Effect of multi-target antidepressant strategies on monoamine systems: electrophysiological studies in the rat brain

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Acknowledgments

The scientific achievements of Dr. Blier and his contributions to the field of psychopharmacology have been well documented. Hence, here I recount the personal experience of having the privilege of being one of his graduate trainees.

From day one I was inspired by Dr. Blier's passion and optimism and his empowering and encouraging attitude toward his students. As my supervisor, I have always admired how Dr. Blier is the most humble individual when it comes to interacting with his students/employees.

I still vividly remember every instance he has lauded me for my good performance, and I remember more clearly how he has most gracefully encouraged and motivated me to be the best version of myself whenever my performance could have been improved.

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Statement of Contributions

Gathering and analysis of the data related to the first and second projects were done primarily by the present author with the support of Dr. El Mansari. In the third project, Michael Iro and Rami Hamati were also engaged in the process of data gathering and analysis. For all three journal publications, the present author has drafted the first manuscripts which were then reviewed and revised by Dr. Blier and Dr. El Mansari.

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino) tetralin
AADC	aromatic L-amino acid decarboxylase
AC	adenylyl cyclase
AMPT	α-methyl-para-tyrosine
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COMT	catechol-O-methyltransferase
CSF	cerebrospinal fluid
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter
DRN	dorsal raphe nucleus
ECT	electroconvulsive therapy
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GIRK channels	G protein-gated inwardly rectifying K^+ channels
GPCR	G protein-coupled receptor

G protein	guanine nucleotide-binding proteins
GRK	G protein-regulated kinase
GTP	guanosine triphosphate
HVA	homovanilic acid
IP_3	inositol triphosphate
i.p.	intraperitoneal
i.v.	intravenous
LC	locus coeruleus
L-DOPA	L-dihydroxyphenylalanine
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
MRN	median raphe nucleus
mTOR	mammalian target of rapamycin
NE	norepinephrine
NET	norepinephrine transporter
NRI	norepinephrine reuptake inhibitor
PCPA	para-chlorophenylalanine
PD	Parkinson's disease
PET	positron emission tomography
PFC	prefrontal cortex

PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PLD	phospholipase D
RD	raphe dorsalis
S.E.M.	standard error of the mean
SERT	serotonin transporter
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
VDCC	voltage dependent Ca^{2+} channel
VMA	vanillylmandelic acid
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area

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Abstract

There is a vast degree of heterogeneity in clinical presentations of major depressive disorder (MDD) symptoms among individual patients. Efficacious treatment strategies for individual MDD patients should address this heterogeneity and if necessary, modulate different neurotransmitter systems based on the individual profile of each patient. The overarching objective of the studies presented in chapters 2-4 was to determine how three different antidepressant strategies modulated the neurotransmission of monoamines.

Single-unit electrophysiological experiments were carried out using adult male Sprague-Dawley rats under chloral hydrate anesthesia. Recordings were obtained from the dorsal raphe nucleus (DRN) serotonin (5-HT), locus coeruleus (LC) norepinephrine (NE), and ventral tegmental area (VTA) dopamine (DA) neurons and the pyramidal neurons of the CA3 region of the hippocampus.

Long-term (14-day) administration of vortioxetine dampened the DA and NE neuronal activity, but to a lesser degree compared to escitalopram while increasing the N-methyl-D-aspartate (NMDA)-evoked responses of CA3 pyramidal neurons.

Long-term combined administration of aripiprazole with escitalopram has been previously shown to recover the escitalopram-induced inhibition of NE and DA neurotransmission. The same regimen (presented in chapter 3) resulted in a synergistic increase in overall 5-HT neurotransmission in the hippocampus.

Long-term individual administration of cariprazine increased the overall 5-HT neurotransmission while its combination with escitalopram was devoid of any effects. Furthermore, long-term administration of cariprazine increased the firing rate and bursting activity of LC NE neurons, and when combined with escitalopram it reversed the escitalopram-induced inhibition of the activity of these neurons.

The results of the abovementioned experiments demonstrated the distinctly heterogeneous effects of three different, yet comparable, pharmacotherapeutic strategies on neurotransmission modulation of monoamines. Based on the hypothesized neurotransmitter-symptom relations for MDD, the current results (along with findings of other preclinical and clinical studies) would offer clinicians potentially valuable information that provides them with the possibility of making evidence-based decisions for devising more efficacious pharmacotherapeutic strategies for individual MDD patients.

Chapter 1 — General Introduction

1. Definition of Major Depressive Disorder

Diagnosis of major depressive disorder (MDD) by th e Diagnostic and Statistical Manual of Mental Disorders (DSM–5; American Psychiatric Association, 2013) requires experiencing, at least, five or more of the following symptoms during the same two-week period. At least one of these symptoms should be depressed mood or loss of interest or pleasure.

1) Depressed mood most of the day, nearly every day.
2) Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day.
3) Significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day.
4) Insomnia or hypersomnia nearly every day.
5) Psychomotor agitation or retardation (observable by others, not merely subjective feelings of restlessness or being slowed down).
6) Fatigue or loss of energy nearly every day.
7) Feelings of worthlessness or excessive or inappropriate guilt nearly every day.
8) Diminished ability to think or concentrate, or indecisiveness, nearly every day.
9) Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

These symptoms have to cause clinically significant distress or impairment in social, occupational, or other important areas of functioning and the symptoms must also not be a result of substance abuse or another medical condition.

2. The Burden of Major Depressive Disorder

Major depressive disorder is ranked as the single most significant contributor to global disability by the World Health Organization (7.5% of all years lived with disability in 2015; World Health Organization, 2017). Selective serotonin (5-HT) reuptake inhibitors (SSRIs) are currently considered as the first line of pharmacotherapy for the treatment of MDD (Koenig et al., 2009), yet treatment resistance and incomplete remission remains to be one of the significant obstacles in the treatment of MDD with SSRIs and other classes of drugs used for the treatment of MDD as well as psychological interventions.

3. Serotonin and Pharmacotherapy of Major Depressive Disorder

SSRIs have been widely considered as the first-line pharmacotherapy of MDD (Koenig et al., 2009). Their popularity stems not only from their efficacy but also from their safe clinical profile since they have a low potential for overdose and are among the therapeutic options for populations at risk of suicide (Möller et al., 2008; Koenig et al., 2009). SSRIs inhibit the activity of the 5-HT transporter (5-HTT), and by doing so, their long-term administration (long-term and sustained administration in the relevant literature generally refers to an administration period of ≥14 days) increases extracellular levels of 5-HT (Wegener et al., 2003). This

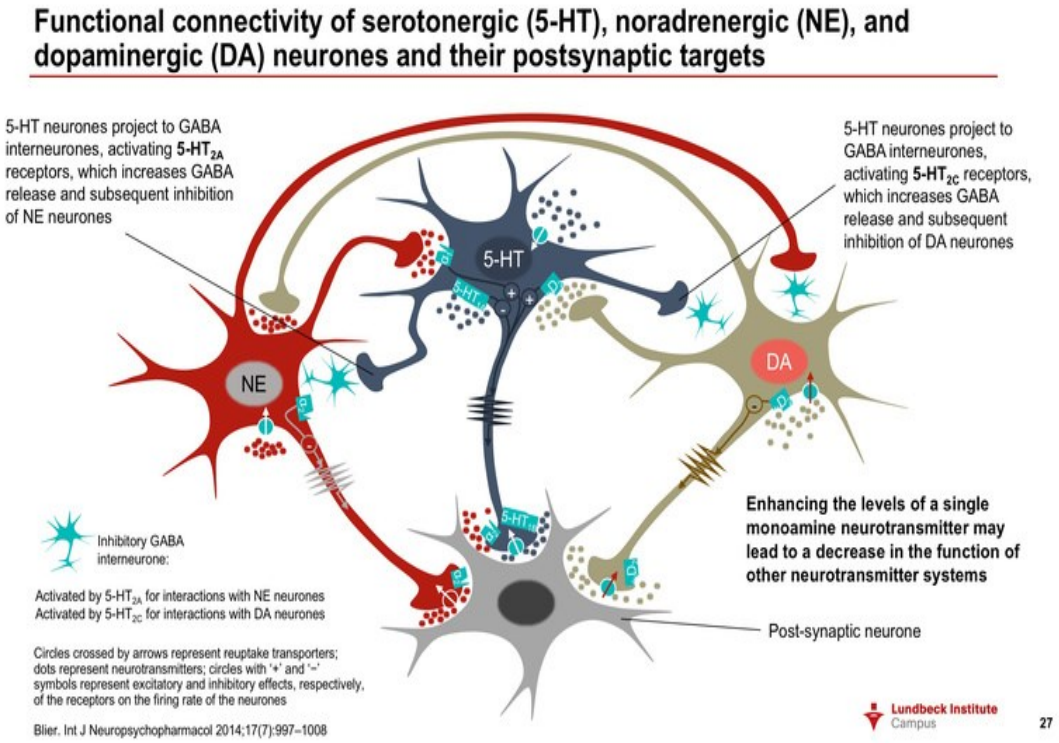
increase initially decreases the firing rate of 5-HT neurons of the dorsal raphe nucleus (DRN) by activating 5-HT_{1A} autoreceptors (Chaput et al., 1986; Blier and El Mansari, 2013). These receptors are desensitized following long-term SSRI administration which leads to the recovery of the firing rate of DRN 5-HT neurons.

4. Functional Interactions of the Serotonin System with Norepinephrine and Dopamine Systems

SSRI-induced increased 5-HT levels also suppress the firing activity of ventral tegmental area (VTA) dopamine (DA) neurons with no effect on the number of spontaneously active DA neurons (Dremencov et al., 2009). This is mediated by activation of 5-HT_{2C} receptors on GABA interneurons within the VTA controlling DA neuronal firing (Figure 1; Blier, 2014; Di Matteo et al., 2001; Dremencov et al., 2009; for more information, please refer to sections 7.1.5.2.3 and 7.4.2 of the current chapter). While 5-HT neurons regain their baseline firing rate with SSRI treatment prolongation, due to the desensitization of 5-HT_{1A} autoreceptors (Blier and de Montigny, 1983), the firing rate of DA neurons remains attenuated (Dremencov et al., 2009).

Sustained SSRI administration also inhibits the firing activity of locus coeruleus (LC) norepinephrine (NE) neurons by activating 5-HT_{2A} receptors, located on GABA interneurons controlling NE neuronal activity (Figure 1; Blier, 2014; Szabo and Blier, 2001a, b; for more information, please refer to sections 7.1.5.2.1 and 7.4.1 of the current chapter).

Figure 1. Schematic representation of the functional interactions of monoaminergic neurons (Blier, 2014).



5. Functional Interactions of Monoamines and Treatment-Resistant Depression

Due to the critical role of DA neuronal activity in motivation, anhedonia, and reward (for more information, please refer to sections 1 and 2 of chapter 5; Nestler and Carlezon, 2006), the SSRI-induced inhibition of DA neuronal firing might contribute, in some patients with MDD, to an incomplete or lack of response to SSRIs. In addition, the SSRI-induced attenuation of NE neuronal firing activity has also been hypothesized to contribute (Blier and El Mansari, 2013; Montoya et al., 2016), at least in part in some patients, to incomplete or lack of response to SSRIs in some patients with MDD.

6. Multi-target Pharmacotherapy for Tackling Treatment-resistant Depression

Due to the abovementioned functional interactions of monoamines, an efficacious treatment strategy should at least address the interconnectedness of these three systems. Such a strategy can be achieved with either a single drug targeting multiple targets (vortioxetine) or by augmentation strategies entailing the combination of two or more drugs (for example escitalopram plus aripiprazole or cariprazine). The reversal of the attenuated firing activity of NE neurons has been documented with quetiapine, aripiprazole, olanzapine, risperidone, and paliperidone (Chernoloz et al., 2012 and 2009; Seager et al., 2005; Dremencov et al., 2007a, b). Furthermore, this effect was shown to be exerted through the

blockade of 5-HT_{2A} receptors, which is a commonality between the abovementioned drugs. This implies that using 5-HT_{2A} receptor antagonists in SSRI-resistant patients might contribute to the therapeutic response.

Similarly, it has been shown that the DA and 5-HT partial agonist agent, aripiprazole, reverses the escitalopram-induced inhibition of the firing activity of VTA DA neurons (Chernoloz et al., 2009). This action is believed to be exerted, at least in part, by the blockade of 5-HT_{2C} receptors. Blockade of these receptors has been shown to stimulate DA outflow, suggesting that 5-HT_{2C} receptors predominantly located on inhibitory interneurons in the VTA are tonically activated (Millan et al., 1998; Di Matteo et al., 2000; Di Giovanni et al., 2001).

In addition, as explained in the DSM definition of MDD, there are multiple diagnostic symptoms of depression and different patients might receive the same diagnosis while experiencing different clusters of symptoms. Hence, given this heterogeneity, it is within the realm of possibility that different combinations of drugs would be beneficial for the treatment of different patients with different profiles of MDD.

As discussed, in the section on the functional interactions of monoamines, different regions and functions of the brain are anatomically and/or functionally interconnected. Hence a plausible strategy for clinical interventions for the dysfunctions of the brain, including MDD, is to address this complexity using a multi-target strategy.

7. Monoamine Systems

7.1 The Serotonin System

7.1.1 Behavioral and Physiological Significance

The 5-HT system is involved in the modulation of mood, sleep, aggression, cognition, memory, emesis, and feeding behavior. It has also been implicated in the pathophysiology of disorders including MDD, schizophrenia, obsessive-compulsive, and anxiety disorders (Aghajanian and Liu, 2009).

7.1.2 Neuroanatomy

5-HT was first discovered in the peripheral system (Rapport et al., 1948) and subsequently in the CNS (Bogdanski et al., 1956).

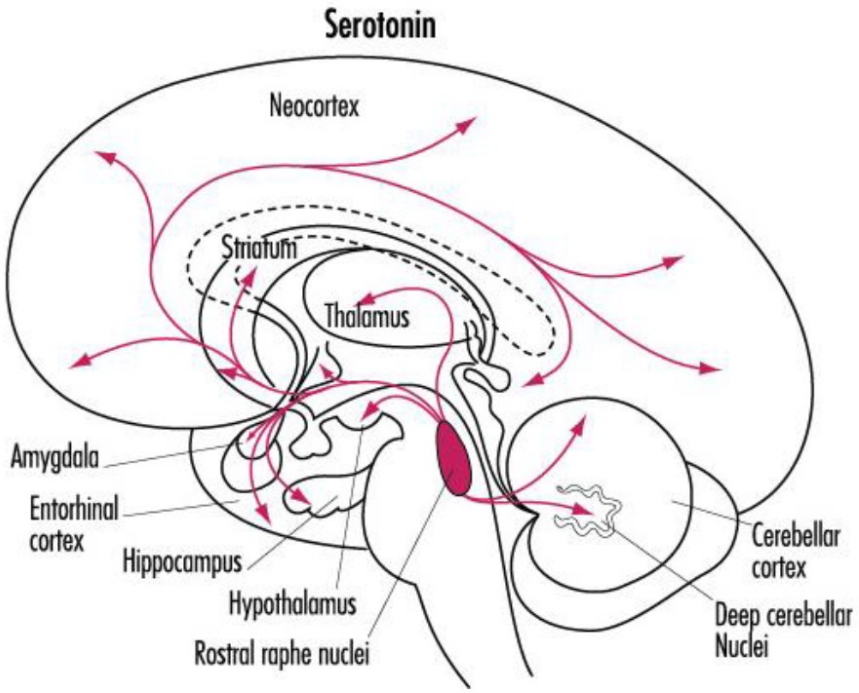
5-HT was initially discovered in the peripheral tissues and subsequently in the brain. In the 1950s, a similarity was discovered between 5-HT and d-lysergic acid diethylamide (LSD), a very potent hallucinogen. This structural similarity generated an interest that 5-HT might have an essential function in the brain and affect cognition and behavior (Aghajanian and Liu, 2009).

The majority of serotonin content in the body is outside of the central nervous system (CNS; 90% - mucous membranes of the gastrointestinal tract, 8% - blood platelets). The first 5-HT neurons were discovered in the brainstem by Ramon Cajal. Raphe nuclei were identified and named following the work of Dahlström and Fuxe (1964) which divided and named 9 units (B1-B9) of serotonin

nuclei along the brain stem midline. Following the work of Törk (1990), they were subdivided into rostral (B5-B9) and caudal (B1-B4) subunit. DRN (B6 and B7) and median raphe nucleus (MRN) are the significant sources of 5-HT innervation for the forebrain. The caudal part provides innervations for the medulla and spinal cord. Not all neurons in the raphe nuclei are serotonergic; for example, 50-75% of DRN and 70-80% of MRN neurons are non-serotonergic (Moss and Glazer et al., 1981; Descarries et al., 1982).

Although 5-HT neurons do not comprise a significant portion of the neuronal population of the brain (Okaty et al., 2019; approximately 20,000 in rats and 450,000 in humans), there are numerous projections from raphe nuclei to other parts of the brain, and there are not many structures in the brain that do not receive 5-HT innervation. Hence the 5-HT system is strategically positioned to influence many functions of the brain including mood, sleep, aggression, cognition, memory, emesis, and feeding behavior, as well as the pathophysiology of disorders including MDD, schizophrenia, obsessive-compulsive, and anxiety disorders.

Figure 2. Major 5-HT pathways in the human brain (Heimer, 1995).



7.1.3 Serotonin Synthesis, Storage, Release, and Metabolism

Since the 5-HT circulating the peripheral tissue cannot cross the brain-blood barrier, neurons in the brain synthesize their 5-HT from tryptophan. Initially, the essential amino acid tryptophan is hydroxylated by tryptophan hydroxylase (TPH). This enzyme is the rate liming enzyme in the synthesis of 5-HT. There are two forms of this enzyme: THP1 and 2. THP1 is present in the periphery and the CNS. On the other hand, THP2 is exclusively present in the CNS (Walther et al.,

2003) and since it is only expressed in 5-HT neurons, it is an excellent chemical marker for the identification of these neurons.

In the 5-HT neurons, there are two pools of 5-HT (Tracqui et al., 1983). The first one, or the functional reserve, contains 20% of newly synthesized 5-HT while the other pool containing 80% of 5-HT, acts as a reservoir. The release of 5-HT from these pools and into the synapse is a Ca^{2+} dependent phenomenon and happens through exocytosis.

Degradation of 5-HT is done by monoamine oxidase (MAO) through oxidative deamination that produces 5-hydroxyindoleacetic acid (5-HIAA) which can find its way into the cerebrospinal fluid (CSF) and for that reason it can act as an indicator for the turnover of 5-HT in the brain. There are two different types of MAO. MAO-A is ten-fold more potent than the MAO-B to catabolize 5-HT, but 5-HT neurons express more of the MAO-B (Westlund et al., 1985; Konradi et al., 1988).

7.1.4 Serotonin Transporter

The primary method for inactivating 5-HT in the synaptic cleft is through reuptake mediated by the 5-HTT. 5-HTTs are present in both the CNS and the periphery, for example in platelets (Qian et al., 1995), lung membrane, and placenta (Cool et al., 1990; Ramamoorthy et al., 1993). There have been several research projects on the regional variations in the binding of different drugs to 5-HTT using radioligands (Langer et al., 1980; Habert et al., 1985; D'Amato et al.,

1987). For example, SSRIs have a lower binding density in postsynaptic areas, like the cortex and hippocampus, compared to the tricyclic drug imipramine. Because of the critical role of 5-HTT in regulating the amount of 5-HT in the synapse, it has been the target of a variety of drugs with antidepressant properties including SSRIs, serotonin, and norepinephrine reuptake inhibitors (SNRIs), and multimodal drugs like vortioxetine. For more information about vortioxetine please refer to chapter 2.

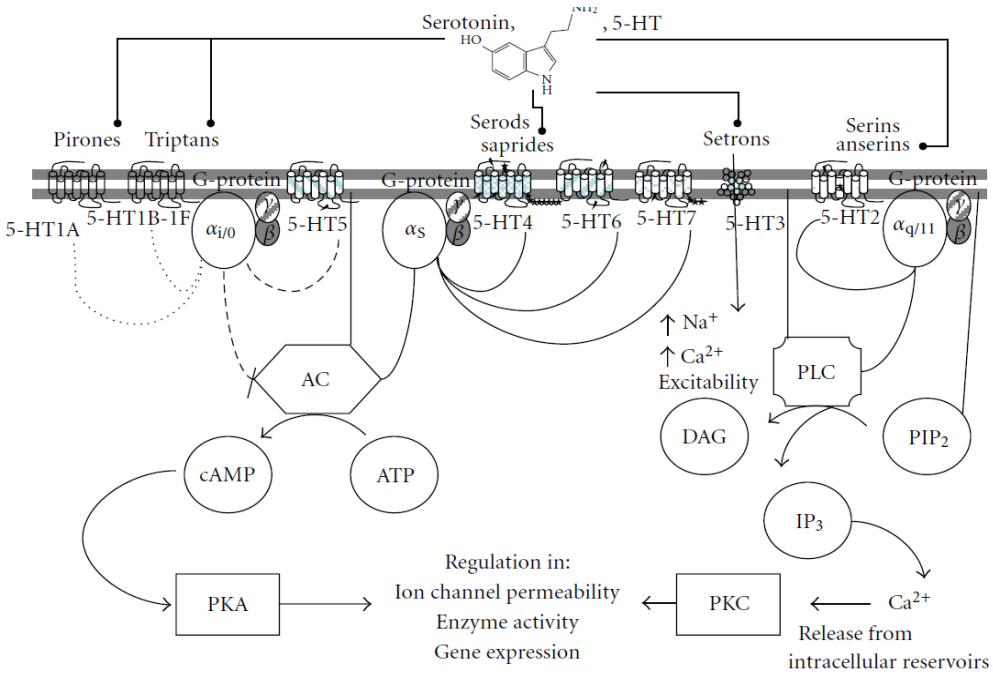
7.1.5 Serotonin Receptors

Seven families of 5-HT receptors have been identified so far. All these identified families of 5-HT receptors are guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) except the 5-HT_3 subgroup, which are ligand-gated ion channels. Among these G proteins, $G_{\alpha s}$ is linked to 5-HT_4, 5-HT_6, and 5-HT_7 receptors, $G_{\alpha i}/G_{\alpha o}$ proteins are linked to 5-HT_1 and 5-HT_5 receptors, and $G_{\alpha q/11}$ and $G\alpha_{12/13}$ proteins to 5-HT_2 receptors (Bockaert et al, 2006).

Activation of $G_{\alpha s}$ proteins increases intracellular levels of cyclic adenosine monophosphate (cAMP) through stimulating the adenylyl cyclase. Increased levels of cAMP activate protein kinase A (PKA) which in turn regulates a variety of cellular functions including ion channel permeability, enzyme activity, and gene expression (Bockaert et al, 2006). On the contrary, activation of $G_{\alpha i}/G_{\alpha o}$ proteins inhibits the activity of adenylyl cyclase, decreasing the production of cAMP which

in turn has a negative impact on the activity of PKA and its downstream targets (Nichols and Nichols, 2008).

Figure 3. Main intracellular 5-HT signaling pathways. AC, adenylate cyclase; PLC, phospholipase C; PKC, protein kinase C; PKA, protein kinase A (Berumen et al., 2012).



$G_{\alpha q/11}$ proteins activate phospholipase C (PLC) which in turn hydrolysis phosphatidylinositol 4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 elevates the intracellular levels of calcium which in turn, among other consequences, leads to activating the protein kinase C (PKC). This protein kinase regulates a variety of cellular functions including, ion channel permeability, enzyme activity, and gene expression (Nichols and Nichols, 2008; Werry et al., 2005). Activation of the $G_{\alpha 12/13}$ proteins leads to structural changes

within the cells via phospholipase A (PLA), and phospholipase D (PLD), and the Rho signaling pathway (Hannon and Hoyer, 2008).

7.1.5.1 Serotonin$_1$ Receptors

These GPCRs provide binding sites for the endogenous neurotransmitter 5-HT and mediate inhibitory neurotransmission through their coupling with G_i/G_o (Hannon and Hoyer, 2008; for more information on G proteins please refer to section 7.1.6. of chapter 1).

7.1.5.1.1 Serotonin$_{1A}$ Receptors

Members of this family are coupled to G_i/G_o G proteins and exert their effects by targeting the G protein-gated inwardly rectifying K^+ (GIRK) channels. 5-HT$_{1A}$ receptors inhibit adenylyl cyclase through coupling with G_i/G_o. High levels of 5-HT$_{1A}$ receptors are expressed in the DRN, hippocampus, lateral septum, and cerebral cortex (Aghajanian and Liu, 2009). 5-HT$_{1A}$ receptors can be divided into autoreceptors and heteroreceptors or postsynaptic receptors.

7.1.5.1.2 Serotonin$_{1A}$ Autoreceptors

Presynaptic 5-HT$_{1A}$ receptors on somatodendritic regions of 5-HT neurons have been named autoreceptors. 5-HT neurons are negatively modulated by these autoreceptors (Aghajanian and Liu, 2009). This negative modulation takes place by decreasing intracellular levels of cAMP which increases the opening of

GIRK channels (William et al., 1998) which in turn leads to hyperpolarization of these neurons (Hannon and Hoyer, 2008).

These autoreceptors desensitize following long-term activation which leads to the recovery of the firing rate of DRN 5-HT neurons (Blier and de Montigny, 1983; please refer to section 7.1.7. of chapter 1). There are three different underlying mechanisms for this desensitization: reduced surface expression (Welner et al., 1989), internalization (Riad et al., 2004), and reduced G protein coupling (Hensler, 2002). These receptors can be activated both directly for example by gepirone, vortioxetine, and aripiprazole, or indirectly by SSRIs and monoamine oxidase inhibitors (MAOIs).

7.1.5.1.3 Postsynaptic Serotonin$_{1A}$ Receptors

These receptors have a significant role in inhibiting the postsynaptic 5-HT projection areas. For example, it has been shown that the micro-iontophoretic application of 5-HT inhibits the firing of pyramidal neurons of the CA3 region of the hippocampus both in anesthetized and awake animals (de Montigny, 1984). This effect can be blocked by the application of the 5-HT$_{1A}$ antagonist WAY 100635. The effect of 5-HT$_{1A}$ autoreceptors in the DRN and 5-HT$_{1A}$ receptors in the hippocampus is mediated by G_i proteins (Blier et al., 1993).

Different drugs have different properties for these two types of 5-H$_{1A}$ receptors. For example, gepirone and ipsapirone act as full agonists at autoreceptors in the DRN but partial agonists at postsynaptic 5-HT$_{1A}$ receptors

(Blier and de Montigny, 1990; Dong et al., 1997). Similarly, cariprazine which acts as a partial agonist at 5-HT_{1A} autoreceptors *in vitro* (Kiss et al., 2010) has been shown *in vivo* to be a full agonist at 5-HT_{1A} receptors in the hippocampus (Herman et al., 2018).

Unlike 5-HT_{1A} autoreceptors, the hippocampus 5-HT_{1A} receptors do not desensitize following long-term administration of a 5-HT_{1A} agonist (Blier and de Montigny, 1990; Godbout et al., 1991) or an SSRIs (de Montigny et al., 1990) and in fact, some antidepressant strategies have been shown to increase the sensitivity of these receptors, including electroconvulsive therapy (ECT) and tricyclic drugs used for the treatment of MDD (de Montigny and Aghajanian, 1978; de Montigny, 1984; please refer to table 1).

It has been shown that increasing 5-HT neurotransmission signaling through postsynaptic 5-HT_{1A} receptors is a mechanism shared by antidepressant strategies and might underlie at least in part the clinical effect of strategies used to treat MDD (Haddjeri et al., 1998).

7.1.5.1.4 Serotonin$_{1D/1B}$ Receptors

Unlike the somatodendritic 5-HT_{1A} autoreceptors, dendrodendritic 5-$HT_{1D/1B}$ receptors are located on the synaptic terminals and control the release of 5-HT into the synaptic cleft (Piñeyro et al., 1995; Piñeyro and Blier, 1996). These receptors have also been shown to form dendrodendritic synapses (Mamounas et al., 1991; Stamford et al., 2000). These two receptors are both targets of

vortioxetine as this drug is a partial agonist of 5-HT_{1B} and an antagonist of 5-HT_{1D} receptors. Similar to 5-HT_{1A} receptors, 5-HT_{1B} autoreceptors also desensitize after long-term SSRI treatment which leads to increased 5-HT release and might contribute to the clinical efficacy of SSRIs (Chaput et al., 1986 and 1991; Blier et al., 1988).

7.1.5.2 Serotonin$_2$ Receptors

5-HT_2 receptors constitute another subfamily of the 5-HT receptors. These receptors are coupled to G_q/G_{11} proteins to exert their excitatory effects. There are three types of these receptors namely 5-HT_{2A}, $_{2B,}$ and $_{2C}$ receptors (Hannon and Hoyer, 2008).

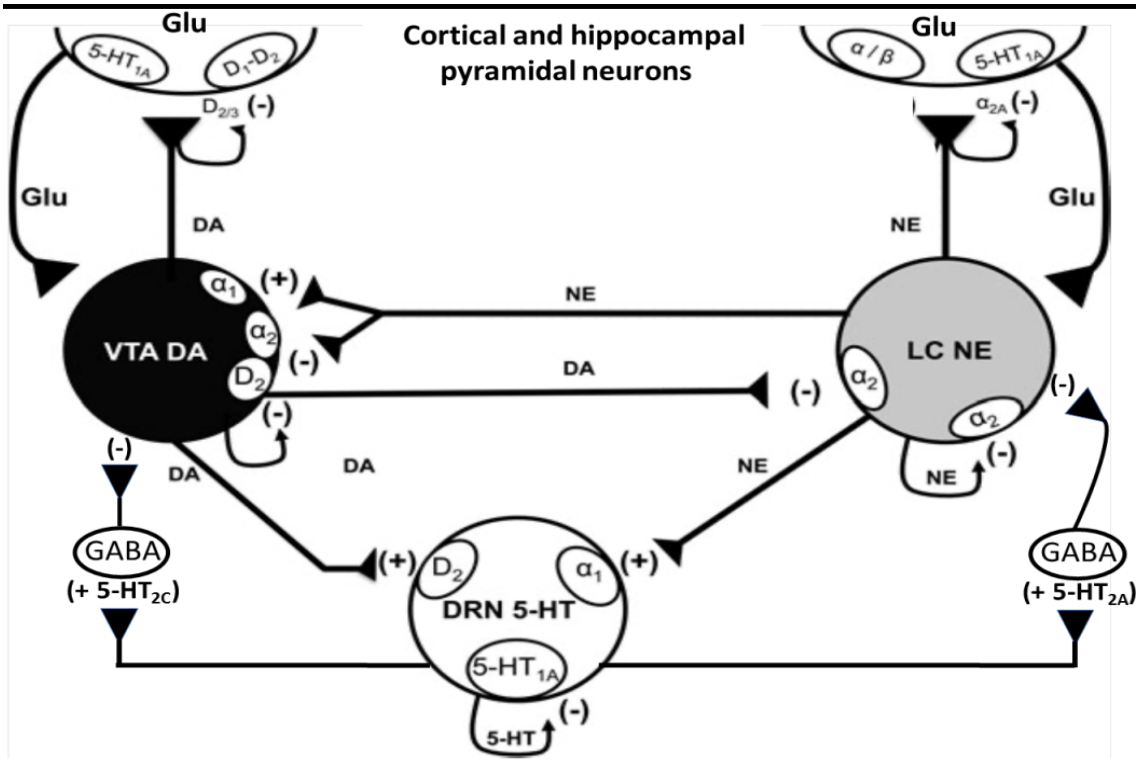
7.1.5.2.1 Serotonin$_{2A}$ receptors

These are postsynaptic receptors expressed in different brain regions including the neocortex, entorhinal and piriform cortices, hippocampus, caudate nucleus, nucleus accumbens, and olfactory tubercles (López-Giménez et al., 1997). These receptors are also expressed on GABA neurons controlling the firing activity of LC NE neurons (Figure 4) and accordingly, it has been shown that blocking these receptors block the SSRI-induced inhibition of the firing activity of these neurons (Szabo and Blier, 2001b). It is worth mentioning that both aripiprazole and cariprazine act as antagonists of these receptors as demonstrated by *in vivo* investigations (Chernoloz et al., 2009; Kiss et al., 2010; Herman et al., 2018).

In the cortex, these receptors are expressed on both glutamatergic pyramidal neurons and GABAergic interneurons. These receptors are also expressed (with a lower density compared to the cortex) on the glutamatergic pyramidal neurons of the hippocampus (Pompeiano et al., 1994; Burnet et al., 1995).

5-HT_{2A} receptors are coupled to PLC which induces mobilization of intracellular Ca^{2+} (Pritchett et al., 1988). These receptors also have a regulatory effect on releasing dopamine, glutamate, and GABA along with a regulatory effect on the firing activity of DA neurons. These receptors increase the release of glutamate from the pyramidal neurons of the prefrontal cortex (PFC; Aghajanian and Marek, 1999) and also release of GABA onto CA1 pyramidal neuron of the hippocampus (Shen and Andrade, 1998).

Figure 4. Schematic representation illustrating part of the interactions between 5-HT neurons of the dorsal raphe nucleus, NE neurons of the locus coeruleus, DA neurons of the ventral tegmental area, and glutamatergic neurons of the hippocampus and the cortex (Farah and Blier, 2020).



7.1.5.2.2 Serotonin$_{2B}$ Receptors

These receptors are expressed on the DRN 5-HT neurons and have been suggested to have a role in the antidepressant response (Auclair et al., 2010; Diaz et al., 2012). It has been shown that activation of these receptors exerts antidepressant-like effects in the forced swim test (FST) and also antagonism of these receptors prevented the antidepressant-like effects of SSRIs in the FST (Diaz et al., 2012). In addition, it has been shown that 2-day administration of the

selective 5-HT_{2B} partial agonist LY266097 increases 5-HT activity (Hamati et al., 2019).

It has been shown that long-term antagonism of these receptors increases the firing activity of VTA DA neurons (Chenu et al., 2014). Besides, acute injection of the 5-HT_{2B} receptor agonist BW72386 has been shown to decrease the bursting but not the firing activity of dopamine neurons (Hamati et al., 2019). In the medial PFC (mPFC) blocking 5-HT_{2B} receptors might potentiate the activity of pyramidal neurons either by targeting these receptors or by facilitating mesocortical dopaminergic neurotransmission (Hamati et al., 2020). The clinical use of 5-HT_{2B} antagonists for the treatment of MDD needs to be examined carefully since antagonizing these receptors can cause pulmonary and cardiovascular side effects (Artigas, 2013).

7.1.5.2.3 Serotonin$_{2C}$ Receptors

These are postsynaptic receptors that have been shown to have a role in regulating DA neurotransmission. These receptors have a widespread expression in different brain areas including the cortex, amygdala, hippocampus, substantia nigra, caudate nucleus, and cerebellum (Abramowski et al., 1995). Activation of these receptors leads to membrane depolarization and may mediate some of the excitatory effects of 5-HT in multiple brain areas including the cortical pyramidal neurons (Sheldon and Aghajanian, 1991).

Stimulation of these receptors in substantia nigra blocks DA release in the striatum. Furthermore, blocking these receptors by atypical drugs used for the treatment of schizophrenia, for example aripiprazole and cariprazine, stimulate DA release in the striatum which could mitigate extrapyramidal symptoms (EPS) caused by typical drugs, or pure DA antagonists, used for the treatment of schizophrenia. 5-HT_{2C} receptors are expressed mostly on the cell bodies of both GABA and DA neurons within VTA (Figure 4, Farah and Blier, 2020; Bubar and Cunningham, 2007). Although these receptors are primarily expressed on the cell's soma (indicating post-synaptic localization) there is evidence suggesting that some of these receptors are expressed on terminal boutons of both GABA and DA neurons (Bubar and Cunningham, 2007).

7.1.5.3 Serotonin$_3$ Receptors

These receptors are ligand-gated ion channels and in that regard, they are structurally different from other 5-HT receptors that are GPCRs. These receptors are cation-selective and regulate the depolarization and excitation in the CNS and peripheral nervous system (PNS; Barnes et al., 2009).

These postsynaptic receptors modulate the release of 5-HT, NE, and DA mainly through regulating inhibitory GABA neurons. These receptors have a role in causing nausea and emesis, therefore blocking these receptors is a strategy to reduce nausea and vomiting caused by cancer chemotherapy, which releases 5-HT due to tissue lysis. Mirtazapine which is used for the treatment of MDD is a

potent 5-HT_3 antagonist, which has been used to reduce nausea produced by inhibition of 5-HT reuptake (Pedersen and Klysner, 1997).

It has also been shown that 5-HT_3 antagonism potentiates increased extracellular levels of 5-HT caused by 5-HTT blockade (Mørk et al., 2012). Accordingly, it is hypothesized that this property might contribute to the 5-HT enhancing capacity of vortioxetine.

7.1.5.4 Serotonin$_6$ Receptors

These receptors are exclusively expressed in the CNS with the highest densities in brain areas involved in cognitive processes (Chaumont-Dubel et al., 2020). 5-HT_6 receptors are postsynaptic receptors coupled to the G_s pathway (Sebben et al., 1994) and stimulation of these receptors activates the Erk 1/2 MAP kinase pathway (Yun et al., 2007). These receptors also interact with Fyn, Jab1 proteins (Yun et al., 2007 and 2010), light chain 1 (LC1) subunit of MAP1B protein (MAP1B-LC1; Kim et al., 2014), and neuro-oncological ventral antigen 1 (Nova-1; Kim et al., 2019).

There is evidence indicating that 5-HT_6-mediated upregulation of the mammalian target of rapamycin (mTOR) might contribute to the cognitive deficit evident in some patients with schizophrenia. Antagonists of these receptors have been extensively investigated and have been shown to possess pro-cognitive properties in rodent models of schizophrenia and Alzheimer's disease (AD) along with human studies (Chaumont-Dubel et al., 2020). The effects of these receptors

on cognition in AD are speculated to be exerted via modulation of the length of cilia and morphology of Axonal Initial Segment (AIS; Yamada and Kuba, 2016).

Agonists of 5-HT_6 receptors have also been investigated for several applications including (but not limited to) cognitive function, mood, depression, and anxiety (Jastrzebska-Wiesek et al., 2014; Nikiforuk et al., 2011). However, the results of such investigations remain inconclusive. These receptors are also believed to be involved in the anti-apoptotic effects of 5-HT (through Jab1 activation) and neuronal migration (Riccio et al., 2009; Vitalis and Parnavelas, 2003).

7.1.5.5 Serotonin$_7$ Receptors

These are postsynaptic receptors coupled to G_s, and hence their activation leads to increased production of cAMP (Bard et al., 1993). They are expressed in a variety of peripheral tissues (smooth muscles and vasculature) and different areas of the brain including the thalamus, hypothalamus, hippocampus, and cortex (Vanhoenacker et al., 2000).

The antagonism of these receptors has been shown to modulate the circadian rhythm, sleep, and mood in animal studies (Hedlund et al., 2005). Several medications used for the pharmacotherapy of MDD, including vortioxetine, have a moderate affinity for these receptors. Also, several DA and 5-HT partial agonists have an affinity for these receptors including aripiprazole, cariprazine (low affinity), and asenapine (Shahid et al., 2009; Kiss et al., 2010).

As is the case with 5-HT_3 receptors, blocking the 5-HT_7 receptors when combined with an SSRI causes an increase in extracellular levels of 5-HT more than the SSRI alone (Bonaventure et al., 2012).

7.1.6 G Proteins: Intracellular Mechanisms of Action

5-HT receptors are GPCRs except for the 5-HT_3 receptors which belong to the Cys-loop superfamily of ligand-gated ion channels (LGICs). 5-HT_1 receptors are coupled to $G_{\alpha i}/G_{\alpha o}$ proteins, 5-HT_2 receptors to $G_{\alpha q/11}$ and $G\alpha_{12/13}$ proteins, 5-HT_4 receptors to $G_{\alpha s}$, 5-HT_5 receptors to $G_{\alpha i}/G_{\alpha o}$ proteins, and 5-HT_6 and 5-HT_7 receptors to $G_{\alpha s}$ proteins (Bockaert et al., 2006).

G proteins belong to the larger group of enzymes called guanosine triphosphate (GTP)ases. These proteins are involved in the transmission of signals from outside to the inside of cells. The activity of G proteins is modulated by factors that affect their propensity to bind and hydrolase GTP to guanosine diphosphate (GDP). The GTP-bound conformation is the “on”, while the GDP-bound conformation is the “off” state of these proteins. G proteins are either monomeric small GTPases or heteromeric G protein complexes. These complexes consist of alpha (α), beta (β), and gamma (γ) subunits. The latter two can form a dimeric complex called the beta-gamma complex (Hurowitz et al., 2000).

The binding of hormones, neurotransmitters, and other signaling factors to the outside domain of the GPCRs results in the activation of G proteins and

cascades of signaling pathways regulating metabolic enzymes, ion channels, transporter proteins, and other parts of the cell machinery (Neves et al., 2002). For example, activation of $G_{\alpha s}$ proteins stimulates adenylyl cyclase and increases intracellular levels of cAMP. Increased levels of cAMP activate PKA which regulates a variety of cellular functions including ion channel permeability, enzyme activity, and gene expression (Bockaert et al., 2006).

On the other hand, $G_{\alpha i}$/$G_{\alpha o}$ proteins inhibit the activity of adenylyl cyclase and consequently decrease cAMP production and have a negative effect on the activity of PKA (Figures 5 and 6; Nichols and Nichols, 2008). Activation of $G_{\alpha q/11}$ proteins has a positive impact on the activity of PLC which leads to increased hydrolysis of PIP_2 into DAG and IP_3. IP_3, in turn, elevates intracellular levels of calcium, which has several consequences including the activation of PKCs. Activation of these proteins affects a variety of cellular functions including ion channel permeability, enzyme activity, and gene expression (Nichols and Nichols, 2008; Werry et al., 2005). When activated, $G_{\alpha 12/13}$ proteins exert structural changes within the cells by modulating the activity of PLA, PLD, and the Rho signaling pathway (Hannon and Hoyer, 2008).

