 6 or 10 sec. 5-HT (serotonin creatinine sulfate monohydrate, Sigma® Life Science) was bath-applied at increasing increments of approximately half-log units. This was done in the presence of fluoxetine (1 µM). The results were fit to the Hill equation ($y=V_{max}((x^n)/(k^n + x^n))$ using Origin® (OriginLab Corporation) and the maximum current response (I_{max}) and the 5-HT concentration needed to produce a half-maximal response (EC_{50}) were analyzed. Any cell whose BHI or R_{access} deviated more than ~30% of the maximum current response was discarded. To facilitate data collection, Pet1-Cre X Ai32 mice were also utilized for these experiments.

Immunohistochemistry

Immunohistochemistry (Fig. 1) was performed on a SERT-Cre X Ai3 mouse. The "Ai3" mouse (B6.Cg-*Gt(ROSA)26Sor* $^{tm3(CAG-EYFP)Hze}$/J) (Jackson Laboratory stock number 007903) (Madisen et al., 2010) contains the CAG-*loxP*-STOP-*loxP*-EYFP-WPRE-BGHpA expression cassette inserted into the *Rosa2*6 locus. The mouse was perfused with 4% paraformaldehyde and 50 micron slices were made with a vibratome (Vibratome® Series 1000 Sectioning System). Slices were washed in PBST (PBS with Triton X) with agitation for 30 minutes and incubated overnight in PBST. They were left in (horse) blocking solution for 1 hour, followed by incubation with polyclonal Goat Anti-SERT (ST(C-20): sc-1458 Santa Cruz Biotechnology), polyclonal Chicken Anti-GFP (catalogue No. GFP-1020, Aves) or monoclonal Mouse Anti-Tryptophan hydroxylase (Clone WH-3 product No. T0678, Sigma®) overnight at 4°C. Slices were rinsed twice

in PBS, twice in PBST (30 minutes per wash), and incubated overnight with Alexa Fluor® 568-conjugated Donkey Anti-Goat (Cat No. A11057 Invitrogen), DyLight™488-conjugated Goat Anti-Chicken (Jackson ImmunoResearch, #102-485-155), or Cy™3-conjugated Goat Anti-Mouse (Jackson ImmunoResearch, #115-165-146) antibodies, respectively. The slices were then rinsed twice in PBS, twice with PBST (30 minutes per wash), and left overnight in ABC solution at 4°C, then washed twice in PBS and twice in PBST (30 minutes per wash). Fluorescence was visualized using an Olympus BX50WI confocal microscope.

Data Analysis and Statistics

In order to preserve biological variability of 5-HT neurons (reviewed in Andrade and Haj-Dahmane, 2013) and thus the physiological relevance of the data, each cell was utilized as a datum point and all cells, even outliers, were included in our dataset. Although qualitative comparison of the 'within-mouse' variance to the 'between-mice' variability within a group suggested mouse-to-mouse variability was not a particularly influential factor, we nevertheless excluded data from mice where <3 data cells were obtained. The average number of cells sampled per SERT-Cre X Ai32 mouse for 5-HT_{1A} R I_{light} analysis was ~4.3, 6, and 6.1 for the no treatment, sham treatment, and chronic fluoxetine treatment groups, respectively.

Results are presented as mean ± SEM unless otherwise noted. Significance was assessed using a two-tailed student's unpaired t-test, one-way ANOVA, or moderated regression analysis, as appropriate. Tukey was used for post-hoc analysis. A value of $p < 0.05$ was set to indicate statistical significance. Both SPSS® (IBM®) and Origin® (8.5, OriginLab Corporation) were used for statistical analysis. IgorPro (4.0.6.1, WaveMetrics, Inc) and CorelDRAW®12 (Corel Corporation) were used to prepare figures.

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ABSTRACT

USING A NOVEL OPTOGENETIC APPROACH TO DIRECTLY ASSESS 5-HT$_{1A}$ SOMATODENDRITIC AUTORECEPTOR FUNCTION IN RESPONSE TO CHRONIC SELECTIVE SEROTONIN REUPTAKE INHIBITOR TREATMENT

by

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Antidepressant drugs are widely used but their mechanism of action remains only partially understood. One leading hypothesis holds that a key effect of chronic treatment with a Selective Serotonin Reuptake Inhibitor (SSRI) is loss of somatodendritic 5-HT$_{1A}$ receptor-mediated autoinhibition in serotonergic neurons of the dorsal raphe nucleus (DRN). However, technical limitations have prevented direct testing of this hypothesis. In the current study we took advantage of optogenetic strategies to assess the effects of the classic SSRI fluoxetine on 5-HT$_{1A}$ receptor-mediated autoinhibition. We conducted these experiments in mice expressing the light-sensitive ion channel Channelrhodopsin (ChR) in 5-HT neurons to facilitate their unambiguous identification and achieve precise temporal control over endogenous 5-HT release and 5-HT$_{1A}$ autoreceptor activation. Whole-cell intracellular recordings of DRN 5-HT neurons in *in vitro* brainstem slices revealed that light-induced 5-HT$_{1A}$ autoreceptor-mediated currents in chronically treated mice (14 days) were smaller in amplitude but longer in duration, thereby resulting in an overall greater charge transfer compared to controls. Consistent with this, 5-HT dose response curves constructed in the presence of bath fluoxetine also provided no evidence for a reduction in autoreceptor sensitivity. To test whether an attenuation of 5-HT clearance could potentially account for the alterations observed from chronic treatment, responses were compared to those obtained following acute or subchronic fluoxetine treatment (bath application or 3 days). In both

conditions the 5-HT_{1A}R I_{light} responses resembled those of chronic treatment and differed substantially from controls, suggesting that reduced 5-HT reuptake was likely to be a contributing factor. Collectively, instead of autoreceptor desensitization, our results suggest that the 5-HT_{1A} autoreceptor-mediated signal is actually preserved after chronic SSRI treatment.

AUTOBIOGRAPHICAL STATEMENT

Kelly Marie McGregor was born in Louisville, Kentucky and grew up in Saline, Michigan. She did her undergraduate work at Michigan State University and received a Bachelor's of Science in Psychology. Kelly attended graduate school at Wayne State University and received her PhD in Pharmacology with a concentration in Molecular Neuropharmacology. She currently resides in Clinton Township, Michigan.