Impact of depressogenic- and antidepressant-like challenges on monoamine system activities: *in vivo* electrophysiological characterization studies

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Statement of contributions

The experiments and data analysis in chapter I-IV were solely performed by the present author. The present author drafted the first manuscript for each of these studies, which were reviewed by Drs. Blier and El Mansari, resulting in the versions as presented in the present thesis. In addition, the present author performed electrophysiological experiments and made intellectual contributions to the manuscripts on vilazodone, ketamine, and olfactory bulbectomy, which can be found in the appendix.

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Glossary

M100907	*(R)*-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]-4-piperidyl]methanol
5,7-DHT	5,7-dihydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin, 5-hydroxytryptamine
6-OHDA	6-hydroxy-dopamine
8-OHDPAT	8-hydroxy-2-(dipropylamino)tetralinhydrobromide
AADC	l-amino acid decarboxylase
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPT	α-methylparatyrosine
ANOVA	analysis of variance
AP	anterior-posterior
APTD	acute phenylalanine/tyrosine depletion
ATP	adenosine triphosphate
BAY 3072	(R)-(−)-2-[4-[(chroman-2-ylmethyl)-amino]-butyl]-1,1-dioxo-benzo[d]isothiazolone
BBB	blood brain barrier
BMY 7378	8-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspiro[4.5]decane-7,9-dione
CA3	cornus ammonis region 3
cAMP	cyclic adenosine monophosphate
CAR	conditioned avoidance response
CMS	chronic mild stress
CNS	central nervous system
COMT	catechol-O-methytransferase
CORT	corticosteroids (cortisol in humans, and corticosterone in rats)
CRH	corticotrophin-releasing hormone
DA	dopamine
DAG	diacylglycerol
DALY	disability adjusted fife years
DBS	deep brain stimulation
DEX	dexamethasone
DOI	2,5-dimethoxy-4-iodoamphetamine
DOS	duration of silence
DRN	dorsal raphe
DßH	DA ß-hydroxylase
DST	dexamethasone suppression test
DV	dorso-ventral
ECT	electroconvulsive therapy
ED100	effective dose for 100% of the maximal response

ED50	effective dose for 50% of the maximal response
FST	forced swim test
GABA	gamma-aminobutyric acid
GD	gestational day
GIRK	inwardly rectifying potassium channels
GR	glucocorticoid
HPA	hypothalamus-pituitary-adrenal
hquetiapine	humanized quetiapine, a 3:1 mixture of quetiapine and norquetiapine
HVA	homovanillic acid
IP3	inositol triphosphate
LC	locus coeruleus
LGN	lateral geniculate nucleus
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAP	mitogen-activated protein
MDD	major depressive disorder
MDMA	3,4-methylene-dimethoxy-methamphetamine
MHPG	3,4-dihydroxymandelic acid, 3-methoxy-4-hydroxyphenylglycol
ML	medio-lateral
MR	mineralocorticoid
MRN	median raphe
mRNA	messenger ribonucleic acid
Nac	nucleus accumbens
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NE	norepinephrine
NET	NE transporter
NMDA	N-methyl-D-aspartate
PCP	phenylcyclidine
PCPA	parachlorophenylalanine
PDSP	Psychoactive Drug Screening Program
PET	positron emission tomography
PKC	protein kinase C
PLC	phospholipase C
PND	postnatal day
PNS	prenatal stress
PVN	paraventricular nucleus
REM	rapid eye movement
RM ANOVA	repeated measures ANOVA
S.E.M.	standard error of the mean
SB242084	6-chloro-5-methyl-*N*-{6-[(2-methylpyridin-3-yl)oxy]pyridin-3-yl}indoline-1-carboxamide
SERT	5-HT transporter
SNRI	serotonin/norepinephrine reuptake inhibitors
SNRI	substantia nigra

SSRI	Selective serotonin reuptake inhibitor
TCAs	tricyclic antidepressants
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
TTX	Tetradotoxin
VMA	vanillylmandelic acid
VMAT	vesicular monoamine transporter
VNS	vagus nerve stimulation
VTA	ventral tegmental area
WAY 100.635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)-cyclohexanecarboxamide
WHO	World Health Organization

Abstract

Introduction: major depressive disorder is a common psychiatric disorder associated with high economic cost, severe human suffering, and low remission rates. Imbalanced neurotransmission of the monoaminergic serotonin (5-HT), dopamine (DA) and norepinephrine (NE) systems is implicated in this disorder. However, the etiology underlying this presumed imbalance and the mechanism by which antidepressant strategies restore this imbalance requires further exploration. Accordingly, the present work assessed the effects of depressogenic and antidepressant-like conditions on these systems.

Methodology: Electrophysiological extracellular single unit recordings from 5-HT, DA, NE, and hippocampal pyramidal neurons were obtained in adult male chloral hydrate anaesthetized Sprague-Dawley rats. Effects on relevant receptors were characterized using established electrophysiological and/or pharmacological strategies. Prenatal stress was used to model depressogenic-like conditions. The effects on monoamine systems of asenapine and brexpiprazole, two atypical antipsychotics with antidepressant potential, were characterized after acute (brexpiprazole) and sustained administration. These sustained regimens resulted in clinically relevant blood plasma levels.

Results: Prenatal stress exposure altered monoamine system activities but did not produce detrimental effects on behavior. Asenapine and brexpiprazole had unique effects on the activities of monoamine systems. Unlike other antipsychotics, both agents did not produce a cessation of the firing of dopamine neurons in the ventral tegmental area, thereby providing novel insights in the role of this system in the treatment of mood

disorders. Furthermore, both agents enhanced the tonic activation of postsynaptic 5-HT_{1A} receptors, similarly to the effects of all antidepressant strategies.

Conclusion: Prenatal stress altered the activities of 5-HT, NE and DA neurons. Since these central changes were obtained in animals displaying normal behavior, they presumably reflect adaptations to depressogenic-like conditions. The characterization of asenapine and brexpiprazole contributed to a further understanding of their mechanisms of action. Together, these studies provide insight in neural substrates presumably relevant to the antidepressant response.

1) Major depressive disorder

Major depressive disorder (MDD) is one of the most common psychiatric disorders. In the United States, lifetime prevalence of MDD was estimated at 9% for men and 17% for women (Hasin *et al.*, 2005). According to the World Health Organization (WHO), MDD has one of the greatest burdens of disease worldwide (Ferrari *et al.*, 2013), and prognoses indicate that MDD will be the greatest cause of Disability Adjusted Life Years (DALYs) by the year 2030. Evidently, the greatest cost of MDD is human suffering; however, the consequences of MDD are not restricted to the individual alone. Because depression often results in loss of productivity, failure to advance in school, substance abuse, suicide, and comorbidity of physical condition, it is clear that MDD negatively affects society both socially and economically (Kessler, 2012).

Although the etiology of MDD requires further exploration, well-known risk factors include genetic makeup (Kendler *et al.*, 2005), life trauma (Gillespie and Nemeroff, 2005), a family history of mood disorders (Reinherz *et al.*, 2003), female sex (Weissman, 1996), age (Hasin *et al.*, 2005), and comorbid medical conditions (Glassman and Shapiro, 1998). Together with the heterogeneous symptomatology in depression, best illustrated by the fact that two individuals diagnosed with depression may have not one single symptom in common, these lines of evidence suggest a complex interplay between genetic, physiological and/or environmental factors in the development of MDD.

Treatment of MDD faces two major challenges; firstly antidepressants have an unwanted and not fully understood delayed onset of action (~4-6 weeks). Second, first-line antidepressants yield an unsatisfactory equal proportion of responders, partial responders, and non-responders. Interestingly, recent studies found a reduction of

depressive symptoms within hours following administration of ketamine at a subanaesthetic dose (Zarate *et al.*, 2006, 2012), indicating that a delayed onset of action might not always be necessary in the treatment of MDD. Furthermore, co-administration of agents with synergistic properties has been demonstrated to have superior therapeutic efficacy compared to monotherapy of either agent alone (Blier *et al.*, 2009, 2010).

Importantly, all clinically efficacious agents in depression treatment alter the activity of the serotonin (5-HT), norepinephrine (NE), and/or dopamine (DA) neurotransmitter systems either directly or indirectly (El Mansari *et al.*, 2010). Conversely, a depletion of these neurotransmitters is known to produce a mood-lowering effect in either healthy controls and remitted MDD patients (Booij, van der Does, *et al.*, 2005; Krishnan and Nestler, 2008). Given their paramount role in MDD, the aim of the present work was twofold; firstly, to study the effects of the novel atypical antipsychotics asenapine and brexpiprazole on monoamine systems following acute (brexpiprazole), subacute and long-term administration, and secondly, to assess the effect of potentially depressogenic conditions in early life and adulthood on monoamine system activities. Before discussing the outcomes of these studies, background information and evidence for involvement in MDD of the 5-HT system (section 2.1), the NE system (section 2.2) the DA system (section 2.3.), interactions between these systems (section 2.4), antidepressant strategies and their effects on monoamine systems (section 2.5), and the interaction between stress, depression, and physiological changes (section 2.6) will be discussed.

2) Monoamine systems

2.1 The 5-HT system

As suggested by its name, the vasoconstrictive property of serotonin (5-hydroxytryptamine, [5-HT]) was discovered (Rapport *et al.*, 1948) before its central action as a neurotransmitter (Bogdanski *et al.*, 1956). Nearly all 5-HT containing neurons (~300.000 cells in humans) are found in the raphe (Greek; "seam") nuclei, a group of brain regions located in the brain stem most directly bordering the cerebral aqueduct. Based on their morphological and anatomical characteristics (Baker *et al.*, 1990; Hornung, 2003), the human raphe nuclei have been subdivided in rostal and caudal groups. The caudal raphe consists of the raphe pallidus (Latin: "pale"), raphe obscurus (Latin; "obscure"), and raphe magnus (Latin: "large"). Together, these cell groups contain ~15% of 5-HT neurons, which predominantly project to the lower brain stem and spinal cord. The other ~85% of 5-HT neurons are located in the rostal raphe nucleus, which can be subdivided into the median raphe (MRN), dorsal raphe (DRN), caudal linear nuclei and supralemniscal (Latin: "above the ribbon") raphe nuclei. In its original characterization, 5-HT neurons in the rat (~20,000 neurons) were divided in 9 groups (B1-9; Dahlström and Fuxe, 1964). Based on their morphology and connectivity, these clusters can be anatomically designated as follows: B1: raphe pallidus, B2 & B4: raphe obscurus, B3, raphe magnus, B5: median raphe nucleus, B6 & B7; dorsal raphe nucleus, B8; caudal linear nucleus, B9, supralemniscal region (Törk, 1990; Jacobs and Azmitia, 1992).

The presence of recurrent axon collaterals, dendrodendritic connections between 5-HT containing neurons within raphe nuclei, and recurrent 5-HT connections between the DRN and MRN clearly demonstrate that multiple levels of communication exist between 5-HT neurons (Wiklund *et al.*, 1981; Kapadia *et al.*, 1985; Chazal and Ralston, 1987). Given the relatively low number of synaptic specializations at 5-HT nerve endings in the raphe nuclei, this communication likely results to a greater extent from extracellular than from transsynaptic neurotransmission (Harandi *et al.*, 1987). Notably, the raphe nuclei are not homogenous; the proportion of 5-HT neurons in the DRN has been estimated one- to two-third. Colocalization of other neurotransmitters and neuromodulators within 5-HT neurons has been demonstrated, including DA (Lindvall and Björklund, 1974), gamma-aminobutyric acid (GABA; Belin *et al.*, 1979), glutamate (Kaneko *et al.*, 1990), and substance P (Chan-Palay *et al.*, 1978). In addition, these neurotransmitters and neuromodulators were also found in non 5-HT neurons within the DRN (Köhler and Steinbusch, 1982; Jacobs and Azmitia, 1992).

Despite the relatively low number of neurons, 5-HT projections densely innervate the brain. The rostal raphe nuclei predominantly innervate cerebral and subcortical areas. In more detail, the prefrontal cortex receives its strongest innervation from the DRN (Törk, 1990; Wilson *et al.*, 1994), while the MRN and supralemniscal nuclei equally project to parietal, occipital, and frontal cortices. Projections from these nuclei are homogenous, and estimates suggest that each 5-HT neuron may be responsible for up to a striking 500,000 cortical varicosities (Audet *et al.*, 1989). Similarly to neurotransmission within the raphe nuclei, the presence of 5-HT containing synaptic vesicles and 5-HT immunoreactivity in the absence of synaptic specialization indicates that extrasynaptic

release contributes to 5-HT neurotransmission, at least in cortical regions (Smiley and Goldman-Rakic, 1996). For an illustration of 5-HT signalling in the human brain, see figure 1.

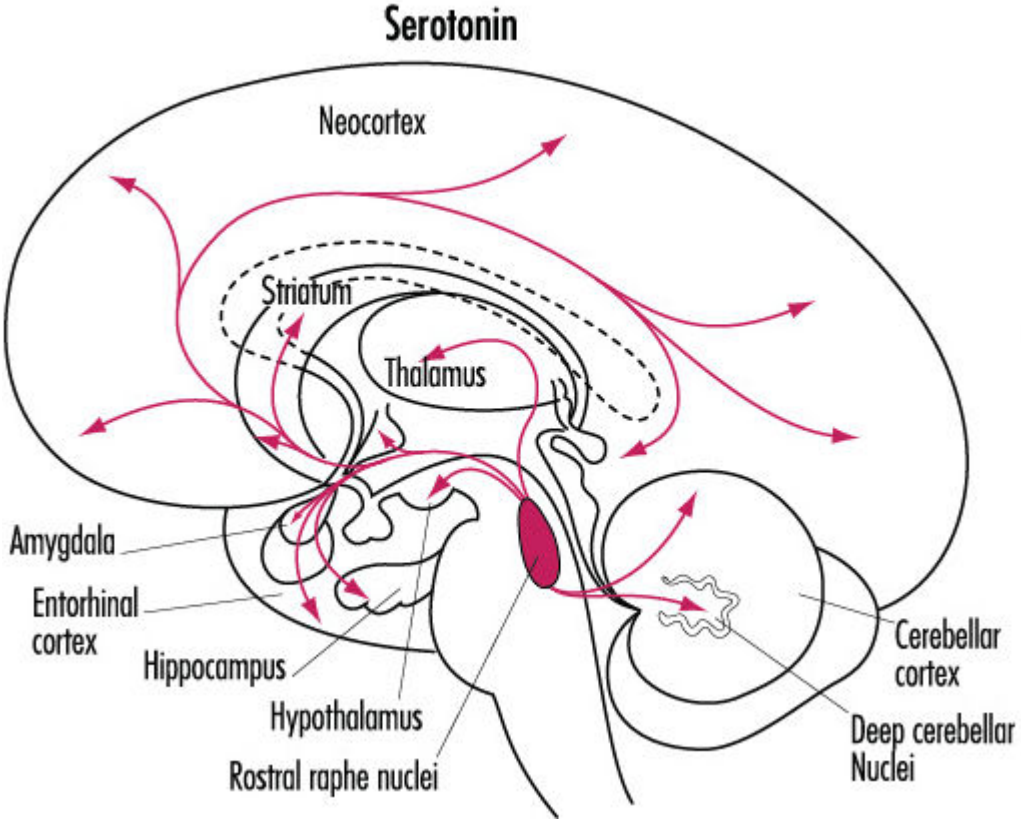

Figure 1: illustration of the major source of 5-HT (the raphe nuclei) and innervation of the brain in humans. Figure adapted from (Heimer, 1995).

2.1.1 5-HT: Synthesis, storage, release, and metabolism

The vast majority of 5-HT (~90%) is located in the periphery. Importantly however, due to its hydrophilic nature, serotonin can not cross the blood brain barrier (BBB). Therefore, all 5-HT in the central nervous system (CNS) is synthesized from the essential amino acid tryptophan. This process is accomplished in two enzymatic steps; first, tryptophan hydroxylase (TPH) produces 5-hydroxytryptophan, which requires vitamin B6 as a co-enzyme , Fe^{2+} as a co-factor, and oxygen and tetrahydrobiopterin as co-substrates (Weissbach *et al.*, 1957; Miwa *et al.*, 1985; Hasegawa and Ichiyama, 2005). 5-hydroxtryptophan is subsequently decarboxylated by aromatic l-amino acid decarboxylase (AADC), resulting in 5-HT. The two forms of TPH are strictly compartmentalized: TPH1 is only found in the periphery, while TPH2 is only found in the CNS (Walther *et al.*, 2003). TPH is an important enzyme for several reasons; its action is the rate-limiting step in 5-HT synthesis. Furthermore, TPH is exclusively expressed in neurons containing 5-HT, and therefore a useful immunohistochemical marker for 5-HT neurons. Moreover, inhibition of TPH with parachlorophenylalanine (PCPA) provides a method to study the effects of central 5-HT depletion (Koe and Weissman, 1966).

Vesicular storage of 5-HT requires its active transport from the cytoplasm by the vesicular monoamine transporters (VMAT), which is coupled to a proton pump and exchanges H^+ for cytoplasmic 5-HT (Brady *et al.*, 2005). Activity of the VMAT can be blocked by reserpine, leading to depletion of 5-HT (as well as NE and DA; Berger *et al.*, 1989). Under physiological conditions, 5-HT is released via a Ca^{2+}-dependent vesicular release mechanism (Bagdy and Harsing, 1995), indicating that neural discharge is

required for 5-HT release. Indeed, perfusion of raphe nuclei with the selective Na^+ channel blocker tetradotoxin (TTX) reliably lowers 5-HT levels in the raphe nuclei and projection areas (Matos *et al.*, 1996). Following its release, active reuptake from the extracellular space by the 5-HT transporter (SERT) is the main route to terminate 5-HT neurotransmission. Following its reuptake, 5-HT can remain in the cytoplasm, be stored in vesicles by action of the VMAT, or be oxidized by monoamine oxidase (MAO; Hare, 1928). Initially based on pharmacological differences, two forms of MAO have been identified; MAO-A and MAO-B (Johnston, 1968; Shih and Thompson, 1999). The Michaelis-Menten kinetics (K_m: a measure of enzyme activity; Michaelis and Menten, 1913) for 5-HT of MAO-A has been estimated around 100 μmol/L, and that of MAO-B around 1170 μmol/L (Fowler and Tipton, 1982; Garrick and Murphy, 1982). Interestingly, the low-affinity MAO-B is the predominant MAO subtype found in 5-HT neurons, suggesting that a certain concentration of cytoplasmic 5-HT might be physiologically desirable (Westlund *et al.*, 1985). Metabolism of 5-HT by MAO produces 5-hydroxyindoleacetic acid (5-HIAA), which is excreted into the cerebrospinal fluid. A summary of the most important intra- and extracellular mechanisms for 5-HT neurotransmission is illustrated in figure 2, and these components will be discussed in more detail in the following sections.

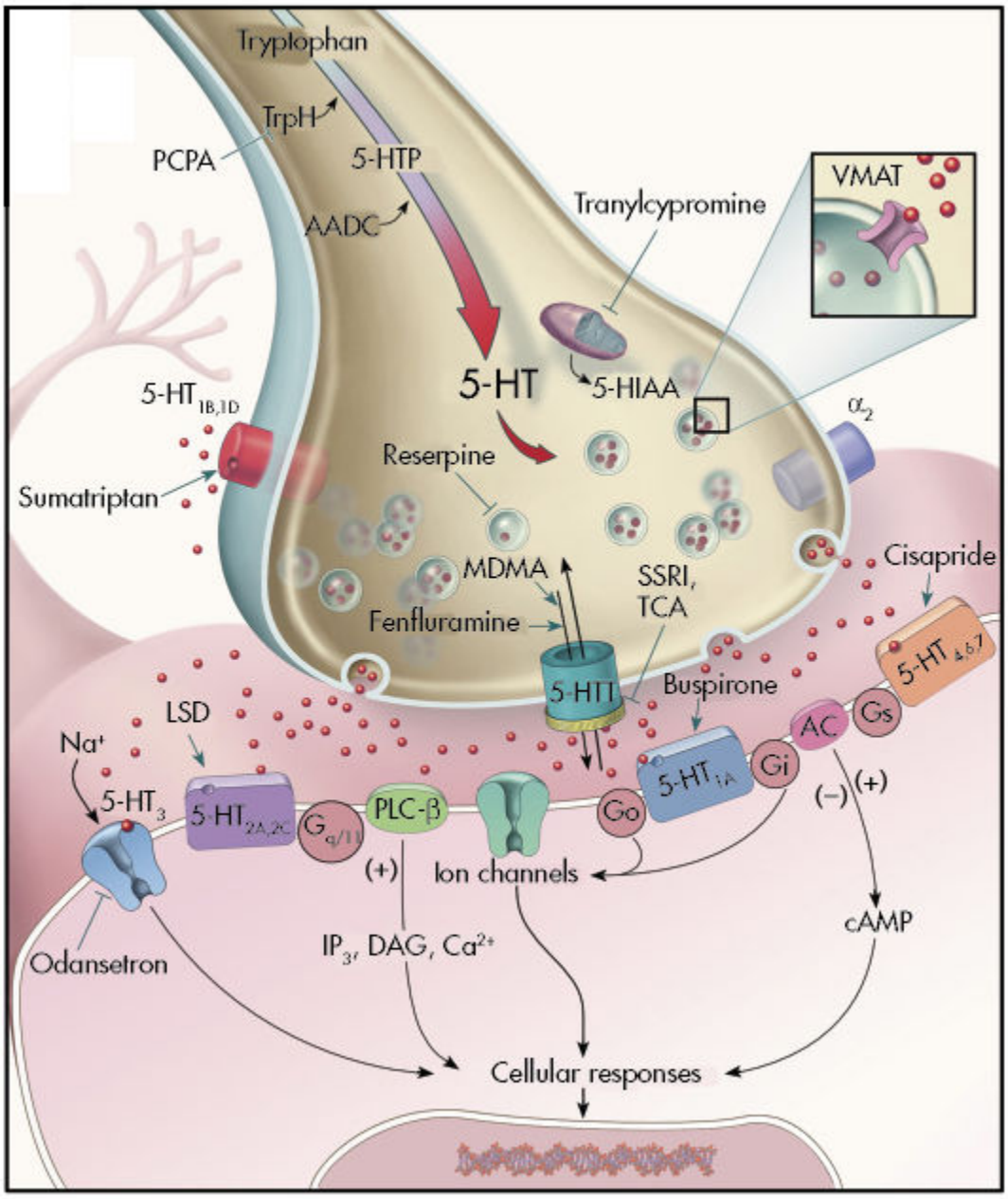

Figure 2: The main intra- and extracellular components involved in 5-HT neurotransmission. Cisapride is a 5-HT_4 receptor agonist that was used for the treatment of hiatal hernias, and was used for irritable bowel syndrome and nausea; fenfluramine is a 5-HT releaser that was used as an appetite suppressor; lysergic acid diethylamine (LSD) is 5-HT_{1A} receptor agonist with hallucinogenic properties; 3,4 methylenedioxy-methamphetamine (MDMA) is a 5-HT releaser mainly used for recreational purposes; ondansetron is a 5-HT_3 receptor antagonist used for the treatment of nausea during chemotherapy, irritable bowel syndrome, and migraine; para-chloropehnylalanine (PCPA) is a selective 5-HT synthesis inhibitor used in research; reserpine is a VMAT blocker used for the treatment of hypertension; selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) are classes of antidepressants more thoroughly discussed in sections 2.5.1 and 2.5.5, respectively; sumatriptan is a 5-$HT_{1B/D}$ receptor agonist used for the treatment of migraine; tranylcypromine is a MAOI used in treatment of MDD. Figure adapted from (Schatzberg and Nemeroff, 2009) with courtesy of S. Szabo.

2.1.2 SERT

In the CNS, SERT is expressed predominantly in 5-HT neurons and to a lesser extend on astrocytes (Kimelberg and Katz, 1985), while in peripheral tissues its expression is found in the gut (Gordon and Barnes, 2003), placenta (Balkovetz *et al.*, 1989), lung (Paczkowski *et al.*, 1996), adrenal chromaffin cells (Schroeter *et al.*, 1997), blood lymphocytes (Faraj *et al.*, 1994) and blood platelets (Carneiro and Blakely, 2006; Ramamoorthy *et al.*, 2011). SERT is a 12-membrane spanning protein, and its genetic basis consists of a single gene in both rodents and humans (Ramamoorthy *et al.*, 1993). Notably, polymorphisms in the SERT gene have functional consequences for 5-HT reuptake dynamics and could play a role in human psychopathologies (Caspi *et al.*, 2003; Kilic *et al.*, 2003), effects that will be discussed more thoroughly in section 2.1.4.

Reuptake of 5-HT by the SERT requires intracellular adenosine triphosphatase (ATPase) and extracellular binding of a sodium ion, a chloride ion, and a protonated 5-HT molecule (Brady *et al.*, 2005). This binding causes a conformational change of the SERT protein and results in cytoplasmic transportation (Brady *et al.*, 2005). The SERT has multiple phosphorylation sites (Hoffman *et al.*, 1991), and its activity can be altered by a variety of intracellular mediators including protein kinase C (PKC), protein kinase G, Ca^{2+}/calmodulin-dependent kinase II, tyrosine kinase, mitogen activated protein kinase (MAPK), and protein phosphotase 2 (Anderson and Horne, 1992; Jayanthi *et al.*, 1994; Helmeste and Tang, 1995; Ramamoorthy *et al.*, 1998; Zhu *et al.*, 2004).

In the CNS, regional differences in SERT binding and –availability exist; these are highest in the mood-regulating midbrain and striatum, lower in cortical regions, and lowest in the cerebellum (Kranz *et al.*, 2010; Savli *et al.*, 2012). Blockade and consequent

downregulation of the SERT increases postsynaptic 5-HT levels (Piñeyro *et al.*, 1994), which is clinically important in the treatment of MDD. Indeed, selective serotonin reuptake inhibitors (SSRIs; citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline) are currently the first-line treatment of MDD; their effects on monoamine systems following acute and sustained are more thoroughly discussed in section 2.5.1.

In addition to blockade, extracellular 5-HT levels can be enhanced by "swapping" the transport direction of the SERT. Indeed, the stimulant 3,4-methylene-dimethoxy-methamphetamine (MDMA, "ecstasy") and the anorexic fenfluramine reverse the action of both the VMAT and the membrane-bound SERT, providing a pharmacological 5-HT release mechanism that is Ca^{2+}-independent (Berger *et al.*, 1992).

2.1.3 5-HT receptors

In mammals, the family of 5-HT receptors consists of 7 receptor families and a total of 14 receptor subtypes. These are, in ascending order for their 5-HT affinity, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, 5-HT_{1D}, 5-HT_3, 5-HT_{1E}, 5-HT_7, 5-HT_{2B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_6, 5-HT_4, 5-HT_{5B} and 5-HT_{5A} receptors (Psychoactive Drug Screening Program [PDSP] Ki database). With the exception of the 5-HT_3 receptor, which constitutes a ligand-gated ion channel, all of these receptors are G-protein coupled receptors. The 5-HT_1 and 5-HT_5 receptor families have an inhibitory effect on cell activity, while the other 5-HT receptor families are generally excitatory. In the next sections, some of these receptors will be discussed in more detail given their important role in mood disorders.

2.1.3.1 5-HT_{1A} receptors

The observation that radioactively labeled 5-HT (^{3}H-5-HT) was replaced by the 5-HT agonist spiperone in a biphasic manner led to the classification of 5-HT_{1A} receptors (Pedigo *et al.*, 1981), and this receptor was the first to be successfully cloned (Fargin *et al.*, 1988). In the CNS, 5-HT_{1A} receptors are the most abundant of all 5-HT receptors. Based on their different electrophysiological and pharmacological properties *in vivo*, 5-HT_{1A} autoreceptors on raphe neurons, those located on the soma of postsynaptic neurons, and those located intrasynaptically on the dendrites of postsynaptic neurons will be discussed separately.

2.1.3.2 5-HT_{1A} autoreceptors

5-HT_{1A} autoreceptors in the DRN are expressed exclusively presynaptically, are found on both the soma and dendrites of 5-HT neurons (Sotelo *et al.*, 1990), and partake in autoregulatory feedback on the discharge activity of these cells (Aghajanian, 1978; Hjorth *et al.*, 1982). These autoreceptors are coupled to a Gi/Go protein; their activation decreases intracellular cyclic adenosine monophosphate (cAMP) levels, which reduces the opening of inwardly rectifying potassium channels (GIRK; Williams *et al.*, 1988) leading to membrane hyperpolarization, hence these receptors have an inhibitory effect on discharge activity of 5-HT neurons. Downstream signaling of 5-HT_{1A} receptors is solely mediated by $G_{i\alpha3}$ subunit (Mannoury la Cour *et al.*, 2006). Accordingly, raphe nuclei application of pertussis toxin which inhibits Gi/Go signaling by catalyzing

adenosine diphosphatase (ADP) ribosylation of $G\alpha_{i/o}$ subunits prevented the inhibitory effect of 5-HT, 8-hydroxy-2-(dipropylamino)tetralinhydrobromide (8-OHDPAT), lysergic acid diethylamide (LSD) and ipsapirone on 5-HT discharge activity (Innis *et al.*, 1988; Blier *et al.*, 1993b). In contrast, application of cholera toxin, which stimulates G_s protein signaling by catalyzing ADP ribosylation of $G_{\alpha s}$ subunits, did not affect the responsiveness of 5-HT_{1A} autoreceptors (Blier *et al.*, 1993b). Finally, 5-HT_{1A} autoreceptors can be pharmacologically blocked by various antagonists, including spiperone and N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)-cyclo-hexanecarboxamide (WAY 100.635; Blier *et al.*, 1993a; Fletcher *et al.*, 1996). Arguably the most important characteristic of 5-HT_{1A} autoreceptors in the treatment of MDD is their desensitization following sustained activation (Blier and de Montigny, 1983), which results from less surface expression (Welner *et al.*, 1989), receptor internalization (Riad *et al.*, 2004), and/or reduced G-protein coupling (Hensler, 2002). Indeed, administration of antidepressant agents that directly (e.g. gepirone, tandospirone; see section 2.5.2) or indirectly (SSRIs; see section 2.5.1) activate 5-HT_{1A} receptors cause an early dampening of 5-HT neural activity. Following their sustained administration, however, 5-HT neural discharge activity recovers due to concomitant reduction of 5-HT_{1A} autoreceptors responsiveness (Blier and de Montigny, 1987; de Montigny *et al.*, 1990; Godbout *et al.*, 1991).

2.1.3.3 Postsynaptic intrasynaptic 5-HT_{1A} receptors

Given their location, the physiological role of intrasynaptic 5-HT_{1A} receptors in 5-HT neurotransmission is best characterized *in vivo* by electrical stimulation of 5-HT

projections, resulting in endogenous 5-HT release where the duration of suppressed postsynaptic activity is proportional to the intrasynaptic 5-HT_{1A}-mediated signal. Indeed, the 5-HT_{1A} receptor antagonist BMY 7378 prevents this neurotransmission (Chaput and de Montigny, 1988). Intriguingly, WAY 100.635 does not have this effect (Haddjeri *et al.*, 1999), suggesting absent access and/or no binding to this receptor population. Furthermore, activation of intrasynaptic 5-HT_{1A} receptors was prevented by cholera, but not pertussis toxin, effects suggestive of predominant signal transduction via G_s proteins and opposite to those on 5-HT_{1A} autoreceptors (Blier *et al.*, 1993b).

2.1.3.4 Postsynaptic extrasynaptic 5-HT_{1A} receptors

Postsynaptic extrasynaptic 5-HT_{1A} receptors are the primary mediators of neural inhibition by extrasynaptic 5-HT in projection areas. Indeed, microiontophoretic application of 5-HT and 8-OHDPAT robustly inhibited hippocampal neurons, an effect blocked by WAY100.635. Interestingly however, spiperone did not block these effects of 5-HT and 8-OHDPAT on postsynaptic 5-HT_{1A} receptors, in contrast to its earlier mentioned blockade of presynaptic 5-HT_{1A} autoreceptors (Blier *et al.*, 1993a). While the activation of DRN 5-HT_{1A} receptors is G_s-mediated, the inhibitory effects of local 5-HT and 8-OHDPAT application were blocked by pretreatment of both pertussis and cholera toxin, demonstrating that 5-HT_{1A} receptors in the hippocampus are coupled to both G_i and G_s proteins (Blier *et al.*, 1993b). Functionally, distinguishable effects have been demonstrated for gepirone and ipsapirone, which acted as full agonists on 5-HT_{1A} receptors in the DRN, but as partial agonists on post-synaptic 5-HT_{1A} receptors (Blier and

de Montigny, 1990; Dong *et al.*, 1997). In the orbitofrontal cortex, chronic administration of the SSRI paroxetine significantly decreased the responsiveness of 5-HT_{1A} receptors to 8-OHDPAT but not 5-HT, demonstrating ligand-specific effects on this receptor population (El Mansari and Blier, 2005). Postsynaptic 5-HT_{1A} receptors couple to $G_{i\alpha 0}$ in addition to $G_{i\alpha 3}$ subunits, indicating different downstream signal transduction from their presynaptic counterparts (Mannoury la Cour *et al.*, 2006).

Notably, lesion of the prefrontal cortex potently reduced the inhibiting effect of 8-OHDPAT on DRN 5-HT neurons by approximately 10-fold, demonstrating afferent modulation of 5-HT firing activity by prefrontal neurons expressing 5-HT_{1A} receptors (Ceci *et al.*, 1994). This effect was ligand-dependent, as the sensitivity of 5-HT neurons to elevated levels of 5-HT, both following iontophoresis or SSRI administration, was unaltered after lesion of the prefrontal cortex (Hajós *et al.*, 1999).

Importantly, hippocampal 5-HT_{1A} receptors do not desensitize following their sustained activation by either 5-HT_{1A} agonists (Blier and de Montigny, 1990; Godbout *et al.*, 1991) or SSRIs (de Montigny *et al.*, 1990). Furthermore, some antidepressant strategies (electroconvulsive therapy [ECT] and tricyclic antidepressants [TCAs]) increase the sensitivity of postsynaptic 5-HT_{1A} receptors (de Montigny and Aghajanian, 1978; de Montigny, 1984). As will be discussed in more detail in section 2.5, enhanced signaling through postsynaptic 5-HT_{1A} receptors is a common effect of antidepressant treatments (Haddjeri *et al.*, 1998b) which might be of clinical relevance in the treatment of MDD.

