DYNAMIC REGULATION OF SYNAPTIC TRANSMISSION ONTO SEROTONIN NEURONS BY ANTIDEPRESSANTS

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ABSTRACT

Antidepressants are generally believed to exert their clinical efficacy by enhancing 5-HT transmission. However, they do so with a delay of several weeks that broadly matches the delay of therapeutic benefits in humans. For example, sustained administration of selective serotonin (5-HT) reuptake inhibitors (SSRIs) strongly suppresses in the first few days the firing activity of 5-HT neurons in the dorsal raphe nucleus (DRN), thereby severely hampering the increase of 5-HT in target regions. Remarkably, the firing activity of 5-HT neurons gradually recovers over the time course of treatment and this recovery is believed to be accounted for by the desensitization of 5-HT_{1A} somatodendritic autoreceptors. Here, we sought to investigate whether additional mechanisms might contribute to the dynamic regulation of excitability of 5-HT neurons during the course of SSRI treatments. Borrowing from the well-described homeostatic strengthening of glutamatergic synapses onto cortical pyramidal neurons following prolonged periods of inactivity, we hypothesized that a similar homeostatic-like regulation of synaptic strength might be operant on 5-HT cells during an SSRI treatment. To test this possibility, we used whole-cell electrophysiological recordings on acute midbrain slices to monitor glutamatergic synapses onto 5-HT neurons. We found that a two-day treatment with the SSRI citalopram induced a robust reduction in both the amplitude and frequency of AMPAR-mediated mEPSCs. We also show that this depression in synaptic strength, induced by an SSRI, is transient since excitatory drive onto 5-HT neurons was enhanced by 7 days of treatments. Altogether, these results document a dynamic regulation of glutamatergic synaptic transmission during the time course of a prolonged treatment with an SSRI. Further elucidation of the cellular and molecular mechanisms driving this synaptic plasticity might identify novel pharmacological target to shorten the delay of antidepressant action.

RÉSUMÉ

Il est généralement admis que l'augmentation de la transmission des neurones secrétant la sérotonine (5-HT) par les antidépresseurs sous-tend leur efficacité clinique dans le traitement de la dépression majeure. Des études fondamentales principalement chez le rat ont démontrées que cette augmentation de la transmission sérotoninergique induite par les antidépresseurs se développe avec un délai de plusieurs semaines, délai qui concorde avec le délai thérapeutique chez les humains. Ainsi, il a été démontré qu'une administration soutenue d'un inhibiteur sélectif de la recapture de la sérotonine (ISRS) supprime fortement le taux moyen de décharge des neurones 5-HT dans le raphé dorsal (RD) au début du traitement. Par conséquent, l'augmentation des niveaux de 5-HT dans les régions cibles de l'innervation sérotoninergique se trouve sévèrement entravée. Remarquablement, le taux de décharge des neurones 5-HT retourne graduellement jusqu'au niveau normal au cours d'un traitement prolongé, menant éventuellement à une nette augmentation de la transmission sérotoninergique par les ISRS. Ce rétablissement du taux de décharge est généralement associé à une désensibilisation des autorécepteurs inhibiteur 5-HT_{1A} somatodendritiques. Ici, nous cherchions à déterminer si d'autres mécanismes neuronaux contribueraient à la régulation dynamique de l'excitabilité des neurones 5-HT durant un traitement antidépresseur. En se basant sur le phénomène de renforcement homéostatique des synapses glutamatergiques décrites dans des neurones corticaux suite à une période d'inactivité prolongée, nous avons supposé qu'une régulation homéostatique similaire pourrait être opérante sur les cellules 5-HT durant un traitement d'ISRS. Pour tester cette possibilité, nous avons utilisé des enregistrements électrophysiologiques en mode cellule-entière sur des tranches aigües du mésencéphale pour étudier les synapses glutamatergiques sur les neurones 5-HT. Nous avons observé qu'un traitement de deux jours avec l'ISRS citalopram induit une réduction robuste de l'amplitude et de la fréquence des courants excitateurs postsynaptiques miniatures induits

par les récepteurs AMPA. Nous avons également démontré que cette diminution de la force synaptique, induit par un ISRS, est transitoire puisque la force excitatrice sur les neurones 5-HT est revenu au niveau normal lorsque déterminée à sept jours de traitements. En tout, ces résultats documentent une remarquable régulation dynamique de la transmission synaptique glutamatergique sur les neurones 5-HT au cours d'un traitement prolongé avec un ISRS. La clarification des mécanismes cellulaires et moléculaires qui promeuvent cette plasticité synaptique pourrait mener à l'identification de nouvelles cibles pharmacologiques pour raccourcir le délai de l'activité antidépressive.

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Ach	Acetylcholine
ACSF	artificial cerebrospinal fluid
ADP	after-depolarizing potential
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
AP-V	(2*R*)-amino-5-phosphonopentanoate
cAMP	cyclic adenosine monophosphate
Ca^{2+}	Calcium Ion
Cl^{-}	Chloride Ion
CNS	Central nervous system
5-CT	5-carboxyaminotryptamine
DRN	Dorsal Raphe Nucleus
EAA	Excitatory amino acid
eCB	Endocannabinoid
GABA	γ-aminobutyric acid
GPCR	G-protein-coupled receptors
5-HT	5-Hydroxytryptamine, Serotonin
K^{+}	Potassium Ion
KAR	Kainate Receptor
LH	Lateral Habenula
LTD	Long-term Depression
LTP	Long-term Potentiation
MFB	Medial Forebrain Bundle

mPFC	medial prefrontal cortex
MRN	Median Raphe Nucleus
NASPM	1-naphthyl acetyl spermine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NE	Norepinephrine
NMDAR	N-methyl-D-aspartate receptor
6-OHDA	6-hydroxydopamine
QX-314	Lidocaine N-ethylbromide
S.C.	Subcutaneous
SERT	Serotonin Transporter
SSRI	Selective Serotonin Reuptake Inhibitor
TPH	Tryptophan Hydroxylase
TTX	Tetrodotoxin

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INTRODUCTION

1. THE SEROTONINERGIC SYSTEM

In 1964, using a process known as the Falk-Hillarp method (Dahlstroem & Fuxe 1964), Dahlström and Fuxe provided the first detailed anatomical map of the serotoninergic system in a mammalian brain. The later development of immunohistochemical techniques permitted a more precise visualization and rigorous mapping of the serotoninergic system. Most notably, the use of specific antibodies selective for serotonin (5-HT) (Steinbusch et al 1981), tryptophan hydroxylase (TPH), the serotonin transporter (SERT) (Ovalle et al 1995, Sur et al 1996) and serotonin receptors (*e.g.* 5-HT_{1A}) have greatly contributed to defining the extent of the brain projections of this monoaminergic system of the brainstem. Together, these experimental techniques have shown the 5-HT system pertains to a dense population of 5-HT neurons located in the center and most medial portions of the brainstem known as the raphe nuclei. With this detailed map of the serotoninergic system, a classification system was introduced to divide 5-HT containing cells of the raphe nuclei into nine subdivisions (B1 to B9) (see Table 1; (Dahlstroem & Fuxe 1964, Tork 1990). This "coding" system is organized from caudal to rostral, where B1 corresponds to the most caudal 5-HT cell group (Tork 1990).

1.1. Dorsal Raphe Nucleus

The dorsal raphe nucleus (DRN) corresponds largely to subdivision B7 while extending caudally into the B6 subdivision (Dahlstroem & Fuxe 1964). The DRN in mammals is located in the ventral part of the periaqueductal gray matter of the midbrain with a caudal projection which extends into the periventricular gray matter of the rostral

pons (Tork 1990). When cross-sectioned, the DRN maintains a distinct bilateral symmetry and demonstrates a highly characteristic "fountain-like" shape conserved across many species (Hornung & Fritschy 1988, Takahashi et al 1986, Tillet 1987, Tork 1990). Furthermore, studies have highlighted various sub-regions located within the DRN including medial, lateral and caudal divisions (Tork 1985, Baker et al 1990). The medial portion is further divided into ventral and dorsal sub-sections whereas the lateral component of the DRN is not further divided in the rodent brain, as it is not as prominent compared to other species (such as in the human brain).

The DRN is the largest of all raphe nuclei and contains over 50% of all serotoninergic cell bodies in the mammalian brain (Azmitia & Segal 1978, Descarries et al 1990, Descarries et al 1982, Jacobs & Azmitia 1992, Molliver 1987, Wiklund et al 1981). Admittedly, amongst the 5-HT expressing neurons in the DRN, there are also non-5-HT expressing neurons including glutamate (Kaneko et al 1990), GABA (Belin et al 1979) and dopamine (Nagatsu et al 1979), among other neurotransmitters and modulators (Jacobs & Azmitia 1992). However, in comparison to the other raphe nuclei subdivisions, the DRN contains the highest ratio of 5-HT neurons to non-5-HT neurons (Molliver 1987). It is noteworthy that over two-thirds of all 5-HT afferents in the brain originate specifically from 5-HT neurons contained within the DRN. Thus, the DRN provides more afferents compared to the combination of all the other raphe nuclei subdivisions. Together, these features have made the DRN a compelling area of the brain to study the function of 5-HT neurons in the mammalian brain.

1.2. Efferent Projections of the DRN

The raphe nuclei provide sparse and expansive innervations to diverse brain regions

in the mammalian brain. The fact that the raphe nuclei innervate many diverse brain regions is not to say that this extensive monoaminergic system is non-specific. In fact, there are specific brain regions that receive highly conserved 5-HT innervations whereas abutting regions receive limited innervations from distinct raphe nuclei. The innervation pathways arising from the raphe nuclei are divided into ascending and descending projections. However, the majority of efferent projections from the DRN are ascending projections with very limited projections through a descending pathway towards other brainstem regions and the spinal cord.

1.2.1. Ascending Efferent Projections of the DRN

The ascending projections of the raphe constitute the majority of the main efference pathway of the DRN. These projections traverse through the medial forebrain bundle (MFB) and provide many diverse axons and collaterals that follow different fiber pathways to their specific areas of innervation. Anterograde labeling of 5-HT neurons in the raphe nuclei, using Phaseolus vulgaris Lectin, has indicated that there are two types of 5-HT axons that are of different origins within the raphe and both target diverse brain regions (Kosofsky & Molliver 1987).

First, fine varicose axons with a diameter of less than 1 μm arise from the DRN (Kosofsky & Molliver 1987). These fine varicose axons appear to be the most abundant and effusive of 5-HT neuronal projections to the forebrain. In contrast, the second type of origin-specific axons discovered arise from MRN. These axons appear “beaded” due to the fact that they have large round and oval varicosities arising from relatively thin axons. Interestingly, immunocytochemical methods have revealed that, when stained, the varicosities of MRN axons stain densely for 5-HT whereas the axons react rather weakly.

Further investigation has indicated that these two types of axons not only originate from distinct raphe nuclei, but follow separate axonal tracts to various target brain regions (Mulligan & Tork 1988).

Following the development of numerous novel fluorescent tracers in the 1980s, many of the DRN-specific target regions were identified. These studies were performed using retrograde-tracers that were injected into terminal fields and then, by endocytosis, incorporated into the axons and transported to serotoninergic cell bodies in the DRN. It became evident that the DRN densely innervates, amongst other regions, the prefrontal cortex (Puig et al 2005), nucleus accumbens (Bjorklund et al 1971, Descarries et al 1975, Li et al 1989), amygdala (Li et al 1993), paraventricular nucleus of the hypothalamus (PVN) (Petrov et al 1992) and the lateral parabrachial nucleus (LPB) (Petrov et al 1992). For the purpose this thesis, I have focused on the projections and details of the DRN. At these various target regions, 5-HT neuronal projections exert effector responses onto target neurons by activating a wide range of serotonin receptors within the CNS.

2. SEROTONIN RECEPTORS AND TRANSPORTER

In the 1980s, radioligand binding studies rapidly began to identify numerous 5-HT receptor subtypes within the CNS. Over the following two decades, seven 5-HT receptor families containing 14 receptor subtypes had become recognized. 5-HT receptors in the CNS have been associated with modulatory roles in neuronal excitability, neurotransmitter release, behaviour, as well as psychiatric disorders. The 5-HT receptors have been extensively characterized (Barnes & Sharp 1999); however, for the purpose of this thesis we will focus solely on the $5HT1_A$ somatodendritic receptor, as it specifically pertains to my research paradigm. The remaining 5-HT receptors have been summarized

in a chart with regards to their receptor classification, G-protein coupling (with the exception of the 5-HT_3), second messenger responses and location at central synapses (Table 2; but see:(Barnes & Sharp 1999).

2.1. 5-$HT1_A$ Somatodendritic Autoreceptors

The 5-HT_{1A}-receptor was the first of the many 5-HT receptor subtypes to be sequenced and cloned (Albert et al 1990, Fargin et al 1988). Like all 5-HT receptors (with the exception of 5-HT_3 receptors), the 5-HT_{1A} receptor is a G-protein coupled receptor (GPCR) with 7 transmembrane domains. Its distribution within the CNS has been extensively studied through receptor autoradiography using selective $5HT_{1A}$ receptor ligands such as [^{3}H]-5-HT, [^{3}H]-8-OH-DPAT, [^{3}H]-ipsapirone, [^{125}I]-BH-8-MeO-N-PAT and [^{3}H]-WAY 100635 (Barnes & Sharp 1999, Hoyer et al 1986, Pazos & Palacios 1985, Verge et al 1986, Weissmann-Nanopoulos et al 1985). The receptor is particularly localized in forebrain regions such as the cingulate cortex, entorhinal cortex, and hippocampus, as well as densely populating the DRN (Barnes & Sharp 1999). Indeed, $5HT_{1A}$ binding studies and immunocytochemistry have indicated that, in the forebrain regions the $5HT_{1A}$ receptors are located postsynaptically on pyramidal neurons whereas in the DRN they exist as autoreceptors located somatodendritically on 5-HT neurons (Miquel et al 1992, Radja et al 1991). Interestingly, the presence of the densely populated 5-HT_{1A} receptors in the dorsal medial portion of the DRN can act as an identifiable characteristic (both immunochemically and electrophysiologically) of 5-HT neurons (Calizo et al 2011, Francis et al 1992, Weissmann-Nanopoulos et al 1985).

Functionally, the $5HT_{1A}$ receptor is linked to a G_i-protein and when activated, independent of diffusible second messengers, opens a G-protein inwardly rectifying

potassium (GIRK) channel (Nicoll et al 1990). Electrophysiologically, due to GIRK channel activation, activation of the $5HT_{1A}$ receptor *in vitro* results in neuronal hyperpolarization in all brain regions expressing this receptor of interest (Andrade & Nicoll 1987, Araneda & Andrade 1991).

2.2. Serotonin Transporter

The serotonin transporter (SERT) was first cloned in rats in 1991 (Blakely et al 1991, Hoffman et al 1991). The SERT has 12 membrane-spanning domains and a molecular mass of approximately 71 kDa. The SERT is Na^+/Cl^- dependent and utilizes their electrochemical gradient in order to transport serotonin back into 5-HT neurons (Yamashita et al 2005). These transporters play an active role in regulating the levels of extracellular serotonin released from 5-HT neurons. The SERT also plays a role in recycling released serotonin to be re-packaged into vesicles for future release from the presynaptic terminal. Similar to determining the distribution of $5\text{-}HT_{1A}$ receptors in the CNS, radioligand studies have revealed specific distributions of the SERT within the CNS (Hrdina et al 1990, Kovachich et al 1988). Although the SERT has been observed in locations such as the substantia nigra and amygdala nuclei, they are known to be densely located in the raphe nuclei.

