Alteration of Monoaminergic Neuronal Firing by Acute Administration of Cariprazine: an *In Vivo* Electrophysiological Study

Anna Herman

University of Ottawa, Institute of Mental Health Research

Department of Cellular and Molecular Medicine, Neuroscience Program

Faculty of Medicine

University of Ottawa

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Abstract

Cariprazine is a novel dopamine (DA) and serotonin (5-HT) partial agonist with an *in vitro* receptor affinity profile that endows it with the potential to be used successfully in the treatment of both unipolar and bipolar disorders. The objective of this study was to determine whether *in vitro* findings with cariprazine lead to functional alterations of monoamine systems in the intact rat brain. *In vivo* electrophysiological recordings were carried out in male Sprague-Dawley rats under chloral hydrate anesthesia. Dorsal raphé nucleus (DRN), locus coeruleus (LC), and hippocampus cornu ammonis region 3 (CA3) pyramidal neurons were recorded and cariprazine was administered systemically by intravenous injection or locally through iontophoresis. In the DRN, cariprazine induced a complete inhibition of the firing of 5-HT neurons, which was fully reversed by the selective 5-HT_{1A} antagonist WAY100.635. In the LC, the inhibitory effect of the preferential 5-HT_{2A} agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) was reversed by cariprazine with an ED_{50} value of 67 µg/kg, i.v., and it did not block the inhibitory effect of the α_2-adrenergic agonist clonidine. In the hippocampus, when cariprazine was administered by iontophoresis, it inhibited the firing of pyramidal neurons, but it did not dampen the suppressant effect of 5-HT. These results indicate that, *in vivo,* cariprazine acts as a 5-HT_{1A} agonist in the DRN, as an antagonist on 5-HT_{2A} receptors controlling the firing of NE neurons, and is a full agonist at 5-HT_{1A} receptors located on pyramidal neurons of the hippocampus. The modulatory actions of cariprazine on the 5-HT and NE systems may contribute to its reported effectiveness in depressive episodes.

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5-HIAA	5-hydroxyindoleacetic
5-HT	5-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
AP	anterior-posterior
CA3	cornu ammonis region 3
cAMP	cyclic adenosine phosphate
CNS	central nervous system
COMT	catechol-O-methyl transferase
DA	dopamine
DAT	dopamine transporter
DRN	dorsal raphé nucleus
DOI	2,5-dimethoxy-4-iodoamphetamine
DV	dorsal-ventral
GABA	γ-aminobutyric acid
GI	gastro-intestinal
HVA	homovanillic acid
i.p.	intraperitoneal
IP3	inositol trisphosphate
i.v.	intravenous

LC	locus coeruleus
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
ML	medial-lateral
NAcc	nucleus accumbens
NGF	nerve growth factor
NE	norepinephrine
NET	norepinephrine transporter
NRI	norepinephrine reuptake inhibitor
PNS	peripheral nervous system
S.E.M.	standard error of mean
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WHO	World Health Organization

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1. Introduction

1.1. Major depressive disorder

Major depressive disorder (MDD) is a psychiatric disorder characterized by extreme emotional suffering that can lead tragically to suicide. More specifically, when patients are diagnosed with clinical depression, they must show five or more of the following symptoms on a somewhat daily basis over the same 2-week period: depressed or irritable mood and/or decrease interest or pleasure, changes in appetite, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue, feelings of guilt and worthlessness, decreased ability to concentrate, and/or thoughts of death or suicide (American Psychiatric Association, 2013).

MDD is an increasingly pressing issue weighing on the collective conscious. Public understanding and sympathy for the disorder has been increasing, and the stigma around mental health disorders has lessened, but the prognosis of MDD in particular is only getting worse. Currently, it is designated the leading cause of disability when measured by years lived with disability (WHO, 2012). When quantifying the overall level of disease burden by years of life lost to suicide and disability, depression is currently ranked third worldwide. By 2030, the World Health Organization estimates that MDD will rise to be the number one cause of disease burden worldwide (WHO, 2008). Additionally, reports say that rates of depression in current youth, aged 12 to 20, are higher than in previous generations (Mojtabai et al, 2016). The reasons for this increase

are not within the scope of this thesis, but it indicates the importance of research in all aspects of the disorder, and mental health in general.

It remains true that approximately one third of patients that have undergone multiple treatments for MDD will not achieve remission (Rush et al, 2006). Patients are generally referred to as inadequate responders if they do not respond to the first line therapy, which is often an SSRI, but they may respond to another drug with a different mode of action. If a patient does not respond to two separate classes of antidepressant therapy, they are qualified as treatment-resistant (Souery et al, 1999). Lack of response typically means that the patient has no response at all to an attempted therapy, but many patients do not achieve a full remission in terms of the number of persistent symptoms. Even in cases where antidepressants are initially helpful, there is evidence that the quality of recovery can be limited, both in terms of patients that have reached remission, but report a number of residual symptoms, as well as those patients that respond, but do not achieve remission due to an even greater number of adverse residual symptoms (McClintock et al, 2011, Fava et al, 2007).

In cases of inadequate response and treatment resistance, it is often helpful to use a combination of drugs, in a process known as augmentation therapy. While many patients find it troubling to be taking multiple drugs, it is very difficult to develop a drug with enough multi-modal properties to fully treat all the symptoms of psychiatric disorders because of the complexity of the networks involved, Additionally, antidepressants, and SSRIs in particular often have side effects that are so adverse to the daily functioning of depressed patients that

another drug is necessary to counteract them. This can greatly alleviate the number of patients that discontinue the use of antidepressant drugs. When augmentation therapy has been put into clinical practice, for example, approximately one third of patients that were resistant to initial SSRI treatment were further treated to remission with bupropion or buspirone (Trivedi et al, 2006).

There are cases where a single drug is adequate for an individual, but the widespread neurodiversity of patients with mental health disorders suggests the need for continual development of novel drugs with different modes of action, and neuroscientists and clinicians are increasingly aware of the need for biomarkers and personalized medicine.

1.2. Monoamine hypothesis

The neurological basis of clinical depression is still not fully understood. The current working hypothesis, however, is that levels of monoamines are dysregulated in patients with clinical depression, and that increasing them can effectively improve mood. In fact, all currently approved treatments for depression have the net effect of enhancing one monoamine or another, either directly or indirectly (Berton and Nestler, 2006, El Mansari et al, 2010). The monoamines in question are serotonin (5-HT), norepinephrine (NE) and dopamine (DA).

Table 1. Effect of long-term administration of various antidepressant medications in rats (adapted from El Mansari et al, 2010).

	Cell body α_2 autoreceptor	Cell body D_2 autoreceptor	Cell body 5-HT_{1A} autoreceptor	Net NE transmission	Net DA transmission	Net 5-HT transmission
Pramipexole	↓	↓	↓	N.D.	↑	↑
NRI	Ø	N.D.	Ø	↑	↑*	↑
Nomifensine	Ø	↓	↓	↑*	↑*	↑*
Bupropion	↓	N.D.	↓	↑	↑*	↑
Mirtazapine	Ø #	N.D.	↓	↑	↑	↑
SSRI	N.D.	N.D.	↓	↓	↓	↑

N.D., not determined; ø, no change; ↑, increased; ↓, decreased; *, presumed from their acute effect; #, these experiments were carried out after a washout, but in the presence of mirtazapine this receptor is antagonized.

1.2.1. Dopamine system

DA is a neurotransmitter which is involved in three key networks within the brain, known as the mesolimbic pathway, the mesocortical pathway, and the nigrostriatal pathway. It is primarily synthesized in a pathway beginning with the essential amino acids phenylalanine and tyrosine, and is produced by midbrain dopaminergic cells in areas such as the ventral tegmental area (VTA), nucleus accumbens (NAcc), and the substantia nigra (SNc), which are important areas with respect to the three main DA pathways (Cooper et al, 2003). The mesolimbic pathway is involved in reward and pleasure and projects from the VTA to the NAcc. The mesocortical pathway projects from the VTA to the prefrontal cortex, and is involved in cognition. Finally, the nigrostriatal pathway projects from the SNc to the dorsal striatum and is heavily involved in motor control and movement, evidenced by the fact that a loss of DA neurons in the SNc leads to the extrapyramidal symptoms common to Parkinson's disease (Burns et al, 1983).

In a set of mechanisms common to all monoamines, DA is stored in

presynaptic vesicles and released into the synaptic cleft by the protein vesicular monoamine transporter (VMAT). It can then be moved back into the presynaptic terminal by the dopamine transporter (DAT), or alternatively it can be degraded and metabolized by the enzymes monoamine oxidase (MOA), catechol-O-methyl transferase (COMT), and aldehyde dehydrogenase (ALDH), into the inactive metabolite homovanillic acid (HVA) (Schatzberg and Nemeroff, 2009). Known DA receptors include the D_1-like receptors (D_1 and D_5) and the D_2-like receptors (D_2 to D_4). The D_2-like receptors are separated from the D_1-like receptor system because they generally inhibit the second messenger cyclic adenosine phosphate (cAMP) system, rather than activating it (Jaber et al, 1996).

1.2.2. Serotonin system

5-HT plays a substantial role as a signaling molecule throughout the central nervous system (CNS) as well as in the digestive system in the GI (gastro-intestinal) tract. It is synthesized by conversion of the essential amino acid tryptophan through a short enzymatic pathway. In the CNS, 5-HT is mainly synthesized in a group of cell bodies known as the raphé nuclei in the brainstem, and it is involved in networks throughout the brain that regulate mood, appetite, and sleep. It is stored in presynaptic vesicles and released into the synaptic cleft by the protein VMAT. It can then be moved back into the presynaptic terminal by the serotonin transporter (SERT), or alternatively it can be degraded and metabolized by the enzyme MAO, into the metabolite 5-hydroxyindoleacetic (5-

HIAA). Known receptors for 5-HT include: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{2B}, 5-HT_3, 5-HT_4, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6, and 5-HT_7.

Table 2. A review of serotonin receptors and their function

Receptor	***Coupled to***	***Signaling Pathway***	***Potential***	***Distributions***	***Functions***
5-HT_1	$G_{i/o}$ proteins	Decreases cAMP concentration	Inhibitory	Blood vessels, CNS	Anxiety, sexual behaviour, hypothermia
5-HT_2	$G_{q/11}$ proteins	Increases IP3 concentration	Excitatory	Blood vessels, CNS, PNS, GI tract, Platelets, Smooth muscle	Appetite, sexual behaviour
5-HT_3	Ion Channels	Depolarization	Excitatory	GI tract, CNS, PNS	Nausea and vomiting, anxiety, cognition
5-HT_4	G_s proteins	Increases cAMP concentration	Excitatory	GI tract, CNS, PNS	Appetite, anxiety, cognition
5-HT_5	$G_{i/o}$ proteins	Decreases cAMP concentration	Inhibitory	CNS	Anxiety
5-HT_6	G_s proteins	Increases cAMP concentration	Excitatory	CNS (limbic)	Learning, memory, mood
5-HT_7	G_s proteins	Increases cAMP concentration	Excitatory	CNS (limbic), blood vessels, GI tract	Learning, memory, mood

Adapted from Barnes and Sharp, 1999

1.2.3. Norepinephrine system

NE is a monoaminergic neurotransmitter/hormone that is involved mainly in arousal of the sympathetic nervous system. Interestingly, NE is mainly synthesized through a direct conversion of DA by the enzyme dopamine-β-monooxygenase. In the CNS, NE is mainly produced in areas such as the LC, which is a very small area of tightly packed nuclei in the pons region. Inside these nuclei, it is stored in presynaptic vesicles and released into the synaptic cleft by the protein VMAT. It can then be moved back into the presynaptic terminal by the norepinephrine transporter (NET), or alternatively it can be degraded and metabolized by the enzymes MAO and COMT, into various inactive metabolites (Szabadi et al, 2013). As it relates to depression and other psychiatric disorders, NE is often involved in processes that produce intense anxiety and stress, but it is also important for general arousal, alertness, and motivation. The NE receptors fall into two subtypes: the alpha receptors and the beta receptors. The alpha receptors are involved in NE control in the CNS and includes the α_1 receptors (α_{1A}, α_{1B}, and α_{1D}) and the α_2 receptors (α_{2A}, α_{2B}, and α_{2C}). The beta receptors are involved in the flight or fight response in the peripheral nervous system and includes the β_1, β_2, and β_3 receptors (Szabadi et al, 2013).