2.1.3.5 5-$HT_{1B/D}$ receptors

Despite their different genetic basis in human and rodents, the distribution and function of 5-HT_{1D} and 5-HT_{1B} terminal autoreceptors are largely similar across species (Galzin *et al.*, 1992; Saxena *et al.*, 1998). In the rat raphe nuclei, 5-HT_{1D} receptor are the predominant subtype, and activation of these receptors decreased terminals 5-HT release, thereby inhibiting 5-HT release in concert with 5-HT_{1A} autoreceptors (Piñeyro *et al.*, 1995; Piñeyro and Blier, 1996). In the hippocampus, *in vivo* activation of 5-HT_{1B} terminal autoreceptors reduced 5-HT release while 5-HT_{1B} receptor blockade potently increased 5-HT release, indicating that these receptors are tonically activated under physiological conditions (Chaput, Blier, *et al.*, 1986). Although the precise intracellular mechanism by which 5-HT_{1B} receptors provide autoinhibitory feedback on intrasynaptic 5-HT release in the hippocampus remains to be established, the absent effect of both pertussis and cholera toxin pretreatment suggest that these receptors do not couple to Gi/Go or G_s proteins (Blier, 1991).

Importantly, 5-HT_{1B} receptors desensitize following sustained SSRI administration, a unique effect of this drug class (Chaput, Blier, *et al.*, 1986; Chaput *et al.*, 1991; Blier *et al.*, 1988) that enhances 5-HT neurotransmission, and therefore has therapeutic implications for treatment of MDD.

2.1.3.6 5-HT_{2A} receptors

5-HT_{2A} receptors are predominantly expressed on postsynaptic neurons, generally more on soma than on dendrites, with the largest pool of receptors located in the cytoplasm (Cornea-Hébert *et al.*, 1999). 5-HT_{2A} receptors are excitatory; their activation

activates a $G_{q\alpha}$ subunit which stimulates the phospholipase C (PLC) cleavage of phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate (IP_3; Nichols and Sanders-Bush, 2001). DAG activates the PKC pathway and downstream transcription factors, while IP_3 (amongst others) triggers downstream signaling including intracellular release of Ca^{2+}. Interestingly, 5-HT_{2A} receptor activation by endogenous 5-HT activates a ß-arrestin-dependent pathway while the preferential 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) signaling is ß-arrestin independent (Schmid *et al.*, 2008). Thus, activation of the same receptor can, at least in some cases, activate different downstream signaling pathways. Activation of 5-HT_{2A} receptors on different neuronal subpopulations has distinguishable electrophysiological effects; for example, excitation of nucleus accumbens (NAc) neurons predominantly involves a 5-HT_{2A} receptor mediated decrease in K^+ conductance, while that of interneurons in the periform cortex is predominantly Ca^{2+} dependent (North and Uchimura, 1989; Sheldon and Aghajanian, 1991).

Behaviorally, 5-HT_{2A} receptor activation plays a role in the hallucinogenic effects of psychedelic drugs such as LSD, DOI and psilocybin (Nichols, 2004; Kometer *et al.*, 2013), and induces "wet dog shakes" in animal models (Bedard and Pycock, 1977). In part, these effects could be DA-mediated via 5-HT_{2A} receptors located on ventral tegmental area (VTA) DA neurons (Nocjar *et al.*, 2002), which activation has an excitatory effect on their discharge activity and causes elevated DA release in projection areas regions (Bortolozzi *et al.*, 2005). In the treatment of schizophrenia, the lower incidence on motor side effects by atypical antipsychotics has been attributed to 5-HT_{2A} receptor antagonism (Kuroki *et al.*, 2008). Furthermore, blockade of 5-HT_{2A} receptors is

thought to be important for augmentation strategies in depression following an incomplete response, as will be discussed in more detail in section 2.5.12 (Szabo and Blier, 2001b; Blier and Szabo, 2005).

2.1.3.7 5-HT_{2B} receptors

Although 5-HT_{2B} receptor expression was previously reported to be absent in the CNS (Hoyer *et al.*, 1994; Pompeiano *et al.*, 1994), recent studies not only demonstrated expression of this receptor type on DRN 5-HT neurons, but also suggested a role for these receptors in the antidepressant response (Auclair *et al.*, 2010; Diaz *et al.*, 2012). Indeed, pharmacological and genetic inactivation of 5-HT_{2B} receptors prevented the antidepressant-like action of SSRIs in the FST, while conversely, 5-HT_{2B} receptor agonism exerted an antidepressant-like effect in this behavioral test (Diaz *et al.*, 2012). In addition, sustained 5-HT_{2B} receptor antagonism increased the firing activity of VTA DA neurons (Chenu *et al.*, 2014), in line with an acute modulatory effect of 5-HT_{2B} receptor blockade on DA outflow in VTA projection areas (Auclair *et al.*, 2010). Since targeting 5-HT_{2B} receptors could potentially produce pulmonary and cardiovascular side effects (Artigas, 2013), this potential target for antidepressants clearly requires further investigation.

2.1.3.8 5-HT_{2C} receptors

Similarly to 5-HT_{2A} receptors, 5-HT_{2C} receptors are excitatory proteins that are widely expressed in the brain, with high densities in cortical areas, hippocampus, choroid plexus, and striatum (Abramowski *et al.*, 1995). However, in addition to G_q coupling and

PLC activation, 5-HT_{2C} receptor also couple to G_{13} and activate phospholipase D (McGrew *et al.*, 2002). In further contrast to 5-HT_{2A} receptors, 5-HT_{2C} receptor agonists decreased VTA DA neuronal firing and DA output while increasing the discharge activity of non-DA neurons (Di Matteo *et al.*, 2000). Conversely, blockade of 5-HT_{2C} receptors stimulated DA outflow, suggesting that 5-HT_{2C} receptors in the VTA are tonically activated and predominantly located on inhibitory interneurons (Millan *et al.*, 1998; Di Matteo *et al.*, 2000; Di Giovanni *et al.*, 2001). Based on the antagonistic properties at 5-HT_{2C} receptors of the antidepressants agomelatine (Millan *et al.*, 2003), this receptor type might play a role in the treatment of mood disorders.

Although 5-HT_3-5-HT_7 receptors might partake in the antidepressant response (Glennon, 2003; Lucas *et al.*, 2007; Sanchez *et al.*, 2011), their function was not studied the present work, hence their discussion is beyond the scope of the present work.

2.1.4 Abnormalities in the 5-HT system; relevance to MDD

One of the first direct clinical observations linking depression and the 5-HT system stems from studies showing that acute administration of the tryptophan hydroxylase inhibitor PCPA rapidly reinstated depressive symptoms in patients responding to the TCAs imipramine and the MAOI tranylcypromine (Shopsin *et al.*, 1975; Shopsin, 1976). Furthermore, a large proportion of MDD patients were shown to have abnormally low baseline tryptophan plasma content (Møller *et al.*, 1980; Quintana, 1992). Moreover, an amino acid rich, tryptophan-free drink – which decreases central 5-HT synthesis by producing competition for transport between tryptophan and other

amino acids across the BBB – produced low mood, particularly in remitted MDD patients (Delgado, 1990; Smith *et al.*, 1997) or healthy controls with a genetic predisposition towards development of this disorder (Benkelfat *et al.*, 1994). In addition, a higher degree of tryptophan depletion was shown to lower mood more severely (Booij, Van der Does, Haffmans, and Riedel, 2005; Booij, Van der Does, Haffmans, Riedel, *et al.*, 2005). Interestingly, a positron emission tomography (PET) study demonstrated that decreased neural activity in the dorsolateral PFC, thalamus, and anterior cingulate cortex of remitted patients following tryptophan depletion correlated with depressive symptoms, while these physiological and emotional effects were not observed in healthy controls (Bremner *et al.*, 1997).

Female gender is known to be a risk factor for MDD, particularly unipolar depression (Nolen-Hoeksema, 1987). In part, this might be due to sex-specific variations in the 5-HT system; for example, central synthesis of 5-HT occurred at a lower rate in females (Nishizawa *et al.*, 1997), 5-HT transporter expression was decreased in females but not males (Staley *et al.*, 2006), and 5-HT_{1A} receptor binding in forebrain regions was related to MDD in males only (Kaufman *et al.*, 2015).

One of the most robust findings in postmortem studies using brains of depressed individuals is abnormal low 5-HIAA content, suggesting less 5-HT metabolism in MDD patients – a trait also linked to suicide (Mann and Malone, 1997; Placidi *et al.*, 2001). Interestingly, binding potential of 5-HT_{1A} autoreceptors in the dorsal raphe nucleus was shown to be increased, while decreased 5-HT_{1A} receptor binding was found in prefrontal areas, together indicating that 5-HT neurotransmission in postsynaptic areas of depressed individuals was dampened (Arango *et al.*, 1995; Stockmeier *et al.*, 1998). Other

postmortem studies assessing the expression of SERT have shown abnormal low levels of this protein in prefrontal regions of depressed individuals, possibly reflecting a compensatory mechanism for decreased postsynaptic 5-HT neurotransmission (Leake *et al.*, 1991; Owens and Nemeroff, 1994).

Functional studies, using the kinetics of thrombocyte SERT as a proxy for central SERT activity (Pletscher, 1987), repeatedly found reduced SERT activity in depressed patients (Paul, 1981; Meltzer et al, 1981). Decreased central 5-HT transport in depression has also been demonstrated using PET (Malison *et al.*, 1998) and single-proton emission computed tomography (Joensuu *et al.*, 2007). Importantly, a common polymorphism in the SERT promoter region has been shown to reduce 5-HT reuptake, decrease the size of brain regions implicated in depression, to predispose to development of MDD, and to predict the response to antidepressants (Lesch *et al.*, 1996; Arias *et al.*, 2003; Caspi *et al.*, 2003; Pezawas *et al.*, 2005; Karg *et al.*, 2011). Interestingly, brain volume increased in patients remitting with antidepressants, but decreased in non-remitters (Phillips *et al.*, 2012). Together with the obvious argument that SSRIs are clinically efficacious, several lines of evidence suggest that abnormalities in synthesis, transport, neurotransmission, and/or genetic basis of the 5-HT system are implicated in MDD.

2.2 The norepinephrine system

As its name suggests, the elevating effect of adrenal gland extract of norepinephrine on blood pressure (Greek; epi = above, nephros = kidney) was discovered before its central activity as a neurotransmitter (Vogt, 1954). Approximately 40% of NE neurons are located in the lateral tegmental fields (composed of the intermediate reticular zone, lateral paragigantocellularis nucleus and nucleus ambiguous; Dahlström and Fuxe, 1964). These fields project exclusively to lower brain regions and the spinal cord in primates, while they also project to subcortical regions in rats (Delfs *et al.*, 2000). The majority of central NE neurons is situated in the in locus coeruleus (LC; Latin: blue spot, caused by neuromelanin, a by-product of NE synthesis), a brain region located in the dorsal pons just below the floor of the 4th ventricle and directly medial from the trigeminal nerve (Foote *et al.*, 1983). The LC consists of approximately 40,000 neurons in humans and (Mouton *et al.*, 1994) 3,000 cells in the rat (Foote *et al.*, 1983). Main projection areas of LC NE neurons are brain regions involved in sensory processing (e.g. the somatosensory and visual cortices) and the cerebral cortex, hippocampus, thalamus, hypothalamus and amygdala (Gatter and Powell, 1977; Gaspar *et al.*, 1989). The main NE projections in the human brain are illustrated in figure 3**.**

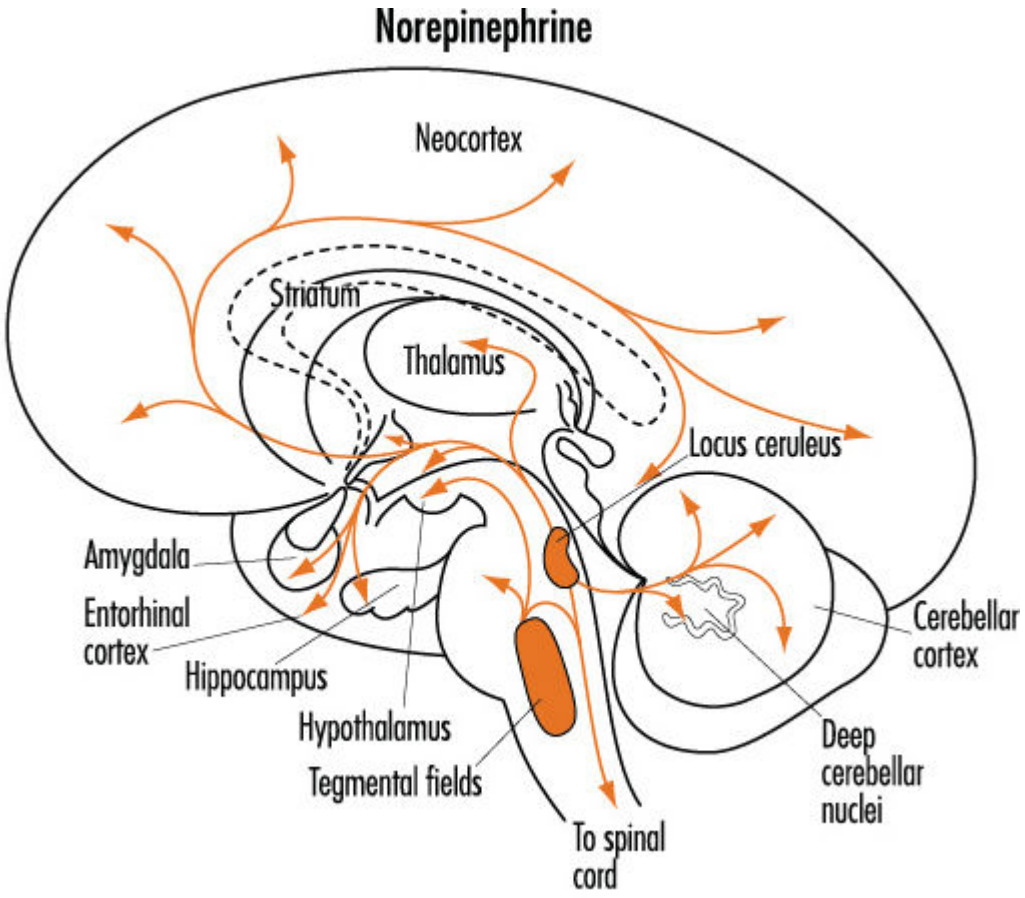

Figure 3: illustration of the major source of NE and innervation of the brain in humans. Figure adapted from (Heimer, 1995).

2.2.1 NE: Synthesis, storage, release, and metabolism

NE is synthesized from the non-essential amino acid L-tyrosine (Greek "tyros": cheese, the medium where it was first isolated from) in three steps: tyrosine hydroxylase (TH) produces L-dihydroxyphenylalanine which is decarboxylated to DA, whereupon DA ß-hydroxylase (DßH) generates NE. TH requires Fe^{2+}, oxygen, and tetrahydrobiopterin as cofactors, and is the rate-limiting step of both NE and DA synthesis (Udenfriend, 1966; Friedman *et al.*, 1972). Consequently, inhibition of TH activity by α-methylparatyrosine (AMPT) non-selectively depletes both of these catecholamines. Following cytoplasmic DA synthesis and storage, NE synthesis occurs in vesicles (Brady *et al.*, 2005). Similarly to 5-HT, NE storage is an active process that requires VMAT coupled to a proton pump, and its vesicular release is Ca^{2+} dependent (Weinshilboum *et al.*, 1971; Blaustein *et al.*, 1972). Following its release, NE can diffuse into the extracellular space, be metabolized by catechol-O-methytransferase (COMT) to normetanephrine, or re-enter the presynaptic terminal following reuptake by the NE transporter (NET). Similarly to 5-HT, the latter route is the main pathway to terminate the action of NE (Brady *et al.*, 2005). In the cytoplasm, NE is repackaged in vesicles by action of the VMAT, or oxidized by MAO-A resulting in 3,4-dihydroxymandelic acid, 3-methoxy-4-hydroxyphenylglycol (MHPG), and vanillylmandelic acid (VMA; Blinc *et al.*, 1989). In the CNS, MHPG is the main metabolite and therefore a commonly used measure for NE metabolism. A summary of the most important intra- and extracellular mechanisms for NE neurotransmission is illustrated in figure 4, and electrophysiological findings relevant to the present work on these components will be discussed in more detail in the next sections.

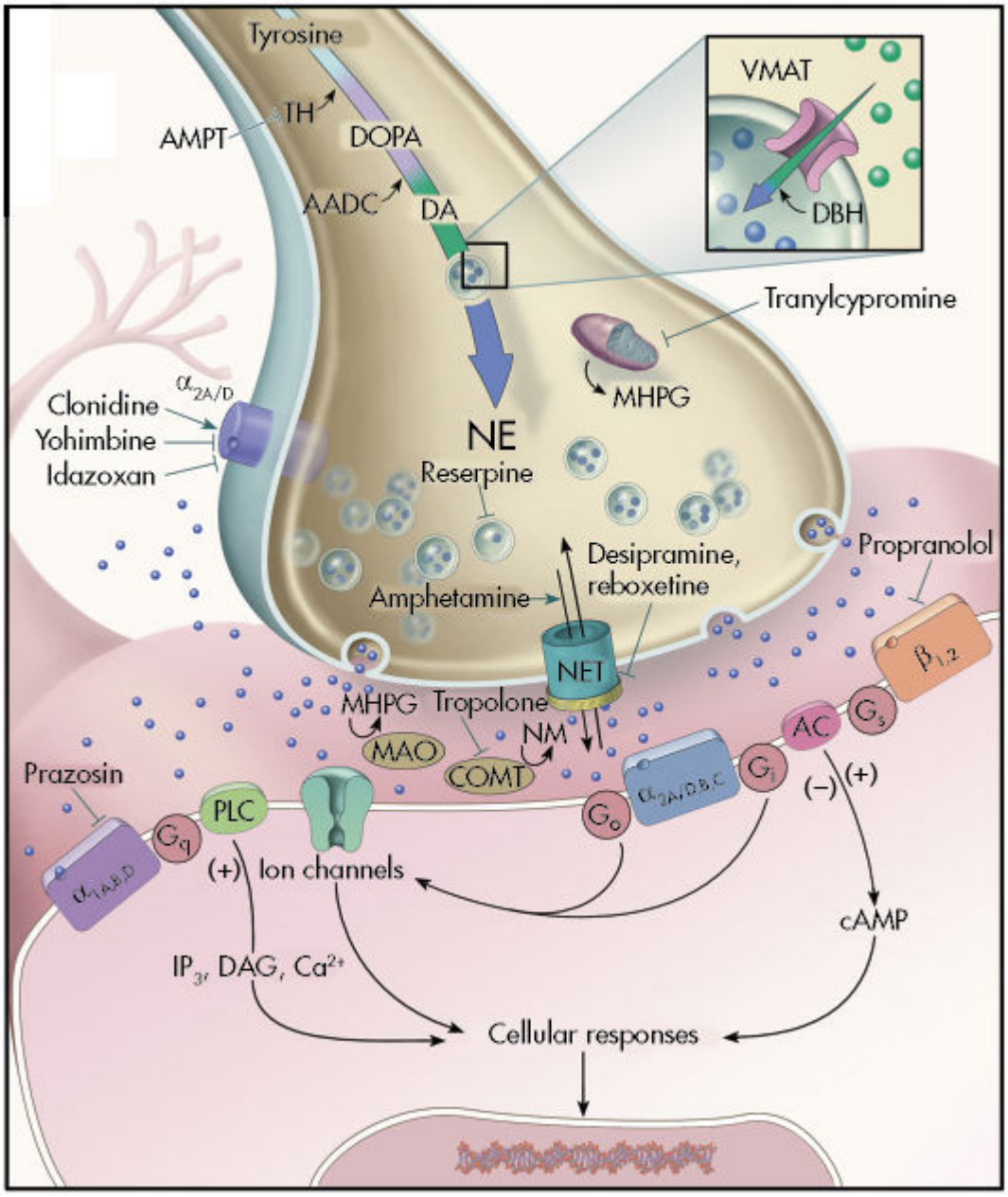

Figure 4: The main intra- and extracellular components involved in NE neurotransmission. Amphetamine is an NE releasing agent used for treatment of attention deficit hyperactivity disorder; alpha-methyl-p-tyrosine (AMPT) is a tyrosine hydroxylase inhibitor that is used for treatment of pheochromocytoma; clonidine is an α_2-adrenoceptor agonist used for treatment of hypertension; desipramine is a NET inhibitor used in the treatment of MDD; idazoxan is an α_2-adrenoceptor antagonists mainly used for experimental purposes; prazosin is an α_1-adrenoceptor antagonist used in treatment of hypertension and nightmares in post traumatic stress disorder; propranolol is a ß-adrenergic receptor antagonist used in treatment of hypertension and migraine prophylaxis; reboxetine is a NET inhibitor used in the treatment of MDD; reserpine is a VMAT blocker that was used for the treatment of hypertension; tranylcypromine is a MAO inhibitor used in the treatment of MDD; yohimbine clonidine is an α_2-adrenoceptor agonist used for treatment of erectile dysfunction. Figure adapted from (Schatzberg and Nemeroff, 2009) with courtesy of S. Szabo.

2.2.2 NET

The NET is a 12-transmembrane protein belonging to the monoamine transporter superfamily (Pacholczyk *et al.*, 1991). In the CNS, expression of the NET is restricted to NE neurons (Lorang *et al.*, 1994). NE transport by the NET - similarly to that of 5-HT - is ATP, Na^+ and Cl^- dependent (Bönisch *et al.*, 1999). In addition to NE, the NET is also involved in DA reuptake (Horn, 1973), demonstrating an overlap of these systems at their transport level. Accordingly, the selective NET blocker atomoxetine was shown to increase both NE and DA levels in the rat prefrontal cortex and hippocampus (Bymaster *et al.*, 2002; Swanson *et al.*, 2006).

The NE transporter is saturable and follows K_m kinetics (Brady *et al.*, 2005). The best characterized intracellular regulator of NET activity is arguably PKC, which has been shown to regulate NET surface density (Apparsundaram *et al.*, 1998), and to reduce its activity by phosphorylation (Bönisch *et al.*, 1997). In addition, tyrosine kinase, nitric oxide, and MAPKs play a regulatory role in NET activity (Zahniser and Doolen, 2001). Interestingly, long-term pharmacological blockade of the NET reduces its activity and expression (Zhu and Ordway, 2002) possibly due to increased intracellular degradation (Torres *et al.*, 2003).

2.2.3 Adrenergic receptors

Based on their different affinities for agonists, adrenergic receptors were originally divided into α-adrenergic and ß-adrenergic receptors (Ahlquist, 1948). Further pharmacodynamic and genetic studies have led to the identification of three families (α_1-,

α_2-, and ß-adrenoceptors), and their final subdivision (in descending order for their affinity for NE) into α_{2C}, α_{2A}, α_{2B}, $ß_1$, $ß_3$, α_{1B}, α_{1A}, α_{1D}, and $ß_2$ adrenoceptor subtypes (PDSP Ki Database; Langer, 1974; Bylund *et al.*, 1994). All of these receptors belong to the superfamily of 7-transmembrane G-protein coupled receptors. Although these receptors are expressed throughout the brain, the most relevant for the present work are those located in the rat DRN, LC, hippocampus, and lateral geniculate nucleus (LGN).

2.2.3.1 α_1-adrenergic receptors

Based on messenger ribonucleic acid (mRNA) expression, α_1-adrenergic receptors are found virtually everywhere in the brain, yet large regional differences exist in subtype expression patterns (Day *et al.*, 1997). For example, the α_{1B}-adrenoceptor is the exclusive subtype in the LGN, α_{1D} is predominant in the hippocampus, and both α_{1A} and α_{1B} are expressed in the DRN and LC. Most α_1-adrenoceptors are excitatory G_q/G_{11} receptors, and their activation causes the earlier described PLC → IP_3 → DAG pathway, activating downstream signals leading to membrane depolarization. Notably, electrophysiological studies have demonstrated region-specific functional differences between α_1-adrenoceptor populations. For example, blockade of α_1-adrenergic receptors had no effect on the firing activity of LC NE neurons *in vivo*, while *in vitro* these receptors reduced membrane depolarization following α_2-adrenergic activation (Marwaha and Aghajanian, 1982; Osborne *et al.*, 2002). Furthermore, α_1-adrenoceptor agonism increase the neural activity of LGN and DRN neurons, however, these receptors are tonically activated only in the DRN (Baraban and Aghajanian, 1980; Rogawski and Aghajanian, 1980). In the

hippocampus, activation of α_1-adrenergic receptors - which are located intrasynaptically on postsynaptic dendrites - by endogenous NE has an inhibitory action on postsynaptic neurons, suggesting that activation of this receptor population causes intracellular mechanisms other than or additional to G_q/G_{11} activation (Curet and de Montigny, 1988b).

2.2.3.2 α_2-adrenergic receptors

Similarly to α_1-adrenergic receptors, the distribution of α_2-adrenoceptor subtype mRNA is heterogeneous; α_{2A} is the predominant subtype in the LC, amygdala, and prefrontal areas, α_{2B} expression is only found in thalamic regions, and α_{2C} is common in the basal ganglia and olfactory regions (Scheinin *et al.*, 1994). To the best of knowledge, α_2-adrenergic receptors are all Gi/Go-coupled; α_2-adrenoceptor agonism reduces adenylyl cyclase activity, which decreases cAMP levels (Andrade and Aghajanian, 1985).

In the LC, α_2-adrenoceptor activation by endogenous NE decreases potassium conductance, which decreases membrane potential and plays an important role in providing autoinhibitory feedback on NE neurons (Williams *et al.*, 1985). These α_2-adrenergic autoreceptors are pertussis-toxin sensitive and furthermore, tonically activated as their inactivation and pharmacological blockade increases the firing activity of LC NE neurons (Simson *et al.*, 1988).

NE neurons express, in addition to autoreceptors at the cell body level, α_2-adrenergic autoreceptors at nerve terminals. The properties of these autoreceptors have been thoroughly characterized by stimulating NE afferents – thereby evoking release of

endogenous NE – while recording pyramidal neurons in the hippocampus. Using this paradigm, pharmacological blockade enhanced inhibition of postsynaptic neurons even under low stimulation frequencies, demonstrating that α_2-adrenergic terminals autoreceptor are heavily tonically activated (Curet and Montigny, 1989). Compared to their 5-HT counterpart, α_2-adrenergic autoreceptors play a larger quantitative role in controlling the release of their endogenous ligand compared to 5-HT_{1B} autoreceptors both *in vitro* and *in vivo*. Indeed, increasing the frequency of electrical stimulations from 1 to 5 Hz in the NE system caused a greater decrease in NE release from NE terminals (~80%) than 5-HT release from 5-HT (~30%; Chaput *et al.*, 1986; Curet and Montigny, 1989; Blier *et al.*, 1990).

In addition to their function as autoreceptors, α_2-adrenergic heteroceptors are also located on 5-HT terminals and those on cornus ammonis region 3 (CA3) pyramidal neurons. On 5-HT terminals, activation of these receptors results in attenuated 5-HT release (Mongeau *et al.*, 1993). Similar to terminal α_2-adrenergic autoreceptors, these heteroceptors are tonically activated; however, the latter receptors have a considerably lower affinity for the α_2-adrenoceptor agonist clonidine (Mongeau *et al.*, 1993). Clearly, the presence of NE receptors on 5-HT terminals provides an interaction node between these systems; the significance of this will be discussed in more detail in sections 2.4.1.1.

On CA3 pyramidal neurons, α_2-adrenergic heteroceptors have been shown to be located extrasynaptically. Indeed, the inhibitory effect of microiontophoretic application of NE on these neurons is potently blunted by α_2-, but not by α_1-adrenoceptor blockade (Curet and de Montigny, 1988a).

Interestingly, administration of agents that act on α_2-adrenoceptors causes region-specific functional changes in α_2-adrenoceptor populations. For example, the α_2-adrenoceptor antagonist mirtazapine (see section 2.5.6) acutely blocked both α_2-adrenergic autoreceptors on LC NE neurons, heteroceptors on 5-HT terminals, and heteroceptors on CA3 pyramidal neurons (Haddjeri *et al.*, 1996). Following sustained mirtazapine administration, the responsiveness of α_2-adrenerig heteroceptors on 5-HT terminals remained blunted to systemic administration clonidine, while α_2-adrenergic autoreceptors on LC NE neurons and heteroceptors on CA3 pyramidal neurons were normosensitive (Haddjeri *et al.*, 1998a). Furthermore, sustained administration of desipramine, a TCA with potent NET blocking properties, desensitized the responsiveness of terminal α_2-adrenergic autoreceptors but not heteroceptors on CA3 pyramidal neurons to NE (Lacroix *et al.*, 1991). In addition, sustained desipramine reduced the responsiveness of α_2-adrenergic autoreceptor on LC NE neurons to iontophoretic application of clonidine, but not to NE, demonstrating that α_2-adrenergic activation can be ligand-specific (Lacroix *et al.*, 1991).

2.2.3.3 ß-adrenoceptors.

Based on their responsiveness to pharmacological challenges, ß-adrenoceptors have been divided into $ß_1$-$ß_3$ subtypes (Bylund *et al.*, 1994). Of these, $ß_1$ and $ß_2$ are the predominant subtypes found in the CNS (but see Claustre et al., 2008) where they are expressed strictly postsynaptically in distribution patterns similar between humans and rodents (Nicholas *et al.*, 1993, 1996). NE has a higher affinity for the $ß_1$ subtype

(Molinoff, 1984), and ß-adrenoceptor subtype expression is brain region specific; $ß_1$-adrenoceptors are predominantly found in the cerebral cortex, oculomotor complex, and thalamus, whereas dense $ß_2$-adrenoceptor expression has been demonstrated in the olfactory bulb, hippocampus, and cerebellum (Nicholas *et al.*, 1993). Notably, the LC and DRN are devoid of ß-adrenergic receptor expression (Nicholas *et al.*, 1996). All ß-adrenergic receptors are excitatory, and activate adenylyl cyclase via G_s-proteins (Bylund *et al.*, 1994).

In the hippocampus, the status of ß-adrenergic receptors can be determined using electrophysiological techniques. Indeed, the previously described inhibitory effect of α_1-adrenoceptor activation on CA3 pyramidal neurons by endogenous NE is followed by a delayed neural activation, an effect blocked by the ß-adrenoceptor antagonist propranolol (Curet and de Montigny, 1988b).

2.2.4 Abnormalities in the NE system; relevance to MDD

NE levels can be depleted with an amino-acid rich, tyrosine and phenylalanine-devoid drink (Moja *et al.*, 1996) or inhibition of TH by AMPT. Based on the common synthesis pathway of DA and NE however, the effects have to be attributed to catecholamine depletion rather than depletion of either DA or NE. Other hand, evidence from animal studies indicated that AMPT is more effective in NE depletion while acute phenylalanine/tyrosine depletion (APTD) has a stronger effect on DA levels (McTavish *et al.*, 1999). Similar to the effects of 5-HT depletion, mood-lowering effects following AMPT administration are most pronounced in remitted MDD patients that responded to

antidepressants that primarily target the NE, but not the 5-HT system (Delgado *et al.*, 1993; Miller *et al.*, 1996a; Ruhé *et al.*, 2007). Interestingly, an AMPT-PET study demonstrated a positive correlation between activity of the ventromedial prefrontal cortex and depressive symptoms in MDD patients (Hasler *et al.*, 2008).

Post-mortem studies have suggested abnormal pre- and postsynaptic α_2-adrenergic receptor signaling in depressed patients that committed suicide. At the cell body level, elevated α_2-adrenergic autoreceptor binding in the LC has been demonstrated, which would theoretically result in dampened NE neurotransmission in projection areas (Ordway *et al.*, 2002, 2003). Postsynaptically, depression-related elevations of α_2-adrenoceptor binding and density were demonstrated in the temporal cortex, prefrontal cortex, and hippocampus (Meana and Garcia-Sevilla, 1987; Javier Meana *et al.*, 1992; De Paermentier *et al.*, 1997; Callado *et al.*, 2002). Upregulation of ß-adrenergic receptor expression and/or binding has also been proposed to play a role in MDD (Mann, 1986; Arango *et al.*, 1990). In keeping with this idea, antidepressants have been shown to downregulate ß-adrenergic receptors in forebrain regions (Anand and Charney, 2000). On the other hand, since blockade of these receptors is anti-therapeutic, the role of ß-adrenergic receptors in mood disorders remains to be fully elucidated (Blier and de Montigny, 1994).

Several studies have assessed whether genetic variation in components of the NE system such as the NET (Ryu *et al.*, 2004; Chang *et al.*, 2007; Buttenschøn *et al.*, 2013), α-adrenergic (Wakeno *et al.*, 2008; Lee *et al.*, 2009) and ß-adrenergic receptors (Zill *et al.*, 2003) could underlie depressive symptoms. However, a strong genetic correlate between altered NE function and depressive symptoms remains yet to be established. On

the other hand, the therapeutic effects of various antidepressants that alter NE neurotransmission (e.g. desipramine, reboxetine, venlafaxine, mirtazapine, and bupropion) indicate that antidepressant effects are mediated through the NE system at least in some patients.

2.3 The dopamine system

In the adult brain, a total of 9 DA containing cell groups has been distinguished and labeled A9-A16 (Dahlström and Fuxe, 1964). In the human brain, the total number of DA neurons has been estimated at 600,000, while in rats this number is approximately 40,000 (Chinta and Andersen, 2005). Based on anatomical and functional characteristics, three midbrain DA systems are distinguishable; the tuberoinfundibular, nigrostriatal, and mesocorticolimbic system. DA neurons of the tuberoinfundibular system are located in the arcuate nucleus of the hypothalamus, and are best characterized for their function in prolactin release (MacLeod and Lehmeyer, 1974; Ojeda *et al.*, 1974). Cell bodies of the nigrostriatal system project from the substantia nigra to the caudate nucleus and putamen, and are crucial for proper voluntary movements. Despite their important roles in everyday life, the present work will focus on the DA neurons in the ventral tegmental area (VTA) due to their important role in psychiatric disorders, including MDD.