3. PROPERTIES OF 5-HT NEURONS OF THE DRN

Following the identification of distinct aggregations of 5-HT neurons within the DRN, there quickly became a need for cellular and biophysical characterization of 5-HT neurons. Subsequent studies emerged characterizing the electrophysiological, pharmacological and morphological properties of these neurons in order to accurately

identify 5-HT neurons. Indeed, recent studies have indicated heterogeneity within 5-HT neurons in the raphe nucleus (Calizo et al 2011, Gartside et al 2000, Kocsis et al 2006). Even though 5-HT neurons of the raphe nuclei are not collectively homogenous, neurons restricted to specific raphe nuclei and subdivisions of the respective nuclei are in fact quite uniform with respect to their electrophysiological and morphological properties (Calizo et al 2011). The focus of this section will be on 5-HT neurons in the dorsal medial subdivision of the DRN (dmDRN) as they pertain to the neurons recorded for and presented within this thesis.

3.1. Electrophysiological characteristics

In vivo extracellular and intracellular electrophysiological recordings performed in combination with selective lesioning studies, histoflourescence and antidromic stimulation were the first to begin describing the properties of 5-HT neurons in the DRN (Aghajanian & Haigler 1974, Aghajanian et al 1978, Wang & Aghajanian 1977a). Extracellular recordings revealed that 5-HT neurons have a firing rate of 1-3 Hz, a biphasic or triphasic action potential with a duration between 1.8 - 3 milliseconds, and selective responsiveness to $5HT_{1A}$ receptor agonists (such as LSD) resulting in a hyperpolarizing effect (Aghajanian 1982, Aghajanian & Lakoski 1984, Aghajanian & Vandermaelen 1982, Penington et al 1992, Vandermaelen & Aghajanian 1983). In fact, whole-cell current clamp recordings have revealed that serotoninergic neurons in the dmDRN (such as the neurons recorded from for this thesis) are almost exclusively responsive to $5HT_{1A}$ receptor agonists compared to non-serotonin cells located within this region (Calizo et al 2011). Additionally, the recorded 5-HT neurons in the dmDRN were observed to have low resting membrane potentials of approximately -75 mV and a large

~20 mV slow after hyperpolarization (AHP) lasting 200-1000 milliseconds (Calizo et al 2011). An apparent difference in recording from 5-HT cells *in vitro*, compared to *in vivo* recordings, is that there are fewer spontaneously active 5-HT neurons (Aghajanian & Lakoski 1984), likely due to severing the afferent projections to the DRN that have been shown to have modulatory roles in the firing of 5-HT neurons.

Immunohistochemistry experiments combined with whole-cell recordings from midbrain slices containing the DRN has provided further insight into the properties of 5-HT neurons of the dmDRN. For example, 5-HT neurons from the dmDRN display high input resistances of approximately 600 – 800 MΩ (Calizo et al 2011). This is unusually high when compared to a hippocampal pyramidal neurons at this age, which is approximately 100 MΩ. One possible explanation for this high input resistance could be due to lower expression levels of leak-potassium channels of the TASK1 and TASK3 subclasses (Washburn et al 2002).

3.2. Morphology

The basic morphology of 5-HT neurons in the DRN was originally established using the Golgi method (Diaz-Cintra et al 1981). Recent intracellular biocytin labelling combined with confocal microscopy has provided excellent insight into the morphological characteristics of 5-HT neurons in the DRN (Calizo et al 2011, Li et al 2001). The cell bodies in the raphe nuclei vary in size and shape. It has been documented that in comparison to other raphe nuclei, the DRN contains medium size neurons (~ 1500 μM^2; diameter 15-30 μm) that range in shapes from round, fusiform and oval (Calizo et al 2011, Hale & Lowry 2011, Li et al 2001). Furthermore, it has been observed that 5-HT

neurons of the DRN have few dendritic projections (2-3) and spare dendritic spines that actually seem to become more densely populated on distal portions of dendrites.

4. AFFERENT PROJECTIONS OF SEROTONIN NEURONS IN THE DRN

The serotoninergic system is evidently a very expansive and anatomically well-studied network. In conjunction with having many defined efferent projections, the DRN also receives a large amount of afferent projections from various neurotransmitter systems including, (but not exclusively) glutamate (Kalen et al 1986, Soiza-Reilly & Commons 2011), GABA (Park 1987, Stern et al 1981, Wang & Aghajanian 1977b) and norepinephrine (NE) (Baraban & Aghajanian 1981).

4.1. Norepinephrine Afferent Projections

The majority of NE afferents to the DRN arise from the locus coeruleus, which is located along the lateral portion of the fourth ventricle. The NE system has been suggested to play a role in tonic modulation of 5-HT neurons in the DRN by activating specific subtypes of norepinephrine receptors located on 5-HT neurons. This is based on *in vivo* recordings from 5-HT neurons in the DRN displaying a reduction in firing activity following microiontophoretic and systemic administration of α_1-norepinerphine receptor antagonists (Baraban & Aghajanian 1980a, Baraban & Aghajanian 1980b, Hertel et al 1997). Researchers reasoned that if the NE system were the main input driving 5-HT firing activity, selective lesioning of NE neurons would decrease the firing activity of 5-HT neurons. In fact, it has been observed that 5-HT neuronal firing activity can be suppressed by the selective lesioning of the norepinephrine neurons using the toxin 6-OHDA (Baraban & Aghajanian 1980a). Although, at first glance, the NE system may appear to be a main drive to 5-HT neurons in the DRN, these 5-HT neurons regain

normal firing activity 4 – 7 days after 6-OHDA injections by an unknown mechanism (Baraban & Aghajanian 1980a). Perhaps this recovery is due to an intrinsic mechanism, however it is important to note that these neurons are receiving various inputs, as previously covered, from various neurotransmitter systems, which could be contributing to the firing activity of these neurons.

4.2. GABAergic Afferent Projections

The lateral habenula (LH) is believed to provide a major inhibitory input to the DRN targeting $GABA_A$ and $GABA_B$ receptor subtypes on 5-HT neurons (Kalen et al 1985, Nishikawa & Scatton 1985, Wang & Aghajanian 1977b). Indeed, electrical stimulation of the LH markedly suppresses 5-HT neuronal firing activity in the DRN (Wang & Aghajanian 1977b). This was subsequently confirmed to be $GABA_A$ receptor mediated, as the suppression of 5-HT neurons via this electrical stimulation was blocked by the systemic or microiontophoretic administration of picrotoxin (a $GABA_A$ selective antagonist). Additionally, intracellular recordings from 5-HT neurons revealed that electrical stimulation of the LH lead to $GABA_A$ mediated synaptic potentials (Park 1987). Although, the LH has been widely considered as the major GABAergic input to the DRN, the ventral tegmental area and substantia nigra have also been recently shown to provide GABAergic input to the DRN (Kirouac et al 2004).

4.3. Glutamatergic Afferent Projections

Identification of glutamatergic terminals, using the vesicular glutamate transporters (VGLUT 1, 2, and 3) as markers, has indicated specific and diverse glutamatergic inputs to the DRN (Amilhon et al 2010, Commons 2009, Crawford et al 2011, Hioki et al 2010). Retrograde tracing from the DRN has revealed numerous glutamatergic afferent inputs to

5-HT neurons and can moreover be specific to the individual subdivisions it is divided into. Amongst the various brain regions from which these afferent projections originate, Phaseolus vulgaris Lectin anterograde tracing has confirmed that the major input to all subdivision of the DRN collectively originates from the medial prefrontal cortex. Utilizing a combination of WGA-apo-HRP-gold retrograde tracing and immunocytochemical detection of glutamate containing neurons has provided a detailed map of glutamatergic inputs to 5-HT neurons of the DRN. Consequently, it has been observed that the majority of glutamatergic afferents to the DRN arise from the lateral tegmental nuclei, LH, arcuate nuclei and dense innervation from the medial prefrontal cortex (Celada et al 2001, Hajos et al 1999, Lee et al 2003). For the purpose of this thesis I have listed the main glutamatergic afferents to the dorsal medial subdivision of the DRN, the subdivision from which my recordings were performed.

5. GLUTAMATE RECEPTORS

Glutamate is the most abundant excitatory neurotransmitter in the brain, activating ligand-gated ionotropic glutamate receptors of the α-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), N-methyl-D-aspartate (NMDAR) and kainate (KAR) subtypes. Through cation permeability, all of these ligand-gated ionotropic receptors act to depolarize neurons in the CNS; however, they play separate and distinct roles in synaptic transmission. In addition, these receptors have been implicated in synapse history as well as neurological and psychiatric disorders including depression.

5.1. AMPA Receptors

AMPARs are ligand-gated ionotropic glutamate receptors; specific subunit composition (GluA1, GluA2, GluA3 and GluA4) of these receptors is dependent on cell type, developmental epoch as well as synapse history (Beique & Huganir 2009, Cull-Candy et al 2006). The GriA1- GriA4 genes encode the GluA1- GluA4 AMPAR subunits respectively. AMPAR subunits can exist as both homo- and heteromers. It is their composition that in turn dictates AMPAR channel biophysical properties. For example, GluA2-containing AMPARs constitute the majority of AMPARs in the CNS and are permeable to sodium and potassium. In comparison, AMPARs of the GluA2-lacking subtype are bestowed with calcium permeability, while still remaining permeable to sodium and potassium (Cull-Candy et al 2006, Traynelis et al 2010). Whole-cell recording reveals that GluA2-containing AMPARs exhibit a characteristic linear current-voltage relationship. In contrast, GluA2-lacking AMPARs exhibit an electrophysiological signature of an inwardly rectifying current-voltage relationship as a result of an intracellular voltage-dependent polyamine block (Bowie & Mayer 1995, Traynelis et al 2010). Apart from being able to identify these GluA2-lacking receptors by examining their current-voltage relationship, these receptors can also be targeted pharmacologically using selective antagonists such as NASPM and philanthotoxin.

5.2. NMDA Receptors

NMDARs are also ionotropic glutamate receptors and are permeable to sodium, calcium and potassium. Functioning as coincidence detectors, NMDARs activation requires a voltage-dependent removal of a magnesium block as well as co-agonists glutamate and glycine (or D-serine) (Mayer et al 1984). NMDARs exist as heterotetrameric structures, consisting of two obligatory GluN1 subunits coupled with

two GluN2 (A-D subtypes) or GluN3 (A-B subtypes) subunits. NMDA receptor subunits are encoded by the genes Grin1 (GluN1), Grin2 A-D (GluN2A-D) and Grin3A-B (GluN3A-B). The expression of the specific GluN2 (A-D) or GluN3 (A or B) subunits dictates the biophysical properties of NMDARs such as decay kinetics and cation permeability, as well as interactions with downstream signalling molecules and pathways (Foster et al 2010, Hardingham & Bading 2002, Hardingham & Bading 2003, Hardingham et al 2002, Lau & Zukin 2007, Martel et al 2009, Pokorska et al 2003, Soriano et al 2008). In addition, NMDARs have been found to play critical roles in brain functions such as synaptic plasticity and brain development, while also being linked to numerous neurological and psychiatric disorders (Cull-Candy et al 2001, Lau & Zukin 2007).

5.3. Kainate Receptors

High-affinity binding studies performed in 1982, by Monaghan and Cotman, were the first to indicate the potential presence of kainate receptors in the CNS. It wasn't long before the first kainate receptor subunit (GluK1) was cloned and isolated in 1990 (Bettler et al 1990). Further biochemical studies and subsequent cloning then confirmed that there are 5 kainate receptor subunits designated as Gluk1-5 and encoded by the GriK1-5 genes respectively (Contractor et al 2011). Kainate receptors form heterotetrameric receptor complexes composed of the required GluK1-3 subunits incorporated with the high-affinity kainate subunits GluK4-5 (Contractor et al 2011). These receptors are permeable to sodium, potassium and calcium. Furthermore, GluK4-5 subunits have been suggested to play a role in the modulation of the receptor's ion permeability and biophysical properties (Contractor et al 2011). Moreover, when comparing AMPAR- and KAR-

mediated currents, KAR currents have a much longer rise time and decay time (Cossart et al 2002, Perrais et al 2010). Presently, the most widely studied evoked-kainate currents are at hippocampal mossy fiber-CA3 synapses and thalamocortical synapses.

5.4. Integrative Aspect

AMPAR- and NMDAR-mediated synaptic currents in the DRN were first observed by Pan & Williams, in 1989, using electrical stimulation of afferent projections in slice preparations and performing whole-cell intracellular recordings of pharmacologically isolated excitatory synaptic currents (Pan & Williams 1989). Additionally, they isolated each respective current pharmacologically and performed current-voltage (IV) curves to determine the IV relationship of both currents (Pan & Williams 1989). KAR-mediated currents have been quite difficult to observe in the CNS due to the requirement of brief stimuli-trains to evoke a current, however, KAR currents have been observed in the DRN through application of its respective agonist kainate (Tao & Auerbach 2000)(Samir Haj-Dahmane, personal communication). Moreover, KAR activation in the DRN appears to have positive-effects on 5-HT neuronal activity and subsequently 5-HT release as well (Tao & Auerbach 2000).

The Allen Mouse Brain Atlas has outlined the gene expression of specific ion channels throughout the CNS. A recent study has indicated that there appears to be pronounced expression of the Gria1, Gria3, Gria4, Grik2 and Grik5 genes specifically within the DRN. This is not to say the other glutamate receptor subunit genes were not expressed; in fact, they are expressed simply at lower levels compared to the genes previously listed (Templin et al 2012). Expression studies have also indicated that GluN1

and GluN2D NMDA receptors subunits are predominantly expressed within the DRN compared to other NMDAR subunits (Pallotta et al 1998, Tolle et al 1993).

Although it has received little attention in the past, researchers have recently begun to investigate the glutamatergic input to 5-HT neurons of the DRN and the potential modulatory roles it could play in 5-HT neuronal excitability (Haj-Dahmane & Shen 2009, Haj-Dahmane & Shen 2011, Jacobs & Azmitia 1992, Kirby et al 2007, Lemos et al 2006, Liu et al 2002, Soiza-Reilly & Commons 2011, Tao & Auerbach 2000). Interestingly, these ligand-gated ionotropic glutamate receptors have even been suggested to play roles in the development of depression and its subsequent treatment (Autry et al 2011, Cryan & O'Leary 2010, Duman et al 2012, Jernigan et al 2011, Li et al 2010).