Figure 5. Signaling of 5-HT_{1A} receptors in 5-HT neurons. The net effect of the activation of these receptors is reducing neuronal activation. In these neurons, signal transduction occurs primarily via $G_{\alpha i3}$. These subunits inhibit the activity of adenylyl cyclases (AC) and protein kinase A (PKA) which, in turn, reduces the opening of L-type voltage-dependent Ca^{2+} channels (VDCCs). The Gβγ subunit has a variety of effects including the closing of N-type VDCCs, opening of G protein inwardly rectifying K^{+} (GIRK) channels, inhibition of ERK1/2 signaling, and increasing intracellular Ca^{2+} levels by activating PLCβ2 (Albert and Vahid-Ansari, 2019).

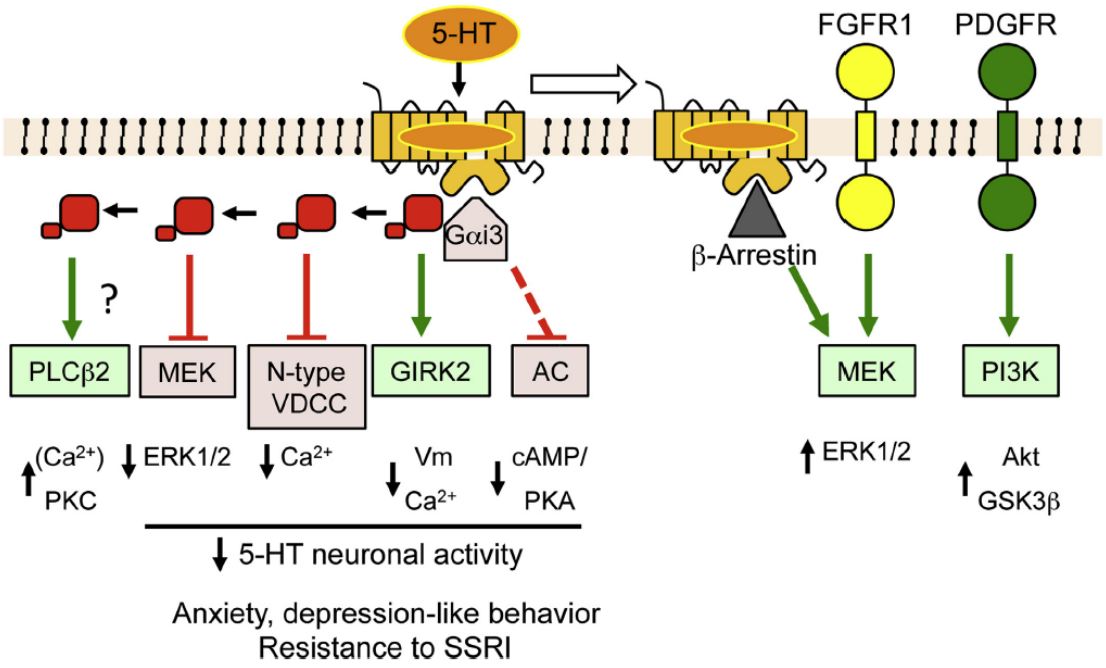
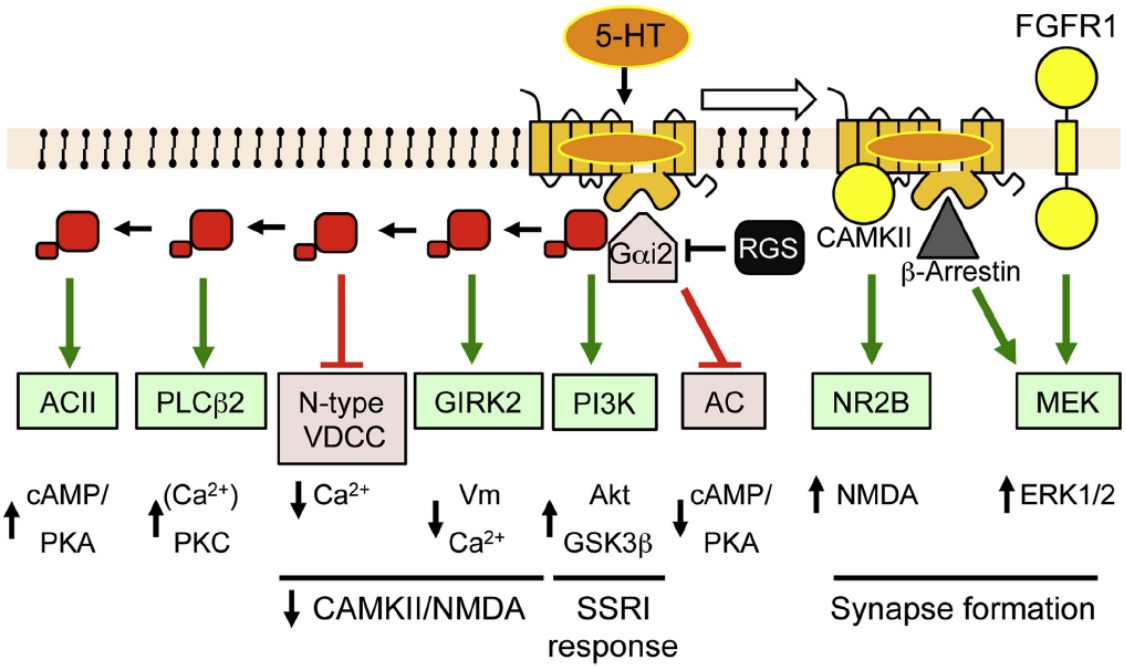


Figure 6. Signaling of 5-HT_{1A} receptors in the hippocampal and cortical neurons. In these neurons, transduction happens primarily via $G_{\alpha i2}$ which inhibits the activity of adenylyl cyclase (AC) and PKA which leads to the reduced opening of L-type VDCCs. In the hippocampus, the Gβγ subunit activates Akt through activation of phosphatidylinositol 3-kinase (PI3K) which is hypothesized to contribute to the antidepressant effects of SSRIs. Other effects of the Gβγ subunit include the opening of GIRK channels, closing of N-type VDCCs, and reducing Ca^{2+} levels leading to reduced excitability. However, these subunits may also activate AC and PKA, and increase Ca^{2+} levels by activating PLCβ2 and PKC in specific neurons (Albert and Vahid-Ansari, 2019).



7.1.7 Desensitization of Serotonin$_{1A}$ Receptors

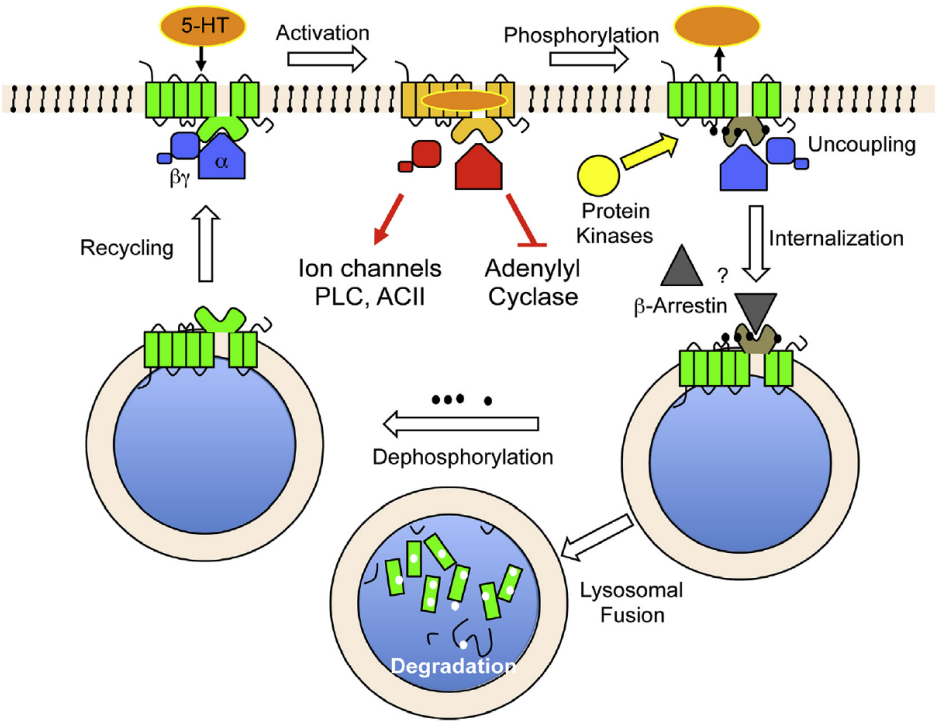
Several mechanisms could be involved in agonist-induced desensitization of 5-HT$_{1A}$ receptors. One of these mechanisms, which happens seconds after the activation of these receptors by an agonist, is the rapid and reversible uncoupling of these receptors. The G protein-regulated kinase (GRK), PKC, and PKA are hypothesized to be involved in the acute agonist-induced desensitization of these receptors. 5-HT has been shown to cause decoupling of these receptors from G proteins and induce receptor hyperphosphorylation (Nebigil et al., 1995). This phosphorylation is not blocked by PKA and PKC inhibitors, and GRK is hypothesized to be recruited for this process. Experiments in transformed cell lines with overexpression of β-arrestins have indicated the involvement of β-arrestin in agonist-induced desensitization of 5-HT$_{1A}$ receptors. Co-expression of β-arrestin and GRK2 increases agonist-induced internalization of 5-HT$_{1A}$ receptors in HEK-293 cells (Figure 7; Heusler et al., 2008).

PKA, PKC, or both, can uncouple 5-HT$_{1A}$ signaling in different cell types (Albert and Vahid-Ansari, 2019). The evidence for the involvement of PKA and PKC has been, partly, obtained from studies indicating that PKA and PKC phosphorylate 5-HT$_{1A}$ receptors, and PKA increases PKC-dependent desensitization of these receptors in non-neuronal cells (Raymond, 1991; Raymond and Olsen, 1994). The PKC-dependant phosphorylation process uncouples 5-HT$_{1A}$ receptors from inhibition of N-type voltage-dependent Ca^{2+}

channels (VDCCs) leading to increased calcium mobilization (Lembo and Albert, 1995; Chen and Penington, 1996; Wu et al., 2002).

Seconds to minutes following the uncoupling, the internalization of receptors into clathrin-coated vesicles begins. This internalization is initiated by receptor phosphorylation, recruitment of β-arrestins, and formation of clathrin-coated pits. It is worth mentioning that these internalized receptors can be involved in signaling pathways including coupling to mitogen-activated protein kinase (MAPK; Ferguson, 2001; Shenoy and Lefkowitz, 2003; Albert and Lemonde, 2004). Finally, following hours of activation by the agonist, the internalized receptors are fused with lysosomes and get degraded (Figure 7; Shenoy and Lefkowitz, 2003; Albert and Vahid-Ansari, 2019).

Figure 7. Internalization of 5-HT_{1A} receptors. The binding of 5-HT to these receptors and the consequent GTP exchange leads to the activation of the receptor-G protein complex (illustrated in orange). The subunits of the G protein exert multiple effects including inhibition of adenylyl cyclases (via $G_{\alpha i/o}$) and regulation of K^+ and Ca^{2+} ion channels to inhibit neuronal activity (for more information, please refer to figures 5 and 6). Agonist-induced internalization of 5-HT_{1A} receptors in raphe neurons is hypothesized to be due to the recruitment of G protein-regulated kinase (GRK) and β-arrestins. Through a process of dephosphorylation, these receptors can be recycled back to the plasma membrane. However, downregulation of these receptors might also be done via lysosomal fusion and consequent degradation of these receptors (Albert and Vahid-Ansari, 2019).



7.1.7.1 Physiological and Clinical Relevance of the Changes in the Sensitivity of Serotonin$_{1A}$ and Serotonin$_{1B}$ Receptors

The therapeutic benefits of a large variety of antidepressant strategies are hypothesized to be exerted, at least in part, through increasing 5-HT neurotransmission (Blier and El Mansari, 2013). Alterations in the sensitivity of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors can contribute to this increased 5-HT neurotransmission. 5-HT$_{1A}$ autoreceptors are desensitized with a variety of antidepressant treatment strategies including SSRIs, SNRIs, MAOIs, 5-HT$_{1A}$ agonists, mirtazapine, bupropion, and vortioxetine. On the other hand, the sensitivity of postsynaptic 5-HT$_{1A}$ receptors is increased with tricyclic antidepressants (TCAs) and electroconvulsive shocks (ECS). 5-HT$_{1B}$ autoreceptors are desensitized following long-term administration of SSRIs and vortioxetine (Table 1; Blier and El Mansari, 2013; El Mansari et al., 2015).

5-HT$_{1A}$ autoreceptors partake in autoregulatory negative feedback on the discharge activity of 5-HT neurons (Aghajanian, 1978; Hjorth et al., 1982) and desensitization of these receptors recovers the reduced firing rate of DRN 5-HT neurons. Unlike the 5-HT$_{1A}$ autoreceptors, 5-HT$_{1B}$ receptors are located on synaptic terminals and provide autoinhibitory feedback on the intrasynaptic release of 5-HT (Piñeyro et al., 1995; Piñeyro and Blier, 1996). Desensitization of 5-HT$_{1B}$ autoreceptors in concert with desensitization of 5-HT$_{1A}$ autoreceptors increases the terminal release of 5-HT. Increased sensitivity of postsynaptic 5-HT$_{1A}$ receptors (by TCAs and ECS) increases the responsiveness of forebrain

structures to synaptically released 5-HT (de Montigny and Aghajanian, 1978; Wang and Aghajanian, 1980; Menkes and Aghajanian, 1980 and 1981; de Montigny, 1984).

Desensitization of presynaptic and hypothalamic post-synaptic 5-HT_{1A} receptors is hypothesized to contribute to behavioral and physiological responses (including hypothermia and neuroendocrine changes) following long-term administration of SSRIs (Goodwin et al., 1987; Hensler et al., 1991; Li et al., 1996).

Table 1. Effects of different antidepressant treatment strategies on the responsiveness of 5-HT_{1A} and 5-HT_{1B} receptors and their effect on net 5-HT transmission. Arrows pointing up and down represent increased and decreased parameters, respectively. Open circles represent no change and “n.d.” not determined (Blier and El Mansari, 2013; El Mansari et al., 2015).

	cell body 5-HT_{1A} autoreceptor responsiveness	terminal 5-HT_{1B} autoreceptor responsiveness	terminal α_2-adrenoceptor responsiveness on 5-HT terminals	postsynaptic 5-HT_{1A} receptor responsiveness	net effect on 5-HT transmission
SSRI	↓	↓	○	○	↑
SNRI	↓	○	↓	○	↑
MAOI	↓	○	↓	○ or ↓	↑
5-HT_{1A} agonists	↓	○	n.d.	○	↑
tricyclics	○	○	↓	↑	↑
ECS	○	○	○	↑	↑
mirtazapine[a]	↓	○	↓	○	↑
bupropion[a]	↓	○	↓	○	↑
vagus nerve st.[a]	○	n.d.	n.d.	○	↑
agomelatine	○	n.d.	n.d.	○	↑
vortioxetine	↓	↓	n.d.	○	↑

7.1.7.2 SRI-induced Desensitization of Serotonin$_{1A}$ Receptors and Internalization of 5-HTTs

Minutes following agonist-induced uncoupling of 5-HT_{1A} receptors (with the hypothetical involvement of GRK, PKA, and PKC), the process of internalizing these receptors begins. Acute SSRI administration leads to fast and reversible desensitization of 5-HT_{1A} autoreceptors through internalization of the (functional

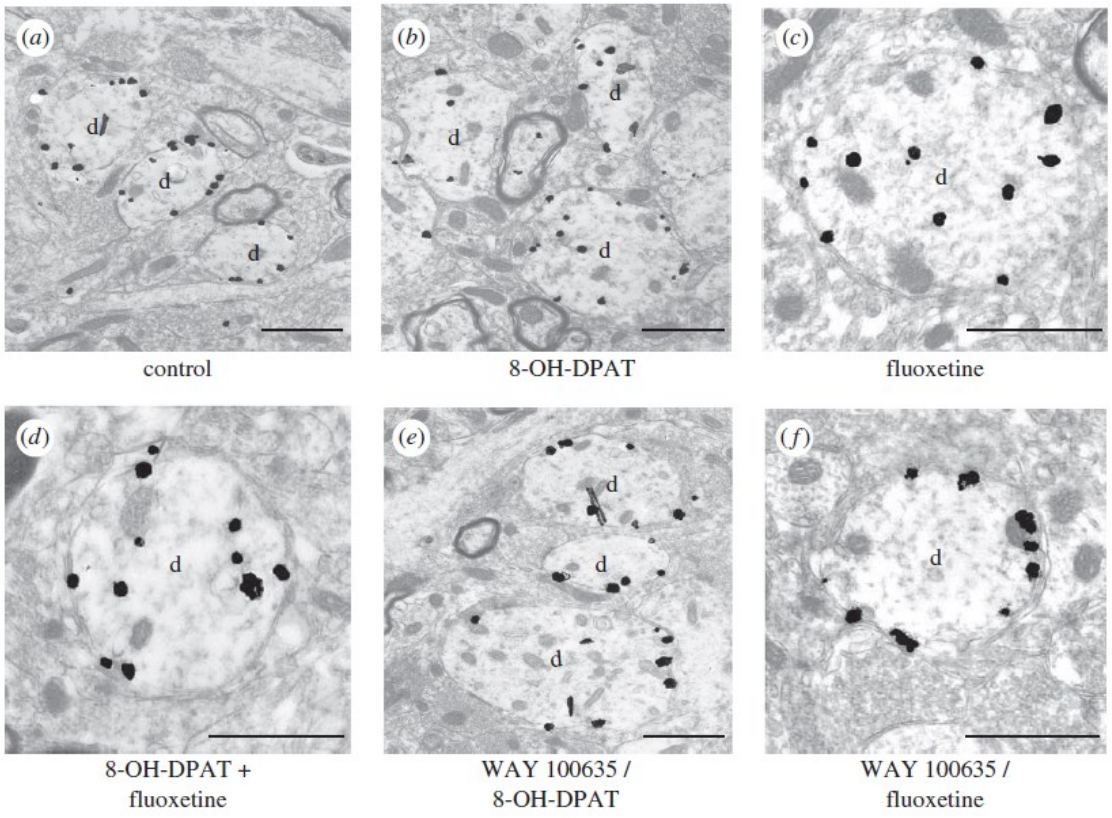
pool of membrane-bound) receptors (Figures 5, 7, and 8; Descarries and Riad, 2012). This desensitization is evidenced by the transient decreased responsiveness of 5-HT neurons to the 5-HT_{1A} receptors agonist 8-OH-DPAT, an effect that can be blocked by the antagonist of these receptors WAY 100635 (Beer et al., 1990; Kennett et al., 1987; Seth et al., 1997). The internalization process is indicated by the combination of the decreased density of 5-HT_{1A} receptors immunogold labeling of the plasma membrane in DRN 5-HT cell bodies and increased labeling in the cytoplasm of these neurons one hour following the administration of the SSRI fluoxetine (or 8-OH-DPAT; Figure 8; Descarries and Riad, 2012). The internalization of these receptors can be detected 15 minutes after the injection and these receptors are re-sensitized 24 hours following the injection.

Chronic SSRI administration, however, leads to progressive and long-lasting desensitization of 5-HT_{1A} receptors. These receptors will re-sensitize six weeks (but not 24 h or one week) after the cessation of chronic fluoxetine treatment (Descarries and Riad, 2012). 5-HT_{1A} receptors expressed in the hippocampus do not desensitize in such circumstances (probably due to different G protein interactions compared to receptors expressed in the DRN) and the internalization process does not happen for these receptors.

5-HTTs also desensitize with the chronic administration of certain antidepressants such as chronic administration of the SSRI paroxetine (Piñeyro et al., 1994). Further investigations suggest that 5-HTTs are internalized and

degraded in cell bodies and dendrites of DRN neurons as well as their axon terminals in the hippocampus following chronic treatment (Descarries and Riad, 2012). These transporters are reported to be re-sensitized 10 days following the cessation of treatment with the SSRI sertraline (Benmansour et al., 1999).

Figure 8. Immuno-electron microscopic visualization of internalization of 5-HT_{1A} autoreceptors following acute administration of fluoxetine and/or the 5-HT_{1A} agonist 8-OH-DPAT. Note the relatively higher distribution of silver-intensified immunogold particles on the plasma membrane in the control (a) compared to animals that have received acute administration of 8-OH-DPAT and/or fluoxetine (b-d). Prior administration of the 5-HT_{1A} antagonist WAY 100635 prevents the process of internalization by 8-OH-DPAT and fluoxetine (e and f; Descarries and Riad, 2012).



7.1.7.3 Therapeutic Effects of Drugs Used for the Treatment of MDD: Evidence for the Involvement of Serotonin$_{1A}$ Receptors and 5-HTT

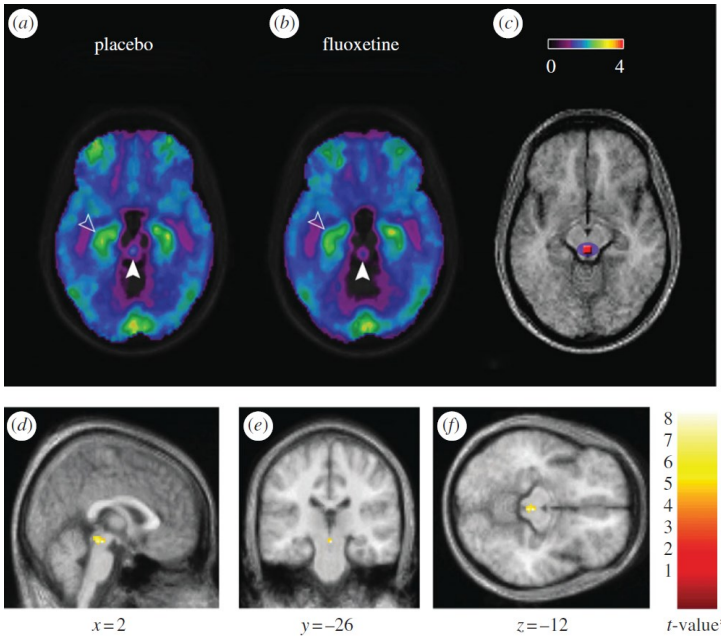
Preclinical investigations have indicated that engaging 5-HT$_{1A}$ auto- and hetero-receptors are likely to be involved (at least in part) in the therapeutic effects of SSRIs and various other treatment strategies used for the treatment of MDD. Long-term administration of SSRIs, the 5-HT$_{1A}$ agonist gepirone, and MAOIs desensitize somatodendritic 5-HT$_{1A}$ autoreceptors on DRN 5-HT neurons (Blier and Montigny, 1985; Chaput et al., 1988; Blier and de Montigny, 1987; for a more detailed list please refer to table 1 of chapter 1). Using positron emission tomography (PET) imaging, it has been shown that following the acute administration of fluoxetine to cats, there is a decrease in the binding potential of the specific 5-HT$_{1A}$ radioligand [^{18}F] MPPF in the DRN, but not in other regions of the brain (Aznavour et al., 2006) indicating the decreased availability of these receptors only in the DRN. This decreased availability, along with the results from immuno-electron microscopic results in rats (Figure 8), provides further evidence for the internalization of DRN 5-HT$_{1A}$ receptors following fluoxetine administration.

Similarly, clinical evidence from healthy human subjects has indicated that following a single dose of fluoxetine (20 mg) there is a 44% decrease in the binding potential of [^{18}F] MPPF only in the DRN (Figure 9; Sibon et al., 2008; Descarries and Riad, 2012) providing further evidence for fluoxetine-induced internalization of these receptors in the DRN. Imaging investigations of subjects suffering from MDD and postmortem studies of victims of suicide have reported

reduced 5-HT_{1A} binding in the hippocampus and cortex (Bhagwagar et al., 2004; Hirvonen et al., 2008; Savitz et al., 2009) and increased expression and binding of 5-HT_{1A} autoreceptors in the raphe nuclei (Stockmeier et al., 1998; Hesselgrave et al., 2013) indicating decreased and increased availability of these receptors in the hippocampus-cortex, and DRN, respectively.

Delayed desensitization of the 5-HT_{1A} receptor along with alterations in gene transcription of these receptors has been hypothesized to be involved in the delayed onset of clinical actions associated with SSRI treatment in MDD (Blier et al., 1998; Albert and Lemonde, 2004). More evidence for the potential involvement of 5-H_{1A} receptors in the antidepressant effect of different therapeutic strategies is provided by investigations showing that a variety of these strategies increase tonic activation (for more detailed information please refer to figure 18 and section 7.6. of chapter 1) of 5-HT_{1A} receptors in the hippocampus (Haddjeri et al., 1998; El Mansari et al., 2015; Ebrahimzadeh et al., 2018; for a detailed list and more information, please refer to table 1 of chapter 1 and the study presented in chapter 3).

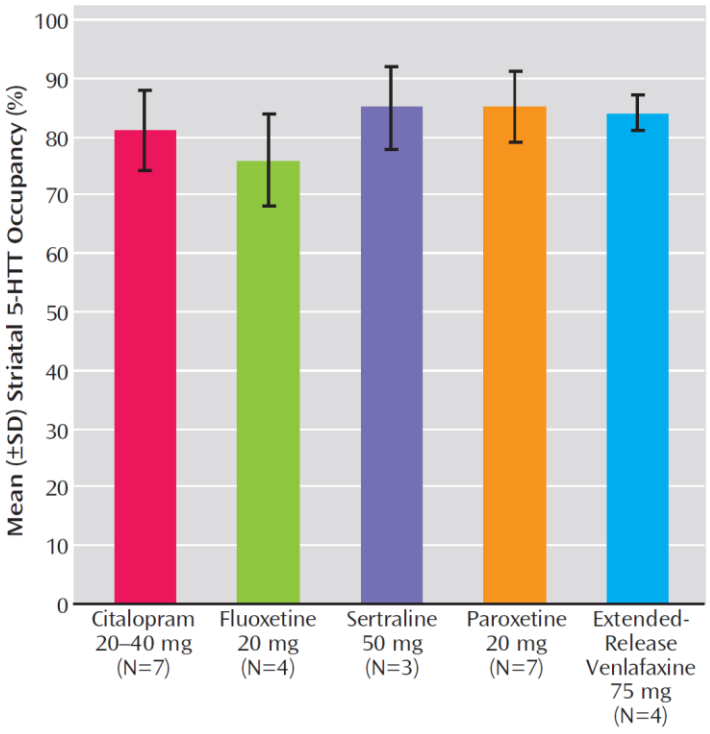
Figure 9. PET imaging illustrations indicating internalization of 5-HT_{1A} autoreceptors in the human brain. These images reflect the decreased binding potential of the specific 5-HT_{1A} radioligand [^{18}F] MPPF in the DRN following the acute administration of fluoxetine (20 mg) to healthy human subjects. Parametric images of the average [^{18}F] MPPF binding potential in the subjects who were administered placebo or fluoxetine are represented in sections (a) and (b), respectively. Note the decreased density of [^{18}F] MPPF binding in the DRN pointed by a white arrow in (b). Section (c) is an averaged MRI image illustrating the mesencephalic area encompassing the DRN in blue and a 27-voxel cubic region of interest around the most active voxels within this area in red. Sections d-f represent the statistical parametric mapping (SPM) analysis of the data. Each of these sections illustrates a contrast map (with corresponding color-coded t-values) which visualizes the difference between fluoxetine vs. placebo (Sibon et al., 2008).



(Asterisk: spm2, k = 25 voxels, p = 0.001 uncorrected, FWHM = 8 mm isotropic.)

Using PET imaging substantial occupancy (up to 60-80%) of 5-HTTs have been shown following the treatment of healthy subjects with sub-therapeutic doses of five SSRIs for four weeks (Meyer et al., 2004). In subjects with mood and anxiety disorders, minimum therapeutic doses of these SSRIs resulted in 76-85% occupancy of striatal 5-HTTs. The same study, along with a follow-up investigation, indicated higher 5-HTT occupancies with higher doses of several SSRIs (Figure 10; Meyer et al., 2004; Voineskos et al., 2007).

Figure 10. Occupancy of striatal 5-HTTs in MDD subjects following four weeks of treatment with the minimum therapeutic dose of five SSRIs. Several SSRIs including venlafaxine and paroxetine also inhibit the norepinephrine transporter but in orders of magnitude much less than their effect on 5-HTT (Meyer et al., 2004).



7.2 The Norepinephrine System

7.2.1 Behavioral and Physiological Significance

The NE system is involved in modulating stress responses, anxiety, fear, motivation, alertness, sleep, and “fight or flight” responses (Aston-Jones et al., 2010).

7.2.2 Neuroanatomy

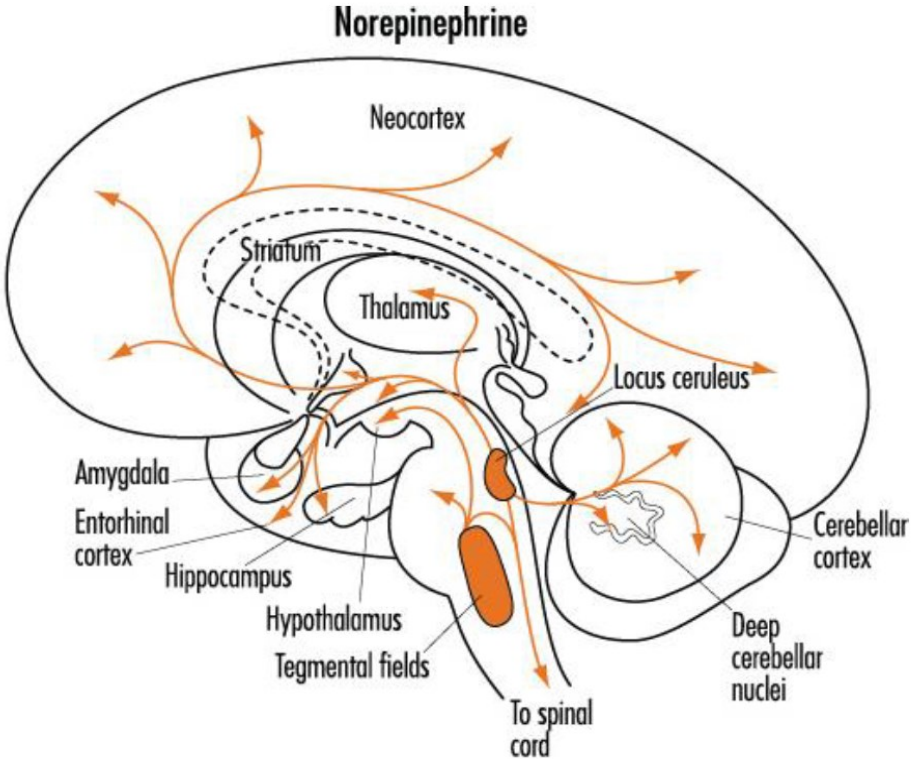
There are two major NE centers in the mammalian brain. These two centers contain 7 clusters; LC (A6), and lateral tegmental system (areas A1-A5 and A7; Paxinos and Watson, 2006). LC contains the majority of (90%) of the NE innervations of the brain (Fuxe and Sedvall, 1965; Foote et al., 1983). Despite having numerous projections to different parts of the brain (Foote et al., 1983), this nucleus only contains 3000 neurons in rats and around 26,000 in humans (Mouton et al., 1994).

The projections from LC have a modulatory role in areas associated with stress response, anxiety, fear, motivation, alertness, and sleep. Hence, the peripheral and central NE systems are the biological underpinning of what is referred to as the “fight or flight” response.

The lateral tegmental system has two different projections. The rostral projections act as a bridge between the central and peripheral nervous systems.

The caudal projections project to the thalamus, among other regions, and have a role in regulating homeostasis (Guyenet and Cabot, 1981).

Figure 11. Major NE pathways in the human brain (Heimer, 1995).



7.2.3 Norepinephrine Synthesis, Storage, Release, and Metabolism

The highest levels of NE are in the LC, followed by the dentate gyrus, hippocampus, and DR (Cheetham et al., 1996). NE in the brain is produced by hydroxylating L-tyrosine by tyrosine hydroxylase (TH,) and turning it into 3,4-dihydroxy-L-phenylalanine (L-DOPA). In the next step, L-DOPA is decarboxylated into DA by aromatic L-amino acid decarboxylase (AADC) and

finally, DA is hydroxylated into NE by DA-β-hydroxylase (Schatzberg and Nemeroff, 2009; please refer to figure 15).

NE is synthesized in nerve terminals and stored in vesicles by the vesicular monoamine transporter (VMAT2). DA and 5-HT are also packaged by this same protein (Erickson et al., 1992). NE is released in a stimulus-evoked Ca^{2+} dependent manner (Thureson-Klein, 1983). In addition, membrane transport proteins can pump out NE from the cytoplasm (Raiteri et al., 1979).

NE is metabolized both extracellularly and intracellularly. Catechol-O-methyltransferase (COMT) metabolizes NE extracellularly and turns it into 3-methoxy-4-hydroxyphenylglycol (MHPG). Intracellular degradation of NE is done by MAO, which turns NE into vanillylmandelic acid (VMA).

7.2.4 Norepinephrine Transporter

Re-uptake by the NE transporter (NET) is one of the primary mechanisms for the inactivation of NE that is released into the synapse. This transporter is a 12 membrane-spanning glycoprotein with a significant degree of similarity to 5-HT, DA, GABA, and glycine transporters (Amara and Kuhar, 1993). The NE transported by the NET is either degraded by MAO or repackaged for future release. mRNA of NET is expressed primarily in the LC, lateral tegmentum, and nucleus tractus solitarius (Lorang et al., 1994; Eymin et al., 1995). NET also transports DA and in fact in the prefrontal cortex is the primary source of DA reuptake (Di Chiara et al., 1992).

7.2.5 Norepinephrine Receptors

The adrenergic receptors are all GPCRs. Adrenergic receptors are divided into α and β sub-groups which are further divided into $\alpha_{1A/B/D}$, $\alpha_{2A/B/C}$, and β_1, β_2, β_3 receptors (Bylund et al., 1994). β receptors activate G_s to stimulate adenylate cyclase. On the other hand, $\alpha_{1A/B/D}$ receptors are coupled to G_q and stimulate PLC, and $\alpha_{2A/B/C}$ receptors are coupled to G_i and inhibit adenylyl cyclase.

7.2.5.1 Norepinephrine α Receptors

These receptors are expressed in the LC, raphe dorsalis (RD), and hippocampus and are divided into the two α_1 and α_2 sub-groups (Unnerstall et al., 1985; Palacios et al., 1987). α_1-adrenoceptors are further sub-divided to α-$_{1A,1B,\ 1D}$ and predominantly act as excitatory heteroreceptors. These receptors activate the PLC through their coupling with G_i/G_q proteins.

Activating PLC generates two second messengers, IP_3 and DAG. IP_3 increases intracellular concentrations of Ca^{2+} which has further regulatory effects on protein kinases (Berridge, 1993). DAG activates PKC which in turn activates downstream targets (Timothy and Casey, 1997). These events make neurons more excitable by decreasing K^+ conductance and pushing the neurons to a more depolarized state. The pharmacological benefits of targeting these receptors have been limited by the fact that activating the peripheral α_1 receptors increases blood pressure.

α_2-adrenoceptors are further divided into 2A, B, C, and D sub-groups. These receptors are expressed in the LC, DR, cortex, and hippocampus (Bruning et al., 1987). α_2-adrenoceptors are expressed both post- and pre-synaptically (Boehm and Kubista, 2002) and are coupled to $G_{i/o}$ to reduce the accumulation of cAMP (Neer, 1995). Activation of these receptors increases K^+ conductance by activation of G protein-gated K^+ channels, which hyperpolarizes neurons and makes them less excitable. Antagonists of these receptors (for example mirtazapine and clozapine) increase extracellular levels of NE and 5-HT by blocking α_2-auto and heteroreceptors, respectively.

7.2.5.2 Norepinephrine β receptors

β-adrenoceptors are expressed postsynaptically and are divided into 1, 2, and 3 sub-groups. β_1 and β_2 are the sub-types mostly expressed in the CNS whereas β3-adrenergic receptors are mainly present in the adipose tissue. β_1 receptors are mostly expressed in the cortex, hippocampus, and caudate putamen, while β_2 receptors are expressed in the cerebellum, thalamus, and olfactory bulb (Nicholas et al., 1993).

β-adrenergic receptors are coupled to G_s and stimulate adenylyl cyclase (Bylund et al., 1994). Since these receptors have a modulatory role in the regulation of emotional memories, blockade of these receptors has been proposed to be a potential treatment strategy for post-traumatic stress disorder (PTSD: Brunet et al., 2008).

7.3 The Dopamine System

7.3.1 Behavioral and Physiological Significance

The DA system is involved in modulating emotional control, motivation, cognition, sensorimotor coordination, and hormonal regulation (Grace et al., 2009; Nestler et al., 2006).

7.3.2 Neuroanatomy

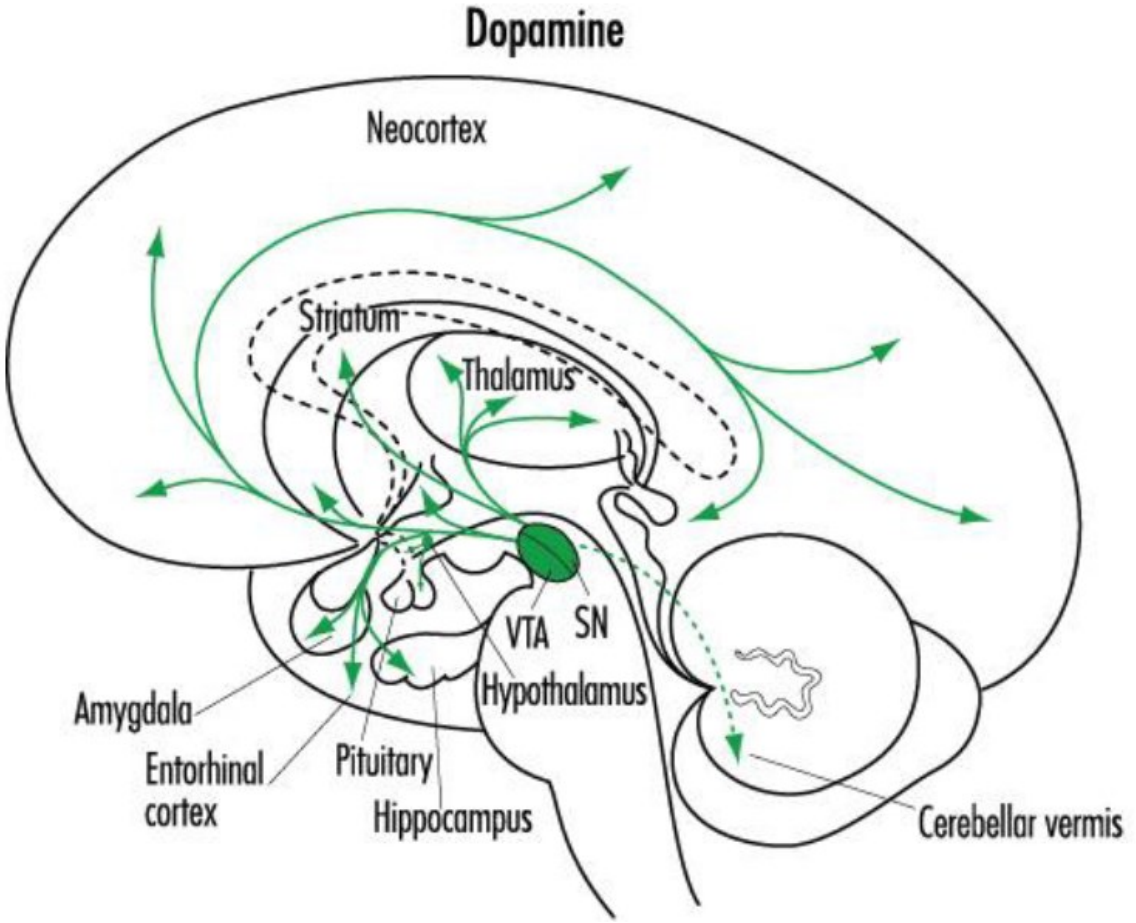
In the human brain, the total number of DA neurons has been estimated to be close to 600,000 (Chinta and Andersen, 2005). However, this number in rats is estimated to be around 30,000-40,000 neurons (Hedreen and Chalmers, 1972; Swanson, 1982). From this population, roughly 9,000 are in the VTA, and the rest in the substantia nigra zone compacta and retrorubral field. In the rat brain, there are four major DA pathways: mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular. The mesocortical pathway is comprised of the VTA (A8, A10) projections to the cingulate and mPFC.

The mesolimbic pathway consists of the VTA projections to the nucleus accumbens, amygdala, hippocampus, and olfactory bulb. These two pathways modulate emotional control, motivation, cognition, and their malfunctions are believed to be involved in some psychiatric disorders like addiction, schizophrenia, and MDD (Nestler et al., 2006).

The nigrostriatal pathway consists of the projections from substantia nigra pars compacta to caudate, putamen, and globus pallidus and is involved in sensorimotor coordination and has been implicated in the pathophysiology of Parkinson's disease (PD).

The DA projections from the hypothalamus arcuate and periventricular nuclei to the median eminence form the tuberoinfundibular pathway which is involved in hormonal regulation.

Figure 12. Major DA pathways in the human brain (Heimer, 1995).



7.3.3 Dopamine Synthesis, Storage, Release, and Metabolism

DA is synthesized from L-tyrosine in nerve terminals. TH hydroxylates L-tyrosine to L-DOPA, and in the next step L-aromatic amino acid decarboxylase turns L-DOPA into DA (Deutch and Roth, 1987). Expression of TH can be up- or down-regulated by different drugs including the drugs used for the pharmacotherapy of MDD, nicotine, caffeine, and morphine. DA is stored in vesicles by VMAT2 (Weihe and Eiden, 2000).