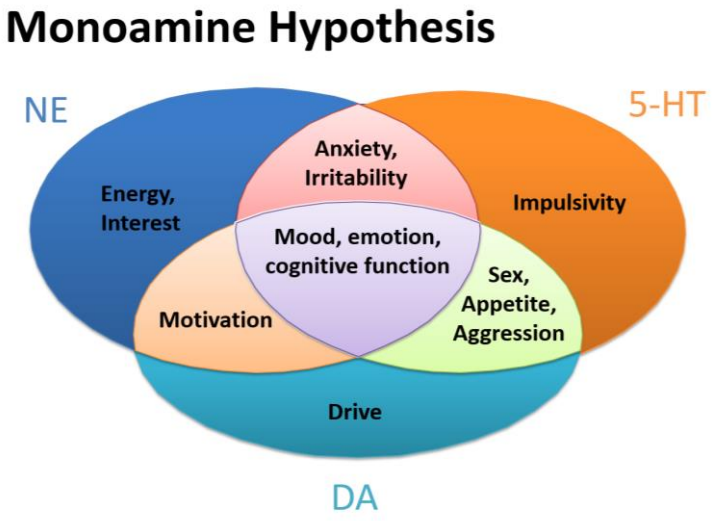

Figure 1. Involvement of 5-HT, NE, and DA in particular brain functions (adapted from Stahl, 2001).

1.2.4. Network Interactions

A key component of ones understanding of these three systems, and their involvement in mood, is that they are not independent players. These three systems work very closely together, and altering the activity of one system can have profound effects on the others. This is known as functional connectivity and is best explained in terms of experiments showing quantitative changes in the brain, both directly and indirectly, because of manipulation of one or more of these systems. For example, an *in vivo* electrophysiological study in rats using selective lesions of 5-HT, NE, or DA neurons, found that rats with DA-depletion had decreased spontaneous 5-HT neuron firing by 60%, and the reverse, 5-HT-depletion lead to a 36% enhancement in DA neuron firing. Additionally, DA-depletion increased NE firing in the locus coeruleus by 47%, and the opposite

manipulation lead to an increase of DA neuron firing by 70% (Guiard et al, 2008).

The inhibitory effect of 5-HT on DA neurons occurs through 5-HT_{2C} receptors, and the excitatory effect of DA on 5-HT occurs through D_2 receptors (Millan et al, 1998). The inhibitory effect of DA on NE neurons occurs through α_2-adrenergic receptors, and the inhibitory effect of NE on DA neurons occurs through α_2-adrenergic receptors as well, although the evidence is less clear regarding the latter, and there is additional evidence of an enhancing effect through α_1 -adrenoceptors (El Mansari et al, 2008; El Mansari et al, 2010). In terms of the interaction between 5-HT and NE, 5-HT has been shown to have inhibitory effects on the NE system, through 5-HT_{2A} receptors (Dremencov et al, 2007; Szabo et al, 2002). When 5-HT neurons are lesioned, NE firing increased 70% (Dremencov et al, 2007; Haddjeri et al, 1997). NE can also have an excitatory effect on 5-HT neurons through α_1-adrenergic receptors (Svensson et al., 1975; Baraban and Aghajanian, 1980).

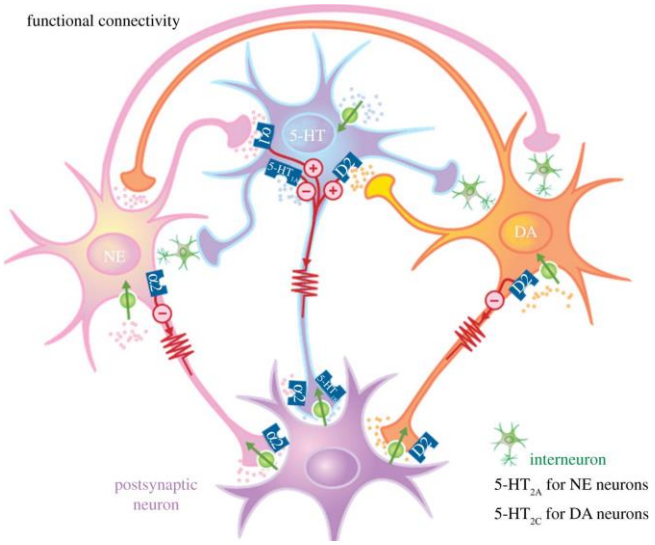

Figure 2. Graphical representation of reciprocal interactions between 5-HT system, NE system, and DA system (adapted from Blier et al, 2012).

1.3. Mechanisms involved in dopamine-serotonin receptor ligands

In order to thoroughly discuss the mechanisms of action involved in drugs that are potentially strong candidates for use in psychiatric disorders such as MDD, it is important to first discuss the various receptor subtypes involved, and the effects of either activation or blockade, whether these actions are beneficial in mood regulation or not, and how they can be beneficial.

1.3.1. D_2-like receptors

The D_2-like receptor system includes the D_2 receptor, the D_3 receptor, and the D_4 receptor. These receptors are separated from the D_1-like receptor system (D_1 and D_5) because they generally inhibit the second messenger cAMP system, rather than activating it. Pure antagonists at D_2 receptors, specifically, have been

developed and used for many years in the treatment of schizophrenia. Schizophrenia is characterized by an overactive DA system in the mesolimbic pathway, in association with the positive symptoms, and an underactive DA system in the mesocortical pathway, in association with the negative and cognitive symptoms (Meltzer and Stahl, 1976; Howes and Kapur, 2009). D_2 antagonists, therefore, have been very helpful in the treatment of the positive symptoms of schizophrenia. For example, amisulpride is a high affinity $D_{2/3}$ receptor antagonist that has been shown to be effective in the treatment of schizophrenia, particularly in patients with primarily negative symptomology (Danion et al, 1999). Importantly, however, D_2 receptor antagonists can have adverse effects in patients with schizophrenia if they exacerbate the negative and cognitive symptoms, and because they can cause extrapyramidal side effects by decreasing DA transmission in the nigrostriatal system. Amisulpride has the benefit of preferentially binding in limbic regions rather than cortical or striatal regions (Perrault et al, 1997). Additionally, when given at a lower dose, amisulpride has been shown to block D_2 autoreceptors controlling negative feedback of DA transmission, thereby facilitating the DA system and inducing improvements in patients that have predominantly negative symptomology, and also potentially having some benefit in depressive symptomology in patients with MDD and bipolar disorders (Perrault et al, 1997).

The role of DA receptor activation in the treatment of depression was first suggested in the early use of monoamine oxidase inhibitors (MAOIs), which prevent the breakdown of monoamines, and are effective antidepressants

(Quitkin et al, 1979). More specifically, studies of a potent $D_{2/3}$ agonist, bromocriptine showed it to be as effective as therapeutic regimens of tricyclic agents (Waehrens and Gerlach, 1981, Theohar et al, 1981). In recent studies, the selective $D_{3/2}$ agonist drug pramipexole that has been shown to be effective in mood on its own, and as an augmentation strategy alongside SSRIs (Cusin et al, 2012; Corrigan et al, 2000).

Thought to be an improvement on the pure D_2 antagonism of drugs used for psychosis and mania, it is suggested that D_2 partial agonists -- that is, drugs that bind to the receptor but do not produce as much activation -- such as aripiprazole, brexpiprazole, can effectively regulate the DA system by enhancing transmission in the presence of low endogenous levels and dampening transmission in the presence of high endogenous levels, thereby inducing balance throughout the brain (Stahl, 2001). These kinds of dialed-down agonists may be more beneficial and/or tolerable for some patients with schizophrenia and depression because they can have a general balancing effect on the dopamine system.

The effect of aripiprazole and brexpiprazole has been studied through *in vivo* electrophysiological studies looking at the acute and long term effects of these drugs on the firing rate of DA neurons in rats. Both drugs were shown to occupy D_2 receptors in that acutely, they reverse the effect of the D_2-like full agonist, apomorphine. When administered long term, there was no change in the intrinsic firing rate of DA neurons (Chernoloz et al, 2009, Dahan et al, 2009, Oosterhoff et al, 2014, Oosterhoff et al, 2015). Theoretically, it could be said that

these D_2 partial agonists have a stabilizing effect on the DA system, but the precise mechanism of action requires further characterization.

1.3.2. 5-HT$_{1A}$ receptors

5-HT$_{1A}$ receptors are very important in regulating the 5-HT system. They play a key role in that they can be autoreceptors on serotonergic neurons, acting as negative feedback on the cell body in order to dampen the release of 5-HT whenever it is too high (Aghajanian, 1972, Haigler and Aghajanian, 1974, Blier and Montigny, 1987). Interestingly, SSRIs can take weeks to exert their effects in depressed patients because 5-HT cannot freely increase until such time as the 5-HT$_{1A}$ autoreceptors themselves are desensitized (Blier and Montigny, 1983, El Mansari et al, 2008). There are also 5-HT$_{1A}$ receptors on postsynaptic terminals in the limbic system in areas including the prefrontal cortex, the hippocampus, and the amygdala that when activated can have a variety of long-term effects downstream (Barnes and Sharp, 1999).

Blockade of these 5-HT$_{1A}$ receptors can have drastic effects throughout the brain. Pindolol is an example of a 5-HT$_{1A}$ antagonist, and there is placebo-controlled evidence indicating that this mechanism accelerates the onset of action of SSRIs on antidepressant response (Portella et al, 2011). Presumably, this could result from a blockade of the negative feedback action of 5-HT$_{1A}$ autoreceptors, facilitating 5-HT transmission (Blier and Bergeron, 1995). Alternatively, the usefulness of activating 5-HT$_{1A}$ receptors in depressed patients has been demonstrated by findings that buspirone and gepirone, known 5-HT$_{1A}$

agonists, are effective either on their own or in combination with SSRIs (Blier and Ward, 2003; Robinson et al, 2003, Bielsky et al, 2008). Evidence suggests that this is the result of repeated activation leading to the desensitization of 5-HT_{1A} autoreceptors, and therefore a theoretically similar mechanism of action to pindolol and other antagonists (Blier and Montigny, 1987, El Mansari et al, 2008). However, pindolol and buspirone would have drastically different effects on postsynaptic 5-HT_{1A} receptors. The activation of postsynaptic 5-HT_{1A} receptors by selective agonists such as 8-OH-DPAT, but also by aripiprazole increases DA release in the prefrontal cortex and the hippocampus (Arborelius et al, 1993, Assié et al, 2005, Li et al, 2004), whereas blockade of these receptors would not have this effect. There is evidence that the long-term postsynaptic activation of 5-HT_{1A} receptors in the forebrain may play a part in the beneficial action of 5-HT_{1A} agonists, as well, given that these 5-HT_{1A} receptors do not desensitize (Haddjeri et al, 1998).