6. Depression and Antidepressant Medication

Depression is one of the most prevalent and life-threatening forms of mental illness associated with significant disabilities and mortality (Chopra et al 2011). It is generally defined as a state of decreased mood, feelings of hopelessness and aversion to activity, all of which have been linked to changes in the serotonergic system (Chase et al 2010). Currently, 21% of the world's population is inflicted with depression, which has led to an increase in antidepressant prescriptions (Chopra et al 2011). The discovery of antidepressant medication occurred quite serendipitously, in 1952, when treating tuberculosis with an antimycobacterial agent known as iproniazid (Lieberman, 2003). The patients treated with iproniazid became "more cheerful, more optimistic, and more physically active" (Crane, 1956; Lieberman, 2003). In 1955, Ernst Zeller discovered that iproniazid blocked the enzymatic breakdown of monoamines such as norepinephrine, dopamine and serotonin (Zeller et al 1955). Thus, the "monoamine hypothesis of

depression" arose which suggests that depression can be treated by enhancing monoaminergic activity in the CNS. Among the many modern antidepressant medications clinically available, selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed. Although modern SSRIs provide much welcomed therapeutic benefits, they are unfortunately plagued by a relatively long delay of action (weeks) and by a lack of efficacy in a high proportion of patients. Significant research efforts in both academia and industry have therefore been devoted to understanding their mechanisms of action with the hope of circumventing the cellular/molecular mechanisms responsible for their slow onset and partial effectiveness. Unfortunately, since the spectacular introduction of selective serotonin re-uptake inhibitors (SSRIs) in the late 1980's, progress along this front has, arguably, been only incremental.

6.1. Selective Serotonin Reuptake Inhibitors (SSRIs)

As described in section 2.2, serotoninergic neurons actively control extracellular 5-HT levels through serotonin transporters (SERTs) that re-uptake 5-HT into the 5-HT neurons. SSRIs disrupt this active re-uptake process, by blocking SERTs, resulting in higher levels of extracellular 5-HT. It is generally thought that this rise in extracellular 5-HT levels, in part, underlies the therapeutic efficacy of SSRIs in treating depression and anxiety. Interestingly, it is well established that sustained administration of SSRIs initially, with in 2 days, suppresses the firing activity of 5-HT neurons in the DRN (Blier & De Montigny 1983, Blier et al 1984, Chaput et al 1986a, Gartside et al 1995). This reflects the enhanced activation of inhibitory 5-HT_{1A} somatodendritic autoreceptors by the elevation of extracellular 5-HT (Hensler et al 1991, Verge et al 1985, Weissmann-Nanopoulos et al 1985). Although sustained SSRI administration suppresses the firing

activity of 5-HT neurons within 2 days, remarkably, the firing activity gradually recovers over the time course of sustained SSRI treatments. This recovery in serotoninergic neuronal firing activity is accompanied by a desensitization of 5-HT_{1A} somatodendritic autoreceptors (Blier & De Montigny 1983, Blier & de Montigny 1994, Blier et al 1984, Chaput et al 1986b). Although the reason for this delay is not completely understood, the most commonly used explanation is a gradual desensitization of the 5-$HT1_A$ somatodendritic autoreceptors over the time course of SSRI treatments. This has been described by a reduced responsiveness of 5-HT neurons to the direct microiontophoretic application of $5HT_{1A}$ receptor agonists (Chaput et al 1986b). Superficially, we were struck by the resemblance between the gradual recovery of firing activity of 5-HT neurons during prolonged SSRI treatment (from quasi complete silence early in the treatment towards normal levels by 2-3 weeks) and a form of plasticity called homeostatic synaptic plasticity (Turrigiano 2008, Turrigiano et al 1998).

7. HOMEOSTATIC SYNAPTIC PLASTICITY

Homeostatic synaptic plasticity (HSP) is a phenomenom which refers to the bi-directional ability of the overall synaptic strength of a neuron to actively compensate for changes in overall excitability levels (Beique et al 2011, Davis 2006, O'Brien et al 1998, Turrigiano 2008, Turrigiano et al 1998). Homeostatic synaptic plasticity was first described in cultured neocortical neurons, where the authors observed adaptations in glutamatergic synaptic strength following perturbations of neuronal network activity (Turrigiano et al 1998). Since its discovery, HSP has been regarded as an attractive potential candidate to address the stability issues involved with the simple implementation of models of Hebbian-type LTP or LTD processes (Beique et al 2011,

Davis 2006, Nelson & Turrigiano 2008, Turrigiano 2008). For example, when network activity is enhanced for a prolonged period of time (24-48 hours), neurons compensate by, in part, reducing synaptic strength. Conversely, prolonged blockade of network activity results in an enhancement in synaptic strength. At least in part, these bi-directional homeostatic adjustments are carried out by changes in the number and/or function of excitatory synaptic glutamate receptor of the AMPA subtype (Beique et al 2011, O'Brien et al 1998, Shepherd et al 2006, Turrigiano 2008, Turrigiano et al 1998). In particular, the GluA2-lacking AMPARs are thought to play a role in HSP as they appear to be preferentially inserted in some, but not all cases.

Over the past decade, key experiments have shone light onto the mechansitic details of homeostatic synaptic plasticity. Core features of homeostatic synaptic plasticity have been observed following the inhbition in firing activity of single neurons within an otherwise unperturbed network (i.e., receiving normal ongoing synaptic input) (Burrone et al 2002, Ibata et al 2008). This observation highlights a widely acknowledged parameter of homeostatic synaptic plasticity: neuronal firing activity is subject to homeostatic pressure (Burrone et al 2002, Ibata et al 2008, Turrigiano 2008). Collectively, these studies provide important insight into mechanisms which neurons have been endowed with. These mechanisms include the ability: 1) to "sense", and integrate over time, their own level of activity by a mechanism that is likely continuously operant and, 2) to actively control this firing activity, presumably to maintain neuronal firing activity. Here, we wondered whether a homeostatic-like mechanism is operant to maintain (or more specifically, "recover") the firing activity of 5-HT neurons during treatments with an SSRI.

8. AIM OF STUDY

Taking together the knowledge that, (*i)* 5-HT neurons of the DRN receive many active excitatory glutamatergic inputs, (*ii)* 5-HT neuronal firing activity is initially shut-down following 2-days of SSRI treatments, (*iii)* 5-HT neurons are able to gradually regain firing activity over 3 weeks of treatment and, (*iv)* the well characterized homeostatic plasticity phenomenon stating that neurons have the ability to actively compensate the overall synaptic strength of a neuron in order to compensate for changes in firing activity, we hypothesize that a homeostatic-like regulation of glutamatergic synaptic strength might be operant on 5-HT cells during an SSRI treatment. We posit that a homeostatic-like upregulation in synaptic strength will be observed as an attempt to maintain 5-HT neuronal firing activity (Figure 2.). Therefore, utilizing whole-cell electrophysiological recordings from 5-HT neurons, we proposed to monitor glutamatergic synaptic strength onto 5-HT neurons during sustained SSRI treatments and determine if these synapses are being dynamically regulated.

MATERIALS AND METHODS

ANIMALS

Male Sprague Dawley rats (90 – 150 g; Charles River, St. Constant, Quebec, Canada) were received at least 6 days prior to surgery and housed two to three per cage. They were kept on a 12:12 hour light/dark cycle, with free-range access to food and water.

TREATMENTS

Citalopram was delivered to animals via subcutaneously implanted osmotic minipumps (Alzet; Cupertino, California) at a daily dose of 20 mg/kg/day. Animals were 26 – 29 days old and anesthetized with isofluorane at the time of minipump implantation. Citalopram was dissolved in 40% w/v 2-Hydroxypropyl-β-cyclodextrin to enhance drug solubility in water. Citalopram or 40% w/v 2-Hydroxypropyl-β-cyclodextrin (vehicle) was administered for 2 or 7 days.

SLICE PREPARATION

All experiments were performed in accordance with guidelines and approved procedures set forth by the University of Ottawa Animal Care and Veterinary Services. Brainstem slices containing the DRN were prepared from 28 – 36 days old Sprague Dawley rats. Rats were anesthetized with isofluorane and killed by decapitation. The brain was then quickly removed and placed in ice-cold choline chloride-based cutting solution of the following composition: 119 mM choline-Cl, 2.5 mM KCl, 1 mM $CaCl_2$, 4.3 mM $MgSO_4$-$7H_2O$, 1mM NaH_2PO_4, 1.30 mM sodium L-ascorbate, 26.20 mM $NaHCO_3$, and 11 mM glucose, and equilibrated with 95% O_2, 5% CO_2. A block of brain tissue containing the DRN was dissected out and affixed to a stage with superglue. Two

or three coronal slices (300 μm thick) containing the DRN were sectioned in ice-cold choline chloride-based cutting solution using a Vibratome slicer Series 1000 Plus (Shepreth, England). Slices were then transferred into a recovery chamber containing standard ACSF of the following composition: 119 mM NaCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$-$7H_2O$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose, at a temperature of 37°C for 5 minutes, continuously bubbled with a mixture of 95% O_2, 5% CO_2. Slices were then allowed to recover for 1 hour in the recovery chamber and equilibrate to a temperature of 25°C. Following recovery, slices were transferred one at a time to a recording chamber mounted on a fixed upright microscope and continuously perfused with standard ACSF saturated with 95% O_2, 5% CO_2 at a temperature of 25°C. In experiments using 0 Mg^{2+} ACSF, 1.3 mM $MgSO_4$-$7H_2O$ was omitted and replaced with 2.6 mM $CaCl_2$.

WHOLE-CELL RECORDINGS

DRN neurons were visualized using an upright microscope (Examiner D1; Zeiss, Oberkochen, Germany) equipped with a Dodt-contrast and infrared imaging system. 5-HT neurons were identified by morphological and biophysical characteristics using previously established criteria described in Section 4.1 & 4.2. Whole-cell recordings were performed using borosilicate glass patch electrodes (3-5 MΩ; World Precision Instruments) pulled on a Narishige PC-10 pipette puller. Excitatory post-synaptic currents (EPSCs) were recorded using an intracellular solution of the following composition: 115 mM cesium methane-sulfonate, 5 mM tetraethylammonium-Cl, 10 mM sodium phosphocreatine, 20 mM HEPES, 2.8 mM NaCl, 5 mM QX-314, 0.4 mM EGTA, 3 mM ATP(Mg^{2+}), and 0.5 mM GTP, pH 7.25 (adjusted with CsOH; osmolarity, 280–290

mOsmol/L). Due to the blockade of GIRK channels by cesium, 5-HT_{1A} mediated-currents were recorded using a potassium gluconate-based internal solution of the following composition: 115 mM potassium gluconate, 20 mM KCl, 10 mM sodium phosphocreatine, 10 mM HEPES, 4 mM ATP(Mg^{2+}), and 0.5 mM GTP, pH 7.25 (adjusted with KOH; osmolarity, 280–290 mOsmol/L). Liquid junction potential was not compensated for.

STIMULATION AND RECORDINGS

A monopolar borosilicate glass stimulating electrode (WPI) was placed 50 to 100 μm dorsal to dorsolateral to the recorded neurons. EPSCs were evoked with a single square pulse (intensity, 0.5 – 10 V; duration, 100 μs) delivered at 0.067 Hz to neurons voltage clamped at -70 mV or +40 mV (holding potential is indicated in figures). All recordings were performed in the presence of the $GABA_A$ receptor antagonist bicuculline (20 μM) or picrotoxin (100 μM), unless otherwise indicated. Paired-pulse ratios (PPRs) were obtained with an inter-stimulus interval (ISI) of 50 msec. For NMDAR/AMPAR ratios (N/A ratios), mixed NMDAR and AMPAR currents were evoked and recorded at +40 mV. NBQX (20 μM) was then bath applied in order to isolate the NMDA receptor-mediated component. The AMPAR component was determined by subtracting the NMDAR component from the mixed current traces.

$5HT_{1A}$-mediated currents were recorded while holding the cells at -55 mV and bath applying 5-carboxyaminotryptamine (5-CT) for 3 minutes. During current-clamp recording of serotonergic neurons, action potential discharge was induced by a constant small current injection and 5-CT (100 nM) was bath-applied for 3 minutes.

Miniature EPSCs (mEPSCs) were acquired at -70 mV in the presence of TTX (1

μM) and bicuculline (20 μM). Membrane currents were amplified using Multiclamp 700B amplifier (Molecular Devices, Sunnydale, CA), filtered at 2 kHz, digitized at 10 kHz with a Digidata 1440A Data Acquisition System (Molecular Devices), and acquired using the pClamp 10.3 software (Molecular Devices). Access resistance was continuously monitored by applying a 125 msec, 2 mV hyperpolarizing pulse 245 msec prior to stimulation. Recordings were discarded when the access resistance changed by >20%.

TWO-PHOTON MNI-GLUTAMATE UNCAGING AND IMAGING

Electrophysiological recordings were performed from DRN 5-HT neurons in similar conditions described above except the cesium methanesulfonate intracellular solution contained Alexa Fluor 594 (30 μM; for dendritic visualization and 2-Photon imaging). In addition, 2.5 mM MNI-Glutamate was added to the extracellular solution similar to the one described above. ACSF-containing MNI-Glutamate was continuously circulated by a fluid pump (World Precision Instruments). The imaging was obtained on an Olympus FV 1000 with a 60x/1.0 NA objective, and excitation was obtained with a Mai Tai Deep See Ti:Sapphire laser (Spectra-Physics) tuned at 810 nm. Two-photon uncaging was performed using a Mai Tai Deep See Ti:Sapphire laser (Spectra-Physics) tuned at 720 nm.

DATA ANALYSIS

Analysis was performed offline using Clampfit 10.3 software (Molecular Devices). The amplitude of eEPSCs and uncaged-EPSCs were determined by measuring the peak amplitude current in relation to the baseline current during a 5 msec window prior to the stimulus artifact. The paired pulse experiments at an ISI of 50 msec

(PPR=$EPSC_2/EPSC_1$) were averaged for at least 15 - 20 sweeps. mEPSCs were analyzed using a mEPSC current-template search through Clampfit 10.3 software (Molecular Devices). The current-template was created based on mEPSCs recorded in the DRN in control conditions. Furthermore, the same template was used for all mEPSC recordings. All data are presented as means +/- SEM. *N* refers to the number of cells recorded. Differences between treatment and vehicle groups were analyzed using a standard Student's *t*-test ($P < 0.05$).

DRUGS AND CHEMICALS

Bicuculline, NBQX di-sodium salt and D,L-AP5 were purchased from Abcam (Cambridge, MA). Tetrodotoxin (TTX), GYKI 52466, 2-Hydroxypropyl-β-cyclodextrin, and 5-Carboxyaminotryptamine (5-CT) were purchased from Tocris Cookson (Ellisville, MO). Citalopram was purchased from Toronto Research Chemicals (Toronto, ON).

RESULTS

To determine whether glutamatergic synapses onto 5-HT neurons are being dynamically regulated during sustained SSRI treatments, we treated animals with the SSRI Citalopram (20 mg/kg/day) and performed *ex vivo* recordings from 5-HT neurons in midbrain slice preparations of the DRN. We sought to, in our hands (*i*) identify 5-HT neurons in the DRN using 2-Photon imaging for visualization by morphology as well as observing hyperpolarizing currents mediated by $5HT_{1A}$ receptors known to densely and selectively populate 5-HT neurons of the DRN, (*ii*) identify glutamatergic synapses onto 5-HT neurons by electrical stimulation and 2-Photon MNI-glutamate uncaging (*iii*) during the time course of citalopram treatments, determine changes in presynaptic glutamate release and postsynaptic AMPAR and NMDAR function/number on 5-HT neurons using electrophysiological voltage clamp recordings combined with electrical stimulation.