There are two vesicular DA pools, one containing 5-20% of DA for rapid release and the other containing the majority of DA for storage and is mostly an inactive pool. The Ca^{2+}-dependent release of DA is dependent both on the firing rate and pattern of action potentials of DA neurons. In the burst mode, more DA is released per action potential compared to the single spike pattern (Gonon, 1988; Garris et al., 1994; for more information, please refer to section 7.5.3. of chapter 1 and figure 16 of the same chapter). DA is also released through reverse transport by the DA transporter (DAT; Raiteri et al., 1979).

DA is metabolized by COMT and MAO. Metabolizing DA by MAO ultimately leads to the formation of 3,4 dihydroxyphenylacetic acid (DOPAC). Forty percent of DOPAC is eliminated from the brain while the rest is metabolized by COMT into homovanillic acid (HVA). DOPAC and HVA concentrations in the brain and CSF are used as indicators of DA activity in the brain.

7.3.4 Dopamine Transporter

Reuptake of DA by DAT is the primary mechanism of DA removal from the synapse. DAT is a 12 membrane-spanning glycoprotein. Because uptake of DA by DAT is sensitive to Na^+ gradient drugs that inhibit the transport of Na^+/K^+ or open Na^+ channels decrease DA reuptake (Cooper et al., 2003).

DAT is 66% similar to NET in terms of its molecular structure. The distribution of DAT follows the localization of DA neurons. However, the hypothalamic tuberoinfundibular DA neurons that release DA into the bloodstream do not have DAT. In addition, in the cortex DA is transported by NET (Gresch et al., 1995). Similarly, it has been shown that in the hippocampus both the NE and DA transporters were involved in DA reuptake (Guiard et al., 2008a).

The function of DAT has an essential role in psychiatric disorders. Cocaine and some other drugs of abuse block DAT and increase extracellular DA levels. However, these drugs bind to a different site than the DA. Drugs that bind to the DA binding site do not have addictive properties and have potential antidepressant properties (Schatzberg and Nemeroff, 2009).

7.3.5 Dopamine Receptors

DA receptors are also a class of GPCRs. These receptors are divided into two sub-groups, D_1-like (D_1 and D_5) and D_2-like (D_2, $_3$, and D_4) receptors (Rashid et al., 2007; Garau et al., 1978; Kebabian and Calne, 1979). D_1-like receptors are coupled to G_s and activate AC and increase cAMP formation while D_2-like

receptors are coupled to G_i and inhibit AC and either decrease cAMP or increase IP3 production (Kebabian and Calne, 1979).

7.3.5.1 Dopamine D_1 Receptors

D_1 receptors have the highest expression in the nucleus accumbens, caudate putamen, olfactory tubercle, substantia nigra, thalamus, hypothalamus, and cortex. D_1 receptors in the frontocortical regions have an essential role in cognitive function. D_5 receptors are expressed in the hypothalamus, thalamus, and hippocampus (Schatzberg and Nemeroff, 2009).

7.3.5.2 Dopamine D_2 Receptors

These are somatodendritic receptors acting as autoreceptors. Activation of these receptors decreases the firing activity of DA neurons and the release of DA depending on where the receptor is expressed. D_2-like receptors are further divided into D_{2S} receptors that mostly function as autoreceptors and D_{2L} receptors that act as heteroreceptors. D_2 receptors have been implicated in therapeutics of MDD, schizophrenia, PD, and attention deficit hyperactivity disorder (ADHD; Schatzberg and Nemeroff, 2009).

7.4 Structural and Functional Interactions between the Monoaminergic Systems

7.4.1 Serotonin-norepinephrine Interactions

LC NE neurons send projections to the DRN and lesions of these neurons initially decrease the firing activity of DRN 5-HT neurons (Svensson et al., 1975). The modulation of 5-HT neuronal activity by NE is mainly done by α_1-adrenoceptors expressed on 5-HT neurons (Baraban and Aghajanian, 1981). Accordingly, the systemic administration of α_1-adrenoceptor antagonist prazosin decreases 5-HT release in the hippocampus (Rouquier et al., 1994).

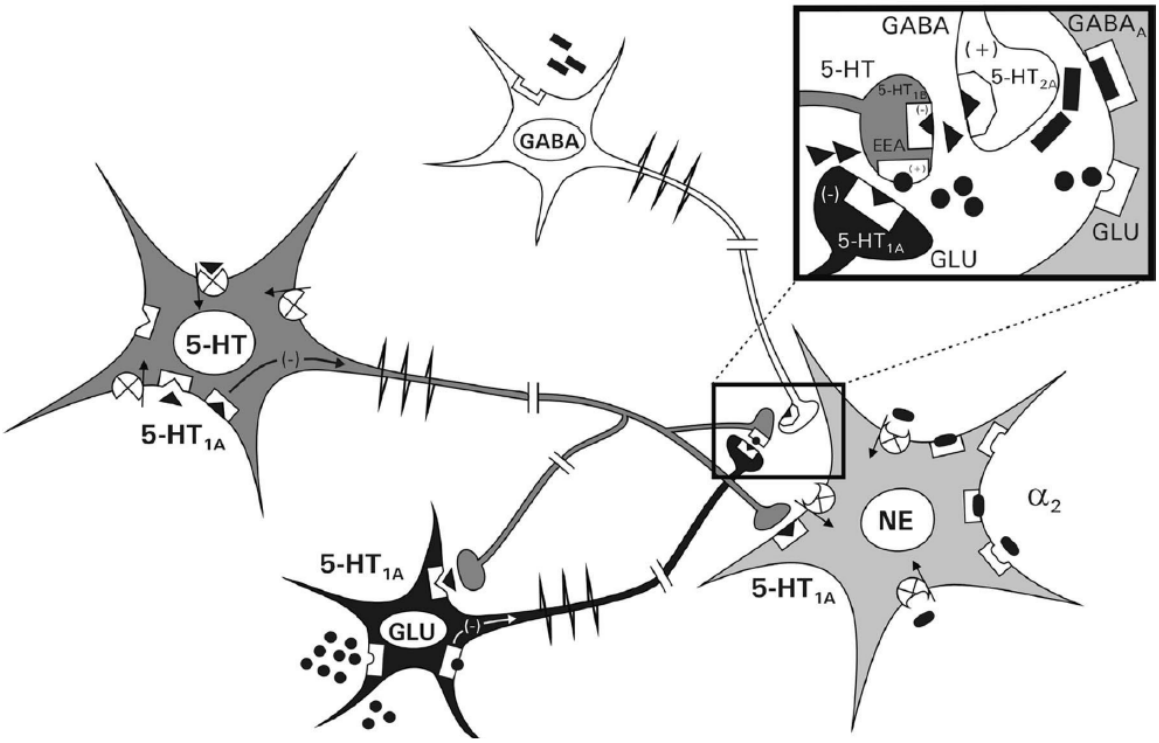
Alpha$_2$-adrenoceptors are expressed on 5-HT terminals projecting to the hippocampus, cortex, and hypothalamus, and consequently, activation of these receptors decreases the release of 5-HT in these areas (Tao and Hjorth, 1992). It has been shown that prolonged administration of mirtazapine, a drug used for the treatment of depression and a potent α_2-adrenoceptor antagonist, increases the firing rate of DRN 5-HT neurons (Haddjeri et al., 1995).

Pharmacological lesions of RD or reducing 5-HT synthesis by the tryptophan hydroxylase inhibitor para-chlorophenylalanine (PCPA) increase the firing activity of LC NE neurons suggesting that 5-HT has inhibitory effects on LC NE neuronal firing activity (please refer to figures 1 and 4 of chapter 1; Reader et al., 1986; Crespi et al., 1980; Haddjeri et al., 1997).

Activation of 5-HT_{2A} receptors has been reported to inhibit the activity of LC NE neurons, an effect that has been reported to be blocked by the selective 5-HT_{2A} antagonist R-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]-4-piperidyl]-methanol (M100907; Szabo and Blier, 2001b). These receptors have been suggested to be expressed on GABAergic interneurons (Chiang and Aston-Jones, 1993; Haddjeri et al., 1997). Accordingly, the decrease in the activity of LC NE neurons after administration of SSRIs and the increase in the activity of these neurons following pharmacological lesions of DRN is suggested to be due to the increased and decreased activation of 5-HT_{2A} receptors on GABA neurons, respectively (Figure 4; Farah and Blier, 2020).

On the other hand, activation of 5-HT_{1A} receptors through the systemic administration of an agonist increases the firing activity of LC NE (Figure 4; Farah and Blier, 2020) neurons and NE metabolite levels in the LC (Engberg, 1989). Blocking these receptors with the 5-HT_{1A} antagonist WAY 100635 decreases the firing activity of these neurons (Szabo and Blier, 2001c). These receptors are presumably located on glutamatergic neurons in the paragigantocellularis nucleus (Figures 4 and 13; Bobker and Williams, 1989; Farah and Blier, 2020).

Figure 13. The speculative neuroanatomical basis for the interplay between dorsal raphe nucleus 5-HT and locus coeruleus NE neurons (Szabo and Blier, 2001b).



7.4.2 Serotonin-dopamine Interactions

DRN receives input from DA neurons of the VTA (Suzuki et al., 1998; Mansour et al., 1990). These neurons have an overall excitatory effect on DRN 5-HT neurons as evidenced by the 60% decrease in the firing activity of these 5-HT neurons following lesions of VTA DA neurons (Guiard et al., 2008a). Further evidence of this excitatory effect has been provided by *in vivo* micro dialysis studies with freely moving animals showing that DA uptake inhibitors and DA receptors agonists increase the release of 5-HT in the DRN (Ferre and Artigas,

1993; Ferre et al., 1994) and its projection areas (Matsumoto et al., 1996; Mendlin et al., 1998).

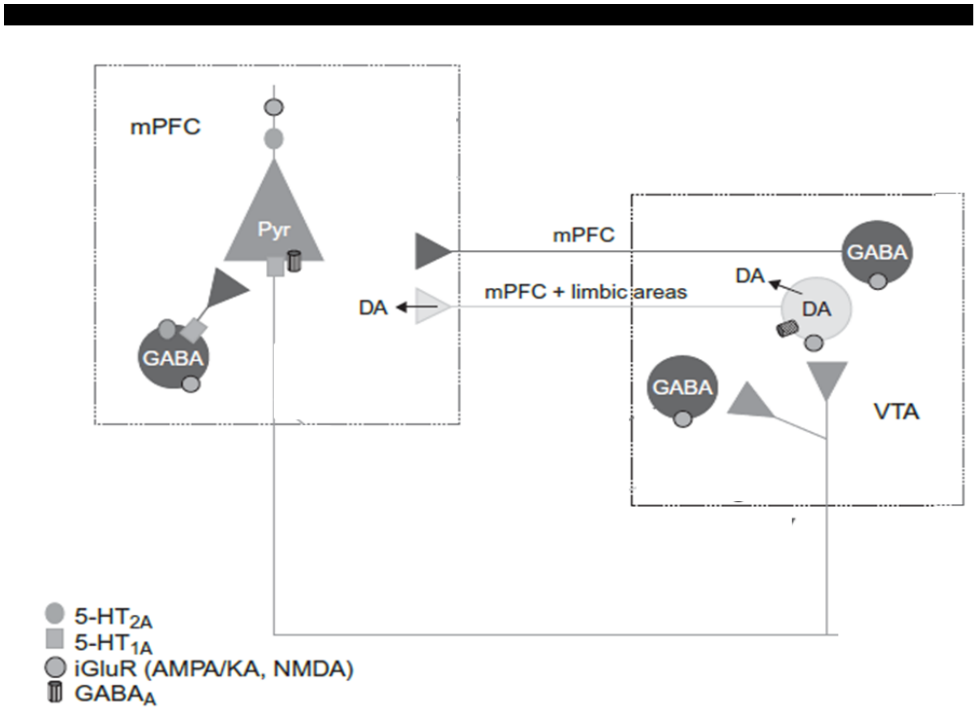
D_2 receptors are expressed on the cell body of DRN 5-HT neurons (Mansour et al., 1990; Suzuki et al., 1998) and activation of these receptors is believed to have an excitatory effect on the activity of DRN 5-HT neurons (Figures 1 and 4, Martín-Ruiz et al., 2001; Farah and Blier, 2020). It has been shown that stimulation of these receptors depolarizes the DRN 5-HT neurons, not through decreasing potassium current, but probably through activation of a nonselective cation current (Haj-Dahmane, 2001).

On the other hand, several 5-HT receptors modulate DA activity. It has been shown that the intravenous administration of the 5-HT_{1A} agonist BAY x 3702 increases the firing activity of VTA DA neurons (Díaz-Mataix et al., 2006). This effect can be blocked by the systemic administration of the 5-HT_{1A} antagonist WAY 100635 as well as frontocortical transection. These effects have been reported to be the consequence of modulating 5-HT_{1A} receptors that are presumed to be expressed on GABAergic interneurons of the mPFC (Figure 14; Díaz-Mataix et al., 2006; Santana et al., 2004).

5-HT_{1B} receptors also modulate DA neurotransmission and it has been shown that in 5-HT_{1B} receptor knockout mice extracellular level of DA is increased in the nucleus accumbens (Shippenberg et al., 2000).

In addition, 5-HT_{2C} receptors have an inhibitory effect on mesolimbic and mesocortical DA neurons through the excitation of GABA neurons in the VTA (Figures 1 and 4). Hence administration of SB 206553, a 5-$HT_{2B/2C}$ antagonist, increases the firing activity of VTA DA neurons (Di Giovanni et al., 2000; Alex et al., 2005), and lesioning of 5-HT neurons enhances the firing activity of these neurons (Guiard et al., 2008b). In conclusion, the 5-HT system has an inhibitory effect on the DA system predominantly through 5-HT_{2C} receptors. The effect of targeting 5-HT_{2B} receptors on the DA activity has been explained in detail in the section dedicated to 5-HT_{2B} receptors (7.1.5.2.2.).

Figure 14. Schematic representation of the interactions between DA neurons of the ventral tegmental area, glutamatergic, and GABAergic neurons of the frontal cortex (Díaz-Mataix et al., 2005).



7.4.3 Dopamine-norepinephrine Interactions

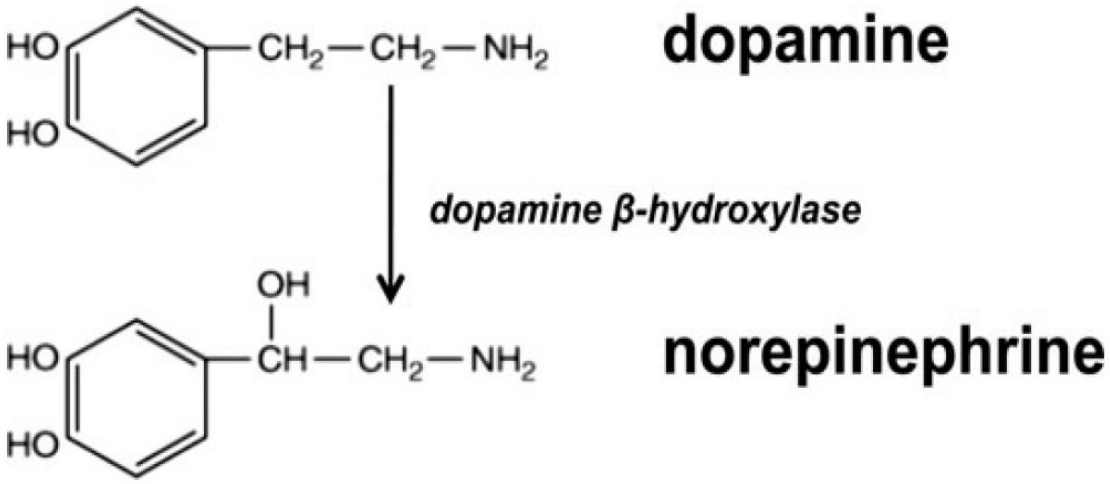
It has been shown that VTA DA neurons have an inhibitory effect on the activity of LC NE neurons. Microiontophoretically ejected DA has been shown to inhibit the activity of LC NE neurons and blockade of DA receptors enhances the firing activity of these neurons (Elam et al., 1986; Cedarbaum and Aghajanian, 1977; Guiard et al., 2008a; Nilsson et al., 2005; Piercey et al., 1994). The inhibitory effects of the VTA DA neurons on the firing activity of LC NE neurons are exerted through the activation of α_2-adrenergic receptors (El Mansari et al., 2010).

Direct application of NE has been shown to inhibit the activity of VTA DA neurons, an effect which is reversed by blockade of α_2-adrenergic receptors (Guiard et al., 2008a). Electrophysiological studies have indicated that the inhibitory effect of NE on VTA DA neurons can be blocked by the D_2 receptor antagonist sulpiride (Aghajanian and Bunney, 1977; White and Wang, 1984a, and b). Overall, the effect of the NE system on the DA system appears to be inhibitory and through activation of α_2-adrenergic and D_2 receptors.

Given the structural similarity of DA and NE molecules (El Mansari et al., 2010, Figure 15) the above-mentioned involvement of α_2-adrenergic receptors in the inhibitory effects of DA on the activity of LC NE neurons, and involvement of D_2 receptors in the inhibitory effects on NE on the firing activity of VTA DA neurons

is not unexpected. Another instance of this functional overlap is the transfer of DA molecules by NET in the prefrontal cortex (Yamamoto and Novotney, 1998).

Figure 15. Conversion of DA to NE by DA β-hydroxylase. Note the only difference between the molecular structures of DA and NE is an additional hydroxy group (El Mansari et al., 2010).



7.5 Electrophysiological Properties of Monoaminergic Neurons

The electrophysiological recordings of research projects presented in chapters 2-4 have been conducted in male Sprague-Dawley rats under chloral hydrate anesthesia (please refer to section 7.5.1. of chapter 1 for the effects of chloral hydrate anesthesia on the firing activity of DRN 5-HT neurons). The use of this strain of rats is fairly common (Wang et al., 2017) in preclinical research partly due to their calm demeanor, ease of handling, and their longer body length compared to other strains. Accordingly, there is a plethora of data from previous

electrophysiological and behavioral studies (chronic restrain stress, chronic unpredictable stress, and genetic manipulations) using this strain of rats.

The factors that are often assessed in single-unit electrophysiological recordings include the firing frequency, bursting activity, the population activity of the recorded neurons (for more information, please refer to section 7.5.3. and figure 16 of chapter 1), RT50, and tonic activation of 5-HT and NE receptors (for more information, please refer to section 7.6. and figures 17 and 18 of chapter 1).

7.5.1 Serotonin

5-HT has a role in the inhibition of sensory input processing and the facilitation of motor output. The activity of 5-HT neurons is absent during rapid eye movement (REM) sleep, while it is low during slow-wave sleep, quiet waking and it is maximum during active waking (Jacobs and Fornal, 1993). In anesthetized rats, the firing activity of 5-HT neurons has been well characterized. Two of the most commonly used anesthetic agents for the electrophysiological investigations of these neurons have been chloral hydrate and urethane (McCardle and Gartside, 2012). *In vitro* electrophysiological experiments of dorsal raphe slices have demonstrated that although urethane increases the basal firing rate of 5-HT neurons, chloral hydrate did not have such an effect (McCardle and Gartside, 2012). Using chloral hydrate (400 mg/kg, i.p.) it has been shown that 5-HT neurons of dorsal raphe have a slow firing rate (0.5-2.5 Hz) and biphasic long-duration action potentials (2-5 ms; Aghajanian and Vandermaelen, 1982b).

The pacemaker-like activity of 5-HT neurons is related to Ca^{2+}-dependent K^+ current in these neurons. After depolarization Ca^{2+} ions enter the neuron which activates outward K^+ conductance, which itself causes an afterhyperpolarization period (Figure 3; Burlhis and Aghajanian, 1987).

7.5.2 . Norepinephrine

The firing rate of NE neurons is virtually absent during REM sleep, while it is low during slow-wave sleep and quiet waking, and is the highest in active waking. In anesthetized rats, these neurons have a firing rate of 0.5-5 Hz with action potentials with a long duration (0.8-1.2 ms; Aghajanian and Vandermaelen, 1982a). These neurons discharge in bursts followed by a brief quiescent period after noxious pinching of the collateral paw (Chiang and Aston-Jones, 1993).

7.5.3 Dopamine

The firing frequency of these neurons is virtually absent during REM sleep, while it is low in slow-wave sleep, quiet waking, and is the highest in active waking. These neurons have a firing frequency of 0.5-7 Hz with long action potentials (>2.6 ms) and often have a notch in the rising phase of their action potentials.

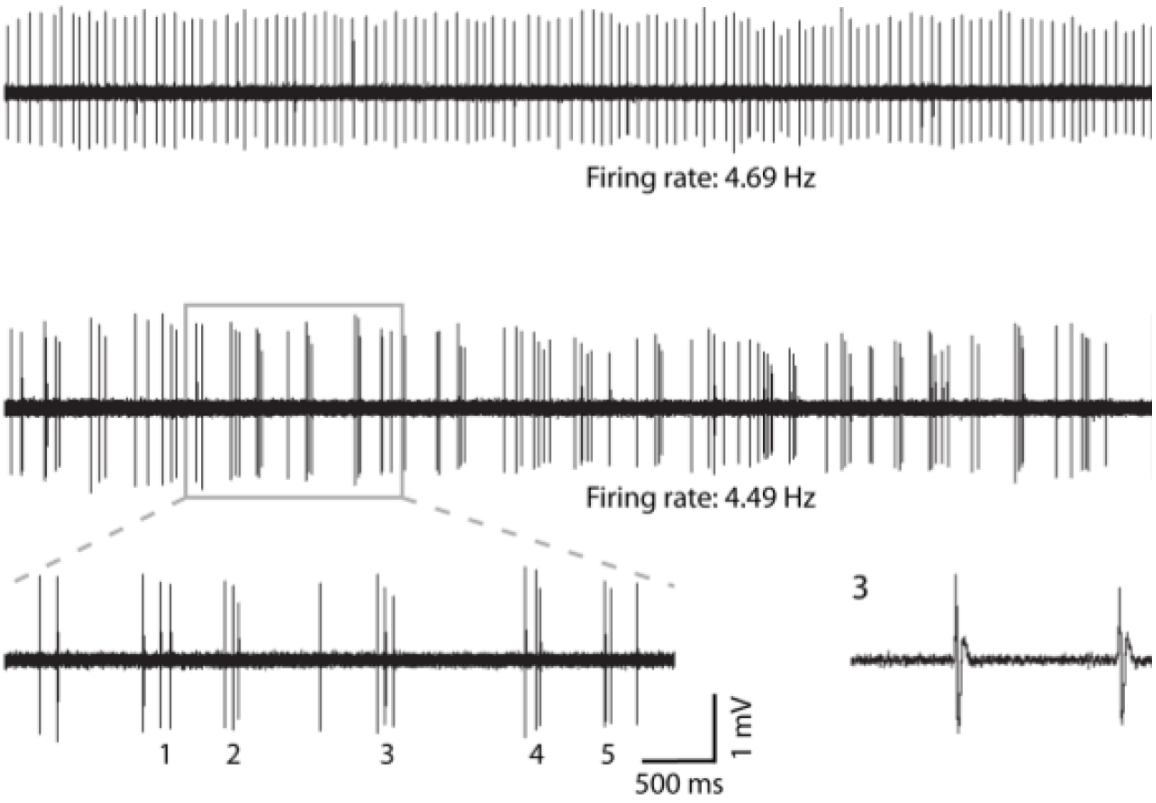
In vivo electrophysiological recordings have demonstrated that DA neurons have three main patterns of firing activity. The first pattern is an inactive hyperpolarized state while the second one is a slow, single-spike, or “tonic” firing

pattern and the third pattern is the bursting or “phasic” mode (Grace and Bunney, 1983).

It has been shown that independent of the state of anesthesia 50% of mesencephalic DA neurons are in a hyperpolarized inactive state (first pattern of activity; Grace et al., 2007). Different drug regimens have been shown to modulate the spontaneous firing activity of DA neurons (Figure 3B of chapter 4). For example, acute administration of some of the dopamine modulators, used for the pharmacotherapy of schizophrenia, increases the spontaneous firing activity of these neurons while a 21-day regimen of the same drugs decreases the spontaneous activity to levels lower than control (Grace et al., 1997).

The third pattern of activity or the bursting mode has been reported to cause an increase in the release of DA (and also NE and 5-HT in bursting NE and 5-HT neurons) per action potential at the terminal level, and hence it is one of the important factors in the regulation of DA neurotransmission (Figure 3C of chapter 4; Grace et al., 2007). The bursting activity of DA neurons has been suggested to occur in response to stimuli that might predict reward (Schultz, 1998). The bursting pattern in these neurons has been defined as trains of 2-10 action potentials with decreasing amplitudes and increasing durations. The interspike interval within a burst has been defined to have a value of 80-160 ms (Figure 16; Ungless and Grace., 2012).

Figure 16. Electrophysiological recordings of the firing activity of ventral tegmental area DA neurons. The top trace illustrates a regular firing pattern (also referred to as single-spike, or tonic firing) while the middle trace illustrates an irregular firing pattern (including bursts which are illustrated in the lower traces). Note the decreasing amplitudes of action potentials in each train of bursts (Ungless and Grace, 2012).



7.6 Hippocampus: A Region of Interest for the Electrophysiological Investigations on Pharmacotherapy of Major Depressive Disorder

Hippocampus is involved in the regulation of mood (Soares and Mann, 1997) and various cognitive functions (Learning and Memory; Fanselow, 2000; Squire, 2004) which are among the diagnostic symptoms of MDD (please refer to section 1 of this chapter; DSM–5; American Psychiatric Association, 2013).

Imaging studies not only have shown a reduction in the hippocampus volume in MDD patients (Campbell et al., 2004; Videbech and Ravnkilde, 2004; Koolschijn et al., 2009; McKinnon et al., 2009; Arnone et al., 2012) but also an increase of this volume in remitted MDD patients following pharmacotherapy (Phillips et al., 2015).

NE neurons of LC and 5-HT neurons of raphe nuclei both send projections to the hippocampus and take part in modulating the activity of this region (Mongeau et al., 1997). Accordingly, the clinical efficacy of drugs used for the pharmacotherapy of MDD has been hypothetically linked to the effects of these drugs on the modulation of 5-HT and NE neurotransmission in the hippocampus (Mongeau et al., 1997).

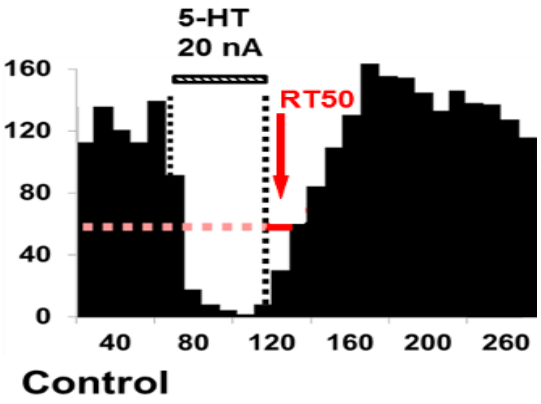
Needless to say, this hypothesis does not assume that a single brain structure or a limited number of neurotransmitters are solely involved in the pathophysiology and therapeutics of MDD. However, electrophysiological investigations of the effect of the drugs used for the pharmacotherapy of MDD on

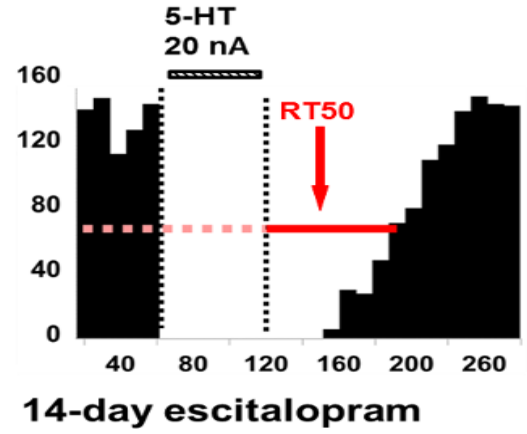
the modulation of the hippocampus activity by 5-HT and NE systems have provided a simplified model which hypothetically serves as a stepping stone for more complicated models.

It is worth mentioning that the relative lack of technical difficulties for electrophysiological investigations in the hippocampus and the existence of a plethora of evidence on topics closely tied to MDD (cognitive functions and regulation of mood, Mongeau et al., 1997; neurogenesis, Martinowich et al., 2007) has had a positive effect on choosing this structure for the electrophysiological recording investigating the effect of drugs used for the treatment of MDD.

Two of the valuable indices that can be acquired by the electrophysiological recording of pyramidal neurons of the CA3 region of the hippocampus are RT50 (Figures 17 of chapter 1; 1 of chapter 2; 1 and 6 of chapter 3; 2A and 5A of chapter 4) and tonic activation of certain 5-HT and NE receptors (Figures 18 of chapter 1; 4 and 5 of chapter 2; 3, 4, 5, 8, 9 of chapter 3; 2B, 5B and C of chapter 4). RT50 is an index for the relative activity of 5-HTT and NET. Measuring tonic activation of 5-HT_{1A}, α_1-, and α_2-adrenoceptors provides us with valuable insights into possible elevations in 5-HT and NE neurotransmission through these receptors.

Figure 17. RT50, an index for the activity of 5-HT and NE transporters. RT50 values are the time needed for pyramidal neurons to recover to 50% of their baseline firing rate after being inhibited by microiontophoretically ejected 5-HT or NE.

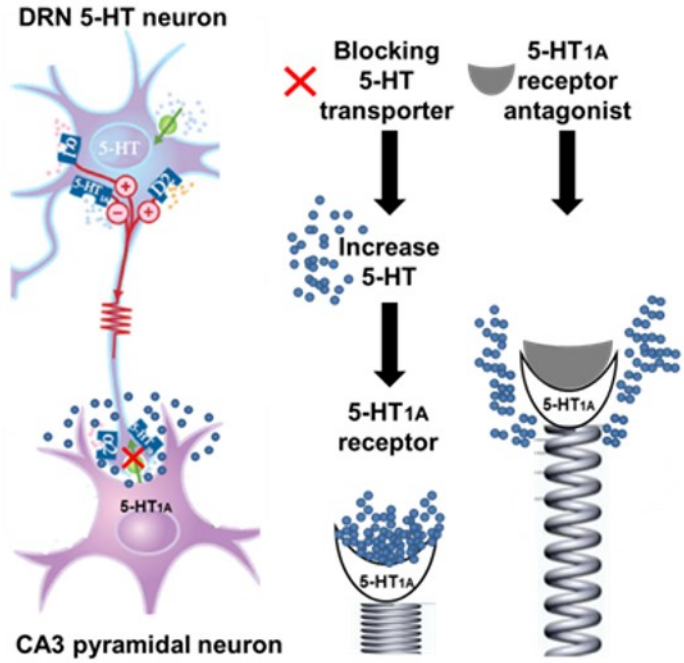




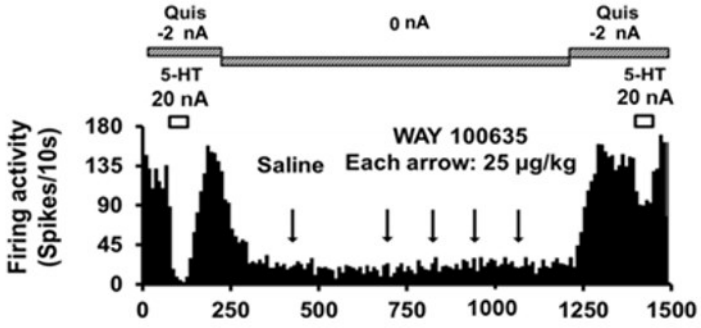
The current figure represents actual recordings of two pyramidal neurons (in the CA3 region of hippocampus) for comparing RT50 values (for 5-HTT) in a control rat and a rat that was treated 14 days with escitalopram. The increased RT50 value in the escitalopram-treated rat suggests a decrease in the activity of 5-HTT indicating the inhibition of 5-HTT by escitalopram.

Figure 18. Tonic activation of 5-HT and NE receptors, an index for quantifying neurotransmission alterations in postsynaptic areas. (A) Increased extracellular levels of 5-HT following the inhibition of 5-HTT (and/or direct stimulation of 5-HT_{1A} receptors by agents like vortioxetine, aripiprazole, and cariprazine) results in inhibition of neuronal activity (the left loaded spring in section A; the spring represents the firing activity of the recorded neurons and changes to its length represent increased or decreased firing activity). Hence, blocking the 5-HT_{1A} receptors (with the 5-HT_{1A} receptor antagonist WAY 100635) would lead to disinhibition of the neuronal firing activity (the right expanded spring in section A). Integrated firing rate histograms of CA3 pyramidal neurons showing their responsiveness to the 5-HT_{1A} receptor antagonist WAY 100635 in (B) a vehicle-administered rat and (C) a rat administered for 14 days with the combination of escitalopram and aripiprazole. Note the disinhibition of the firing activity of the pyramidal neuron following the injection of WAY 100635 in section C (similar to the right expanded spring in section A). WAY 100635 also blocks the inhibitory effect of 5-HT on the activity of the recorded neurons (the right side of B and C histograms). The ejection currents for quisqualate or a leak (Quis; -2 and 0 nA, respectively) and 5-HT (+20 nA) are indicated above the horizontal bars, which correspond to the duration of the drug ejection. In each rat, only one neuron was tested. For measuring NE neurotransmission the same experimental paradigm but with adrenergic receptor antagonists (idazoxan and prazosin) can be used.

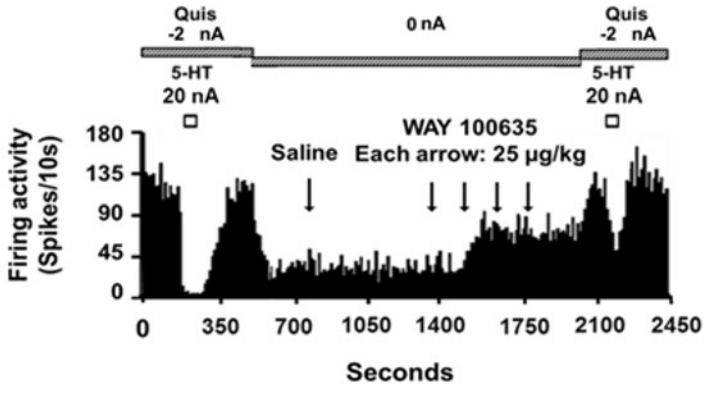
A.



B.14-day vehicles



C.14-day escitalopram + aripiprazole



7.7 Abnormalities in Monoamines and Relevance to Major Depressive Disorder

7.7.1 Serotonin

It has been reported that 5-HT depletion via acute administration of PCPA leads to reinstatement of depressive symptoms in patients who responded to the tricyclic drug imipramine and the MAOI tranylcypromine (Shopsin et al., 1975; Shopsin, 1976). Besides, the tryptophan plasma concentrations have been shown to be lower in the vast majority of MDD patients (Møller et al., 1980; Quintana, 1992; Ogawa et al., 2014).

It has also been shown that a tryptophan-free amino acid-rich diet, which decreases 5-HT synthesis by producing competition for transport between tryptophan and other amino acids across the blood-brain barrier (BBB), lowers the mood especially in MDD patients who are in remission (Delgado, 1993; Smith et al., 1997) or healthy controls with a genetic predisposition towards development of this disorder (Benkelfat et al., 1994).

A PET study has shown that in remitted patients the depressive symptoms following the tryptophan depletion were correlated with decreased activity in the dorsolateral PFC, thalamus, and anterior cingulate cortex (Bremner et al., 1997).

There is also evidence from post mortem studies showing that brains of individuals who had suffered from MDD have a lower 5-HIAA content, inferring a lower degree of 5-HT metabolism which is an attribute that has been associated

with suicide (Mann and Malone, 1997; Placidi et al., 2001). These post mortem studies of individuals with MDD have also shown that the expression of 5-HTT is increased in prefrontal brain regions that might be a compensatory mechanism in reaction to reduced neurotransmission of 5-HT (Leake et al., 1991; Owens and Nemeroff, 1994).

It also has been shown that in depressed individuals the binding potential of 5-HT_{1A} autoreceptors is increased in the DRN while the binding potential is decreased for 5-HT_{1A} receptors in prefrontal areas which together suggests a dampened 5-HT neurotransmission (Arango et al., 1995; Stockmeier et al., 1998). The purported decrease in 5-HT neurotransmission has been corroborated in other studies using PET (Malison et al., 1998) and single-photon emission computed tomography (Joensuu et al., 2007).

According to previous research, a polymorphism in the serotonin transporter promoter (5-HTTLPR) causing reduced 5-HT reuptake predicts the response to drugs used for the pharmacotherapy of MDD. The same polymorphism has also been reported to be correlated to the decreased size of brain regions implicated in MDD (Lesch et al., 1996; Arias et al., 2003; Caspi et al., 2003; Pezawas et al., 2005; Karg et al., 2011). However, while this polymorphism has been reported to be associated with increased responsiveness to life stressors (Caspi et al., 2003), a similar study (Fergusson et al., 2011) has not replicated these findings. In conclusion, so far, candidate gene, linkage, or genome-wide association studies have not indicated a single locus of major effect

in MDD, and it is hypothesized that thousands of loci may contribute to the manifestations of the symptoms of MDD (Ripke et al., 2013).

7.7.2 Norepinephrine

Catecholamine depletion by an amino-acid-rich, tyrosine and phenylalanine-deficient drink, or inhibition of TH by α-methyl-p-tyrosine (AMPT), has been shown to lower the mood more pronouncedly in remitted MDD patients who primarily responded to drugs that affect NE but not 5-HT neurotransmission (Delgado et al., 1993; Miller et al., 1996; Ruhé et al., 2007).

A strong genetic link between the NE system and MDD has not been established yet. However, post mortem studies have shown increased levels of α_2-adrenergic autoreceptors binding in the LC in MDD patients and also increased binding of the α_2-adrenergic receptors in the temporal cortex, prefrontal cortex, and the hippocampus (Meana and Garcia-Sevilla, 1987; Javier Meana et al., 1992; De Paermentier et al., 1997; Ordway et al., 2002 and 2003).

7.7.3 Dopamine

As previously mentioned, it has been shown that catecholamine depletion may decrease mood. In MDD patients DAT activity is enhanced in limbic regions (Laasonen-Balk et al., 1999; Newberg et al., 2007; Amsterdam et al., 2012). The only genetic risk factor for the development of MDD so far in the DA system is a genetic variation in the gene encoding D_4 receptors (López León et al., 2005).

8. Glutamate System: A Target for Drugs Used for the Treatment of Major Depressive Disorder

The glutamate system is involved in a variety of important brain functions including cognition, learning, memory, and neurogenesis. In addition to innervating cortical structures, glutamate neurons also send projections to the monoaminergic subcortical structures including the DRN, LC, and VTA (Catena-Dell'Osso et al., 2013).

Glutamate binds to ionotropic and metabotropic glutamate receptors. The ionotropic receptors are ligand-gated non-selective cation channels encompassing three types of receptors; the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-Methyl-D-aspartic acid (NMDA), and kainate receptors (Mathews et al., 2012). Metabotropic receptors, on the other hand, are GPCRs (Meldrum, 2000).

One of the initial insights into the potential involvement of the glutamate system in MDD was provided with the report of increased levels of glutamate in serum (Kim et al., 1982) and CSF (Levine et al., 2000) of MDD patients. Studies using PET imaging have shown a reduced density of metabotropic glutamate receptors (type 5) in MDD patients (Deschwanden et al., 2011). In addition, results of investigations using magnetic resonance spectroscopy have indicated reduced Glx levels (a combined measure of glutamate and glutamine) in patients with MDD (Yüksel and Öngür, 2010). More recent meta-analyses have also reported that

levels of Glx are decreased in the PFC (Arnone et al., 2015) and mPFC of patients with depression (Moriguchi et al., 2019). Further, reduced Glx levels have been related to the number of failed trials with drugs used for the treatment of depression (Arnone et al., 2015). ECT and antidepressant pharmacotherapy have been shown to increase Glx levels in the mPFC of patients with depression (Pfleiderer et al., 2003; Chen et al., 2014). In summary, these pieces of evidence point to a possible dysregulation of the glutamate system in MDD.

The research on targeting the glutamate system for the pharmacotherapy of MDD has implicated AMPA potentiators and NMDA antagonists as potential therapeutic agents. Monoaminergic antidepressants have been reported to potentiate AMPA receptor-mediated neurotransmission (Barbon et al., 2011; Murrough, 2011). In addition, AMPA potentiators have produced antidepressant-like effects in preclinical models of MDD (Li et al., 2001; Andreasen et al., 2013). However, the potential to utilize AMPA receptors as drugs to treat MDD has to be further investigated in preclinical studies and clinical trials.

Monoaminergic antidepressants have also been shown to reduce the synaptic release of glutamate in the frontal cortex (Barbon et al., 2011; Tokarski et al., 2008). Ketamine, a non-competitive NMDA antagonist (Katalinic et al., 2013), has been shown to produce antidepressant-like effects in preclinical models of depression (Chung, 2012).

It has been shown that the glutamine/glutamate ratio is decreased in the pregenual ACC (pgACC) of patients with depression, and ketamine has been shown to increase this ratio in the pgACC of healthy individuals (Li et al., 2016). In the clinical setting, ketamine produces remarkably fast antidepressant effects even in treatment-resistant patients (Blier et al., 2012; Zarate et al., 2006) and has been shown to produce sustained antidepressant effects following repeated infusions (Phillips et al., 2019). Esketamine, the s-enantiomer of ketamine, has been the first non-competitive NMDA antagonist to be approved (U.S. Food and Drug Administration, March 2019; European Medicines Agency, December 2019; Health Canada, May 2020) as adjunctive therapy for adults with treatment-resistant MDD (Sancar, 2019).