Recently, drugs have been developed that are partial agonists at the 5-HT_{1A} receptor, rather than full agonists. These are the same drugs that are designed to be partial agonists at the $D_{2/3}$ receptors – aripiprazole, and most recently, cariprazine (Shapiro et al, 2003; Kiss et al, 2010). So far it is not clear whether there is an important difference between partial and full 5-HT_{1A} agonists regarding treatment outcomes in patients with depression.

1.3.3. 5-HT_{2A} receptors

5-HT_{2A} receptors exert mainly an excitatory effect on neuronal function,

and are found throughout the brain, but highly concentrated in areas associated with cognition, memory, and attention (Barnes and Sharp, 1999). When 5-HT_{2A} receptors are activated, a hallucinogenic effect is produced. The psychedelic drug, DOI, for example, is a preferential 5-HT_{2A} agonist, and has been shown to induce decreased locomotor and investigatory activity in rats – a behavioural model considered characteristic of hallucinogens -- and these effects were negated by the selective 5-HT_{2A} antagonist, MDL100907 (Krebs-Thomson et al, 1998). This mechanism of action is also involved in the psychotropic action of LSD and psilocybin mushrooms. Interestingly, there is some evidence that psychoactive drugs can improve depressive symptoms (Carhart-Harris et al, 2016, Carhart-Harris and Goodwin, 2017) but this may have less to do with the activation of 5-HT_{2A} receptors, and more to do with 5-HT_{1A} agonism, which also occurs at the typical dose range of LSD (Reissig et al, 2005).

Conversely, there is some evidence that depressed patients have an over-activity of these receptors, in that they have been found in high concentrations in people that have died by suicide (Turecki et al, 1999; Pandey et al, 2002). Several lines of evidence suggest that the blockade of 5-HT_{2A} receptors may contribute to the therapeutic benefits of SSRIs in MDD (Szabo and Blier, 2005). For instance, agents that block 5-HT_{2A} receptors, such as aripiprazole, mirtazapine, quetiapine, risperidone, and olanzapine are effective augmentation strategies in combination with SSRIs (Wen et al 2014, Kennedy et al, 2016). Although mirtazapine is thought to exert its antidepressant effect through the antagonism of α_2-adrenoceptors, it is also a 5-HT_{2A} antagonist. The only property

that the aforementioned drugs have in common is their capacity to block 5-HT$_{2A}$ receptors. It is likely that this is partially related to the inhibitory effect of SSRIs on the NE system, which has been shown acutely and chronically in rats (Szabo and Blier, 2002). In fact, the role of the 5-HT$_{2A}$ receptor in this process has been shown through experiments that block the SSRI-induced inhibition of the NE system with a highly selective 5-HT$_{2A}$ antagonist, MDL100907 (Dremencov et al, 2007).

When synthesizing this conflicting data, it is possible that 5-HT$_{2A}$ agonism is more-so beneficial in a portion of the treatment-resistant depressed population, and that perhaps there is a phenotypic variation, as well, in terms of whether there is an over activity of 5-HT$_{2A}$ receptors, or not.

1.3.4. 5-HT$_{2B}$ receptors

While it has only been in the last decade that 5-HT$_{2B}$ receptors were discovered to exist in the brain (Auclair et al, 2010, Diaz et al, 2012, Duxon et al, 1997), there is now compelling evidence to suggest that 5-HT$_{2B}$ receptors are involved in mood disorders in humans. For example, when 5-HT$_{2B}$ receptors are knocked out in a mouse model, the effectiveness of SSRIs is significantly reduced in terms of behavioral enhancement, as well as the SSRI induced increase in hippocampal 5-HT concentration (Diaz et al, 2012). Interestingly, the same study found that it was a 5-HT$_{2B}$ receptor agonist that directly mimicked the effects of SSRIs in mice. This may be because the 5-HT$_{2B}$ receptor is an important 5-HT regulator via the serotonin transporter within 5-HT neurons, thus

acting as an autoreceptor (Launay et al, 2006).

A good example of an antagonist at these receptors, is the previously mentioned compound, agomelatine. Aside from activity at melatonin receptors, agomelatine acts as an antagonist of 5-HT_{2B} and 5-HT_{2C} receptors (Millan et al, 2003). It is not known whether the activity of agomelatine at the 5-HT_{2B} receptor is responsible for its effects on mood, but there has been some evidence to suggest that antagonism of these receptors could play a role in enhancing DA signaling. For example, in an *in vivo* electrophysiological study in rats that replicated the properties of agomelatine through a combination of melatonin, a selective 5-HT_{2C} antagonist (SB242084), and a selective 5-HT_{2B} antagonist (LY266097), it was shown that the 5-HT_{2B} antagonist, LY266097 was necessary to achieve a combined effect of increasing the number of bursts per minute and the percentage of spikes in bursts for DA neurons, which equates to an overall increased release of DA. Additionally, the study showed a small but significant enhancement in DA firing rate with LY266097 alone, after 14 days of administration, compared to controls (Chenu et al, 2013).

Overall, further study is required to elucidate the involvement of 5-HT_{2B} receptors in improving depressive symptoms.

1.3.5. 5-HT_{2C} receptors

5-HT_{2C} receptor agonists do not induce hallucinations like 5-HT_{2A} receptor agonists, but they are widely known to reduce appetite, making them potentially important drugs in the treatment of obesity (Bickerdike, 2003). SSRIs can

indirectly act as 5-HT$_{2C}$ receptor agonists because they increase the amount of 5-HT available to bind to and activate such receptors. There is some evidence that this can have a number of detrimental effects to the patient, including inhibition of sleep and sexual behaviour, and increased anxiety (Millan, 2005). In some cases, patients may prefer a drug that has little to effect on the 5-HT$_{2C}$ receptors in general, because of the adverse effects of both agonism and antagonism. Because 5-HT$_{2C}$ receptors are functionally connected to the DA and NE systems through 5-HT signaling, activating these receptors through increased 5-HT levels can also lead to inhibition of DA and NE release in the frontal cortex of rats (Millan et al, 1998). The effects of decreased DA and NE release could hinder the recovery of the patient because of their involvement in mood and motivation – reward and arousal, respectively – separate from that of 5-HT. Conversely, there is some evidence that 5-HT$_{2C}$ receptor agonists may have antidepressant effects. For instance, the 5-HT$_{2C}$ selective agonist, WAY-163909 was shown to reduce immobility in the forced swim test in rats, decreased escape latency in the learned helplessness model, and also reduced hyperactivity in olfactory-bulbectomized rats even more rapidly than SSRIs (Dunlop et al, 2006).

Like 5-HT$_{2A}$ receptors, there is some evidence of greater expression of 5-HT$_{2C}$ receptors in the prefrontal cortices of people that have died by way of suicide (Shelton et al, 2009, Niswender et al, 2001) indicating that over-activity may in fact lead to a depressive phenotype, and that blocking 5-HT$_{2C}$ receptors may be therefore preferable. Known antagonists of the 5-HT$_{2C}$ receptor include

the compounds agomelatine, as well as aripiprazole, which have both been found to be useful in the treatment of depressive symptomology (Millan et al, 2003, Kennedy and Emsley, 2006; Shapiro et al, 2003, Berman et al, 2007).

1.3.6. α_2-adrenergic receptors

α_2-adrenergic receptors are one of the main receptors involved in NE signaling in the CNS. Activation of α_2-adrenergic receptors generally leads to sedation in mammals (England et al, 1992; Scheinin et al, 1987). The α_2-adrenergic receptors are divided into the subtypes α_{2A}, α_{2B}, and α_{2C}. These receptors are highly homologous but there are also a few important differences. For example, the α_{2A} receptors seem to be most abundantly found in the LC, whereas α_{2C}-adrenergic receptors are more located in the basal ganglia, hippocampus, and cerebral cortex, and α_{2B} receptors are found mainly in the thalamus (Scheinin et al, 1994, Nicholas et al, 1993). α_2-adrenergic receptors in general act as presynaptic autoreceptors controlling negative feedback of NE release, either located on the cell body, or at the axon terminal. As evidenced by findings that knock-out of α_{2A}-adrenergic receptors severely impaired, but did not completely abolish the negative feedback mechanism (Hein and Kobilka, 1995), α_{2A}-adrenergic receptors seem largely responsible for this mechanism, with $\alpha_{2C/2B}$ receptors being only partially responsible. Interestingly, in a study of rat cells transfected with human α_2-adrenergic receptor subtype genes, and differentiation induced by nerve growth factor (NGF), it was found that α_{2A}-adrenergic receptors mainly localized at distal axon terminals, whereas α_{2C}-adrenergic receptors were

distributed more intracellularly (Olli-Lähdesmäki et al, 1999). There is also some evidence that α_{2A}-adrenergic receptors mainly inhibit release at high frequencies, and α_{2C}-adrenergic receptors mainly inhibit release at low frequencies (Starke, 2001). The importance of α_{2A}-adrenoceptors in the negative feedback of NE release may indicate its overall importance in mood disorders. Interestingly, it has been reported that post-mortem analysis of suicide victim's brains showed a selective increase in α_{2A}-adrenoceptors (Callodo et al, 1998). Additionally, knock-out of this receptor subtype lead to decreased activity in the forced swim test and insensitivity to the antidepressant effects of imipramine (Schramm et al, 2001).

Presynaptic α_2-adrenergic receptors are also involved in the negative feedback of 5-HT transmission, in contrast to the activational effect of α_1-adrenergic receptors, and NE signaling can lead to the inhibition of DA neurons through α_2-adrenoceptors. These receptors are also located extrasynaptically on hippocampal CA3 neurons, where NE has an inhibitory effect, which is reduced by α_2-adrenoceptor blockade (Curet and de Montigny, 1988a).

α_2-adrenergic agonists in general could potentially be beneficial in the treatment of mood disorders in that they could desensitize α_2-adrenergic autoreceptors, and therefore increase the transmission of NE, and this could be particularly useful in patients already taking NRIs, for example. However, interestingly, the antidepressant effect produced in rats by the NRI desipramine, was greatly reduced by pretreatment with the α_2-adrenergic antagonist idazoxan (Zhang et al, 2008), indicating the importance of postsynaptic α_2-adrenergic receptor activation in the regulation of mood.

Alternatively, there is evidence for the efficacy of direct α_2-adrenergic antagonism, rather than indirect desensitization. Additionally, the increase in NE would bind to α_1-adrenergic receptors and increase 5-HT transmission further. Interestingly, it has been shown that the blockade of α_2-adrenergic receptors significantly increases NE and DA levels in the prefrontal cortex in rats (Gobert et al, 1997). Recent examples of α_2-adrenergic antagonists include the compounds risperidone and brexpiprazole, which have both been approved for use in schizophrenia and bipolar disorder, but their effects at α_2-adrenoceptors may help improve mood regulation within these disorders, and potentially also in MDD (Keitner et al, 2009, Dhir and Kulkarni et al, 2008; Oosterhoff et al, 2016; Thase et al, 2015).