IDENTIFICATION OF 5-HT NEURONS IN THE DRN

To study the properties of 5-HT neurons, reliable identification by morphology and electrophysiological characteristics is essential. Using previously established criteria (Aghajanian & Vandermaelen 1982, Calizo et al 2011, Haj-Dahmane 2001), we have selectively targeted 5-HT neurons of the DRN (Figure 3). Consistent with previous findings (Li et al 2001), 2-Photon imaging of neurons filled with Alex-594 (30 μM) has revealed that the 5-HT neurons targeted had few dendritic processes and sparse dendritic spines (Figure 3A). These medium sized (~15-25 uM in diameter) neurons tended to consistently have round and fusiform morphologies (Figure. 3B). In order to ensure that

the neurons recorded from were in fact 5-HT neurons, we required an electrophysiological signature by which we could identify them. From the literature (Calizo et al 2011, Francis et al 1992, Weissmann-Nanopoulos et al 1985), we know that 5-HT neurons specifically in the dmDRN express 5-HT_{1A} somatodendritic receptors whereas non-serotoninergic neurons in the dmDRN do not. Consequently, when activated with a selective 5-HT_{1A}-receptor agonist such as 5-Carboxyamidotryptamine (5-CT), a distinct inward current/hyperpolarization can be observed in 5-HT neurons. While recording from a 5-HT neuron, held at -55 mV in the voltage clamp configuration using a potassium-based internal solution, an inward current of approximately 50 pA was observed following a 3-minute bath application of 5-CT (100 nM; Figure 3C). Additionally, we held 5-HT neurons in current clamp to elicit action potential firing in response to the application of small current injections. Once firing at a steady rate of approximately 1 Hz, we bath-applied 5-CT and observed a hyperpolarizing effect mediated by 5-HT_{1A} somatodendritic autoreceptors which subsequently inhibited the firing activity of the neuron (Figure 3D). This effect recovered as 5-CT was washed out of the recording chamber with regular ACSF (Figure 3D). Taken together, these observations have indicated that we can consistently target and perform whole-cell recordings from 5-HT neurons in a midbrain slice preparation containing the DRN.

GLUTAMATERGIC RECEPTORS ARE LOCATED ON 5-HT NEURONS OF THE DRN

In order to investigate changes in glutamatergic synaptic strength onto 5-HT neurons in response to SSRI administration, we first identified glutamatergic receptors on 5-HT neurons electrophysiologically. As previously reported (Haj-Dahmane & Shen 2005), fast eEPSCs via “local” electrical stimulation can be observed while performing

whole-cell recordings from 5-HT neurons, voltage-clamped at -70 mV, in the DRN. These fast eEPSCs were pharmacologically isolated and determined to be AMPAR-mediated currents (Haj-Dahmane & Shen 2005, Haj-Dahmane & Shen 2009). In our hands, we sought to detect similar currents as previously described. When holding a 5-HT neuron voltage-clamped at -70 mV and electrically stimulating via a monopolar stimulating electrode, we reliably evoked a similar fast eEPSC (Figure 4B). These eEPSCs were isolated by bath application of bicuculline (20 μM) and APV (100 μM), to block $GABA_A$ and NMDA receptors respectively. The isolated eEPSC was determined to be AMPAR-mediated, as it was blocked by the bath application of the selective AMPAR antagonist NBQX (20 μM) (Figure 4B & D).

NMDA receptor-mediated currents recorded from 5-HT neurons, in the DRN, have been observed by both bath application of the respective agonist as well as evoked electrical stimulation (Pan & Williams 1989). Here, analogous to our identification of AMPAR current, we pharmacologically isolated NMDAR-mediated eEPSCs by local electrical stimulation while holding the cells at +40 mV in the presence of bicuculline (20 μM) and NBQX (20 μM) (Figure 4A). As expected, these eEPSCs were abolished by bath application of the selective NMDAR antagonist APV (100 μM) (Figure 4A &C).

Following the identification of both NMDAR and AMPAR currents from 5-HT neurons of the DRN, in our hands, we sought to determine if we could evoke kainate receptor currents. We attempted to evoke kainate receptor currents in the presence of bicuculline (20 μM), APV (100 μM) and the selective AMPAR antagonist GYKI 52466 (50 μM), however our attempts were futile (data not shown). Interestingly, although kainate currents could not be evoked by electrical stimulation, bath application of the

respective agonist results in a large inward current suggesting that these receptors are potentially located extrasynaptically (Samir Haj-Dahmane, personal communication). To address this possibility, we employed 2-Photon imaging combined with uncaging of MNI-glutamate, allowing us to activate spatial restricted areas while not being exclusively restricted to activating synaptic terminals via electrical stimulation (i.e., allowing us to target extrasynaptic regions). As a first test, prior to attempting to elicit kainate receptor currents, we characterized AMPAR-mediated currents in 5-HT neurons via uncaging. Filling 5-HT neurons with Alexa-594 (30 μM), we visualized the soma, dendrites and dendritic spines of 5-HT neurons (Figure 4E). We proceeded to uncage MNI-glutamate at various sites of interest by applying a brief 1-millisecond laser pulse. Our sites of interest initially included dendritic spines and dendritic shafts. While holding the cells at -70 mV, we observed AMPAR-mediated currents on dendritic spines and shafts by 2-Photon uncaging. These experiments were carried out in the presence of 1.3 mM Mg^{2+} to ensure the voltage-dependent block of an NMDAR-mediated inward current at -70 mV (Figure 4E). Conversely, while performing these experiments in the presence of the AMPAR antagonist GYKI 52466 (50 μM), we observed kainate receptor currents almost exclusively on the soma or along the dendritic shaft but never on dendritic spines (Figure 4F). Thus, in our hands, we have reliably elicited currents from glutamatergic receptors of the AMPA, NMDA and kainate subtypes in 5-HT neurons of the DRN. Moreover, we have observed that AMPA and NMDA receptors are located both synaptically and extrasynaptically on dendritic spines whereas kainate receptors are exclusively located extrasynaptically on the dendritic shafts and the soma of 5-HT neurons.

CITALOPRAM DECREASES THE NMDAR/AMPAR RATIO ON 5-HT NEURONS

As covered in section 5.1, it is well established that sustained administration of SSRIs initially suppresses the firing activity of 5-HT neurons in the DRN (Blier & De Montigny 1983, Blier et al 1984, Chaput et al 1986a, Gartside et al 1995). Throughout three weeks of treatments with an SSRI, a complete recovery in the firing activity of 5-HT neurons is observed. This recovery in firing activity has been associated with a desensitization of the $5HT_{1A}$ somatodendritic autoreceptor. Here, we sought to determine whether this recovery in firing activity could be driven by a homeostatic-like regulation of glutamatergic synapses onto 5-HT neurons. To address this possibility, we treated Sprague Dawley rats (day beginning treatments, P26-P30) *in vivo* with the SSRI citalopram (20 mg/kg/day) and performed *ex vivo* recordings from 5-HT neurons of the DRN after 2 and 7 days of sustained treatments.

As a first test, we determined the NMDAR to AMPAR ratio at glutamatergic synapses onto 5-HT neurons. NMDAR to AMPAR ratio provides a measure of synaptic strength with respect to the ratio of number and/or function of AMPA and NMDA receptors. We recorded evoked EPSCs in 5-HT neurons, in the presence of bicuculline (20 μM), while voltage-clamping the cell at +40 mV. In these conditions, mixed AMPAR and NMDAR-mediated outward currents were observed. The respective contribution from NMDA receptors was isolated pharmacologically, from the mixed NMDAR and AMPAR current, by bath applying the AMPAR antagonist NBQX (20 μM) (Figure 5A). Once the NMDAR current was isolated, we subtracted it from the mixed AMPAR and NMDAR current originally recorded to determine the contribution of AMPARs (Figure 5A). With the specific contributions of the AMPA and NMDA receptors, we determined

the ratio between the receptor currents. In these conditions, we observed a significant reduction of the NMDAR to AMPAR ratio in 2-day treated rats compared to age-matched vehicle-treated littermates (citalopram-treated n = 9, vehicle n = 8; $P < 0.05$; paired Student's *t*-test) (Figure 5A & B). Interestingly, the NMDAR to AMPAR ratio was not altered in animals treated for 7 days with citalopram (citalopram-treated n = 16, vehicle n = 11) (Figure 5A & B). These results indicate a dynamic alteration and adaptation of post-synaptic glutamatergic receptors on 5-HT neurons in response to sustained citalopram administration.

AMPAR-MEDIATED MEPSC FREQUENCY AND AMPLITUDES ARE DYNAMICALLY REGULATED BY CITALOPRAM TREATMENTS

In principle, a decrease in NMDAR/AMPAR ratios can indicate a reduction in the number (or function) of NMDARs, an increase in the number (or function) of AMPARs or a combined disproportional reduction in both of the respective receptors. To distinguish between these possibilities, we recorded miniature EPSC (mEPSC) recordings from 5-HT neurons in citalopram and vehicle-treated rats. mEPSCs provide a quantifiable index of glutamatergic synaptic strength. More specifically, the amplitudes of mEPSCs are a metric of the number (or function) of post-synaptic AMPARs, and the frequency of mEPSCs is an index of the number of AMPAR containing synapses or release probability. These experiments were performed in the presence of bicuculline (20 μM), to block $GABA_A$ receptors, and TTX (1 μM) to block sodium channels thereby isolating spontaneous synaptic release of neurotransmitters independent of action potential dependent glutamate release. In 2-day treated rats, we observed a robust reduction in both the amplitude and the frequency of these mEPSCs onto 5-HT cells compared to age-

matched vehicle-treated littermates (citalopram-treated n = 12, vehicle n = 10; $p<0.05$,paired Student's *t*-test) (Figure 6A, C & D). As such, these results suggested that the decrease in NMDAR to AMPAR ratio must be attributed to a decrease in postsynaptic AMPARs as well as a more profound decrease in NMDAR numbers.

Next, we sought to determine whether synaptic transmission onto 5-HT neurons is altered following a longer sustained treatment with citalopram. In 7-day treated rats, we observed a significant increase in the mEPSC amplitude compared to vehicle-treated littermates (citalopram-treated n = 23, vehicle n = 24; $p<0.05$, paired Student's *t*-test) (Figure 6 B & D). Conversely, we observed no difference in the mEPSC frequency in 7-day citalopram-treated rats compared to vehicle-treated littermates (citalopram-treated n = 23, vehicle n = 24; $p<0.05$, paired Student's *t*-test) (Figure 6 B & C). Population data of both mEPSC frequency and amplitude following 2 and 7 days of citalopram or vehicle treatments has been plotted in cumulative distribution plots (Figure 6E, F, G & H). Taken together, these results document a robust decrease in glutamatergic synaptic strength onto 5-HT neurons in 2-day citalopram-treated rats. Furthermore, the initial depression in synaptic strength is followed by a rebound in synaptic strength with an overcompensation in the number (or function) of postsynaptic AMPARs.

CITALOPRAM DECREASES RELEASE PROBABILITY AT GLUTAMATERGIC SYNAPSES ONTO 5-HT NEURONS

In principle, the robust reduction in mEPSC frequency observed following 2 days of citalopram treatment could be attributed to a decrease in presynaptic release probability or the number of AMPAR-containing synapses. To begin discriminating between these possibilities, we carried out paired-pulse ratio analysis of glutamatergic

synapses onto 5-HT neurons. The paired-pulse ratio (PPR) is a measure of presynaptic neurotransmitter release probability at glutamatergic synapses (Dobrunz & Stevens 1997). The PPR is determined by evoking two EPSCs with a designated inter-stimulus interval. Then, the amplitude of the second EPSC (S2) is divided by the amplitude of the first EPSC (S1) resulting in a ratio of S2/S1 known as the PPR. In theory, a higher PPR reflects a lower probability of release whereas a lower PPR would reflect a higher probability of release.

To address any changes in release probability, we evoked pairs of EPSCs with an inter-stimulus interval of 50 milliseconds at a frequency of 0.1 Hz in slices from citalopram and vehicle-treated animals. In 2-day citalopram-treated rats, we observed an increase in the PPR compared to age-matched vehicle-treated littermates (citalopram-treated n = 8, vehicle n = 8; $p<0.05$, paired Student's *t*-test) (Figure 7A & B). As such, this increase in PPR suggests the observed robust reduction in mEPSC frequency is accounted for by a reduction in release probability onto 5-HT neurons following 2 days of citalopram administration.

We next sought to determine if sustained citalopram treatments altered release probability at glutamatergic synapses onto 5-HT neurons. In 7-day citalopram-treated rats, we observed an increase in the paired-pulse ratio (citalopram-treated n = 16, vehicle n = 7; $p<0.05$, paired Student's *t*-test) (Figure 7A & B). This increase in the PPR at glutamatergic synapses onto 5-HT neurons implies that release probability is still significantly reduced following 7 days of citalopram administration. As such, this result suggests that the compensatory increase in the frequency of mEPSCs following 7 days of citalopram treatment (compared to 2 days; see Figure 6A & C) does not appear to be

accounted for by a change in probability of release. Rather, it is possible that the increase in mEPSC frequency reflects a compensatory upregulation of AMPAR-containing synapse number that develops over sustained citalopram administration. Thus, the "recovery" in the NMDAR to AMPAR ratio and overcompensation in mEPSC amplitude must be attributed to postsynaptic mechanisms. Taking together these observations, we have documented a dynamic adaptation of glutamatergic synapses onto 5-HT neurons during the time course of citalopram treatments.

DISCUSSION

Ligand-gated ionotropic glutamate receptors, of the AMPA and NMDA subtypes, are known to play fundamental roles in synaptic function (Adesnik & Nicoll 2007, Beique 2009, Beique & Huganir 2009, Beique et al 2011, Cull-Candy et al 2006, Dingledine et al 1999, Gardner et al 2005, Gray et al 2007, Isaac et al 2007, Liu & Cull-Candy 2002, Liu & Cull-Candy 2000, Plant et al 2006, Turrigiano 2008), brain/synapse development (Cull-Candy et al 2001, Dehorter et al 2012, Hall & Ghosh 2008, Liu et al 2004, Pachernegg et al 2012) and psychiatric disorders (Cull-Candy et al 2001, Lau & Zukin 2007, Yin et al 2012). The highly dynamic nature of glutamatergic synapses has highlighted their decisive influence on neuronal function. To date, there are few studies that have extensively studied glutamatergic synaptic transmission to the DRN (Pan & Williams 1989) and, furthermore, no studies have investigated how it may be regulated during a treatment with a SSRI.

Using a combination of cellular electrophysiology and 2-Photon microscopy, we report that AMPA and NMDA receptors are synaptically expressed on 5-HT neurons and, moreover, these receptors are dynamically regulated during the first week of sustained SSRI (citalopram) treatments. Specifically, following two days of citalopram treatments, glutamatergic synaptic transmission onto 5-HT neurons is robustly depressed. This is due to a concomitant reduction in the number of postsynaptic AMPARs and NMDARs as well as a reduction in presynaptic glutamate release. Remarkably, by a homeostatic-like mechanism, an over compensatory upregulation in synaptic strength was observed over one week of sustained SSRI treatments. This synaptic strengthening was reflected by an enhancement in postsynaptic AMPA and NMDA receptor functions. Interestingly,

despite the overcompensatory upregulation in postsynaptic function, release probability is still reduced at this 7-day treatment time point. Together, these results outline a robust and dynamic regulation of glutamatergic synaptic transmission during the time course of treatments with an SSRI. As such, these results herein hold a framework that is likewise required not only for understanding the biological basis of the antidepressant effect but also to develop novel and informed strategies to accelerate and maximize it.