9. Pharmacotherapy Approaches in Treatment-resistant Major Depressive Disorder

Two of the most common approaches in the pharmacotherapy of treatment-resistant depression are switching to another class of drug or augmenting the effects of the first drug with the addition of an adjunct medication (Thase et al., 2017; Blier and Blondeau, 2011). Switching to another class of drugs is among the recommended courses of action especially in cases that face tolerability issues to the first drug used for the treatment of MDD. Section 9.1 focuses on vortioxetine as an example of drugs that can be used in cases of treatment-resistance or incomplete response to SSRIs and SNRIs. Augmentation and combination strategies are also among the pharmacological approaches for managing

treatment-resistant MDD. Unlike combination strategies, augmentation strategies make use of adjunct medications that are not conventionally used for the monotherapy of MDD. Section 9.2 focuses on DA and 5-HT partial agonists that can be used as adjuncts to other drugs for MDD augmentation strategy (Thase et al., 2017; Blier and Blondeau, 2011).

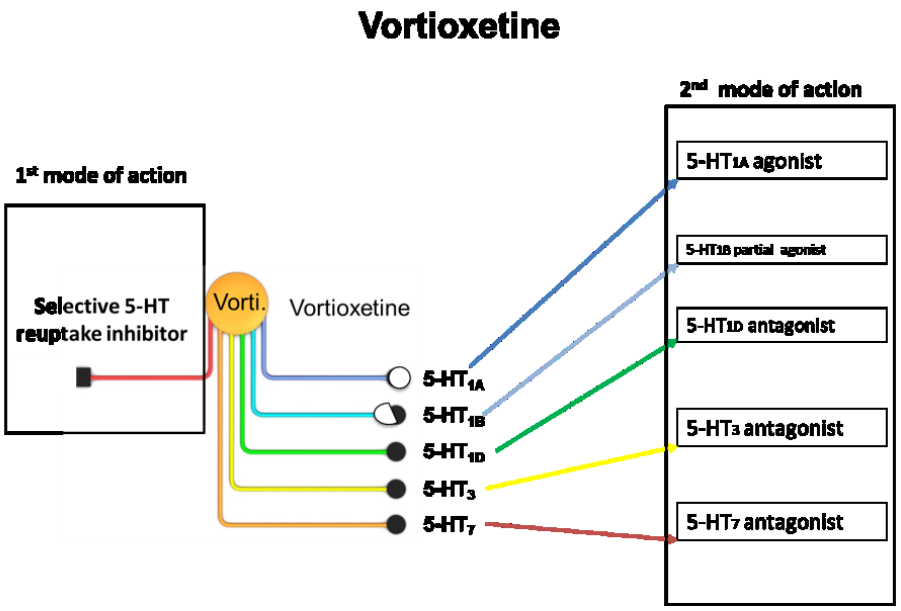
9.1 Vortioxetine

Vortioxetine is a drug used for MDD treatment with a multimodal mechanism of action and has been shown to improve cognitive function independently of its actions on mood (Katona et al., 2012; Mahableshwarkar et al., 2015; McIntyre et al., 2014). Direct comparison of vortioxetine has shown its superiority to agomelatine in MDD patients with previous inadequate response to a single course of SSRI/SNRI monotherapy (Montgomery et al., 2014). Indirect treatment comparisons have indicated a higher probability of remission following vortioxetine treatment compared to sertraline, venlafaxine, bupropion, and citalopram (Thase et al., 2017).

The pharmacological profile of vortioxetine is in some aspects comparable to that of vilazodone. This profile consists of inhibiting 5-HTTs, agonism of 5-HT_{1A} receptors, partial agonism of 5-HT_{1B} receptors, and antagonism of 5-HT_3, 5-HT_7, and 5-HT_{1D} receptors (Sanchez et al., 2015). However, vortioxetine does not interact with 5-HT_6 receptors (Millan et al., 2016).

Acute administration of vortioxetine decreases the firing activity of DRN 5-HT neurons probably due to its excitatory effects on 5-HT_{1A} receptors combined with inhibition of 5-HTT (Bétry et al., 2013). However, the firing rate of these neurons has been shown to recover only after 24 hours (potentially in part due to the antagonism of 5-HT_3 receptors; Bétry et al., 2013; Sanchez et al., 2015). Prolonged administration of vortioxetine has been shown to increase tonic activation of hippocampal 5-HT_{1A} receptors, which is at least in part due to partial agonism, and desensitization, of terminal 5-HT_{1B} receptors by vortioxetine (El Mansari et al., 2015). In contrast to SSRIs, high doses of vortioxetine acutely administered increased the firing activity of LC NE neurons (Pehrson et al., 2013), which is hypothesized to be the results of antagonism of 5-HT_3 receptors and/or agonism of 5-HT_{1A} receptors (Sanchez et al., 2015).

Figure 19. Simplified pharmacological profile of vortioxetine.



9.2 Adjunctive dopamine and serotonin partial agonists

Several drugs used for the treatment of psychosis including aripiprazole and cariprazine can be used as adjunct medications for treatment-resistant MDD. In most cases, the regimens of these drugs (used as adjuncts for the pharmacotherapy of MDD) are less than the dose used for the treatment of psychosis (Blier, 2014).

9.2.1 Aripiprazole

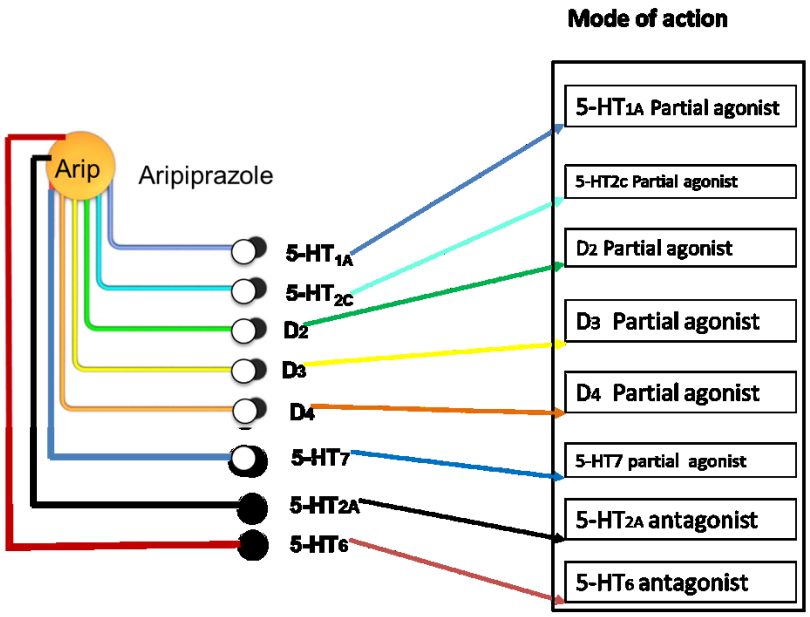
Aripiprazole is a partial DA and 5-HT receptor agonist (formerly known as atypical antipsychotic; Zohar et al., 2015). More specifically aripiprazole is a partial agonist at D_2, D_3, D_4, 5-HT_{1A}, 5-HT_{2C}, 5-HT_7, an inverse agonist of 5-HT_{2B}, and an antagonist at 5-HT_{2A} and 5-HT_6 (Shapiro et al., 2003; Davies et al., 2004) with moderate to low affinity for α_1-adrenergic and H_1-histamine receptors (Burris et al., 2002; Shapiro et al., 2003).

Clinical studies have shown aripiprazole to be an efficacious adjunct to drugs used for the pharmacotherapy of treatment-resistant depression (Barbee et al., 2004; Marcus et al., 2008a; Pae et al., 2007; Papakostas et al., 2005; Patkar et al., 2006; Simon and Nemeroff, 2005; Uher et al., 2020). Preclinical studies have indicated that aripiprazole has a favorable pharmacological profile to be used as an adjunct to other medications in the treatment of MDD. For instance, aripiprazole is a partial agonist of 5-HT_{1A} receptors (Jordan et al., 2002; Bortolozzi et al., 2007; Dahan et al., 2009), a property shared by other medication that are

effective in the treatment of MDD, for example, gepirone and buspirone (Feiger et al., 2003; Rush et al., 2006).

Also, aripiprazole is a 5-HT_{2C} partial agonist and a 5-HT_{2A} antagonist. It has been shown that combining aripiprazole with escitalopram reverses the escitalopram-induced suppression of firing activity of DA and NE neurons which can partly due to the effects of aripiprazole on 5-HT_{2C} and 5-HT_{2A} receptors (Chernoloz et al., 2009). Furthermore, it has been shown that long-term administration of the combination of escitalopram and aripiprazole has a synergistic effect on increasing tonic activation of 5-HT_{1A} receptors of pyramidal neurons in the CA3 region of the hippocampus (Ebrahimzadeh et al., 2019).

Figure 20. Simplified pharmacological profile of aripiprazole. In addition to the receptors presented in this figure, aripiprazole is also an antagonist of 5-HT_{2B} receptors.



9.2.2 Cariprazine

Cariprazine is a DA and 5-HT partial agonist used for the treatment of schizophrenia and bipolar mania (McCormack, 2015). *In vivo* studies have shown that cariprazine acts as a 5-HT_{1A} agonist in the DRN, a full agonist in the hippocampus, and an antagonist on 5-HT_{2A} receptors controlling the firing activity of NE neurons (Herman et al., 2018).

More specifically, cariprazine is a partial agonist at D_3, D_2, D_4, 5-HT_{2C}, 5-HT_7, an inverse agonist of 5-HT_{2B}, and an antagonist at 5-HT_6 receptors (Kiss et al., 2010). Unlike its predecessors, aripiprazole, and brexpiprazole, cariprazine displays a higher affinity for D_3 compared to D_2 receptors.

Similar to aripiprazole and brexpiprazole, cariprazine not only has been shown to be an efficacious adjunct treatment to drugs used for the treatment of MDD (Durgam et al., 2016), but it also possesses a pharmacological profile that might be potentially efficacious as a monotherapy for the treatment of MDD. Cariprazine has been recently approved in the United States as a monotherapy treatment for bipolar mania. The following section outlines the pharmacological properties of cariprazine that might underlie, at least in part, its clinical efficacy as an adjunct treatment for MDD.

Activation of 5-HT_{1A} receptors is a property shared by various drugs used for the treatment of MDD and hence has been hypothesized to be, at least in part, involved in their clinical efficacy (Blier and Ward, 2003). Similar to brexpiprazole,

cariprazine is a full agonist at 5-HT_{1A} receptors of the pyramidal neurons in the CA3 region of the hippocampus (Herman et al., 2018).

Blockade of 5-HT_{2A} receptors is a property shared by various DA and 5-HT partial agonist used as adjuncts to medications used for the treatment of MDD, including aripiprazole and brexpiprazole as well as olanzapine, risperidone, and quetiapine. Medications with this property have been shown to reverse the inhibitory effect of SSRIs on the firing frequency of LC NE neurons (Chernoloz et al., 2009; Seager et al., 2004; Dremencov et al., 2007; Chernoloz et al., 2012a).

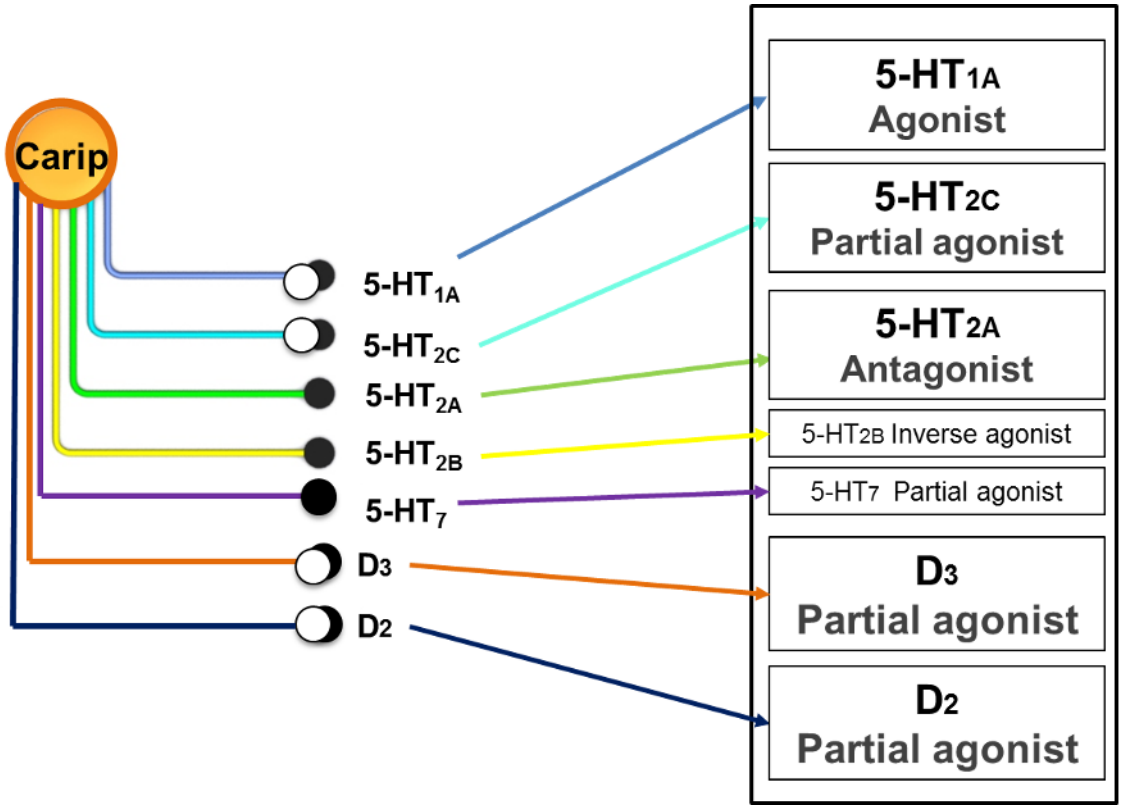
This reversal of SSRI-induced inhibition of NE firing activity might underlie, at least in part, the clinical efficacy of these drugs as adjuncts to SSRIs in patients with treatment-resistant MDD (Keitner et al., 2009; Garakani et al., 2008; Nelson and Papakostas, 2009). Cariprazine has also been shown to block the 5-HT_{2A} receptors controlling the firing activity of NE neurons *in vivo* (Herman et al., 2018).

Activating the D_2 and D_3 DA receptors is a common property between cariprazine, brexpiprazole, and aripiprazole. However, cariprazine, as mentioned above, is a D_3 preferring partial agonist, unlike the two other drugs. Pramipexole, also a D_3 receptor-preferring full agonist, is an efficacious adjunct to drugs used for the treatment of depressive episodes (Cassano et al., 2004; Goldberg et al., 2004; Chernoloz et al., 2012b; Cusin et al., 2013).

Having an affinity of 5-HT_{2C} receptors is also a common property of some DA and 5-HT partial agonists used as adjuncts to medications used for the

treatment of MDD, including aripiprazole, brexpiprazole, and cariprazine (low affinity). Blocking these receptors, for instance by aripiprazole, has been reported to reverse the SSRI-induced reduction of the firing activity of VTA DA neurons (Chernoloz et al., 2009).

Figure 21. Simplified pharmacological profile of cariprazine.



9.2.3 Treatment-emergent Adverse Effects of Adjunctive Aripiprazole and Cariprazine

When selecting DA and 5-HT partial agonists it is imperative to consider the adverse effects associated with this class of medication (including but not limited to weight gain and extrapyramidal symptoms; Durgam et al., 2016). Data from clinical studies on adjunctive aripiprazole for treatment-resistant MDD indicate akathisia, restlessness, insomnia, and blurred vision as adverse effects that are significantly higher than the placebo rate (Blier and Blondeau, 2011). According to data from a clinical study on adjunctive cariprazine for treatment-resistant MDD akathisia, insomnia, and nausea were the adverse effects with higher incidences than the placebo (Durgam et al., 2016).

10. Study rationale

It has been hypothesized that different monoamine systems possibly have different degrees of involvement in specific symptoms of MDD (Figure 22; Nutt, 2008). Accordingly, it may be possible to match the neurotransmission modulation profile of drugs to the clinical profile of individual patients in specific circumstances (Blier, 2014; Nutt et al., 2007).

However, the effects of drugs (used for the pharmacotherapy of MDD) on neurotransmission of monoamines are more complex than their pharmacological profile and their affinities for specific monoamine transporters and receptors indicated by *in vitro* experiments and can be further elucidated by conducting *in*

vivo investigations (including the experiments presented in chapters 2-4 of the present dissertation).

Despite the ample evidence about the clinical efficacy of the antidepressant strategies presented in chapters 2-4, the distinct effects of each of these strategies on neurotransmission of monoamines had yet to be fully investigated. Accordingly, the current reverse-translational studies investigated the net monoamine neurotransmission modulation by these comparable, yet distinct pharmacological strategies.

This goal was achieved by conducting three different scientific projects. The first project measured the effects of administration of the multimodal drug vortioxetine, an inhibitor of the 5-HTT with add-on pharmacological properties that had the potential to modulate NE and DA neurotransmission. The second and third projects measured the effect of combining the 5-HTT inhibitor escitalopram with either aripiprazole or cariprazine, which are both DA and 5-HT partial agonists with favorable pharmacological profiles to modulate 5-HT, NE, and DA neurotransmission. Aripiprazole and cariprazine have been used clinically as adjuncts to medications used for the pharmacotherapy of MDD.

The first study investigated the effects of vortioxetine on neurotransmission modulation of monoamines. Vortioxetine is a novel multimodal drug that inhibits the activity of 5-HTT with add-on properties for directly targeting 5-HT receptors. This unique pharmacological profile of vortioxetine combined with its pro-cognitive

clinical profile and its utility for the pharmacotherapy of MDD and treatment-resistant MDD necessitated the characterization of its effects on neurotransmission modulation of monoamines and glutamate. Since vortioxetine (in addition to inhibiting the activity of 5-HTT) activates 5-HT_{1A} receptors, it was hypothesized that it would either not have an inhibitory effect or have a milder effect (compared to a pure 5-HTT inhibitor) on NE and DA neurotransmission. Given the pro-cognitive clinical profile of vortioxetine and since it has been reported that vortioxetine, but not the SSRI escitalopram, increases the firing activity of glutamate pyramidal neurons in the prefrontal cortex this study investigated the effects of vortioxetine administration on AMPA- and NMDA-evoked responses of the pyramidal neurons in the CA3 regions of the hippocampus.

The second and third studies focused on the DA and 5-HT partial agonists aripiprazole and cariprazine. The clinical antidepressant augmentation strategies using the combination of the 5-HTT inhibitor escitalopram with aripiprazole or cariprazine motivated these preclinical investigations into the effects of combining escitalopram with aripiprazole or cariprazine on neurotransmission modulation of monoamines in the rat brain. Since aripiprazole and cariprazine directly engage the 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, it was hypothesized that the addition of these agents to escitalopram would potentiate the increasing effects of escitalopram on 5-HT neurotransmission while at the same time counteract the escitalopram-induced inhibition of NE and DA neurotransmission.

The effects of these drugs or combination of drugs on monoaminergic systems were assessed with *in vivo* single-unit electrophysiological recording of individual neurons in anesthetized male rats. These recordings were carried out following short- and long-term administration of drug regimens to determine the short-term and also clinically-relevant long-term effects of these regimens. The process of obtaining and analyzing the results of the abovementioned experiments and discussions on the relevance of their findings are outlined in the following chapters.

The main hypothesis of these studies was that complementing 5-HTT inhibition with add-on pharmacological features (for directly targeting of 5-HT and DA receptors) would distinctly modulate neurotransmission of monoamines in ways that might correspond to the clinical antidepressant effects of such pharmacotherapeutic strategies.

Chapter 2 — Partial Inhibition of Catecholamine Activity and Enhanced Responsiveness to NMDA after Sustained Administration of Vortioxetine

1. Title Page

Partial Inhibition of Catecholamine Activity and Enhanced Responsiveness to NMDA after Sustained Administration of Vortioxetine

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2. Abstract

Vortioxetine is a multimodal drug that blocks serotonin (5-HT) reuptake and directly modulates 5-HT receptors. The effects of subacute and long-term administration of vortioxetine on various aspects of catecholamine and glutamate systems were investigated using single-unit extracellular recordings and microiontophoresis in the rat brain. The firing rate of dopamine (DA) neurons was significantly decreased (26%) after 14, but not 4 days of vortioxetine administration (vortioxetine-containing chow, 1.8 g/kg vortioxetine). Same 14- and 4-day regimens of vortioxetine decreased the firing activity of norepinephrine (NE) neurons (by 27% and 41%, respectively). For DA and NE neurons, 14-day vortioxetine exposure also decreased the number of bursts per minute, without changing the number of spikes per burst, percentage of spike firing in burst, and the number of spontaneously active neurons per track. However, this vortioxetine-induced suppression of DA and NE neuronal activity is less than that obtained in previous studies with the selective 5-HT reuptake inhibitor (SSRI) escitalopram. In the CA3 region of the hippocampus, 14 days of vortioxetine exposure did not change the sensitivity of postsynaptic α_2-adrenoceptors nor did it increase the tonic activation of α_1- and α_2-adrenoceptors. Vortioxetine administration for 14 days increased the N-methyl-D-aspartate (NMDA)-, but not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-evoked responses of CA3 pyramidal neurons. Taken together, the results of the current study suggest that vortioxetine

might produce a lesser inhibition of DA and NE neuronal activity when compared to previous studies on the SSRI escitalopram.

3. Introduction

Sustained administration of selective serotonin (5-HT) reuptake inhibitors (SSRIs) elevates extracellular 5-HT concentrations, which initially decreases the firing rate of 5-HT neurons of the dorsal raphe nucleus (DRN) by activating 5-HT_{1A} autoreceptors (Blier and El Mansari, 2013). Such increased 5-HT levels also suppress the firing activity of ventral tegmental area (VTA) dopamine (DA) neurons with no effect on the number of spontaneously active DA neurons (Dremencov et al., 2009). This has been shown to be mediated by activation of 5-HT_{2C} receptors controlling DA neuronal firing (Di Matteo et al., 2001; Dremencov et al., 2009). Hence, while 5-HT neurons regain their firing baseline rate with SSRI treatment prolongation, due to desensitization of 5-HT_{1A} autoreceptors (Blier and de Montigny, 1983), the firing rate of DA neurons remains attenuated (Dremencov et al., 2009). Due to the critical role of DA neuronal activity in motivation, anhedonia, and reward (Nestler and Carlezon, 2006), the inhibition of DA neuronal firing might contribute, in some patients with major depressive disorder (MDD), to an incomplete or lack of response to SSRIs.

Sustained SSRI administration also inhibits the firing activity of locus coeruleus (LC) norepinephrine (NE) neurons by activating 5-HT_{2A} receptors, located on GABA neurons controlling NE neuronal activity (Szabo and Blier,

2001a, b). This persistent attenuation of NE neuronal firing activity has been hypothesized to contribute (Blier and El Mansari, 2013; Montoya et al., 2016), at least in part, to incomplete or lack of response to SSRIs in some patients with MDD. The reversal of the attenuated firing activity of these neurons has been documented with aripiprazole, olanzapine, risperidone, and paliperidone (Chernoloz et al., 2009; Seager et al., 2005; Dremencov et al., 2007a, b). Furthermore, this action was shown to be exerted through the blockade of 5-HT_{2A} receptors, which implies that using 5-HT_{2A} receptor antagonists in SSRI-resistant patients could produce a therapeutic response.

Beyond the role of monoamines, the glutamate system has also been implicated in the therapeutics of MDD. In particular, the N-methyl-D-aspartate (NMDA) antagonist ketamine has been shown to exert a fast onset of therapeutic response, even in cases of treatment-resistant MDD (Sanacora et al., 2017). Based on findings of preclinical studies, the potential contribution of increased throughput of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to NMDA receptors has been hypothesized to underlie the rapid antidepressant effect of ketamine (Du et al., 2006). Consistent with this hypothesis, it has been reported that acute low doses of ketamine increased the responsiveness of AMPA receptors of the pyramidal neurons in the rat hippocampus (El Iskandrani et al., 2015). It has been reported that the multimodal serotonergic agent vortioxetine, but not the SSRI escitalopram, increases the firing activity of glutamate pyramidal neurons in the prefrontal cortex (Riga et al., 2016).

Vortioxetine is a 5-HT transporter (5-HTT) inhibitor, a 5-HT_{1A} receptor agonist, a 5-HT_{1B} receptor partial agonist, and a 5-HT_3, 5-HT_7, and 5-HT_{1D} receptor antagonist that can improve cognitive function independently of its actions on mood (Katona et al., 2012; Mahableshwarkar et al., 2015; McIntyre et al., 2014). Because of inhibition of 5-HT reuptake, chronic vortioxetine may dampen the firing activity of NE and DA neurons. However, this dampening may not occur because of the vortioxetine's 5-HT_{1A} agonism, known to increase NE and DA neurons firing (Diaz-Mataix et al., 2005; Piercey et al., 1994). The present study was thus aimed at assessing the effects of vortioxetine on catecholamine neurons, and on glutamatergic pyramidal neurons in the hippocampus, using electrophysiological paradigms in rats.

4. Materials and Methods

4.1 Experimental Preparations

The experiments were conducted on male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada). Animals were kept (two per cage) in a controlled environment with (12:12 light-dark cycle) and ad libitum access to food and water. Following arrival, rats went through a treatment-free acclimatization period for 5-7 days. Animals weighed between 280-320 g at the time of electrophysiological recordings. Rats were assigned, randomly, to the treatment or the control group (4 days or 14 days). The experiments were approved by the local Animal Care Committee (University of Ottawa, Institute of Mental Health

Research, Ottawa, Ontario, Canada) and were carried out in accordance with the Canadian Council on Animal Care, for the care and use of laboratory animals.

For electrophysiological recordings, rats were anesthetized with intraperitoneal (i.p.) injection of chloral hydrate (400 mg/kg) and supplemental doses (100 mg/kg, i.p.) were given to keep the animal anesthetized. Following anesthesia, rats were mounted on a stereotaxic apparatus and an intravenous catheter was inserted in a lateral tail vein for acute drug delivery. During the electrophysiological recordings, body temperature was maintained at 37°C using a temperature-controlled heating pad.

4.2 Treatment

Based on the results of previous experiments (Wallace et al., 2014), vortioxetine administration was done via ad libitum access to vortioxetine-containing chow. No significant difference in intake of chow was found in rats that received vortioxetine compared to vehicle (Smagin et al., 2016). This chow diet was chosen in order to reach 5-HTT occupancy (Wallace et al., 2014) comparable to that achieved with therapeutic doses of SSRIs (Meyer, 2007). Moreover, the chow diet was used to achieve pharmacologically relevant occupancies of 5-HT_{1A} (Ki = 230 nM) and 5-HT_7 (Ki = 200 nM) receptors because of the low affinity of vortioxetine to these receptors in rats (Pehrson et al., 2014). Purina 5001 rodent chow (control) had the same nutritional content as the one containing vortioxetine.

4.3 Compounds

Purina rodent chow 5001 with 1.8 g/kg vortioxetine or vehicle was manufactured by Research Diet, Inc (New Brunswick, NJ, US) and provided to us by Lundbeck A/S pharmaceutical company, Ltd. (Valby, Denmark). All other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada). Norepinephrine (noradrenaline) bitartrate (4-[(1R)-2-amino-1-hydroxyethyl]-1,2-benzenediol (L-(+))-bitartrate salt), 5-HT creatinine sulfate (3-[2-aminoethyl]-5-hydroxyindole creatinine sulfate complex), quisqualic acid (b-(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine), AMPA hydrobromide ((±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide) and NMDA (N-methyl-D-aspartate) were dissolved in 0.2 M NaCl. The α_1-adrenoceptor antagonist prazosin (100 µg/kg) and the α_2-adrenoceptor antagonist idazoxan (1 mg/kg) were dissolved in distilled water.

4.4 *In vivo* Electrophysiological Recordings

Firing activity of VTA DA and LC NE neurons were recorded extracellularly using a single-barrel glass micropipette, with an impedance of 2 to 6 MΩ, which was filled with 2 M NaCl. Recordings of hippocampus pyramidal neurons were done with a five-barrel glass micropipette.

4.5 Electrophysiological Recording of DA Neurons

Recordings of putative DA neurons were obtained by lowering (6-8.5 from the surface of the brain) a single barrel microelectrode in the following

coordinates: 3.2-3.6 mm anterior to lambda and 0.6-1.0 mm to the midline suture (Paxinos and Watson, 1998). DA neurons were identified by their established electrophysiological criteria: a regular firing rate (2-10 Hz), an irregular single spiking pattern, a long duration (> 2.5 ms) action potential often with a notch on the rising phase and a slow bursting activity with the amplitude of the action potentials progressively decreasing in a given burst and a low pitch sound on the audiometer (Ungless and Grace, 2012). As previously described (Valenti et al., 2011) the number of neurons per track was identified by recording multiple tracks in a 400 μm x 400 μm grid.

4.6 Electrophysiological Recording of LC NE Neurons

For assessing the firing activity of NE neurons, a single barrel microelectrode was lowered (4.5-6.0 mm from the surface of the brain) in the following coordinates: 0.9-1.2 mm posterior to lambda and 0.9-1.3 mm to the midline suture. NE neurons were identified by their regular firing rate (0.5-5 Hz), long duration (~2 ms), and biphasic action potentials. Another identifying factor was a quiescent period succeeding a volley of spike discharges following a nociceptive pinch of the contralateral hind paw (Marwaha and Aghajanian, 1982).

4.7 Extracellular Recording and Microiontophoresis of Dorsal Hippocampus CA3 Pyramidal Neurons

Recordings of hippocampal pyramidal neurons were carried out with a five-barrel glass micropipette. The NaCl-filled (2 M) central barrel of the pipette, with

an impedance of 2-5 M Ω, was used for extracellular unitary recording and a side barrel, also filled with 2 M NaCl solution was used for automatic current balancing. The other side barrels were filled with the following solutions depending on each experiment: quisqualic acid (1.5 mM in 0.2 M NaCl, pH 8), norepinephrine bitartrate (10 mM in 0.2 M NaCl, pH 4), 5-HT creatinine sulfate (15 mM in 0.2 M NaCl, pH 4), AMPA hydrobromide (5 mM in 0.2 M NaCl, pH 8) and NMDA (10 mM in 0.2 mM NaCl, pH 8).

The micropipette was lowered (4.0 ± 0.5 mm from the surface of the brain) into the hippocampus CA3 region at the following coordinates: 4.0-4.2 mm anterior to lambda and 4.2 mm to the midline suture. Since these pyramidal neurons do not discharge spontaneously under chloral hydrate anesthesia, a small current of quisqualic acid was used to activate them within their physiological range (10-15 Hz; Ranck, 1975). The used identification criteria for pyramidal neurons included: long duration, large amplitude, and simple action potentials alternating with complex spike discharges (Kandel and Spencer, 1961).

4.8 *In vivo* Determination of 5-HT and NE Reuptake

Microiontophoretic ejection of 5-HT creatinine sulfate and NE bitartrate was used to reduce the firing activity of CA3 pyramidal neurons to approach near-complete suppression. Following the termination of the microiontophoretic ejection, pyramidal neurons gradually regained their firing activity due to the active reuptake process of 5-HT and NE, by the 5-HTT and NE transporter (NET)

respectively. The time bracket from the end of the microiontophoretic ejection to where the activity of neuron has recovered to half of its initial firing activity is defined as the RT50 value and is used as a reliable *in vivo* index for the activity of the 5-HTT and NET (de Montigny et al., 1980; Piñeyro et al., 1994; El Mansari et al., 2015a). The RT50 values which measure 5-HTT and NET blockade were determined to ensure that the ad libitum regimen of vortioxetine-containing chow has resulted in relevant concentrations of vortioxetine and a significant blockade of 5-HTT.

4.9 Determination of Sensitivity of α_2- and Tonic Activation of α_2- and α_1-Adrenoceptors in the Hippocampus

The sensitivity of the α_2-adrenoceptors was assessed by determining the neuronal responsiveness to the iontophoretic application of NE (Curet and de Montigny, 1988). After establishing a proper baseline firing activity, NE was iontophoretically ejected for 50 seconds to inhibit the firing activity of pyramidal neurons. The responsiveness of the recorded neuron was measured by calculating the number of spikes within the 50-second period prior to NE ejection minus the number of spikes during the ejection divided by the applied current. This gives the number of spikes suppressed by the amount of current ejected.

The selective adrenoceptor antagonists, idazoxan, and prazosin were used for investigating a possible tonic activation of postsynaptic α_2- and α_1-adrenoceptors, respectively. After obtaining a lowered and steady-state firing rate,

idazoxan (1 mg/kg) and prazosin (100 µg/kg) were sequentially administered intravenously to investigate possible disinhibition of pyramidal neuronal activity. The used doses of idazoxan and prazosin have been reported as physiologically effective in an electrophysiological setting (Chernoloz et al., 2012).

4.10 Determination of AMPA- and NMDA-evoked Activity of CA3 Pyramidal Neurons

The drug effect was assessed by measuring the degree of excitation of pyramidal neurons (measured as the number of spikes generated for 60 s ejection) induced by iontophoretic application of NMDA and AMPA. Two of the side barrels of the electrode were filled with AMPA hydrobromide and NMDA and another one was filled with quisqualate. Both the duration (60 seconds) and ejection currents of AMPA and NMDA were kept constant in control and vortioxetine-administered rats. To activate and locate the neurons, a small current of quisqualate was used (-2 to -5 nA). Once a pyramidal neuron was found, the quisqualate pump was stopped and AMPA and NMDA were applied. When AMPA and NMDA were not ejected, a retention current of +15 nA was applied to prevent leakage from the barrels.

Effect of acute intravenous (i.v.) injection of vortioxetine (6 mg/kg; El Mansari et al., 2015b) on the responsiveness of neurons was measured by recording the firing activity of neurons in response to iontophoretically ejected AMPA (-1 nA) and NMDA (-8 nA) before and after the injection. Prior to the

vortioxetine injection, rats received an i.v. injection of saline followed by measuring the AMPA- and NMDA- evoked firing activity of pyramidal neurons. Two minutes after vortioxetine injection, recording the responsiveness of neurons to AMPA and NMDA was started and continued for thirty minutes.

The effects of long-term vortioxetine exposure (vortioxetine-containing chow, 1.8 g/kg) were assessed by comparing the responsiveness of pyramidal neurons to 60 s of AMPA (0,-1,-2 nA) and NMDA (-7,-8,-9 nA) ejection in control and vortioxetine-exposed rats. The acquired data were used to construct a dose-response curve representing the iontophoretic ejection current and frequency of firing of pyramidal neurons (Hz ± S.E.M.).

4.11 Data Analysis

The acquired data are presented as mean values ± S.E.M. In VTA and LC, comparisons between controls and treated groups were carried out using Kruskal-Wallis One-Way analysis of variance (ANOVA) on ranks followed by Dunn's method. Interspike interval (ISI) burst analysis was used for analyzing the firing patterns of DA and NE neurons. The initiation of a burst was defined as the occurrence of two spikes with ISI < 0.08 s and the termination of the bursts were defined as ISI > 0.16 s (Grace and Bunney, 1984). The burstiDAtor software was used in this project for burst analysis (https://github.com/nno/burstiDAtor).

Paired t-test and Mann-Whitney U tests were used for measuring the effect of acute i.v. injection of vortioxetine on AMPA- and NMDA-evoked firing activity of

pyramidal neurons. For analyzing, the data related to tonic activation of adrenoceptors and comparing AMPA- and NMDA-evoked firing of pyramidal neurons in control and 14-day vortioxetine administered rats, two-way analyses of variance with repeated measures were used, with treatment as the main factor. Throughout the analyses, statistical significance was taken as $p < 0.05$. These statistical comparisons were analyzed using the software Graphpad (Prism Software Inc, La Jolla, CA).

5. Results

5.1 Effects of Sustained Vortioxetine Exposure on the Activity of the 5-HTT and NET

The RT50 values were determined as an *in vivo* measure for activity of 5-HTT and NET. RT50 values were significantly increased in rats exposed to vortioxetine for 4 (240%) and 14 days (650%), compared to control rats indicating a potent blockade of the 5-HTT (Fig. 1A). This increase of the recovery time of firing activity of pyramidal neurons after 5-HT ejection (increased RT50 value) indicated the presence of physiologically relevant doses of vortioxetine, enough to inhibit the activity of 5-HTT.

Microiontophoretic applications of NE were carried out in the vehicle group and in rats exposed to vortioxetine for 14 days. As illustrated in Figure 1B, the RT50 values did not significantly change in the controls and rats exposed to vortioxetine for 14 days, indicating that NET was not blocked.

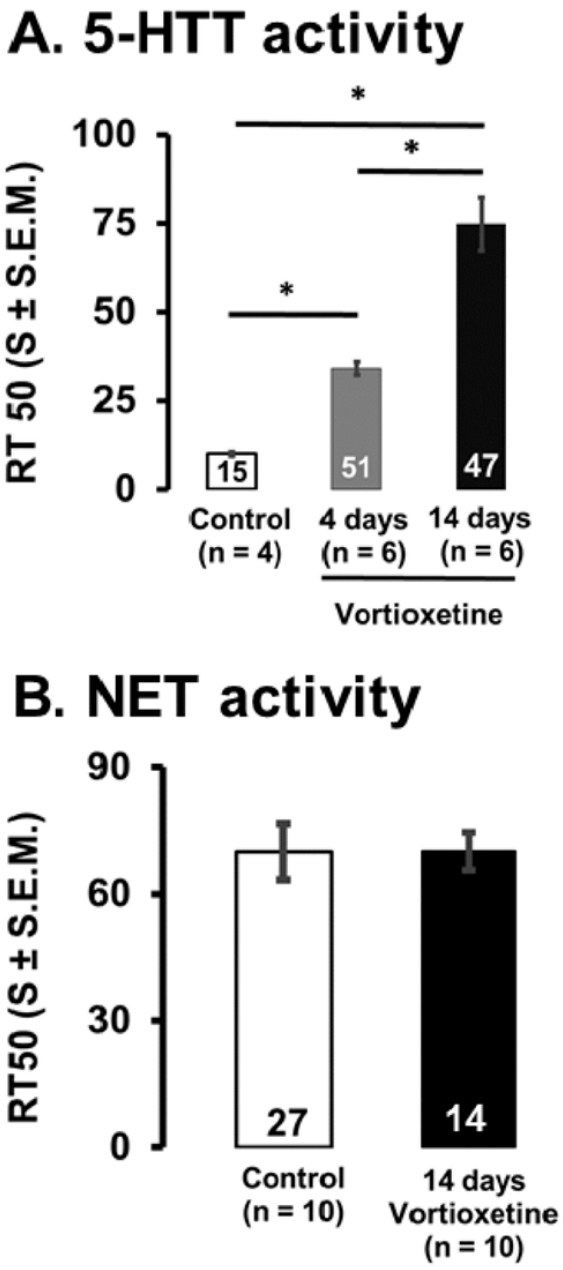


Figure 1. Effect of 4- and 14-day vortioxetine exposure on the recovery time, expressed as RT50 values, from microiontophoretic applications of 5-HT (A) and NE (B) with 20 nA. (A)There was a significant increase in 5-HTT-related RT50 values in 4- and 14-day vortioxetine administered rats when compared to the control group. (B) Vortioxetine administration for 14 days did not have any statistically significant effect on NET-related RT50 values when it is compared to the control group. The numbers of neurons are indicated at the bottom of each graph. The numbers of rats are indicated in the brackets. * $p <$ 0.05.

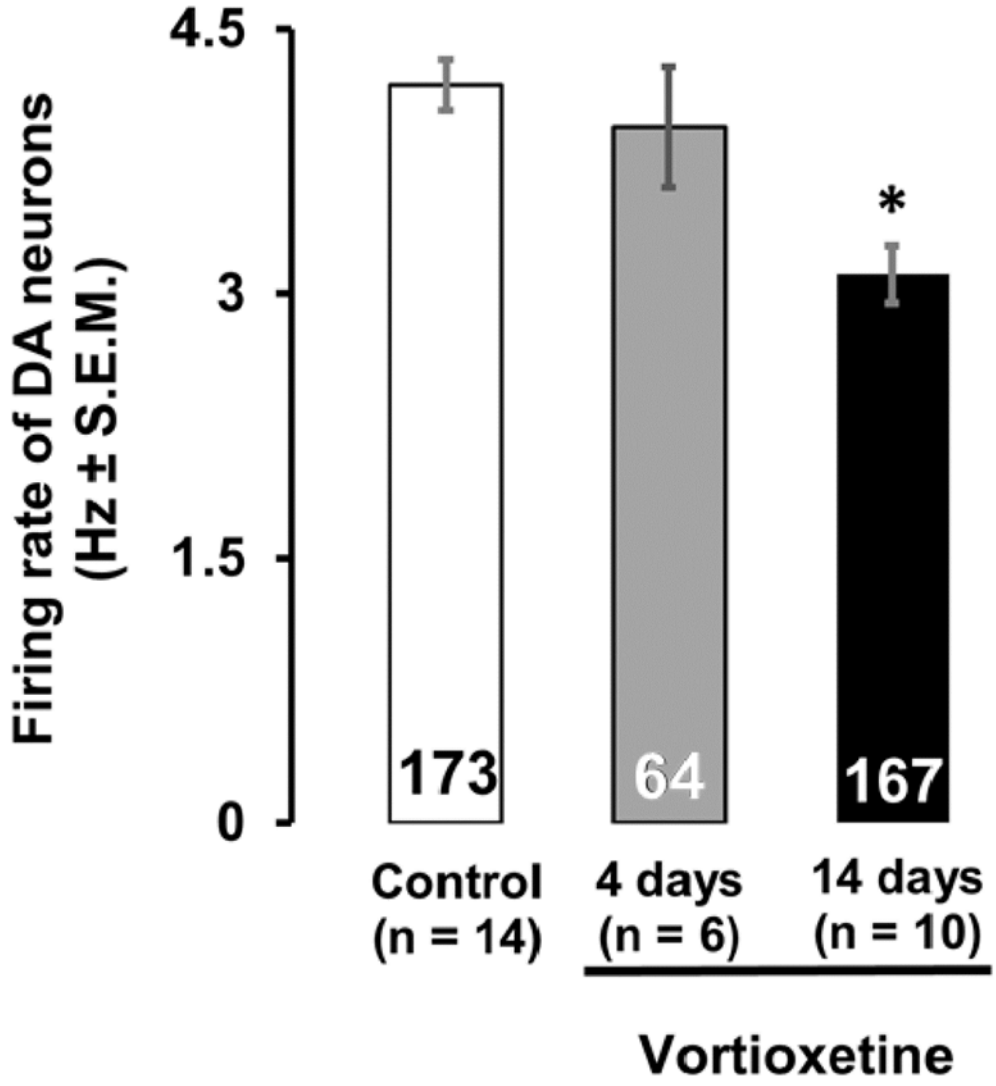
5.2 Effects of 4- and 14-day Vortioxetine Administration on the Firing Activity of VTA DA Neurons

The mean firing activity of DA neurons in the 4- and 14-day control groups were not significantly different (4-day control group: 4.6 ± 0.2 Hz, n = 93 versus 14-day control group: 4.2 ± 0.2 Hz, n = 80) and consequently these two groups were combined.