1.3.7. 5-HT$_7$

Researchers have more recently become aware of the importance of the lesser known 5-HT$_7$ receptors in the treatment of depression. In fact, agents that block 5-HT$_7$ receptors have been shown to induce a rapid antidepressant response in rats (Mnie-Filali et al, 2011, Guscott et al, 2005). Similarly, 5-HT$_7$ knockout mice displayed reduced immobility in tests of depressive behaviour such as the forced swim test and the tail suspension test (Hedlund et al, 2005). It is thought that 5-HT$_7$ receptors agonists have a similar mechanism of action to 5-HT$_{1A}$ agonists in that 5-HT$_7$ receptors act as negative feedback on 5-HT transmission. The main difference is that rather than being autoreceptors like presynaptic 5-HT$_{1A}$ receptors, 5-HT$_7$ receptors innervate gamma-amino butyric

acid (GABA) neurons in the raphé nuclei, releasing GABA, which inhibits 5-HT release (Fink and Gothert, 2007).

Known 5-HT_7 antagonists include those agents formerly known as atypical antipsychotics such as clozapine, olanzapine, and lurasidone, as well as the novel multimodal agent, vortioxetine (Roth et al, 1994; Ishibashi et al, 2010; Stahl, 2015). Additionally, aripiprazole is an example of a weak partial agonist at these receptors, but little research has been done to elucidate the potential role partial agonism may play in its mood regulating effects (Shapiro et al, 2003).

1.4. Cariprazine

Cariprazine (Vraylar®) is a novel DA and 5-HT partial agonist that has been approved by the FDA for use in schizophrenia and bipolar mania (Citrome, 2013). However, its *in vitro* affinity profile and early clinical trials indicate that it could also have benefits in the treatment of schizophrenia with mainly negative symptoms (Nemeth et al, 2017), as well as in unipolar and bipolar depression.

1.4.1. Affinity in vitro

Like other agents formerly classified as atypical antipsychotics; cariprazine has affinity at both 5-HT_{2A} and D_2 receptors. Like more recently developed drugs in this category, such as aripiprazole and brexpiprazole, it acts as a partial D_2 agonist *in vitro*, and an antagonist at 5-HT_{2A} receptors, but with a higher affinity for DA receptors. What separates cariprazine from its two partial DA agonist predecessors is that it displays a much higher affinity at D_3 receptors. In addition

to its affinity for 5-HT_{2A} and $D_{2/3}$ receptors, cariprazine was shown *in vitro* to be a partial agonist at 5-HT_{1A} receptors in the hippocampus, a high affinity antagonist at 5-HT_{2B} receptors, and to have relatively low affinity for α_2-adrenergic receptors, 5-HT_{2C} and 5-HT_7 receptors (Kiss et al, 2010).

1.4.2. Rodent behavior

There has been convincing evidence through behavioural alterations in rodents that cariprazine has antidepressant utility. In studies involving the chronic mild stress model of anhedonia, it has been shown that cariprazine had antidepressant-like effects in that the consumption of sucrose solution were increased significantly, with cariprazine being more potent in relation to aripiprazole (Papp et al, 2013, Duman et al, 2012). Interestingly, when the study is replicated in D_3 receptor knockout mice, the antidepressant effect of cariprazine is negated (Duman et al, 2013, Adham et al, 2014). D_3 receptors are preferentially located in regions involved in mood such as the nucleus accumbens, and corticolimbic regions, involved in cognition (Joyce and Millan 2005; Sokoloff et al., 2006). In fact, a study has shown that D_3 knockout mice show depressive-like symptomology on their own (Moraga-Amaro et al, 2014).

In addition to the antidepressant-like effects of cariprazine in rats, studies have shown that cariprazine can induce procognitive and anxiolytic effects (cognitive impairments and increased anxiety are common symptoms in MDD) and that at least the cognitive effects are also dependent on D_3 receptors (Adham et al, 2014).

1.4.3. Clinical Evidence

Cariprazine has shown promising results in recent clinical trials that were conducted from 2011 to 2014 at multiple institutions but headed by Forest Industries Inc. The first was a study conducted on patients with Bipolar I disorder, and found that a dose of 1.5-3.0 mg/day of cariprazine was significantly better than placebo at improving symptoms in depressive episodes, specifically Durgam et al, 2015). The second study was conducted on patients that had inadequate response to antidepressants, and found that a dose of 2.0-4.5 mg/day of cariprazine was significantly better than placebo as an augmentation therapy alongside current antidepressant treatment (Durgam et al, 2016). In terms of negative symptomology in patients with schizophrenia, it was shown recently that cariprazine (3.0-6.0 mg/day) induced a greater improvement in quality of life than the D_2 receptor antagonist risperidone (3.0-6.0 mg/day) in patients with significant negative symptoms (Nemeth et al, 2017).

1.5. The present study

While *in vitro* studies are necessary to identify potentially therapeutic compounds, to have a full mechanistic understanding of a drug, it is important to confirm that the activity also occurs *in vivo.* To this end, the objective of the present study was to determine whether *in vitro* findings with cariprazine lead to functional alterations of monoamine systems in the intact rat brain. More specifically, we wanted to determine the *in vivo* effect of acute cariprazine

application at 5-HT$_{1A}$ receptors in the DRN, the 5-HT$_{2A}$ receptors controlling LC firing, as well as α_2-adrenergic receptors in the LC, to investigate the effect of cariprazine on 5-HT$_{2B}$ receptors in the VTA, and to determine the intrinsic activity of cariprazine on post-synaptic 5-HT$_{1A}$ receptors in the hippocampus.

1.5.1. Hypothesis

a. Cariprazine, a moderate affinity 5-HT$_{2A}$ antagonist *in vitro*, will partially reverse DOI-induced inhibition of NE neurons in the LC.
b. Cariprazine, a high affinity 5-HT$_{1A}$ partial agonist *in vitro*, will inhibit the firing rate of 5-HT neurons in the DRN.
c. Cariprazine, a high affinity 5-HT$_{1A}$ partial agonist *in vitro*, will attenuate the 5-HT induced inhibition of pyramidal neurons in the hippocampus, when applied concomitantly with 5-HT.
d. BW 723C86, a selective 5-HT$_{2B}$ agonist, will inhibit the firing of VTA DA neurons, and cariprazine, a high affinity 5-HT$_{2B}$ antagonist *in vitro*, will reverse this effect

2. Materials and Methods

2.1. Animals

Experiments were carried out in male Sprague-Dawley rats (Charles River Laboratories, St. Constant, QC, Canada) weighing 250-400g housed in groups of two per cage, under standard laboratory conditions (12-hour light/dark cycle with

food and water ad libitum). *In vivo* extracellular recordings were carried out in chloral hydrate–anesthetized rats (400 mg/kg i.p.) that were mounted in a stereotaxic apparatus. Supplemental doses of the anesthetic (100 mg/kg, i.p.) were given to maintain constant anesthesia and prevent nociceptive reaction to a pinching of the hind paws. Body temperature was maintained at 37°C throughout the experiment via a thermistor-controlled heating pad. If applicable, prior to the electrophysiological recordings, a catheter was inserted in a lateral tail vein for systemic intravenous injection of pharmacologic agents. All experiments were carried out in accordance with the Canadian Council on Animal Care and the local Animal Care Committee (University of Ottawa, Institute of Mental Health Research, Ottawa, ON, Canada).

2.2. Compounds

The preferential 5-HT_{2A} receptor agonist DOI (100 µg/kg), the α_2-adrenergic agonist clonidine (10 µg/kg), the α_2-adrenergic antagonist idazoxan (1 mg/kg) and the 5-HT_{1A} receptor antagonist WAY100.635 (100 µg/kg), were dissolved in distilled water. Cariprazine (50 µg/kg) and the 5-HT_{2B} receptor agonist BW 723C86 (200 µg/kg) were dissolved in lactic acid and distilled water for i.v. injection. Cariprazine was provided by Forest Laboratories Inc. (New York City, New York, USA); all other compounds were purchased from Tocris Bioscience (Bristol, United Kingdom).

2.3. *In Vivo* Electrophysiological Recordings

A burr hole was drilled at the stereotaxic coordinates corresponding to the brain structure of interest (Paxinos, 2007). Extracellular recordings of neurons in the DRN and LC were carried out with a single-barrel glass micropipette (Stoelting, Spencerville, MD) preloaded with 2 M NaCl and with impedance between 2 and 6 MΩ. Neurons in the cornu ammonis layer 3 (CA3) region of the hippocampus were recorded with a five-barrel micropipette. The central barrel, used for unitary recordings, and one side barrel, used for automatic current balancing, were filled with 2 M NaCl; the other barrels were filled with cariprazine (10 mM in distilled water and acetic acid, pH 4), 5-HT creatinine sulfate (15 mM in 0.2 M NaCl, pH 4), or quisqualic acid (1.5 mM in 0.2 M NaCl, pH 4). 5-HT and cariprazine were ejected as cations and retained with a negative current; quisqualate was ejected as anions and retained with a positive current.

2.4. Recording of DRN 5-HT Neurons

Putative 5-HT neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in millimeters from lambda): anterior/posterior (AP), 1.0–1.2; mediolateral (ML), 0; and dorsal/ventral (DV), 5.0–7.0. 5-HT neurons were identified using the following criteria: a bi- or triphasic extracellular waveform with a long-duration (0.8–1.2 milliseconds) positive phase and regular firing in the range of 0.8–2Hz were recorded (Vandermaelen and Aghajanian, 1983). To test the effect of cariprazine on 5-HT_{1A} receptors, cariprazine was injected intravenously following a 50 second

period to establish baseline firing activity. If cariprazine produced an inhibitory dose-response relationship, the selective 5-HT_{1A} antagonist, WAY100.635 would then be administered to determine if the inhibition was related to binding at 5-HT_{1A} receptors. The inhibitory, and reversal effects described here were quantified in percentage of firing frequency (Hz) relative to the established baseline.

2.5. Recording of LC NE Neurons

NE neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in millimeters from lambda): AP, 21.0 to 21.2; ML, 1.0–1.3; and DV, 5.0–7.0. NE neurons were identified using the following criteria: regular firing rate (1–3 Hz), a long duration (0.8–1.2 milliseconds) of the rising phase of the action potential, and a brisk excitatory response followed by a short period of inhibition (~1 second) in reaction to a nociceptive pinch of the contralateral hind paw (Vandermaelen and Aghajanian, 1983). To test the effect of cariprazine on 5-HT_{2A} receptors, NE neurons were inhibited by the preferential 5-HT_{2A} receptor agonist DOI (Szabo and Blier, 2001). Following a 120-second inhibition period, cumulative doses of cariprazine were administered to reverse the inhibitory effect of DOI. The reversing effect of cariprazine was quantified relative to baseline firing activity.

2.6. Recording of VTA DA Neurons

Putative DA neurons were recorded by positioning single-barrel glass

micropipettes at the following coordinates (in millimeters from l): AP, 3.2–3.6; ML, 0.6–1.0; and DV, 7.0–9.0. At these coordinates, neurons with a long duration (3–5 milliseconds) triphasic action potential with a marked negative deflection, an inflection or "notch" on the rising phase, an irregular spontaneous single-firing pattern (3–6 Hz), and slow bursting activity with decrementing action potential amplitude were recorded (Grace and Bunney, 1983). To test the effect of cariprazine on 5-HT_{2B} receptors, the selective 5-HT_{2B} agonist, BW 723C86 was administered intravenously, with the hypothesis that it would inhibit the firing of DA neurons in the VTA (Chenu et al, 2013). If the compound produced an inhibitory response, cariprazine would then be administered to determine if cariprazine would significantly block 5-HT_{2B} receptors, and what dose would be required to reverse the inhibition. The inhibitory and reversing effects of said compounds were quantified relative to baseline firing activity.