To address the effects of SSRIs on glutamatergic synaptic transmission onto 5-HT neurons, we treated rats with citalopram (an SSRI) for a period of 2 or 7 days. Using whole-cell electrophysiological recordings, we identified a dynamic regulation of glutamatergic synapses over the first week of treatments with citalopram. As a first test, we determined the NMDAR to AMPAR ratio at glutamatergic synapses onto 5-HT neurons following 2 days of citalopram treatments.

NMDAR to AMPAR ratios provide a measure of synaptic strength with respect to the relative number and/or function of AMPA and NMDA receptors. Following 2 days of sustained treatments, citalopram induced a robust reduction in the ratio of synaptic NMDAR to AMPAR on 5-HT neurons. In principle, this reduction in the NMDAR/AMPAR ratio could be accounted for by: *(i)* a reduction in synaptic NMDARs, *(ii)* an increase in synaptic AMPARs, *(iii)* a combinatory reduction in synaptic NMDARS and an increase in synaptic AMPARs, *(iv)* or a disproportional reduction in the number both receptor subtypes. To distinguish between these possibilities, we recorded AMPAR-mediated mEPSC from 5-HT neurons following 2 days of citalopram treatments. The amplitudes of mEPSCs provided a quantifiable index of the number and/or function of AMPARs. Interestingly, 2 days of sustained treatment with citalopram led to a reduction

in the number and/or function of synaptic AMPARs as made evident by a reduction in the amplitude of AMPAR-mediated mEPSCs. These results bear consequential implications on the number and/or function of synaptic NMDARs. As a decrease in the number of AMPARs was reported (by a reduction in the mEPSC amplitude), we must infer that the reduction in the NDMAR/AMPAR ratio is also due to a disproportional (in comparison to the contribution from AMPARs) concomitant reduction of NMDARs.

Interestingly, the observed reduction in the amplitude of AMPAR-mediated mEPSCs was accompanied by a reduction in the frequency of mEPSCs as well. In principle, this implies that either release probability at glutamatergic synapses onto 5-HT neurons is reduced or there is an overall reduction in the number of AMPAR-containing synapses. In addressing this issue, we carried out paired-pulse ratio analysis of evoked-EPSCs on 5-HT neurons. Paired-pulse ratios provide a quantifiable metric of presynaptic neurotransmitter release probability (Dobrunz & Stevens 1997). The paired-pulse ratio indicated that there is a reduction in the release probability at AMPAR-containing synapses onto 5-HT neurons following 2 days of treatments with citalopram.

Functionally, the subunit composition of NMDA and AMPA receptors dictates the biophysical properties of the channel, such as activation/deactivation/desensitization parameters, single channel conductance and ionic permeability (Dingledine et al 1999, Liu & Cull-Candy 2000, Swanson et al 1997). We sought to determine whether the subunit composition of AMPARs is altered following 2 days of citalopram treatments. Beyond the identification of AMPA receptors on 5-HT neurons by biochemical and basic electrophysiological means, relatively little work has been done to elucidate the exact properties of these receptors. To address this, we obtained preliminary current to voltage

relationship plots from AMPAR-mediated eEPSCs. GluA2-lacking AMPARs exhibit higher single-channel conductance and possess an identifiable electrophysiological signature of an inwardly rectifying IV curve signature consequent of a polyamine-block (Beique & Huganir 2009, Bowie & Mayer 1995, Dingledine et al 1999, Isaac et al 2007, Panicker et al 2008, Shepherd & Huganir 2007). Our preliminary results from IV plots of AMPAR-mediated eEPSCs have revealed that glutamatergic synapses onto 5-HT neurons preferentially, in control conditions of 3 to 4-week-old rats, contain GluA2-lacking AMPARs. The resulting IV plot exhibited the characteristic inwardly rectifying relationship expected from GluA2-lacking AMPAR-mediated currents (preliminary data; data not shown). These observations complemented molecular and biochemical studies indicating preferential expression of GluA1 and GluA4 compared to GluA2 AMPAR subunits in the DRN (Soiza-Reilly & Commons 2011, Templin et al 2012). Remarkably, following 2 days of citalopram treatments, the I-V relationship of AMPAR-mediated eEPSCs is linear (preliminary data; data not shown). A linear IV relationship is indicative of predominantly synaptic GluA2-containg AMPARs. Thus, this suggests that we have observed a change in AMPAR number (decrease in mEPSC amplitude) and function (IV relationship) following 2 days of citalopram treatments. It appears that the reduction in the mEPSC amplitude is accounted for by a specific reduction in the number of GluA2-lacking AMPARs. Thus, leaving predominantly GluA2-containing AMPARs remaining on 5-HT neurons following 2 days of citalopram treatments.

Glutamatergic synaptic transmission has been shut down within 2 days of treatments with citalopram. More intriguingly, this depression in glutamatergic synaptic transmission is present in experimental conditions where citalopram is likely no longer

present within the slice preparations. Thus, this may reflect the presence of long-term functional changes at glutamatergic synapses on 5-HT neurons. Thus, we posit that the observed citalopram-induced depression in synaptic transmission onto 5-HT neurons (following 2 days) could be accounted for by a Hebbian-type plasticity phenomenon known as long-term depression.

Long-term depression (LTD) is characterized as a long-lasting decrease in glutamatergic synaptic strength (Collingridge et al 2010). LTD was first described at Schaffer collateral-CA1 glutamatergic synapses of the hippocampus (Lynch et al 1977). Over the past 35 years, LTD has been described in widespread brain regions and, moreover, has been divided into various types with distinct loci of expression. Intriguingly, some modes of LTD are expressed presynaptically as a reduction in presynaptic glutamate release. As we observed a robust reduction in release probability following 2 days of citalopram treatments, we sought to find a mode of LTD that was accompanied by a reduction in release probability. One appealing mode of presynaptically expressed LTD, to explain our citalopram treatment-induced depression in synaptic strength, is endocannabinoids-mediated LTD (eCB-LTD).

The eCB-LTD phenomenon was first reported in the dorsal striatum in 2002 (Gerdeman et al 2002), and since then eCB-mediated forms of synaptic plasticity have been described at synapses in various brain regions such as the nucleus accumbens (Robbe et al 2002), amygdala (Azad et al 2004, Marsicano et al 2002), cerebellum (Soler-Llavina & Sabatini 2006) and VTA (Pan et al 2008). eCB-LTD results from an endogenous release of endocannabinoids and the subsequent activation of presynaptic type-1 endocannabinoid receptors (CB1Rs) (Gerdeman et al 2002). CB1R activation

leads to inhibition of presynaptic glutamate release by modulating various presynaptic effectors (depending on the system) such as voltage-dependent calcium channels, PKA activity and MAPK (Chevaleyre et al 2006, Howlett 2005, McAllister & Glass 2002, Mukhopadhyay et al 2002, Schlicker & Kathmann 2001).

Specifically, eCB-mediated LTD provides an attractive mode for the observed depression in presynaptic glutamate release as CB1Rs are known to be expressed in the DRN (Haring et al 2007, Herkenham et al 1991, Matsuda et al 1993, Tsou et al 1998) and, moreover, eCBs have been shown to depress presynaptic glutamate release onto 5-HT neurons of the DRN (Haj-Dahmane & Shen 2009, Haj-Dahmane & Shen 2011). The authors of this study found that depolarization of 5-HT neurons resulted in the release of endogenous eCBs acting retrogradely on the presynaptic terminals of glutamatergic afferents to shut down neurotransmitter release. This phenomenon is known as depolarization-induced suppression of excitation (DSE) (Kreitzer & Regehr 2001). Despite DSE being a short-term eCB-mediated effect, lasting only minutes, these observations nevertheless provide an integral framework where an eCB-induced long-term functional depression in synaptic strength could occur at glutamatergic inputs onto 5-HT neurons during citalopram treatments for 2 days.

We reasoned that if eCB signalling was mediating the observed synaptic depression, we would be able to prevent it by blocking the presynaptic type-1 endocannabinoid receptor (CB1R). In parallel with both citalopram and vehicle treatments, we likewise administered the CB1R-antagonist AM-251 (3mg/kg/day; via osmotic minipumps). As a first test, we recorded AMPAR-mediated mEPSCs from 5-HT neurons. Excitingly, we observed no significant reduction in mEPSC frequency and

amplitude (preliminary data, data not shown). This suggests that there is no reduction in synaptic strength when citalopram was administered with AM-251 for 2 days (preliminary data, data not shown). As eCB-LTD has predominantly been described as a presynaptic mechanism, the lack of reduction in postsynaptic AMPAR receptor number and function (mEPSC amplitude) was admittedly unexpected. However, there have been recent reports in the hippocampus (Han et al 2012) and VTA (Edwards et al 2012) that indicate eCBs may play some role in AMPAR internalization and postsynaptically expressed LTD. With these results in mind, we posit that 2 days of citalopram treatments results in an increase in eCB release (by an unknown mechanism) thereby inducing an endocannabinoid-mediated LTD at glutamatergic synapses onto 5-HT neurons. This synaptic depression is composed of an eCB-dependent reduction in presynaptic glutamate release accompanied by a disproportional reduction in the number of postsynaptic NMDA and AMPA receptors, and a functional subunit shift from GluA2-lacking to GluA2-containg synaptic AMPARs (Figure 8).

Based on our observations, it is evident that glutamatergic synaptic transmission onto 5-HT neurons is being dynamically regulated after just 2 days of citalopram treatments. We were intrigued by this initial regulation of synaptic transmission and wondered what changes might these synapses undergo over prolonged treatments with citalopram. Would glutamatergic synapses onto 5-HT neurons be further depressed? Would they recover? Would they strengthen? To address the effect of prolonged antidepressant treatments, we treated animals with citalopram for 7 days. Here, we employed a similar experimental scheme (as at the 2-day treatment time point) to study

any changes in glutamatergic synaptic transmission onto 5-HT neurons following 7 days of citalopram treatments.

Contrary to the initial depression in synaptic strength following 2 days of citalopram treatments, we observed a very different regulation of glutamatergic synaptic transmission onto 5-HT neurons after 7 days of sustained treatments. As a first test to determine any changes in glutamatergic synaptic transmission, following 7 days of citalopram treatments, we performed NMDAR to AMPAR ratio analysis on 5-HT neurons. We observed no significant change in the NMDAR to AMPAR ratio between 7-day vehicle and citalopram-treated animals. However, following 2 days of citalopram treatments we had observed a reduction in the NMDAR to AMPAR ratio. Therefore, we must infer that there has been a recovery in the ratio of NMDAR to AMPARs between 2 days to 7 days of sustained citalopram treatments. Following 2 days of treatments, we reasoned that the observed reduction in the NMDAR/AMPAR ratio was due to a disproportional reduction in both NMDAR and AMPAR numbers. But what is accounting for this apparent recovery after 7 days of citalopram treatments? In principle, this recovery in the NMDAR/AMPAR ratio could be accounted for by: *(i)* an increase in synaptic NMDARs, *(ii)* an decrease in synaptic AMPARs, *(iii)* a combinatory increase in synaptic NMDARS and a decrease in synaptic AMPARs, *(iv)* or a disproportional increase in the number of both receptor subtypes. To distinguish these possibilities, we performed AMPAR-mediated mEPSC recordings.

As covered, mEPSC recordings provide a quantifiable measure of postsynaptic AMPAR number and/or function (mEPSC amplitude) as well as a measure of release probability and/or the number of AMPAR-containing synapse numbers (mEPSC

frequency). Following 7 days of citalopram treatments, we observed a significant increase in the amplitude of AMPAR-mediated mEPSCs and no significant difference in their frequency. The significant increase in the mEPSC amplitude, following 7 days of citalopram treatments, is indicative of an over compensatory increase in the number and/or function of synaptic AMPARs (from the initial reduction following 2 days of citalopram treatments). Thus, we reasoned that if we know that there is an increase in AMPAR number and/or function, the recovery in the NMDAR to AMPAR ratio must be due to a disproportional increase in both receptor subtypes.

The observed increase in the amplitude of AMPAR-mediated mEPSCs was not accompanied by a significant change in the frequency of mEPSCs between 7-day vehicle and 7-day citalopram-treated littermates. Although, following 2 days of treatments, we had observed a robust reduction in the mEPSC frequency. With this is in mind, these results imply that there is a recovery in the mEPSC frequency following 7 days of citalopram treatments (compared to 2-day treatments). Following 2 days of citalopram treatments, using paired-pulse ratio analysis, we reasoned that the reduction in mEPSC frequency was due to a robust decrease in release probability. Here, we employed paired-pulse ratio analysis to examine whether a recovery in release probability could account for this apparent recovery in the frequency of mEPSCs following 7 days of treatments. Interestingly, similar to in 2-day treated animals, we observed a sustained reduction in release probability made evident by an increase in the paired-pulse ratio. If release probability is still reduced in 7-day treated animals, what accounts for this recovery in the mEPSC frequency? mEPSC frequency provides a metric of release probability or the number of AMPAR-containing synapses. Therefore, because release probability is still

reduced (as made evident by an increase in the paired-pulse ratio), the recovery in the mEPSC frequency must be due to an overall increase in the number of AMPAR-containing synapses. Together, these observations suggest that we have a postsynaptic strengthening made evident by an increase in the number of AMPARs as well as the number of AMPAR-containing synapses.

We have preliminary indications towards a functional change in the subunit composition of synaptic AMPARs on 5-HT neurons following 2 days of citalopram treatments. We sought to determine whether the subunit composition of AMPARs is altered following 7 days of citalopram treatments. As mentioned, the subunit-composition of AMPARs consequently dictates their biophysically characteristics. Interestingly, following 7 days of citalopram treatments, IV relationships of synaptic AMPARs on 5-HT neurons have a characteristic inwardly rectify IV plot. This implies that glutamatergic synapses onto 5-HT neurons preferentially contain GluA2-lacking AMPARs following 7 days of citalopram treatments (Beique & Huganir 2009, Bowie & Mayer 1995, Dingledine et al 1999, Isaac et al 2007, Panicker et al 2008, Shepherd & Huganir 2007). Conversely, we had observed linear IV plots following 2 days of treatments. This suggests that synaptic GluA2-lacking AMPARs are selectively removed following 2 days of citalopram treatments. Thus, following 7 days of citalopram treatments, we observed an increase in AMPAR number that is accompanied by a functional shift from predominantly GluA2-containing synapses (following 2 days of treatments) to synapses mainly populated by GluA2-lacking AMPARs.