While vortioxetine administration for 4 days did not significantly change the firing activity of DA neurons (Figure 2), its administration for 14 days, however, significantly decreased their activity by 26% (Figure 2).

Vortioxetine administration for 14 days, but not 4 days, decreased the burst frequency of DA neurons without changing the bursting pattern (spikes/burst and percentage of spikes in burst) and the number of spontaneously active DA neurons per track (Table 1).

Figure 2. Firing rates of VTA DA neurons (mean ± S.E.M.) in control, 4- and 14-day vortioxetine-administered rats (1.8 g/kg, vortioxetine-infused chow). The numbers of neurons are indicated at the bottom of each histogram. The numbers of neurons are indicated at the bottom of each graph. The numbers of rats are indicated in the brackets. * $p < 0.05$ compared to the control group.



	Burst/min	Spikes/burst	% spikes in burst	Neurons/track
Control (n = 151)	**22 ± 2**	**3.2 ± 0.2**	**29 ± 2**	**1.5 ± 0.1 (14 rats)**
4-day vortioxetine (n = 54)	**19 ± 3**	**2.8 ± 0.2**	**26 ± 4**	**1.6 ± 0.3 (6 rats)**
14-day vortioxetine (n = 91)	**14 ± 2***	**2.8 ± 0.1**	**21 ± 3**	**1.5 ± 0.2 (10 rats)**

Table 1: Bursting activity (mean ± S.E.M.) of VTA DA neurons. * $p < 0.05$, compared to the control group; n indicates the number of neurons.

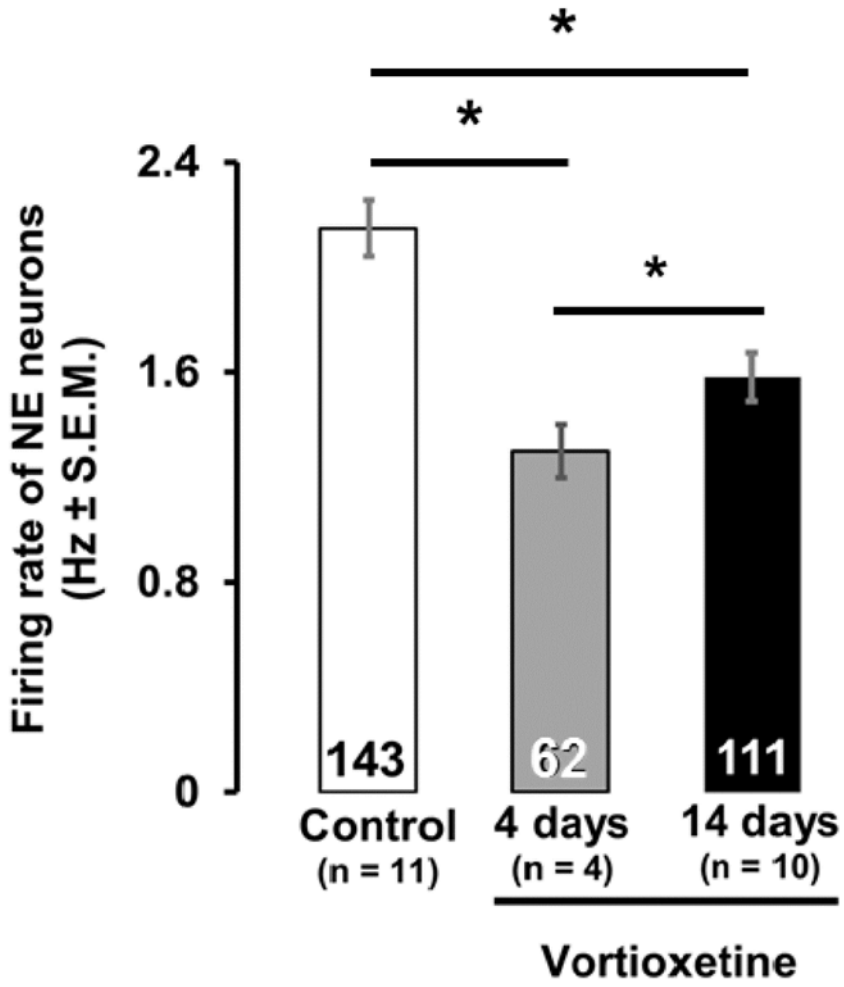
5.3 Effects of 4- and 14-day Vortioxetine Administration on the Firing Activity of LC NE Neurons

The firing activity of NE neurons in the 4- and 14-day control groups were not statistically different (4-day control group: 2.0 ± 0.1, n = 65 versus 14-day control group: 2.4 ± 0.2 Hz, n = 78). Hence, the data from these two groups were combined.

Vortioxetine administration for 4 days significantly decreased the firing rate of NE neurons by 41% (Fig. 3). Following the administration of vortioxetine for 14 days, the firing rate of NE neurons remained dampened when compared to the control group, but to a significantly lesser extent in comparison to the 4-day group (27%; Figure 3).

As illustrated in table 2, vortioxetine administration for 14 days, decreased the burst frequency of NE neurons without changing the bursting pattern (spikes/burst and percentage of spikes in burst) and the number of spontaneously active NE neurons per track.

Figure 3. Mean (± S.E.M.) firing rate of LC NE neurons in control and vortioxetine-administered rats for four and fourteen days. The numbers of neurons are indicated at the bottom of each graph. The numbers of rats are indicated in the brackets. * $p < 0.05$.



	Burst/min	Spikes/burst	% spikes in burst	Neurons/track
Control (n = 80)	**5.8 ± 1.2**	**2.2 ± 0.0**	**6 ± 1**	**2.9 ± 0.3 (11 rats)**
4-day vortioxetine (n = 19)	**4.5 ± 2.8**	**2.1 ± 0.1**	**6 ± 4**	**3.1 ± 0.5 (4 rats)**
14-day vortioxetine (n = 37)	**1.9 ± 0.4***	**2.5 ± 0.3**	**4 ± 1**	**3.4 ± 0.3 (10 rats)**

Table 2: Bursting activity of LC NE neurons (mean ± S.E.M.). * $p < 0.05$, compared to the control group; n indicates the number of neurons.

5.4 Effect of Prolonged Administration of Vortioxetine on the Sensitivity of α_2- and Tonic Activation of α_2- and α_1-adrenoceptors of the Pyramidal Neurons of the Hippocampus

The sensitivity of the α_2-adrenoceptors, measured by the number of spikes inhibited by iontophoretic ejection of NE per nanoampere, was not altered by vortioxetine exposure for 14 days.

In 14-day vortioxetine-administered rats, systemic administration of the α_2- and α_1-adrenoceptor antagonists, idazoxan (1 mg/kg) and prazosin (100 µg/kg) respectively (Figure 4), did not result in a change in the firing activity of pyramidal neurons of the hippocampus compared to the control group (Figure 5). These

results indicate that there was no increase in NE neurotransmission in the hippocampus after 14 days of vortioxetine administration.

Figure 4. Integrated firing rate histograms of CA3 pyramidal neurons of the dorsal hippocampus illustrating its responsiveness to microiontophoretic ejection of NE (20 nA) and systemic administration of idazoxan (1 mg/kg; Idaz) and prazosin (100 µg/kg; Praz) in a control rat (A) and a 14-day vortioxetine-treated rat (B). Note the decreased inhibition of firing produced by NE following the injection of the noradrenergic antagonists.

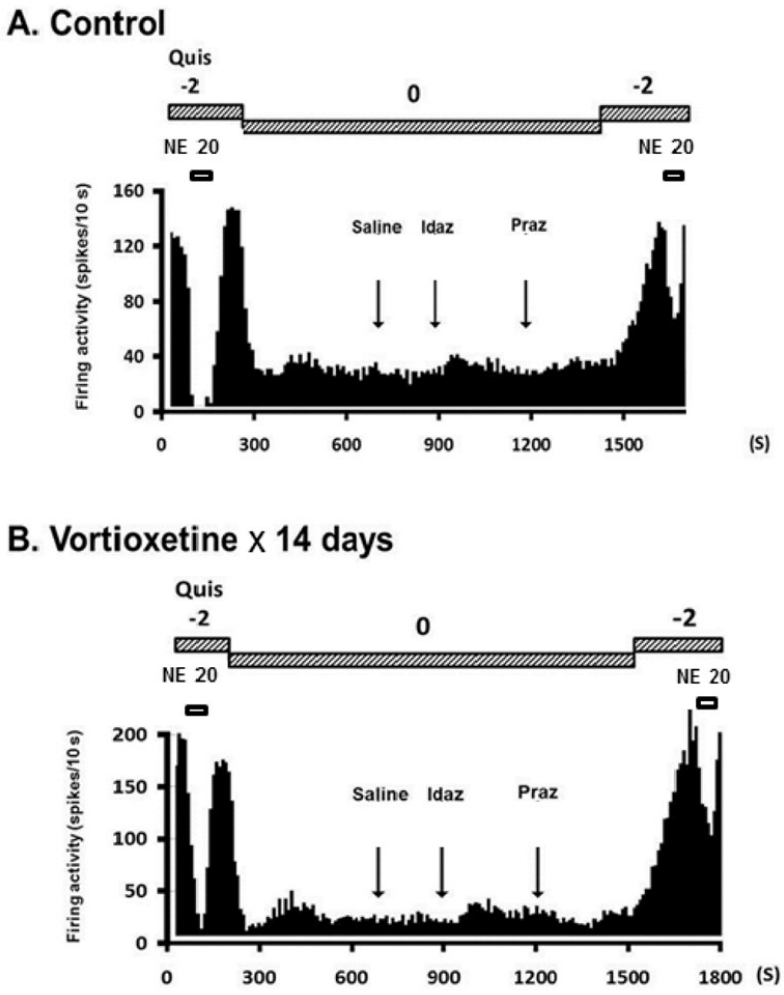
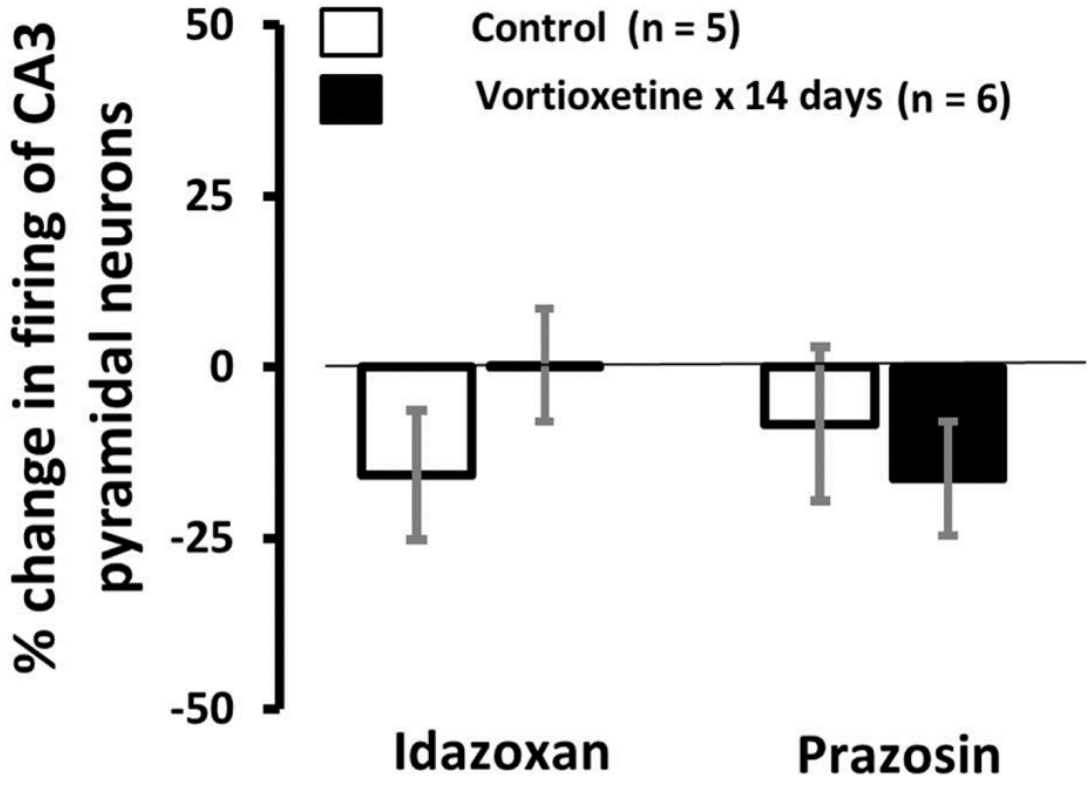


Figure 5. Percentage of change in firing frequency of pyramidal neurons in response to systemic administration of idazoxan (1 mg/kg) and prazosin (100 µg/kg) in control and 14-day vortioxetine treated rats. The numbers of rats are indicated at the top of the graph.



5.5 Effects of Acute Injection and Sustained Exposure to Vortioxetine on AMPA- and NMDA-induced Firing Activity of Pyramidal Neurons of the CA3 Region of the Hippocampus

Intravenous vortioxetine administration (6 mg/kg; El Mansari et al., 2015b) significantly decreased AMPA-evoked firing activity in 7 out of 11 pyramidal neurons (mean firing activity prior to vortioxetine injection was 13 ± 1.4 versus 7 ± 1.9 Hz after vortioxetine injection; $p = 0.02$). In the remaining four neurons, vortioxetine did not cause a significant change in the AMPA-evoked firing activity.

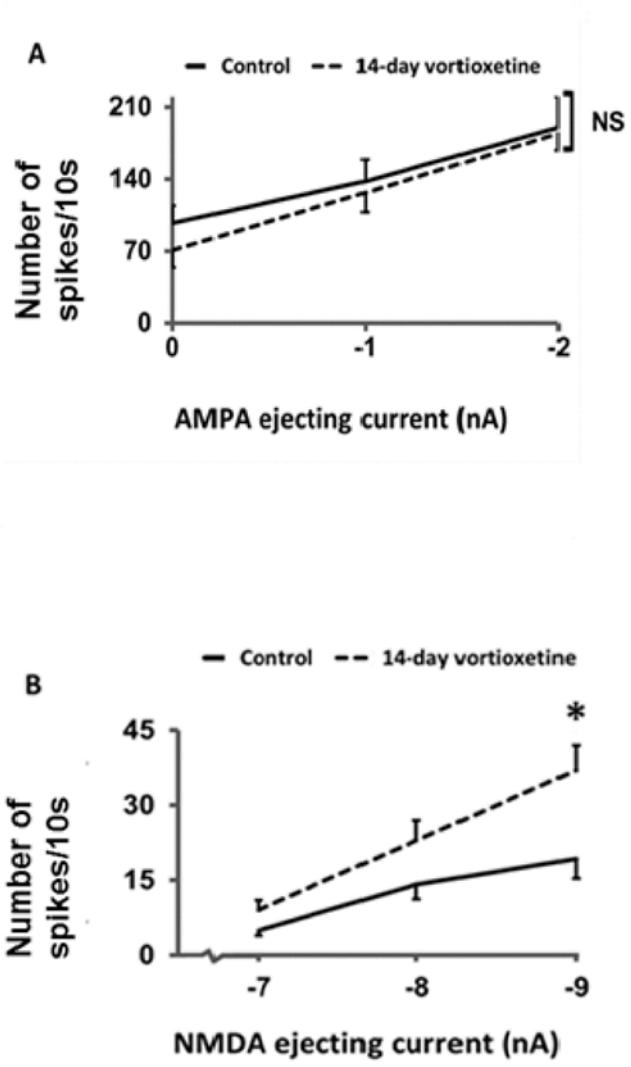
However, in 14-day vortioxetine administered rats, there was no alteration in the responsiveness of pyramidal neurons to iontophoresed AMPA, using incremental currents of ejection (Fig. 6A).

Intravenous administration of vortioxetine (6 mg/kg) significantly decreased NMDA-induced firing activity of pyramidal neurons in 8 out of 10 tested pyramidal neurons (mean firing rate prior to vortioxetine injection was 12 ± 1.2 versus 6 ± 1.1 Hz after vortioxetine injection; $p = 0.01$). In the two other neurons, injection of vortioxetine had no effect on the NMDA-evoked firing activity (mean firing rate prior to vortioxetine injection was 12 ± 1.3 versus 13 ± 2.3 Hz after vortioxetine injection).

Fourteen days of vortioxetine exposure, however, significantly enhanced NMDA-induced firing of pyramidal neurons (Fig. 6B, $F [1, 103] = 5.2$; $p = 0.03$).

Moreover there was a significant effect of current (F [1, 103] = 36.9; p = 0.001) as well as a significant interaction (treatment x current, F [1, 103] = 3.7; p = 0.03).

Figure 6. The effects of 14-day vortioxetine administration on the responsiveness of AMPA and NMDA receptors. Responsiveness is measured by calculating the number of spikes (per 10 seconds) generated by microiontophoretic application of AMPA (0, -1, and -2 nA) and NMDA (-7,-8, and -9 nA). NS: non-significant. * p < 0.05.



6. Discussion

The present *in vivo* electrophysiological experiments showed that prolonged vortioxetine administration dampened the firing activity of catecholamine neurons, although to a lesser degree than that observed with the SSRI escitalopram. Following this same regimen, the current results also showed that the tonic activation of the α_1- and α_2-adrenoceptors on hippocampus pyramidal neurons was not changed. On the other hand, while 14 days of vortioxetine exposure did not change the AMPA-evoked firing activity of pyramidal neurons, the NMDA-evoked activity of these neurons was increased after 14 days of vortioxetine regimen.

The current study showed an increase of the RT50 values for 5-HT in the hippocampus indicating blockade of 5-HTT by vortioxetine. The RT50 values also significantly increased from 4- to 14-day vortioxetine administration, suggesting that the steady-state level of vortioxetine was not achieved after 4 days of treatment.

Acute administration of vortioxetine has been shown to cause no change in hippocampal and cortical DA levels as well as VTA DA neuronal firing activity (Pehrson et al., 2013). In the present study, although 4-day vortioxetine administration did not change the firing activity of DA neurons, its sustained administration significantly dampened the firing activity of these neurons by 26%. Since vortioxetine has no significant affinity for any DA receptors or the DA

transporter (Bang-Andersen et al., 2011), the latter effect may be due to an increase of 5-HT levels around DA neuron cell bodies. Indeed, evidence has shown that 5-HT neurons exert an inhibitory effect on DA neurons through 5-HT_{2C} receptors (Di Matteo et al., 2001; Gobert et al., 2000; Prisco et al., 1994). In addition, this suppression of DA neurons firing was blocked by the selective 5-HT_{2C} receptor antagonist SB 242084 (Dremencov et al., 2009). Although in the current study vortioxetine exposure has suppressed the activity of DA neurons (26%), this decrease was lower compared to one induced by the SSRI escitalopram, as found in previous studies (40-50%; Chernoloz et al., 2009; Dremencov et al., 2009). Likewise, a lower decrease of firing activity of DA neurons compared to escitalopram was found with vilazodone (28%), which is an inhibitor of 5-HTT (Ki = 0.5 nM) and an agonist with high affinity for 5-HT_{1A} receptors of rats (Ki = 0.2 nM; Dawson and Watson, 2009; El Mansari et al., 2015a). It might be argued that this relatively weak suppression of DA neuronal activity by vortioxetine and vilazodone may be due to the activation of 5-HT_{1A} receptors by these compounds. Indeed activation of 5-HT_{1A} receptors by 5-HT_{1A} receptor agonists was shown to markedly increase firing and bursting activities of DA neurons in the VTA, much more so than for NE neurons (Gronier, 2008; Lejeune and Millan, 1998). This enhancing action of a 5-HT_{1A} receptor agonist is abolished by 5-HT_{1A} receptor antagonist WAY 100635 (Ichikawa et al., 2001). However, due to the weak occupation of 5-HT_{1A} receptors by vortioxetine in rats (Ki = 230 nM) compared to humans (Ki = 15 nM), the involvement of the latter receptor in this effect remains uncertain.

In the LC, the current study showed that the firing activity of NE neurons was significantly dampened after 4 days of exposure to vortioxetine as it was reported in another study after acute injection (Pehrson et al., 2013). In the current study, after 14 days of exposure, the firing activity of NE neurons remained decreased but to a lesser degree. The inhibitory action of vortioxetine on NE neuronal firing may be due to an enhancement of 5-HT levels resulting from a potent blockade of the 5-HTT (Fig. 1A), as shown in the present study and previous microdialysis experiments (Pehrson et al., 2013). Interestingly, it was shown that the inhibitory effect obtained with the SSRI escitalopram is mediated via activation of 5-HT_{2A} receptors since it is reversed by injection of the selective 5-HT_{2A} receptor antagonist M100907 (Dremencov et al., 2007a; Haddjeri et al., 1997). These results are similar to those obtained with several SSRIs that share blockade of 5-HTTs with vortioxetine (Chernoloz et al., 2009; Dremencov et al., 2007a; Ghanbari et al., 2010; Szabo et al., 2000). The vortioxetine-induced inhibition of NE neuronal firing activity (27%) in the present study was, however, less than the reported decrease following administration of escitalopram, found in earlier studies (40-50%; Chernoloz et al., 2009; Ghanbari et al., 2010). Although vortioxetine and escitalopram were not directly compared, the magnitude of their effect on DA and NE firing activity appears to be different. On the one hand, such a comparison does not take into consideration that vortioxetine has an agonistic activity at 5-HT_{1A} receptors and may have exerted an excitatory influence on NE neuronal activity. Indeed, previous studies have shown that 5-HT_{1A} receptor agonists increase neuronal firing rate and burst activity of NE neurons (Piercey et

al., 1994; Szabo and Blier, 2001a, b). On the other hand, although vortioxetine has a high affinity for the human 5-HT_{1A} receptors, it has a weaker affinity to these receptors in rats (Pehrson et al., 2014). By increasing the dose of vortioxetine in the present study, occupancy of 5-HTT was aimed to reach a level (Wallace et al., 2014), that is reached in patients with MDD treated with SSRIs (Meyer, 2007). It is possible that a lack of recovery of NE firing activity to normal after 14 days of vortioxetine administration may have stemmed from the desensitization of the 5-HT_{1A} receptors. This argument is strengthened by a previous report demonstrating that the increasing effect of the 5-HT_{1A} receptor agonist 8-OH-DPAT on NE neuronal firing was abolished in rats that received the SSRI citalopram, due to desensitization of the 5-HT_{1A} receptor (Szabo et al., 2000). Furthermore, it was shown that long-term administration of vilazodone, a 5-HTT inhibitor that possesses high affinity to 5-HT_{1A} receptors in rats, also resulted in a significant decrease (33%) of neuronal activity of NE neurons, when 5-HT_{1A} receptors were desensitized (El Mansari et al., 2015a). Nonetheless, the contribution of these 5-HT_{1A} receptors to this effect remains uncertain due to the weak occupation of 5-HT_{1A} receptors by vortioxetine in rats.

Despite an increase in NE levels in the hippocampus after a 3-day administration of vortioxetine (Pehrson et al., 2013), the present electrophysiological study did not show an increase in the tonic activation of α_1- and α_2-adrenoceptors following a 14-day administration of this compound. This lack of increase cannot be due to a desensitization of α_2-adrenoceptors as these

receptors are normosensitive under a prolonged vortioxetine regimen as shown in the present work. Although vortioxetine, which possesses no affinity for either adrenoceptors or NET (Bang-Andersen et al., 2011), induced no change in tonic activation of α_2- and α_1-adrenoceptors, an increase in tonic activation of these receptors was present with drugs that have an affinity for adrenoceptors or NET, such as bupropion and quetiapine (Chernoloz et al., 2012; Ghanbari et al., 2010).

It is noteworthy that the measure of tonic activation in this study reflects the net effect of NE transmission because it is determined in postsynaptic neurons, while NE levels measured by microdialysis reflect the changes that take place in the extracellular compartment. In addition, the mentioned microdialysis study (Pehrson et al., 2013) was done with vortioxetine administered sub-acutely, while tonic activation in the current study was measured after long-term vortioxetine administration, which is more relevant to clinical conditions.

Previous studies have shown that acute and sub-chronic administration of the SSRI fluoxetine increases phosphorylation of the AMPA receptor subunits (Svenningsson et al., 2002). In the present study, acute administration of vortioxetine, which is also a 5-HTT inhibitor, had mixed effects whereby it decreased AMPA-evoked firing of CA3 pyramidal neurons in some neurons and did not alter it in others. However, following long-term administration of vortioxetine, the NMDA- but not AMPA-induced firing of these glutamatergic neurons increased. It is thus possible that this direct effect of vortioxetine contributes to the pro-cognitive effects of vortioxetine, which was reported to be

ameliorated following vortioxetine administration in rats (du Jardin et al., 2014; Jensen et al., 2014; Mørk et al., 2013) and in patients with MDD (Katona et al., 2012; Mahableshwarkar et al., 2015; McIntyre et al., 2014). Interestingly, a recent electrophysiological study using the same regimen of vortioxetine as herein (Riga et al., 2017) has shown that prolonged administration of vortioxetine increased the firing activity of glutamatergic pyramidal neurons in the rat medial prefrontal cortex, while the SSRI escitalopram had no effect (Riga et al., 2016).

In summary, the results of the present study in comparison with those obtained in previous studies using escitalopram suggest that vortioxetine might have a different effect on the basis of its weaker inhibitory activity on DA and NE neurons. However, only a head-to-head clinical trial would be able to determine whether these drugs have differential effectiveness on various symptom domains.

7. Conflict of Interest

M. El Mansari and M. Ebrahimzadeh declare no conflict of interest. P. Blier received grant funding and/or honoraria for lectures and/or participation in advisory boards for Allergan, Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Euthymics, Janssen, Lundbeck, Merck, Otsuka, Pfizer, Pierre Fabre, Servier, Shire, Takeda, and Valeant.

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design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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Chapter 3 — Synergistic Effect of Aripiprazole and Escitalopram in Increasing Serotonin but not Norepinephrine Neurotransmission in the Rat Hippocampus

1. Title Page

Synergistic Effect of Aripiprazole and Escitalopram in Increasing Serotonin but not Norepinephrine Neurotransmission in the Rat Hippocampus

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2. Abstract

In addition to schizophrenia and bipolar disorder, aripiprazole is approved as an adjunct for major depressive disorder (MDD). Adding aripiprazole to the 5-HT reuptake inhibitor escitalopram reverses the inhibitory action of escitalopram on the firing activity of rat 5-HT, norepinephrine (NE), and DA neurons. This study investigated how aripiprazole, escitalopram, and their combination affect the net effect of 5-HT and NE neurotransmission in the rat hippocampus. Electrophysiological recordings of hippocampus CA_3 pyramidal neurons were conducted in anesthetized Sprague-Dawley rats after 2- and 14-day administration regimens. Aripiprazole and escitalopram (2 and 5 mg/kg/day, respectively) were delivered alone or in combination through subcutaneous injections and implanted osmotic minipumps, respectively. Overall neurotransmission of 5-HT and NE were assessed by determining possible enhancements in tonic activation of 5-HT_{1A} receptors and α_1- and α_2-adrenoceptors. This was achieved by assessing increases in the firing rate of pyramidal neurons due to disinhibition induced by injections of antagonists for these three types of receptors. While neither 2- and 14-day administration of escitalopram nor aripiprazole significantly altered the firing rate of pyramidal neurons following injection of 5-HT_{1A} antagonist WAY 100635, their combination for 14 days significantly increased this parameter. Fourteen days of the same drug regimens did not change firing following injection of the α_1- and α_2-adrenoceptor antagonists prazosin and idazoxan, respectively. A synergy between aripiprazole and escitalopram was thus documented by an

increase in the tonic activation of 5-HT_{1A} receptors after 14 days of administration that may account, at least in part, for the benefits of this strategy in MDD.

3. Introduction

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) are currently the first line of pharmacotherapy for the treatment of major depressive disorder (MDD), yet treatment resistance and incomplete remission remains a major obstacle in the treatment of MDD not only with various medications but also psychological therapeutic interventions. Catecholamine (i.e. norepinephrine (NE) and dopamine (DA)) systems play an important role in the response to drugs used to treat MDD. They have been direct targets of a variety of antidepressant compounds (Dunlop and Nemeroff, 2007; Moret and Briley, 2011). In the presence of an SSRI, the 5-HT transporter (5-HTT) is blocked, which results in an increase in extracellular concentrations of 5-HT that exerts an inhibitory effect on the DA and NE systems (Chernoloz et al., 2009, 2012; Blier and El Mansari, 2013). This translates into a reduction of firing activity of ventral tegmental area (VTA) DA neurons via activation of 5-HT_{2C} receptors (Di Matteo et al., 2001; Dremencov et al., 2009; Demireva et al., 2018) and of locus coeruleus (LC) NE neurons via activation of 5-HT_{2A} receptors (Szabo and Blier, 2001a, b). The decrease in catecholamine activity may be one of the main causes of treatment resistance or incomplete remission following treatment with SSRIs (Blier and Blondeau, 2011; Blier and El Mansari, 2013), thereby justifying several strategies to overcome inadequate response to treatments of MDD with SSRIs. Switching to a medication with a

different mechanism of action and adding another drug for augmentation of antidepressant response are two examples of these strategies (Blier and Blondeau, 2011). One of these augmentation strategies is combining an SSRI with 5-HT and partial DA agonists, like aripiprazole or brexpiprazole that have been shown to be effective in treatment-resistant patients with MDD. It is important to mention that as an adjunct medication for MDD, the dosage of these drugs is less than the doses used for schizophrenia or mania (Nelson and Papakostas, 2009; Rogóż 2013; Citrome 2015) implying that the therapeutic benefits of these adjunct agents do not rely on decreasing DA neurotransmission.

Aripiprazole is a DA and 5-HT partial agonist (formerly known as atypical antipsychotic, Zohar et al., 2015). More specifically, aripiprazole is a partial agonist at D_2, D_3, D_4, 5-HT_{1A}, 5-HT_{2C}, 5-HT_7, an inverse agonist of 5-HT_{2B}, and an antagonist at 5-HT_{2A} and 5-HT_6 (Shapiro et al., 2003; Davies et al., 2004). Aripiprazole also has a moderate to low affinity for α_1–adrenergic and H_1-histamine receptors (Burris et al., 2002; Shapiro et al., 2003). The ability of aripiprazole to modulate several 5-HT receptors provides favorable pharmacologic properties to be an adjunct to other medications in the treatment of MDD. In particular, aripiprazole is a 5-HT_{1A} receptor partial agonist (Jordan et al., 2002; Bortolozzi et al., 2007; Dahan et al., 2009), similar to 5-HT_{1A} receptor agonists, like gepirone and buspirone, that have been shown to be effective in the treatment of MDD (Feiger et al., 2003; Rush et al., 2006). Aripiprazole is also a partial agonist at 5-HT_{2C} and an antagonist at 5-HT_{2A} receptors and by targeting these

receptors it has the potential to block the suppressant effect of SSRIs on the activity of DA and NE neurons (Di Matteo et al., 2001; Dremencov et al., 2009; Szabo and Blier, 2001a, b). Indeed, a previous study has shown that combining aripiprazole with escitalopram reverses the escitalopram-induced suppression of the firing activity of DA and NE neurons (Chernoloz et al., 2009).

Although the effect of combining aripiprazole with escitalopram on VTA DA and LC NE neurons was studied (Chernoloz et al., 2009), it is important to note, however, that despite the importance of changes in the neural activity of presynaptic components, overall alterations of neurotransmission in the projection areas are still among most important factors underlying the effectiveness of a particular treatment. In the current study, the effects of the combination of escitalopram and aripiprazole were investigated on tonic activation of postsynaptic 5-HT_{1A} receptors and α_1- and α_2-adrenoceptors in the CA3 region of the hippocampus, in order to document potential changes in overall synaptic transmission.

4. Materials and Methods

4.1 Experimental Preparation

Male Sprague-Dawley rats (Charles River, St. Constant, Québec, Canada) were kept (two per cage) in a controlled environment with (12:12) light-dark cycle and *ad libitum* access to food and water. Rats went through a treatment-free acclimatization period for 5-7 days following arrival. At the time of the

electrophysiological recordings, each animal weighed 280-320 g. Rats were randomly assigned to the control group or the drug-administered (2- and 14-day) groups. The experiments were reviewed and approved by the local Animal Care Committee (University of Ottawa, Institute of Mental Health Research, Ottawa, Ontario, Canada) and were conducted in accordance with the Canadian Council on Animal Care, for the care and use of laboratory animals.

4.2 Treatments

Escitalopram (5 mg/kg/day; s.c.; El Mansari et al., 2015) was administered using implanted Alzet minipumps. For escitalopram administration, two different types of minipump were used: Alzet osmotic pump model 1003D for 2-day (mean pumping rate =0.98 µL/hr; mean fill volume = 98.7 µL) and Alzet osmotic pump model 2ML2 for 14-day administration (mean pumping rate = 4.93 µL/hr; mean fill volume = 2103.4 µL).

Aripiprazole was injected s.c. daily at 2 mg/kg/day as previously reported (Chernoloz et al., 2009) for 2 and 14 days either alone or in combination, with the last dose administered one hour prior to the experiment.

4.3 *In vivo* Electrophysiological Recordings

Rats were anesthetized with intraperitoneal (i.p.) injections of chloral hydrate (400 mg/kg) and supplemental doses (100 mg/kg) were to keep the animal anesthetized. After reaching a deep state of anesthesia, rats were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and

an intravenous (i.v.) catheter was inserted in their lateral tail vein, for the purpose of acute drug delivery. Body temperature was maintained at 37 °C during the electrophysiological recordings.

For recording pyramidal neurons five-barrel glass micropipettes were used. The central barrel of the pipette was filled with a 2 M NaCl solution and was used for the purpose of extracellular unitary recording (impedance 2-5 M Ω). One of the side barrels was also filled with 2 M NaCl solution and served as an automatic current balancer. The remaining three side barrels were filled with quisqualic acid (1.5 mM in 200 mM NaCl, pH 8), NE bitartrate (20 mM in 200 mM NaCl, pH 4), and 5-HT creatinine sulfate (20 mM in 200 mM NaCl, pH 4). 5-HT and NE were ejected as cations and retained with a negative current (-15 nano ampere [nA]); quisqualate was ejected as an anion (-2 nA or less).

The following coordinates were used for guiding the micropipette to the hippocampus CA3 region: 4.0-4.2 mm anterior to lambda, 4.2 mm lateral from the midline, and 4.0 ± 0.5 mm lower than the surface of the brain. Since pyramidal neurons do not discharge spontaneously under chloral hydrate anesthesia, quisqualic acid was used to activate these neurons within their physiological range (10-15 Hz; Ranck, 1975). The identification criteria for the pyramidal neurons were based on the following criteria: large amplitude (0.5-1.2 mV), long duration (0.8-1.2 ms) simple action potentials alternating with complex spike discharges (Kandel and Spencer, 1961).

4.4 *In vivo* Determination of 5-HT and NE Reuptake

Iontophoretic ejection of 5-HT and NE for 50 seconds (s) suppresses the firing activity of pyramidal neurons of the CA3 region of the hippocampus. However, the inhibited pyramidal neurons gradually regain their initial firing activity after the completion of ejection due to the reuptake of 5-HT and NE. To reliably determine *in vivo* the activity of 5-HTT and NE transporter (NET), the RT50 index was used. It is defined as the time elapsed from the cessation of iontophoretic application of 5-HT or NE to 50% recovery of the initial firing rate (de Montigny et al., 1980; Piñeyro et al., 1994).

4.5 Determination of Responsiveness of 5-HT_{1A} and α_2-adrenoceptors in the Hippocampus

The sensitivity of the 5-HT_{1A} receptors and α_2-adrenoceptors was assessed by determining the neuronal responsiveness to the iontophoretic application of 5-HT and NE (Hadrava et al., 1994; Curet and de Montigny, 1988). After establishing a proper baseline firing activity, 5-HT or NE was iontophoretically ejected for 50 seconds to inhibit the firing activity of pyramidal neurons. The responsiveness of the recorded neuron was measured by calculating the number of spikes suppressed by the amount of ejected current in nA. This index was computed by subtracting the number of spikes during the ejection period from the number of spikes in the period prior to 5-HT/NE ejection, divided by the ejection current (nA).

4.6 Determination of Tonic Activation of 5-HT_{1A} Receptors in the Hippocampus

In order to assess the degree of activation of the postsynaptic 5-HT_{1A} receptors exerting an inhibitory influence on the firing activity of CA_3 pyramidal neurons, WAY 100635 was administered intravenously to disinhibit the hippocampal neurons resulting in an increase in their firing activity (Haddjeri et al., 1998). The best condition to measure this disinhibition is when the neurons were not firing at a high rate. The ejection current of quisqualate was hence reduced which in turn decreases the firing rate of CA3 neurons. Any effect of the injection by itself was accounted for by i.v. injection of saline prior to incremental doses of WAY 100635 (25 µg/kg at time intervals of 2 minutes; Haddjeri et al, 1998; Besson et al, 2000). Only one neuron per rat was recorded after the injection. The alteration of firing activity was assessed by calculating the percent of change in the firing rate of neurons before and after each injection.

4.7 Determination of Tonic Activation of α_2- and α_1-adrenoceptors in the Hippocampus

The α_2-adrenoceptor antagonist idazoxan (1 mg/kg) and the α_1-adrenoceptor antagonist prazosin (100 µg/kg) were sequentially injected for investigating respectively a possible increase in tonic activation of the postsynaptic α_2- and α_1-adrenoceptors. At these doses, these drugs have been reported to be effective in detecting overall NE synaptic transmission alterations in

the same electrophysiological setting (Ghanbari et al., 2011; Chernoloz et al., 2012).

4.8 Drugs

Escitalopram was provided by Lundbeck A/S pharmaceutical company, Ltd. (Valby, Denmark) and aripiprazole was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). All other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada). WAY 100635, escitalopram, prazosin, and idazoxan were dissolved in distilled water. Aripiprazole was dissolved in distilled water acidified with lactic acid (5%). NE bitartrate (4-[(1R)-2-Amino-1-hydroxyethyl]-1, 2-benzenediol (L-(+))-bitartrate salt), 5-HT creatinine sulfate (3-[2-Aminoethyl]-5-hydroxyindole creatinine sulfate complex) and quisqualic acid (b-(3, 5-Dioxo-1, 2, 4- oxadiazolidin-2-yl)-L-alanine) were dissolved in 200 mM NaCl.

4.9 Statistical Analysis

The results of the current study are presented as mean values ± S.E.M. RT50 values for 5-HTT and NET were analyzed by one-way ANOVA followed by Dunn's method. Kruskal-Wallis one-way ANOVA on ranks was used for measuring the responsiveness of 5-HT and NE receptors. For analyzing the data related to tonic activation of 5-HT_{1A} receptors and α-adrenoceptors, two-way analysis of variance (ANOVA) with repeated measures, with treatment as the main factor, and Holm-Sidak as the post-hoc test have been used. Throughout the analyses,

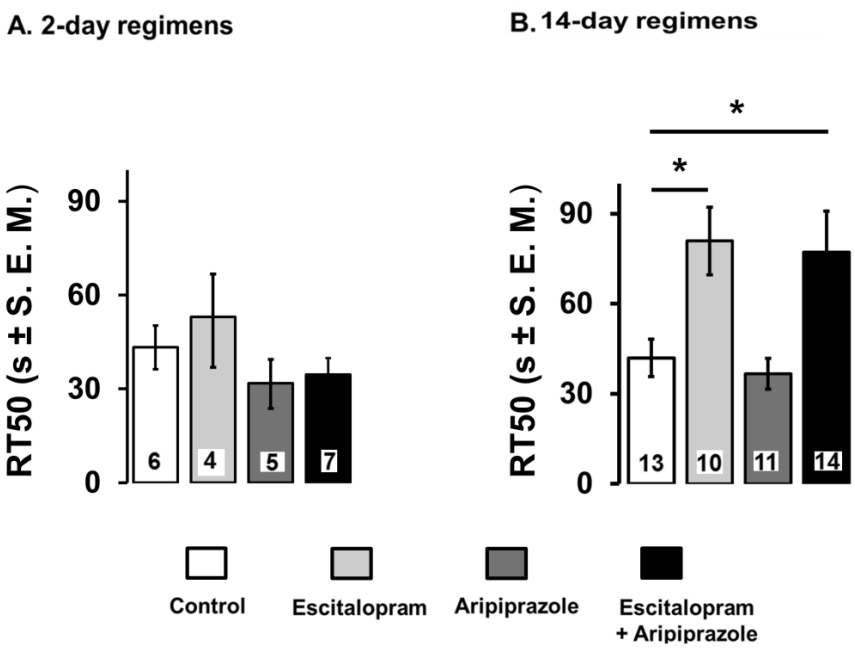
statistical significance was taken as $p < 0.05$. Statistical analyses were done using the software SigmaPlot 12.5 (Systat Software, Inc., San Jose California USA).

5. Results

5.1 Effect of 2- and 14-day Administration of Escitalopram, Aripiprazole and their Combination on the Activity of the 5-HTT

To determine the effect of different drug administration regimens on the activity of the 5-HTT, RT50 values were assessed in the 2- and 14-day drug-administered groups. Suppression of firing activity in the current study was done via iontophoretic ejection of 5-HT. As illustrated in Fig. 1A, 2-day administration of the 3 regimens did not result in any significant changes in the values of RT50. However, 14-day administration of escitalopram and its combination with aripiprazole produced a significant increase in RT50 values when compared to the control group (14-day escitalopram: 93%; 14-day escitalopram-aripiprazole: 83%. Kruskal-Wallis one way ANOVA on ranks followed by Dunn's method; $p < 0.05$; Fig. 1B), thus indicating the ability of escitalopram to inhibit the activity of the 5-HTT either alone or in the presence of aripiprazole.