VTA DA neurons receive abundant input from neighboring DA cells, however, recurrent axonal projections appear to be absent (Wassef *et al.*, 1981; Grace and Onn, 1989). A large proportion of neurons in the VTA is GABAergic; these neurons locally synapse on DA neurons and provide substantial regulatory input (Omelchenko and Sesack, 2009). VTA DA projections to limbic structures (including NAc, amygdala, and hippocampus) constitute the mesolimbic pathway, and those projecting to the cingulate and prefrontal cortex composing the mesocortical pathway. In addition, VTA DA neurons strongly innervate the LC and to a lesser extent, the DRN (Swanson, 1982; Kalén *et al.*, 1988). An overview of DA pathways in the human brain is illustrated in figure 5**.**

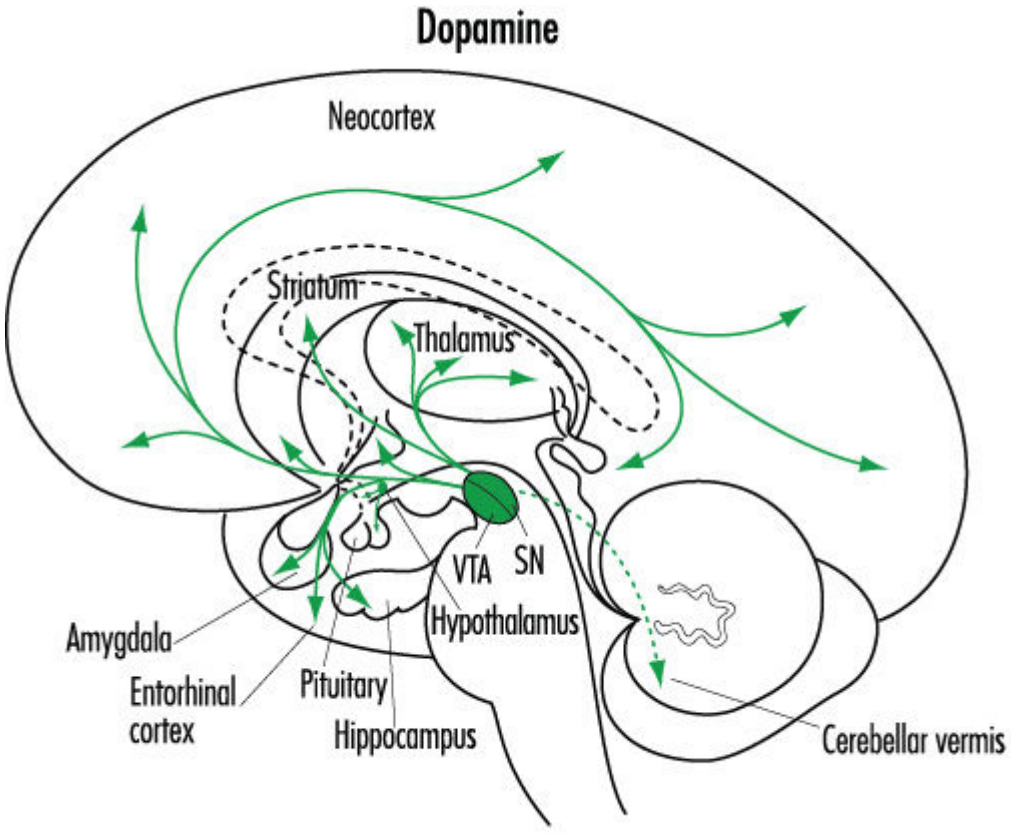

Figure 5: illustration of the major source of DA (VTA and substantia nigra [SN]) and innervation of the human brain. Figure adapted from (Heimer, 1995).

2.3.1 DA: Synthesis, storage, release, and metabolism

As mentioned in section 2.2.1, DA is metabolized in two steps from tyrosine, with enzymatic action of TH being the rate-limiting step (Nagatsu *et al.*, 1964). Although the estimates of intracellular DA concentrations together with enzymatic activity suggest saturation of TH under physiological conditions, a recent microdialysis study demonstrated that local perfusion with L-tyrosine increased DA levels two- to three-fold, suggesting that TH occupancy might be submaximal under some conditions (Brodnik *et al.*, 2012). Notably, the activity of TH is affected by a manifold of intracellular mechanisms; for example, phosphorylation by PKA increases TH activity 20-fold *in vivo* (Daubner *et al.*, 2011).

Following its synthesis, DA is stored in vesicles by action of VMAT coupled to a proton pump (Brady *et al.*, 2005). Vesicular release of DA is calcium-dependent, however, intrasynaptic concentrations of DA can be enhanced by several orders of magnitude depending on the neuron firing mode (phasic vs. burst; Woodward *et al.*, 1988; Moore *et al.*, 1999). In addition to a Ca^{2+}-dependent release mechanism, amphetamine causes a channel-like conformational change in the DA transporter (DAT) by which DA can be released independent of neural discharge (Robertson *et al.*, 2009). Termination of DA action is mainly mediated by reuptake and is accomplished by both the DAT and the NET, the latter with a greater affinity for DA that plays an important physiological function in cortical regions (Morón *et al.*, 2002). In the cytoplasm, DA is repackaged in vesicles by action of the VMAT, or oxidized by MAO (A and B), COMT and aldehyde dehydrogenase to its final metabolite homovanillic acid (HVA). An overview of important mechanisms for DA neurotransmission is illustrated in figure 6,

and some of these components relevant to the present work will be discussed in detail in the next section.

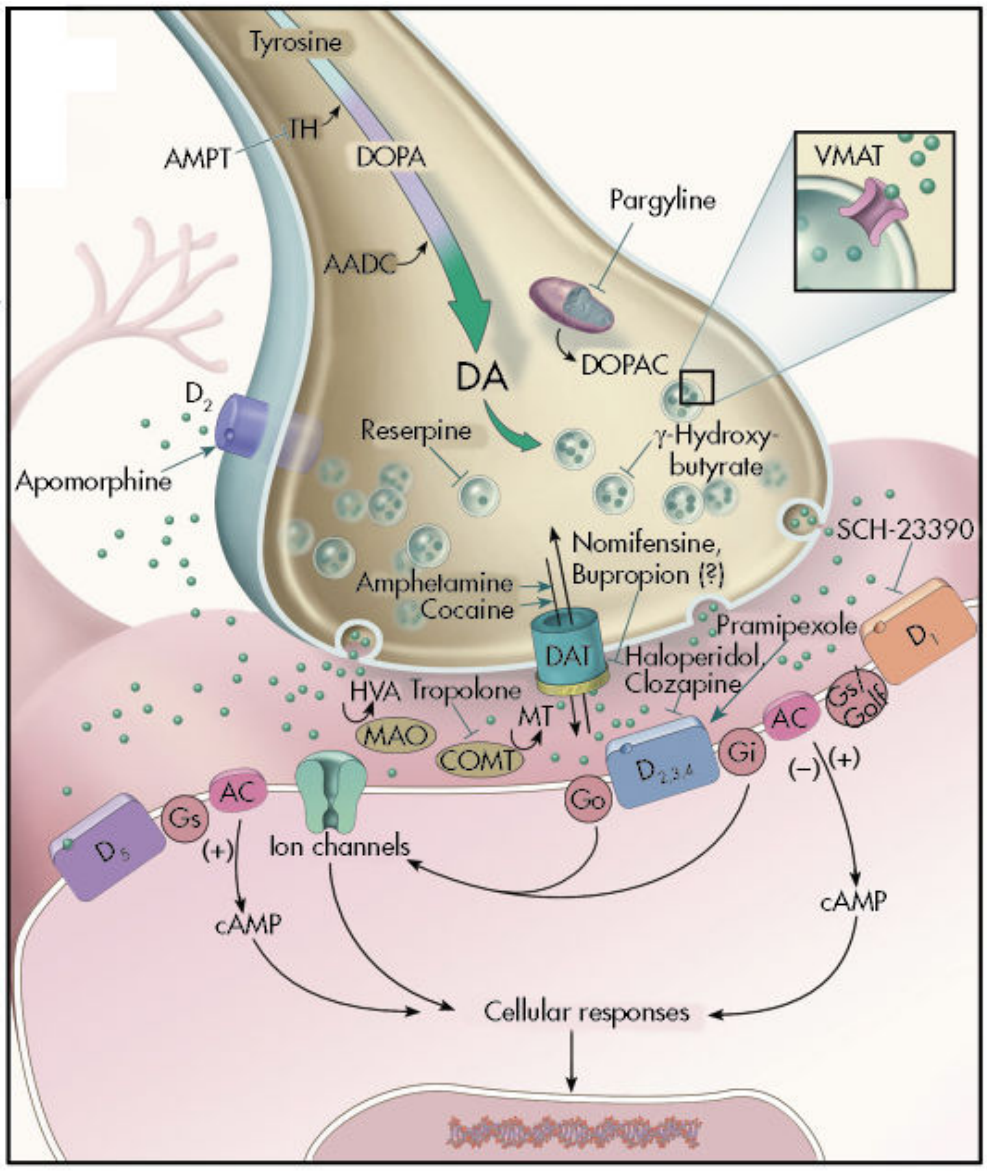

Figure 6: The main intra- and extracellular components involved in DA neurotransmission. Amphetamine is a DA releasing agent used for the treatment of attention deficit hyperactivity disorder and recreational purposes, alpha-methyl-p-tyrosine (AMPT) is a tyrosine hydroxylase inhibitor that is used for treatment of pheochromocytoma; apomorphine is a DA receptor agonist used in the treatment of Parkinson's disease; bupropion is a DA releaser used for smoking cessation and the treatment of MDD; clozapine and haloperidol are DA receptor antagonists mainly used in the treatment of schizophrenia; cocaine is a DAT blocker and DA releasing agent used for topical anesthesia and recreational purposes; γ-hydroxybutyrate is an endogenous impulse propagation inhibitor used in the treatment of narcolepsy; nomifensine is a DAT inhibitor that was used in the treatment of MDD; pramipexole is a DA receptor agonist used in the treatment of Parkinson's disease, restless leg syndrome, and MDD; reserpine is a VMAT blocker that was used for the treatment of hypertension. Figure adapted from (Schatzberg and Nemeroff, 2009) with courtesy of S. Szabo.

2.3.2 DA receptors

Five DA receptors have been identified; in order of their affinity for DA these are D_3, D_5, D_4, D_2 and D_1 (PDSP Ki Database). Based on their genetic and functional characteristics, D_1 and D_5 receptors are excitatory and commonly referred to as "D_1-like" receptors; D_2-D_4 are inhibitory and referred to as "D_2-like".

D_1 receptors are predominantly expressed postsynaptically; and dense populations have been found in limbic regions, including the caudate, putamen, nucleus acumens and amygdala, as well as in the hippocampus and prefrontal cortex (Levey *et al.*, 1993; Jaber *et al.*, 1996; Hurd *et al.*, 2001). Central D_5 receptors are predominantly expressed postsynaptically, at relatively low levels in the prefrontal cortex, hippocampus, and premotor cortex (Bergson *et al.*, 1995). Notably, these receptors are not expressed in the substantia nigra and VTA (Hurd *et al.*, 2001). D_1-like receptors have been grouped based on their adenylyl cyclase-activating properties via coupling to excitatory G_s proteins (Dearry *et al.*, 1990; Jaber *et al.*, 1996). However, recent evidence indicates that they activate alternate G-protein mediated second-messenger cascades (Sidhu, 1998; Mannoury la Cour *et al.*, 2007). Indeed, pertussis toxin prevented activation of selective D_1 receptor agonists in the prelimbic cortex, thus demonstrating Gi/Go coupling of D_1 receptors (Wang *et al.*, 2002).

D_2 receptors are expressed in both pre- and postsynaptically, and are located in the olfactory bulb, caudate, putamen, hippocampus, and in brainstem nuclei, including the VTA and substantia nigra (Levey *et al.*, 1993; Hurd *et al.*, 2001). D_3 receptor expression is restricted to limbic regions and their afferents, e.g. substantia nigra, globus pallidus and NAc, although D_3 receptor expression has been reported to be absent in the

VTA (Gurevich and Joyce, 1999). In contrast to D_3 receptors, D_4 receptors are predominantly found in higher brain regions, while scarce in limbic regions (Primus *et al.*, 1997). D_2-like receptors were originally grouped for their inhibitory effect on adenylyl cyclase and couple to $G_{i/o}$ family of G-proteins (Kebabian and Calne, 1979), and their agonism inhibits calcium channels while activating GIRK channels (Neve *et al.*, 2004).

VTA D_2 autoreceptors are located somatodendritic, are pertussis-toxin sensitive, and tonically activated (Bunney *et al.*, 1973; Missale *et al.*, 1998). Since these receptors are found extrasynaptic, autoinhibition likely occurs via a volume-transmission mediated mechanism (Fuxe and Agnati, 1991). Alternatively splicing of the gene encoding for D_2 receptors gives rise to D_2-short (D_2S) and D_2-long (D_2L) variants, of which D_2S-receptors are more sensitive to agonist activation, inhibit adenylyl cyclase more potently, and presumably are the predominant mediators of VTA DA neuron activity (Hayes *et al.*, 1992; Montmayeur *et al.*, 1993; Centonze *et al.*, 2002). Importantly, sustained excitation of VTA DA neurons causes a depolarized state which renders these neurons inactive, an effect produced by sustained D_2 receptor antagonist administration as well as long-term iontophoretic application of glutamate (Chiodo and Bunney, 1983; Grace and Bunney, 1986). Accordingly, this hyperpolarized state can be reversed by manipulations that normally cause inhibition of VTA DA neurons, such as systemic administration of the D_2 receptor apomorphine, iontophoretic application of GABA, and intracellular injection of a hyperpolarizing current (Grace and Bunney, 1986). Furthermore, D_2 receptors become more sensitive to administration of agonists following their long-term blockade (Vogelsang and Piercey, 1985). Notably, the depolarization block commonly induced by

first- and second-generation antipsychotics might have a greater impact on limbic than cortical areas. Indeed, pertussis toxin infusion of the VTA was shown to decrease DA levels in the NAc but not prefrontal cortex, indicating that DA neurotransmission in the former area is predominantly impulse dependent, while DA levels are largely regulated at the terminal level in cortical regions (Frånberg *et al.*, 2009).

2.2.3 Abnormalities in the DA system; relevance to MDD

With the earlier discussed overlap in synthesis pathways and consequently, depletion pathway of NE and DA in mind (Ruhé *et al.*, 2007), it is noteworthy that APTD was shown to lower mood in healthy females, particularly in combination with acute stress (Leyton *et al.*, 2000). Interestingly, this procedure impaired memory and reward-punishment tasks, but critically not mood in remitted MDD patients, suggesting that DA system alterations could be involved in cognitive and hedonic domains possibly secondary to depressed mood (McTavish *et al.*, 2005; Roiser *et al.*, 2005). In line with this hypothesis, activation of the DA system by the DA releaser amphetamine produced a greater response to reward in depressed patients than controls, and was correlated with altered function of the striatum, putamen, and ventrolateral prefrontal cortex (Tremblay *et al.*, 2002, 2005).

One post-mortem studies found elevated binding and decreased expression of D_2 receptors in the amygdala of MDD patients, although no differences were found in the caudate, putamen and NAc (Bowden *et al.*, 1997) (Klimek *et al.*, 2002). Furthermore, brain content of TH, DA and HVA was generally found to be normal in depressed

patients (Beskow *et al.*, 1976; Bowden *et al.*, 1997; Howes *et al.*, 2013). On the other hand, cerebrospinal fluid and blood plasma of intact depressed patients has repeatedly been shown to contain lower HVA levels than controls (van Praag and Korf, 1971; Mendels *et al.*, 1972; Lambert *et al.*, 2000).

Imaging studies using PET showed enhanced D_2 receptor binding in depressed subjects (D'haenen and Bossuyt, 1994; Shah *et al.*, 1997; Klimke, 1999). Furthermore, DAT activity has also been found to be enhanced in limbic regions of depressed patients (Laasonen-Balk *et al.*, 1999; Newberg *et al.*, 2007; Amsterdam *et al.*, 2012). Although these data do not allow a differentiation between DAT expression and sensitivity, and could be confounded by intrasynaptic competition between endogenous DA and the radioactive tracer, these data suggest a role for imbalanced limbic DA activity in MDD.

Genetic variation in the gene encoding for D_4 receptors has been identified as a risk factor for development of depression (López León *et al.*, 2005). Notably, polymorphisms in the genetic basis of other DA receptors or the DAT have not been related to MDD (Manki *et al.*, 1996; Frisch *et al.*, 1999).

Several antidepressant agents mediate their antidepressant effect in part through the DA system (Dunlop and Nemeroff, 2007). Indeed, the antidepressants bupropion, nomifensine, and pramipexole all enhance DA neurotransmission (Hunt *et al.*, 1974; Goodnick and Goldstein, 1998; Corrigan *et al.*, 2000; Zarate *et al.*, 2004; Katz *et al.*, 2010; Sporn *et al.*, 2011). A similar therapeutic effect has been implicated for the DA agonists bromocriptine (Wæhrens and Gerlach, 1981; Inoue *et al.*, 1996), piribedil (Post, 1978), and pergolide (Izumi *et al.*, 2000) although arguably, interpretation of these study outcomes is limited due to their small samples size and/or comorbidity with other

dopamine-related disorders such as Parkinson's disease and schizophrenia. Finally, therapeutic effects of pergolide and pramipexole were shown when administered in adjunct to antidepressants (DeBattista *et al.*, 2000; Perugi *et al.*, 2001; Goldberg *et al.*, 2004; Roesch-Ely *et al.*, 2006; Sporn *et al.*, 2011; Cusin *et al.*, 2013).

2.4 Monoamine system interactions

The regulation of monoamine systems is in part mediated by autoinhibition, and part by reciprocal interactions between the DA, 5-HT, and NE systems, both at the pre- and postsynaptic level. Some of the most relevant mediators of DRN 5-HT, LC NE, and VTA DA firing activity are summarized in figures 7-9**,** respectively. In the next sections, electrophysiological evidence for and consequences of these interactions will be discussed in detail.

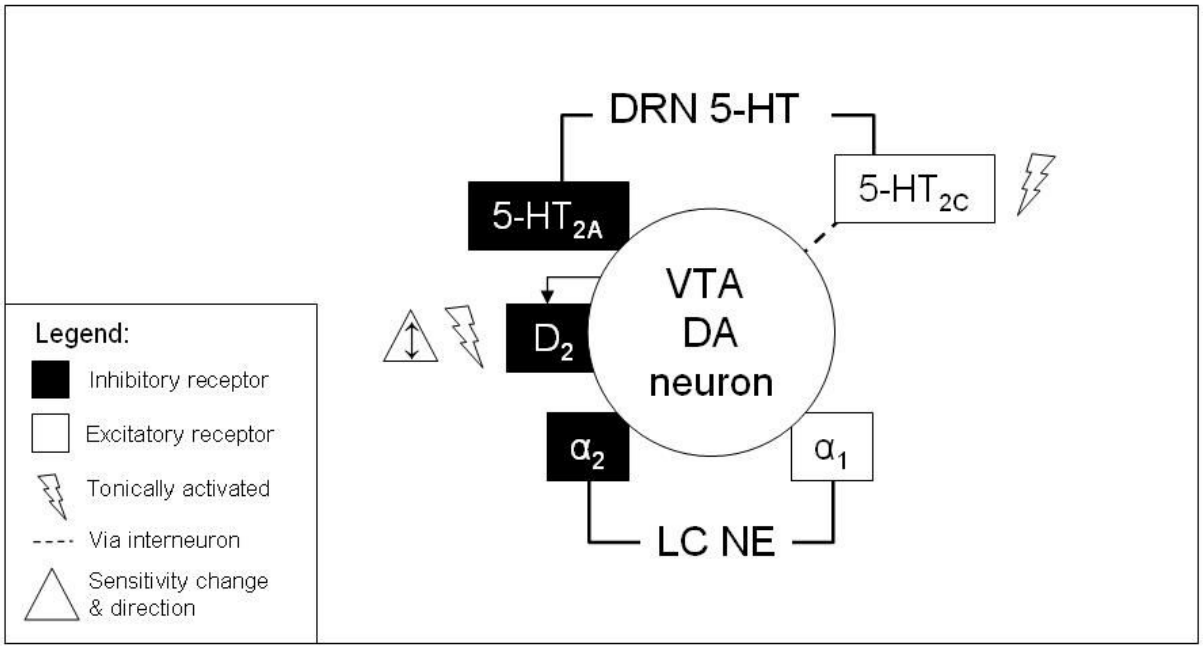

Figure 7: Summary of relevant receptor populations, and their effects on firing activity of VTA DA neurons under baseline conditions. Lightning symbols indicate tonic activation of a receptor population under baseline conditions; triangles illustrate that a receptor population is subject to sensitivity change; ↕ indicates both increased sensitivity and desensitization.

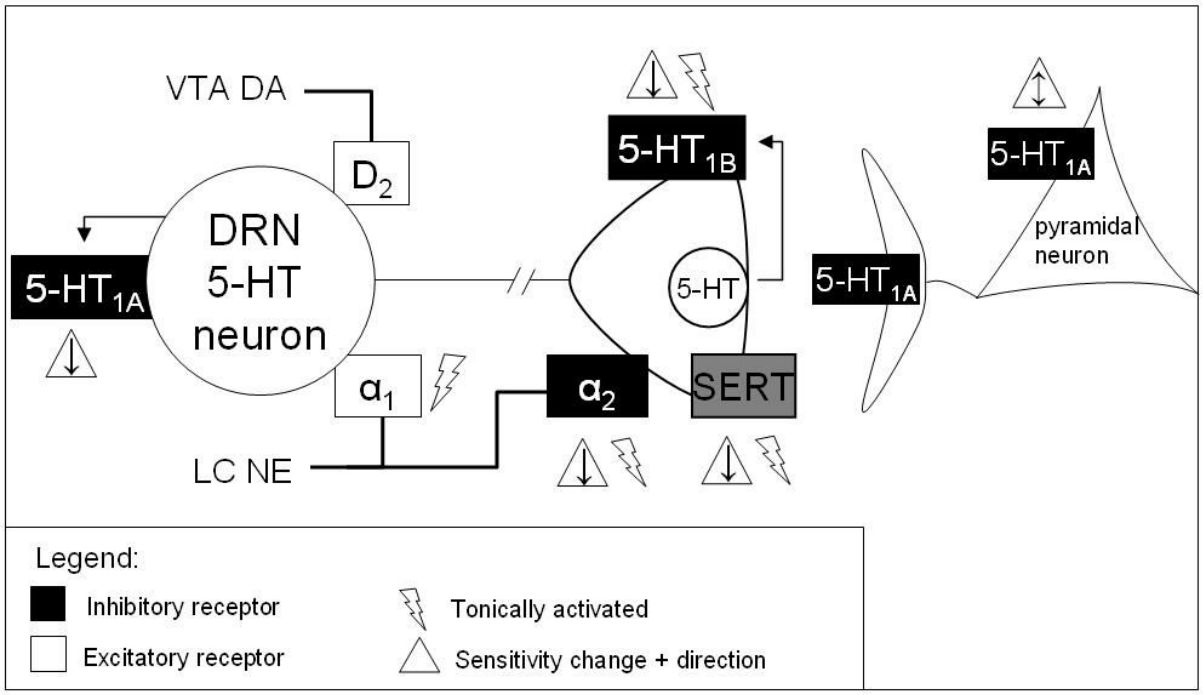

Figure 8: Summary of relevant receptor populations mediating firing activity of DRN 5-HT neurons, postsynaptic 5-HT release, and 5-HT neurotransmission. Lightning symbols indicate tonic activation of a receptor population under baseline conditions; triangles illustrate that a receptor population is subject to sensitivity change; ↓ indicates desensitization, ↕ indicates both increased sensitivity and desensitization.

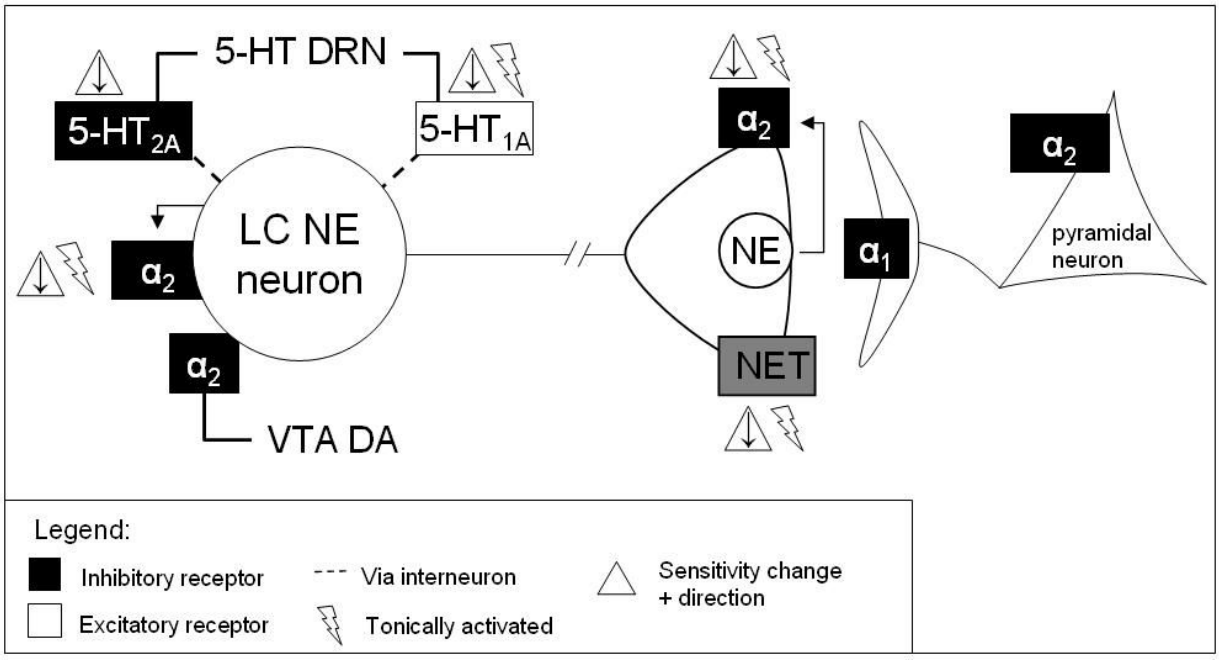

Figure 9: Summary of relevant receptor populations mediating firing activity of LC NE neurons, postsynaptic NE release, and NE neurotransmission. Lightning symbols indicate tonic activation of a receptor population under baseline conditions; triangles illustrate that a receptor population is subject to sensitivity change; ↓ indicates desensitization.

2.4.1.1 NE & 5-HT: presynaptic interactions

One of the first electrophysiological demonstrations for excitatory input to DRN 5-HT neurons from the NE LC ame from the demonstration that systemic clonidine administration inhibited both LC NE and DRN 5-HT neurons, while its microiontophoretic application only inhibited NE neurons (Svensson *et al.*, 1975). Furthermore, lesion of NE neurons by 6-hydroxy-dopamine (6-OHDA) caused that "for the first few days, 5-HT cells fired slowly and erratically, consistent with the loss of adrenergic tone. However, in animals tested 4-7 days after injection [of 6-OHDA, red] the normal rate and pattern of firing resumed" (Baraban and Aghajanian, 1980). In subsequent studies, attenuated firing activity of DRN 5-HT neurons was demonstrated following iontophoretic application of α_1-adrenergic receptor antagonists, indicating that the tonic activation of these adrenergic receptors drives the firing activity of DRN 5-HT neurons (Baraban and Aghajanian, 1980). Electron microscopy data showed that NE terminals directly innervate 5-HT neurons, and that α_1-adrenergic receptors are located on the soma and dendrites of 5-HT neurons, providing a direct pathway from the LC to the DRN (Baraban and Aghajanian, 1981). Interestingly, after lesion of NE neurons the firing activity of 5-HT neurons gradually recovered by a mechanism that remains to be elucidated (Svensson *et al.*, 1975).

Central 5-HT depletion, either by lesion with 5,7-dihydroxytryptamine (5,7-DHT) or synthesis inhibition with PCPA was shown to increase the firing activity of LC NE neurons, indicating tonic inhibition of LC NE neurons by 5-HT (Reader *et al.*, 1986; Haddjeri *et al.*, 1997). Conversely, systemic administration of the 5-HT_{2A} receptor agonist DOI fully inhibited LC NE neurons, an effect reversed by the selective 5-HT_{2A}

receptor antagonist R-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]-4-piperidyl]-methanol (M100907), clearly demonstrating an inhibitory action of this receptor type on NE neurons (Szabo and Blier, 2001b). Since iontophoretic application of DOI and 5-HT did not alter the firing activity of LC NE neurons while iontophoretic application of the $GABA_A$ antagonist bicuculline prevented the inhibitory effect of systemic administration of DOI on these neurons, it was suggested that 5-HT_{2A} receptors are located on GABA neurons outside the LC (Chiang and Aston-Jones, 1993; Haddjeri *et al.*, 1997). In further support of this, 5-HT_{2A} receptor mRNA is absent in the LC (Pompeiano *et al.*, 1994). Lesion of the hypoglossal nucleus prevented the inhibitory effect of DOI on LC NE neurons, while DOI application in the former brain region did not alter the firing activity of NE neurons, suggesting that the inhibitory effect of 5-HT_{2A} receptor agonists on LC NE neurons is produced by activation of excitatory 5-HT_{2A} receptors on GABA terminals (Gorea *et al.*, 1991). Notably, systemic M100907 administration did not alter the firing activity of LC NE neurons, indicating that under baseline conditions, these 5-HT_{2A} receptors are not tonically activated (Szabo and Blier, 2001b). However, 5-HT_{2A} receptors become tonically activated following 5-HT_{1A} receptor manipulation. Indeed, systemic administration of the 5-HT_{1A} receptor agonist 8-OHDPAT increased LC NE firing while the antagonist WAY 100.635 - at a dose that did not change firing of 5-HT neurons (Fletcher *et al.*, 1996) - produced a complete inhibition of these neurons (Szabo and Blier, 2001b). The latter effect was prevented by 5,7-DHT pretreatment and reversed with systemic prazosin, ritanserin and M100970 administration (Haddjeri *et al.*, 1997; Szabo and Blier, 2001b), demonstrating that both intact firing activity of 5-HT neurons and functional 5-HT_{2A} receptors are required for the inhibitory effect of WAY on LC NE

firing. Since M100907 reversed the effect of WAY, it is likely that 5-HT_{1A} receptor blockade enhanced the tonic activation of 5-HT_{2A} receptors. Given the absent effect of iontophoretic application of 5-HT on NE neurons (Pompeiano *et al.*, 1992), together with absent 5-HT_{1A} receptor mRNA expression in the LC (Verge *et al.*, 1985), the data suggested that 5-HT_{1A} receptors are also located outside the LC. Based on anatomical and pharmacological evidence, these 5-HT1A receptors are presumably located on glutamatergic neurons in the paragigantocellularis nucleus (Bobker and Williams, 1989; Szabo and Blier, 2001b).

2.4.1.2 NE & 5-HT: postsynaptic interactions

In the hippocampus, NE tonically activates α_2-adrenergic heteroceptors on 5-HT terminals. Indeed, the effectiveness of 5-HT bundle stimulation to release 5-HT on pyramidal CA3 neurons was significantly attenuated following systemic administration of the NET blocker desipramine, an effect reversed by α_2-adrenoceptor antagonists (Mongeau *et al.*, 1993). 6-OHDA pretreatment attenuated the enhancing effect on 5-HT release of a low dose of clonidine while the dampening effect on 5-HT release of a high dose of clonidine remained intact after lesion of NE neurons, indicating that the low dose of clonidine preferentially activated terminal α_2-adrenergic autoreceptors thereby decreasing NE release and tonic activation of α_2-adrenergic heteroceptors, while the high dose preferentially activated α_2-adrenergic heteroceptors causing decreased 5-HT release per electrical impulse (Mongeau *et al.*, 1993). These α_2-adrenergic heteroceptors are pertussis toxin sensitive, and desensitize following sustained NET blockade (Yoshioka *et al.*, 1992, 1995; Mongeau *et al.*, 1994; Matsumoto *et al.*, 1995).

In addition to these well-established connections, a microdialysis study showed that local DOI perfusion enhanced NE levels in the prefrontal cortex, an effect prevented by M100907. Thus, these data indicate that 5-HT plays a role in NE release at the terminal level (Frånberg *et al.*, 2012). Conversely, local perfusion with clonidine reduced 5-HT output in the prefrontal cortex while α_2-adrenergic antagonists perfusion had no effect on this measure, indicating that α_2-adrenergic receptors partake in local regulation of 5-HT levels (Hertel *et al.*, 1999; Frånberg *et al.*, 2012).

2.4.2.1 5-HT & DA: presynaptic interactions

At the cell body level, the 5-HT system provides both excitatory and inhibitory input to the VTA via 5-HT_{2A} and 5-HT_{2C} receptors, respectively (Millan *et al.*, 1998; Nocjar *et al.*, 2002). The inhibitory input through 5-HT_{2C} receptor is predominant, as acute administration of 5-HT_{2C} receptor antagonists increased VTA DA firing activity (Millan *et al.*, 1998; Blackburn *et al.*, 2006). Furthermore, lesion of the DRN significantly enhanced the firing and burst activity of DA neurons (Guiard, El Mansari, Merali, *et al.*, 2008), and SSRIs produce a significant reduction of VTA DA neuron firing activity (Di Mascio *et al.*, 1998; Dremencov *et al.*, 2009). The latter effect was reversed with the selective 5-HT_{2C} receptor antagonist SB242084 (Dremencov *et al.*, 2009), an agent devoid of effect on the firing activity of VTA DA neurons following its subacute and sustained administration (Chenu *et al.*, 2008, 2014). Moreover, the 5-HT_{1A} receptor agonists 8-OHDPAT, flesinoxan and S15535 were shown to enhance the firing activity of VTA DA neurons at doses that fully inhibit DRN 5-HT neurons (Arborelius, Chergui, *et al.*, 1993; Lejeune and Millan, 1998). The effect of the latter two agents was reversed

with WAY 100.635, which did not alter the firing activity of VTA DA neurons when administered alone, thereby indicating that 5-HT neurons have an overall inhibitory effect on these neurons (Lejeune and Millan, 1998). Although 5-HT_{2A} receptor agonists increased the firing activity of VTA DA neurons and prefrontal DA levels, M100907 had no effect on the firing activity of VTA DA neurons at low doses, indicating that 5-HT_{2A} receptors on VTA DA neurons are not tonically activated by 5-HT (Pessia *et al.*, 1994; Minabe *et al.*, 2001; Nocjar *et al.*, 2002).