2.7. Recording of Pyramidal Neurons in the CA3 Region of the Hippocampus

CA3 pyramidal neurons were recorded by positioning multibarrel micropipettes at the following coordinates (in millimeters from lambda): AP, 3.8–4.2; ML, 4.0–4.2; and DV, 3.5–4.5. Because most CA3 pyramidal neurons are not spontaneously active in chloral hydrate–anesthetized rats, a small ejection current (+2 to -2 nA) was applied to the quisqualate barrel to activate them within their physiologic firing range (10–15 Hz) (Ranck, 1973). Neural responsiveness to 5-HT was assessed by determining the total number of spikes suppressed during a 50-second ejection divided by the ejection current of 5-HT. Partial or full

agonism of cariprazine on 5-HT$_{1A}$ receptors was assessed by comparing the inhibitory effect of ejection of 5-HT alone to the inhibitory effect of concomitant ejection of 5-HT and cariprazine, following restoration of the firing rate to the same level as before ejecting cariprazine by increasing quisqualate ejection. In this paradigm, coapplication of a partial agonist reduces the inhibitory effect of 5-HT, whereas coapplication of a full agonist does not change the inhibitory effect of 5-HT (Blier and de Montigny, 1990; Ghanbari et al., 2010). To ascertain whether the inhibitory effect of 5-HT and cariprazine was mediated by 5-HT$_{1A}$ receptors, the inhibitory effect of iontophoretic 5-HT and cariprazine application was compared before and after administration of the selective 5-HT$_{1A}$ receptor antagonist WAY100.635.

2.8. Data Analysis/Statistics

Electrophysiological recordings were made, and filtered from artifacts by wavemark analysis, using Spike2 software version 6.17 (Cambridge Electronic Design, Cambridge, UK). Quantification of firing activity was performed using Spike2. Experiments with fewer than three experimental groups were analyzed with a paired t test. All data were analyzed with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA). Data are presented as mean ± S.E.M.; $p<0.05$ was considered significant.

3. Results

3.1. Effect of cariprazine on the firing activity of DRN 5-HT neurons: role of 5-HT$_{1A}$ autoreceptors

In the DRN, successive intravenous injections of 50 µg/kg of cariprazine decreased the firing rate of putative 5-HT neurons. This effect was subsequently reversed by the selective 5-HT$_{1A}$ antagonist WAY100.635, indicating that cariprazine was acting as an agonist at 5-HT$_{1A}$ autoreceptors *in vivo* (Figure 3).

Interestingly, the response was not found to be dose-dependent, as the dose required to completely inhibit the firing rate ranged from a minimum of 150 ug/kg to a maximum of 850 ug/kg (Figure 4A). However, the majority of neurons tested (n=12/15) were completely inhibited by cumulative doses of 150-350 ug/kg.

There was no correlation between the initial baseline firing rate of individual 5-HT neurons and the dose required to completely inhibit the firing (Figure 4B).

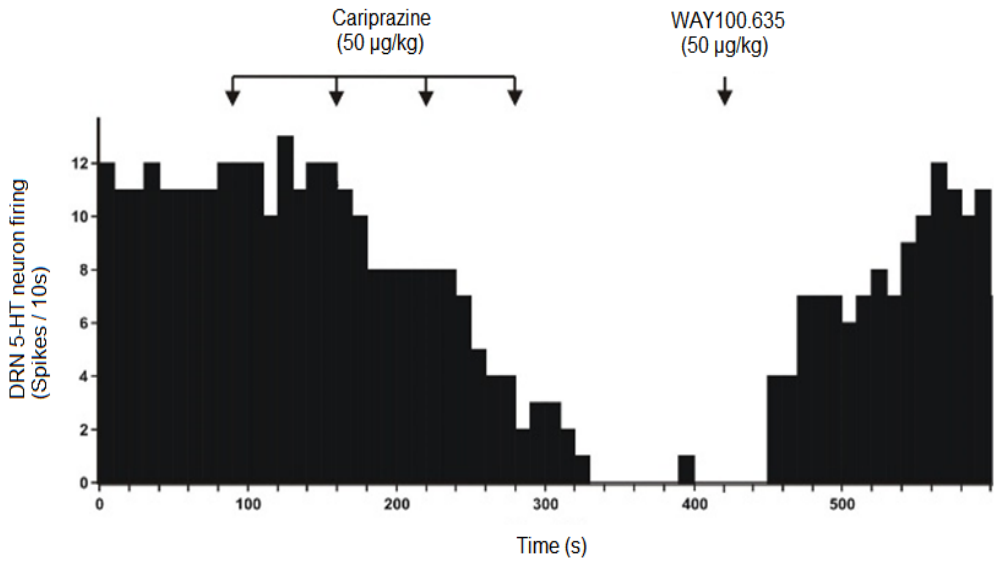

Figure 3. Integrated firing rate histogram of a single 5-HT neuron showing its response to four cumulative intravenous doses of cariprazine and the subsequent reversal with intravenous dose of the selective 5-HT$_{1A}$ antagonist WAY100.635. In all cases only one neuron was recorded per rat.

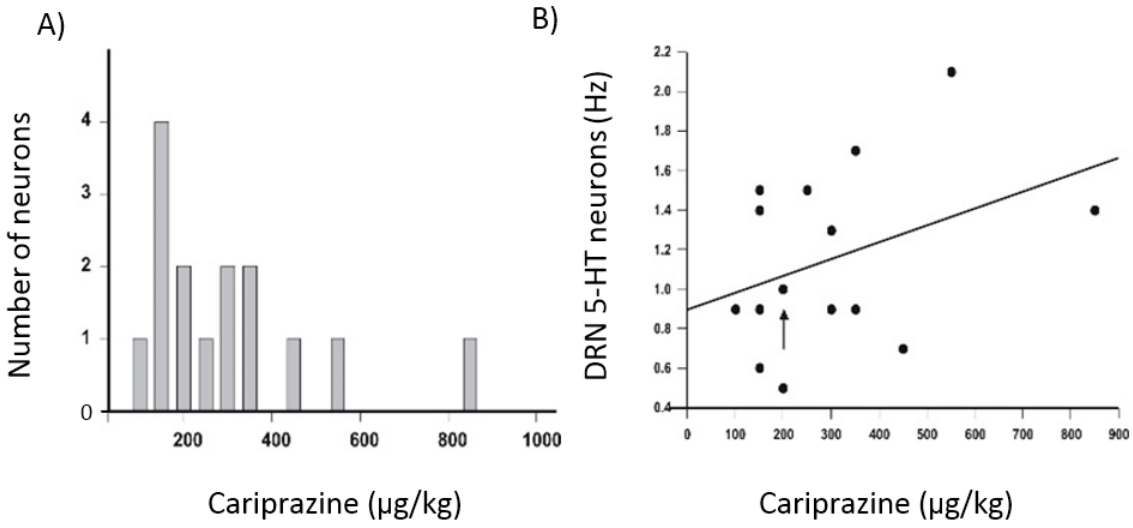

Figure 4. A) Relation between the number of 5-HT neurons showing a 100% inhibition of firing and the intravenous dose necessary to achieve the complete suppression of firing. There was only one neuron recorded in each rat. B) Lack of significant correlation between the basal firing rate of dorsal raphé (DRN) 5-HT neurons and the cumulative dose of cariprazine necessary to achieve a complete inhibition. This figure show lack of correlation between baseline firing level of 5-HT neurons and the dose of cariprazine used to obtain 100% inhibition of firing. Arrow indicates the neuron in figure 3.

3.2. Effect of cariprazine on postsynaptic serotonin 5-HT$_{1A}$ receptors located at pyramidal neurons in the hippocampus.

In the CA3 region of the hippocampus, microiontophoretic application of cariprazine significantly inhibited the firing activity of pyramidal neurons, as did the natural ligand, 5-HT (Figures 5&6). After an intravenous injection of the

selective 5-HT$_{1A}$ antagonist WAY100.635, the amount of inhibition induced by both cariprazine and 5-HT were significantly reduced, indicating that both compounds were acting as 5-HT$_{1A}$ agonists at the postsynaptic receptors of the hippocampal neurons ($p<0.05$, Figure 5).

There was no statistically significant difference between the amount of inhibition induced by 5-HT alone and the amount of inhibition induced by co-application of cariprazine and 5-HT, indicating that cariprazine acted as a full agonist *in vivo* ($p>0.05$, Figure 6 & 7).

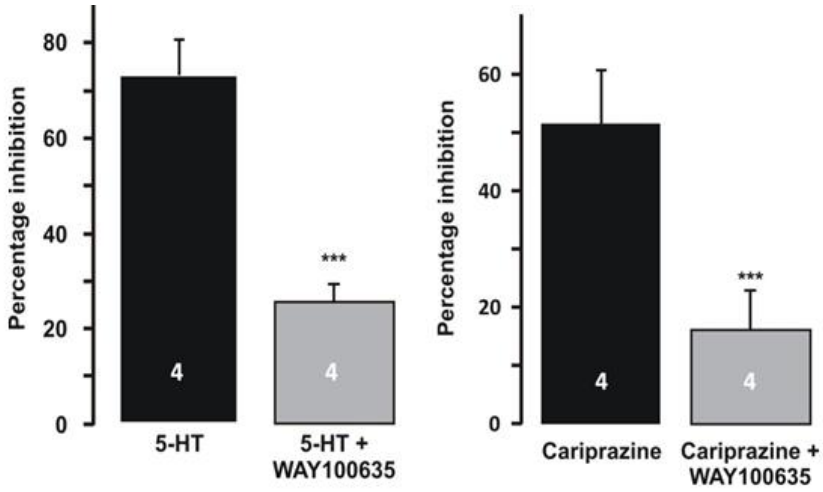

Figure 5. A) Average percent inhibition of pyramidal neuron firing rate in the hippocampus by 5-HT, and the subsequent reduced inhibition induced by 5-HT after an injection of 100 µg/kg of the selective 5-HT$_{1A}$ antagonist WAY100.635. B) Average percent inhibition of pyramidal neuronal firing rate by cariprazine, in comparison to the reduced inhibition by cariprazine after an injection of 100 µg/kg of WAY100.635. Sample sizes refer to the number of neurons. In some cases more than one neuron was recorded per rat.