Figure 1**.** RT50 is the time needed in seconds (s) for a pyramidal neuron inhibited by the iontophoretic ejection of 5-HT, to recover to half of its baseline firing rate after completion of ejection. RT50 value is proportional to the degree of 5-HT transporter (5-HTT) blockade. (A) Although there is no change after a 2-day treatment regimen, (B) a 14-day regimen of escitalopram (5 mg/kg, osmotic minipump) and the combination of escitalopram with aripiprazole (2 mg/kg, s.c. injections) significantly increased RT50 values, indicating a potent blockade of the activity of the 5-HTT. The numbers of rats are indicated at the bottom of the histogram. * $p < 0.05$.



5.2 Effect of 2- and 14-day Administration of Escitalopram, Aripiprazole and their Combination on Tonic Activation of 5-HT_{1A} Receptors

The responsiveness of 5-HT_{1A} receptors to iontophoretically ejected 5-HT was not altered after any of the drug regimens ($p > 0.8$; Fig 2).

The effect of administration of WAY 100635 on the firing activity of pyramidal neurons was assessed following 2- and 14-day administration of escitalopram, aripiprazole, and their combination (Fig. 3, 4, and 5). After a 2-day administration of any of the three regimens, i.v. injection of WAY 100635 (total cumulative dose; 0.1 mg/kg) did not yield any significant alterations in the firing frequency of CA3 pyramidal neurons when compared to the control group. Similarly, following a 14-day administration, neither escitalopram nor aripiprazole induced any alteration in tonic activation of 5-HT_{1A} receptors when administered alone. Their combination, however, significantly increased tonic activation of these receptors (Figures 4 and 5). A two-way repeated-measures ANOVA on tonic activation of 5-HT_{1A} receptors revealed a significant effect of treatment with the combination of escitalopram and aripiprazole (two way repeated measures ANOVA followed by Holm-Sidak method; $F [3, 61] = 4.1$; $p = 0.02$), WAY 100635 ($F [3, 61] = 7.6$; $p < 0.001$) and a significant interaction between both variables ($F [9, 61] = 3.1$; $p = 0.004$). WAY 100635 did not significantly alter the firing activity of pyramidal neurons in control rats (Figures 4A and 5).

Figure 2. The effects of different drug regimens on the responsiveness of 5-HT_{1A} receptors were assessed by calculating the number of spikes suppressed per nanoampere (nA). The number of rats is indicated at the bottom of each histogram.

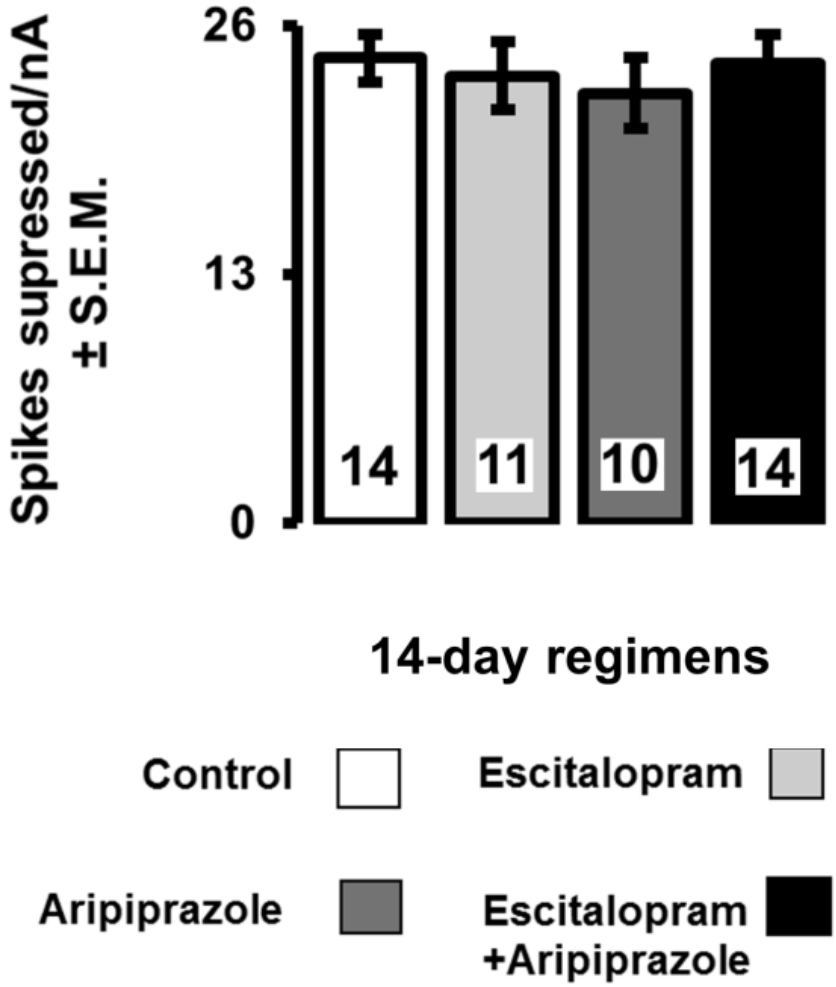


Figure 3. Degree (% ± S.E.M.) of change of the firing activity of pyramidal neurons following the administration of the 5-HT_{1A} receptor antagonist WAY 100635 after 2-day administration of escitalopram, aripiprazole, and their combination. The number of rats (n) is indicated in brackets. In each rat, only one neuron was tested.

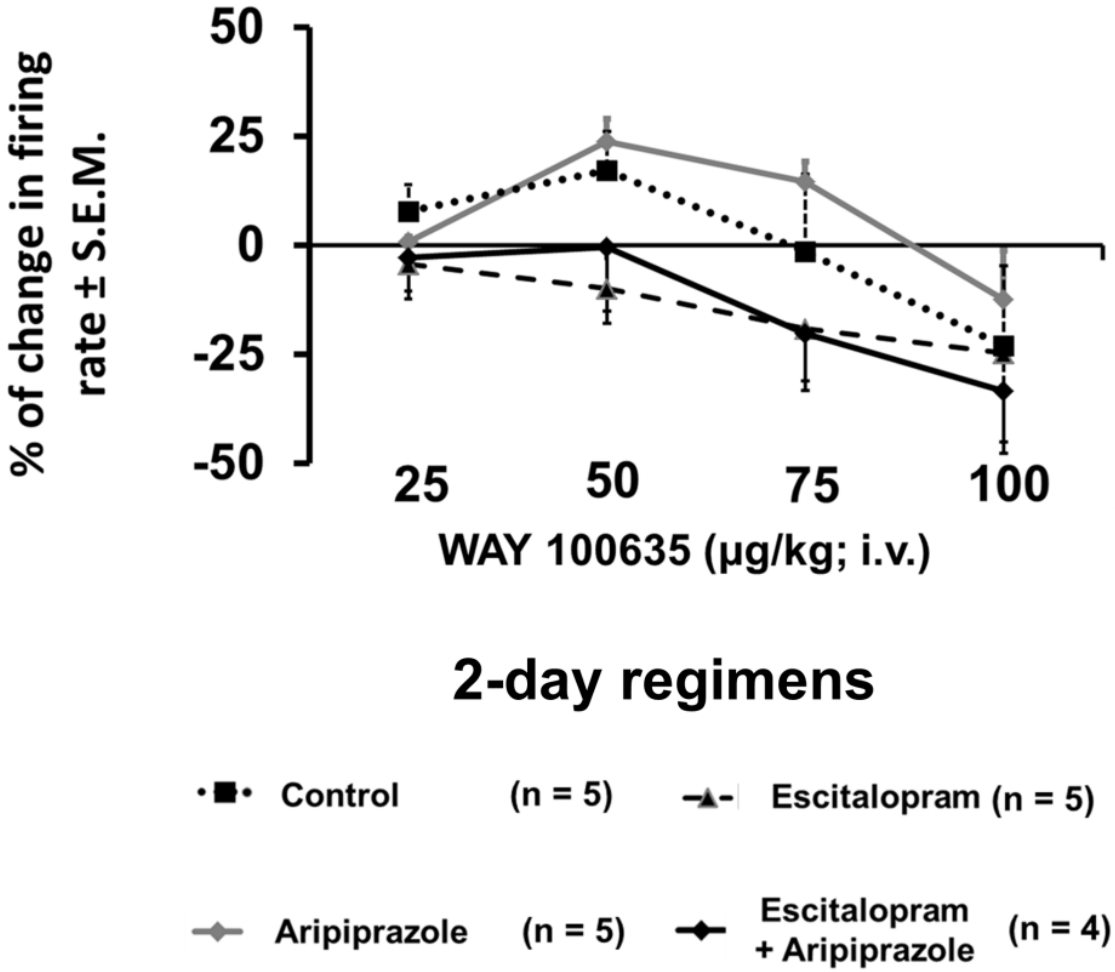


Figure 4. Integrated firing rate histograms of CA3 pyramidal neurons showing their responsiveness i.v. injection of the 5-HT_{1A} receptor antagonist WAY 100635 in (A) a vehicle-administered rat and (B) a rat administered for 14 days with the combination of escitalopram and aripiprazole. Note the increase in the firing activity of pyramidal neurons after the injection of WAY 100635 and the following blockade of the inhibitory effect of 5-HT (B). The ejection currents for quisqualate or a leak (Quis; -2 and 0 nA, respectively) and 5-HT (+20 nA) are indicated above the horizontal bars, which correspond to the duration of the drug ejection. In each rat, only one neuron was tested.

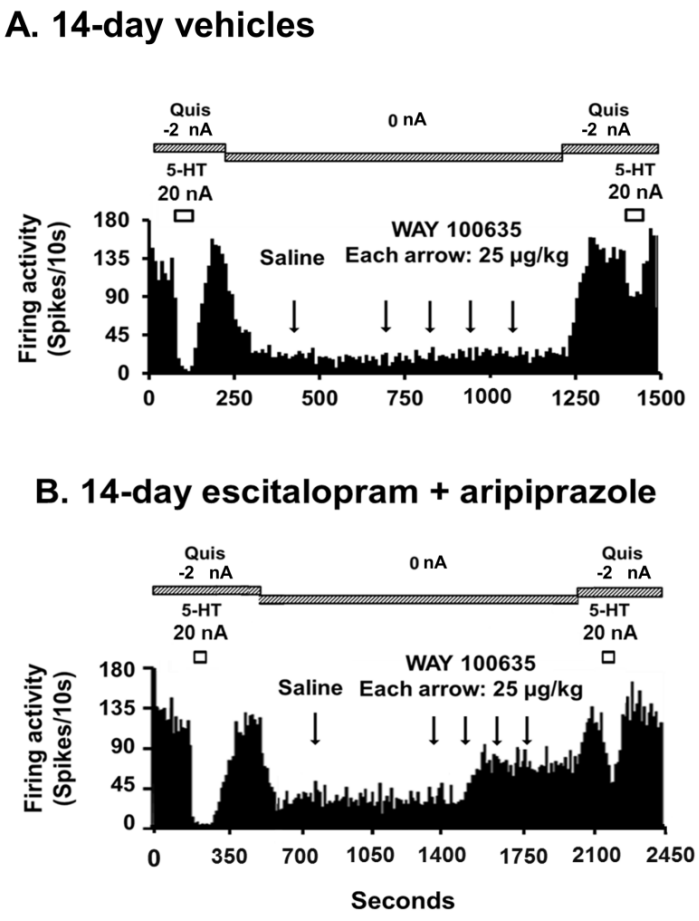
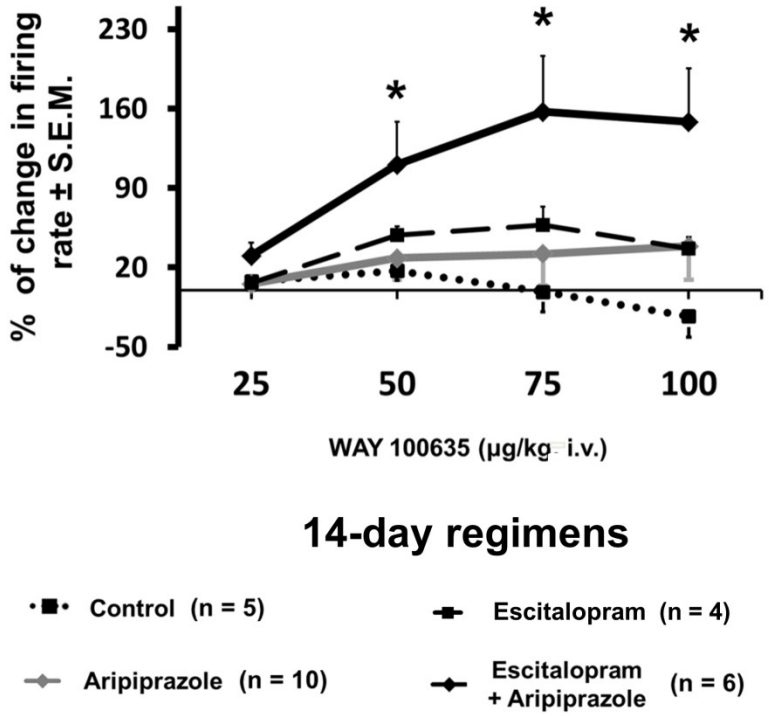


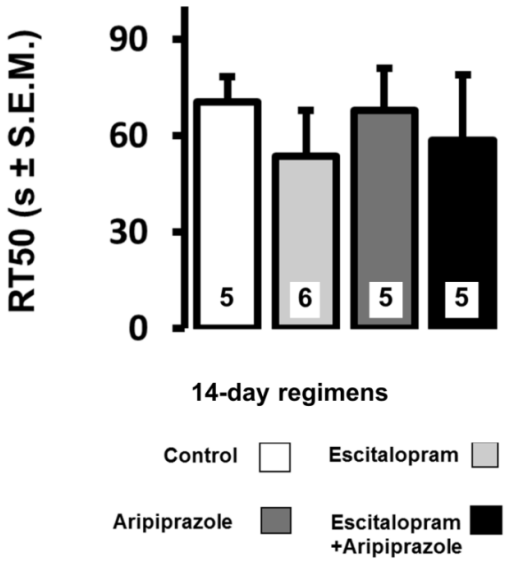
Figure 5. Degree (% ± S.E.M.) of change of the firing activity of pyramidal neurons following the administration of the 5-HT_{1A} receptor antagonist WAY 100635 after 14-day administration of escitalopram, aripiprazole, and their combination. Note that although escitalopram and aripiprazole alone did not cause an increase in the tonic activation of 5-HT_{1A} receptors, their combination caused a significant increase in tonic activation of these receptors. The number of rats (n) is indicated in brackets. In each rat, only one neuron was tested. * $p < 0.05$.



5.3 Effect of 14-day Administration of Escitalopram, Aripiprazole and their Combination on the Activity of the NET

As illustrated in Figure 6, none of the 14-day drug administration regimens resulted in any significant change in the values of RT50, indicating normal functioning of the NET ($F [2, 13] = 0.2$; $p > 0.05$).

Figure 6. Degree (% ± S.E.M.) of change of the firing activity of pyramidal neurons following the administration of the 5-HT_{1A} receptor antagonist WAY 100635 after 14-day administration of escitalopram, aripiprazole, and their combination. Note that although escitalopram and aripiprazole alone did not cause an increase in the tonic activation of 5-HT_{1A} receptors, their combination caused a significant increase in tonic activation of these receptors. The number of rats (n) is indicated in brackets. In each rat, only one neuron was tested. * $p < 0.05$.



5.4 Effect of 14-day Administration of Escitalopram, Aripiprazole and their Combination on Tonic Activation of α_2- and α_1-adrenergic Receptors

The responsiveness of α_2-adrenoceptors to iontophoretically ejected NE was not changed after any of the drug regimens ($p > 0.9$; Figure 7).

Alterations in tonic activation of the α_2- and α_1-adrenergic receptors were also measured in these experiments. Injections (i.v.) of, respectively, the α_2- and α_1-adrenoceptor antagonists idazoxan (1 mg/kg) and prazosin (100 µg/kg), respectively, following 14-day administration of the drug regimens (escitalopram, aripiprazole, and escitalopram-aripiprazole) did not result in any significant disinhibition of the firing activity of CA3 pyramidal neurons compared to the control group (Figures 8 and 9; $F [3, 28] = 0.7$; $p = 0.6$).

Figure 7. The effects of different drug regimens on the responsiveness of α_{-2} adrenoreceptors were assessed by calculating the number of spikes suppressed per nanoampere (nA). The numbers of rats are indicated at the bottom of each histogram.

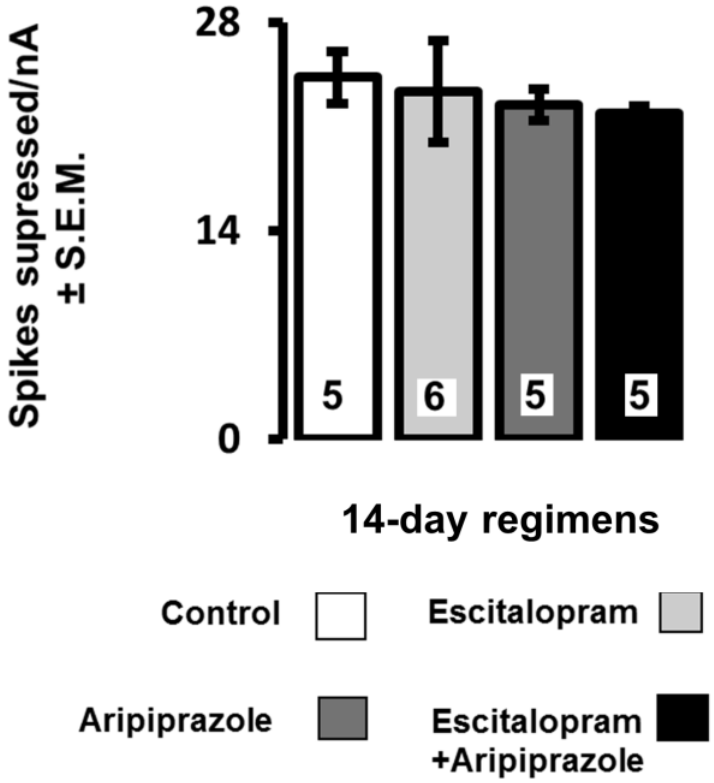


Figure 8. Integrated firing rate histograms of dorsal hippocampus CA3 pyramidal neurons illustrating their response to i.v. injections of the α_{-2} and α_{-1} adrenoceptor antagonists idazoxan and prazosin, respectively, in (A) a vehicle-administered rat and (B) a rat administered for 14 days with the combination of escitalopram and aripiprazole. Note the blockade of the inhibitory effect of NE following the injection of the antagonists. The ejection currents for quisqualate or a leak (Quis; -2 and 0 nA, respectively) and NE (+20 nA) are indicated above the horizontal bars, which correspond to the duration of the drug ejection. In each rat, only one neuron was tested.

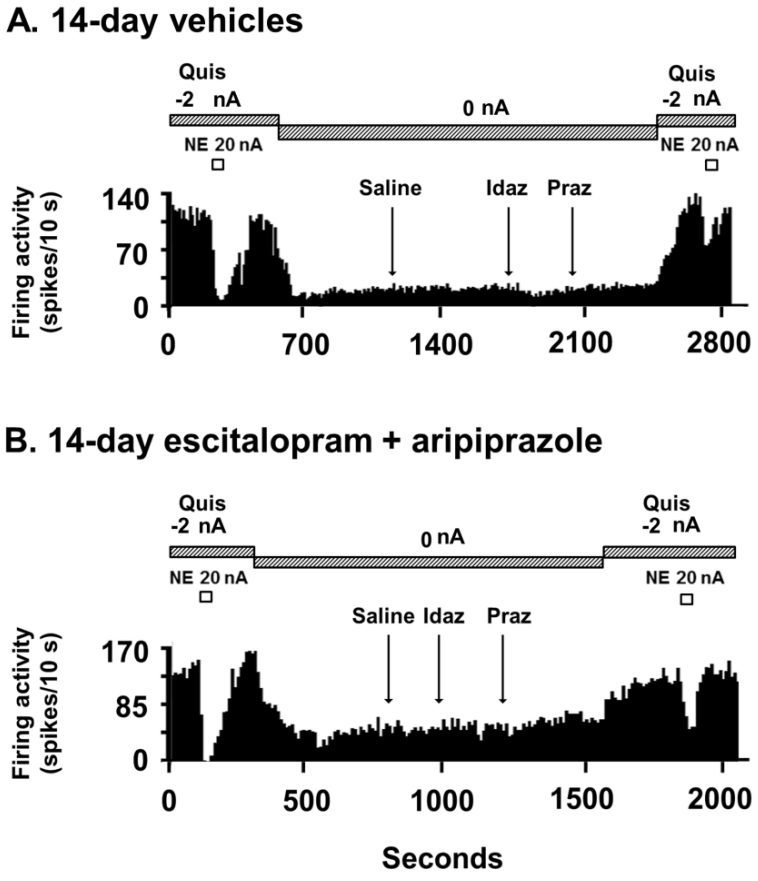
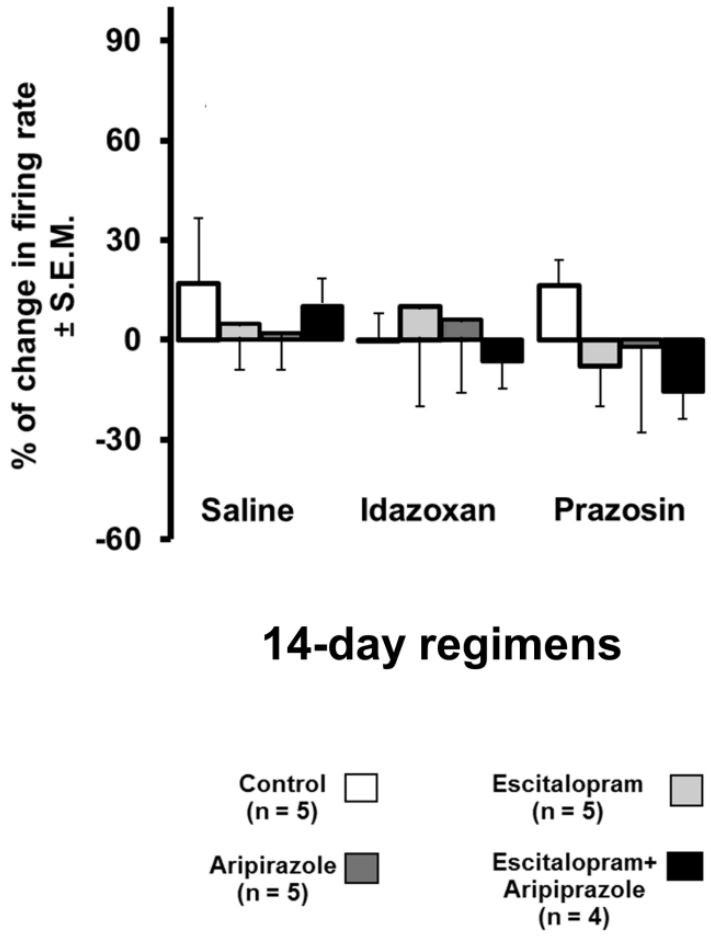


Figure 9. Degree (% ± S.E.M.) of change of the firing activity of pyramidal neurons after the sequential i.v. administration of idazoxan and prazosin to rats administrated with escitalopram, aripiprazole, and their combination for 14 days. The number of rats (n) is indicated in brackets. In each rat, only one neuron was tested.



5.5 Discussion

The results of the current study showed that 2-day administration of escitalopram, aripiprazole, and their combination did not have any effect on the activity of the 5-HTT and tonic activation of 5-HT_{1A} receptors of pyramidal neurons in the CA3 region of the hippocampus. However, 14-day administration of escitalopram, as well as its combination with aripiprazole, resulted in significant inhibition of 5-HT reuptake. While 14-day administration of neither escitalopram nor aripiprazole alone had an effect on tonic activation of 5-HT_{1A} receptors, their combination significantly increased it. Neither the administration of escitalopram, aripiprazole nor their combination for 14 days had any effect on the activity of the NET or tonic activation of the α-adrenoceptors of the CA3 pyramidal neurons.

It was previously reported that 2-day administration of escitalopram significantly decreased the firing activity of 5-HT neurons (Chernoloz et al., 2009; Ghanbari et al., 2010) and doubled extracellular 5-HT levels in the frontal cortex assessed using microdialysis (Ceglia et al., 2004). These results were obtained, however, using a regimen of 10 mg/kg/day. Such a regimen was shown to result in a ceiling effect on the tonic activation of 5-HT_{1A} receptors in the hippocampus (Chernoloz et al., 2012), therefore making it impossible to detect a synergy between escitalopram and another medication, as was shown for quetiapine. Therefore, the daily dose of escitalopram was decreased by half in prior experiments (from 10 to 5 mg/kg/day), which still lead to a significant 5-HT reuptake inhibition without maximizing the enhanced tonic activation of 5-HT_{1A}

receptors (El Mansari et al., 2015). In the present study, however, there was no prolongation of the *in vivo* 5-HT reuptake index after 2 days, but it was prolonged after 14 days of escitalopram administration. These observations indicate that a steady-state level of escitalopram had not been reached after 2 days of administration. Unexpectedly, even after 14 days of escitalopram administration, the tonic activation of 5-HT_{1A} receptors was not enhanced as before, indicating that we were working in this experimental series in sub-threshold conditions to enhance 5-HT transmission with escitalopram alone.

Achieving a robust degree of 5-HT reuptake inhibition is not sufficient *per se* to increase 5-HT neurotransmission after a 2-day regimen, as was shown with 10 mg/kg/day of paroxetine; under such conditions, 14 days of paroxetine administration did enhance this parameter (Besson et al., 2000). In contrast, when aripiprazole was combined with escitalopram for 14 days, but not 2 days, there was a significant enhancement of 5-HT_{1A} receptor neurotransmission as was found with other effective treatments of MDD (Blier and El Mansari, 2013).

These results showed a clear synergy between these two medications: neither of them had a significant impact on 5-HT_{1A} neurotransmission when given alone, but produced a significant increase when administered concomitantly.

Aripiprazole administrated by itself left unaltered the tonic activation of 5-HT_{1A} receptors despite a significant increase in the firing activity of 5-HT neurons (Chernoloz et al., 2009). This was unexpected given that this drug is a potent 5-

HT_{1A} receptor agonist and that it did not change the responsiveness of 5-HT_{1A} receptors (Lawler et al., 1999; Bortolozzi et al., 2007; Dahan et al., 2009). Indeed, a previous study has shown that long-term administration of the 5-HT_{1A} receptor agonist gepirone induces enhanced tonic activation of these receptors in the hippocampus (Haddjeri et al., 1998). It is unclear why aripiprazole on its own did not exert this action. In contrast, brexpiprazole, which is both a DA D_2 agonist and a high-affinity 5-HT_{1A} receptor agonist like aripiprazole was reported to significantly enhance tonic activation of 5-HT_{1A} receptors after both 2 and 14 days of administration (Oosterhof et al., 2016). The main difference between these two drugs with regards to properties that may have an influence on the 5-HT transmission is the potent α_2-adrenergic antagonistic activity of brexpiprazole (Maeda et al., 2014; Oosterhof et al., 2016). This is likely relevant because blockade of α_2- adrenergic receptors on both NE and 5-HT terminals by mirtazapine can produce a small but significant enhancement of the tonic activation of 5-HT_{1A} receptors after two days of exposure, which becomes robust with prolongation of its administration for 14 days (Besson et al., 2000). It is therefore conceivable that a synergy between 5-HT_{1A} receptor activation and α_2-adrenoceptor antagonism is necessary to produce an increase in 5-HT transmission after a short-term period. This is strengthened by the fact that quetiapine administered with its metabolite desalkylquetiapine, which has a high affinity for 5-HT_{1A} and α_2- adrenergic receptors, respectively (Jensen et al., 2008), enhance the tonic activation of 5-HT_{1A} receptors in the rat hippocampus after 14 days of administration (Chernoloz et al., 2012). Interestingly, there are several

studies showing that quetiapine monotherapy is efficacious in the treatment of MDD (Ignácio et al., 2017). However, the clinical extrapolation of the results obtained with aripiprazole and brexpiprazole given alone is not possible since these two drugs have not been tested in monotherapy in MDD, in contrast to quetiapine.

It was previously shown that the increased activation of 5-HT_{1A} receptors in the hippocampus can be due to increased sensitivity of the postsynaptic 5-HT_{1A} receptors (with repeated electroconvulsive shocks or with long-term administration of the tricyclic medications used to treat depression; de Montigny 1984; de Montigny and Aghajanian, 1978) or through enhanced 5-HT signaling in the synapse acting on normosensitive postsynaptic receptors. This explains the enhanced tonic activation that can be achieved with a sufficient regimen of a 5-HT reuptake inhibitor, a direct-acting 5-HT_{1A} agonist, or after sustained monoamine oxidase inhibition (Chaput et al., 1991; Haddjeri et al., 1998; Blier and El Mansari, 2013). In the current study, since 5-HT is inhibitory on the firing of these neurons through 5-HT_{1A} receptors (Piñeyro et al., 1994; Gobbi et al., 2001) their antagonism by WAY 100635 produced disinhibition (i.e. an increase in firing activity) in the rats that received combination regimen, suggesting that there was more 5-HT signaling in the synapse, while the sensitivity of these receptors was unchanged.

The possibility that WAY 100635 can act indirectly on the hippocampus cannot be entirely eliminated. Nevertheless, the observation that the inhibitory

effect of 5-HT applied directly on the pyramidal neurons is markedly diminished by the systemic injection of WAY 100635, both in control and treated rats, strongly supports that the disinhibition of firing is mediated principally by a local phenomenon.

In the present study, 14-day administration of escitalopram, aripiprazole, and their combination neither blocked the activity of NET nor increased tonic activation of α_1- and α_2- adrenoceptors in the hippocampus. The lack of effect on NET activity was expected because these two drugs have no significant affinity for this reuptake transporter (Lawler et al., 1999; Zhong et al., 2012). The present approach of examining the tonic activation of α_1- and α_2- adrenoceptors are adequate to assess an increase of transmission by a medication that increases NE availability, as was demonstrated using a 14-day regimen of bupropion (Ghanbari et al., 2011). It cannot unveil, however, a decreased activation of these receptors because, under such conditions, there is not a marked activation of these receptors in the hippocampus, as is the case for 5-HT_{1A} receptors (Kasamo et al., 2001). This is evidenced by the lack of disinhibition of the firing of the pyramidal neurons neither by the α_1-adrenoceptor antagonist prazosin nor by the α_2- adrenoceptor antagonist idazoxan in control rats. Using this methodology, prolonged administration of bupropion, trazodone, quetiapine, and brexpiprazole was shown to increase the tonic activation of α_2-adrenoceptors in the hippocampus, but not α_1- adrenoceptors because the latter three are α_1-adrenoceptor antagonists (Ghanbari et al., 2011, 2012; Chernoloz et al., 2012;

Oosterhof et al., 2016). The common property of these four drugs is that they all enhance the firing activity of LC NE neurons above normal after 14 days of administration and most also block or desensitize adrenoceptors on NE terminals, thereby attenuating their inhibitory role on NE release. In the case of escitalopram plus aripiprazole combination, the firing rate of NE (and DA) neurons had been shown to be normalized when compared to escitalopram alone (Chernoloz et al., 2009), therefore an increase in tonic activation of α_2- adrenoreceptors was not expected. Nevertheless, a normal or restored firing activity of NE neurons and of DA neurons by this combination may still contribute to its antidepressant action in treatment-resistant patients with MDD, and allow the consistently reported manifestation of its therapeutic benefits.

The need to stimulate hippocampal CA3 cells in our anesthetized rodents could be perceived as a potential limitation in terms of translating the results to the human brain. However, it was previously shown in our laboratory that the enhanced responsiveness of these neurons to 5-HT produced by treatments for depression is the same whether the neurons have to be activated because of anesthesia or studied without anesthesia and any activation (using a cerveau isolé preparation; de Montigny and Aghajanian, 1978; de Montigny, 1984; Chaput et al., 1991).

In conclusion, the combination of escitalopram and aripiprazole produced a synergistic action on the tonic activation of 5-HT_{1A} receptors in the hippocampus. However, overall NE transmission was not enhanced by the aripiprazole-

escitalopram combination, as was the case with quetiapine and brexpiprazole alone. Taken together, these results indicate these three adjunctive drugs, which are approved for inadequate response to SSRIs in MDD, act by different mechanisms to enhance at least 5-HT and/or NE transmission.

6. Conflict of Interest

M. El Mansari and M. Ebrahimzadeh declare no conflict of interest. P. Blier received grant funding and/or honoraria for lectures and/or participation in advisory boards for Allergan, Bristol Myers Squibb, Eli Lilly, Janssen, Lundbeck, Otsuka, Pfizer, Pierre Fabre Médicaments, Takeda, and Valeant.

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Chapter 4 — Long-term Administration of Cariprazine increases Locus Coeruleus Noradrenergic Neurons Activity and Serotonin$_{1A}$ Receptor Neurotransmission in the Hippocampus

1. Title Page

Long-term Administration of Cariprazine Increases Locus Coeruleus Noradrenergic Neurons Activity and Serotonin$_{1A}$ Receptor Neurotransmission in the Hippocampus

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2. Abstract

Background: Cariprazine, the novel dopamine (DA) D_3-preferring D_3/D_2 and serotonin $(5\text{-HT})_{1A}$ partial agonist has demonstrated activity as adjunctive therapy in major depressive disorder (MDD).

Aims: To investigate the effects of chronic cariprazine administration in combination with the selective serotonin reuptake inhibitor escitalopram on the activity of monoaminergic systems.

Methods: Rats received cariprazine alone and adjunctively with escitalopram for 2 and 14 days and the firing activity of dorsal raphe nucleus 5-HT, locus coeruleus norepinephrine (NE), and ventral tegmental area DA neurons were assessed. 5-HT and NE neurotransmission in hippocampus pyramidal neurons was evaluated by assessing tonic activation of their 5-HT_{1A}, and α_1- and α_2-adrenergic receptors, using their selective antagonists.

Results: Two and 14-day cariprazine regimens increased the firing rate of NE, but not 5-HT and DA neurons. The addition of cariprazine to escitalopram reversed the inhibitory effect of escitalopram on NE but not 5-HT and DA neurons. In the hippocampus, there was an increase in neurotransmission at 5-HT_{1A} receptors in cariprazine-treated rats, but no change in overall NE neurotransmission by either regimen was observed.

Conclusion: Cariprazine increased NE neuronal firing and reversed the escitalopram-induced inhibition of these neurons. Despite a lack of effect on 5-HT

neurons firing activity, there was an increase in tonic activation of hippocampus 5-HT_{1A} receptors by cariprazine alone but not with the combination. These effects provide a possible rationale for the clinical efficacy of cariprazine as an adjunctive medication in MDD patients.

3. Introduction

Despite considerable progress in developing therapeutic treatment strategies for major depressive disorder (MDD), incomplete responses remain a major unmet need with a variety of approaches, including first-line selective serotonin (5-HT) reuptake inhibitors (SSRIs) and psychotherapeutic interventions. SSRIs increase extracellular levels of 5-HT by inhibiting the activity of the 5-HT transporter (5-HTT). However, this increase in extracellular levels of 5-HT reduces the firing activity of locus coeruleus (LC) norepinephrine (NE) neurons via activation of 5-HT_{2A} receptors (Szabo and Blier, 2001a, b) and ventral tegmental area (VTA) dopamine (DA) neurons via activation of 5-HT_{2C} receptors (Di Matteo et al., 2001; Dremencov et al., 2009; Demireva et al., 2018). Interestingly, following SSRI treatment, residual symptoms of depression that are related to NE and DA, such as asthenia, fatigue, and anhedonia have been hypothesized to be, at least in part, caused by dampening NE and DA activity through enhanced activation of 5-HT_2 receptors (Blier and Blondeau, 2011; Nutt, 2008). This theoretical framework likely underlies the clinical responses in the treatment of MDD with SSRIs.

Augmentation of the clinical response to an SSRI can be obtained by adding another drug with or without intrinsic antidepressant efficacy. Examples of such medications that can be added to SSRIs include DA and 5-HT antagonists (i.e. olanzapine, quetiapine) and partial agonists (i.e. aripiprazole and brexpiprazole; Citrome, 2015; Nelson and Papakostas, 2009; Rogóż, 2013), all formerly known as atypical antipsychotics (Zohar et al., 2015). These drugs are typically prescribed at lower dosages when used as adjuncts to SSRIs for treatment for depression compared to when used for treatments of schizophrenia or mania (Citrome, 2015; Nelson and Papakostas, 2009; Rogóż, 2013). The usefulness of such lower doses suggests that the therapeutic benefits of these agents for treating MDD probably do not rely on decreasing DA neurotransmission through the direct blockade of D2 receptors or their functional antagonism resulting from partial agonism.

Cariprazine is an orally active and potent DA D_3/D_2 receptor partial agonist with preferential binding to D_3 receptors and partial agonist activity at serotonin 5-HT_{1A} receptors. Cariprazine also has a high and moderate binding affinity, respectively at the 5-HT_{2B} and 5-HT_{2A} receptors, acting as a pure antagonist at both of these receptors (Kiss et al., 2010). The main distinguishing characteristic of cariprazine from the two $D_{2\text{-like}}$ partial agonists, aripiprazole and brexpiprazole, is that it displays a much higher affinity at D_3 compared to D_2 receptors. Similar to the latter two drugs, cariprazine also appears to be clinically efficacious as an adjunct in patients with treatment-resistant depression (TRD; Durgam et al., 2016;

Earley et al., 2018), although more studies are still being carried out. The following section outlines the pharmacological properties of cariprazine that might underlie, at least in part, its potential efficacy for the treatment of MDD.

Increased 5-HT_{1A} receptor activation has been hypothesized to play an important role in the antidepressant properties of various strategies used in MDD (Blier and Ward, 2003), including the selective 5-HT_{1A} receptor agonist gepirone and the multi-targeted agents aripiprazole and brexpiprazole endowed with this property (Blier and de Montigny, 1990; Chernoloz et al., 2009; Ebrahimzadeh et l., 2018; Oosterhof et al., 2016). Furthermore, cariprazine, like brexpiprazole, has been shown to act as a full agonist *in vivo* at 5-HT_{1A} receptors of the pyramidal neurons in the CA3 region of the hippocampus (Herman et al., 2018; Oosterhof et al., 2014).

5-HT_{2A} receptor antagonism is a property shared by DA and 5-HT antagonists and partial agonists used as adjuncts to medications with antidepressant properties. Agents having this property, including the selective 5-HT_{2A} antagonist MDL 100,907, have been shown to reverse the suppressant effect of SSRIs on firing activity of NE neurons (Chernoloz et al., 2009, 2012; Dremencov et al., 2007; Seager et al., 2004; Szabo and Blier, 2002). This reversal of inhibited NE firing activity resulting from SSRIs might underlie, at least in part, their well-documented therapeutic efficacy in TRD. Similar to these drugs, cariprazine also acts *in vivo* as an antagonist on 5-HT_{2A} receptors controlling the firing activity of NE neurons (Herman et al., 2018).

Functional 5-HT_{2C} receptor antagonism is also a feature shared by DA/ 5-HT partial agonists used in combination with medications with antidepressant actions including aripiprazole and brexpiprazole. Agents having this property, including the selective 5-HT_{2C} antagonist SB-242084, have been shown to reverse the suppressant effect of SSRIs on the firing activity of DA neurons (Chernoloz et al., 2009, 2012b; Seager et al., 2004; Dremencov et al., 2007, 2009). This reversal of inhibited DA firing activity might underlie, at least in part, their well-documented therapeutic efficacy in TRD, as was shown using aripiprazole (Chernoloz et al., 2009).

The present study was thus designed to examine the electrophysiological effects of cariprazine alone or in combination with the SSRI escitalopram upon repeated administration in rats. The spontaneous firing activity of LC NE, VTA DA, and DRN 5-HT neurons was recorded and possible control of the above-mentioned monoaminergic receptors on neuronal firing was assessed. Despite the significance of alterations of the neural activity of presynaptic components, the net changes of neurotransmission in their projection areas are also important to determine. Hence, the current study also investigated the effects of cariprazine alone and in combination with escitalopram on tonic activation of postsynaptic 5-HT_{1A} receptors and α_1- and α_2-adrenoreceptors in the CA3 region in the hippocampus.

4. Materials and Methods

4.1 Experimental Preparation

Male Sprague-Dawley rats (Charles River, St. Constant, Québec, Canada) weighing 280-320 g at the time of the recordings were used for these experiments. They were kept in a controlled environment with (12:12) light-dark cycle and *ad libitum* access to food and water. Following arrival rats (two per cage) went through a treatment-free acclimatization period for 5-7 days. Rats were randomly assigned to the drug-administered (2- and 14-day) or control groups. The experimental procedures were reviewed and approved by the local Animal Care Committee (the University of Ottawa, Institute of Mental Health Research, Ottawa, Ontario, Canada) and were conducted in accordance with the Canadian Council on Animal Care, for the care and use of laboratory animals.

Anesthesia was achieved with intraperitoneal (i.p.) injections of chloral hydrate (400 mg/kg) and supplemental doses (100 mg/kg) throughout the experiment. Anesthetized rats were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and an intravenous (i.v.) catheter was inserted in their lateral tail vein, for the purpose of acute drug delivery. Body temperature was maintained at 37°C during the course of the electrophysiological recordings using a heating pad.

4.2 Treatments

Cariprazine was injected subcutaneously (s.c.) daily at 0.6 mg/kg/day for 2 and 14 days, with the last dose administered one hour before the experiment; this regimen was selected on the basis of prior behavioral experiments (Choi et al., 2014; Papp et al., 2014).