Together, we found this synaptic up regulation, and apparent homeostatic-like mechanistic recovery to be analogous to the well-described phenomenon homeostatic

synaptic plasticity (Turrigiano 2008, Turrigiano et al 1998). As covered in the Section 7, homeostatic synaptic plasticity is defined as the bidirectional ability of neurons to alter their synaptic strength in response to changing levels of network activity (Beique et al 2011, Turrigiano 2008). Indeed, neurons are endowed with the ability to sense and integrate their level of firing activity and to actively maintain it within a computationally optimal range (Davis & Bezprozvanny 2001, Marder & Prinz 2003, Turrigiano 1999). More specifically, if you inhibit the firing of individual neurons by overexpressing a hyperpolarizing potassium channel, synapses onto that neuron act to increase glutamatergic synaptic strength and help in reestablishing the neuron's original firing activity (Burrone et al 2002). In some cases, including the study previously described, this strengthening of synapses occurs through increases in postsynaptic AMPAR number and/or function (Burrone et al 2002, O'Brien et al 1998, Shepherd et al 2006, Turrigiano 2008, Turrigiano et al 1998). Here, we see a similar scenario when treating animals with citalopram for 7 days. Indeed, the firing activity of 5-HT neurons is similarly shut down due to the consistent activation of the hyperpolarizing 5-HT_{1A}-somatodendritic autoreceptor (Blier & De Montigny 1983, Blier et al 1984, Chaput et al 1986a, Gartside et al 1995) and these 5-HT neurons are still receiving dense, active glutamatergic afferents (Celada et al 2001, Hajos et al 1999, Lee et al 2003). We observed an overcompensatory increase in the mEPSC amplitudes, indicative of an increase in postsynaptic AMPAR number and/or function analogous to observations in previous homeostatic synaptic plasticity studies (Burrone et al 2002, O'Brien et al 1998, Shepherd et al 2006, Turrigiano 2008, Turrigiano et al 1998).

The results presented here document a dynamic regulation of glutamatergic synaptic strength throughout the first week of treatments with an antidepressant. Our proposed model incorporates an initial LTD-like early phase, followed by a homeostatic-like synaptic strengthening (Figure 8). Further elucidation of the mechanisms of these changes in glutamatergic synaptic strength will not only provide an understanding of the biological basis of the antidepressant effect but also help to develop novel and informed strategies to accelerate and maximize it. Currently, it takes three weeks of sustained antidepressant treatments to reach therapeutic efficacy in humans. We posit that the pharmacological targeting of glutamatergic synapses onto 5-HT neurons during antidepressant treatment may circumvent this delay in therapeutic efficacy.

MODEL FOR ANTIDEPRESSANT–INDUCED SYNAPTIC PLASTICITY (FIGURE 8)

Based on our results presented here, we have devised a hypothesized model of antidepressant-induced synaptic plasticity. We hypothesize that the initial LTD-like effect following 2 days of citalopram treatments is mediated by retrograde signaling of eCBs to presynaptic CB1 receptors thereby hampering synaptic release at glutamatergic synapses onto 5-HT neurons (Haring et al 2007, Matsuda et al 1993, Tsou et al 1998). Additionally, we have observed that this LTD-like phenomenon is also expressed post-synaptically by a selective removal of synaptic NMDAR and GluA2-lacking AMPARs. Such selective removal of GluA2-lacking AMPARs has been previously reported during the induction of LTD at the mossy fiber-CA3 synapses (Ho et al 2007). Studies have also reported LTD of NMDAR-mediated currents in many brain regions including the hippocampus (Morishita & Malenka 2008, Selig et al 1995), VTA (Harnett et al 2009) and nucleus accumbens (Chergui 2011, Kombian & Malenka 1994). We are unsure as to

whether the reduction in NMDAR and AMPAR currents is mediated by eCBs or a separate unknown mechanism. However, there have been recent reports in the hippocampus (Han et al 2012) and VTA (Edwards et al 2012) that indicate eCBs may play some role in the internalization of AMPARs and postsynaptically expressed LTD.

Following 7 days of citalopram treatments there is a homeostatic-like synaptic strengthening at these synapses reflecting an overcompensatory increase in the number and function of postsynaptic AMPA receptors. Such synaptic upregulations in the number of AMPARs have been reported to, in part, account for homeostatic synaptic plasticity mechanisms (O'Brien et al 1998, Shepherd et al 2006, Turrigiano 2008, Turrigiano et al 1998). In addition, we observed an upregulation in the number and/or function of synaptic NMDARs. We propose that the sustained reduction in release probability can be explained by the persistent activation of the presynaptic CB1 receptors, by the endogenous release of endocannabinoids, over 7 days of citalopram treatments. We believe, judging by our preliminary data, that the overcompensation in postsynaptic receptors is due to selective insertion of AMPARs of the GluA2-lacking subtype and synaptic NMDARs. The selective insertion of GluA2-lacking AMPARs has been shown to accompany some, but not all, homeostatic upregulations in synaptic strength (Aoto et al 2008, Ju et al 2004, Thiagarajan et al 2005). Investigating key molecular determinants of homeostatic synaptic plasticity will provide further insight into the exact mechanistic recovery that occurs following 7 days of citalopram treatments. In addition, examination of eCB-LTD during citalopram treatments could help define the unique mechanism responsible for this initial depression in glutamatergic drive to 5-HT neurons following 2 days of citalopram treatments.

FUTURE DIRECTIONS

We will further investigate the role of endocannabinoid signalling on this initial LTD-like effect following 2 days of citalopram treatments. If retrograde eCB signalling is involving presynaptic CB1Rs is playing an active role in the initial LTD-like effect, we should be able to block the LTD mechanism (as our preliminary data suggests) by administering a CB1R antagonist in combination with citalopram. Also, if LTD is dependent on long-term activation of the CB1 receptor we propose that DSE will be altered in animals treated with citalopram (2 days). If this is the case, we hypothesize that in treated animals we will be able to successfully occlude DSE whereas in control treated animals we will still observe a robust DSE effect (Haj-Dahmane & Shen 2009).

We will further elucidate the involvement of homeostatic synaptic plasticity (HSP) following 7 days of citalopram treatments. If homeostatic scaling is playing a role, we reason that perturbations in key determinants of HSP would block this upregulation in synaptic strength following 7 days of citalopram treatment. The pro-inflammatory cytokine tumour necrosis factor-alpha (TNFα) has been shown to play a pivotal role in homeostatic synaptic plasticity (Beattie et al 2002, Stellwagen & Malenka 2006). The authors reported that by knocking out TNFα, homeostatic scaling could be blocked. Thus, we reason that if we treat TNFα knockout mice with citalopram for 7 days, we will be able to successfully block the observed synaptic strength (compared to citalopram-treated wild-type littermates).

As previously covered, 5-HT neurons of the DRN are receiving many glutamatergic inputs from a wide variety of brain regions. This leaves us with a lingering question of where exactly these glutamatergic inputs are coming from, and, is input

specificity carried in this dynamic regulation of synaptic strength in response to treatments of citalopram? To address this, a relatively new and revolutionary technique in neuroscience known as optogenetics will be employed (Deisseroth 2011). Optogenetics is a targetable control tool allowing light to deliver an effector response on a neuron (Deisseroth 2011). As previously mentioned, the DRN receives its most dense glutamatergic input from the prefrontal cortex. As a first test, we will stereotactically inject an adenovirus containing channelrhodopsin 2 (ChR2) into the prefrontal cortex analogous to Admantidis et al (2007) who injected ChR2 into the lateral hypothalamus (Adamantidis et al 2007). ChR2 is a light-activated (blue: 480nm wavelength) non-specific cation channel commonly found in alga bacteria (Nagel et al 2002). Once injected into the prefrontal cortex *in vivo*, we anticipate axonal transport of ChR2 in prefrontal cortex neurons to terminal projections in the DRN. This can be inferred from axonal transport of ChR2 in pyramidal neurons described by Petreanu et al (2007). ChR2 will then be detectable in these neurons through coupling of a red fluorescent protein (RFP) by the absorption of green light. This will suggest that these channels were in fact transported down the axons to the DRN. We can then assess changes in glutamatergic synaptic strength onto 5-HT neurons by performing whole-cell recordings from 5-HT neurons and blue light will be shone to activate ChR2 at glutamatergic terminal afferents from the prefrontal cortex. If a response to the light stimulation is observed, this will confirm that ChR2 was transported through the axon and ChR2 containing neurons are forming synapses with that 5-HT neuron in DRN. Using light activation of that specific ChR2 containing neuron we will assess changes in glutamatergic synaptic strength onto 5-HT neurons from the PFC by performing light activated PPRs, NMDAR/AMPAR

ratios and AMPAR I-V relationships. Investigating these ratios and relationships will provide us with a model that could suggest input specific changes in synaptic strength onto 5-HT neurons in response to citalopram treatments.

CONCLUSION

Recent years have witnessed extraordinary advances in our understanding of the dynamical nature of synapse function and this, in cellular, molecular and computational terms. Such knowledge has already begun to be translated directly into problems relevant to psychiatric conditions. The framework developed in the last 10 years or so on the synaptic basis of addiction (Kauer & Malenka 2007) provides in this respect an explicit illustration. Here, we have observed dynamic changes in glutamatergic synaptic strength onto 5-HT neurons in response to citalopram treatments for 2 and 7 days. Such a background is likewise required not only for understanding the biological basis of the antidepressant effect but also to develop novel and informed strategies to accelerate and maximize it.

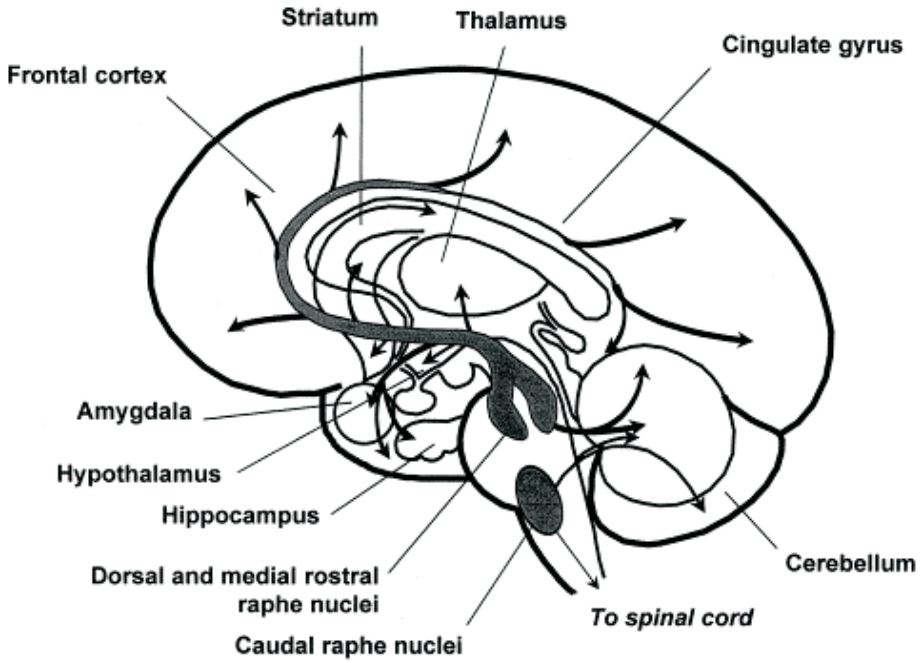
TABLES AND FIGURES

TABLE 1: Anatomical Designation of 5-HT neurons in the Raphe Nuclei (Information obtained from: (TORK 1990)	
B1	Raphe pallidus nucleus Caudal veterolateral medulla
B2	Raphe obscurus nucleus
B3	Raphe magnus nucleus Rostral veterolateral medulla Lateral paragigantocellular reticular nucleus
B4	Raphe obscurus (dorsolateral part)
B5	Median raphe nucleus (caudal part)
B6	**Dorsal raphe nucleus (caudal part)**
B7	**Dorsal raphe nucleus (principle, rostral part)**
B8	Median raphe nucleus (rostral main part) Caudal linear nucleus Nucleus pontisoralis
B9	Supraleminiscal region Nucleus pontisoralis

TABLE 2:
Central 5-HT Receptors
(Information obtained from: (Barnes & Sharp 1999))

FAMILY	COUPLING	SECOND MESSENGER RESPONSES	SYNAPTIC LOCALIZATION
5-HT$_{1A}$	G_i/G_o	↓ cAMP	Pre- and Postsynaptic
5-HT$_{1B}$	G_i/G_o	↓ cAMP	Presynaptic
5-HT$_{1D}$	G_i/G_o	↓ cAMP	Presynaptic
5-HT$_{1E}$	G_i/G_o	↓ cAMP	Postsynaptic
5-HT$_{1F}$	G_i/G_o	↓ cAMP	Pre- and Postsynaptic
5-HT$_{2A}$	G_q/G_{ll}	↑ DAG & IP_3	Postsynaptic
5-HT$_{2B}$	G_q/G_{ll}	↑ DAG & IP_3	Postsynaptic
5-HT$_{2C}$	G_q/G_{ll}	↑ DAG & IP_3	Postsynaptic
5-HT$_3$	Ligand-gated ion channel	Depolarization of postsynaptic neurons	Postsynaptic
5-HT$_4$	G_s	↑cAMP	Postsynaptic
5-HT$_{5A}$	G_i/G_o	↓ cAMP	Postsynaptic
5-HT$_{5B}$	G_s	↑cAMP	N/A
5-HT$_6$	G_s	↑cAMP	Postsynaptic
5-HT$_7$	G_s	↑cAMP	Postsynaptic

A



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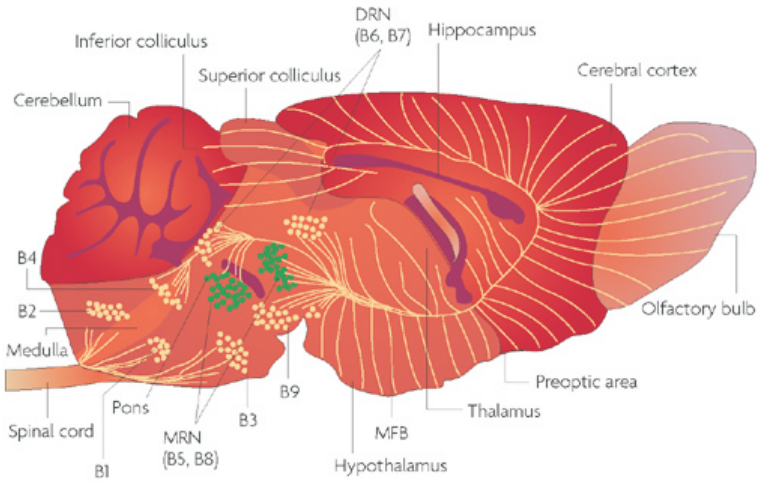

Figure 1: Projections from the raphe nuclei in humans (A) (Nutt et al., 1999) and rodents (B) (Murphy & Lesch, 2008) .