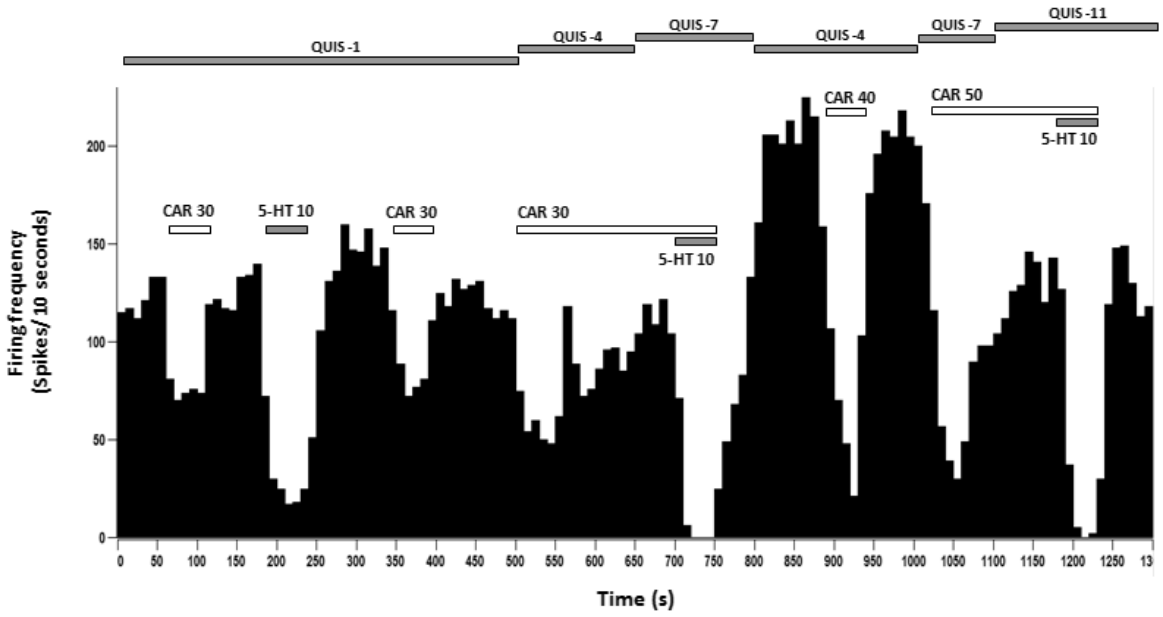

Figure 6. Integrated firing rate histogram of a single pyramidal neuron in the hippocampal CA3 region, showing inhibition by cariprazine at various currents (CAR: 30 nA, 40 nA, 50 nA), inhibition by 10 nA of 5-HT alone, and subsequent full inhibition by concurrent cariprazine and 5-HT application. Quisqualate was applied with variable ejection currents in order to adjust the firing rate to the same level as before cariprazine application for appropriate comparison.

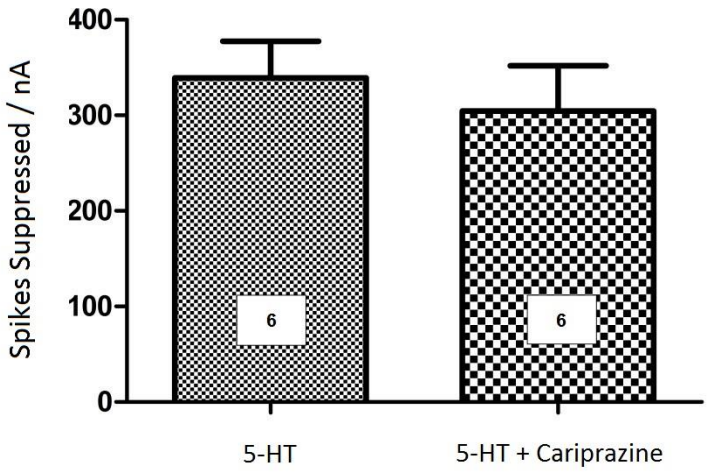

Figure 7. Total number of neuronal spikes suppressed per nA for 5-HT application alone, and concurrent 5-HT and cariprazine application, showing lack of significant difference between 5-HT application alone and concurrent 5-HT and cariprazine application. Sample sizes refer to the number of neurons, in some cases more than one neuron was recorded per rat.

3.3. Effect of cariprazine on the firing activity of LC NE neurons: role of 5-HT$_{2A}$ receptors and α$_2$-adrenergic receptors

In the locus coeruleus, the 5-HT$_{2A}$ agonist DOI (100 µg/kg, i.v.) nearly completely inhibited NE neurons (Figure 8 & 9, n=7). Successive injections of 50 µg/kg of cariprazine reversed the firing rate up to 70%, with an ED$_{50}$ value of 67 µg/kg (Figure 9). Two consecutive injections of 5 µg/kg of clonidine, an α$_2$-adrenoceptor agonist, were sufficient to fully inhibit neurons, and 1 mg/kg of idazoxan, an α$_2$-adrenoceptor antagonist, was sufficient to fully reverse this inhibition, providing further confirmation that recordings were of NE neurons (Figure 8). The effect of clonidine after systemic injections of cariprazine was

compared to its effect under control conditions. As shown in Figure 10A and 10B, there was no significant difference between the effect of clonidine after pre-treatment with cariprazine and the effect of clonidine under control conditions, either at 5 µg/kg or at 10 µg/kg.

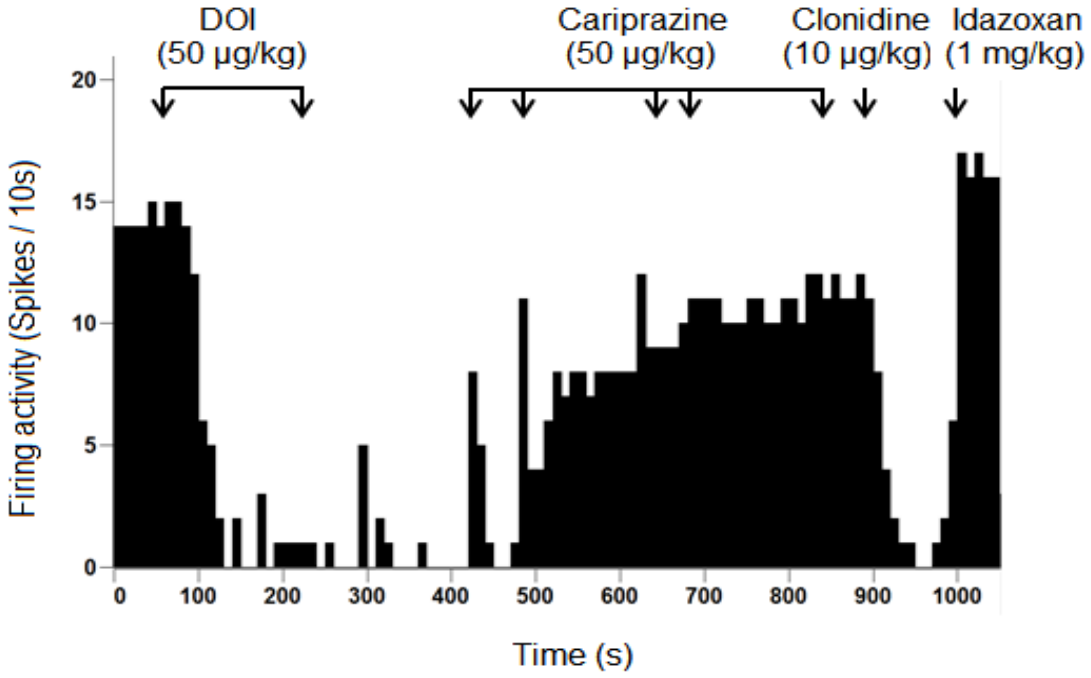

Figure 8. Integrated firing rate histogram of a single NE neuron in the LC showing inhibition by two cumulative doses of 5-HT$_{2A}$ agonist DOI, followed by reversal by cumulative doses of cariprazine, and subsequent inhibition and reversal by clonidine and idazoxan. All drugs were given intravenously, and in all cases only one neuron was recorded per rat.

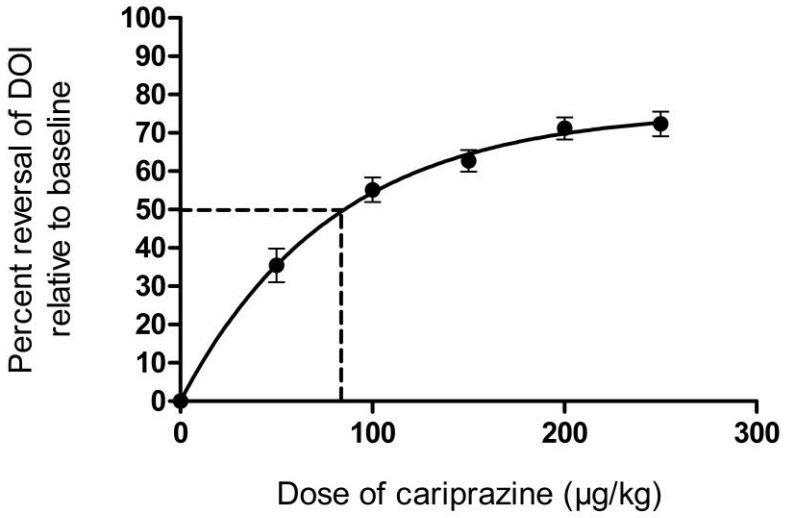

Figure 9. Dose-response curve showing the average percent reversal of the inhibition produced by the 5-HT_{2A} agonist DOI relative to baseline in NE neurons by cumulative doses of cariprazine. N = 7, only one neuron was recorded per rat. Dotted lines indicate the estimated ED_{50} value of cariprazine (ED_{50}=67µg/kg).

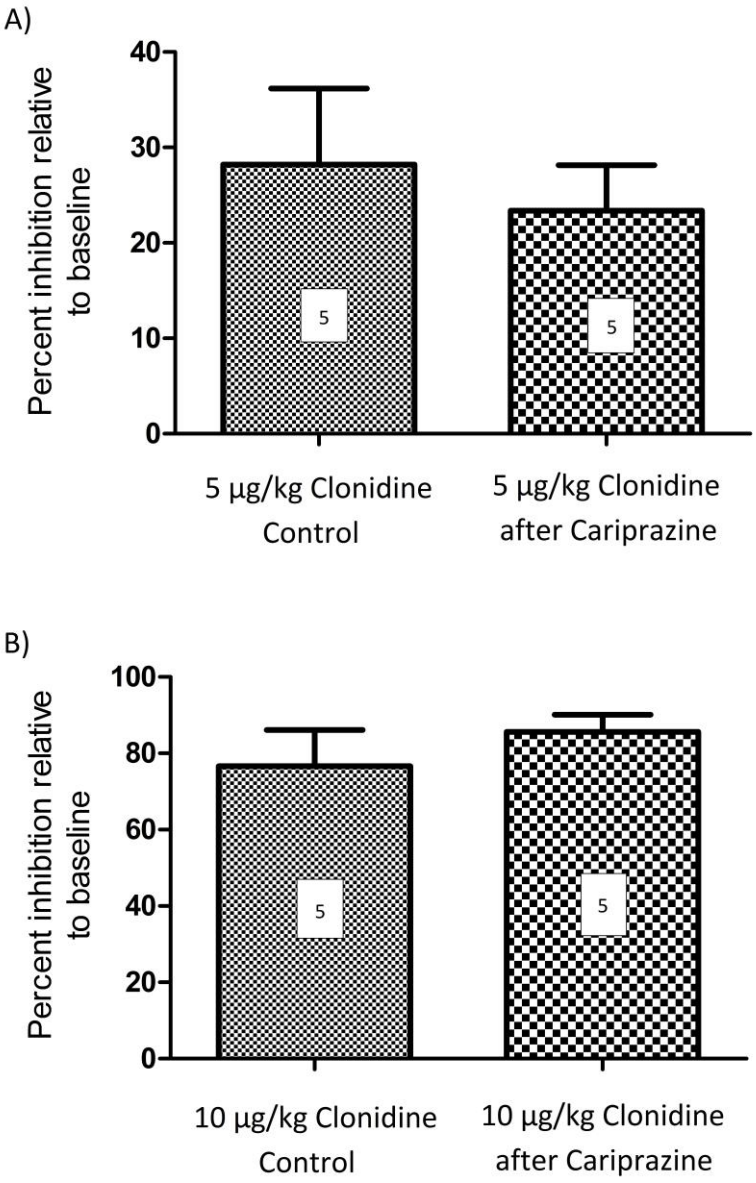

Figure 10. A) Comparison of percent inhibition induced by 5 µg/kg of the α_2-adrenergic agonist clonidine under control conditions, and after the injection of cariprazine. B) Comparison of percent inhibition induced by 10 µg/kg of the α_2-adrenergic agonist clonidine under control conditions, and after pretreatment with cariprazine. All drugs were given intravenously, and in all cases only one neuron was recorded per rat.