From the VTA, DA projections mediate an excitatory input on DRN 5-HT neurons through D_2-like receptors. Indeed, acute systemic apomorphine administration increases the firing activity of (most) 5-HT neurons, an effect prevented by the D_2 receptor antagonist raclopride (Martín-Ruiz *et al.*, 2001). Anatomical and physiological evidence further suggests that this excitatory effect could be mediated through D_2 receptors directly located on DRN 5-HT neurons (Mansour *et al.*, 1990; Haj-Dahmane, 2001), as well as outside the DRN (Martín-Ruiz *et al.*, 2001). Notably, the D_2 receptor antagonist raclopride and paliperidone did not alter the firing activity of DRN 5-HT neurons, suggesting that this D_2 receptor population is not tonically activated (Martín-Ruiz *et al.*, 2001; Chernoloz *et al.*, 2009). However, lesion of VTA DA neurons attenuated the firing activity of DRN 5-HT neurons by 60%, demonstrating an overall excitatory effect of DA neurotransmission on the firing activity of 5-HT neurons (Guiard, El Mansari, Merali, *et al.*, 2008).

2.4.2.2 5-HT & DA: postsynaptic interactions

Application of the 5-HT_{1A} receptor agonist BAY 3702 in the prefrontal cortex activated the firing and burst activity of VTA DA neurons. Since this effect was blocked by WAY 100635 as well as frontocortical transsection, these data demonstrate that 5-HT_{1A} receptors, presumably located on pyramidal neurons (Santana *et al.*, 2004), increase frontal DA levels by activating VTA DA neurons via a cortical loop (Arborelius, Nomikos, *et al.*, 1993; Díaz-Mataix *et al.*, 2005). Similarly, systemic administration of - and prefrontal perfusion with - DOI increased the firing activity of VTA DA neurons, demonstrating excitatory effects of 5-HT_{2A} receptors via corticolimbic projections (Bortolozzi *et al.*, 2005). In addition to reversing these effects, M100907 applied at a high concentration was shown to decrease prefrontal DA levels, suggesting an effect of 5-HT_{2A} receptors on DA release at the nerve terminal level (Pehek *et al.*, 2001; Frånberg *et al.*, 2012).

2.4.3 DA-NE interactions

The LC receives a strong DA projection from the VTA, and expression of D_2 receptors has been reported in this brain region (Swanson, 1982; Kalén *et al.*, 1988; Yokoyama *et al.*, 1994). Lesion of the VTA was shown to increase the firing activity of LC NE neurons by approximately 50%, indicating an overall inhibitory effect of DA on these neurons (Guiard, El Mansari, Merali, *et al.*, 2008). Interestingly, the attenuating effect of DA microiontophoresis on LC NE neurons was blocked to a greater degree by idazoxan than by raclopride and haloperidol, demonstrating that DA signaling in the LC

predominantly occurs via α_2-adrenoceptors (Guiard, El Mansari, and Blier, 2008). It should be noted, however, that acute administration of the D_2 receptor haloperidol - which is known to increase VTA DA neural activity and output (Chiodo and Bunney, 1983; Westerink *et al.*, 1998) - enhanced the activity of LC NE neurons (Ramirez and Wang, 1986). This effect was prevented by pharmacological blockade of N-methyl-D-aspartate (NMDA) receptors, demonstrating an additional indirect, glutamate-dependent effect of DA on LC NE firing (Nilsson *et al.*, 2005).

Projections from the LC to the VTA terminate in close vicinity to DA neurons (Simon *et al.*, 1979). Electric stimulation of the LC causes an early excitation followed by attenuated firing activity of VTA DA neurons, an effect prevented by reserpine pretreatment, thus demonstrating NE neurotransmission on DA neurons (Grenhoff *et al.*, 1993). This excitation was blocked with prazosin, demonstrating α_1-adrenoceptor mediation of this effect, while α_2-adrenoceptor antagonists did not alter the effect of LC stimulation (Grenhoff *et al.*, 1993). Reserpine also prevented the attenuating effect of prazosin on burst-mode firing activity of DA neurons, further demonstrating NE-mediated excitatory input to the VTA (Grenhoff and Svensson, 1993). Notably, iontophoretic application of NE has an inhibitory effect on VTA DA neurons (Guiard, El Mansari, and Blier, 2008). This effect was prevented by systemic idazoxan but not haloperidol administration, indicating α_2-adrenoceptor mediation of this effect, possibly of the α_{2C} subtype (Lee *et al.*, 1998; Inyushin *et al.*, 2010). In keeping with an overall inhibitory effect of NE on DA neurons, LC lesion increased the firing activity of VTA DA neurons by 50% (Guiard, El Mansari, Merali, *et al.*, 2008). Notably however, iontophoretic application of clonidine had a little effect on the firing activity of DA

neurons (Aghajanian and Bunney, 1977; White and Wang, 1984). Together with the aforementioned limited effect of α_2-adrenoceptor blockade on stimulus-evoked inhibition of VTA DA neurons *in vivo*, and little effect of this receptor blockade on the membrane potential of VTA DA neurons *in vitro* (Grenhoff *et al.*, 1995), the precise role of α_2-adrenoceptors on VTA DA firing activity remains to be fully characterized. Notably, idazoxan significantly attenuated the inhibitory effect of iontophoretic DA application in the VTA, suggesting a role for these α_2-adrenoceptors in DA signaling (Guiard, El Mansari, and Blier, 2008).

A similar overlap in receptor activation of DA and NE has been demonstrated in the hippocampus CA3 region, where inhibition of pyramidal neurons by iontophoretic application of these neurotransmitters was blocked by systemic idazoxan, but not haloperidol administration (Guiard, El Mansari, and Blier, 2008). In the same study, NET blockade reduced reuptake of both DA and NE, demonstrating reuptake of both catecholamines by the same transporter protein. This was not so unexpected, given the structural similarities of these neurotransmitters at the molecular level, and the higher affinity of the NET for DA than for NE (Giros *et al.*, 1994). Furthermore, it provides a mechanism by which DA reuptake is accomplished in DA projection areas with little DAT expression (Bymaster *et al.*, 2002; Morón *et al.*, 2002; Carboni and Silvagni, 2004).

2.5 Antidepressants & monoamine systems

Virtually all antidepressant strategies modify the activity of one or more monoamine systems. In figure 10 and table I, the most relevant actions of antidepressants for this work are summarized. In the next sections, these actions are discussed in more detail.

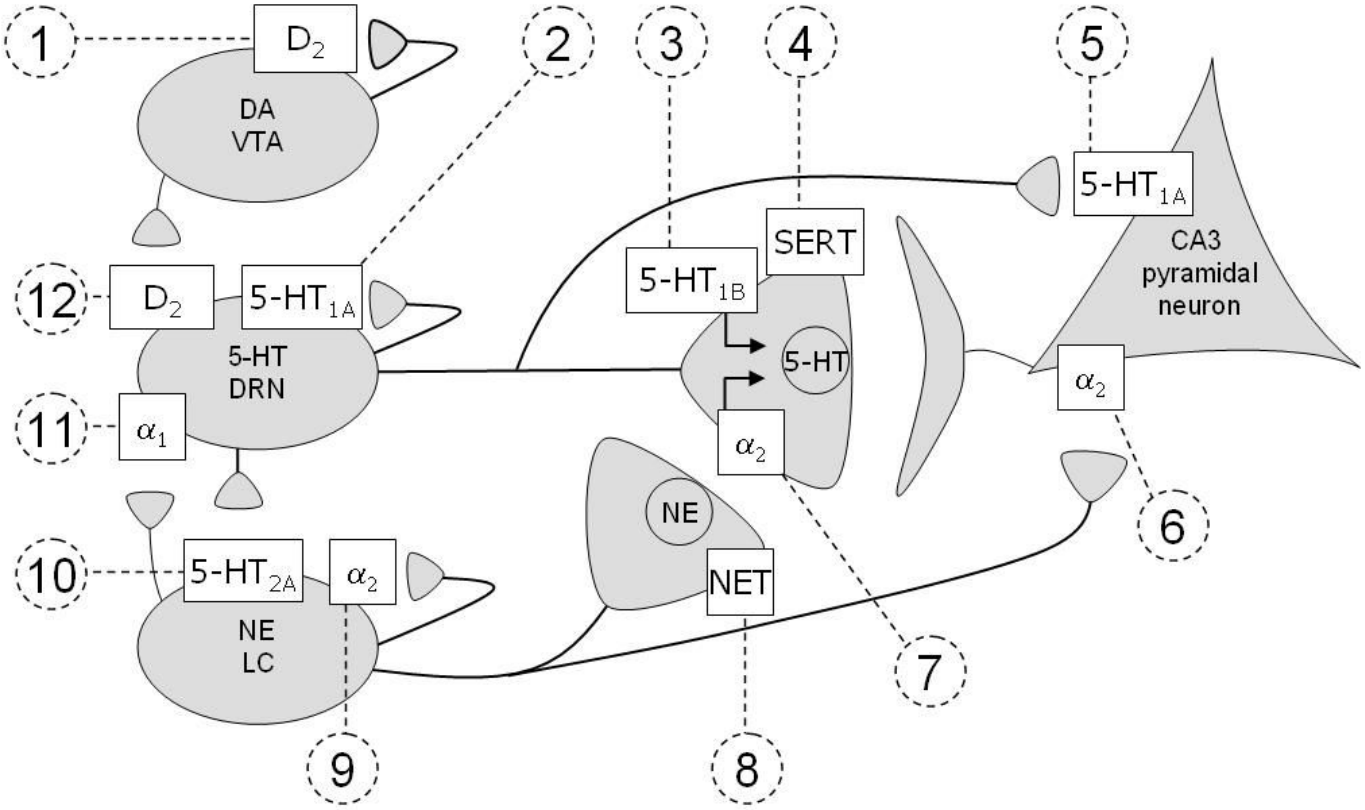

Figure 10: Schematic representation of neural mechanisms regulating the activity of monoamine neurons at the presynaptic level, or altering neurotransmission. Some of the most common effects of sustained antidepressant administration on monoamine systems are depicted by numbers 1-12; see table I for a description of these numbers and their modulation by sustained antidepressant administration.

# in fig. 10	Mechanism	Increased by	Decreased by	Physiological adaptations to sustained administration
1	D_2 autoinhibition	Aripiprazole Pramipexole	Other antipsychotics	- D_2 agonists: desensitization - D_2 antagonists: desensitization & depolarizing block
2	5-HT_{1A} autoinhibition	SSRIS 5-HT_{1A} agonists SNRIs TCAs MAOI Ziprasidone Vortioxetine	-	-Desensitization 5-HT_{1A} (not TCAs and milnacipran) -Recovery of firing 5-HT neurons
3	Autoinhibition 5-HT release by terminal 5-HT_{1B}	-	SSRIs SNRIs Vortioxetine	Desensitization 5-HT_{1B} by SSRIs & SNRIs
4	5-HT transport	-	SSRIs SNRIs TCAs	Desensitization
5	Tonic activation postsynaptic 5-HT_{1A}	SSRIs 5-HT_{1A} agonists SNRIs TCAs Mirtazapine Bupropion MAOI Quetiapine ECT VNS	-	-Sensitization 5-HT_{1A} TCAs & ECT -Desensitization 5-HT_{1A} by clorgyline
6	Tonic activation postsynaptic α_2	SNRIs Bupropion Trazodone	-	-
7	Heteroinhibition 5-HT release by terminal α_2	SNRIs TCAs Mirtazapine Quetiapine Bupropion	-	Desensitization α_2 (not mirtazapine)
8	NE transport	SNRIs TCAs	-	-
9	α_2 autoinhibition	SNRIs TCAs Bupropion	Quetiapine Imipramine 5-HT_{1A} agonists (1-PP)	Desensitization α_2 by bupropion, imipramine, milancipran
10	Inhibition NE neurons by 5-HT_{2A}	SSRIs SNRIs TCAs	Atypical antipsychotics Mirtazapine	Desensitization 5-HT_{2A} by SSRIs & SNRIs
11	Excitation 5-HT neurons by α_1	SNRIs Bupropion VNS	Clozapine Olanzapine	-
12	Excitation 5-HT neurons by D_2	Aripiprazole	-	-

Table I: Common effects of sustained antidepressant administration on monoamine systems. See figure 10 for a schematic representation of numbers 1-12.

2.5.1 Selective serotonin reuptake inhibitors

SSRIs are the first-line treatment for depression, primarily due to their positive safety profile and relatively few side effects. The most commonly prescribed SSRIs are, in descending order for their affinity for the SERT, paroxetine, citalopram, fluvoxamine, sertraline, and fluoxetine (Boyer and Feighner, 1991). These agents have comparable therapeutic efficacy in MDD (Kroenke *et al.*, 2001), and mainly differ in their side effects (Feighner, 1999; Ferguson, 2001). For example, the use of paroxetine or fluoxetine is not recommended in geriatric populations or medically ill patients due to their anticholinergic activity and relatively long half-life, respectively (Feighner, 1999; Ferguson, 2001). Despite some differences in pharmacodynamics and side effects, the mechanism of action of SSRIs in the treatment of depression is thought to be largely similar.

Acute SSRI administration enhances central extracellular 5-HT levels to a larger extent in the DRN than in forebrain regions (Bel and Artigas, 1992; Fuller, 1994), likely reflecting higher SERT density and/or 5-HT neurotransmission at the cell body level of 5-HT neurons (Hrdina *et al.*, 1990). Enhancement of endogenous extracellular 5-HT levels attenuates the firing activity of 5-HT neurons by activating 5-HT_{1A} autoreceptors; indeed, this effect was reversed by 5-HT_{1A} receptor antagonists and prevented by TTX pretreatment (Sharp and Hjorth, 1990; Fuller, 1994; Tao *et al.*, 2000). Notably, acute SSRI administration causes activation of autoreceptors on 5-HT terminals, thereby limiting the release of 5-HT (Rutter *et al.*, 1995).

Following short-term SSRI administration (two-7 days), the firing activity of DRN 5-HT neurons gradually recovered (Chaput, Blier, *et al.*, 1986), and complete recovery was demonstrated after 14-21 days of administration of citalopram, zimelidine,

paroxetine and fluoxetine administration (Blier and de Montigny, 1983; Chaput *et al.*, 1986; de Montigny *et al.*, 1990; Chaput *et al.*, 1991). Importantly, the responsiveness of DRN 5-HT neurons to 5-HT_{1A} agonists was severely diminished by sustained SSRI administration, indicating that the gradual recovery in firing activity of DRN 5-HT neurons is caused by desensitization of 5-HT_{1A} autoreceptors (Blier and de Montigny, 1985). Furthermore, sustained SSRI administration desensitized terminal 5-HT_1 autoreceptors, thereby diminishing negative feedback on 5-HT release at the terminal level, both in the DRN and in projection areas (Blier and de Montigny, 1983; Chaput, Blier, *et al.*, 1986; Blier *et al.*, 1988; Chaput *et al.*, 1991; Piñeyro *et al.*, 1995). Notably, SSRIs do not alter the sensitivity of postsynaptic 5-HT_{1A} receptors (Chaput, Blier, *et al.*, 1986; Chaput *et al.*, 1991). Together, these findings provide a mechanism by which SSRIs enhance tonic activation of postsynaptic 5-HT_{1A} receptors (Haddjeri *et al.*, 1998b).

As a consequence of the reciprocal interactions between monoamine systems, enhanced 5-HT neurotransmission by SSRIs diminishes the firing of LC NE and VTA DA neurons. Importantly, acute SSRI administration had no effect on the firing rate of NE neurons, while a gradual decrease in firing was observed following two, 7 and 14 days to approximately 60% of baseline (Béïque *et al.*, 1998; Szabo *et al.*, 1999). Notably, the excitatory effect on NE neurons of 8-OHDPAT was completely abolished following SSRI administration, indicating desensitization of the earlier described excitatory 5-HT_{1A} receptors that are presumably located on neurons in the paragigantocellularis nucleus (Ennis and Aston-Jones, 1988; Szabo *et al.*, 1999). Furthermore, sustained SSRI administration reduced the inhibitory effect of DOI by approximately two-fold, indicating desensitization of 5-HT_{2A} receptors on terminals of presumably GABA neurons in the

hypoglossal nucleus (Gorea *et al.*, 1991; Szabo *et al.*, 1999). In contrast to NE neurons, acute SSRI administration reduced firing activity of VTA DA neurons, an effect prevented by 5-HT_{2C} receptor blockade (Prisco and Esposito, 1995; Di Mascio *et al.*, 1998). Similarly to NE neurons, attenuated firing of VTA DA neurons does not recover over time, while 5-HT_{2C} antagonists reversed the suppressant effect of SSRIs on these neurons (Dremencov *et al.*, 2009). As will be discussed in more detail in sections 2.5.11 and 2.5.12, nearly all atypical antipsychotics are potent 5-$HT_{2A/2C}$ receptor antagonists, a pharmacological quality contributing to their normalizing effect on the firing activity of catecholamine neurons when co-administered at a sub-antipsychotic dose to an SSRI regimen (Blier and Szabo, 2005).

2.5.2 5-HT_{1A} receptor agonists

The class of 5-HT_{1A} receptor agonists consists of tandospirone, buspirone, gepirone and ipsapirone, of which buspirone and tandospirone in clinical use for treatment of depression. Acute 5-HT_{1A} receptor agonist administration had an inhibitory effect on the firing activity of DRN 5-HT neurons, which gradually recovered after sustained administration due to 5-HT_{1A} autoreceptor desensitization (VanderMaelen *et al.*, 1986; Blier and de Montigny, 1987; Godbout *et al.*, 1991; Dong *et al.*, 1997). In 5-HT projection areas, 5-HT_{1A} receptor agonists had no effect on the sensitivity of terminal 5-HT_{1B} autoreceptors or on 5-HT_{1A} heteroceptors on CA3 pyramidal neurons. (VanderMaelen *et al.*, 1986; Blier and de Montigny, 1987; Godbout *et al.*, 1991; Dong *et al.*, 1997). Therefore, their enhancement of postsynaptic 5-HT_{1A} receptor activation has

been attributed to direct agonism in combination with enhanced 5-HT release following firing recovery of 5-HT neurons (Haddjeri *et al.*, 1998b). Notably, the aforementioned 5-HT_{1A} receptor agonists have a common active metabolite, 1-(2-pyrimidinyl-)-piperazine (1-PP), which exerted appreciable antagonistic action at α_2-adrenergic autoreceptors in the LC and hippocampus, and on α_2-adrenergic heteroceptors on pyramidal neurons both following acute and sustained administration, thereby suggesting an indirect action of these agents on NE neurotransmission (Blier *et al.*, 1991)

2.5.3 Norepinephrine reuptake inhibitors

Currently, reboxetine is arguably the only selective NET inhibitor used in the clinic (Massana *et al.*, 1999). Although metabolites of TCAs have potent NET blocking properties (e.g. desipramine, nortriptyline), these agents will be discussed in section 2.5.5 based on their chemical structure and non-selectivity. In addition to reboxetine, nisoxetine is a selective NET blocker used in basic research.

Acute reboxetine administration dose-dependently decreased the firing activity of LC NE neurons, an effect reversed by α_2-adrenoceptor blockade (Szabo and Blier, 2001a). Firing activity of NE neurons gradually decreased following chronic reboxetine administration by ~85%, while α_2-autoreceptors on LC neurons did not desensitize (Szabo and Blier, 2001a). In contrast, NET blockade desensitized terminal α_2-adrenergic autoreceptors, providing a mechanism in addition to NET blockade for enhanced NE levels in LC NE projections (Sacchetti *et al.*, 1999; Szabo and Blier, 2001a).

Despite receiving an important stimulatory input from the LC, the firing of 5-HT neurons remained unaltered after subacute and sustained NET blockade (Menkes *et al.*, 1981; Szabo and Blier, 2001). Possibly, the enhancement of intrasynaptic NE following NET blockade compensated for decreased NE firing (Szabo and Blier, 2001a). Although the sensitivity of α_2-adrenergic autoreceptors on LC NE neurons was unaltered, chronic reboxetine reduced the potency of clonidine to inhibit 5-HT neurons, possibly by reducing NE input to the DRN to a lesser extent (Szabo and Blier, 2001a). Similarly to the effect of sustained SSRI administration, the excitatory effect of 8-OHDPAT on LC NE neurons was abolished while the inhibitory effect of DOI was blunted, demonstrating region-dependent desensitization of 5-HT_{1A} and 5-HT_{2A} receptors, respectively (Szabo and Blier, 2001a). In the hippocampus, sustained NET blockade desensitized α_2-adrenergic heteroceptors on 5-HT terminals, providing a mechanism by which activation of postsynaptic 5-HT_{1A} receptors was enhanced (Mongeau *et al.*, 1994; Szabo and Blier, 2001). In the VTA, acute reboxetine dose-dependently enhanced burst-firing presumably by increased α_1-adrenoceptor activation (Grenhoff *et al.*, 1993; Linner *et al.*, 2001). Thus, acute NET blockade caused elevated DA levels VTA projection regions, both by reducing its reuptake (see section 2.4.3), and by enhancing VTA firing activity. Notably however, two day administration of reboxetine (2.5 mg/kg/day) decreased the firing and burst activity of VTA DA neurons, of which only the former measure recovered after 14-day administration (Katz *et al.*, 2010). In addition, sustained 21-day reboxetine (1 mg/kg/day) enhanced the population activity of VTA DA neurons, although notably, this result was obtained with a relatively low dose (Sekine *et al.*, 2007).

2.5.4 Serotonin/norepinephrine reuptake inhibitors

The class of serotonin/norepinephrine reuptake inhibitory (SNRIs) consists of venlafaxine, duloxetine, and milnacipran. Venlafaxine and its active metabolite desvenlafaxine are efficacious in the treatment of depression (Septien-Velez *et al.*, 2007; Liebowitz *et al.*, 2008; Nemeroff *et al.*, 2008). Both agents are preferential SERT blockers, but also have NET blocking properties at a higher dosage (Bymaster *et al.*, 2001; Deecher *et al.*, 2006). In accordance, acute venlafaxine administration preferentially increased prefrontal 5-HT levels at low doses, while increasing 5-HT and NE levels to a similar extent at a higher dosage (Koch *et al.*, 2003). *In vivo* electrophysiological studies showed that acute venlafaxine administration inhibited the firing activity of 5-HT and, at a higher dose, NE neurons by activating 5-HT_{1A} and α_2-adrenergic autoreceptors, respectively (Béïque *et al.*, 1999). Subacute administration of venlafaxine attenuated DRN 5-HT firing activity which recovered after a chronic regimen due to 5-HT_{1A} autoreceptor desensitization (Béïque *et al.*, 2000a). In line with more potent action at the SERT than NET, a high (40 mg/kg/d) but not a low dose (10 mg/kg/d) of venlafaxine reduced the firing activity of LC NE neurons after its subacute administration, while both doses decreased the firing activity of NE neurons after sustained administration (Béïque *et al.*, 2000a). In the hippocampus, sustained venlafaxine significantly diminished SERT and terminal 5-HT_{1B} autoreceptor activity, resulting in enhanced 5-HT tone on postsynaptic 5-HT_{1A} receptors (Béïque *et al.*, 2000a; b). Chronic venlafaxine administered at a high dose also reduced the activity of the hippocampal NET and likely enhanced NE neurotransmission, although *in vitro* data suggested that this did not result in altered sensitivity of terminal α_2-adrenergic auto- and

heteroceptors (Béïque *et al.*, 2000a; b). Hence, it has been concluded that the effect of sustained venlafaxine administration on 5-HT neurotransmission is largely similar to that of SSRIs (Béïque *et al.*, 2000b).

The enhanced effectiveness of duloxetine in depression when dosage is increased probably results from a dose-dependent blockade of the NET (Nierenberg *et al.*, 2007; Shelton *et al.*, 2007; Thase *et al.*, 2007; Sagman *et al.*, 2011). Indeed, the affinity of duloxetine is approximately 10-fold higher for the SERT than for the NET (Bymaster *et al.*, 2001; Kuo *et al.*, 2004). In line with this, duloxetine enhanced prefrontal rat 5-HT levels at low doses, while 5-HT and NE levels were both elevated when dosage increased (Koch *et al.*, 2003). Similarly, *in vivo* electrophysiological experiments in the hippocampus demonstrated an appreciable SERT-blocking effect of duloxetine at low doses, while NET-blocking properties became significant only at relatively high dosage (Kasamo *et al.*, 1996). Accordingly, acute duloxetine administration was 5-fold more potent in inhibiting DRN 5-HT compared to LC NE neurons (Kasamo *et al.*, 1996). Subchronic duloxetine attenuated the firing activity of DRN 5-HT neurons, which recovered after its chronic administration due to 5-HT_{1A} autoreceptor desensitization, similarly to the effect of venlafaxine (Rueter *et al.*, 1998).

Milnacipran is a slightly more potent inhibitor of the NET than the SERT (Koch *et al.*, 2003; Vaishnavi *et al.*, 2004). When administered acutely, prefrontal 5-HT and NE levels showed similar dose-dependent elevations (Koch *et al.*, 2003). Acute milnacipran inhibited the firing rate of DRN 5-HT neurons, which gradually recovered when its administration was prolonged (Mongeau *et al.*, 1998). Interestingly, the inhibitory effect of acute milnacipran on 5-HT neurons was abolished by 6-OHDA pretreatment, the

potency to inhibit 5-HT neurons by iontophoretic application of 5-HT and 8-OHDPAT was unaltered after chronic milnacipran administration, and clonidine less potently inhibited 5-HT neurons after chronic milnacipran, together indicating that milnacipran attenuated excitatory input from the LC to the DRN (Mongeau *et al.*, 1998). Possibly, the sustained attenuation of the firing activity of LC NE neurons by milnacipran contributed to this (Mongeau *et al.*, 1998), although this mechanism does not fully explain why firing of DRN 5-HT neurons gradually returned to baseline. Clearly however, the effects of milnacipran on 5-HT firing were primarily due to altered NE function, and not 5-HT_{1A} autoreceptor desensitization (Bel and Artigas, 1999). Finally, sustained milnacipran decreased the sensitivity of α_2-adrenergic autoreceptors to clonidine but not NE, similarly to the effect of sustained desipramine (see section 2.5.5).

2.5.5 Tricyclic antidepressants

Since the discovery of their antidepressant potential in the 1950s, TCAs have been the first-line treatment of MDD for 30 years. Due to their side-effect (including sedation, delirium, seizures, antihistamine and anticholinergic effects), relatively narrow treatment window, toxicity, and potential for suicide when overdosed (Preskorn and Simpson, 1982; Callaham, 1985; Rosenstein *et al.*, 1993), these drugs are nowadays only prescribed in treatment-resistant depressed patients. As suggested by their name, TCAs have a central three-ring chemical structure. This drug class consists of (in descending order of their SERT blocking potential) clomipramine, imipramine, amitriptyline, desipramine, nortriptyline, protriptyline, amoxapine, doxepin, trimipramine, and

maprotiline (Tatsumi *et al.*, 1997). Notably these agents also have potent NET blocking properties. In descending order for their NET affinities these are desipramine, protriptyline, nortriptyline, maprotiline, amoxapine, doxepin amitriptyline, imipramine and clomipramine (Tatsumi, Groshan, Blakely, & Richelson, 1997). The side-chain structure primarily determines SERT vs. NET blocking properties (e.g. imipramine-desipramine and amitriptyline-nortriptyline), while variation in three-ring composition contributes to variable affinities for α_1- and α_2-adrenergic, muscarinic, cholinergic, 5-HT_{1A} and 5-HT_2 receptors (Cusack *et al.*, 1994; Tatsumi *et al.*, 1997; Potter *et al.*, 1998).

Electrophysiological experiments demonstrated that acute administration of imipramine attenuated the firing activity of DRN 5-HT neurons (Bramwell and Straughan, 1972), an effect attributable to its SERT blocking properties which cause 5-HT levels to increase and consequently, enhanced 5-HT_{1A} autoreceptor activation (Wesołowska and Kowalska, 2008). Although this acute effect is similar to that of SSRIs, TCAs have important distinguishable effects following sustained administration. Indeed, despite elevating presynaptic 5-HT levels, TCAs do not produce a desensitization of somatodendritic 5-HT_{1A} autoreceptors or terminal 5-HT_{1B} autoreceptors (Blier and de Montigny, 1980; Chaput *et al.*, 1991). Furthermore, TCAs cause a sensitization of 5-HT_{1A} receptors on postsynaptic receptors in postsynaptic areas, as demonstrated by enhanced responsiveness to 5-HT iontophoresis on CA3 pyramidal neurons (de Montigny and Aghajanian, 1978; Gallager and Bunney, 1979). Accordingly, these neurons were significantly disinhibited by systemic WAY 100.635 administration, demonstrating enhanced 5-HT tone on postsynaptic 5-HT_{1A} receptors (Haddjeri *et al.*, 1998b).

Acute administration of imipramine reduced the firing activity of LC NE neurons, at least in part by NET blockade (Nybäck *et al.*, 1975; Wesołowska and Kowalska, 2008). This firing activity remained attenuated after subacute and sustained imipramine administration and was reversed by acute idazoxan administration, thus demonstrating enhanced NE tone on LC α_2-adrenergic autoreceptors (Linnér *et al.*, 1999). Furthermore, sustained imipramine reduced the responsiveness of LC NE neurons to systemic clonidine inhibition (Svensson and Usdin, 1978). Although the latter effect has been interpreted as α_2-adrenergic autoreceptor desensitization, it needs to be noted that chronic desipramine administration reduced the inhibitory effect of clonidine but not NE iontophoresis on LC NE firing activity (Lacroix *et al.*, 1991), similarly to the effect of sustained milnacipran (Mongeau *et al.*, 1998), thereby indicating that such blunted responsiveness might be ligand-specific. In postsynaptic areas, the sensitivity of α_2-adrenoceptors on CA3 pyramidal neurons did not change by chronic imipramine (de Montigny and Aghajanian, 1978). In contrast, the NET-blocking properties of desipramine acutely activated α_2-adrenergic heteroceptors on 5-HT terminal and terminal α_2-adrenergic autoreceptors (Lacroix *et al.*, 1991; Mongeau *et al.*, 1993), and caused a desensitization of the latter receptor population following sustained administration (Lacroix *et al.*, 1991). In addition to enhancing 5-HT tone, imipramine increased NE levels in the prefrontal cortex (Linnér *et al.*, 1999).

2.5.6 Mirtazapine

Mirtazapine is a tetracyclic antidepressant renowned for its α_2-adrenergic receptor blocking properties. Its acute administration increased the firing activity of 5-HT neurons, an effect primarily mediated by enhanced NE input as it was prevented by 6-OHDA pretreatment but by acute administration of the 5-HT_{1A} autoreceptor antagonist (-) pindolol (Haddjeri *et al.*, 1996). Accordingly, mirtazapine was shown to increase the firing activity of LC NE neurons (Haddjeri *et al.*, 1996), and to dampen and reverse the inhibitory effect of clonidine on LC NE firing, demonstrating α_2-adrenoceptor blockade *in vivo* (Haddjeri *et al.*, 1996). After sustained mirtazapine, firing of 5-HT neurons remained unaltered despite 5-HT_{1A} autoreceptor desensitization (Blier *et al.*, 2003). Furthermore, sustained mirtazapine increased the firing activity of NE neurons while perhaps surprisingly, the sensitivity of α_2-adrenergic autoreceptors remained unaltered (Haddjeri *et al.*, 1998a). In the hippocampus, the status of terminal 5-HT_{1B} autoreceptors remained unaltered while α_2-adrenergic heteroceptors on 5-HT terminals desensitized, and enhanced activation of postsynaptic 5-HT_{1A} receptors was presumably enhanced due to increased firing activity of 5-HT neurons (Haddjeri *et al.*, 1998a; b). Chronic mirtazapine administration was shown to diminish the activity of α_2-adrenergic autoreceptors on NE terminals (Haddjeri *et al.*, 1998a), which contributed to an enhancement of prefrontal NE and DA concentrations (Millan *et al.*, 2000).

2.5.7 Vortioxetine

Vortioxetine is a relatively new, multimodal antidepressant agent (Mahableshwarkar *et al.*, 2013; Boulenger *et al.*, 2014) endowed with SERT blocking properties, 5-HT_{1A} receptor agonism, 5-HT_{1B} receptor partial agonism, and antagonistic

properties at 5-HT_{1D}, 5-HT_3 and 5-HT_7 receptors (Bang-Andersen *et al.*, 2011; Mørk *et al.*, 2012). In line with agonistic action on 5-HT_{1A} receptors, acute vortioxetine attenuated the firing activity of DRN 5-HT neurons (Bétry *et al.*, 2013). Interestingly, firing activity of these neurons completely recovered after 24 hours, an effect at least in part due to the 5-HT_3 receptor antagonism (Bétry *et al.*, 2013). Notably, since the affinity of vortioxetine for 5-HT_{1A} receptors is ~10 fold higher in humans (Bang-Andersen *et al.*, 2011; Mørk *et al.*, 2012), such a rapid recovery of 5-HT firing may not occur in the clinic. Indeed, under conditions where 5-HT_{1A} receptors were more activated by co-administration of the 5-HT_{1A} receptor agonist flesinoxan, vortioxetine did not prevent attenuated firing of 5-HT neurons (Bétry *et al.*, 2013). In the hippocampus, sustained vortioxetine administration increased the degree of tonic activation on 5-HT_{1A} receptors, at least in part by partial terminal 5-HT_{1B} autoreceptor agonism (El Mansari *et al.*, 2015). At high doses, acute vortioxetine increased the firing activity of LC NE neurons, an effect opposite to that of SSRIs (Pehrson *et al.*, 2013) that has been hypothesized to result from 5-HT_3 receptor antagonism and/or 5-HT_{1A} receptor agonism (Sanchez *et al.*, 2015).

2.5.8 Bupropion

Bupropion is a clinically efficacious agent in the treatment of depression with a distinguishable mode of action, as it has little affinity for monoamine receptors and transporters (Richelson and Pfenning, 1984; Cusack *et al.*, 1994; Tatsumi *et al.*, 1997; Sánchez and Hyttel, 1999; Moreira, 2011). However, its acute administration inhibited both LC NE and VTA DA neurons by enhancing NE release (Cooper *et al.*, 1994).