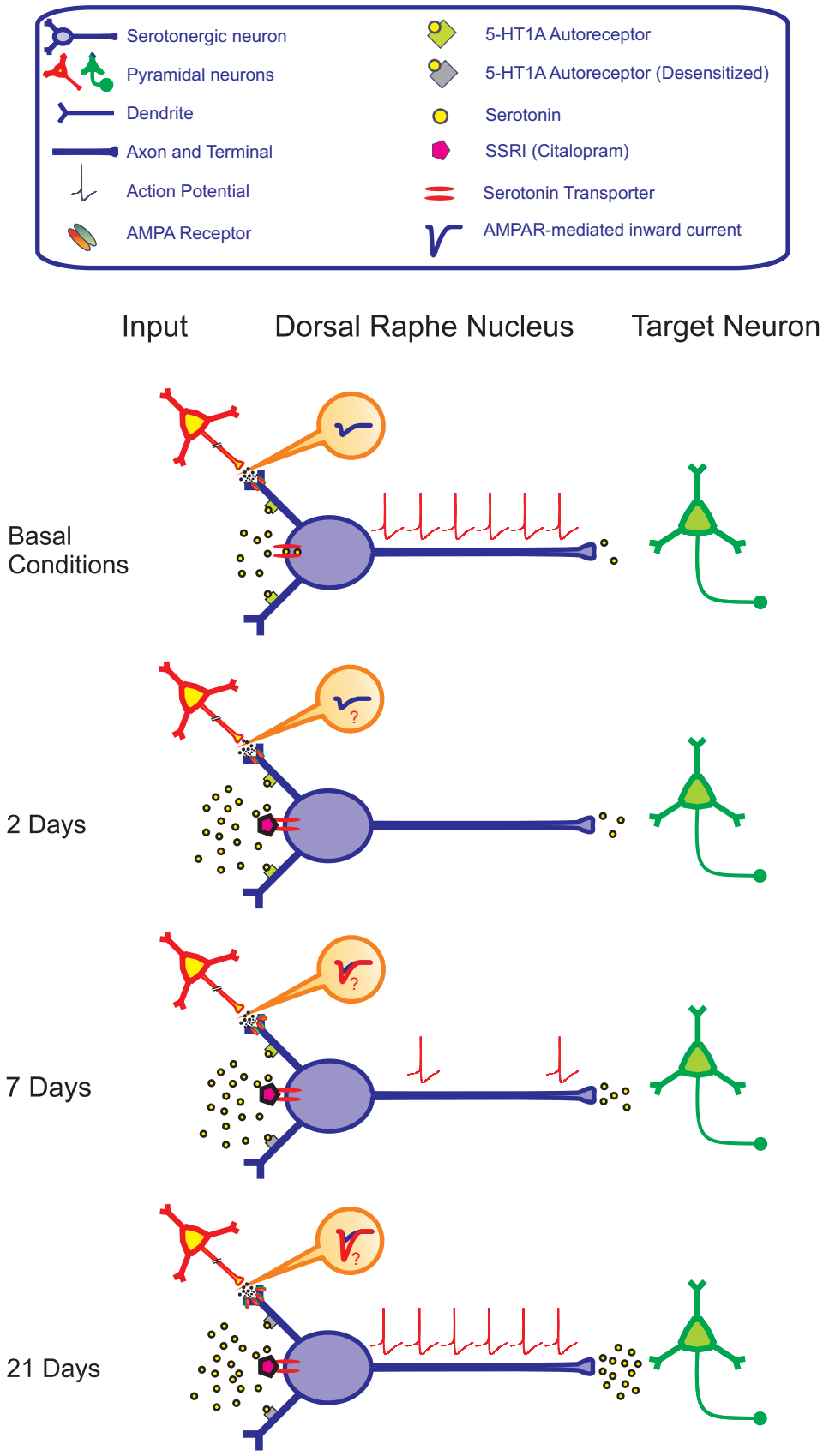

Figure 2: The effects of selective serotonin reuptake inhibtors (SSRIs) on the firing rate of 5-HT neurons. Our overarching hypothesis of changes in glutamatergic synaptic strength onto 5-HT neurons during the time course of treatments with an SSRI is depicted in the yellow bubbles. The legend above clearly defines the components of this cartoon.

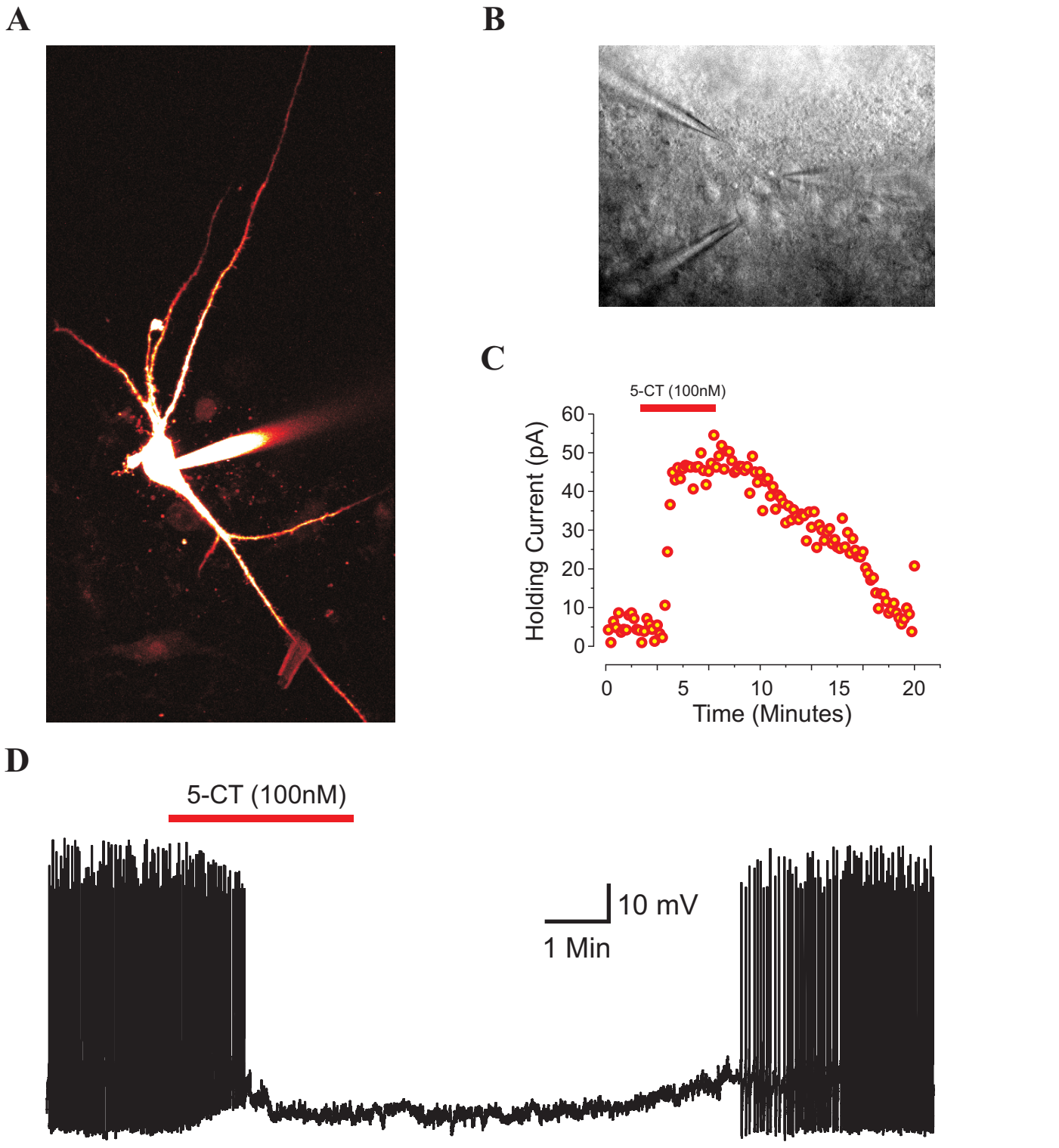

Figure 3: Serotoninergic neurons of dorsal raphe nucleus (DRN). **A.** Two-Photon laser scanning microscopic image of a serotoninergic neuron. This serotonergic neuron was recorded with a recording pipette filled with the fluorescent dye Alexa Fluor 594 (30µM). **B.** Dodt-contrast image of recording from 5-HT neurons in the DRN. **C.** Whole-cell voltage-clamp recording of serotoninergic neuron of the DRN. Bath administration of the 5-HT_{1A}R agonist 5-carboxamidotryptamine (5-CT; 100nM) elicited a robust outward current (Vm, -55mV). **D.** Whole-cell current-clamp recording of a serotoninergic neuron of the DRN. Action potential discharge was induced by a constant small current injection. In these conditions, bath administration of 5-CT (100nM) induced a prominent hyperpolarization of sufficient magnitude to halt action potential discharge in this serotoninergic neuron of the DRN.

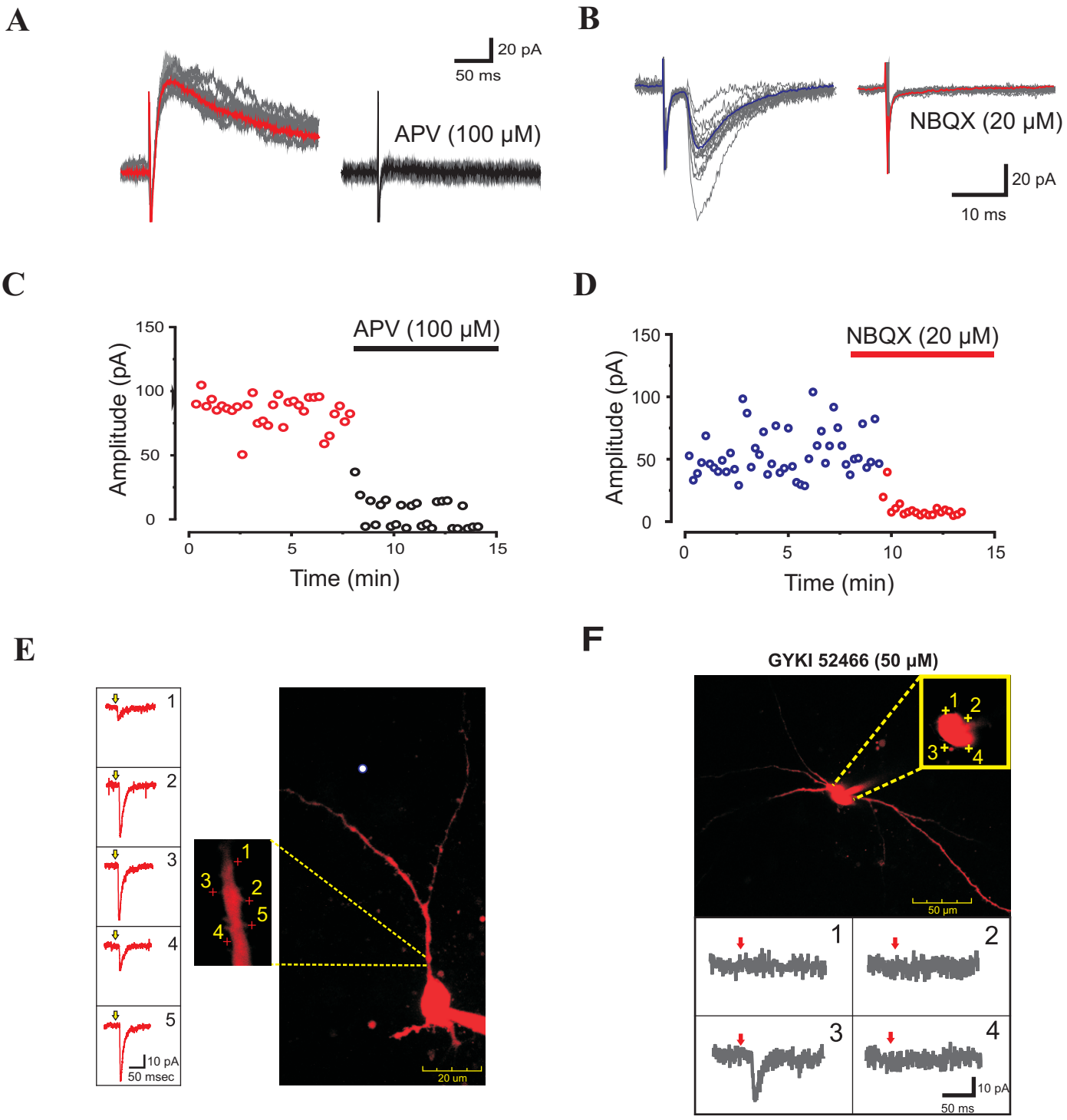

Figure 4: Excitatory post-synaptic currents (EPSCs) recorded from 5-HT neurons. **A.** Representative trace of evoked NMDAR-mediated outward current (Vm: +40mV; left). Representative trace following bath application of APV (100µM) (right). **B.** Representative trace of evoked AMPAR-mediated inward current (Vm: -70mV; left). Representative trace following bath application of NBQX (20µM) (right). **C.** Plot of NMDAR mediated EPSCs amplitudes and abolishment of outward currents with bath application of APV(100µM) **D.** Plot of NMDAR mediated EPSCs amplitudes and abolishment of outward current with bath application of NBQX(20µM). **E & F.** 2-Photon glutamate uncaging of MNI-Glutamate on dendritic spines, shafts, and soma of 5-HT neurons. Neurons (both E & F) were filled with the fluorescent dye Alexa Fluor 594 (30µM) for visualization. **E.** AMPAR-mediated inward currents evoked by glutamate uncaging onto dendritic spines and shaft. **F.** Kainate receptor-mediated inward current evoked by glutamate uncaging onto the soma of a 5-HT neuron in the presence of GYKI 52466 (50µM)

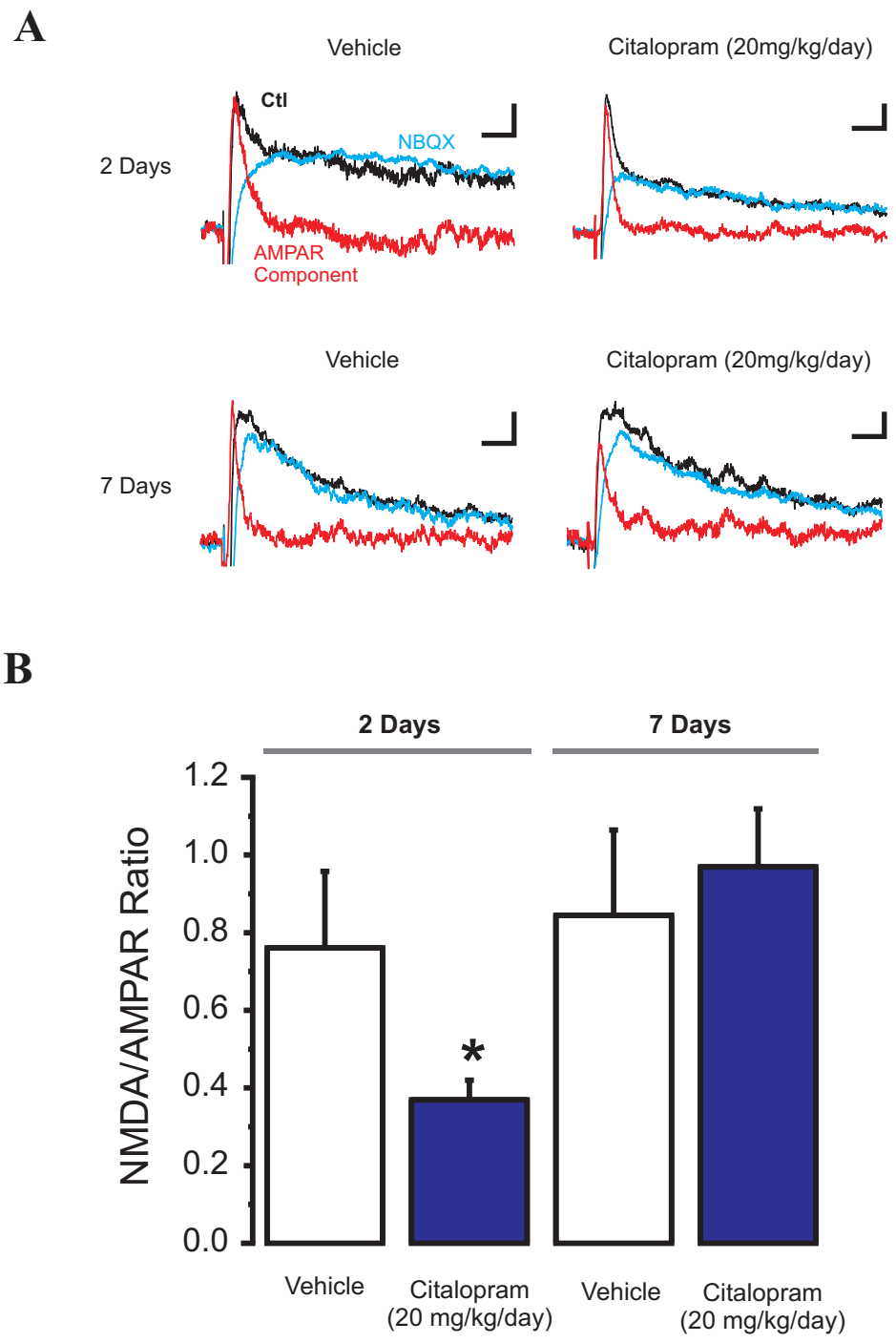

Figure 5: 2 days of citalopram treatment induces a decrease in the NMDAR/AMPAR ratio on 5-HT neurons which is followed by a complete recovery after 7 days of citalopram administration **A.** Representative traces of acquiring NMDAR/AMPAR ratios. Neurons were voltage clamped at +40 mV in 0 Mg^{2+} Ringer. Mixed AMPAR and NMDAR currents were first recorded (Black traces). NBQX (20μM) was then bath applied to isolated the NMDAR component of the mixed current (Blue traces). The isolated NMDAR component was then subtracted from the mixed current trace to acquire an isolated (subtracted) AMPAR-mediated component (Red trace). Calibrated: 10 pA, 25 msec. **B.** Average AMPAR and NMDAR-mediated EPSCs expressed as a ratio following 2 and 7 days of either citalopram or vehicle administration (2 days: Citalopram n = 9, Vehicle n = 8; $P < 0.05$) (7 days: Citalopram n = 16, Vehicle n = 11).