To determine the effect of cariprazine by itself on firing activity of NE neurons, 6 neurons were tested. Out of those NE neurons, only 2 neurons showed a slight increase (30-40%) in their firing activity after administration of cariprazine at a dose of 100 µg/kg (Figure 11). The remaining neurons showed no change (Figure 12). Cariprazine did, however, reduce the effectiveness of DOI to inhibit NE neuron firing in both cases.

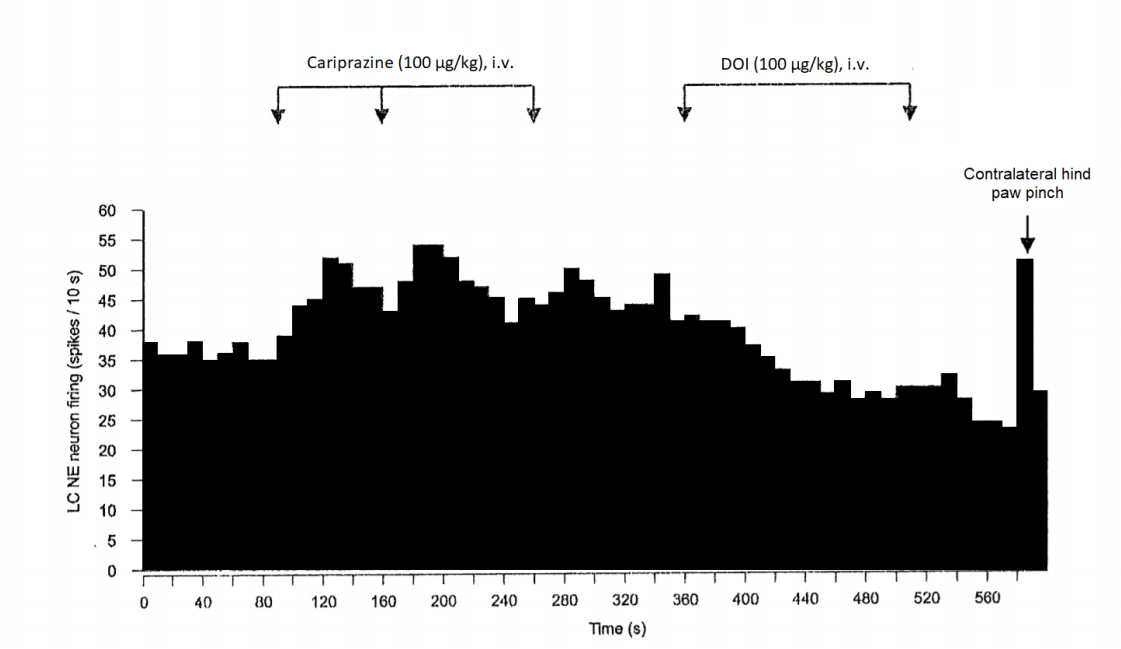

Figure 11. An illustrative example showing the effect of cumulative i.v. doses of cariprazine on one LC NE neuron firing. At the end of the recording, a pinch of the contralateral paw resulting in a volley of discharge followed by a pause, typical of LC NE neurons. All drugs were given intravenously, and in all cases only one neuron was recorded per rat.

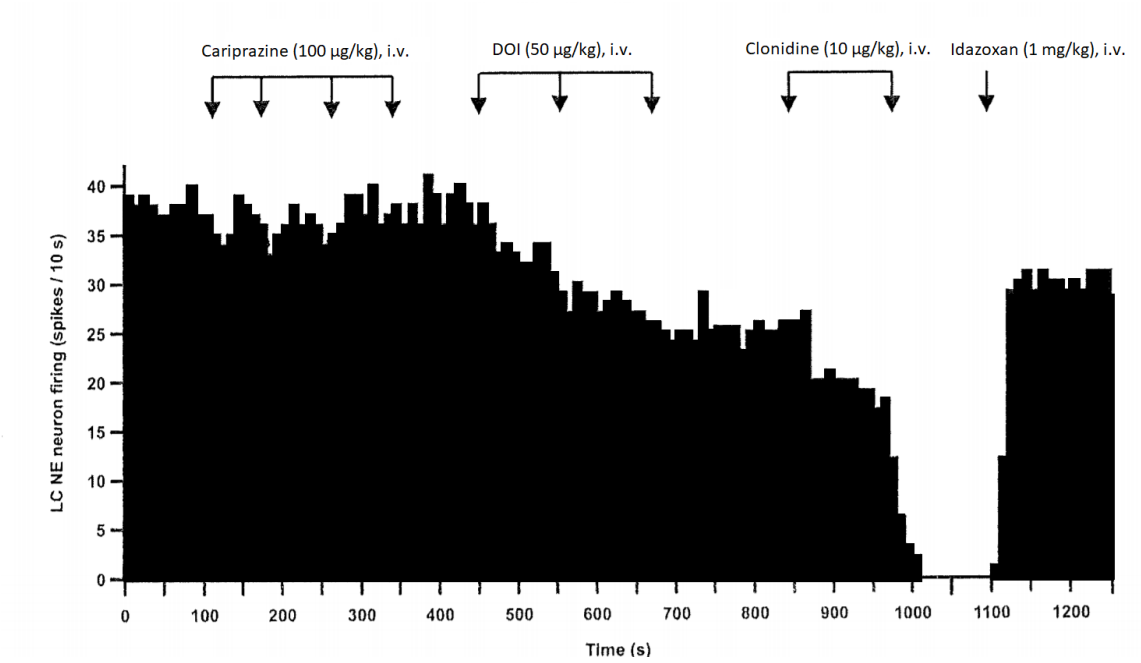

Figure 12. A second trace illustrating an example where cumulative injections of cariprazine (400 ug/kg) did not have any effect on the firing activity of an LC NE neuron. All drugs were given intravenously, and in all cases only one neuron was recorded per rat.

3.4. Effect of cariprazine on the firing activity of VTA DA neurons: role of 5-HT2B receptors

In the VTA, the effect of cariprazine on the 5-HT2B receptors could not be determined. Based on the limited amount of studies on this receptor, it was hypothesized that a 5-HT2B agonist may decrease the firing rate of DA neurons in the VTA. It was found, rather, that a selective 5-HT2B agonist, BW 723C86, at a dose of 200 µg/kg per injection had no significant effect on the firing rate of these neurons, and doses of the drug ranging from 600 µg/kg to 1500 µg/kg were lethal to rats (Fig. 13, n=8).

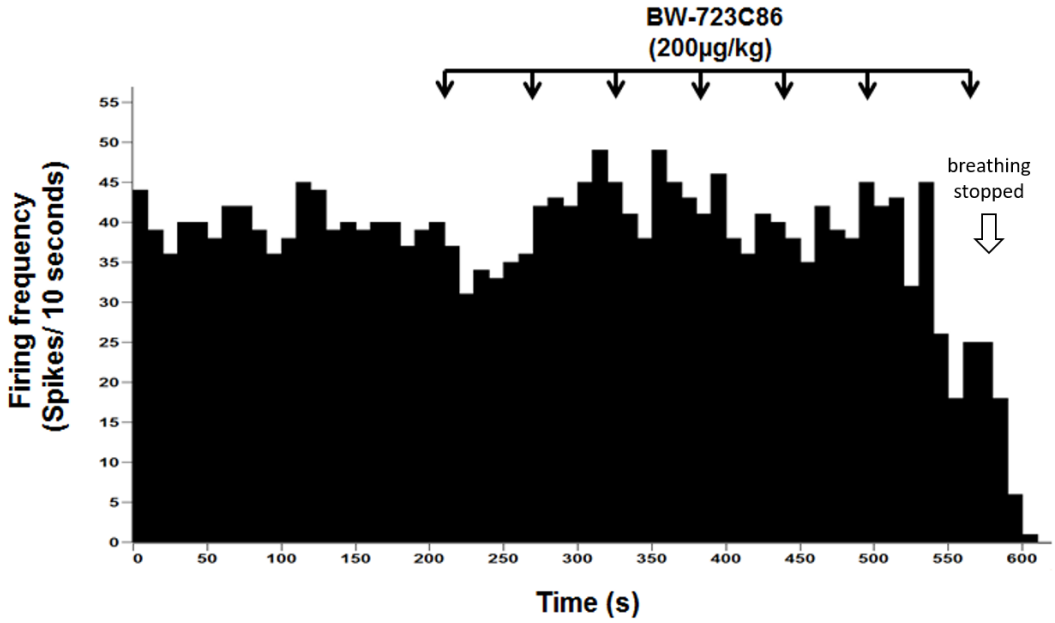

Figure 13. Integrated firing rate histogram of a single DA neuron in the VTA showing its response to seven consecutive intravenous doses of the 5-HT$_{2B}$ agonist BW 723C86. All drugs were given intravenously, and in all cases only one neuron was recorded per rat.

4. Discussion

4.1. Cariprazine: Effect on the 5-HT System

In the DRN, cariprazine fully inhibited the firing of putative 5-HT neurons. This effect was reversed by the selective 5-HT$_{1A}$ antagonist, WAY100.635, indicating that cariprazine acted as an agonist at the 5-HT$_{1A}$ autoreceptors in this part of the brain. The effect of cariprazine was dose-dependent for each individual neuron. In general, these results are consistent with the *in vitro* assays of cariprazine, in that both studies found cariprazine to activate 5-HT$_{1A}$ receptors,

albeit in different parts of the brain. The dose range for full inhibition found here (200-800 µg/kg) was like that of other DA-5-HT partial agonists, with aripiprazole having an ED_{50} of 536 µg/kg in a previous *in vivo* study (Dahan et al, 2009). Alternatively, brexpiprazole has a much more potent effect at these receptors with an ED_{50} of 230 µg/kg (Oosterhoff et al, 2014), and the effect of cariprazine, therefore, seems to be relatively in line with these compounds, with regards to their 5-HT_{1A} receptor affinity.

Interestingly, however, the effect of cariprazine in the present study did not have an overall dose-dependent relationship in that some neurons required more or less of the compound in order to fully inhibit its firing. It could be assumed that this difference stems from variation in baseline firing, as a previous study suggested that 5-HT neurons with slow firing are more sensitive to 5-HT agonists than neurons with faster discharge (Jacobs et al, 1983). However, in the present study, this variability was unrelated to the baseline firing rate of individual neurons. Such variability was unexpected because all 5-HT_{1A} agonists thus far tested in this paradigm yielded tight dose-response relationships (Blier and de Montigny, 1990; Dahan et al, 2009; Dong et al, 1998; Oosterhoff et al, 2014; Reuter et al 1999). A potential explanation of this result is that the balancing of the inhibitory input of 5-HT_{1A} activation and the excitatory input of D_2-like receptor activation (Aman et al, 2007) may lead to such differences if there is some variability in the 5-HT_{1A} to $D_{2/3}$ receptor density ratio from neuron to neuron and/or from animal to animal, especially if it is assumed that the ten-fold greater affinity of cariprazine for D_3 receptors compared to aripiprazole and brexpiprazole

exerts a larger excitatory effect on some 5-HT neurons (Table 3). It is at this point unclear whether the variability translates into a functional difference compared to other DA and 5-HT partial agonists such as brexpiprazole and aripiprazole.