Accordingly, 6-OHDA pretreatment prevented the enhancement of DRN 5-HT neuron firing activity and the attenuation on LC NE neurons following two-day bupropion administration (Dong and Blier, 2001). Idazoxan reversed the latter effect, demonstrating increased activation of α_2-adrenergic autoreceptors on LC NE neurons by bupropion (Dong and Blier, 2001). Following long-bupropion administration, the firing activity of DRN 5-HT neurons remained elevated, and 5-HT_{1A} autoreceptors were desensitized (El Mansari *et al.*, 2008). Interestingly, the firing activity of LC NE neurons gradually returned to baseline levels, an effect due to α_2-adrenergic autoreceptors desensitization (El Mansari *et al.*, 2008). Despite a direct inhibitory effect, sustained bupropion administration did not alter the firing activity of VTA DA neurons (Dong and Blier, 2001; El Mansari *et al.*, 2008). In the hippocampus, bupropion desensitized α_2-adrenergic autoreceptors on NE terminals, and α_2-adrenergic heteroceptors on 5-HT terminals, which together with enhanced firing activity of NE and 5-HT neurons resulted in enhanced tonic activation of α_2-adrenergic and 5-HT_{1A} receptors on CA3 pyramidal neurons (Ghanbari *et al.*, 2011).

2.5.9 Monoamine oxidase inhibitors

The family of monoamine oxidase inhibitor (MAOIs) increases intracellular monoamine levels, which causes adaptive changes after sustained administration that are thought to be important in their therapeutic effect. An early electrophysiology study demonstrated attenuated firing activity of 5-HT neurons after two-day administration of clorgyline (a MAO-A inhibitor), phenelzine (a MAO-A&B inhibitor) but not deprenyl (a

MAO-B inhibitor), which recovered after sustained administration due to 5-HT_{1A} autoreceptor desensitization (Blier and de Montigny, 1985). Firing rate of LC NE neurons was attenuated by subacute and sustained MAO-A inhibition, and α_2-adrenergic autoreceptors did not desensitize (Blier and de Montigny, 1985). In the hippocampus, sustained administration of clorgyline but not deprenyl and phenelzine decreased the responsiveness of 5-HT_{1A} receptors to microiontophoretic application of 5-HT (Blier *et al.*, 1986), indicating desensitization of postsynaptic 5-HT_{1A} receptors. Furthermore, effectiveness of 5-HT bundle stimulation was enhanced by sustained MAO-A inhibition (Blier *et al.*, 1986) by a mechanism other than 5-HT_{1B} autoreceptor desensitization (Blier *et al.*, 1988), at least *in vivo* (Piñeyro and Blier, 1996). The effects of MAO inhibition on postsynaptic components of the NE system was less pronounced, as it did not substantially elevate NE brain content or α_2-adrenergic receptor sensitivity in the hippocampus (Blier *et al.*, 1986). Prolonged but not subacute MAO-A inhibition reduced the firing and burst activity of VTA DA neurons while interestingly, it markedly increased the population activity of VTA DA neurons (Chenu *et al.*, 2008). The timeframe of these changes was paralleled by changes in the 5-HT system and furthermore, reversed by 5-HT depletion, together indicating that the inhibitory effect of clorgyline on VTA neurons is predominantly mediated through the 5-HT system (Chenu *et al.*, 2008).

2.5.10 Ketamine

Although originally used as an anesthetic, recent evidence has demonstrated a rapid yet short-lived antidepressant action of subanaesthetic ketamine administration (Zarate *et al.*, 2006; Murrough *et al.*, 2013). Ketamine primarily acts as a non-competitive antagonist at NMDA receptors. Interestingly, an electrophysiological study showed that acute ketamine upregulated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responsiveness in the hippocampus. Furthermore, ketamine enhanced LC NE firing and bursting activity after acute and two-day ketamine administration while it acutely increased the population activity of VTA DA neurons (El Iskandrani *et al.*, 2015; see appendix). These effects on catecholamine neurons were prevented by pretreatment with the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; El Iskandrani *et al.*, 2015). Since NBQX also prevented the antidepressant effects of ketamine in the forced swim test (FST; Maeng *et al.*, 2008), enhancement of NE and/or DA neurotransmission via AMPA receptor activation might play a role in the antidepressant effects of ketamine.

2.5.11 Atypical antipsychotics

The class of atypical antipsychotics, in descending order of their 5-HT_{2A}/D_2 receptor affinity ratio, consists of clozapine, risperidone, ziprasidone, olanzapine, asenapine, lurasidone, quetiapine, and aripiprazole (Shahid *et al.*, 2009). Based on their chemical structure, these agents can be divided into three groups; dibenzepines (clozapine, olanzapine and quetiapine), benzisoxazoles (lurasidone, risperidone, its

metabolite paliperidone, and ziprasidone), and piperazine derivatives (aripiprazole and brexpiprazole, of which the latter was characterized in the present work; see chapters II and III). Although these agents are not indicated as monotherapy for depression, they are effective agents in adjunct to SSRI regimens (Blier and Szabo, 2005; Blier and Blondeau, 2011). Their clinical and neurobiological effects when combined with SSRIs will be discussed in more details in section 2.5.12; however, a brief overview of effects of these agents on monoamine systems when administered alone is deemed important for the present work.

2.5.11.1 Dibenzepines

Acute clozapine reduces the firing rate of DRN 5-HT neurons, an effect partly reversed by desipramine but not WAY 100.635 (Gallagher and Aghajanian, 1976; Sprouse *et al.*, 1999). Presumably, this inhibitory effect was due to α_1-adrenoceptor blockade, which would be in line with the *in vitro* profile of clozapine (Kroeze *et al.*, 2003). Acute and chronic clozapine administration enhanced the firing rate of LC NE neurons (Souto *et al.*, 1979; Ramirez and Wang, 1986; Nilsson *et al.*, 2005), an effect primarily mediated by α_2-adrenergic autoreceptor blockade (Devoto *et al.*, 2003). Acutely, clozapine enhanced firing, bursting and population activity of VTA DA neurons (Chiodo and Bunney, 1983; Gessa *et al.*, 2000), and elevated cortical DA levels via 5-HT_{1A} receptor activation (Díaz-Mataix *et al.*, 2005). Importantly, sustained clozapine administration potently decreased the population activity of VTA DA neurons, likely by

inducing a depolarizing blockade following sustained D_2 autoreceptor antagonism (Chiodo and Bunney, 1983; Sorensen *et al.*, 1993; Jones-Humble *et al.*, 1996).

Acute olanzapine attenuated the firing activity of DRN 5-HT neurons. In line with little *in vitro* activity at 5-HT_{1A} receptors (Kroeze *et al.*, 2003), WAY 100.635 did not prevent this inhibitory effect (Sprouse *et al.*, 1999). However, desipramine fully reversed the inhibitory effect of olanzapine, indicating that attenuated DRN 5-HT firing was due to reduced NE input in the DRN (Sprouse *et al.*, 1999). Notably, acute olanzapine increased the firing and burst activity of LC NE neurons (Dawe *et al.*, 2001), in line with antagonistic activity at α_2-adrenergic receptors *in vitro* (Kroeze *et al.*, 2003). Given this acute enhancement of LC NE firing activity, the inhibitory effect of olanzapine on the firing activity of 5-HT neurons is likely due to α_1-adrenoceptor blockade. Following sustained olanzapine administration the firing activity of LC NE neurons remained elevated (Seager *et al.*, 2005), similarly to the effect of sustained clozapine administration. In further similarity to clozapine, olanzapine also decreased the firing activity and population activity of VTA DA neurons (Stockton and Rasmussen, 1996; Gessa *et al.*, 2000) and increased prefrontal dopamine levels (Westerink *et al.*, 1998; Koch *et al.*, 2004; Díaz-Mataix *et al.*, 2005). Interestingly, the enhancement of prefrontal DA was lost in 5-HT_{1A} receptor knockout animals despite negligible action of olanzapine at this receptor subtype, an effect not fully understood (Díaz-Mataix *et al.*, 2005). Following sustained administration, olanzapine dose-dependently reduced the population activity of VTA DA neurons by inducing a depolarization block; indeed, acute apomorphine reversed the reduction in active DA neurons (Stockton and Rasmussen, 1996).

In humans, quetiapine metabolism produces norquetiapine, a molecule endowed with partial agonism at 5-HT_{1A} receptors and NET-blocking properties (Jensen *et al.*, 2008). Since this metabolism does not occur in rats, humanized quetiapine (hquetiapine; a 3:1 mixture of quetiapine and norquetiapine) was used to assess its *in vivo* electrophysiological effects. Acute hquetiapine administration inhibited the firing rate of DRN 5-HT neurons, an effect in part mediated by 5-HT_{1A} receptor activation and partly by diminished α_1-adrenoceptor mediated NE input, as demonstrated by the complete reversal inhibition by combined WAY 100.635 and desipramine administration (Chernoloz, El Mansari, *et al.*, 2012). Notably, the firing activity of DRN 5-HT neurons remained attenuated following sustained hquetiapine administration, despite an enhancement of LC NE firing activity after two- and 14-day quetiapine administration (Chernoloz, El Mansari, *et al.*, 2012). This enhanced firing of LC NE neurons was at least in part due to α_2-adrenergic autoreceptor blockade and/or desensitization, as sustained hquetiapine administration reduced the inhibitory effect of acute clonidine on LC NE neurons (Chernoloz, El Mansari, *et al.*, 2012). Despite having the lowest *in vitro* affinity for 5-HT_{2A} receptors of all atypical antipsychotics (Shahid *et al.*, 2009), hquetiapine dampened the 5-HT_{2A} receptor mediated inhibitory effect of DOI on LC NE neurons (Chernoloz, El Mansari, *et al.*, 2012). In the hippocampus, hquetiapine enhanced the tonic activation of 5-HT_{1A} receptors on pyramidal neurons despite diminished firing activity of 5-HT DRN neurons, in part by direct activation of postsynaptic 5-HT_{1A} receptor activation by norquetiapine (Chernoloz, El Mansari, *et al.*, 2012). It also enhanced NE tone on α_2-adrenoceptor due to combined NET blockade and α_2-adrenergic autoreceptor blockade and/or desensitization (Chernoloz, El Mansari, *et al.*, 2012).

Sustained quetiapine in absence of norquetiapine produced a dose-dependent decrease on the population activity of VTA DA neurons (Skarsfeldt, 1995).

2.5.11.2 Benzisoxazoles

Acute risperidone administration decreased the firing activity of 5-HT neurons by blocking terminal 5-HT_{1D} autoreceptors in the DRN, which elevated 5-HT levels and subsequently enhanced activation of 5-HT_{1A} autoreceptors (Hertel *et al.*, 1998, 2001). Indeed, local perfusion with the a 5-HT_{1D} receptor agonist prevented the acute rise in 5-HT levels in the DRN, while PCPA and WAY 100.635 pretreatment prevented the inhibitory effect of risperidone on 5-HT neurons (Hertel *et al.*, 1998, 2001). After both two- and 14-day risperidone administration the firing activity of 5-HT neurons remained attenuated, while the firing activity of LC NE neurons was not altered by risperidone (Dremencov *et al.*, 2007a; b). Acute risperidone elevated prefrontal DA concentrations (Westerink *et al.*, 1998), however, its sustained administration caused a dose-dependent reduction in the population activity of VTA DA neurons (Skarsfeldt, 1995).

Paliperidone, one of the main metabolites of risperidone, had no effect on the firing activity of DRN 5-HT neurons when administered acutely (Dremencov *et al.*, 2007a). After two- and 14-day administration, paliperidone did not alter the firing activity of DRN 5-HT neurons, indicating a different effect on these neurons compared to risperidone (Dremencov *et al.*, 2007a). Although sustained paliperidone did not alter the firing rate or bursting activity of LC NE neurons, it produced a blockade on 5-HT_{2A} receptors (Dremencov *et al.*, 2007a).

Ziprasidone is the only benzisoxazole with marked direct agonistic activity at 5-HT_{1A} receptors (Schmidt *et al.*, 2001). Indeed, its inhibitory effect on DRN 5-HT neurons was prevented by acute WAY 100.635 but not desipramine administration (Sprouse *et al.*, 1999). Acute ziprasidone administration also increased the firing activity of VTA DA neurons and enhanced DA levels in the PFC (Díaz-Mataix *et al.*, 2005). Both of these effects were prevented by prefrontal lesion, and the elevation of prefrontal DA levels was not observed in 5-HT_{1A} receptor knockout mice, together demonstrating that the enhancing effect of ziprasidone on the DA system is at least in part mediated by prefrontal 5-HT_{1A} receptors (Díaz-Mataix *et al.*, 2005). Similarly to other atypical antipsychotics, chronic ziprasidone administration dose-dependently decreased the population activity of VTA DA neurons, presumably by inducing a depolarization block (Skarsfeldt, 1995).

2.5.11.3 Aripiprazole

Aripiprazole has some unique pharmacological characteristics in the class of other atypical antipsychotics; it is the only agent with a lower *in vitro* affinity for 5-HT_{2A} than D_2 receptors, and with partial agonistic action (rather than antagonism) at the latter receptor type (Shapiro *et al.*, 2003). Acute administration of aripiprazole inhibited the firing activity of DRN 5-HT neurons by direct 5-HT_{1A} autoreceptor agonism (Shapiro *et al.*, 2003; Dahan *et al.*, 2009). Despite this agonism, two- and 14-day aripiprazole administration enhanced the firing activity of 5-HT neurons, an effect attributed a combination of (rapid) 5-HT_{1A} autoreceptor desensitization and direct activation of

excitatory D_2 receptors on DRN 5-HT neurons (Chernoloz *et al.*, 2009). Aripiprazole did not alter the firing activity of LC NE neurons after subacute and sustained administration, in line with a relatively low affinity for α_2-adrenergic receptors (Shapiro *et al.*, 2003; Chernoloz *et al.*, 2009). Acute aripiprazole administration attenuated the firing activity of VTA DA neurons to approximately 50% of baseline, while it reversed the full inhibitory effect of apomorphine on these neurons to approximately 60% of baseline activity; results demonstrating partial agonistic action at D_2 autoreceptors *in vivo* (Dahan *et al.*, 2009). Interestingly, long-term aripiprazole administration did not alter the firing activity of VTA DA neurons (Chernoloz *et al.*, 2009).

2.5.12 Augmentation strategies

When the response to an antidepressant treatment is insufficient, a clinician has three main treatment options: increase the dose of the antidepressant, switch to a different antidepressant, or augment the antidepressant response by co-administration of agents that could work synergistically with the ongoing antidepressant regimen. In the following section, some of these augmentation strategies are discussed in more detail.

2.5.12.1 Mirtazapine augmentation

Mirtazapine co-administration to an existing regimen of antidepressants is an effective strategy to augment efficacy in patients with an incomplete response (Carpenter *et al.*, 1999, 2002; Hannan *et al.*, 2007; Ozmenler *et al.*, 2008; Blier *et al.*, 2009, 2010). In rats, co-administration of mirtazapine caused a small yet significant reduction in the

attenuating effect of two-day paroxetine administration on the firing rate of DRN 5-HT neurons (Besson *et al.*, 2000). Presumably, blockade of the inhibitory 5-HT_{2A} and α_2-adrenergic receptors in the LC reduced the dampening effect of paroxetine on LC NE neurons, thereby enhancing NE input to the DRN (Besson *et al.*, 2000). After 21-day administration, paroxetine dampened the elevating effect of mirtazapine administration on the firing activity of 5-HT neurons, presumably by enhanced tone of 5-HT_{1A} autoreceptors (Besson *et al.*, 2000). Importantly, the combination of mirtazapine and paroxetine produced a greater tonic activation of postsynaptic 5-HT_{1A} heteroceptor on CA3 pyramidal neurons after two and 21 days of administration than either agent alone (Besson *et al.*, 2000). Likely, this synergistic effect on 5-HT neurotransmission is due to a combination of increased extracellular 5-HT following SERT blockade, 5-HT_{1B} terminal autoreceptor desensitization, and blockade of α_2-adrenergic heteroceptors on 5-HT terminals (Chaput *et al.*, 1991; Piñeyro *et al.*, 1994; Haddjeri *et al.*, 1998a).

2.5.12.2 Bupropion augmentation

Bupropion addition to an ongoing antidepressant regimen is an efficacious strategy to increase therapeutic efficacy in the treatment of MDD (Blier, 2006; Trivedi *et al.*, 2006; Blier and Blondeau, 2011; Stewart *et al.*, 2014). In rats, the bupropion combination with escitalopram markedly increased the firing activity of DRN 5-HT neurons, both after two- and 14 day administration (Ghanbari *et al.*, 2010). Importantly, two-day bupropion + escitalopram administration caused 5-HT_{1A} autoreceptors to be less responsive to LSD, demonstrating a desensitization of 5-HT_{1A} autoreceptors following

combination of these agents (Ghanbari *et al.*, 2010). After two days, this combination attenuated the firing activity of LC NE neurons to a similar extent as each agent alone, however, firing partly recovered after 14-day bupropion + escitalopram administration due to desensitization of α_2-adrenergic autoreceptors on LC NE neurons (Ghanbari *et al.*, 2010).

2.5.12.3 Quetiapine augmentation

Quetiapine augmentation in patients with an inadequate response to ongoing antidepressant treatment is an effective therapeutic strategy (McIntyre *et al.*, 2007; Olver *et al.*, 2008; Anderson *et al.*, 2009; El-Khalili *et al.*, 2010). In rats, two-day hquetiapine + escitalopram administration caused an overall attenuated firing activity of DRN 5-HT neurons, to a similar extent as when either agent was administered alone (Chernoloz, El Mansari, *et al.*, 2012). Following 14-day administration, this firing activity did not recover. Importantly, however, the combination of these agents produced a significantly greater tonic activation of 5-HT_{1A} receptors on CA3 pyramidal neurons than hquetiapine alone (Chernoloz, El Mansari, *et al.*, 2012). Furthermore, hquetiapine co-administration prevented the attenuated firing activity of LC NE neurons typically observed after sustained SSRI administration, an effect at least in part due to 5-HT_{2A} and/or α_2-adrenoceptor blockade (Chernoloz, El Mansari, *et al.*, 2012). Based on their individual effects, it is likely that their combined administration caused enhanced NE neurotransmission by blockade of α_2-autoreceptors and the NET, while 5-HT neurotransmission was elevated due to direct 5-HT_{1A} receptor agonism and SERT

blockade. Although under baseline conditions an enhancement of NE dampens 5-HT neurotransmission (Mongeau *et al.*, 1993), the blockade of α_2-adrenergic heteroceptors on 5-HT terminals by hquetiapine likely prevented this dampening, thereby further contributing to a synergistic effect of this combination (Chernoloz, Mansari, *et al.*, 2012).

2.5.12.4 Risperidone augmentation

A few studies showed improved remission in patients incompletely responding to an antidepressant treatment after co-administration of risperidone (Mahmoud *et al.*, 2007; Reeves *et al.*, 2008; Keitner *et al.*, 2009). Similarly to the effect of quetiapine, risperidone + escitalopram administered for two and 14 days caused a marked attenuation of the firing activity of 5-HT neurons, which was indistinguishable from the individual effects of these agents (Dremencov *et al.*, 2007b). Interestingly, two-day risperidone + escitalopram administration markedly increased the firing and burst activity of LC NE neurons. Furthermore, risperidone co-administration prevented the attenuating effect of 14-day escitalopram on LC NE neurons, an effect likely due to the potent 5-HT_{2A} receptor blocking properties of risperidone (Schotte *et al.*, 1996; Dremencov *et al.*, 2007b)

2.5.12.5 Aripiprazole augmentation

Aripiprazole as add-on agent is an effective treatment strategy for MDD patients that show an inadequate response to antidepressants (Berman *et al.*, 2007, 2009; Marcus *et al.*, 2008; Kamijima *et al.*, 2013). Aripiprazole co-administration for two days prevented the attenuating effect of escitalopram on the firing activity of DRN 5-HT neurons, an effect attributed to a rapid desensitization of 5-HT_{1A} autoreceptors by this combination (Chernoloz *et al.*, 2009). Similarly to other atypical antipsychotics, aripiprazole prevented the attenuating effect of escitalopram on the firing activity of LC NE neurons, an effect likely due to 5-HT_{2A} receptor blockade. Presumably similarly, aripiprazole co-administration neutralized the attenuating effect of escitalopram on the firing activity of VTA DA neurons, possibly due to blockade of 5-HT_{2C} receptors (Shapiro *et al.*, 2003; Chernoloz *et al.*, 2009).

2.5.13 Non-pharmacological interventions

Non-pharmacological antidepressant strategies are an alternative treatment option, especially when MDD patients are resistant to pharmacological antidepressant strategies. Some of these options, such as ECT or deep brain stimulation (DBS), have a superior efficacy in treatment-resistant patients compared to first-line antidepressants. However, their level of invasiveness and profound side-effects can be important trade-offs. In the next section, some of these antidepressant strategies will be discussed in more detail with an emphasis on their effects on monoamine systems.

2.5.13.1 Deep brain stimulation

DBS is a relatively novel antidepressant strategy with a clinical efficacy of ≥ 50% remission (Anderson *et al.*, 2012), a relatively high percentage considering that these data were obtained in severely depressed patients (Trivedi *et al.*, 2006). Clearly, this clinical effect comes with the trade-off that the intracranial implantation of stimulation electrodes is a highly invasive procedure. Antidepressant effects of DBS are best explored in the anterior cingulate cortex, ventral capsule/ventral striatum and NAc (Lozano *et al.*, 2008; Malone *et al.*, 2009; Anderson *et al.*, 2012; Bewernick *et al.*, 2012; Holtzheimer *et al.*, 2012). Although the mechanism by which DBS has a therapeutic effect remains to be fully established, a common denominator is their connectivity to the medial forebrain bundle, implying an altered signal transduction on this pathway connecting the forebrain to mid- and hindbrain structures (Coenen *et al.*, 2011).

In animals, DBS applied in the subcallosal cingulate gyrus has been shown to be an effective strategy in various rodent models for depressive-like behavior (Hamani *et al.*, 2010). Interestingly, 5,7-DHT pretreatment prevented these effects, demonstrating that integrity of the 5-HT system is required for an antidepressant-like effect of DBS (Hamani *et al.*, 2010). Conversely, lesion of the NE system did not alter the effect of DBS (Hamani *et al.*, 2010). Notably, the recent demonstration that subcallosal DBS enhanced the firing rate of 5-HT neurons is fully in line with the enhancing effect of DBS on 5-HT levels in forebrain regions of both primates and rodents (Juckel *et al.*, 1999; Hamani *et al.*, 2010; Etievant *et al.*, 2015). Together, these data indicate that enhancement of 5-HT neurotransmission in forebrain regions at least in part underlies the therapeutic-like effect of DBS, similarly to the effect of all other antidepressant strategies (Blier and El Mansari, 2013).

2.5.13.2 Vagus nerve stimulation

Electrical stimulation of the vagus nerve is an effective adjunctive strategy for treatment of MDD (Schlaepfer *et al.*, 2008; Bajbouj *et al.*, 2010). Vagus nerve afferents terminate in the nucleus of the solitary tract, and VNS enhances the activity of LC NE neurons presumably by action on the paragigantocellularis and hypoglossal nuclei (Manta *et al.*, 2009). This enhancement of LC NE firing is presumably a direct effect of VNS, as its acute delivery increased LC c-Fos expression (an indirect marker of neural activity; Cunningham *et al.*, 2008). Following 14-day VNS, the α_2-adrenoceptor mediated signal in the hippocampus was enhanced, consistent with microdialysis data showing elevated NE levels in the hippocampus and prefrontal cortex under these conditions (Manta *et al.*, 2013). Enhanced NE neurotransmission by sustained VNS presumably increased the firing activity of DRN 5-HT neurons, as the dose-effectiveness of prazosin to inhibit DRN 5-HT neurons was significantly elevated after VNS administration (Manta *et al.*, 2009). In line with these results, sustained (but not acute) VNS enhanced c-Fos expression in the DRN (Furmaga *et al.*, 2011), and enhanced the tonic activation of 5-HT_{1A} receptors on CA3 pyramidal neurons independent of altered 5-HT reuptake or postsynaptic 5-HT_{1A} receptor sensitivity (Manta *et al.*, 2013). Possibly, enhanced 5-HT neurotransmission caused the observed decrease in firing rate of VTA DA neurons (Manta *et al.*, 2013), similarly to the effect of SSRIs. On the other hand, VNS increased DA levels in the prefrontal cortex and nucleus accumbens (Manta *et al.*, 2013). Behaviorally, sustained VNS improved depressive-like behavior in the FST and anxiety-

like behavior (Cunningham *et al.*, 2008; Furmaga *et al.*, 2011). Interestingly, these effects were prevented by 5,7-DHT but not 6-OHDA pretreatment, indicating that an intact 5-HT system is crucial for the therapeutic effect of VNS (Furmaga *et al.*, 2011).

2.5.13.3 Electroconvulsive therapy

Based on its superior remission rates, ECT remains the "golden standard" for therapeutic efficacy (Kho *et al.*, 2003; Dierckx *et al.*, 2012). Notably, this antidepressant strategy comes with the trade-off of profound memory loss in a large proportion of subjects (Burt *et al.*, 1995). Electrophysiological studies demonstrated that repeated – but not single – electroconvulsive shock administration caused a sensitization of 5-HT_{1A} receptors on CA3 pyramidal neurons in the rat hippocampus (de Montigny, 1984) and consequently, enhanced tonic activation of this receptor population (Haddjeri *et al.*, 1998b). Notably, other components of the 5-HT system, including firing activity of DRN 5-HT neurons, and sensitivity of somatodendritic 5-HT_{1A}- and terminal 5-HT_{1B} autoreceptors, remained unaltered by repeated electroconvulsive shocks (Chaput *et al.*, 1991; Blier and Bouchard, 1992). Furthermore, repeated ECT enhanced the bursting activity of LC NE neurons while it did not alter the firing activity of VTA DA neurons (Tsen *et al.*, 2013).

2.5.13.4 Sleep deprivation

Sleep disturbances in MDD are common, and generally include a delayed onset of sleep, attenuated sleep continuity, reduced total sleeping time, diminished latency to first rapid eye movement (REM) sleep, increased duration and density of REM sleep, and diminished slow wave sleep (Nutt *et al.*, 2008). Interestingly, approximately 50% of depressive patients was shown to significantly benefit from one night sleep deprivation (Boivin, 2000; Adrien, 2002). Although the precise mechanism by which sleep deprivation has this antidepressant effect, monoamine systems likely play a role herein. Indeed, DRN 5-HT neurons are fully active during wakefulness, while their discharge activity is attenuated during slow wave sleep and absent during REM sleep (Trulson and Jacobs, 1979; Guzmán-Marín *et al.*, 2000). Accordingly, intra-DRN infusion of 8-OHDPAT enhanced sleep, while tryptophan depletion promoted wakefulness (Adrien, 2002; Ursin, 2002). Notably, sleep deprivation was shown to enhance firing of 5-HT neurons (Trulson and Jacobs, 1979), to cause a desensitization of 5-HT_{1A} autoreceptors (Maudhuit *et al.*, 1996) while it downregulated SERT density (Hipólide *et al.*, 2005) and MAO-A activity (Thakkar and Mallick, 1993), all of which presumably lead to enhanced central 5-HT levels.

Similarly to the activity of 5-HT neurons, firing activity of LC NE neurons is attenuated during slow-wave sleep, and virtually absent during REM sleep (Aston-Jones and Bloom, 1981). Following sleep deprivation, the expression of ß-adrenergic receptors were downregulated (Pedrazzoli and Benedito, 2004), similarly to the effect of some antidepressants (Anand and Charney, 2000). Similarly to the effect on the 5-HT system, attenuated MAO-A (Thakkar and Mallick, 1993) activity and NET expression (Hipólide *et al.*, 2005) presumably enhance NE levels after sleep deprivation. Interestingly, the

antidepressant-like effect of sleep deprivation was lost after NE depletion (Asakura *et al.*, 1994).

2.5.14 Study rationale chapter I-III

This section described modulatory effects of antidepressant strategies on 5-HT, NE and/or DA systems. Particularly, the ubiquitous enhancement of tonic action on postsynaptic 5-HT_{1A} receptors by these strategies suggests an important role for enhanced 5-HT neurotransmission in the antidepressant response. Given the complex interaction between monoamine systems, and temporal changes in function of pharmacological targets by antidepressants, it is clear that the sustained effects of medications cannot be predicted based on its pharmacological signature. Accordingly, to gain more insight in the neurobiological basis of the antidepressant response and to prevent unwanted polypharmacy, *in vivo* characterization studies of antidepressant agents in the intact brain and after sustained administration are of crucial importance.

Asenapine is a tetracyclic atypical antipsychotic endowed with antidepressant potential (Szegedi *et al.*, 2011; Buchanan *et al.*, 2012). *In vitro*, it had potent antagonistic action at many 5-HT, NE and DA receptors, including 5-HT_{2A}, α_2-adrenergic, and D_2 autoreceptors (Shahid *et al.*, 2009). These actions were largely confirmed in a previous acute *in vivo* characterization study, with the exception that asenapine displayed partial agonism – rather than antagonism – at both pre- and postsynaptic 5-HT_{1A} receptors (Ghanbari *et al.*, 2009). To gain insight in dynamic changes induced by asenapine, the activity of 5-HT, NE, and DA neurons were determined following two- and 21-day

asenapine administration. Furthermore, the status of 5-HT_{2A} receptors, 5-HT_{1A}, D_2, and α_2-adrenergic autoreceptors were assessed to provide a neural framework for the effects of asenapine on monoamine neurons at the presynaptic level. In the hippocampus, 5-HT and NE terminal receptors, transporter proteins, postsynaptic receptors, an overall neurotransmission were determined to assess the effects of sustained asenapine on monoamine neurotransmission in projection areas. Results of this study are presented in chapter I.

Brexpiprazole is a novel agent currently under investigation for the treatment of schizophrenia, and as add-on for treatment-resistant depression. It shares structural and pharmacological characteristics with aripiprazole, an agent in wide clinical use. Together with cariprazine, an agent in phase III of clinical trials, these agents represent the class of "untypical" atypical antipsychotics, firstly because they are partial D_2 receptor agonists – rather than antagonists – and secondly, since their affinity for D_2 receptors is higher than for 5-HT_{2A} receptors (Shapiro *et al.*, 2003; Maeda *et al.*, 2014). *In vitro* data indicated that compared to aripiprazole, brexpiprazole is a more potent 5-HT_{1A} agonist with less intrinsic activity at D_2 receptors (Maeda *et al.*, 2014). Furthermore, brexpiprazole had potent *in vitro* antagonism at 5-HT_{2A}, α_{1B}- and α_2-adrenergic receptors, pharmacological qualities that might be of therapeutic relevance for the treatment depression and schizophrenia. Accordingly, the effect of brexpiprazole on these – and various other – pharmacological targets were assessed following acute brexpiprazole administration in chapter II.

The outcome of the acute study characterization served to design a further characterization of brexpiprazole following sustained administration. This study is

amongst the first to characterize the effects of sustained partial D_2 receptor agonism on D_2 receptor status and VTA DA population activity. Furthermore, modulation of pre- and postsynaptic components of the 5-HT and NE system were assessed, thereby facilitating comparison of the pharmacological properties of this agent to other atypical antipsychotics, and neural framework that might be relevant to the therapeutic effects of brexpiprazole is proposed in chapter III.

3) Stress and depression

3.1 What is stress?

Excessive exposure to threatening and/or painful conditions, which for the remainder of this work is referred to as "stress" (although this is a simplification, as the definition of stress remains a matter of debate; Anisman & Merali, 1999; Chrousos, 2009; Koolhaas et al., 2011; Levine & Ursin, 1991; Selye, 1950) can have detrimental effects on an organism. The eventual effect of a stressor depends on a complex interplay between the perception of a stressor and the susceptibility to stress at the individual level. Animal studies have shown that in particular, predictability and controllability are important modulators of stressor perception (Abbott *et al.*, 1984; De Boer *et al.*, 1989). For example, rats subjected to controllable tailshocks developed significantly less stomach ulcers than their yoked counterparts (Weiss, 1968). Hence, the contextual perception of a stressor rather than the stressor per se determines its impact. Chronicity is another important factor for stress outcome. Indeed, an organism will initially adapt to repeated stress exposure. However, when a stressor exceeds the adaptive capacity, maladaptations occur that might be detrimental; a cumulative effect of stress that is commonly referred to as "allostatic load" (McEwen, 1998). A striking example of such detrimental effects was first obtained in dogs, where uncontrollable shock exposure caused a lethargic acceptance when subsequently presented with an escapable shock a maladaptive coping style was coined "learned helplessness", and has been demonstrated to occur in many animals including humans (Overmier and Seligman, 1967; Seligman

and Maier, 1967). Notably, learned helpless behavior has been hypothesized to be closely linked to depression (Miller and Seligman, 1975). However, individuals vary greatly in the allostatic load required to cause maladaptations. This individual variation is due to susceptibility components, including genetic makeup, environmental factors, and their interaction. In this context, the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis is thought to play an important role.

3.2 The HPA axis

Stress causes an activation of cells containing corticotrophin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus (Swanson *et al.*, 1983). PVN CRH cells project to the anterior lobe of the pituitary gland, where CRH activates CRH1 receptors on corticotrophin neurons, which release adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH activates melanocortin receptors on the adrenal gland, resulting in enhanced synthesis and release of corticosteroids (CORT; cortisol in humans, and corticosterone in rats). CORT has two important physiological functions: first, it allocates metabolic resources (e.g. glucose, oxygen) to active organs and tissues, thereby facilitating the "fight or flight" response to stress. Secondly, CORT activates low affinity glucocorticoid receptor (GR) and high affinity mineralocorticoid receptor (MR) in the hippocampus, PVN and pituitary gland, leading to attenuated release of CRH and ACTH. Thus, CORT attenuates HPA axis activity in a negative-feedback fashion (de Kloet *et al.*, 1991; Joëls and de Kloet, 1994). Importantly, HPA axis malfunction –

particularly hyperactivity and/or diminished negative feedback regulation - has been linked to MDD.