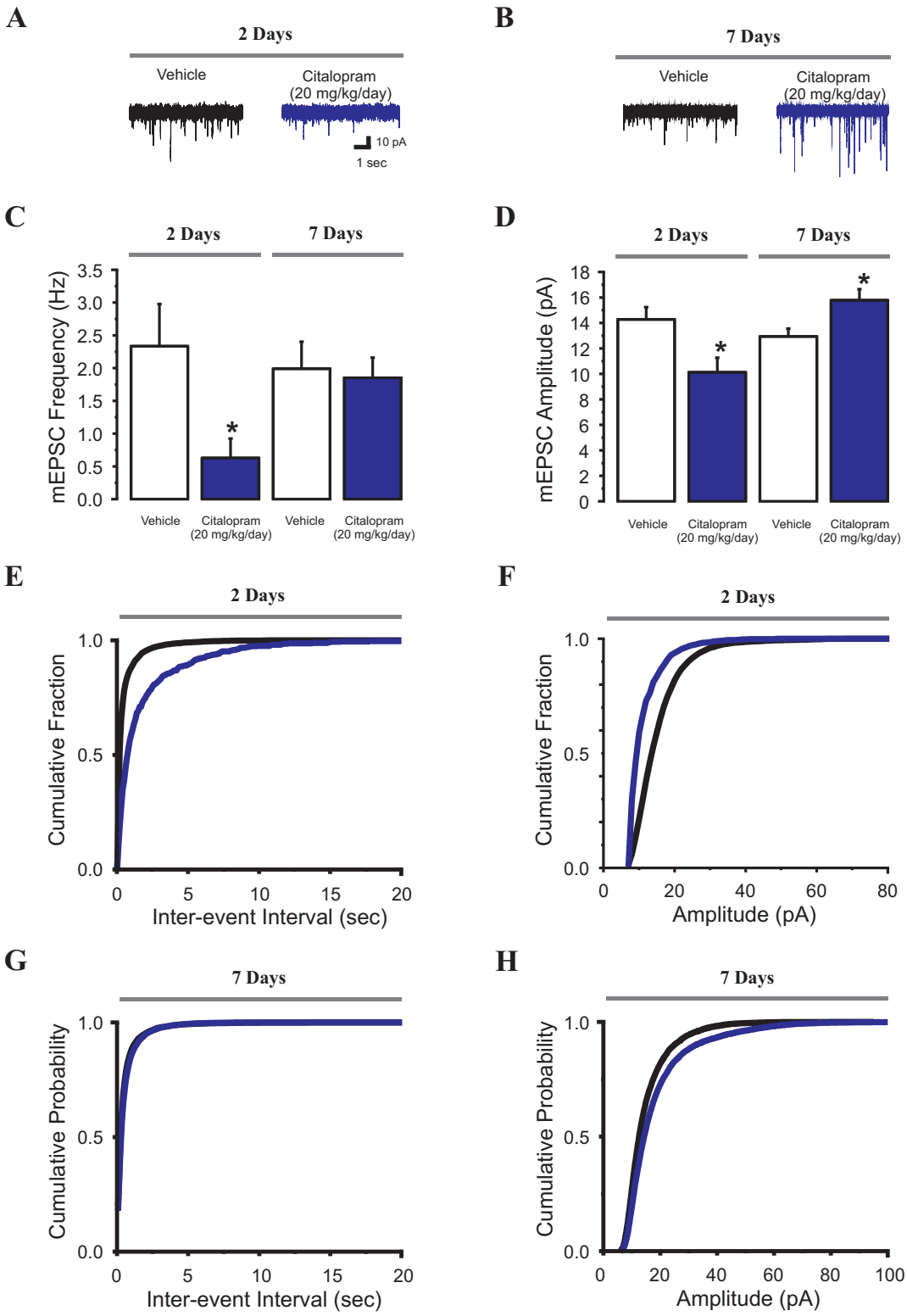

Figure 6: 2 days of citalopram administration depresses glutamatergic synaptic transmission in the DRN followed by an overcompensatory recovery in synaptic strength after 7 days of citalopram administration. A & B. Sample current traces showing mEPSCs recorded in vehicle (black traces) and citalopram treated animals (purple trace) following 2 and 7 days, respectively. C. Histogram of the average frequency of mEPSCs recorded in both vehicle (white) and citalopram (purple) treated animals following 2 (vehicle: n=12; citalopram: n=9; $p < 0.05$) and 7 days (vehicle: n= 24; citalopram: n=23; $p < 0.05$). D. Histogram of the average amplitude of mEPSCs recorded in both vehicle (white) and citalopram (purple) treated animals following 2 days (vehicle: n=12; citalopram: n=9; $p < 0.05$) and 7 days (vehicle: n= 24; citalopram: n=23; $p < 0.05$). E & G. Plots of the cumulative probability of the inter-event interval of neurons from vehicle and citalopram treated animals following 2 and 7 days, respectively. F & H. Plots of the cumulative probability of the mEPSC amplitude of neurons from vehicle and citalopram treated animals following 2 and 7 days, respectively.

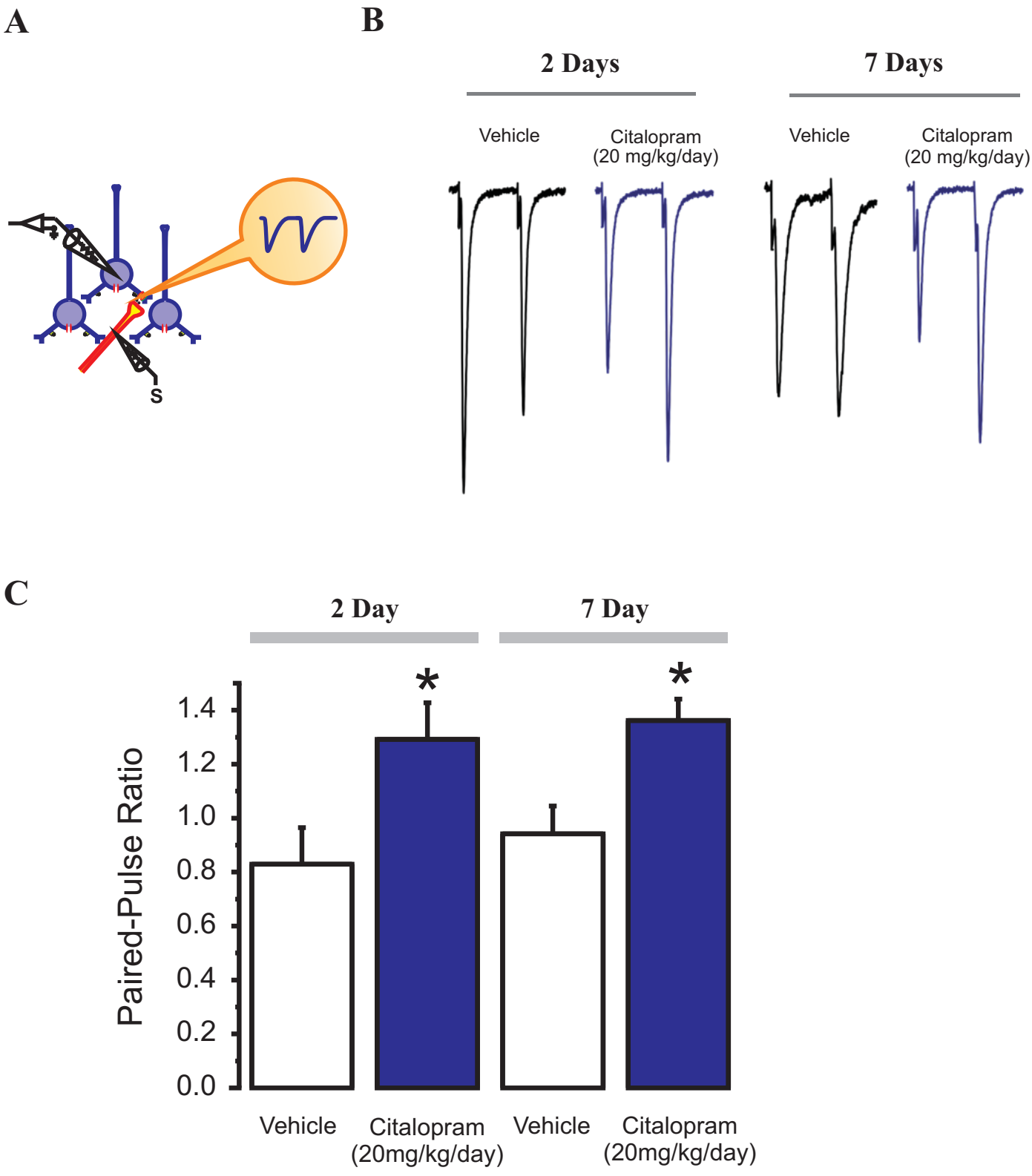

Figure 7: Citalopram induces an increase in paired-pulse ratio in animals following 2 and 7 days of treatment. **A.** Illustration of experimental outline. **B.** Representative paired-pulse traces of evoked AMPAR-mediated inward currents (ISI: 50 msec) following 2 and 7 days of vehicle (black) or citalopram (purple) treatments. **C.** Summary histogram depicting a citalopram induced increase in PPR following 2 and 7 days. (2 days: Citalopram n = 8, Vehicle n = 8; $P < 0.05$) (7 days: Citalopram n = 16, Vehicle n = 7; $p < 0.05$)

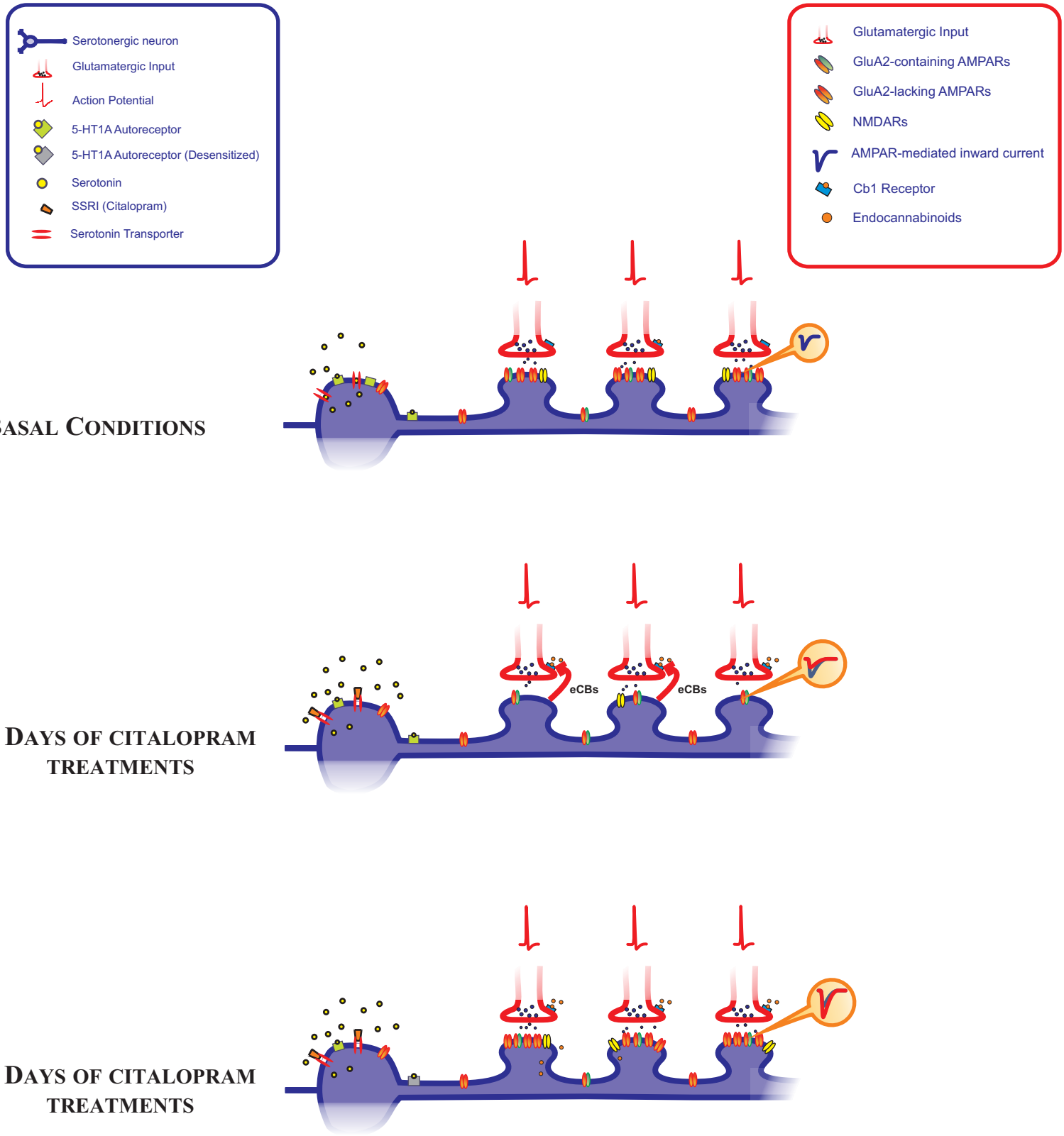

Figure 8:
PROPOSED MODEL OF ANTIDEPRESSANT-INDUCED SYNAPTIC PLASTICITY.

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