Escitalopram was administered s.c. at doses of 5 and 10 mg/kg/day, using implanted Alzet minipumps. Two different types of minipump were used: Alzet osmotic pump model 1003D for 2-day (mean pumping rate = 0.98 μL/hr; mean fill volume = 98.7 μL) and Alzet osmotic pump model 2ML2 for 14-day administration of escitalopram (mean pumping rate = 4.93 μL/hr; mean fill volume = 2103.4 μL).

4.3 *In vivo* Electrophysiological Recordings

4.3.1 Electrophysiological Recording of LC NE Neurons

Firing activity of NE neurons was recorded by lowering (4.5-6.0 mm from the surface of the brain) a single barrel microelectrode filled with 2 M NaCl with an impedance of 6 MΩ, according to the following coordinates: 0.9-1.2 mm posterior to lambda and 0.9-1.3 mm to the midline suture. NE neurons were identified by the following criteria: their regular firing rate (0.5-5 Hz), long duration (~2 ms), and biphasic action potentials. Another identifying factor was a quiescent period following a volley of spike discharges in response to a nociceptive pinch of the contralateral hind paw (Marwaha and Aghajanian, 1982).

4.3.2 Electrophysiological Recording of VTA DA Neurons

Putative DA neurons were recorded by lowering (6-8.5 mm from the surface of the brain) a single barrel microelectrode in the following coordinates: 3.2-3.6 mm anterior to lambda and 0.6-1.0 mm to the midline suture (Paxinos and Watson, 1998). These neurons were identified by their established electrophysiological criteria: a regular firing rate (2-10 Hz), an irregular single spiking pattern, a long duration (>2.5 ms) action potential often with a notch on the rising phase. These neurons have a slow bursting activity with the amplitude of the action potentials progressively decreasing in a given burst and a low pitch sound on the audiometer (Ungless and Grace, 2012). The number of spontaneously active neurons per track was identified by recording multiple tracks in a 400-400 μm grid (Valenti et al., 2011).

4.3.3 Electrophysiological Recording of DRN 5-HT Neurons

Using a single-barreled micropipette, recordings were made according to the following coordinates (in millimeters from lambda): AP, 1.0 to 1.2; L, 0 ± 0.1; V, 5 to 7. The following criteria were used for identifying presumed 5-HT neurons: a slow (0.5–2.5 Hz) and regular firing rate and long-duration (2–5 ms) bi- or triphasic extracellular waveform (Aghajanian and Vandermaelen, 1982).

Firing patterns of NE, DA, and 5-HT neurons were analyzed by interspike interval (ISI) burst analysis. For DA and NE neurons, the initiation and termination of a burst were defined, respectively, as the occurrence of two spikes with ISI <

0.08 and ISI > 0.16 s (Grace and Bunney, 1984). Some 5-HT neurons discharge in brief bursts of action potentials, mostly doublets, and a very short interspike time interval (typically < 10 ms; Hajos and Sharp, 1996). The burstiDAtor software was used in this project for burst analysis (https://github.com/nno/burstiDAtor).

4.3.4 *In vivo* Electrophysiological Recording and Microiontophoresis in Dorsal Hippocampus CA3 Pyramidal Neurons

The firing activity of pyramidal neurons was recorded using a five-barrel glass micropipette. The central barrel of the pipette was filled with a 2 M NaCl solution and was used for the purpose of extracellular unitary recording (impedance 2-5 M Ω) while one of the side barrels was also filled with 2 M NaCl solution and served as an automatic current balancer. The remaining three side barrels were filled with quisqualic acid (1.5 mM in 200 mM NaCl, pH 8), NE bitartrate (20 mM in 200 mM NaCl, pH 4), and 5-HT creatinine sulfate (20 mM in 200 mM NaCl, pH 4). 5-HT and NE were ejected as cations (+20 nano amperes [nA]) and retained with a negative current (-15 nA) while quisqualate was ejected as an anion (-2 nA or less).

For guiding the micropipette to the hippocampus CA3 region, the following coordinates were used: 4.0-4.2 mm anterior to lambda, 4.2 mm lateral from the midline, and 4.0 ± 0.5 mm lower than the surface of the brain (Paxinos and Watson, 1998). Since pyramidal neurons do not discharge spontaneously under chloral hydrate anesthesia, a very small amount of quisqualic acid was ejected to

activate these neurons within their physiological range (10-15 Hz; Ranck, 1975). Pyramidal neurons were identified based on the following criteria: large amplitude (0.5-1.2 mV), long duration (0.8-1.2 ms) simple action potentials alternating with complex spike discharges (Kandel and Spencer, 1961).

4.3.5 *In vivo* Determination of NE and 5-HT Reuptake

Iontophoretic ejection of NE and 5-HT (50 s) suppresses the firing activity of pyramidal neurons in the CA3 region of the hippocampus. However, after the completion of the ejection, the inhibited pyramidal neuron gradually regains its initial firing activity due to reuptake of NE and 5-HT, respectively, by NE transporter (NET) and 5-HTT. RT50 index was used to reliably determine the activity of NET and 5-HTT. This index is defined as the time (s) elapsed from the cessation of iontophoretic application of NE or 5-HT to 50% recovery of the initial firing rate (de Montigny et al., 1980; Piñeyro et al., 1994).

4.3.6 Determination of Responsiveness of 5-HT_{1A} Receptors and α_2-adrenoceptors in the Hippocampus

The sensitivity of the 5-HT_{1A} receptors and α_2-adrenoceptors was determined by calculating the neuronal responsiveness to the iontophoretic application of 5-HT and NE (Curet and de Montigny, 1988; Hadrava et al., 1994). Following a proper baseline firing activity, 5-HT or NE was iontophoretically ejected for 50 s to inhibit the firing activity of pyramidal neurons. The responsiveness of these neurons was measured by determining the number of

spikes suppressed by the amount of ejected current in nA. This was achieved by subtracting the number of spikes during the 5-HT/NE ejection period from the number of spikes in the period prior to ejection, divided by the ejection current (nA).

4.3.7 Determination of Tonic Activation of α_2- and α_1-adrenoceptors in the Hippocampus

Investigating a possible increase in tonic activation of the postsynaptic α_2- and α_1-adrenoceptors was done by sequential i.v. injections of the α_2-adrenoceptor antagonist idazoxan (1 mg/kg) and the α_1-adrenoceptor antagonist prazosin (100 µg/kg) respectively. Only one neuron was tested in each rat. In similar electrophysiological experiments, these doses of idazoxan and prazosin have been reported to be effective in detecting alterations in overall NE synaptic neurotransmission (Chernoloz et al., 2012; Ghanbari et al., 2011).

4.3.8 Determination of Tonic Activation of 5-HT_{1A} Receptors in the Hippocampus

For assessing the degree of tonic activation of the postsynaptic 5-HT_{1A} receptors, which exert an inhibitory influence on the firing activity of pyramidal neurons, the 5-HT_{1A} receptor antagonist WAY 100635 was administered (i.v.) to disinhibit the activity of these neurons (Haddjeri et al., 1998). The ideal condition to measure this disinhibition is when the neurons are not firing at a high rate. For achieving this condition, the ejection current of quisqualate was reduced to

decrease the firing rate of pyramidal neurons. WAY 100635 (100 µg/kg) was injected i.v. consecutively (4 injections; each 25 µg/kg at 2-minute intervals) via the tail vein (Haddjeri et al, 1998; Besson et al, 2000). An i.v. injection of saline prior to incremental doses of WAY 100635 was carried out to account for any effect of the injection by itself. Only one neuron was tested in each rat. Any alterations of the firing activity were assessed by calculating the percent of change in the firing rate of the neuron prior to and after each injection.

4.4 Drugs

Cariprazine was provided by Allergan and escitalopram was generously provided by Lundbeck A/S pharmaceutical company, Ltd. (Valby, Denmark). All other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada). WAY 100635, escitalopram, prazosin, and idazoxan were dissolved in distilled water. Cariprazine was dissolved in distilled water acidified with lactic acid (5%). NE bitartrate (4-[(1R)-2-amino-1-hydroxyethyl]-1, 2-benzenediol (L-(+))-bitartrate salt), 5-HT creatinine sulfate (3-[2-aminoethyl]-5-hydroxyindole creatinine sulfate complex) and quisqualic acid (b-(3, 5-dioxo-1, 2, 4- oxadiazolidin-2-yl)-L-alanine) were dissolved in 0.2 M NaCl.

4.5 Statistical Analyses

Throughout this study, data are presented as mean values ± S.E.M. In the LC, VTA, and DRN comparisons between control and treated groups were carried out using one-way analysis of variance (ANOVA) followed by Dunn's method,

Dunnett's method, and Holm-Sidack tests and Kruskal-Wallis followed by Dunn's method.

RT50 values for NET and 5-HTT were analyzed by one-way ANOVA followed by Dunn's method. For analyzing the data related to tonic activation of 5-HT_{1A} receptors and adrenoceptors, two-way ANOVA with repeated measure, with treatment as the main factor have been used. Statistical significance was taken as $p < 0.05$. These statistical comparisons were analyzed using the software SigmaPlot 12.5 (Systat Software Inc, San Jose, California).

5. Results

5.1 NE System

5.1.1 Effect of 2- and 14-day Administration of Escitalopram, Cariprazine and their Combination on LC NE Neurons

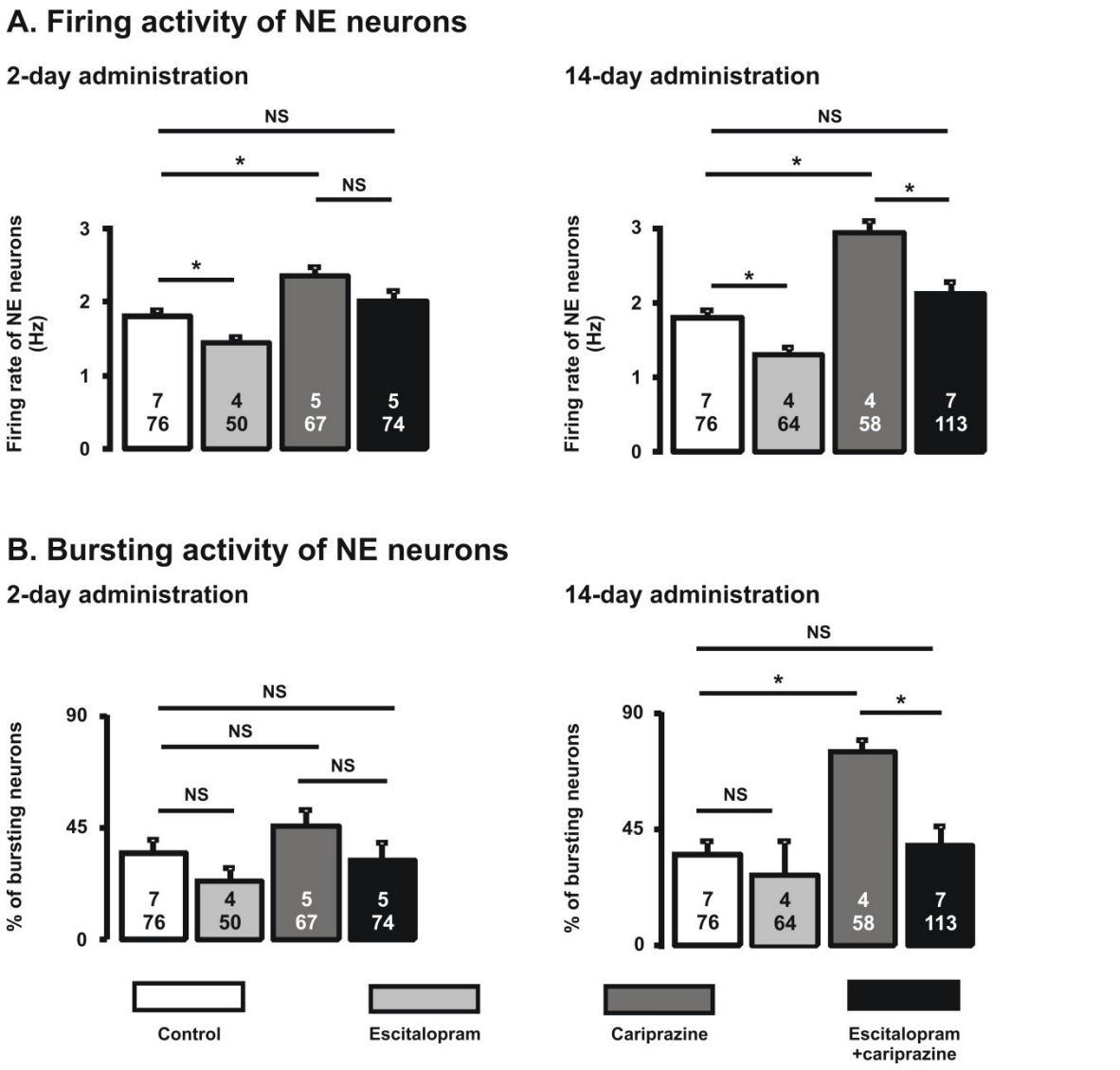
There was no statistically significant difference in the firing activity of LC NE neurons in the 2- and 14-day control groups; hence the data for these two groups were combined.

Because cariprazine is a 5-HT_{2A} receptor antagonist, we tested whether its combination can rescue escitalopram-induced inhibition of NE neurons firing. After 2- and 14-day administration of escitalopram (10 mg/kg/day) the firing activity of NE neurons was significantly attenuated, respectively, by 20% and 27% when compared to controls (2-day: H = 29.5, Q = 2.8; 14-day: H = 63.8, Q = 3.6;

Kruskal-Wallis One-Way ANOVA followed by Dunn's method; $p < 0.05$; Figure 1A).

Two and 14-day administration of cariprazine (0.6 mg/kg/day) significantly increased the firing activity of LC NE neurons, respectively by 33% and 61% when compared to controls (2-day: $H = 29.5$, $Q = 3.0$; 14-day: $H = 63.8$, $Q = 4.8$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; $p < 0.05$; Figure 1A). In addition, the percentage of neurons firing in bursts increased significantly after 14-, but not 2-day cariprazine administration when compared to control (113%; $F[3, 18] = 5.6$, $P<0.01$; One-Way ANOVA followed by Holm-Sidak method; Figure 1B). Cariprazine, when combined with escitalopram for 2 days, resulted in full recovery of decreased firing activity of NE neurons induced by escitalopram. This restoration of firing activity was also present following 14-day administration ($p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 1A).

Figure 1. (A) Firing rate of locus coeruleus (LC) norepinephrine (NE) neurons in rats administered with vehicle, escitalopram (10 mg/kg/day; s.c.), cariprazine (0.6 mg/kg/day; s.c.) and their combination for 2 and 14 days. (B) Percentage of NE neurons firing in bursts after these same regimens. At the bottom of each histogram, the number of rats (top) and neurons recorded (bottom) are indicated. Data are presented as mean values ± S.E.M. * $p < 0.05$, statistical significance is indicated where it applies.



5.2 Effect of 14-day Administration of Escitalopram, Cariprazine and their Combination on Tonic Activation of α_2- and α_1-adrenergic Receptors

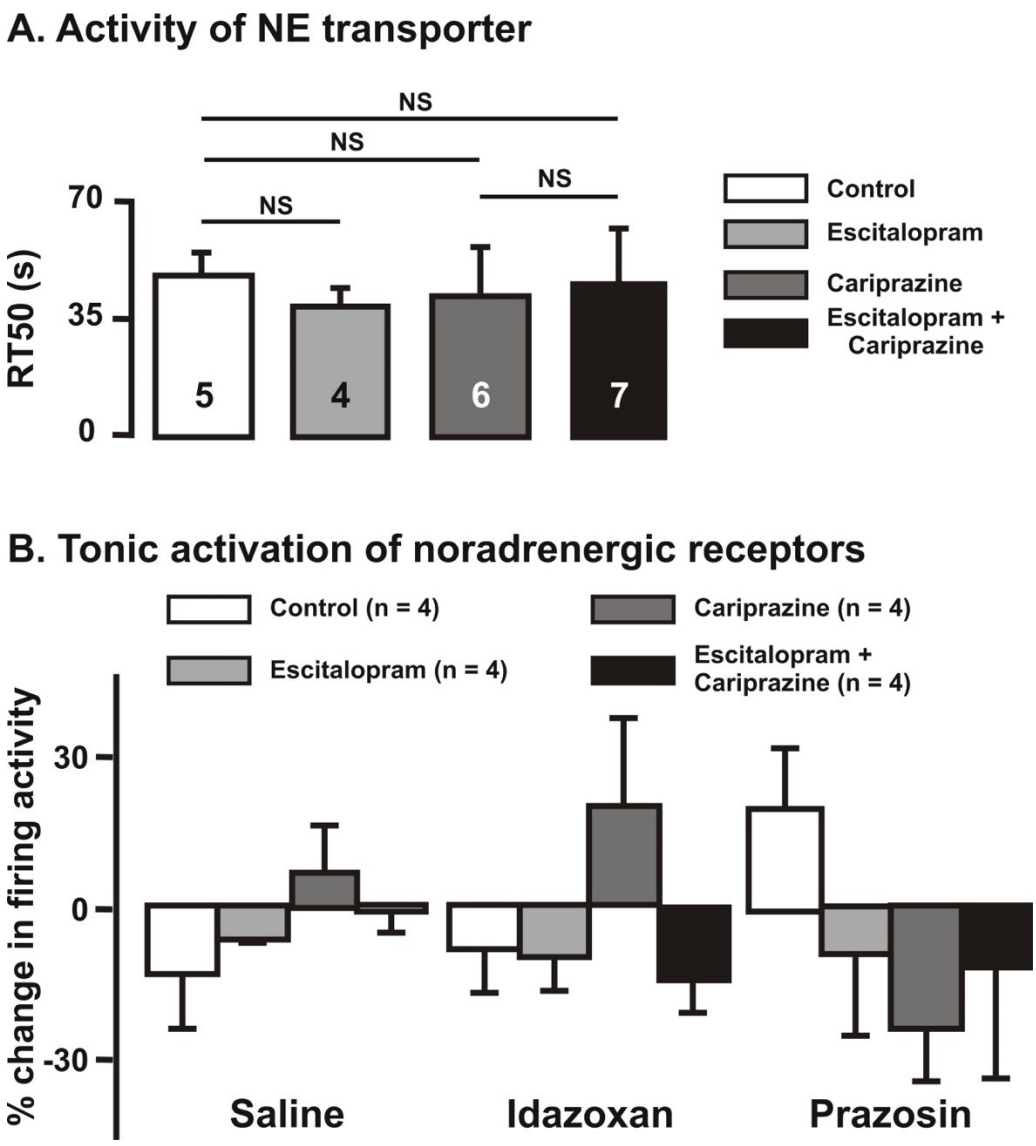
Since the firing activity of NE neurons was enhanced by cariprazine, we tested whether it can result in an increase in tonic activation of α-adrenoceptors in the hippocampus.

As illustrated in Figure 2A, none of the 14-day regimens produced any significant change in the RT50 values, indicating normal functioning of the NET under these treatments ($p > 0.05$).

The responsiveness of α_2-adrenoceptors to iontophoretically ejected NE was not changed after any of the drug regimens (data not shown). In rats that received escitalopram, cariprazine, or their combination, i.v. administration of the α_2- and α_1-noradrenergic receptor antagonists, idazoxan (1 mg/kg) and prazosin (100 μg/kg) respectively, did not significantly modify the firing activity of CA3 hippocampal pyramidal neurons compared to controls (Figure 2B; $p > 0.05$), while decreasing the effect of microiontophoretically applied NE (data not shown).

Figure 2. (A) Histograms illustrating time (s) elapsed from the cessation of microiontophoretic application of norepinephrine (NE) to 50% recovery of the initial firing rate (RT50) following administration of vehicle, escitalopram, cariprazine, and their combination. There was no significant change in RT50 values between control and drug regimens, indicating the normal functioning of the NE transporter (NET). (B) Overall change (%) in the firing activity of CA3 pyramidal neurons after

the sequential i.v. administration of idazoxan and prazosin to rats administrated with the vehicle, escitalopram, cariprazine, and their combination for 14 days. The Number of rats is indicated at the bottom of each histogram (A) and in parentheses in (B). In each rat, only one neuron was recorded. Data are presented as mean values ± S.E.M; Statistical significance is indicated where it applies.



5.3 DA system

5.3.1 Effect of 2- and 14-day Administration of Escitalopram, Cariprazine and their Combination on VTA DA Neurons

There was no statistically significant difference in the firing activity of VTA DA neurons in the 2- and 14-day control groups; hence the data for these two groups were combined.

Since cariprazine is a 5-HT_{1A} receptor agonist, we tested whether its combination can reverse escitalopram-induced inhibition of DA neurons firing. Following 2- and 14-day administration of escitalopram (5 mg/kg/day), the firing activity of DA neurons was significantly decreased by 28% and 18%, respectively (2-day: H = 30.9, Q = 4.2; 14-day: H = 23.6, Q = 2.7; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; $p < 0.05$; Figure 3A). The population activity of DA neurons, as measured by the number of neurons recorded per track, was increased after 2- and 14-day escitalopram administration (2-day: F[3, 19] = 4, $p<0.05$; 14-day: F[3, 20] = 4.8, P = 0.01; One-Way ANOVA followed by Dunn's method; Figure 3B).

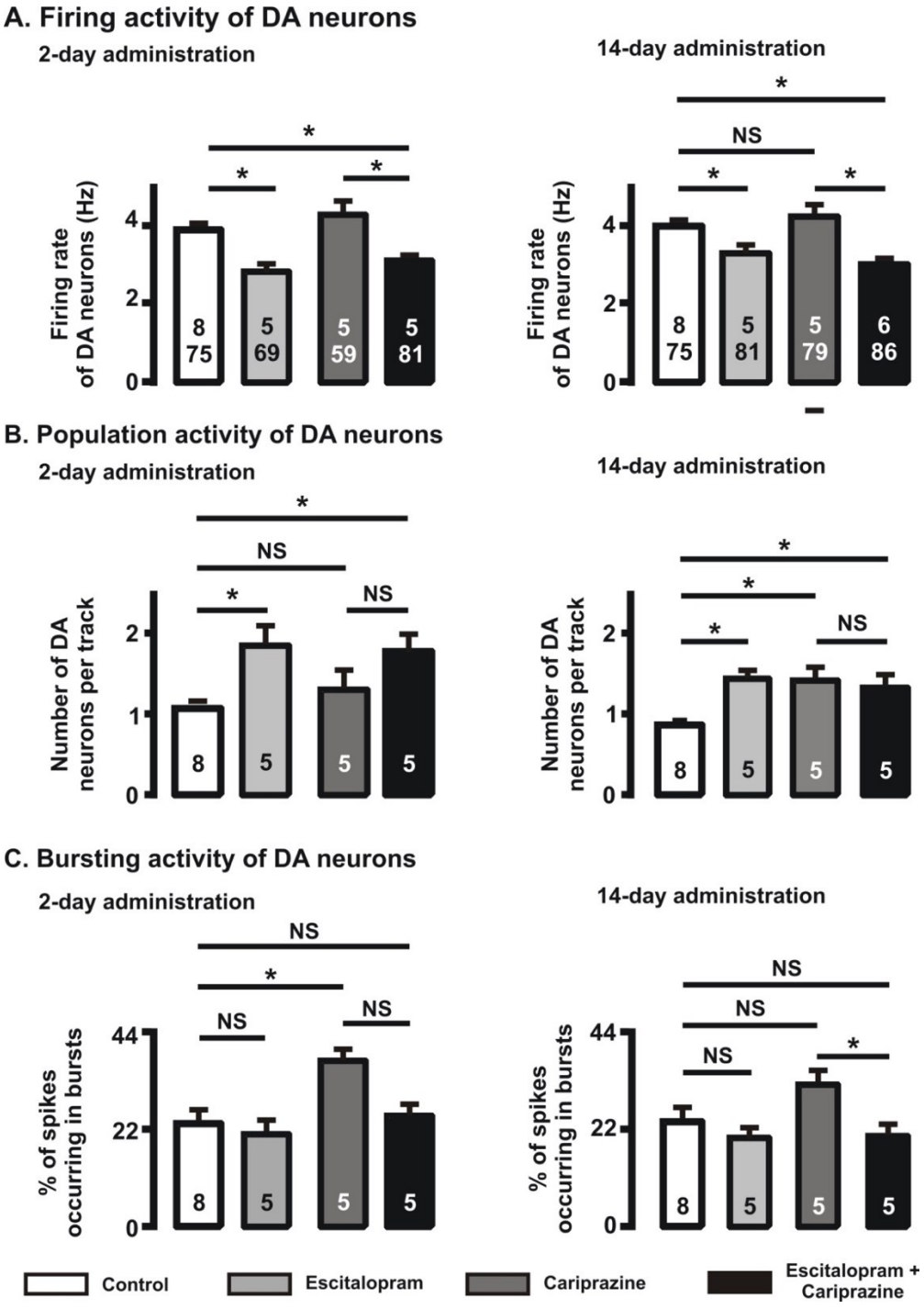
There was no alteration in the frequency of DA neurons following 2-day and 14-day cariprazine administration when compared to control (Figure 3A**)**. Administration of cariprazine for 14 days increased the number of spontaneously active DA neurons per track (2-day: F[3, 19] = 4; 14-day: F[3, 20] = 4.8; $p < 0.05$; One-Way ANOVA followed by Dunnett's; Figure 3B). The percentage of spikes

occurring in bursts was increased after 2- but not sustained after long-term cariprazine administration compared to control ($H = 12.7$, $Q = 3.0$, $p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 3C). In addition, after 14-day administration, there was a significant increase in the percentage of spikes firing in bursts between the cariprazine-treated group compared to the group treated with the combination of escitalopram and cariprazine ($H = 8.7$, $Q = 2.8$, $p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 3C).

The addition of cariprazine to escitalopram for 2 and 14 days did not reverse the escitalopram-induced inhibition of the firing activity of DA neurons (2-day: $H = 30.9$, $Q = 2.9$; 14-day: $H = 23.6$, $Q = 3.7$, $p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 3A). Two- and 14-day co-administration of escitalopram and cariprazine increased the number of spontaneously active DA neurons per track compared to control (2-day: $F[3, 19] = 4$; 14-day: $F[3, 20] = 4.8$; $p < 0.05$; Figure 3B). The percentage of spikes occurring in bursts was not changed following 2- and 14-day co-administration of escitalopram and cariprazine ($P>0.05$; Figure 3C).

Figure 3. (A) Firing rate of ventral tegmental area (VTA) dopamine (DA) neurons in rats administered with vehicle, escitalopram (5 mg/kg/day; s.c.), cariprazine (0.6 mg/kg/day; s.c.) and their combination for 2 and 14 days. (B) Effects of these drug regimens on the number of spontaneously active DA neurons recorded in each descent of electrode and on the number of spikes occurring in bursts (C). At the

bottom of each histogram are indicated the number of rats (A-C) and neurons recorded (A). Data are presented as mean values ± S.E.M; * $p < 0.05$, statistical significance is indicated where it applies.



5.4 5-HT system

5.4.1 Effect of 2- and 14-day Administration of Escitalopram, Cariprazine and their Combination on DRN 5-HT Neurons

There was no statistically significant difference in the firing activity of DRN 5-HT neurons in the 2- and 14-day control groups; hence the data for these two groups were combined.

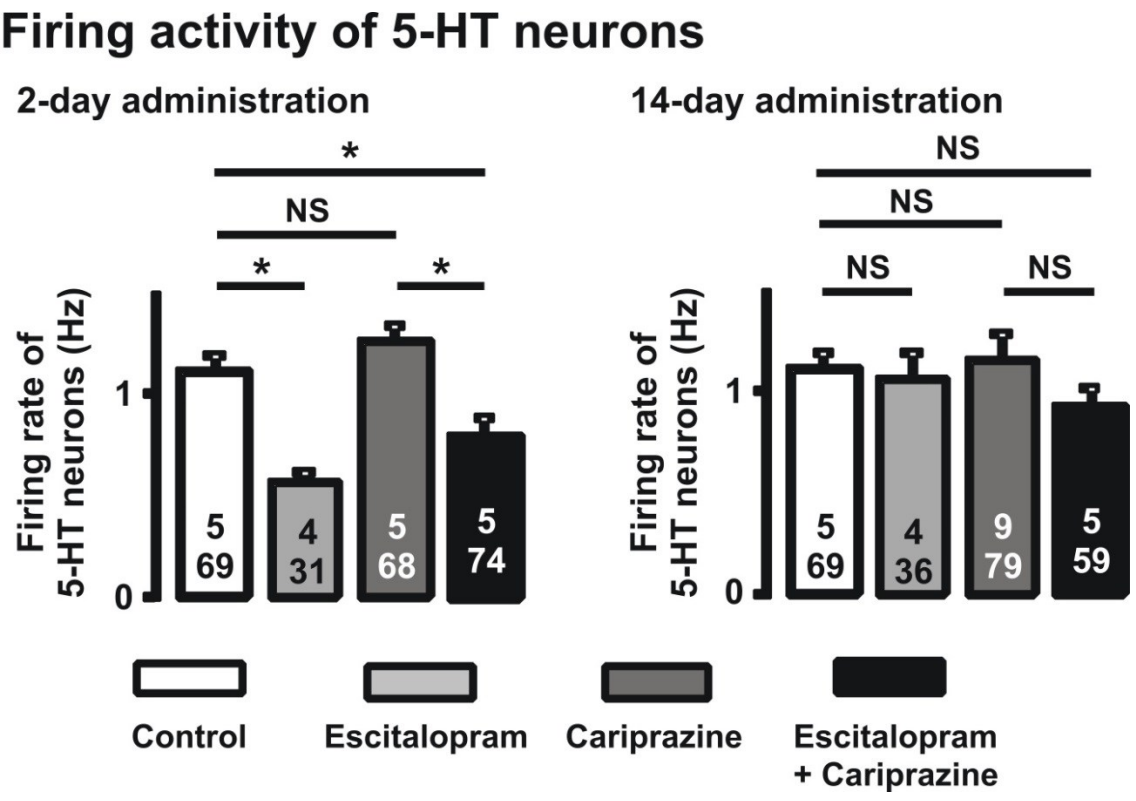
Because cariprazine is a 5-HT_{1A} autoreceptor agonist, we tested whether its addition can rescue escitalopram-induced inhibition of 5-HT neuronal firing. Escitalopram administered at 5 mg/kg/day significantly decreased the firing activity of 5-HT neurons after 2-day administration (60%; $H = 40.7$, $Q = 3.8$; $p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 4). The firing rate of these neurons recovered to baseline following 14-day escitalopram administration, as shown in prior studies (El Mansari et al., 2005; Ghanbari et al., 2010).

The firing activity of 5-HT neurons was not changed following 2- and 14-day administration of cariprazine (0.6 mg/kg/day) compared to controls (Figure 4).

The addition of cariprazine to escitalopram for 2 days did not reverse escitalopram-induced suppression of firing activity of these neurons ($H = 40.7$, $Q = 3.5$; $p < 0.05$; Kruskal-Wallis One-Way ANOVA followed by Dunn's method; Figure 4). Following 14-day administration, 5-HT neurons were firing at the control level

in rats that received a combination of escitalopram and cariprazine similar to those receiving escitalopram alone (Figure 4).

Figure 4. Firing rate of dorsal raphe nucleus (DRN) serotonin (5-HT) neurons in rats that received escitalopram (5 mg/kg/day; s.c.), cariprazine (0.6 mg/kg/day; s.c.), and their combination for 2 and 14 days. The number of rats (top) and neurons (bottom) recorded are indicated at the bottom of each histogram. Data are presented as mean values ± S.E.M; * $p < 0.05$, statistical significance is indicated where it applies.



5.4.2 Effect of 2- and 14-day Administration of Escitalopram, Cariprazine and their Combination on the Activity of the 5-HTT

In order to determine the effect of different drug regimens on the activity of the 5-HTT, RT50 values were assessed in 14-day drug-administered groups. Iontophoretic ejection of 5-HT induced a relatively complete inhibition of the firing activity of pyramidal neurons.

As illustrated in Figure 5A, 14-day administration of escitalopram (5 mg/kg/day) alone or in combination with cariprazine produced a significant increase in RT50 values when compared to control (14-day escitalopram: 171%; 14-day combination: 124%. One-Way ANOVA followed by Holm-Sidak method; $F[3, 20] = 15.9$; $p < 0.001$). This indicated the ability of escitalopram to inhibit the activity of the 5-HTT, either alone or in the presence of cariprazine.

5.4.3 Effect of 2- and 14-day Administration of Escitalopram, Cariprazine and their Combination on Tonic Activation of 5-HT_{1A} Receptors

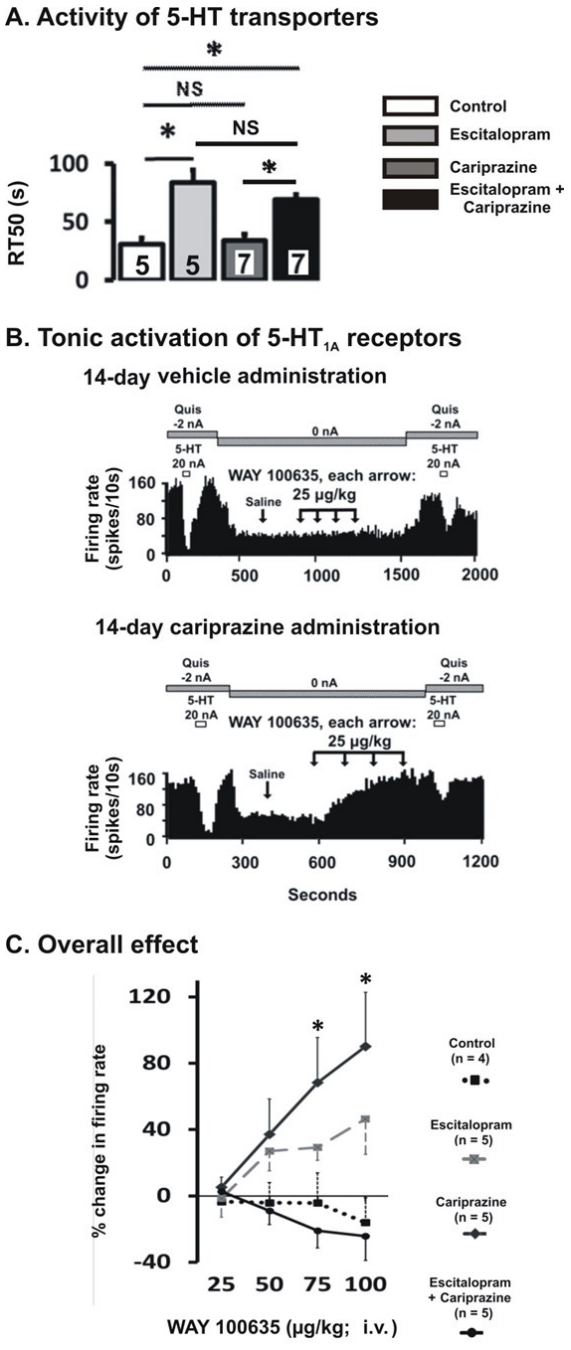
It is possible that simultaneous blockade of 5-HTT by escitalopram combined with cariprazine agonistic activity on 5-HT_{1A} receptors can result in a greater tonic activation than when administered alone.

The responsiveness of 5-HT_{1A} receptors to iontophoretically ejected 5-HT was not altered after any of the drug regimens ($p > 0.05$; data not shown). The effect of administration of the 5-HT_{1A} receptor antagonist WAY 100635 on the firing activity of pyramidal neurons was assessed following 14-day administration

of escitalopram, cariprazine, and combination. A two-way repeated-measures ANOVA on tonic activation of 5-HT_{1A} receptors revealed a statistically significant effect of treatment ($F[3, 15] = 3.7$; $p < 0.05$) and interaction between WAY 100635 and treatment ($F[3, 15] = 3$; $p < 0.05$). Dunnett's posthoc analysis showed a statistically significant increase in the tonic activation of 5-HT_{1A} receptors in the cariprazine-administered group when the dose of WAY 100635 reached 75 and 100 µg/kg (Figures 5B, C). It is important to note that 100 µg/kg of WAY 100635 is usually necessary to block enough 5-HT_{1A} receptors and induce disinhibition of firing activity of CA3 pyramidal neurons.

Figure 5. (A) Effects of 14-day regimen of escitalopram (5 mg/kg/day; s.c), cariprazine (0.6 mg/kg/day; s.c.), and the combination of escitalopram with cariprazine on the serotonin (5-HT) transporter (5-HTT). Note that escitalopram alone as well as in the presence of cariprazine significantly increased RT50 values, indicating a potent blockade of the activity of the 5-HTT. * $p < 0.05$; NS, non-significant. (B) Integrated firing rate histograms of CA3 pyramidal neurons elucidating their response to cumulative i.v. injections of the 5-HT_{1A} receptor antagonist WAY 100635 in a vehicle-administered rat and a rat that has received cariprazine for 14 days. Note the increase in the firing activity of pyramidal neurons for the latter after the injection of WAY 100635 and the subsequent attenuation of the inhibitory effect of 5-HT. (C) Overall change (%) in the firing activity of pyramidal neurons after the administration of WAY 100635 in vehicle rats and those administered with escitalopram, cariprazine, and their combination

for 14 days. Only one neuron was tested in each rat. The number of rats is indicated in parentheses. Data are presented as mean values ± S.E.M; * $p < 0.05$ compared to the control, statistical significance is indicated where it applies.



6. Discussion

The results of the present study showed that 2- and 14-day administration of cariprazine increased the firing activity of LC NE neurons and also reversed escitalopram-induced inhibition of NE neurons firing. Despite these changes, there was no increase in tonic activation of α-adrenoceptors in the hippocampus by cariprazine alone and in combination with escitalopram. The same treatments with cariprazine and its combination with escitalopram enhanced the population activity of DA neurons in the VTA. The addition of cariprazine did not result, however, in the recovery of escitalopram-induced inhibition of firing activity of DA neurons. Although DRN 5-HT neuronal firing was not altered by cariprazine, it did increase tonic activation of postsynaptic 5-HT_{1A} receptors in the hippocampus when administered alone but not in combination with escitalopram.

The clear increase in the mean firing activity of NE neurons, as well as the doubling of the percentage of neurons firing in burst (to nearly 70%) following 2- and 14-day administration of cariprazine, does not appear to directly result from α_2-adrenergic antagonism because it lacks significant affinity to the receptor (Kiss et al., 2010), neither does it show the functional property *in vivo* (Herman et al., 2018). In contrast, mirtazapine and brexpiprazole, which share α_2-adrenergic antagonism, have been shown to produce such enhancement of NE firing activity (Haddjeri et al., 1998; Oosterhof et al., 2016). This increase in firing of NE neurons is also unlikely to result from cariprazine releasing NE, thereby eventually indirectly desensitizing α_2-adrenergic autoreceptors on the cell body of NE

neurons, as does bupropion, because the latter initially suppresses the firing rate of NE neurons before a recovery takes place (El Mansari et al., 2008), whereas cariprazine does not. Therefore, the increase in firing of NE neurons remains to be explained and could manifest itself through the activation of an excitatory input to the LC, such as its glutamatergic innervation (Szabadi, 2013).

The restoration of the SSRI-attenuated firing activity of NE neurons by cariprazine can be explained by its 5-HT_{2A} receptor blocking property. Indeed, cariprazine reversed the suppression of NE neuronal firing by the preferential 5-HT_{2A} receptor agonist DOI (Herman et al., 2018). Such a reversal of SSRI-induced suppression had been initially established using the selective 5-HT_{2A} receptor antagonist MDL 100,907 (Szabo et al., 2000). Subsequently, it was also demonstrated using a variety of agents endowed with this property; these include olanzapine, risperidone, quetiapine, aripiprazole, and brexpiprazole (Chernoloz et al., 2009, 2012; Dremencov et al., 2007; Oosterhof et al., 2015; Seager et al., 2005). This characteristic of such drugs, and perhaps for cariprazine as well, may significantly contribute to their beneficial action in depressed patients presenting an inadequate response to an SSRI.

What is unclear from the present experiments is why cariprazine did not result in an enhancement of the tonic activation of postsynaptic α_1- and/or α_2-adrenoceptors in the hippocampus, especially after its 14-day administration given its capacity to robustly increase NE neurons firing and bursting. Similar to these findings with cariprazine, long-term administration of quetiapine enhanced the

tonic activation of α_2-adrenoceptors (Chernoloz et al., 2012), whereas bupropion increased tonic activation of both α_1- and α_2-adrenoceptors (Ghanbari et al., 2011). In this perspective, it is important to mention that quetiapine does block terminal α_2-adrenoceptors and that bupropion desensitizes these NE-inhibiting autoreceptors. Presumably, for NE transmission to be increased in the dorsal hippocampus, the terminal α_2-adrenoceptors have to be inactivated to allow an increase in NE release, even if the rate of impulse flow is enhanced. In support of this notion, the selective NE reuptake inhibitor reboxetine desensitizes terminal α_2-adrenoceptors and increases NE transmission in the hippocampus, despite a marked attenuation of NE neurons firing (Szabo and Blier, 2001a, 2001b). Clearly, other regions of the forebrain have to be studied following prolonged cariprazine administration, as a microdialysis study has recently shown that acute injection of cariprazine increases levels of extracellular NE in the nucleus accumbens and the ventral hippocampus (Huang et al., 2019).