3.3 The HPA axis in MDD

Some of the first data (Carroll *et al.*, 1968a; b) indicating concomitant MDD and HPA axis hyperactivation stems from data obtained with the dexamethasone (DEX) suppression test (DST), a tool originally used for diagnosis of hypercortisolemia (or Cushing's syndrome). DEX is a synthetic glucocorticoid which diminishes biosynthesis of the pro-opiomelanocortin (POMC) gene, the precursor for (amongst others) ACTH. Consequently, DEX administration reduces circulating CORT levels in healthy subjects on the following day. However, a subpopulation of MDD patients (20-30%) did not show this reduction, indicating reduced negative feedback on HPA axis activity in MDD (Carroll *et al.*, 1968b; Holsboer, 2001). Selectivity of the DST has been refined by the addition of a CRH challenge on the day after DEX administration, which was found to produce a greater and longer increase of circulating CORT and ACTH levels in up to 80% of depressed patients (Heuser *et al.*, 1994; Schmider *et al.*, 1995). Elevated HPA axis activity after the DST is especially prevalent in depressed patients with psychotic or bipolar features (Evans and Nemeroff, 1983; Schatzberg *et al.*, 1984; Nelson and Davis, 1997; Stetler and Miller, 2011), and has been related to poor treatment outcome (Greden *et al.*, 1980; Nemeroff *et al.*, 1984; Arana, 1985). In addition to HPA axis hyperreactivity following glucocorticoid challenges, measures of baseline HPA axis activity, such as plasma CORT and ACTH levels (Stetler and Miller, 2011; Lok *et al.*, 2012), and

cerebrospinal fluid CRF content (Nemeroff *et al.*, 1984; Hartline *et al.*, 1996), were elevated in MDD patients under baseline conditions. Accordingly, a high comorbidity exists between MDD and Cushing's syndrome, suggesting that elevated CORT levels might contribute to depressive symptoms (Dorn *et al.*, 1995, 1997). Furthermore, elevated transcription and expression levels of CRH in the PVN were demonstrated in brains of depressed individuals (Raadsheer *et al.*, 1994, 1995), further suggesting deregulation of HPA axis activity in MDD.

Activity of the adult HPA axis is in part regulated by exposure to maternal CORT during the fetal life stage (Deroche *et al.*, 1995; Maccari *et al.*, 1995). From an evolutionary point of view, this maternal regulation could serve as a telegraphic mechanism that allows adaptation of an organism to its future environment (Pike, 2005; Love and Williams, 2008), as proposed by the fetal programming hypothesis (Zagron and Weinstock, 2006). However, such adaptations can also be detrimental, and might lead to susceptibility for development of psychopathologies including MDD. There are two opposite hypotheses on the interaction between early and late life stress; the "mismatch" and the "two-hit" hypotheses (Nederhof and Schmidt, 2012). According to the "mismatch" hypothesis, altered HPA axis activity is maladaptive when the adult environment differs from the fetal conditions in terms of stress exposure. Alternatively, the "two-hit" hypothesis suggests that excessive fetal stress exposure causes a maladaptive response to stress in adulthood. Although it is clear that maternal trauma can increase the susceptibility for development of psychopathologies, including MDD, the large heterogeneity of the human population makes it difficult to dissect which of these hypotheses applies at the individual level in humans. To overcome this heterogeneity, as

well as ethical limitations, animal models are a valuable tool to assess central, peripheral and behavioral consequences of depressogenic-like conditions.

3.4. Prenatal stress: an animal model for depression

Ideally, animal models for human disorders should have identical causality (etiological validity), sympotomatology (face validity), and response to treatment (predictive validity; Willner, 1984).

Although the etiology of depression remains to be fully established, it is clear that prenatal stress (PNS) exposure in humans can contribute to maladaptive traits in adulthood, including increased risk for MDD (Brown *et al.*, 2000; Beydoun and Saftlas, 2008; Glover, 2014). Notably, these maladaptations are not specific to MDD, but have been linked to a wider spectrum of disorders (van Os and Selten, 1998; Lupien *et al.*, 2009). Accordingly, maternal stress in rats has been shown to not only induce a depressive-like phenotype (Barbazanges *et al.*, 1996; Drago *et al.*, 1999; Szymańska *et al.*, 2009), but also to impaired learning (Lemaire *et al.*, 2000; Bingham *et al.*, 2013), anxiety (Sun *et al.*, 2013; Van den Hove *et al.*, 2014), and increased risk for addiction (Yang *et al.*, 2006; Gao *et al.*, 2011). In light of these findings, PNS appears to induce a general risk factor for behavioral abnormalities.

Symptomatology of MDD consists of "a depressed mood or loss of interest or pleasure in daily activities for more than two weeks", accompanied by at least five of the following symptoms: irritable or depressed mood, decreased interest or pleasure, weight change or change in appetite, altered sleep architecture, changed motor function, fatigue or loss of energy, inappropriate feelings of guilt or worthlessness, diminished cognitive

function, and suicidal ideation (American Psychiatric Association, 2013). Clearly, some of these symptoms can solely be diagnosed verbally (e.g. feelings of low mood, worthlessness, guilt, or suicidal ideation), hence animal models cover the spectrum of depressive symptoms only partially. With this limitation in mind, it is noteworthy that PNS caused various depressive-like symptoms, including altered sleep (Dugovic *et al.*, 1999), anhedonia (Borges *et al.*, 2013; Sun *et al.*, 2013), impaired social behavior (Wilson and Terry, 2013), and locomotor impairments (Nagano *et al.*, 2012). Furthermore, PNS causes a lower birth weight (Drago *et al.*, 1999; Hausknecht *et al.*, 2013; Modir *et al.*, 2014), which by itself is a risk factor for MDD (Modir *et al.*, 2014). Moreover, PNS caused a hyperactivity of the HPA axis both under baseline (Van den Hove *et al.*, 2013; Wilson and Terry, 2013) and following stressor exposure (Maccari *et al.*, 1995; Dugovic *et al.*, 1999; Szymańska *et al.*, 2009), presumably reflecting blunted HPA axis regulation in MDD. Interestingly, PNS decreased central GR and MR expression, providing a mechanism for HPA axis hyperactivity that could be relevant to the human situation (Maccari *et al.*, 1995; Barbazanges *et al.*, 1996).

The predictive validity of PNS has been extensively assessed using the FST, a common screening model for the activity of antidepressants (Porsolt *et al.*, 1978). In this test, the time spent immobile is commonly used as a measure of behavioral despair, and has been related to depressive-like behavior. Accordingly, various studies demonstrated that increased immobility by PNS was reversed by administration of antidepressants, including agomelatine, aripiprazole, fluoxetine, imipramine, and mirtazapine (Morley-Fletcher *et al.*, 2004, 2011; Szymańska *et al.*, 2009; Ratajczak *et al.*, 2013). Interestingly, antidepressants normalized central glucocorticoid receptor expression and HPA axis

activity, suggesting that abnormal HPA axis activity contributed to the depressive-like phenotype induced by PNS (Morley-Fletcher *et al.*, 2004; Pawluski *et al.*, 2012). However, since both these antidepressants and stress 5-HT, NE and DA neurotransmission, altered activity of these monoaminergic systems are likely to contribute to the effects of PNS. Since these effects remain to be fully explored in vivo, an electrophysiological study was designed to gain more insight herein.

3.5 Study rationale chapter IV

The relatively high etiological, face, and predictive validity make PNS an attractive animal model for depression. However, little electrophysiological studies assessed the effect of such depressogenic-like conditions on monoamine system activities. Also, it remains to be established whether antidepressant agents have a different effect on monoamine systems in animals exposed to depressogenic conditions compared to controls. To gain more insight herein, the present study aimed to establish a prenatal stress protocol causing high fetal corticosterone exposure and lower birth weight, since these measures are amongst the most robust predictors of depressive-like behavior. In adulthood, the effect of PNS on the firing activity of 5-HT, DA and NE neurons was assessed. Furthermore, to determine if PNS had a depressogenic-like effect, swim behavior was scored in the FST. To assess whether PNS caused diminished negative feedback on the HPA axis, the CORT response to FST exposure was quantified. Finally, the effect of FST exposure on monoamine system activities following PNS was assessed

to determine a possible interaction between early and late life stress on a neural level. The results of this study are presented in chapter IV.

Chapter I: Sustained asenapine

Asenapine is a tetracyclic atypical antipsychotic agent approved for treatment of mixed or manic episodes in bipolar 1 disorder as monotherapy, or in combination with lithium or sodium valproate. In vitro, asenapine is an antagonist at various 5-HT, NE and DA receptors (Shahid *et al.*, 2009). In a previous electrophysiological characterization on the acute effects of asenapine in vitro antagonism at α_2-adrenergic and D_2 autoreceptors, and 5-HT_{2A} receptors were confirmed *in vivo* (Ghanbari *et al.*, 2009). However, acute asenapine acted as a partial agonist on pre- and postsynaptic 5-HT_{1A} receptors *in vivo*, despite potent antagonism at this receptor type *in vitro*. To gain insight in potential adaptive changes following long-term asenapine administration, its effects of two- and 21-day administration were assessed on various components of the 5-HT, NE and DA systems, including firing activity, their autoreceptors, 5-HT_{2A} receptors, and neurotransmission of 5-HT and NE in the hippocampus.

The experimental design of this study was drafted by Dr. Blier, Dr. El Mansari, and the present author. All electrophysiological experiments and their analysis were conducted by the present author. All authors contributed to and approved of the final manuscript. Preliminary data of this study were presented at the annual meeting of the Society of Biological Psychiatry in May 2013, and the manuscript was published in the European Journal of Neuropsychopharmacology in April 2015.

Asenapine alters the activity of monoaminergic systems following its subacute and long-term administration: an *in vivo* electrophysiological characterization

Running title: Asenapine and monoamine systems

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Abstract

Asenapine is a tetracyclic atypical antipsychotic used for treatment of schizophrenia and mania. Previous *in vivo* electrophysiological studies demonstrated antagonistic action of asenapine at dopamine D_2, serotonin $(5\text{-HT})_{2A}$, and α_2-adrenergic receptors. Here, we assessed monoamine system activity after two-day and 21-day asenapine administration at a dosage (0.1 mg/kg/day) resulting in clinically relevant plasma levels. In the ventral tegmental area (VTA), asenapine increased the number of spontaneously active dopamine neurons, while firing parameters remained unchanged. Asenapine partially prevented the D_2 autoreceptor-mediated inhibitory response to apomorphine after two days of administration. This effect was lost after 21 days of administration, suggesting adaptive changes leading to D_2 receptor sensitization. Asenapine increased the firing activity of noradrenergic neurons in the locus coeruleus (LC) after 21, but not two days of administration. Furthermore, it potently blocked 5-HT_{2A} receptors while α_2-adrenergic receptors were unaffected by this drug regimen. While noradrenergic tone on α_1-adrenergic receptors remained unchanged, both acute and long-term asenapine administration partially blocked α_2-adrenergic receptors in the CA3 region of the hippocampus. In the dorsal raphe nucleus, asenapine increased the firing rate of 5-HT neurons after two, but not 21 days of administration. In addition, responsiveness of 5-HT_{1A} autoreceptors was unaltered by asenapine. In the hippocampus, 21-day asenapine administration increased serotonergic tone by partial agonistic action on postsynaptic 5-HT_{1A} and terminal 5-HT_{1B} receptors. Taken together, asenapine had profound effects on both catecholamine systems, potently blocked 5-HT_{2A} receptors, and enhanced 5-HT tone, effects that could be important in treatment of mood disorders and schizophrenia.

Introduction

Asenapine (ORG-5222) is indicated for treatment of schizophrenia and acute manic or mixed episodes associated with bipolar 1 disorder as monotherapy or in combination with lithium or sodium valproate. It has a similar affinity for dopamine (DA) D_2 receptors as the prototypical antipsychotic haloperidol, and a higher affinity for several other receptors compared to D_2 receptor affinity. These are, in descending order, serotonin $(5\text{-HT})_{2C}$, 5-HT_{2A}, 5-HT_7, 5-HT_{2B}, 5-HT_6, α_{2B}-adrenoceptors, D_3, H_1, D_4, α_2, α_{1A} and α_{2C} receptors (Shahid *et al.*, 2009). In accordance with negligible action of asenapine on cholinergic receptors, asenapine has little metabolic side-effects in the clinic (Schoemaker *et al.*, 2012) – in contrast to the effects of clozapine, olanzapine and risperidone (Kinon *et al.*, 2001; Potkin *et al.*, 2007). Furthermore, the risk for development of dopaminergic side effects (extrapyramidal symptoms) commonly associated with D_2 receptor antagonism is relatively benign for asenapine, possibly due to its abovementioned activities at a variety of non-dopaminergic receptors acting within the striatonigral pathway (Kane *et al.*, 2010; Tarazi and Stahl, 2012). In this context, stronger antagonistic action on 5-HT_{2A} compared to D_2 receptors is thought to be an important pharmacological feature, which is shared by all atypical antipsychotics (Meltzer *et al.*, 2003).

These clinical findings are consistent with results from animal studies. For example, asenapine suppressed escape behavior in the conditioned avoidance response (CAR; a model for antipsychotic action) while it failed to induce catalepsy (a model for extrapyramidal symptoms) when administered in the dose range of 0.05-0.2 mg/kg (Frånberg *et al.*, 2008). Furthermore, asenapine effectively reversed apomorphine-induced prepulse inhibition and amphetamine-induced locomotion at a dose as low as 0.03 mg/kg, suggesting potent antagonistic action at D_2 receptors (Marston *et al.*, 2009). Subchronic asenapine (0.2 mg/kg/day for 5 days) reduced escape behavior in the CAR up to 40 days after drug administration, a result attributable to D_2 receptor sensitization associated with antipsychotic action (Gao and Li, 2013). In addition, four-week asenapine administration restored phenylcyclidine (PCP)-induced impairments in reversal learning at a dose of 0.075 mg/kg/day and restored sucrose consumption in animals subjected to

chronic mild stress (CMS) at a dose of 0.6 mg/kg/day, suggesting therapeutic potential of asenapine in hedonic and cognitive domains (McLean *et al.*, 2010; Marston *et al.*, 2011).

Previous *in vivo* electrophysiological studies showed that acute asenapine administration increased the firing activity of DA neurons in the ventral tegmental area (VTA) and reversed the inhibitory effect of the DA agonist apomorphine (ED_{50} = 40 ± 2 µg/kg), demonstrating *in vivo* antagonistic action of asenapine at D_2 autoreceptors (Ghanbari et al. 2009; Frånberg et al. 2009). Similarly, asenapine increased the firing activity of locus coeruleus (LC) norepinephrine (NE) neurons and reversed the inhibitory effect of the α_2-adrenoceptor agonist clonidine on these neurons (ED_{50}= 85 ± 2 µg/kg), putting antagonistic activity at α_2-adrenergic autoreceptors into evidence (Ghanbari et al. 2009; Frånberg et al. 2009). Asenapine also reversed the inhibitory effect of the preferential 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI; ED_{50} = 75 ± 2 µg/kg), indicating antagonistic action of asenapine at this receptor subtype (Ghanbari *et al.*, 2009). In support of this, local perfusion of the prefrontal cortex with asenapine blocked both α_2–adrenergic and 5-HT_{2A} receptors (Frånberg et al. 2012). Although acute systemic administration of asenapine did not alter the firing rate of 5-HT neurons in the dorsal raphe nucleus (DRN; Frånberg et al. 2009; Ghanbari et al. 2009), it acted as a partial 5-HT_{1A} agonist when applied microiontophoretically in both the DRN and hippocampus (Ghanbari *et al.*, 2009). Agonistic activity at 5-HT_{1A} receptors together with D_2 and α_2-adrenergic receptor antagonism provides a mechanism by which asenapine increases prefrontal DA and NE levels (Frånberg *et al.*, 2009), a common effect of acute antipsychotic drug administration (Westerink *et al.*, 1998).

To extend insight in the long-term effects of asenapine on therapeutically relevant pharmacological targets, the present study investigated the effects of two and 21 day asenapine administration (0.1 mg/kg/day) on the discharge activity of VTA DA, LC NE, and DRN 5-HT neuron populations. In these brain regions, the status of D_2, 5-HT_{1A}, 5-HT_{2A}, and α_2-adrenergic receptors was characterized using pharmacological tools. In the CA3 region of hippocampus, a major monoaminergic projection site, the effect on 5-HT and NE neurotransmission was assessed following 21-day asenapine administration.

Experimental procedures

Animals

Experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 300-450 g housed under standard laboratory conditions (12:12 light-dark cycle with food and water ad libitum). *In vivo* extracellular unitary recordings were carried out in chloral hydrate anaesthetized rats (400 mg/kg; i.p.) that were mounted in a stereotaxic apparatus. Body temperature was maintained at 37 °C throughout the experiment utilizing a thermistor-controlled heating pad. If applicable, prior to the electrophysiological recordings a catheter was inserted in a lateral tail vein for systemic intravenous (i.v.) injection of pharmacologic agents. At the end of experiments, animals were euthanized by a lethal dose of chloral hydrate (4 % solution, i.p) and a blood and brain samples were collected. 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, Ontario, Canada).

Treatment

Using sterile surgery techniques, isoflurane-anaesthetized animals were implanted with an subcutaneous ALZET osmotic minipump (Durect Corp, Cupertino, CA) that delivered saline (vehicle) or asenapine (0.1 mg/kg/day) for 2 or 21 days at a constant rate. Temgesic (0.1 mg/kg, s.c.) was administered for post-surgical analgesia.

Compounds

Asenapine (0.1 mg/kg), the 5-HT_{1A} receptor agonist flesinoxan (100 µg/kg), the 5-HT_{1A} receptor antagonist WAY 100.635 (100 µg/kg), the preferential 5-HT_{2A} receptor agonist DOI (0.1 mg/kg), the 5-HT_{2A} receptor antagonist MDL 100.907 (200 µg/kg), the α_1-adrenoreceptor antagonist prazosin (100 µg/kg), the α_2-adrenoreceptor agonist clonidine (5 µg/kg), and the α_2-adrenoreceptor antagonist idazoxan (1 mg/kg) were dissolved in distilled water. The DA agonist apomorphine (40 µg/kg) and the D_2 receptor antagonist haloperidol (200 µg/kg) were dissolved in a 0.5 % lactic acid solution in distilled water; pH of the solution was adjusted to 4.5 by addition of NaOH. Asenapine

was provided by Merck (Montreal, QC); all other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada).

In vivo electrophysiological recordings

Extracellular recordings of neurons in the VTA, DRN and locus coeruleus (LC) were carried out with a single-barrel glass micropipette (Stoelting, Spencerville, MD) preloaded with 2 M NaCl and with impedance between 2-6 MΩ. Neurons in the CA3 region of the hippocampus were recorded with a 5-barrel micropipette (impedances: central barrel 2-5 MΩ, side barrels 20-30 MΩ). The central barrel, used for unitary recordings, and one sidebarrel, used for automatic current balancing, were filled with 2 M NaCl; the other barrels were filled with 5-HT creatinine sulfate (10 mM in 0.2 M NaCl, pH=4), NE bitartrate (10 mM in 0.2 M NaCl, pH=4) or quisqualalic acid (1.5 mM in 0.2 M NaCl, pH=4). 5-HT and NE were ejected as cations (+10 to +20 nA) and retained with a current of -20 nA; quisqualate was ejected as an anion (-1 to +1 nA) and retained with a current of +10 nA.

Recording of VTA DA neurons

Putative DA neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP 3.2 to 3.6, ML 0.6 to 1.0, DV 7.0 to 9.0. At these coordinates, neurons with a triphasic action potential with a marked negative deflection, long duration (3-5 ms) often with an inflection or "notch" on the rising phase, irregular spontaneous single firing pattern (1-10 Hz) and slow bursting activity with decrementing action potential amplitude were recorded (Grace et al. 1983). The start of a burst was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an interspike interval (ISI) >160 ms (Grace and Bunney, 1984). Inter-spike interval (ISI) analysis was used to determine the following burst parameters: bursts/min, % of spikes in burst, number of spikes/burst, and ISI within bursts. To quantify of the number of active DA neurons per rat, 9 tracts spaced by 200 μm were performed in standardized grid. The total number of DA neurons recorded divided by the number of tracts was used as a measure for dopamine population activity (Chenu *et al.*, 2013).

Recording of LC NE neurons

NE neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP -1.0 to -1.2, ML 1.0 to 1.3, DV 5.0 to 7.0. NE neurons were identified using the following criteria: regular firing rate (0.1-5 Hz) and a long duration (0.8-1.2 ms) of the positive phase of the action potential (Vandermaelen & Aghajanian 1982), and a brisk excitatory response followed by a short period of inhibition (~1 s) in reaction to a nociceptive pinch of the contralateral hind paw. ISI analysis with the same criteria as for DA neurons was used to determine burst activity of NE neurons (Chenu *et al.*, 2013).

Recording of 5-HT neurons

Putative 5-HT neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP 1.0 to 1.2, ML 0, DV 5.0 to 7.0. At these coordinates, only neurons with a bi- or triphasic extracellular waveform with a long-duration (0.8-1.2 ms) positive phase, and regular firing in the range of 0.1-5 Hz were recorded (Vandermaelen & Aghajanian 1982).

Recording of pyramidal neurons in the CA3 region of the hippocampus

CA3 pyramidal neurons were recorded by positioning multibarrel glass micropipettes at the following coordinates (in mm from lambda): AP: 3.8-4.2, L: 4.0-4.2, DV 3.5-4.5. Since most CA3 pyramidal neurons are not spontaneously active in chloral hydrate anesthetized rats, a small current of quisqualate (-1 to +1 nA) was used to activate them within their physiological firing range (10-15 Hz, Ranck, 1973). The current and duration of 5-HT and NE ejection was kept constant before and after each i.v. administraition of pharmacological compounds. Neuronal responsiveness to the iontophoretic application of 5-HT was quantified as the total number of spikes inhibited during a 50-second ejection period of 5-HT divided by the ejection current, and expressed as spikes suppressed/nA. Activity of the 5-HT transporter (SERT) was assessed by determining the recovery time (in seconds) of CA3 pyramidal neurons to 50% of baseline following complete suppression of firing by a 50-second ejection period of 5-HT ejected

at 20 nA, and expressed as RT_{50} (Piñeyro *et al.*, 1994). Responsiveness to NE was assessed by determining the total number of spikes suppressed from start of NE ejection to recovery to 80% of baseline firing rate at an ejection current of 10 nA. To determine the degree of tonic activation of postsynaptic 5-HT_{1A} receptors, firing activity of CA3 pyramidal neurons was decreased to ~4 Hz and a physiological saline injection was administered (100 μL, i.v.) followed by four consecutive injections of the 5-HT_{1A} receptor antagonist WAY 100.635 (25 μg/kg at two-minute intervals). The average firing rate (spikes/s) of neurons during the second half of the 120 second period following each WAY 100.635 injection was as percentage change from baseline. At the end of each experiment, 5-HT (20 nA) was ejected to confirm blockade of 5-HT_{1A} receptors. Using the same procedure, saline, the α_2-adrenoceptor antagonist idazoxan (1 mg/kg, i.v.), and the α_1-adrenoceptor antagonist prazosin (100 μg/kg) were administered to assess the degree of tonic activation by NE on CA3 pyramidal neurons; NE was ejected to confirm postsynaptic adrenoceptor blockade at the end of each experiment.

Electrical stimulation of afferent 5-HT projections to hippocampus

A bipolar electrode (NE-110, David Kopf, Tujanga, CA) was inserted at the following coordinates (in mm from lambda): AP +1.0, L 0.0, DV 7.8-8.2. A stimulator (S8800, Grass instruments, Quincey, MA) was used to deliver 200 square pulses (0.5 ms, 1 or 5 Hz) at 300 μA. Duration of inhibition per stimulation was plotted in a peristimulus time histogram (PSTH) with 2 ms bin size. The inhibitory effect on CA3 neurons of 5-HT fiber stimulation was expressed as duration of silence (DOS, in ms), defined as the period from the first bin showing a 50% reduction in the number of events per bin from the prestimulus value and the first subsequent bin attaining a 90% recovery of the number of events per bin from prestimulus values (Chaput *et al.*, 1986). By applying low- and high-frequency stimulations onto the same neuron (1 and 5 Hz, respectively), low and high levels of 5-HT are produced in the biophase of the terminal 5-HT autoreceptor. Under normal conditions, 1 Hz stimulation results in more 5-HT release and a longer DOS than 5 Hz stimulation due to stronger activation of terminal 5-HT_{1B} autoreceptors with the latter stimulation frequency (Chaput *et al.*, 1986; Blier *et al.*, 1989). However, when 5-HT_{1B} receptors are blocked with a selective antagonist, 5 Hz stimulation produces a

longer DOS than 1 Hz stimulation , as more 5-HT is released in absence of terminal 5-HT_{1B} autoreceptor mediated negative feedback (Chaput *et al.*, 1986).

Data analysis

Recordings of single units were made using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Recordings were filtered from noise using post-recording waveform analysis. Presynaptic recordings were exported as text files and analyzed using The BurstiDAtor software (Oosterhof and Oosterhof, 2013) to obtain firing and burst parameters of individual neurons. To determine dose-response relation between firing activity and administration of pharmacological tool compounds, the slope for individual neurons was calculated using linear regression and analyzed using one-way ANOVA followed by Bonferroni post-hoc analysis. Statistical analysis of firing rate, burst parameters, and number of neurons/tract/rat were analyzed with a two-way ANOVA. When two observations were made within the same subject, data were analyzed with a paired t-test. When three or more observations were made within the same subject, data were analyzed using repeated measurement ANOVA and Bonferroni *post-hoc* analysis. Results are expressed mean, error bars represent S.E.M. The “a” in histograms indicates the number of animals per group, the “n” indicates number of neurons recorded.

Results

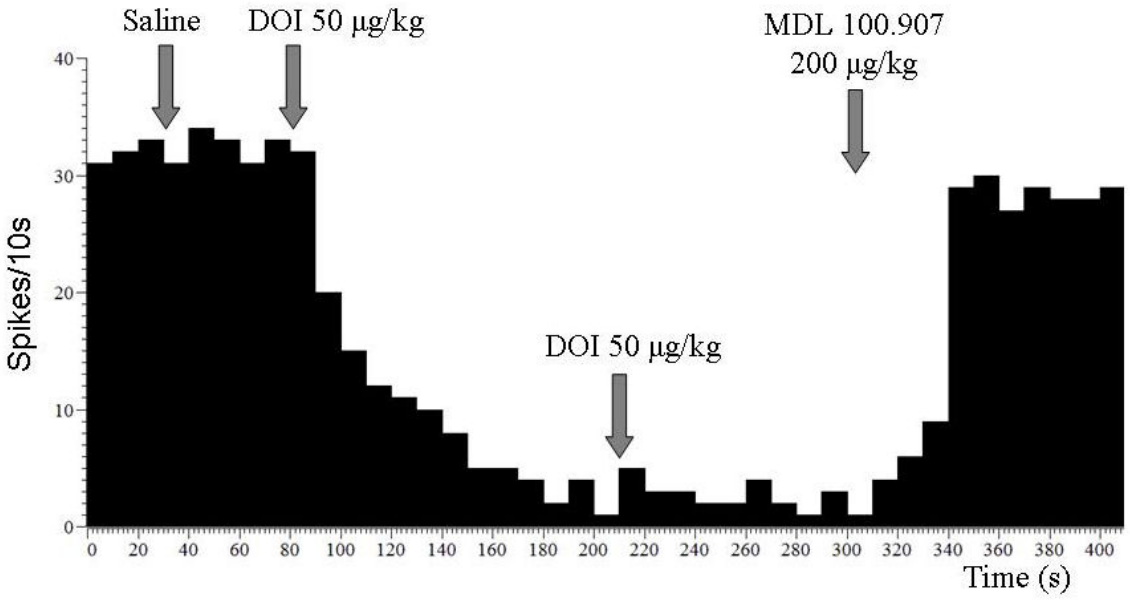
Blood plasma levels following sustained asenapine administration

Following two days of asenapine administration, blood plasma levels were 2.3 ± 0.3 ng/ml (n=6) and 2.5 ± 0.5 ng/ml after 21 days of administration (n=10), values that correspond to blood plasma levels following 5 mg twice daily asenapine treatment in humans at steady state (Chapel *et al.*, 2009).

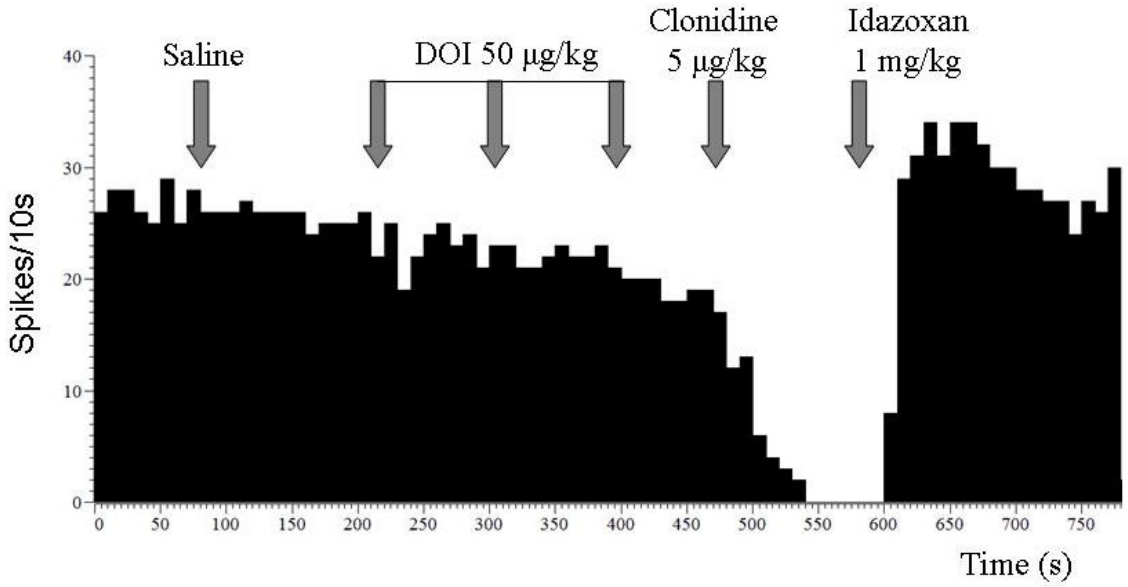
Effect of asenapine on 5-HT_{2A} receptors in LC after two and 21 days of administration

There was no difference in the baseline firing activity of LC NE neurons used for assessment of the dose-response effect to DOI, and injection of saline did not change the firing rate from baseline in either group ($p>0.05$). In animals receiving vehicle, DOI had a potent inhibitory effect on the firing activity of LC NE neurons (ED_{50} = 44 µg/kg). This inhibitory effect was lost following two and 21 days asenapine administration ($F_{1,15}$=53.2, $p<0.0001$, figure 1D). For illustrative firing histograms of the response of a LC NE neuron to DOI in a vehicle, two-day asenapine and 21-day asenapine administered animal see figure 1A, 1B and 1C, respectively.

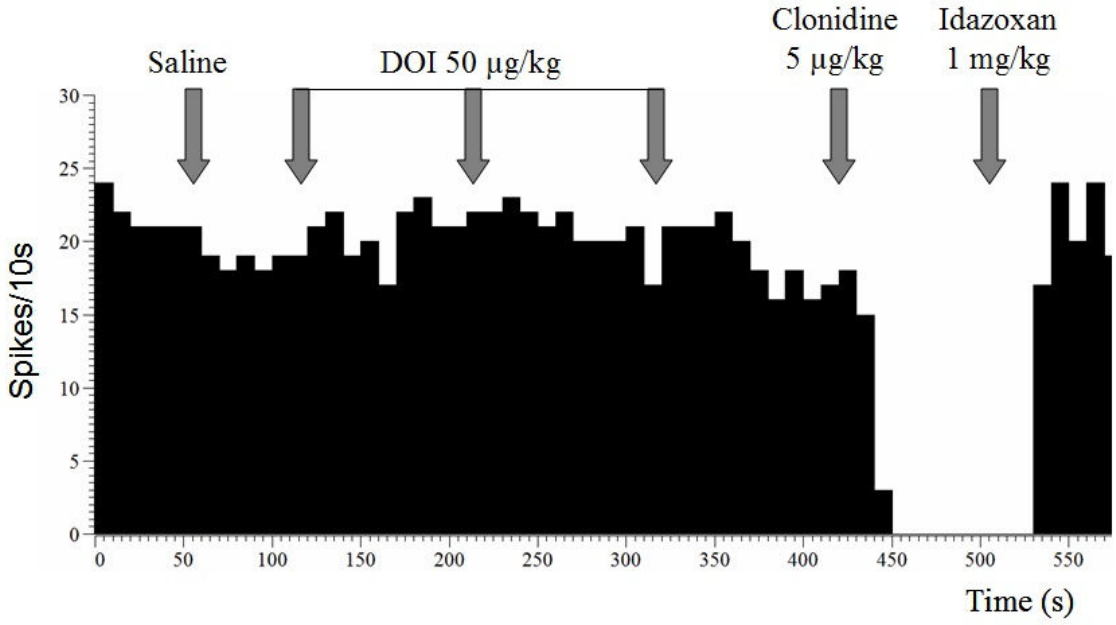
1A



1B


Saline
DOI 50 µg/kg
Clonidine
5 µg/kg
Idazoxan
1 mg/kg
Spikes/10s
Time (s)

1C


Saline
DOI 50 µg/kg
Clonidine
5 µg/kg
Idazoxan
1 mg/kg
Spikes/10s
Time (s)

1D

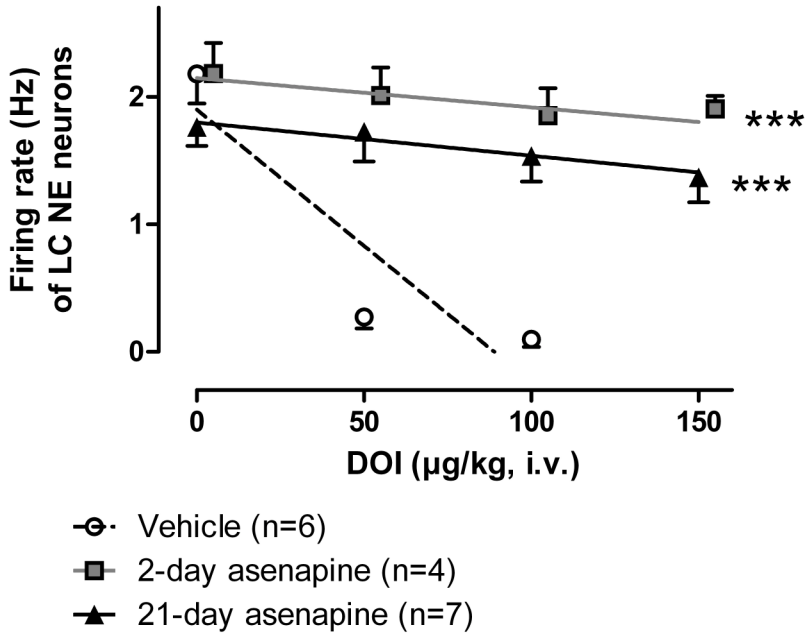


Figure 1: Assessment of antagonistic properties of asenapine on 5-HT_{2A} receptors. (A, B, C) Representative firing rate histograms of a LC NE neuron illustrating the effect of cumulative doses of the 5-HT_{2A} agonist DOI on firing rate in a vehicle (A) and two-day asenapine (B) and a 21-day asenapine (C) administered animal. D) Relationship between the average suppression of firing of LC NE neurons by DOI in vehicle- and asenapine-administered animals. Note in (B) and (C) the complete inhibition of the LC NE neuron after administration of clonidine at a dose of 5 µg/kg, indicating functional α_2-adrenoceptors.
Data were analyzed with a one-way ANOVA followed by Bonferroni post-hoc analysis. Error bars represent S.E.M. *Significant effect of asenapine compared to vehicle administration; ***p<0.001

Effects of two- and 21-day asenapine administration on VTA DA neurons

There was no difference in the baseline firing activity of VTA DA neurons used for assessment of the dose-response effect to apomorphine, and injection of saline did not change their firing rate from baseline activity in either group ($p>0.05$). There was a significant difference in the response to administration of 40 µg/kg ($F_{2,18}=10.2$, $p<0.001$). Post-hoc testing revealed a significant difference between vehicle and two-day asenapine administered animals ($p<0.001$, figure 2D), while there was no difference between vehicle and 21-day asenapine administered animals ($p>0.05$). In two-day asenapine administered animals, a second injection of apomorphine (40 µg/kg) did not further reduce the firing rate of neurons (paired t-test, $p=0.10$). Acute asenapine administration (500 µg/kg, i.v.) fully reversed the inhibitory effect of apomorphine on firing rate (two-day asenapine group, n=3; 21-day asenapine group; n=4, paired t-test, $p>0.05$). For illustrative firing histograms of the response of a VTA DA neuron to apomorphine in a vehicle, two-day and 21-day asenapine administered animal, see figure 2A, 2B and 2C, respectively.