In the hippocampus, cariprazine did not reduce the effectiveness of the endogenous ligand 5-HT at postsynaptic 5-HT$_{1A}$ receptors, when the two compounds were applied concomitantly, indicating that cariprazine acted as a full agonist in this part of the brain, as opposed to a partial agonist. This property has previously been assessed in compounds such as trazodone, gepirone, and flibanserin, with the first being a full agonist like cariprazine and the latter two being partial agonists in this brain region (Ghanbari et al, 2010, Blier and de Montigny, 1990, Rueter and Blier, 1999). It is interesting to note that these kinds of drugs can often be heterogeneous regarding their effect at postsynaptic receptors in different brain areas. For example, flibanserin has undergone thorough studies of this nature, revealing it to act as a full agonist at presynaptic 5-HT$_{1A}$ receptors in the DRN, and as a full agonist at postsynaptic receptors in the mPFC, but as a partial agonist at the postsynaptic receptors in the CA3 region of the hippocampus (Rueter and Blier, 1999). Overall, selective activation of these postsynaptic receptors enhances 5-HT transmission and DA release in the mPFC and hippocampus (Chung et al, 2004).

The present finding that cariprazine acted here as a full agonist in the hippocampus contradicts the results of a previous *in vitro* [^{35}S]GTPγS binding assay, which found cariprazine to be a partial agonist at 5-HT$_{1A}$ receptors in a rat hippocampal membrane preparation (Kiss et al, 2010). Although it was assessed

in the DRN, discrepancy in pharmacological effects *in vivo* versus *in vitro* has been previously reported. For example, with the 5-HT_{1A} receptor agonist NAN-190 where it was shown to act as an agonist *in vitro* but as an antagonist *in vivo* (Greuel and Glaser, 1992). There are a few possible explanations to account for such divergent *in vitro* and *in vivo* results in the present study. It is suggested by some, for instance, that a drug can act as a partial agonist in a tissue with no receptor reserve (spare receptors) while it will behave as a full agonist where high receptor reserve levels are present (Kenakin, 1987). It is also possible that this response is in part dependent on the degree of coupling between receptor reserve and signal transduction (see Cox et al, 1993). This kind of difference could also relate to the small degree of measured response in specific *in vitro* assays (Kenakin, 2003), or that cariprazine operates differently depending on the system involved in a phenomenon known as functional selectivity (Kenakin, 2008). Indeed, in the previous *in vitro* study, cariprazine acted either as a typical $D_{3/2}$ antagonist or as a partial agonist depending on the preparation involved. For example, cariprazine inhibited DA-stimulated binding in a GTPγS binding assay, indicating antagonism, and did not stimulate binding. However, in assays involving the stimulation of IP3 (inositol trisphosphate) or inhibition of forskolin-stimulated cAMP formation, cariprazine did display partial agonist activity as high as 70% (Kiss et al, 2010).

Table 3. Pharmacodynamic profile of *in vitro* binding affinities for human receptors for various DA-5-HT agonist compounds (in nM) and ED_{50} profile for DOI reversal (µg/kg).

Compounds	Human receptor affinities (nM)						ED_{50} to reverse DOI (µg/kg)
	D_3	D_2	5-HT_{2B}	5-HT_{1A}	5-HT_{2A}	α_{2A}	
Aripiprazole	0.8	0.34	0.36	1.7	3.4	74	n.a.
Brexpiprazole	1.1	0.3	1.9	0.12	0.47	15	110
Cariprazine	0.085	0.49	0.58	2.6	18.8	<1000	67
Trazodone	n.a.	4142	78.4	42	20	728	63
Asenapine	0.42	1.3	0.18	2.7	0.07	1.2	75

References: aripiprazole (Shapiro et al, 2003), brexpiprazole (Maeda et al, 2015; Oosterhoff et al, 2014), cariprazine (Kiss et al, 2015), trazodone (Roth and Driscol, 2011; Ghanbari et al, 2012), asenapine (Shadid et al, 2009; Ghanbari et al, 2009)

4.2. Cariprazine: Effect on the NE System

Cariprazine acted as a potent antagonist at 5-HT_{2A} receptors in the LC, in that it reversed the inhibitory effect of the preferential agonist, DOI. These receptors are located on GABA neurons that control NE neurons (Haddjeri et al, 1997; Szabo and Blier, 2002), and blockade of 5-HT_{2A} receptors by various

compounds previously reversed the inhibitory effect of SSRIs on LC NE neurons (Dremencov et al, 2007; Chernoloz et al, 2009; Seager et al, 2004). Cariprazine had a lower *in vivo* ED_{50} value compared to brexpiprazole (67 versus 110 µg/kg) (Oosterhoff et al, 2014), which is discrepant from their respective *in vitro* affinities, but a similar phenomenon been seen previously in drugs such as trazodone (Table 3). It is important to note that *in vitro* affinity data and *in vivo* analysis are not directly comparable because of the additional networks involved in the scope of *in vivo* analysis, differences in subpopulations of NE neurons tested, and other factors that should be taken into account such as dose, and intrinsic activity levels.

Nevertheless, with regards to the α_2-adrenergic receptors, there was no significant activity of cariprazine, based on its inability to block the inhibitory effect of clonidine on NE neurons, and this follows from findings that cariprazine has a pKi value smaller than 6 for these receptors in *in vitro* assays (Kiss et al, 2010). Interestingly, the lack of effect on α_2-adrenergic receptors does constitute a difference compared to several other drugs in the class formerly known as atypical antipsychotics (Oosterhoff et al, 2014; Ghanbari et al, 2009; Dremencov et al, 2007).

Finally, experiments were conducted to determine the effect of acute administration of cariprazine on baseline NE neuron firing. The results were inconclusive in that 2 neurons displayed a 30-40% increase in firing rate, whereas 4 other neurons displayed no change from baseline (Figure 5A and 5B). Previously, it has been shown that a D_2 antagonist, haloperidol, increased the

firing rate of NE neurons (Piercey et al, 1994), and perhaps this would play a role in the excitatory effect of cariprazine in these few neurons. However, based on this limited testing, it can only be concluded that cariprazine does not have a significant effect on NE neuron firing rate, positively or negatively, unlike the inhibitory effect of the 5-HT_{2A} agonist, DOI, for example. This is in line with *in vitro* studies showing negligible intrinsic activity of cariprazine at 5-HT_{2A} receptors, since it was shown to lack a stimulatory effect on inositol phosphate formation (Kiss et al, 2010).

4.3. Cariprazine: Effects on the DA system

In the VTA, the effect of cariprazine on 5-HT_{2B} receptors could not be assessed because the initial hypothesis in itself was not supported. The initial hypothesis was that a 5-HT_{2B} agonist would inhibit the firing of DA neurons, and that therefore cariprazine, as a 5-HT_{2B} antagonist, would reverse this effect. In fact, the 5-HT_{2B} agonist had no significant effect on DA firing.

Interestingly, a recent study by Devroye et al out of Dr. Haddjeri's lab in Lyon, France, presented findings on the effects of 5-HT_{2B} agonists and antagonists on the DA neurons of the VTA, and found that the 5-HT_{2B} antagonist, RS 127445 decreased the firing of DA neurons (Devroye et al, 2016). With this information, there is the potential for cariprazine to have a similar effect to RS 127445, as an inhibitor of DA neuron firing through the antagonism of 5-HT_{2B} receptors.

As it pertains to the lack of overall dose-response relationship for

cariprazine on 5-HT neurons in the DRN, it was also found that RS 127445 has an excitatory effect on the firing of these neurons (Devroye et al, 2017), indicating a functional interaction between the inhibitory effects of 5-HT_{1A} activation and the excitatory effects of 5-HT_{2B} antagonism, in addition to the potential excitatory effect of D_2 agonism.

In the present work, the effects of cariprazine on $D_{2/3}$ receptors were not studied because these experiments were carried out in another lab. The results of their work, will, however, be discussed as it pertains to this thesis. The study similarly involved *in vivo* electrophysiological recordings of DA neurons in the VTA in naive, anesthetized rats. It was found that, acutely, cariprazine partially inhibited the firing of DA neurons, and that this was prevented by a D_2 antagonist, indicating that cariprazine activated D_2 autoreceptors. Additionally, cariprazine seemed to act as an antagonist of $D_{3/2}$ receptors in that it prevented the inhibition of DA firing by a preferential D_3 receptor agonist, and reversed the effect of the D_2 selective agonist, apomorphine, in the SNc (Delcourte et al, 2017). The findings of this study show that cariprazine seems to have partial agonist properties at D_2 receptors and antagonist properties at D_3 receptors, *in vivo.* This is in agreement with *in vitro* studies of cariprazine, where cariprazine also acted as an antagonist at D_3 receptors, and a partial agonist at D_2 receptors. *In vitro* studies, however, indicate that cariprazine may also have agonist/partial agonist properties at D_3 receptors (Kiss et al, 2015). Further studies may be necessary to determine the intrinsic activational effects of cariprazine at D_3 receptors *in vivo,* and the relative percentage of agonism to antagonism. These

relative amounts could have important implications for the differential response of individual patients. While the various symptomology for D_3 receptors are not known, the degree of activation of D_2 receptors has been important in that if a drug is too activating it can lead to agitation and akathisia (Stahl, 2016).

4.4. Conclusions

In this study, cariprazine shows acute *in vivo* activity on the 5-HT and NE systems that supports the *in vitro* analysis of the compound, namely the effective agonism of 5-HT_{1A} receptors and the effective antagonism of 5-HT_{2A} receptors. The present results support the notion that cariprazine has benefit in the treatment of depressive symptomology in various mood disorders. In fact, this is supported by two recent studies that took place between 2011 and 2014. The first was a study conducted on patients with bipolar I disorder, and found that a dose of 1.5-3.0 mg/day of cariprazine was significantly better than placebo at improving symptoms in depressive episodes (Durgam et al, 2016). The second study was conducted on patients that had inadequate response to antidepressants, and found that a dose of 2.0-4.5 mg/day of cariprazine was significantly better than placebo as an augmentation therapy alongside current antidepressant treatment (Durgam et al, 2016).

4.5. Future Directions

The primary future directions relating to the study of cariprazine include basic and clinical research. In terms of basic research, the next step should be a

study of the effect of long term treatment with cariprazine on the firing of 5-HT, DA, NE, and hippocampal neurons. Since adaptations observed during DA and 5-HT agonist administration take time to develop, the effects of long-term administration of cariprazine on monoaminergic systems need to be investigated, especially as it compares to the long term effects of an SSRI such as escitalopram, as well as the effects of long term treatment with a combination of cariprazine and escitalopram. In terms of clinical research, the focus of future studies should be to test the effect of cariprazine monotherapy in a placebo controlled double-blind trial, in patients with MDD.

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