In the current study, while the 2-day administration of escitalopram decreased DA neuronal firing activity (Chernoloz et al., 2009; Dremencov et al., 2009), it did increase their population activity, as previously reported (Sekine et al., 2007). Although this decrease in DA neuronal firing is due to enhancement of 5-HT through action on 5-HT_{2C} receptors (Dremencov et al., 2009), it remains to be determined whether increased population activity occurs through action on 5-HT and/or DA receptors. It is important to note that cariprazine increased population activity when administered alone or in combination with escitalopram,

but it did not reverse the escitalopram-induced inhibition of firing, in contrast to aripiprazole when combined with escitalopram (Chernoloz et al., 2009). This could be due to cariprazine having a lower affinity for 5-HT_{2C} receptors (pKi = 6.87) than aripiprazole (pKi = 7.81). The overall effect on DA levels stemming from decreased firing activity but increased population activity of DA neurons following the combination of cariprazine and escitalopram remains to be determined. However, it was previously found that the increase in firing and bursting activity of DA neurons (phasic), which regulate reward-related cues, does not induce a significant change in DA efflux in the nucleus accumbens, as it is rapidly taken up by DA transporters (Floresco et al., 2003; Schultz, 1998). However, the increase in population activity of DA neurons (tonic), which modulates novelty-gated information storage, produces a significant enhancement in DA concentrations (Floresco et al., 2003; Lisman and Grace, 2005). It is thus probable that the increase in DA neurons population activity found here results in an enhancement of DA levels in projection areas.

A previous study had shown, however, that population activity of DA neurons was decreased by cariprazine, in opposition to the present data (Delcourte et al., 2018). The exact cause for this discrepancy remains to be clarified, all the more so as the daily drug regimens were in the same range (0.6 vs. 1 mg/kg). However, cariprazine in these two studies was administered via different routes (subcutaneously and orally, respectively). It is interesting to note that an increase in population activity of DA neurons had also been documented in

our laboratory with asenapine, a drug approved for the treatment of psychosis that has subnanomolar affinities for a variety of monoaminergic receptors (Oosterhof et al., 2014). In contrast, no significant change of this parameter has been reported using quetiapine, aripiprazole, and brexpiprazole (Moreines et al., 2017; Oosterhof et al., 2016; Sonnenschein et al., 2018). In the case of clozapine, olanzapine, and risperidone, it was initially hypothesized that they exert an antipsychotic action primarily by a depolarization block which, by blocking D_2 receptors on the DA cell body, induces an over-excitation of DA neurons resulting in their cessation of firing following repeated administration (Grace and Bunney, 1984; Grace et al., 1997). Taken together, these results show that the population activity of DA neurons can be markedly influenced by the receptor profile of such medications beyond simply their capacity to act as pure antagonists or partial agonists of DA receptors.

While cariprazine did not increase the firing activity of 5-HT neurons after 2- and 14-day administration in the present study, a similar regimen of aripiprazole did so (Chernoloz et al., 2009). This increase was shown to be due to 5-HT_{1A} autoreceptor desensitization and enhanced activation of D_2 receptors on the cell body of 5-HT neurons, which exert an excitatory activity on these neurons (Aman et al., 2007; Chernoloz et al., 2009). As cariprazine and aripiprazole possess comparable affinities for 5-HT_{1A} and D_2 receptors, the lack of alteration in 5-HT neuronal firing after 2-day administration of cariprazine cannot be due to the difference in affinity for these receptors (Kiss et al., 2010). A similar lack of increase in the firing activity of 5-HT neurons was also found with chronic

asenapine (Oosterhof et al., 2015), which did not desensitize 5-HT_{1A} autoreceptors. Interestingly, asenapine acted as a partial agonist on these autoreceptors, which contrasts with the effects of full agonists like gepirone, BAY3702, and ipsapirone, which rather inhibit the firing activity of 5-HT neurons after a 2-day regimen (Blier and de Montigny 1990; Dong et al., 1997, 1998). In the absence of 5-HT_{1A} autoreceptor desensitization, it is possible that excitation exerted by cariprazine on D_2 receptors kept 5-HT neuronal firing at the baseline level. Differences in the effect of cariprazine on 5-HT neurons could not stem either from its preferential affinity for D_3 receptors, because the D_3 receptor agonist CGS15855 did not modify the activity of 5-HT neurons (Lejeune et al., 1997).

In the present study, the addition of cariprazine resulted in a partial, albeit not complete, recovery of inhibition induced by a 2-day administration of escitalopram on the firing activity of 5-HT neurons. A similar escitalopram-induced inhibition, however, was completely reversed following the addition of aripiprazole (Chernoloz et al., 2009). Although cariprazine and aripiprazole possess comparable affinities for 5-HT_{1A} and D_2 receptors controlling the firing activity of 5-HT neurons, it is unclear at this point why aripiprazole but not cariprazine increase firing activity in the 5-HT neurons. Nevertheless, after 14 days, 5-HT neuronal firing returned to normal when escitalopram was given alone or in combination with cariprazine similar to effects obtained with aripiprazole.

The current results showed that there was an increase in tonic activation of postsynaptic 5-HT_{1A} receptors in the hippocampus after long-term administration of cariprazine, but not when administered concomitantly with escitalopram. Since the firing activity of 5-HT neurons was not increased by cariprazine, this increase in tonic activation of postsynaptic 5-HT_{1A} receptors could thus be due to its direct agonistic action on these receptors (Herman et al., 2018), similar to the effect of the 5-HT_{1A} receptor full agonist gepirone (Haddjeri et al., 1998). In addition, similarly to cariprazine the DA/5-HT receptor partial agonist brexpiprazole also increased tonic activation of 5-HT_{1A} receptors in the rat hippocampus (Oosterhof et al., 2016). Therefore, it remains unexplained why repeated administration of aripiprazole, which has a comparable affinity for 5-HT_{1A} receptors as cariprazine, did not produce these effects on its own but did so in combination with escitalopram (Ebrahimzadeh et al., 2018). As DA/5-HT partial agonists have activity on multiple receptors, it is possible that other receptors may have influenced the outcome.

In summary, a large body of experimental evidence shows that a drug or combination of drugs with an affinity for different receptors, and possibly different downstream pathways, may result in different physiological outcomes in preclinical investigations. Similar discrepancies are also encountered in clinical investigations. For instance, aripiprazole has failed to show clear sustained therapeutic efficacy in depressive episodes in patients with bipolar I disorder (Thase et al., 2012), whereas the use of cariprazine has now an indication in such

patients. Aripiprazole and brexpiprazole have demonstrated consistent efficacy as an adjunct in patients with MDD (Citrome, 2015), whereas more clinical studies with cariprazine are ongoing to determine its efficacy (Durgam et al., 2016; Earley et al., 2018). These results underscore the need for studies on the mechanisms of action of these psychotropic medications, especially in animal models attempting to best mimic the pathophysiology of subtypes of patients with mood disorders.

7. Conflict of Interest

M. Ebrahimzadeh, R. Hamati, M Iro, and M. El Mansari, declare no conflict of interest. P. Blier received grant funding and/or honoraria for lectures and/or participation in advisory boards for Allergan, Bristol Myers Squibb, Eli Lilly, Janssen, Lundbeck, Otsuka, Pfizer, Pierre Fabre Médicaments, Takeda, and Valeant. B. Kiss and B. Farkas are employees of Gedeon Richter Plc. N. Adham is an employee of Allergan.

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Chapter 5 — General Discussion

1. Monoamines and Symptoms of Major Depressive Disorder

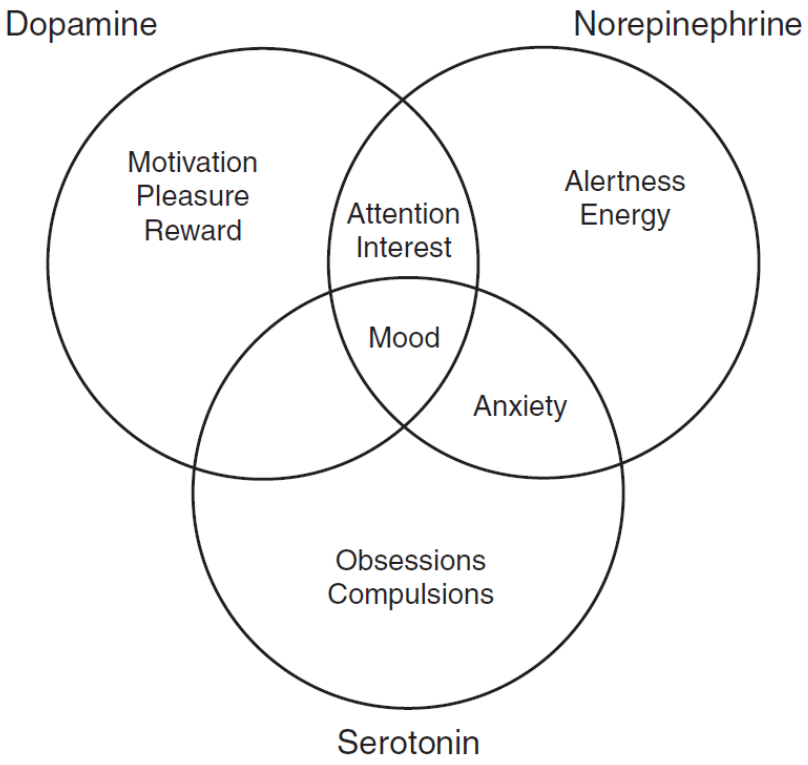
There is a vast degree of heterogeneity in clinical presentations of MDD symptoms among individual patients (Blier, 2014; Fried and Nesse, 2015). It is most likely that such a clinical heterogeneity is the manifestation of the heterogeneity of malfunctions across multiple neurotransmitter systems and brain circuits in individuals diagnosed with MDD. Efficacious treatment strategies for individual MDD patients should address this heterogeneity and if necessary target different neurobiological substrates based on the clinical profile of each patient.

The results of preclinical research projects (including studies presented in chapters 2-4) have demonstrated the distinctly heterogeneous effects of comparable pharmacotherapeutic strategies on neurotransmission modulation of monoamines (and other relevant neurotransmitters) and complement the clinical evidence indicating subtle differences in the effect of these strategies on clinical biomarkers and specific symptoms of MDD (Uher et al., 2020).

These results would offer clinicians potentially valuable information that provides them with the possibility of making evidence-based decisions for devising more efficacious pharmacotherapeutic strategies for individual MDD patients (Blier, 2014; Nutt, 2008; for more information, please refer to section 2 of chapter 5).

It has been hypothesized that different neurotransmitter systems have different degrees of involvement in regulating specific symptoms of MDD (Figure 22; Nutt, 2008). Accordingly, 5-HT has been hypothesized to be more closely related to mood, anxiety, obsessions, and compulsions; NE to mood, anxiety, loss of attention, interest, alertness, and energy; and DA to mood, loss of attention, interest, motivation, and pleasure (Figure 22; Argyropoulos et al., 2007; Foote and Aston-Jones, 1995; Shelton and Tomarken, 2001; Stahl, 2000, 2003).

Figure 22. Regulation of mood and behavior by monoamine neurotransmitters (Nutt, 2008).

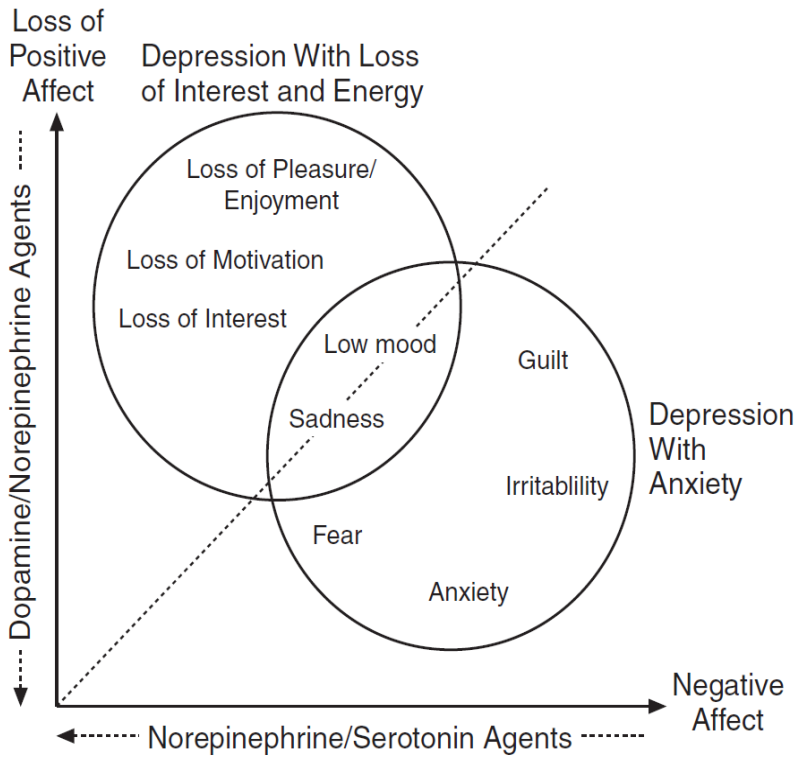


Built upon these neurotransmitter-symptom relationships, the two-dimensional model of neurotransmitter functions (Figure 23) in depression provides an opportunity to match the neurotransmission modulation profile of drugs with the clinical profile of individual patients. This model describes depression as a mixture of increased negative and decreased positive affect (Nutt, 2008; Figure 23).

In this framework, negative affect is defined as having an unpleasant and threatening perspective of the world and considering the surrounding environment as hostile and disturbing. Positive affect is defined as the propensity to enjoy normally rewarding activities such as social interactions. Accordingly, some MDD patients may particularly suffer from increased negative affect and experience symptoms such as anxiety while others may suffer more pronouncedly from decreased positive affect and experience symptoms of anhedonia (Nutt et al., 2007).

In cases where clinical assessments can distinguish whether each patient is particularly suffering from increased negative affect or decreased positive affect, symptom-specific pharmacological agents can be used to address these problems. Consequently, drugs targeting NE and/or 5-HT systems can be used to alleviate symptoms of anxiety, fear, irritability, and guilt. On the other hand, agents that more specifically target the DA and/or NE systems can be used to address the loss of motivation and pleasure (Blier, 2014; Nutt et al., 2007; Nutt, 2008).

Figure 23. The two-dimensional model of neurotransmitter functions in depression (Nutt, 2008).



2. Pharmacological Antidepressant Strategies and Functional Interactions of Monoamines

The effects of drugs on different neurotransmitter systems go beyond their pharmacological profile and their affinities for specific transporters and receptors. For instance, the NE and DA releasing agent bupropion has been shown to increase the firing activity of DRN 5-HT neurons (Ghanbari et al., 2010). And serotonergic agents, such as the SSRIs, have been shown to suppress the firing

activity of LC NE and VTA DA neurons through activation of 5-HT_{2A} and 5-HT_{2C} receptors, respectively (for more information, please refer to section 4 of chapter 1). Moreover, activation of 5-HT_{1A} receptors (along with blocking the 5-HT_{2A} and 5-HT_{2C} receptors) has been shown to counteract the 5-HTT inhibitor-induced suppression of LC NE and VTA DA neuronal activity (Piercey et al., 1994; Szabo and Blier, 2001 b, c; Di Matteo et al., 2001; Díaz-Mataix et al., 2005; Dremencov et al., 2009).

The pharmacological strategies investigated in the current studies supplement the therapeutic profile of 5-HTT inhibitors by incorporating add-on features, mainly, agonism of 5-HT_{1A} receptors and antagonism of 5-HT_{2A} and 5-HT_{2C} receptors. These add-on features have been integrated with inhibition of 5-HTT in a single multimodal molecule, for example, vortioxetine, or by devising combined regimens of the 5-HTT inhibitor escitalopram with the DA and 5-HT partial agonists aripiprazole and cariprazine (Sánchez et al., 2015; Ebrahimzadeh et al., 2019; Durgam et al., 2016; El Mansari et al., 2020).

However, despite the ample evidence about the clinical efficacy of these antidepressant strategies, the distinct effects of each of these strategies on neurotransmission of monoamines had yet to be fully investigated. Accordingly, the current reverse-translational studies investigated the net monoamine neurotransmission modulation by these comparable, yet distinct pharmacological strategies.

3. Vortioxetine

As discussed in section 9.1. of chapter 1, clinical evidence has indicated that administration of vortioxetine is superior to agomelatine in MDD patients with a previous inadequate response to a single course of SSRI/SNRI monotherapy (direct comparison; Montgomery et al., 2014) and also leads to a greater probability of remission compared to sertraline, venlafaxine, bupropion, and citalopram (indirect comparison; Thase et al., 2017).

The first *in vivo* electrophysiological study focused on vortioxetine, a multimodal 5-HTT inhibitor that also directly targets a variety of 5-HT receptors including 5-HT_{1A} receptors. Due to the low affinity of vortioxetine for the 5-HT_{1A} receptor in the rat brain (compared to the human brain), vortioxetine-containing chow was used in this study to deliver a sufficiently high dose of vortioxetine (1.8 g vortioxetine/kg food) ensuring the proper engagement of 5-HT_{1A} receptors and 5-HTTs (Pehrson et al., 2016). The results of this study showed that 14-day vortioxetine administration dampened the firing activity of catecholamine neurons, although to a lesser degree than that observed with the SSRI escitalopram. Following this same regimen, it was also shown that tonic activation of the α_1- and α_2-adrenoceptors on hippocampus pyramidal neurons was unchanged (Ebrahimzadeh et al., 2018).

In the LC, 4-day vortioxetine administration significantly dampened the firing activity of NE neurons. Following 14 days of vortioxetine administration, the

firing activity of NE neurons remained decreased but to a lesser degree compared to the 4-day regimen. The inhibitory action of vortioxetine on NE neuronal firing may be due to an enhancement of 5-HT levels resulting from a potent blockade of the 5-HTT. It has been previously shown that the escitalopram-induced inhibition of the firing activity of LC NE neurons is mediated via activation of 5-HT_{2A} receptors (expressed on GABAergic interneurons; for more information, please refer to sections 7.1.5.2.1 and 7.4.1 of chapter 1) and it can be reversed by injection of the selective 5-HT_{2A} receptor antagonist M100907 (Dremencov et al., 2007a; Haddjeri et al., 1997).

The suppression of firing activity of LC NE neurons by vortioxetine (27%) was, however, less than the reported inhibition by the administration of escitalopram, reported by other studies (40-50%; Chernoloz et al., 2009; Ghanbari et al., 2010). Although vortioxetine and escitalopram were not directly compared in this study, the magnitude of their effect on the firing activity of NE neurons seems to be different. For this comparison, it has to be taken into consideration that vortioxetine, unlike escitalopram, is also an agonist at 5-HT_{1A} receptors which may have exerted an excitatory influence on the firing activity of NE neurons (please refer to section 7.4.1 of chapter 1). Indeed, it has been shown that 5-HT_{1A} receptor agonists increase neuronal firing rate and bursting activity of NE neurons (Piercey et al., 1994; Szabo and Blier, 2001a, b). However, due to the lower affinity of vortioxetine to 5-HT_{1A} receptors in the rat brain (Ki = 230 nM; Sanchez et al., 2015) compared to the human brain (Ki = 15 nM), the contribution of these

receptors in modulation of the firing activity of LC NE neurons in the rat brain remains uncertain.

Although 4-day vortioxetine administration did not change the firing activity of DA neurons, its sustained administration significantly dampened the firing activity of these neurons by 26%. The latter effect may be due to an increase of 5-HT levels around DA neuron cell bodies caused by the inhibition of 5-HTT by vortioxetine. Indeed, evidence has shown that 5-HT exerts an inhibitory effect on DA neurons through activation of 5-HT_{2C} receptors (Di Matteo et al., 2001; Gobert et al., 2000; Prisco et al., 1994). Although in this study vortioxetine administration inhibited the activity of DA neurons (26%), this decrease was lower compared to the effects of the SSRI escitalopram, as reported by previous studies (40-50%; Chernoloz et al., 2009; Dremencov et al., 2009).

This relatively weak inhibition of the activity of DA neurons by vortioxetine might be the result of the direct activation of 5-HT_{1A} receptors. It has been shown that activation of 5-HT_{1A} receptors markedly increases the firing and bursting activities of VTA DA neurons (for more information, please refer to section 7.4.2 of chapter 1; Gronier, 2008; Lejeune and Millan, 1998). However, due to the already discussed weak occupation of 5-HT_{1A} receptors by vortioxetine in rats compared to humans, the involvement of these receptors in this effect remains uncertain. It is worth reiterating that the effects of vortioxetine on NE and DA neurotransmission were not directly compared to the effects of escitalopram in this study but rather to the effects of escitalopram reported in previous studies. Accordingly, differences in

experimental settings (including, but not limited to the experimental animals, experimenter, different drug batches, and laboratory environment) are among the limiting factors for this comparison.

An electrophysiological study using the same regimen of vortioxetine as herein (Riga et al., 2017) has shown that long-term vortioxetine administration increased the firing activity of glutamatergic pyramidal neurons in the rat medial prefrontal cortex, while the SSRI escitalopram did not alter the firing activity of these neurons (Riga et al., 2016; for more information on the glutamate system, please refer to section 8 of chapter 1). In the present study, long-term vortioxetine administration increased the NMDA-, but not AMPA-evoked, firing of glutamatergic pyramidal neurons in the CA3 region of the hippocampus. This direct effect of vortioxetine might be a contributing factor to its pro-cognitive effects, which have been reported following vortioxetine administration in rats (du Jardin et al., 2014; Jensen et al., 2014; Mørk et al., 2013) and in patients with MDD (Katona et al., 2012; Mahableshwarkar et al., 2015; McIntyre et al., 2014).

4. Aripiprazole Combined with Escitalopram

The efficacy of adjunctive aripiprazole for treatment-resistant MDD has been reported in multiple studies (Barbee et al., 2004; Marcus et al., 2008a; Pae et al., 2007; Papakostas et al., 2005; Patkar et al., 2006; Simon and Nemeroff, 2005; Uher et al., 2020). The results of the second study (presented in chapter 3) showed that 2-day administration of escitalopram, aripiprazole, and their

combination did not have any effect on the activity of the 5-HTT and tonic activation of 5-HT_{1A} receptors of pyramidal neurons in the CA3 region of the hippocampus. However, 14-day administration of escitalopram, as well as its combination with aripiprazole, resulted in significant inhibition of 5-HT reuptake. While 14-day administration of neither escitalopram nor aripiprazole alone affected tonic activation of 5-HT_{1A} receptors, their combination significantly increased this parameter. Neither the administration of escitalopram, aripiprazole nor their combination for 14 days had any effect on neither the activity of the NET nor tonic activation of the α-adrenoceptors of the CA3 pyramidal neurons.

In this study following 4- and 14-day administration of a low dose of escitalopram, the tonic activation of 5-HT_{1A} receptors was not enhanced, indicating sub-threshold escitalopram concentrations to increase 5-HT neurotransmission. This is in line with the results obtained in the third study presented in the current dissertation (please refer to chapter 4 and section 5 of chapter 5). It is worth mentioning that achieving a high degree of 5-HT reuptake inhibition does not necessarily result in increased 5-HT neurotransmission (as indicated by a study using a 2-day regimen of paroxetine, 10 mg/kg/day; Besson et al., 2000).

In this study, administration of aripiprazole did not increase the tonic activation of 5-HT_{1A} receptors. It is unclear why aripiprazole on its own did not exert this effect since it has previously been shown that long-term administration of gepirone, a 5-HT_{1A} receptor agonist, enhanced tonic activation of 5-HT_{1A}

receptors in the hippocampus (Haddjeri et al., 1998). In addition, brexpiprazole and cariprazine which are also DA and 5-HT partial agonists with an affinity for 5-HT_{1A} receptors have been shown to significantly increase tonic activation of these receptors after 14 days of administration (Oosterhof et al., 2016; El Mansari et al., 2020).

Although neither escitalopram nor aripiprazole increased tonic activation of 5-HT_{1A} receptors there was a significant enhancement of this parameter following their combined administration which indicated a clear synergy between these two medications for increasing 5-HT neurotransmission in the hippocampus.

Following 14-day administration of escitalopram, aripiprazole, and their combination there was no increase in the tonic activation of α_1- and α_2-adrenoceptors in the hippocampus. The experimental paradigm used for measuring tonic activation of α_1- and α_2- adrenoceptors in the current studies is adequate to assess an increase of NE neurotransmission. However, it cannot unveil decreased or normalized NE neurotransmission, as is the case for measuring 5-HT neurotransmission using the 5-HT_{1A} receptor antagonist WAY 100635 (Kasamo et al., 2001). This is evidenced by the lack of disinhibition of the firing of the pyramidal neurons neither by the α_1-adrenoceptor antagonist prazosin nor by the α_2- adrenoceptor antagonist idazoxan in control rats. Hence, an undetected normalized or restored NE neurotransmission following combined administration of escitalopram with aripiprazole or cariprazine may contribute to the clinical antidepressant efficacy of these regimens. It has been shown that

aripiprazole augmentation of escitalopram monotherapy is more beneficial in patients with prominent loss of interest and activity (Uher et al., 2020). Considering the two-dimensional model of neurotransmitter functions in depression (please refer to sections 1 and 2 of chapter 5) this finding is in line with the preclinical evidence indicating that the addition of aripiprazole to escitalopram recovers the escitalopram-induced inhibition of VTA DA and LC NE neuronal firing activity (Chernoloz et al., 2012).

5. Cariprazine Combined with Escitalopram

The results of a randomized, double-blind, placebo-controlled study in adults with MDD indicated the efficacy of adjunctive cariprazine in non-responders to standard antidepressants (Durgam et al., 2016). The results of the last study (presented in chapter 4) indicated that the administration of cariprazine for 2 and 14 days did not change the firing activity of DRN 5-HT neurons and when added to escitalopram it did not block the inhibitory effect of escitalopram on the firing activity of these neurons. However, 14-day administration of cariprazine, but not escitalopram, increased tonic activation of postsynaptic 5-HT_{1A} receptors on pyramidal neurons of the CA3 region of the hippocampus, indicating increased 5-HT neurotransmission in this region. On the other hand, 14-day combined administration of escitalopram and cariprazine did not increase 5-HT neurotransmission in this region. The neurobiological basis for this absence of increased 5-HT tonic activation remains to be elucidated.

In the LC, administration of cariprazine for 2 and 14 days not only increased the firing rate of NE neurons but also when added to the escitalopram regimen it reversed the escitalopram-induced inhibition of the activity of these neurons. In addition, 14-, but not 2-day, cariprazine administration also increased the percentage of bursting LC NE neurons. However, in the CA3 region of the hippocampus 14-day administration of cariprazine, escitalopram, and their combination did not have any effect on tonic activation of postsynaptic α_1- and α_2-adrenoceptors of pyramidal neurons. This indicates a lack of increase in NE neurotransmission, but as already explained, cannot rule out the probability that a conceivably, escitalopram-induced, decreased NE neurotransmission has been recovered by the addition of cariprazine. In the VTA, administration of cariprazine for 2- and 14-day did not have an effect on the firing rate of DA neurons, and when added to the escitalopram regimen, it did not restore the escitalopram-induced inhibition of these neurons.

Electrophysiological recording in the DRN indicated that 2- and 14-day cariprazine administration did not have any effect on the activity of 5-HT neurons. However, it has been shown that administration of aripiprazole for 2 and 14 days and brexpiprazole for 2, but not 14 days, increases the firing activity of these neurons above the control group (Chernoloz et al., 2009; Oosterhof et al., 2016) which have been stipulated to be due to desensitization of 5-HT_{1A} autoreceptors by these drugs.

In the CA3 region of the hippocampus, 14-day administration of a low dose of escitalopram did not increase tonic activation of 5-HT_{1A} receptors. This is similar to the results obtained in the second study (Ebrahimzadeh et al., 2019; investigating the combination of escitalopram and aripiprazole) and can be attributed to the sub-threshold concentration of escitalopram. However, 14-day administration of cariprazine increased tonic activation of these receptors in the same region. This effect can be attributed to the direct activation of 5-HT_{1A} receptors of pyramidal neurons in this region by cariprazine (Herman et al., 2018), similar to the results obtained with the DA and 5-HT receptors partial agonist brexpiprazole (Oosterhof et al., 2016) and the 5-HT_{1A} receptor partial agonist gepirone (Haddjeri et al., 1998). However, combined administration of escitalopram and cariprazine for 14 days did not cause an increase in tonic activation of 5-HT_{1A} receptors in the hippocampus which stands in contrast with the documented synergy between escitalopram and aripiprazole in increasing 5-HT neurotransmission in the same regions (Ebrahimzadeh et al., 2019). Given the similarities between the pharmacological profiles of cariprazine and aripiprazole, their distinct effect on the modulation of tonic activation of 5-HT_{1A} receptors points to the already mentioned (section 1 of chapter 5) heterogeneous neurotransmission modulation profiles of strategies with comparable pharmacological profiles.

Administration of cariprazine for 2 and 14 days increased the firing activity of LC NE neurons. Combined administration of cariprazine with escitalopram for 2

and 14 days recovered the escitalopram-induced inhibition of NE firing activity, similar to the effect of 14-day (but not 2-day) co-administration of aripiprazole with escitalopram (Chernoloz et al., 2009) which might be due to blockade of 5-HT_{2A} receptors by cariprazine (Kiss et al., 2010).

Neither escitalopram, cariprazine, nor their combination increased tonic activation of α_1- and α_2-adrenoceptors in the CA3 region of the hippocampus, similar to the results obtained with aripiprazole (Ebrahimzadeh et al., 2019). However, due to the already-mentioned inability of the experimental paradigm used in the current studies to detect decreased or normalized neurotransmission in the hippocampus, it is still possible that the addition of cariprazine to escitalopram has recovered a conceivably, escitalopram-induced, reduced NE neurotransmission as it has been the case for the firing rate of NE neurons in the LC.

Administration of cariprazine for 2 and 14 days did not change the firing activity of DA neurons in the VTA which was in line with the results obtained with 2- and 14-day administration of aripiprazole (Chernoloz et al., 2009) and brexpiprazole (Oosterhof et al., 2016). Combined administration of cariprazine and escitalopram for 2 and 14 days did not restore the escitalopram-induced inhibition of DA neuronal activity. It has been shown that antagonism of 5-HT_{2C} receptors counteracts the SSRI-induced inhibition of DA neuronal activity (Gobert et al., 2000; Lucas et al., 2000). However, the current results are probably due to the low affinity of cariprazine for 5-HT_{2C} receptors. Nonetheless, it is still possible that

cariprazine increases postsynaptic DA neurotransmission by directly activating postsynaptic D_3 and D_2 DA receptors.

6. Conclusion

Despite the commonalities in the pharmacodynamic profiles of the three MDD treatment strategies presented in chapters 2-4, combining the inhibition of 5-HTT activity with direct targeting of 5-HT and DA receptors has distinctly modulated neurotransmission of monoamines in each study. These findings, along with the results of other preclinical and clinical studies on different pharmacotherapeutic strategies, offer clinicians potentially valuable information that provides them with the possibility of devising optimal evidence-based pharmacological treatment strategies for individual MDD patients. The interpretation of the present results is that these strategies produce different effects on monoaminergic systems that are not redundant. Until we have predictors of positive response, clinicians can take advantage of neuroscience-informed treatment algorithms to utilize the synergetic effects between different medications and avoid antagonistic effects and sequential steps that might engage the same mechanisms.

7. Limitations

According to the already-mentioned differences in the physiology of Sprague-Dawley rats and humans, including different receptors affinities for the same drugs (for example please refer to section 3 of chapter 5), the results of the

current studies should be considered in the larger context of the evidence gathered from other preclinical and clinical studies and caution should be taken in regards to the clinical implications of the current data.

In addition, the possibility that the 5-HT_{1A} antagonist WAY 100635 (used to estimate the degree of 5-HT_{1A} receptors throughput) can act indirectly on hippocampus pyramidal neurons cannot be entirely discounted. Nevertheless, the observation that the inhibitory effect of 5-HT applied directly on the pyramidal neurons is markedly diminished by the systemic injection of WAY 100635, both in control and treated rats, supports that the disinhibition of firing is mediated principally by a local phenomenon. The effects of anesthesia, sex differences, and lack of experiments with animal models of MDD are among the other limiting factors in these studies which are discussed in the following sections.

7.1 Anesthesia

It can be argued that since these experiments have been conducted in anesthetized rats, their findings are not relevant to clinical settings. However, although data from awake animals might be more relevant, gathering this type of data requires invasive experimental techniques, which necessitate pre-and post-surgical procedures (including the handling required for habituation of animals to the testing environment, use of analgesics, and wound healing), that introduce additional confounds (for example acute stress associated with the experimental procedure), likely to affect neuronal activities. In addition, conducting

electrophysiological experiments in awake animals creates technical challenges in regards to gathering viable data which might, in turn, lead to an increase in the number of animals needed for the experiments, which stands in contrast to the ethical use of experimental animals.

The need to activate the firing of hippocampus pyramidal neurons in the CA3 region in the anesthetized rats could be perceived as a potential limitation. However, it has been previously shown that the enhanced responsiveness of studied neurons to 5-HT produced by MDD treatment regimens is the same whether the neurons have to be activated (because of anesthesia) or were studied without anesthesia and the need for activation (using a low cerveau isolé preparation; de Montigny and Aghajanian, 1978; de Montigny, 1984; Chaput et al., 1991).

7.2 Sex Differences

The results of the current studies were gathered using male Sprague-Dawley rats. However, evidence from preclinical and clinical studies indicates the involvement of gonadal hormones in depression, which might contribute to resilience and treatment efficacy (Eid et al., 2019). In addition, there are other reported sex-specific differences in MDD including (but not limited to) the higher rate of comorbidity with anxiety in women and a higher rate of comorbid substance use disorder in men (Schuch et al., 2014; Marcus et al., 2008b; Eid et al., 2019).

7.3 Animal Models of Depression

Despite the steady progress in developing therapeutic strategies for the treatment of MDD, the precise underlying neurobiology of MDD remains to be fully elucidated. Although clinical studies on the underlying mechanisms of MDD have provided immense insights, these studies face several different challenges with regards to a variety of hard to control factors including (but not limited to) medication history, age, sex, race, and living conditions.

In this regard, preclinical investigations (such as the studies presented in chapters 2-4) provide us with a constructive approach that not only creates more controlled experimental environments but also enable us to investigate the effect of different variables using pharmacological, behavioral, and genetic manipulations (Wang et al., 2017). However, the studies presented in chapters 2-4 were undertaken in naïve rats and more insight can be gained from further elucidating the effects of such drug regimens in animal models of MDD. A brief overview of the most commonly used animal models of depression can be found in the following sections.

7.3.1 Learned Helplessness

This model of depression was initially developed by the American psychologist Martin Seligman and his colleagues (Seligman, 1972). In this model, animals are exposed to highly stressful uncontrollable events which lead to the development of a learned helpless phenotype manifested in decreased motor

activity, decreased eating and drinking, weight loss, or lack of weight gain, decreased grooming, decreased competitive behavior, increased errors in a choice/discrimination task, decreased response to rewarding brain stimulation, and sleep disturbances (Chourbaji et al., 2005).

7.3.2 Chronic Unpredictable Stress

Chronic unpredictable stress (CUS) is among the most widely used models of depression in rodents. In this paradigm, rodents are exposed to systematic, repeated, unpredictable, variable, and uncontrollable stressors. These exposures culminate in the development of anhedonia and reduced reward sensitivity which can be quantified by the decrease of sugar consumption, the increase of intracranial self-stimulation threshold, and the loss of weight and appetite. These stressors include food and water deprivation, overnight illumination, cage tilt, and other similar unpredictable stressors (Sequeira-Cordero et al., 2019).

7.3.3 Maternal Deprivation

Separating the pups from their mothers during the postnatal period (maternal deprivation) is among the early life stress models that have been shown to affect emotionality and stress responsiveness later in life. The interruption of mother-pup interactions is shown to disrupt the responsiveness of the HPA axis (Nishi et al., 2013).

7.3.4 Olfactory Bulbectomy

The disruption of the limbic-hypothalamic axis caused by olfactory bulbectomy in rodents leads to the manifestation of behavioral, neurochemical, neuroendocrine, and neuroimmune alterations which resemble the changes in humans suffering from MDD. These symptoms are relieved by chronic, but not acute, treatments with drugs used for the pharmacotherapy of MDD. It has been shown that administration of bupropion, but not paroxetine, normalizes the 5-HT neuronal activity and increases 5-HT neurotransmission (through 5-HT_{1A} receptors) in the hippocampus of bulbectomized rats (El Mansari et al., 2016).

7.3.5 Social Defeat

In the social defeat model (also referred to as the resident-intruder test) social conflict is used as a stressor to induce behavioral and physiological changes including the emergence of anhedonia, decreased sexual behavior and increased defensive behavior, increased anxiety, decreased locomotor or exploratory activity, changes in circadian rhythmicity, alterations in feeding and body weight, sleep disturbances, and impaired immune functions. Stress in this model is induced by introducing a male rodent to older, aggressive, and dominant male rodents for several sessions (Bohus et al., 1993; Koolhaas et al., 1997; Martinez et al., 1998; Takahashi et al., 2018).

7.3.6 Chronic Restrain Stress

In this model, animals are restrained at least twice daily for a period of 14 to 21 days resulting in manifestation of depressive behavior and electrophysiological changes, including decreased baseline firing rate of DRN 5-HT neurons in rats (Oosterhof et al., 2016).

7.3.7 Glucocorticoid/corticosterone Model

Pharmacological manipulation of corticosteroids can be used to induce depressive-like states in animals. Rats in this model have been reported to show slowed weight gain, longer immobility periods in the FST, and decreased sucrose preference (Gregus et al., 2005).

7.3.8 Genetic Models

7.3.8.1 Congenital Learned Helplessness

Congenital learned helplessness strain rats are produced by selective breeding of rats that show the most depressive-like behaviors in response to inescapable shocks. These rats fail the escape test without the need for a trigger and are resistant to drugs used for the pharmacotherapy of MDD (except high doses of MAOIs; Willner and Belzung, 2015).

7.3.8.2 Wistar-Kyoto Strain

Although these rats are mainly used for studies on PTSD and anxiety, they exhibit some features of depressive-like behavior including psychomotor retardation, behavioral inhibition, learned helplessness, and social withdrawal (Nam et al., 2014). Although these rats have high variability in their behavioral profile, they can be selectively bred to produce animals with a more homogenous behavioral profile. For instance, Wistar-Kyoto most immobile and least immobile sub-strains are selectively bred based on their immobility in the FST (Nam et al., 2014).

7.3.8.3 Flinders Sensitive Line

Flinders sensitive line (FSL) rats, a genetic animal model of depression, are selectively bred from the Sprague-Dawley strain (Overstreet et al., 2005). These rats exhibit hypoactivity in the FST and the open field arena tests and decreased saccharin preference (Overstreet et al., 2005; Pucilowski and Overstreet, 1993).

8. Potential Future Studies

8.1 Experiments with Maternal Deprivation and Chronic Unpredictable Stress Models of Depression

It has been shown that aripiprazole augmentation of escitalopram monotherapy is more beneficial in patients with prominent loss of interest and activity (Uher et al., 2020). Considering the two-dimensional model of

neurotransmitter functions in depression (Nutt, 2008; please refer to section 1 of chapter 5) this finding is in line with the preclinical evidence indicating that the addition of aripiprazole to escitalopram recovers the escitalopram-induced inhibition of VTA DA and LC NE neuronal firing activity in naïve rats (Chernoloz et al., 2009). Previous preclinical studies have shown that while escitalopram ameliorates depression-like and anxiety-like behaviors induced by CUS or maternal deprivation, it does not attenuate anhedonia in rats that have undergone both maternal deprivation and CUS protocols (Zhang et al., 2015).

Further insight would be gained by investigating the effects of aripiprazole, alone and in combination with escitalopram, on anhedonia induced by maternal deprivation + CUS and the correlation of the obtained results with monoamine neurotransmission profiles of these strategies. In these experiments, anhedonia would be measured by the sucrose preference rate (Zhang et al., 2015). In naïve rats, escitalopram does not affect sucrose intake (Jayatissa et al., 2006), and a transient reduction it causes in caloric intake from high-fructose corn syrup is not altered by co-administration of aripiprazole (Hudson et al., 2017).

Although the combination of cariprazine with escitalopram does not reverse the escitalopram-induced inhibition of the VTA DA neuronal activity, cariprazine is a partial DA agonist and also increases the firing activity of LC NE neurons. Similar to the addition of aripiprazole to escitalopram, the addition of extra pharmacological features to 5-HTT inhibition in vortioxetine counteracts the inhibition of LC NE and VTA DA neuronal activity induced by inhibition of 5-HTT.

Accordingly investigating the effects of cariprazine (alone and in combination with escitalopram) and vortioxetine in the same experimental protocols would also be insightful.

8.2 Experiments with the Flinders Sensitive Line Model of Depression

Vortioxetine administration inhibits the firing activity of LC NE and VTA DA neurons less than escitalopram. The combination of aripiprazole with escitalopram recovers the escitalopram-induced inhibition of the firing activity of LC NE and VTA DA neurons, and cariprazine recovers the escitalopram-induced inhibited firing activity of LC NE neurons (for more detailed information please refer to chapters 2-4). Further insights could be gained by conducting catecholamine depletion in FSL rats under vortioxetine, aripiprazole/cariprazine + escitalopram regimens, to investigate the possible dependence of the antidepressant-like effect of the above-mentioned regimens on catecholamine systems. Catecholamine depletion in these studies will be done using α-methyltyrosine which is one of the most potent and also the most extensively studied tyrosine hydroxylase inhibitors and has been shown to decrease the synthesis of NE and DA in Sprague-Dawley rats (Widerlöv and Lewander, 1978).

In conclusion, it is important to emphasize that MDD is an extremely heterogeneous condition. Different individuals exhibit different symptoms with various intensities and even with diametrically opposite directions (for example insomnia vs hypersomnia, weight loss vs weight gain, agitation vs psychomotor

retardation; Krishnan and Nestler, 2008). To make matters more complicated, these symptoms (often being related to the functions of different neurobiological systems) have also been hypothesized to be mutually reinforcing and form interactive networks that can be unique to individual patients (Robinaugh et al., 2020). There are a number of great animal models for depression (for more information, please refer to section 7.3. of the current chapter) that are extremely useful in specific aspects of translational and reverse translational studies. However, given the above-mentioned heterogeneous nature of MDD, it might not be entirely practical to expect a single animal model to encapsulate this vast degree of heterogeneity and provide definite conclusions when studying therapeutic strategies. Hopefully, initiatives like the Canadian Biomarker Integration Network for Depression will pave the way to construct individualized profiles of biomarkers for each patient that will help us tailor personalized strategies to optimize treatment response (Uher et al., 2020).

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