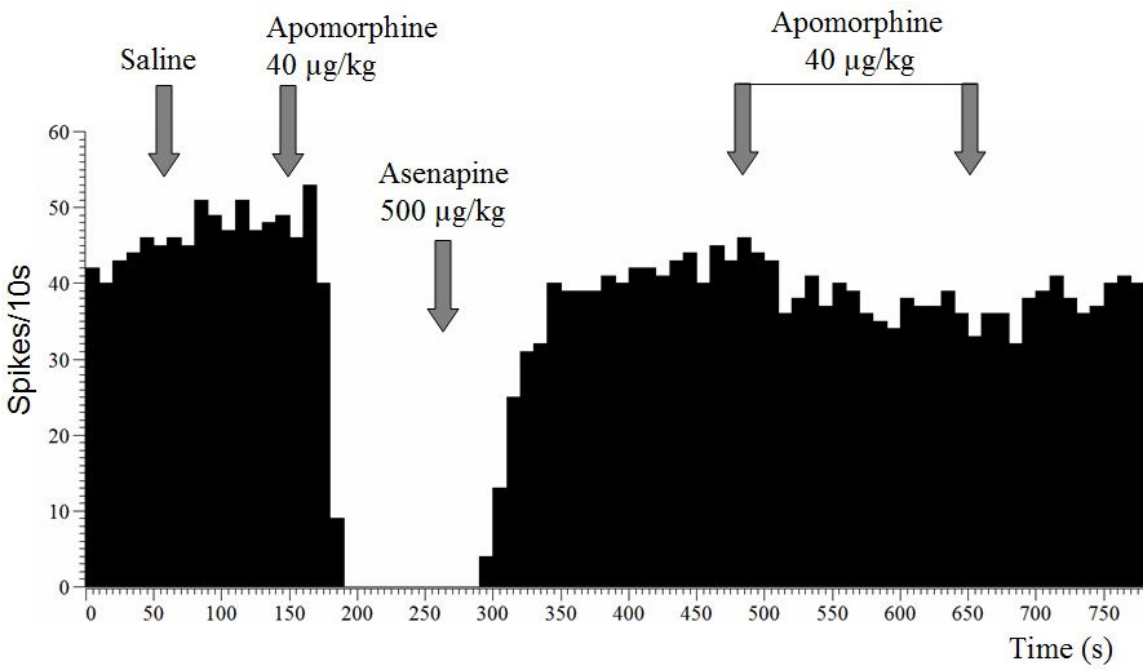
Administration of asenapine significantly increased the number of spontaneously active VTA DA neurons after 2 and 21 days of administration (one-way ANOVA; treatment, $F_{1,16}=189$, $p=0.026$, figure 2E). In vehicle but not asenapine administered animals, the percentage of spikes in burst increased after 21 days compared to two-day administration (two-way ANOVA; time x treatment, $F_{1,250}=5.37$, $p=0.021$). Asenapine administration did not alter firing rate, percent of spikes in burst, spikes per burst, ISI and number of neurons displaying bursting activity (two-way ANOVA, $F_{1,250}$, $p>0.05$, table 1).

Table 1: Effect of asenapine administration on discharge parameters of VTA DA neurons

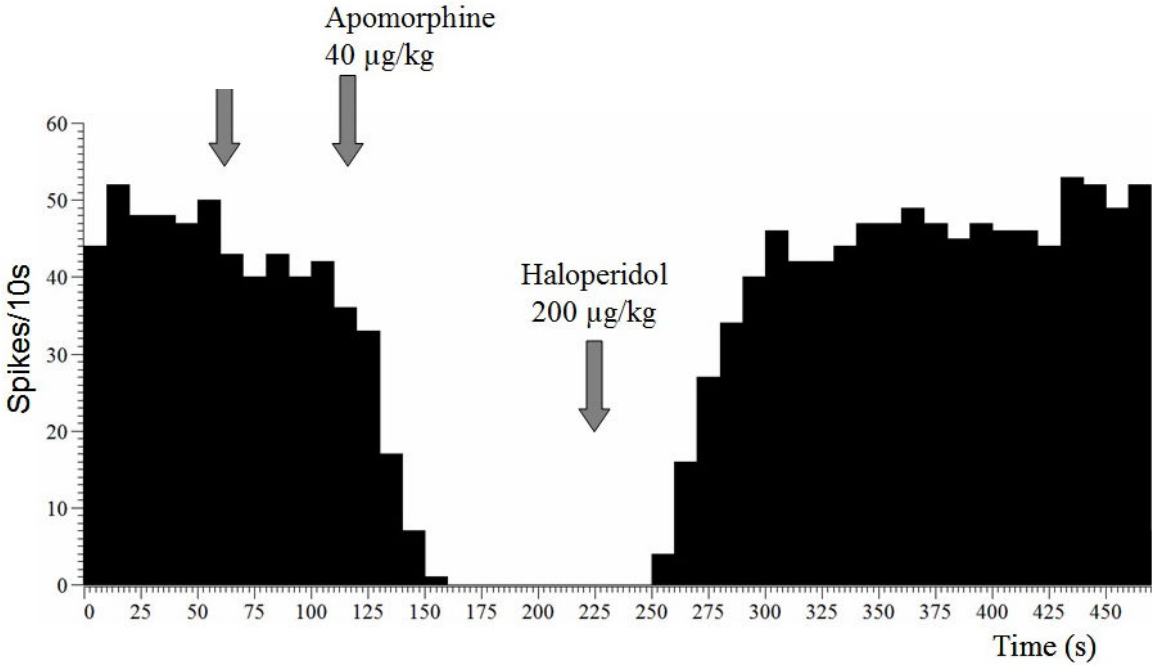
Parameter	Firing rate (Hz)	Bursts/min	% spikes in burst	Spikes /burst	ISI	# of neurons	# of neurons bursting
Vehicle x 2 days	3.8 ± 0.2	16.8 ± 3.5*	18.9 ± 3.7	2.6 ± 0.1	68.6 ± 3.1	53	39
Asenapine x 2 days	3.7 ± 0.1	23.1 ± 1.9	29.2 ± 2.4	2.7 ± 0.1	67.8 ± 1.1	121	112
Vehicle x 21 days	3.9 ± 0.3	27.4 ± 3.5	35.4 ± 4.7	2.9 ± 0.2	62.5 ± 2.4	43	39
Asenapine x 21 days	3.5 ± 0.2	23.3 ± 2.7	29.1 ± 3.1	2.8 ± 0.1	63.8 ± 2.0	82	68

Data are presented as mean ± SEM, * ANOVA, $p<0.05$.

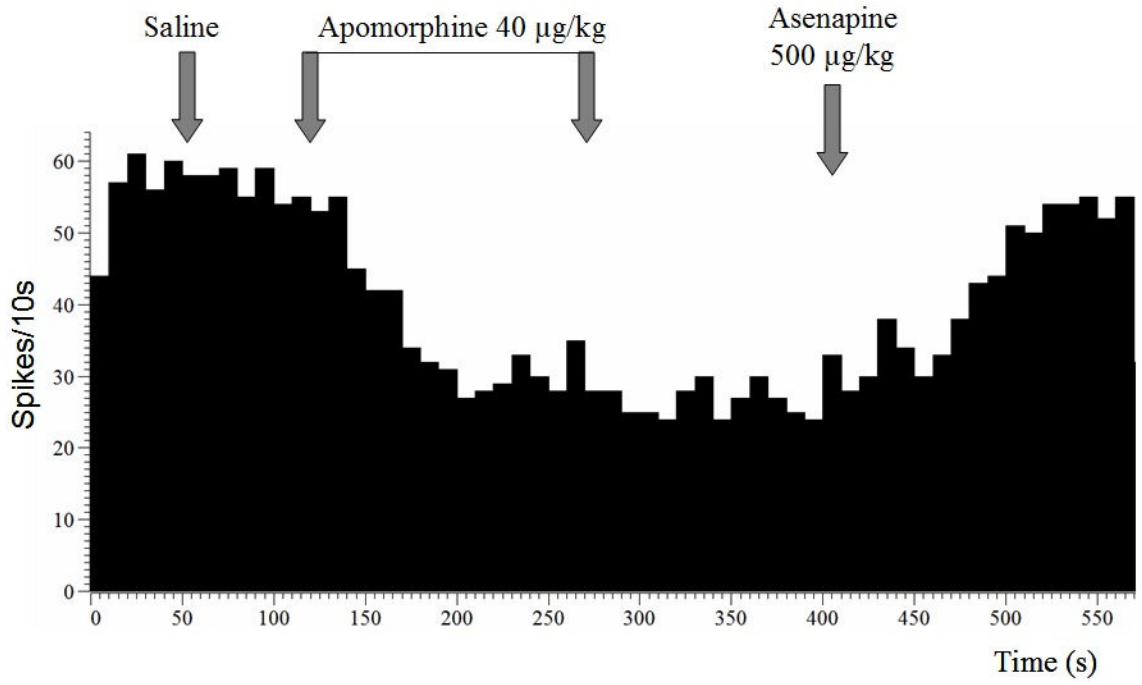
2A


Saline
Apomorphine
40 µg/kg
Asenapine
500 µg/kg
Apomorphine
40 µg/kg
Spikes/10s
Time (s)

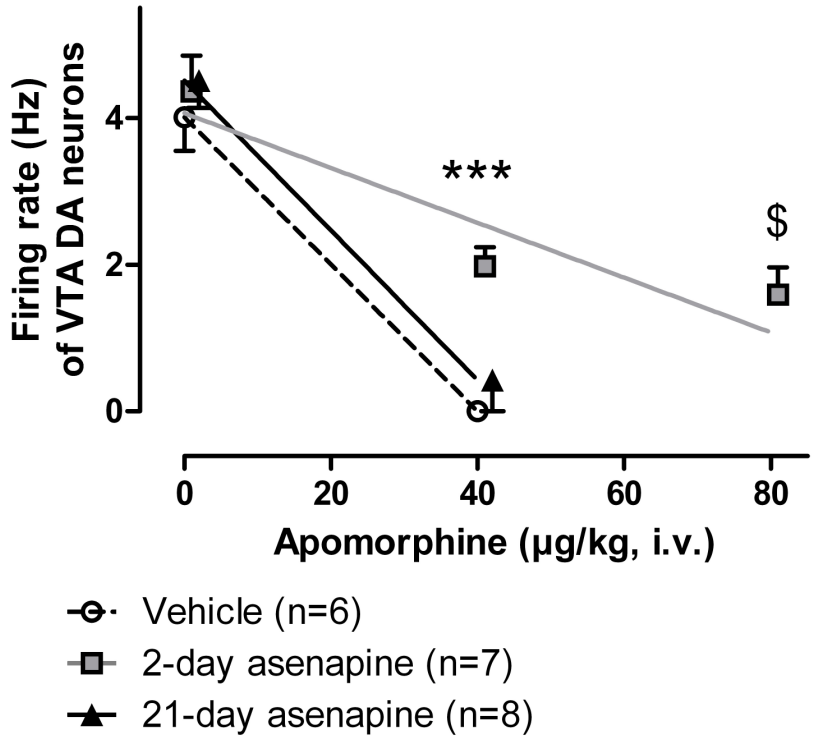
2B


Apomorphine
40 µg/kg
Haloperidol
200 µg/kg
Spikes/10s
Time (s)

2C


Saline
Apomorphine 40 µg/kg
Asenapine
500 µg/kg
Spikes/10s
0
10
20
30
40
50
60
0
50
100
150
200
250
300
350
400
450
500
550
Time (s)

2D


Firing rate (Hz)
of VTA DA neurons
0
2
4
***
$
0
20
40
60
80
Apomorphine (µg/kg, i.v.)
Vehicle (n=6)
2-day asenapine (n=7)
21-day asenapine (n=8)

2E

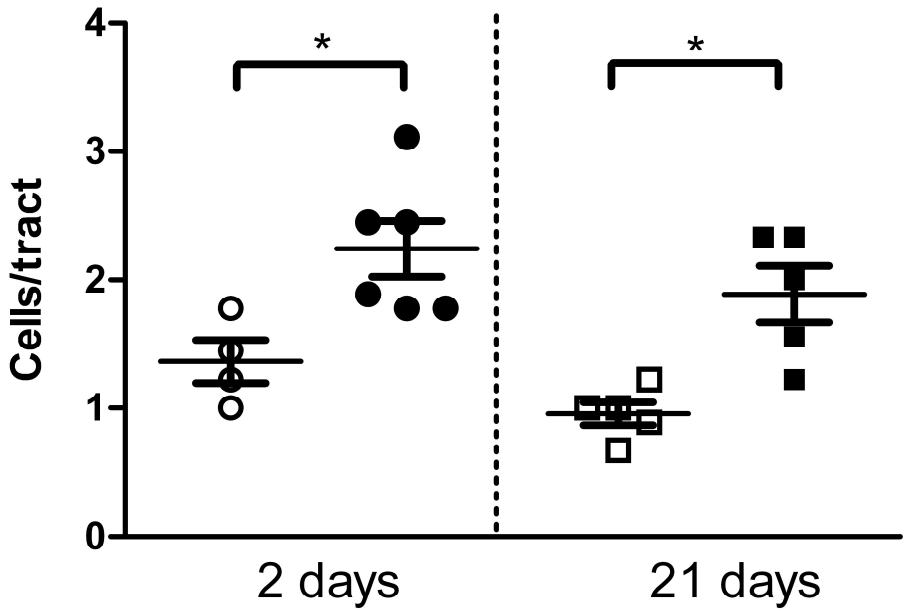


Figure 2 Effect of asenapine administration on VTA DA neurons. (A, B, C) Representative firing histograms of a VTA DA neuron illustrating the effect of acute administration of the dopamine agonist apomorphine in a vehicle (A), two-day asenapine (B) and 21-day asenapine (C) administered animal. D) Relationship between the average suppression of firing of VTA DA neurons by apomorphine. E) Average number of active DA neurons per 9 electrode tracts in animals administered vehicle (open symbols) or asenapine (closed symbols) for 2 and 21 days. Data were analyzed with one-way ANOVA (D,E) followed by Bonferroni post-hoc analysis (D). Error bars represent S.E.M.
***Significant effect of asenapine compared to vehicle administration, *p<0.05, ***p<0.001.**
[$]Nonsignificant effect compared to 40 μg/kg.

Effects of two- and 21-day asenapine administration on NE neurons in the LC

In the LC, asenapine administration significantly increased the firing rate of NE neurons after 21 days of administration (two-way ANOVA, time x treatment; $F_{1,407}$=7.0, p=0.009, figure 3A). The number of bursts per minute, percent of spikes in burst, spikes/burst, ISI and number of neurons displaying bursting activity were unaltered by asenapine administration for 2 and 21 days (two-way ANOVA, $F_{1,407}$, p>0.05, table 2).

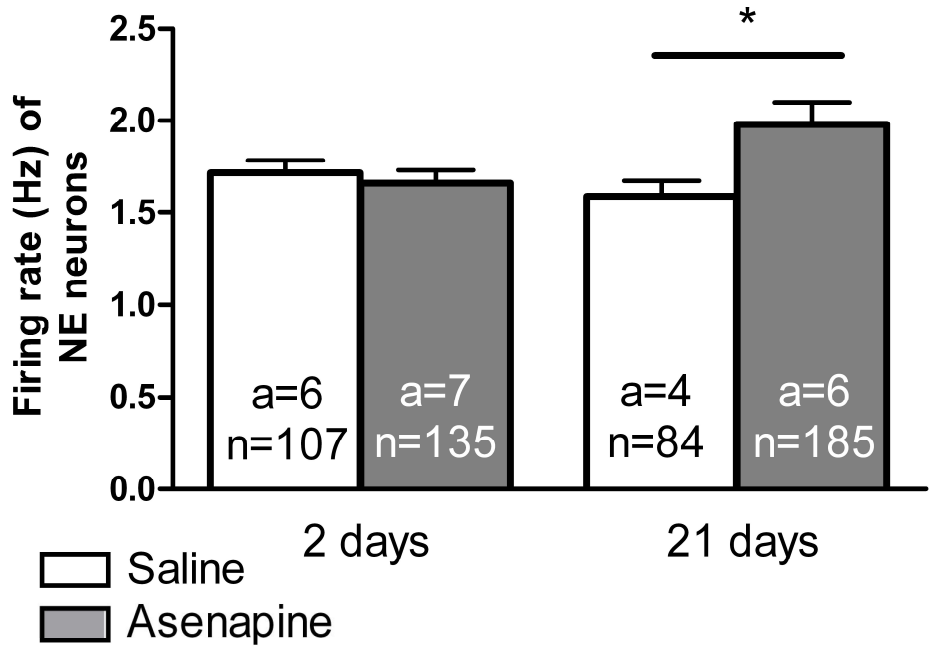
Following two-day and 21-day asenapine administration, the inhibitory response of LC NE neurons to clonidine did not differ from control levels (p>0.05, figure 3B). For an illustration of the inhibitory effect of clonidine after asenapine administration see figure 1B and 1C.

Table 2: Effect of asenapine administration on discharge parameters of LC NE neurons

Parameter	Bursts/min	% spikes in burst	Spikes/burst	ISI	# of neurons	# of neurons bursting
Vehicle x 2 days	4.4 ± 0.7	2.8 ± 0.5	2.0 ± 0.0	65.7 ± 2.1	107	22
Asenapine x 2 days	10.2 ± 1.2	5.5 ± 0.9	2.1 ± 0.1	67.9 ± 1.3	135	45
Vehicle x 21 days	6.5 ± 2.8	4.2 ± 1.2	2.1 ± 0.1	63.3 ± 4.3	84	16
Asenapine x 21 days	8.3 ± 1.4	4.6 ± 0.9	2.1 ± 0.0	66.1 ± 2.2	85	34

Data are presented as mean ± SEM.

3A



3B

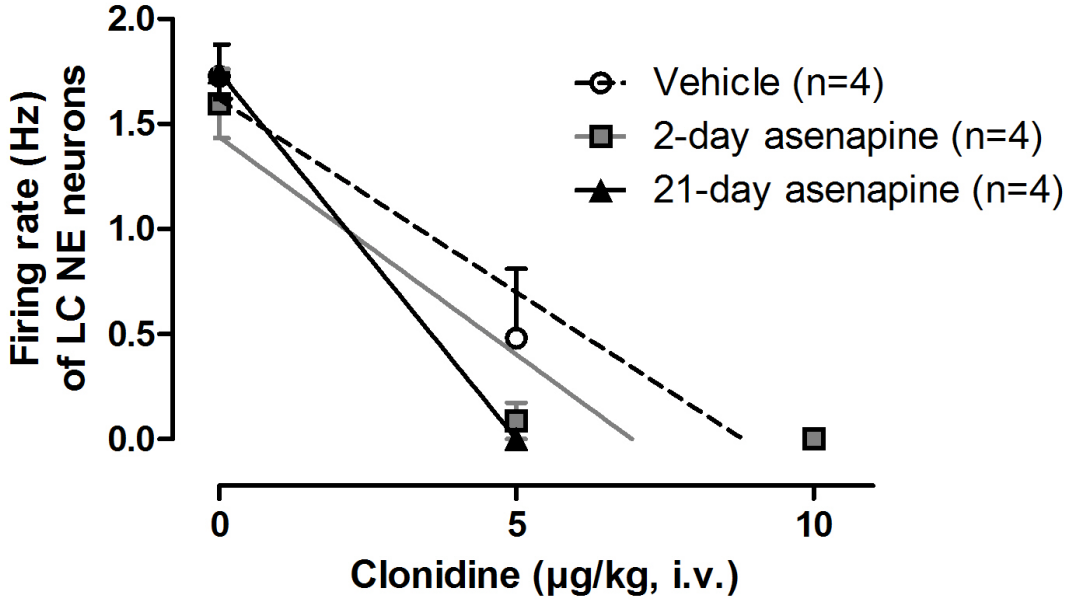


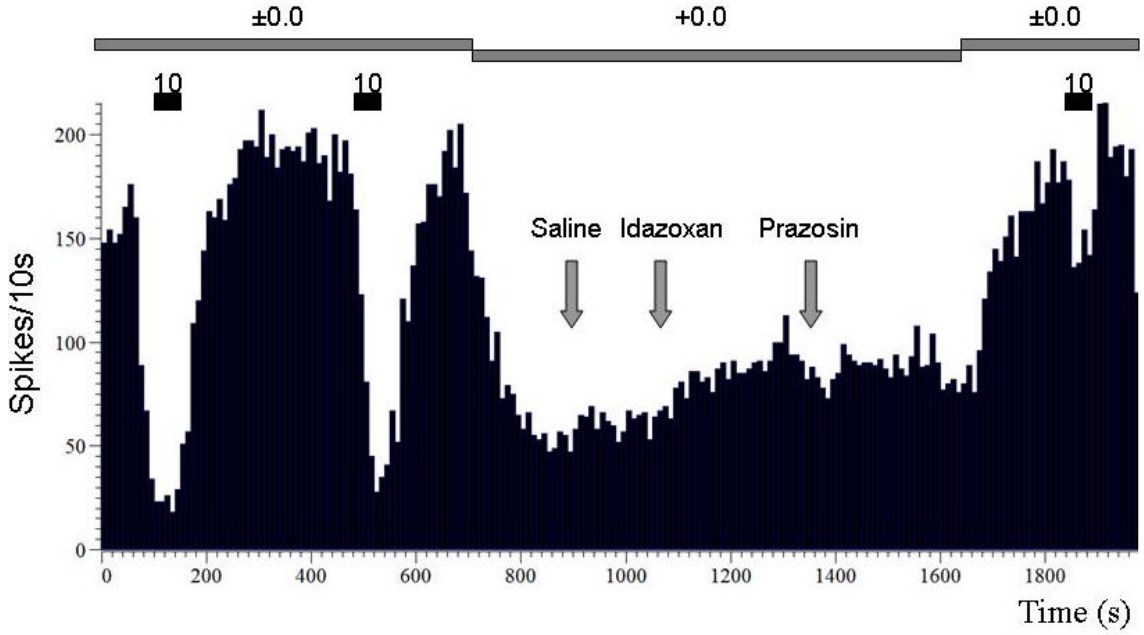
Figure 3 (A) Effect of 2 and 21 days of asenapine administration on the firing activity LC NE neurons. The number of animals (a) and neurons (n) is provided within the histograms. (B) Relationship between the suppression of firing of LC NE neurons by the α_2-adrenoceptor agonist clonidine in vehicle, two-day asenapine and 21-day asenapine-administered animals.
Data were analyzed with two way (A) and one-way (B) ANOVA. Error bars represent S.E.M.
***Significant effect of asenapine compared to vehicle administration, *p<0.05**

Effects of asenapine administration on postsynaptic α_1- and α_2-adrenoceptors in the CA3 region of hippocampus

Acute administration of asenapine (0.1 mg/kg, i.v.) in naïve rats significantly decreased the inhibitory response to microiontophoretic application of NE on CA3 pyramidal neurons by 40 percent (data from 6 animals; 117 *vs* 70 spikes inhibited / nA before and after asenapine administration, respectively, paired t-test, p=0.014, data not shown). Similarly, 21-day asenapine administration significantly blunted the inhibitory response of iontophoretically applied NE on the firing activity of CA3 pyramidal neurons (two-way ANOVA, $F_{1,7}$=6.7, p=0.036, figure 4D).

The degree of tonic activation of adrenoceptors receptors on CA3 pyramidal neurons following 21-day asenapine administration was assessed by i.v. injection of the selective α_1- and α_2-adrenoceptor antagonist prazosin (0.1 mg/kg) and idazoxan (1 mg/kg), respectively. Baseline firing rate of neurons in control and 21-day asenapine administered animals did not differ (4.8 ± 0.5 *vs* 4.3 ± 0.4 Hz, respectively, t-test, p>0.05). Administration of prazosin and idazoxan did not alter the firing rate of CA3 pyramidal neurons in control and 21-day asenapine administered animals (paired t-test, p>0.05, figure 4C; for illustrative firing histograms see figure 4A and 4B). After idazoxan administration, the number of spikes suppressed / nA of NE ejection was significantly reduced in both groups (two-way ANOVA, $F_{1,7}$=17.6, p=0.004, figure 4D). To further strengthen the notion that the inhibitory response to iontophoretically applied NE was reduced by asenapine and not a possible artifact caused by variability in iontophoresis pipettes, a subset of these data was obtained using the same iontophoresis pipette. Statistical analysis of these data confirmed a decreased inhibitory response of CA3 neurons to NE after 21-day asenapine administration (data from 6 and 7 neurons in two vehicle and two asenapine administered animals, average spikes suppressed/nA; 102 ± 15 and 42 ± 9, respectively, t-test; p=0.004).

4A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+0.0
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10
10
10
Saline
Idazoxan
Prazosin
Spikes/10s
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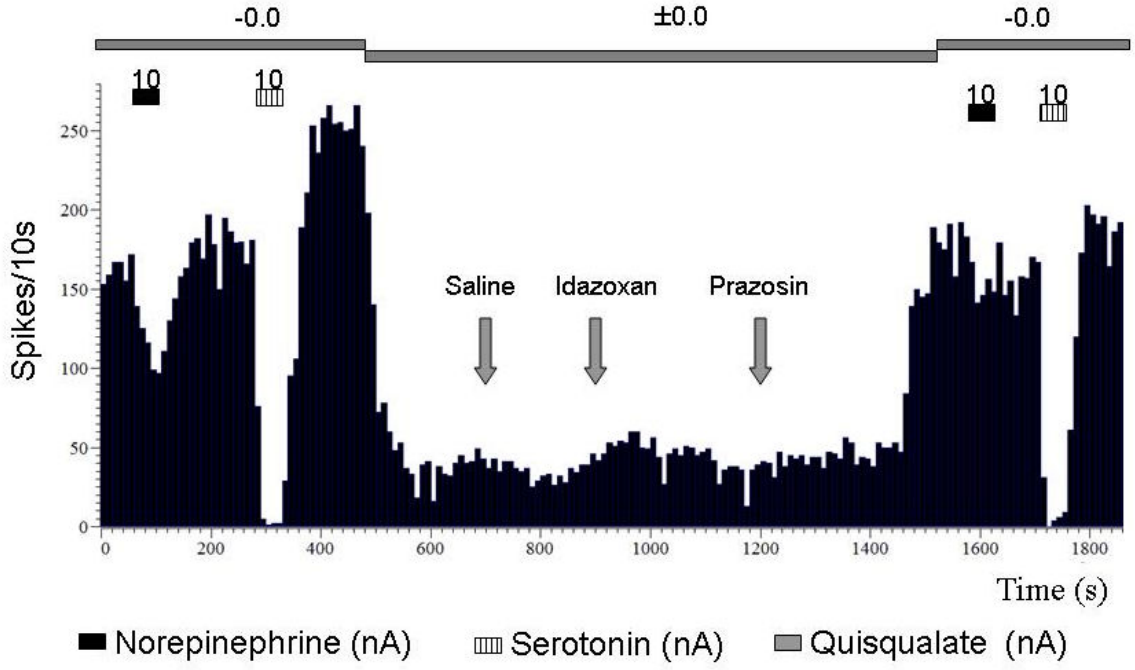
1800
Time (s)

4B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Time (s)
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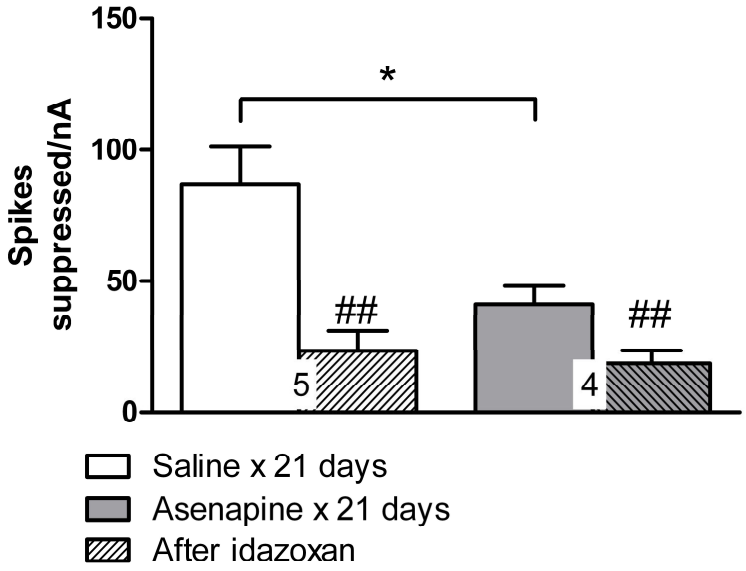
Serotonin (nA)
Quisqualate (nA)

4C



4D

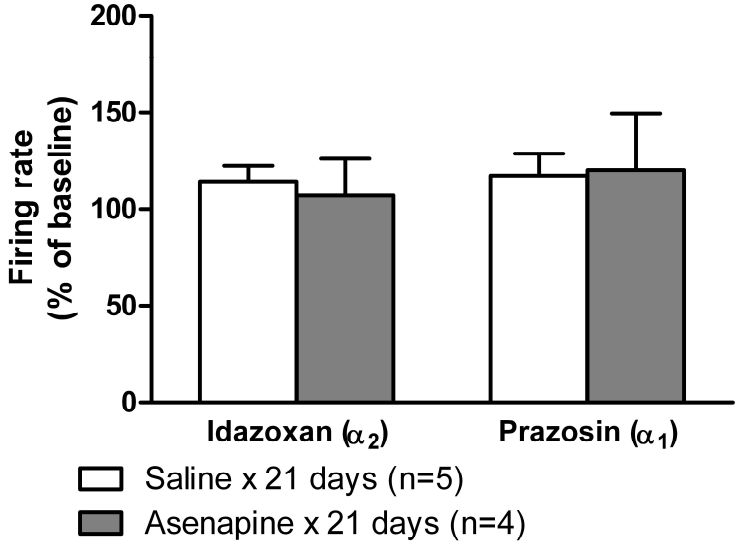


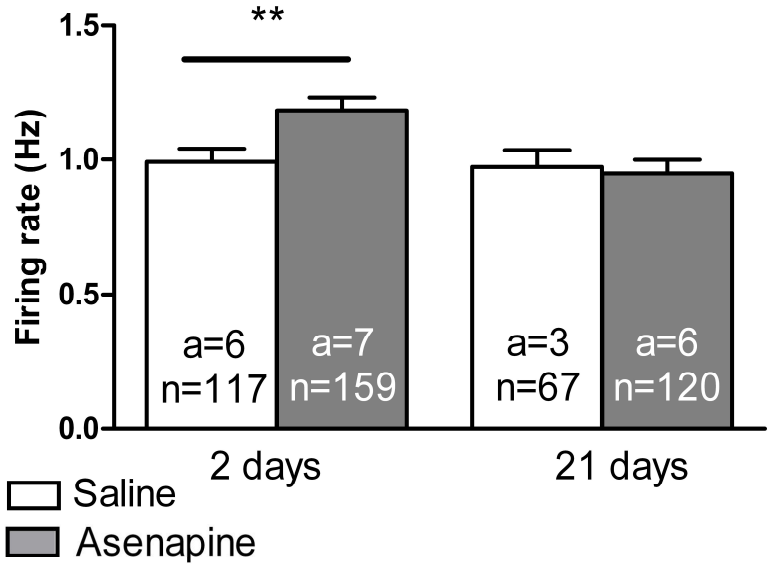
Figure 4: Effect of 21-day asenapine administration on the status of α-adrenoceptors in the CA3 region of the hippocampus (A, B) Illustrative trace of the effect of idazoxan and prazosin administration on the firing activity of a CA3 pyramidal neuron in a 21-day vehicle (A) and 21-day asenapine administered animal (B). C) Effect of the α_2-adrenoceptor antagonist idazoxan (1 mg/kg, i.v.) and the α_1-adrenoceptor antagonist prazosin (0.1 mg/kg, i.v.) on firing rate of CA3 pyramidal neurons after 21-day vehicle or asenapine administration. D) Inhibitory effect of iontophoretically applied NE on CA3 pyramidal neurons in control and 21-day asenapine administered animals before and after administration of idazoxan. Note the blunted response to iontophoretically applied NE in (B) compared to (A).
Data were analyzed with a t-test (C) and two-way ANOVA, number of animals is provided within the histograms, error bars represent S.E.M.
***Significant effect of asenapine compared to vehicle administration; *p<0.05.**
#Significant effect of idazoxan administration; ##p<0.01)

Effects of 2- and 21-day asenapine administration on 5-HT neurons in the DRN

Asenapine administration for two days, but not 21 days, significantly increased the firing rate of DRN 5-HT neurons (two-way ANOVA, $F_{1,459}$=4.0, p=0.045, figure 5A).

There was no difference in dose-effectiveness of the 5-HT_{1A} receptor agonist flesinoxan to inhibit the firing activity of DRN 5-HT neurons between vehicle, two-day and 21-day asenapine administered animals (p>0.05, figure 5B).

5A



5B

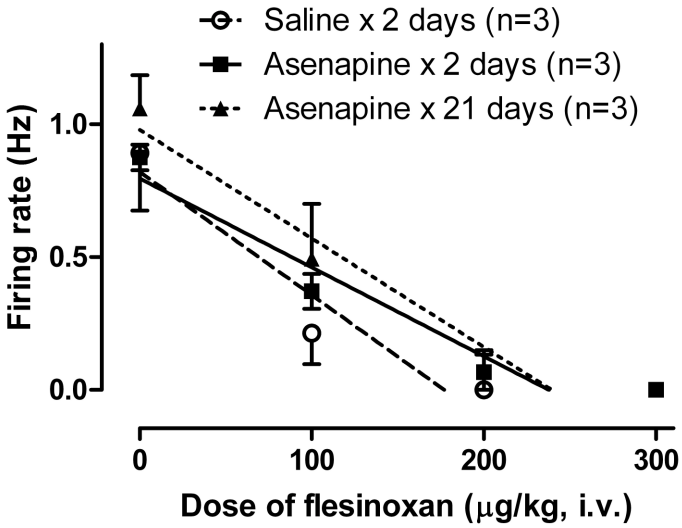


Figure 5: (A) Effect of 2- and 21-day asenapine administration on the firing activity of DRN 5-HT neurons. B) Relationship between the suppression of firing of 5-HT neurons by the 5-HT_{1A} agonist flesinoxan. Data were analyzed with a two-way (A) and one-way (B) ANOVA; the number of animals (a) and neurons (n) is provided within the histograms. Error bars represent S.E.M.
*Significant effect of asenapine compared to vehicle administration; **p<0.01

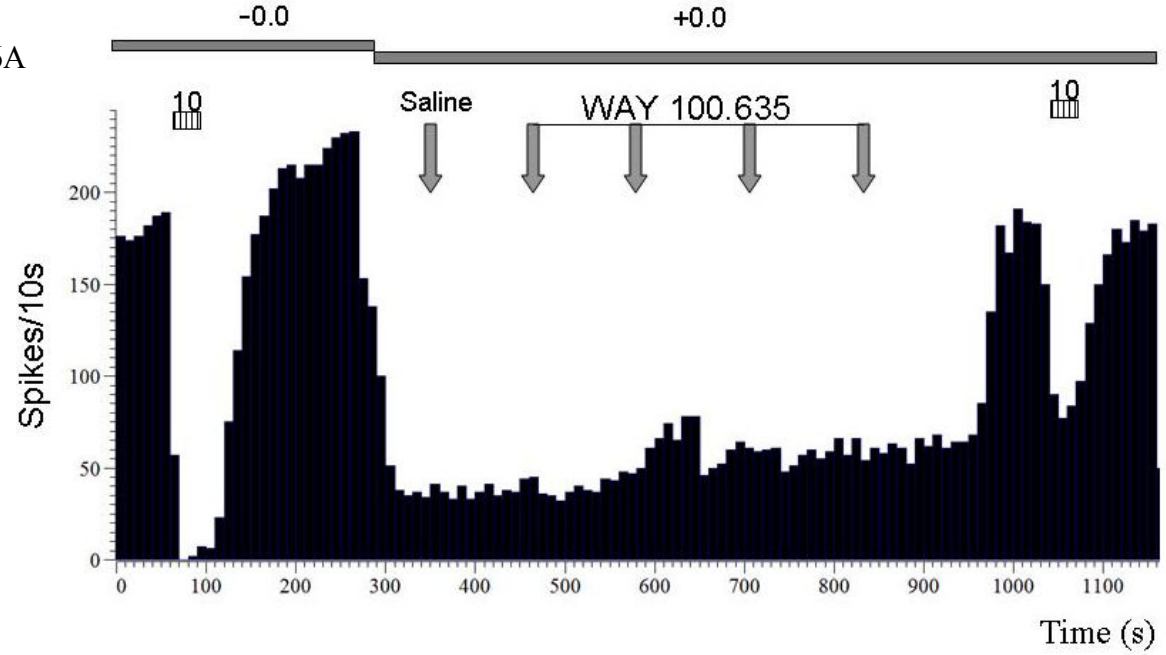
Effects of 21-day asenapine administration on 5-HT transmission in the CA3 region of hippocampus

In the hippocampus, the degree of tonic activation of 5-HT_{1A} receptors following 21-day asenapine administration was assessed by administration of the 5-HT_{1A} receptor antagonist WAY 100.635. At baseline, firing rate between control and 21-day asenapine administered animals did not differ (4.1 ± 0.5 and 3.9 ± 0.3 Hz, respectively, t-test, $p>0.05$). Intravenous administration of cumulative doses of the selective 5-HT_{1A} receptor antagonist WAY 100.635 significantly increased the firing rate of CA3 pyramidal neurons in 21-day asenapine administered animals compared to controls (repeated measures ANOVA, $F_{1,13}=5.3$, $p=0.038$, figure 6C; for illustrative firing histograms see figure 6A and 6B). A Bonferroni *post-hoc* test revealed a significant effect of 21-day asenapine administration of WAY 100.635 at cumulative doses of 75 and 100 μg/kg ($p<0.05$ and $p<0.01$, respectively).

The responsiveness of pyramidal neurons to iontophoretically applied 5-HT was not changed by 21-day asenapine administered animals (spikes inhibited / nA = 47 ± 5 *vs* 46 ± 4 in 21 day vehicle and asenapine administered animals, respectively, $p>0.05$). Also, there was no difference in the RT_{50} index following 21 days of asenapine administration (RT_{50}: 22 ± 3 *vs* 23 ± 3 seconds in 21-day vehicle and asenapine administered animals, respectively, $p>0.05$).

Electrical stimulation of 5-HT afferents at 1 Hz produced a similar DOS in control and 21-day asenapine administered animals (one-way ANOVA, $F_{1,39}=1.60$, $p>0.05$, figures 6B and 6C). In control animals, 5 Hz stimulation significantly decreased the DOS by 35% compared to 1 Hz stimulation (paired t-test, $p<0.0001$, figure 6D). In 21-day asenapine administrated animals, the DOS produced by 1 and 5 Hz stimulations did not differ (paired t-test, $p>0.05$, figure 6E).

6A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+0.0
10
Saline
WAY 100.635
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Spikes/10s
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400
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600
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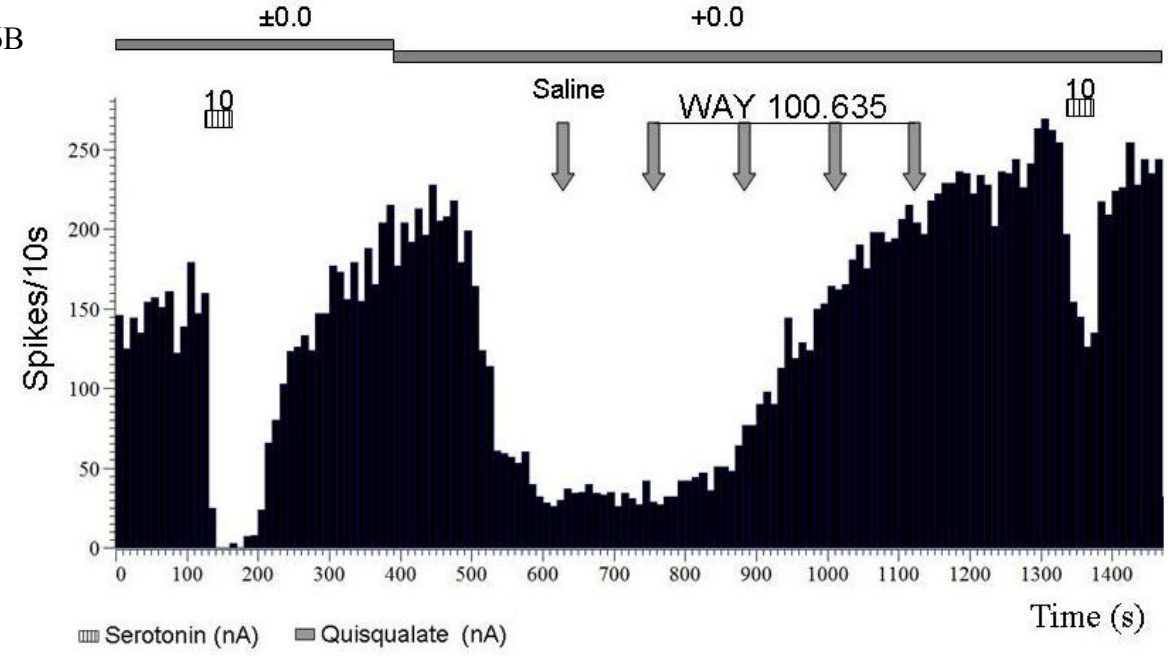
800
900
1000
1100
Time (s)

6B


±0.0
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Time (s)
Serotonin (nA)
Quisqualate (nA)

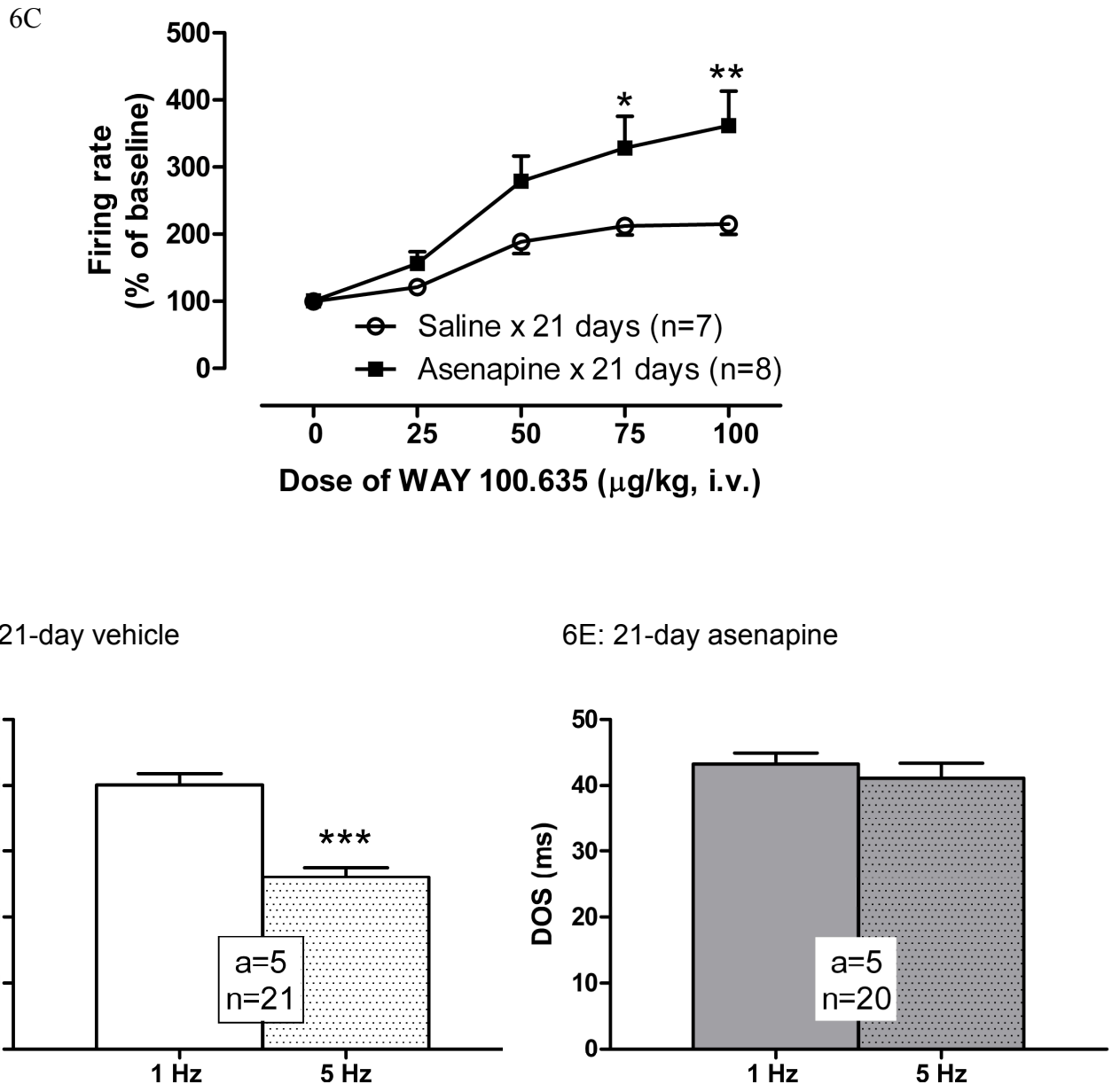

Figure 6: Effect of 21-day asenapine administration on the status of 5-HT_1 receptors in the CA3 region of the hippocampus. (A,B) Illustrative trace of the effect of cumulative doses of WAY 100.635 (WAY) administration on the firing activity of a CA3 pyramidal neuron in a 21-day vehicle (A) and 21-day asenapine administered animal (B). C) Quantification of the effect of WAY 100.635 on basal firing rate in rats administered with vehicle or asenapine for 21 days. (D,E) DOS of CA3 pyramidal neurons following 5-HT fiber bundle stimulation in vehicle (D) and 21-day asenapine (D) administered animals.
Data were analyzed with repeated measures ANOVA (C) followed by Bonferroni post-hoc analysis or with a paired t-test (D,E). The number of animals (a) and neurons (n) is provided within the histograms; error bars represent S.E.M.
*Significant effect of asenapine compared to vehicle administration; *$p<0.05$, **$p<0.01$, ***$p<0.001$

Discussion

The present results were obtained following sustained administration of asenapine (0.1 mg/kg/day) that result in blood plasma levels corresponding to the range obtained with its lowest clinically effective dose in treatment of schizophrenia and mania (5 mg/kg twice daily; Chapel et al. 2009). In animal models, chronic asenapine administered in this dose range was shown to induce behavioral changes related to antipsychotic action (Gao and Li, 2013) as well as restorative effects on cognitive function (McLean *et al.*, 2010), findings contributing to the clinical relevance of the drug regimen used in the present report.

Effect of asenapine on 5-HT_{2A} receptors

5-HT_{2A} receptors are excitatory 5-HT receptors which are expressed on GABA neurons that innervate LC NE neurons (Szabo and Blier, 2001). Stimulation of these receptors by 5-HT increases GABA release which inhibits NE neurons, an effect that may not be desirable in all patients. Therefore, blockade of these receptors provides a rationale for augmentation strategies using 5-HT_{2A} antagonists in depression (Blier and Szabo, 2005; Dremencov *et al.*, 2007). Consistent with prior reports, systemic administration of the 5-HT_{2A} receptor agonist DOI potently suppressed LC NE firing in vehicle administered animals. Two-day and 21-day administration of asenapine significantly dampened this inhibitory effect of DOI, demonstrating potent *in vivo* antagonism at 5-HT_{2A} receptors by this agent (see figure 1D). In line with this finding, previous reports showed that acute asenapine administration reversed the inhibitory effect of DOI on LC NE neurons, and that local application of asenapine prevented prefrontal DOI-induced elevations in 5-HT, DA and NE (Ghanbari *et al.*, 2009; Frånberg *et al.*, 2012). Thus, the current observations support potent antagonistic properties of asenapine on 5-HT_{2A} receptors, a common pharmacological property of atypical antipsychotics that could be clinically relevant in adjunct to treatment of mood disorders (Blier and Szabo, 2005).

Effect of asenapine on DA neurons

Administration of the dopamine receptor agonist apomorphine (40µg/kg, i.v.) completely inhibited the firing activity of VTA DA neurons in control animals, an effect

mediated by D_2 autoreceptor activation. This inhibitory effect of apomorphine was blunted after two-day asenapine administration, suggesting incomplete blockade of D_2 autoreceptors. Indeed, since a second administration of apomorphine did not further inhibit DA neurons, it is likely that the antagonistic properties of asenapine prevented further activation of D_2 receptors. Interestingly, the inhibitory effect of apomorphine returned to control level after 21 days of asenapine administration (see figure 2D). Because blood plasma levels did not differ after two and 21 days of asenapine administration, drug concentration is unlikely to underlie this difference. Rather, this result suggests that D_2 autoreceptors were supersensitized after 21-day asenapine administration, an effect known to occur after chronic haloperidol (Vogelsang and Piercey, 1985). Importantly, *in vivo* demonstration of D_2 receptor supersensitization by haloperidol requires a washout period, because its chronic administration concurrently produces a depolarization block (Chiodo and Bunney, 1983). Similarly, a depolarization block has been demonstrated following chronic chlorpromazine, olanzapine, quetiapine, clozapine, ziprasidone, and paliperidone administration (Chiodo and Bunney, 1983; Sorensen *et al.*, 1993; Skarsfeldt, 1995; Jones-Humble *et al.*, 1996; Stockton and Rasmussen, 1996), all agents with antagonistic action at D_2 receptors. Based on this evidence, it has been suggested that a depolarizing block could underlie the therapeutic effect of antipsychotic drugs (Grace *et al.*, 1997). Asenapine, instead of sharing this effect, nearly doubled the population activity of VTA DA neurons (see figure 1E), while it did not change their firing and burst activity.

Since this study is the first to demonstrate increased population of VTA DA neurons after chronic administrating of a D_2 receptor antagonist, the mechanism(s) for this result remains to be established. One possibility is that the relatively low dose of asenapine was insufficient to occupy D_2 autoreceptors to an extent that produces a depolarizing block. Instead, incomplete D_2 receptor blockade could have activated normally silent neurons while endogenous DA provided a sufficient inhibitory signal to to prevent a depolarization block in already active neurons. In line with this hypothesis, acute administration of 0.5 mg/kg asenapine fully reversed the inhibitory effect of apomorphine in 2- and 21-day asenapine administered animals, suggesting that asenapine administered at 0.1 mg/kg/day did not fully occupy D_2 receptors. Dose-dependent effects

on VTA population activity are known to exist following chronic quetiapine, risperidone, olanzapine, and ziprasidone administration (Skarsfeldt, 1995; Stockton and Rasmussen, 1996), indicating that drug dosage modulates VTA population activity. Notably, since increases in population activity by these agents have not been reported, it would be interesting to assess whether a sufficiently low dose of other antipsychotics, or a higher dose of asenapine, would produce effects opposite to what it is currently known.

In addition, the high affinity, multireceptor binding profile of asenapine (Shahid *et al.*, 2009) suggests that it could act by mechanisms secondary to incomplete D_2 autoreceptor blockade. In this context, multisynaptic GABAergic and glutamatergic projections from the ventral subiculum of the hippocampus, nucleus accumbens, and ventral pallidum are known to modulate VTA DA population activity (Floresco *et al.*, 2001). Also, postsynaptic D_2 receptors and various 5-HT receptors have an effect on this (Blackburn *et al.*, 2006; Valenti and Grace, 2010), while chronic administration of drugs devoid of affinity for D_2 receptors such as desipramine (Chiodo and Bunney, 1983) and agomelatine (Chenu *et al.*, 2013) were shown to increase VTA population activity by currently unknown mechanisms. Clearly, additional experiments are required to fully explain the present results.

Effect of asenapine on the NE system

Asenapine administered for two and 21 days did not change the inhibitory effect of the α_2-adrenoceptor agonist clonidine. In contrast, acute asenapine reversed the inhibitory effect of clonidine on NE neurons (ED_{50} = 85 µg/kg), demonstrating potent *in vivo* antagonistic properties at α_2-adrenergic autoreceptors (Ghanbari *et al.*, 2009). Notably, mirtazapine had a similar dynamic effect on α_2-autoreceptors; it reversed clonidine-induced inhibition on NE neurons acutely (Haddjeri *et al.*, 1996), whereas after 21 days of administration the dose-response effect of clonidine to inhibit NE neurons did not differ from controls (Haddjeri *et al.*, 1998a). Thus, both tetracyclic medications blocked α_2-adrenergic autoreceptors on LC neurons acutely, whereas the function of these receptors was restored after their long-term administration. In further similarity, 21-day administration of both asenapine and mirtazapine induced a small yet significant increase in the firing rate of LC NE neurons.

In the CA3 region of the hippocampus, acute administration of asenapine (0.1 mg/kg) significantly blunted the inhibitory response of pyramidal neurons to NE, strongly suggesting blockade of α_2-adrenoceptors on pyramidal neurons (Curet and de Montigny, 1988). Interestingly, acute mirtazapine administration had this same effect (0.5 mg/kg; Haddjeri et al. 1996), while long-term mirtazapine administration did not occupy postsynaptic α_2-adrenoceptors to a significant extent (Haddjeri *et al.*, 1998a). In contrast to this latter result, sustained asenapine administration reduced the inhibitory response of iontophoretically applied NE, indicating that postsynaptic α_2-adrenoceptors were blocked by asenapine – in line with a previous report (Frånberg *et al.*, 2012). Importantly, this blockade was incomplete since acute idazoxan administration reduced the neural response to NE application even further (figure 4D). In addition, idazoxan and prazosin administration did not have a disinhibitory effect on CA3 pyramidal neurons (figure 4C). This indicates that NE tone on α_1- and α_2-adrenoceptors was not enhanced by 21-day asenapine administration, despite the increased firing rate of LC NE neurons after this drug regimen (figure 3A).

Effect of asenapine on the 5-HT system

Two-day asenapine administration induced a small increase in the firing rate of 5-HT neurons, which was independent of altered 5-HT_{1A} function. Indeed, the inhibitory effect of the 5-HT_{1A} agonist flesinoxan was not different after vehicle, 2-, and 21-day asenapine administration. In line with a limited effect of asenapine on 5-HT neurons in the DRN, acute asenapine was previously shown to have no effect on their discharge properties, and did not reverse the inhibitory effect of the 5-HT_{1A} receptor agonist 8-OH-DPAT (Ghanbari *et al.*, 2009).

In the hippocampus, sustained asenapine administration increased the activation of 5-HT_{1A} receptors on CA3 pyramidal neurons, as demonstrated by a stronger disinhibitory effect of the 5-HT_{1A} receptor antagonist WAY 100.635 (figure 6C). Since 21-day asenapine administration did not change the firing activity of DRN 5-HT neurons, this increase in 5-HT tone was independent of a mechanism at the cell body level. To explore the mechanism by which asenapine increased 5-HT tone, the sensitivity of 5-HT_{1A} receptors and the action of the SERT were assessed, as other drugs increase 5-HT

neurotransmission by action on these pharmacological targets (Haddjeri *et al.*, 1998b). Using iontophoretic application of 5-HT, no effect of chronic asenapine on postsynaptic 5-HT_{1A} receptor sensitivity and 5-HT reuptake was detected. However, since electrical stimulation of 5-HT afferents at 1 and 5 Hz stimulation produced a similar DOS value (figure 6E), the function of terminal 5-HT_{1B} autoreceptors was clearly attenuated by asenapine administration. Here, asenapine likely acted as a partial 5-HT_{1B} receptor agonist, as complete blockade of 5-HT_{1B} autoreceptors would have resulted in a greater DOS after 5 Hz compared to 1 Hz stimulation (Chaput *et al.*, 1986). Diminished function of terminal 5-HT_{1B} autoreceptors can enhance 5-HT tone, as demonstrated following chronic administration of the SSRIs paroxetine, fluoxetine and vortioxetine (Piñeyro and Blier, 1999). Notably, while paroxetine and fluoxetine do not have appreciable affinity for 5-HT_{1B} autoreceptors, *in vitro* and *in vivo* data suggest that vortioxetine and asenapine both act as partial agonists at this receptor subtype (Shahid *et al.*, 2009; Bang-Andersen *et al.*, 2011; El Mansari *et al.*, 2015). In addition to partial 5-HT_{1B} agonism, the partial agonistic action at postsynaptic 5-HT_{1A} receptors on pyramidal neurons (Ghanbari *et al.*, 2009) could have contributed to the disinhibitory effect of acute 5-HT_{1A} receptor blockade, as observed following chronic administration of gepirone (Haddjeri *et al.*, 1998b).

In summary, the present study showed that at clinically relevant blood plasma levels, sustained asenapine administration potently blocked 5-HT_{2A} receptors. Asenapine produced an incomplete blockade on D_2 receptors after two-day administration, while these receptors were sensitized after 21-day administration. Furthermore, its chronic administration increased the population activity of VTA DA neurons and partially blocked postsynaptic α_2-adrenergic receptors, effects that have not been previously observed following sustained antipsychotic drug administration. Asenapine acted as a partial 5-HT_{1A} and 5-HT_{1B} receptor agonism, which enhanced 5-HT neurotransmission. Together, these results provide further insight in the long-term effects of asenapine administration on therapeutically relevant targets, and could contribute to a better understanding of the neural mechanisms by which asenapine improved negative symptoms in schizophrenia (Buchanan *et al.*, 2012) and exerted greater alleviation of depressive scores in bipolar patients with mixed features (McIntyre *et al.*, 2013).

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El Mansari and Oosterhof have no conflicting interests

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Chapter II: Acute brexpiprazole

Brexpiprazole is currently in phase III of clinical trials, where it showed efficacy in the treatment of schizophrenia (Kane *et al.*, 2015) and as add-on in treatment-resistant depression (Thase *et al.*, 2014). Brexpiprazole shares structural and pharmacological characteristics of aripiprazole, an agent in wide clinical use. Of these characteristics, D_2 receptor partial agonism is arguably their most distinguishable feature, which has been implicated in buffering the DA signal in schizophrenia and depression (Stahl, 2008). Compared to aripiprazole, brexpiprazole had more potent 5-HT_{1A} receptor agonism, and less intrinsic activity at D_2 receptors in vitro (Maeda, Sugino, *et al.*, 2014). Accordingly, the present study aimed to compare the *in vivo* effects of acute brexpiprazole and aripiprazole administration on DRN 5-HT and VTA DA neurons. In addition, the acute effects of brexpiprazole on α_1- and α_2-adrenoreceptors, postsynaptic 5-$HT_{1A,}$ terminal 5-HT_{1B}, 5-HT_{2A} and the SERT and NET were assessed to assess the acute in vivo actions of brexpiprazole on therapeutically relevant targets.

The experimental design of this study was drafted by Dr. Blier, Dr. El Mansari, and the present author. All electrophysiological experiments and their analysis were conducted by the present author. All authors contributed to and approved of the final manuscript. Preliminary data of this study were presented at the annual meeting of the Society of Biological Psychiatry in May 2014 and the European College of Neuropsychopharmacology in October 2014. The manuscript was published in the Journal of Pharmacology and Experimental Therapeutics in December 2014.

Acute effects of brexpiprazole on serotonin, dopamine, and norepinephrine systems: an *in vivo* electrophysiological characterization

Running title: Acute brexpiprazole and monoamines

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Abstract

Brexpiprazole, a compound sharing structural molecular characteristics with aripiprazole, is currently under investigation for the treatment of schizophrenia and depression. Using electrophysiological techniques, the present study assessed the *in vivo* action of brexpiprazole on serotonin (5-HT)$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$ receptor subtypes, dopamine (DA) D_2 autoreceptors, and α_1- and α_2-adrenergic receptors. In addition, the effects on 5-HT$_{1A}$ autoreceptors in the dorsal raphe nucleus (DRN) and D_2 autoreceptors in the ventral tegmental area (VTA) were compared to those of aripiprazole, an agent in wide clinical use. In the DRN, brexpiprazole completely inhibited the firing of 5-HT neurons via 5-HT$_{1A}$ agonism, and was more potent than aripiprazole (ED_{50}=230 and 700 μg/kg, respectively). In the locus coeruleus, brexpiprazole reversed the inhibitory effect of the preferential 5-HT$_{2A}$ receptor agonist DOI on norepinephrine neuronal firing (ED_{50}=110 μg/kg), demonstrating 5-HT$_{2A}$ antagonistic action. Brexpiprazole reversed the inhibitory effect of the DA agonist apomorphine on VTA DA neurons (ED_{50}=61 μg/kg), whereas it was ineffective when administered alone, indicating partial agonistic action on D_2 receptors. Compared to aripiprazole, which significantly inhibited the firing activity of VTA DA neurons, brexpiprazole displayed less efficacy at D_2 receptors. In the hippocampus, brexpiprazole acted as a full agonist at 5-HT$_{1A}$ receptors on pyramidal neurons. Furthermore, it increased 5-HT release by terminal α_2-adrenergic heteroceptor, but not 5-HT$_{1B}$ autoreceptor antagonism. In the lateral geniculate nucleus, brexpiprazole displayed α_{1B}-adrenoceptor antagonistic action. Taken together, these results provide insight in the *in vivo* action of brexpiprazole on monoamine targets relevant in the treatment of depression and schizophrenia.

Introduction

Brexpiprazole (OPC-34712) is a compound currently under the investigation for the treatment of depression and schizophrenia. Antipsychotic medications from the first and second generation are efficacious antagonists at dopamine (DA) D_2 receptors. Indeed, D_2 receptor antagonism is the effective strategy for treatment of positive symptoms in schizophrenia (Seeman and Lee, 1975; Rao and Remington, 2013). However, it is accompanied by unwanted motor side-effects leading to extrapyramidal symptoms, at least in part by decreasing dopaminergic transmission in the striatum (Glazer, 2000; Kapur *et al.*, 2000). These side-effects are dampened when combined with serotonin $(5\text{-HT})_{2A}$ receptors antagonism, a defining pharmacological characteristic of second generation antipsychotics (Stockmeier *et al.*, 1993) that may be of therapeutic benefit in the treatment of both schizophrenia and mood disorders (Blier and Szabo, 2005; Kuroki *et al.*, 2008).

Most second generation antipsychotics have higher *in vitro* affinity for 5-HT_{2A} than D_2 receptors; brexpiprazole and aripiprazole are different in this regard (5-HT_{2A} Ki=0.47 and 4.7 nM; D_2 Ki=0.30 and 0.87 nM, respectively; Maeda, Sugino, *et al.*, 2014). Whereas most other atypical antipsychotics are D_2 receptor antagonists, *in vitro* data indicates that brexpiprazole is a D_2 partial agonist with lower intrinsic activity at D_2 receptors than aripiprazole (Maeda, Sugino, *et al.*, 2014). Partial D_2 receptor agonism is thought to have a buffering action on DA neurotransmission by stimulating D_2 receptors under low DA conditions, while dampening their activation when DA levels are high (Burris, 2002). Indeed, *in vivo* systemic administration of aripiprazole has been shown to decrease the firing activity of ventral tegmental area (VTA) DA neurons to approximately 70% of baseline, whereas complete inhibition of these neurons by the DA agonist apomorphine was reversed by aripiprazole to a similar degree (Dahan *et al.*, 2009). In contrast, acute haloperidol and clozapine administration increases firing of VTA DA neurons by D_2 autoreceptors antagonism (Hand *et al.*, 1987). Despite a different action on D_2 autoreceptors, brexpiprazole improved behavioral measures predictive for antipsychotic efficacy, such as apomorphine-induced stereotypy and the conditioned avoidance response (Maeda, Lerdrup, *et al.*, 2014). Furthermore, brexpiprazole reduced

head-twitches induced by the preferential 5-HT_{2A} receptor agonist DOI and restored phencyclidine (PCP)-induced cognitive impairments to greater extent than the 5-HT_{2A} receptor antagonist M100907, indicating *in vivo* antagonistic action at 5-HT_{2A} receptors (Maeda, Lerdrup, *et al.*, 2014).

Aripiprazole and brexpiprazole are *in vitro* 5-HT_{1A} receptor agonists (Ki=1.3 and 0.12 nM, respectively; Maeda, Sugino, *et al.*, 2014), a relevant pharmacological characteristic in treatment of mood disorders and schizophrenia (Blier and Ward, 2003; Newman-Tancredi, 2010). For aripiprazole, *in vivo* electrophysiology studies showed acute agonistic action at 5-HT_{1A} autoreceptors in the dorsal raphe nucleus (DRN; Stark *et al.*, 2007; Dahan *et al.*, 2009). Interestingly, these autoreceptors were desensitized after only two-day aripiprazole administration (Chernoloz *et al.*, 2009). Desensitization of 5-HT_{1A} receptors could increase 5-HT neurotransmission, a common effect of long-term antidepressant administration thought to be therapeutically beneficial in the treatment of mood disorders (Blier and El Mansari, 2013). The recent demonstration that the restorative effect of both acute and chronic brexpiprazole on impaired cognitive function was lost by 5-HT_{1A} receptor blockade further suggests *in vivo* 5-HT_{1A} receptor agonism by this compound (Maeda, Lerdrup, *et al.*, 2014; Yoshimi *et al.*, 2014).

Antagonism of α-adrenoceptors is a pharmacological feature that may have therapeutic implications for schizophrenia and depression (Fawcett and Barkin, 1998; Arnsten, 2004; Marcus *et al.*, 2010). *In vitro,* brexpiprazole has selective affinity for α_{1B}-adrenoceptors (Ki = 0.17 nM) over α_{1A} (Ki = 3.8 nM) and α_{1D}-subtypes (Ki = 2.6 nM; Maeda, Sugino, *et al.*, 2014). Furthermore, it may have antagonistic properties on α_2-adrenoceptors (Maeda, Sugino, *et al.*, 2014), a pharmacological characteristic known to increase neurotransmission of NE (Ghanbari *et al.*, 2011; Chernoloz *et al.*, 2012) and 5-HT (Mongeau *et al.*, 1993; Haddjeri *et al.*, 1998) by blockade of terminal α_2-adrenergic autoreceptors and -heteroceptors, respectively.

Compared to aripiprazole, brexpiprazole has a 3-4 fold higher *in vitro* affinity for the serotonin transporter (SERT; IC50=29 nM) and norepinephrine transporter (NET; IC=140 nM; Maeda, Sugino, *et al.*, 2014). Blockade of these transporters is known to enhance 5-HT and NE neurotransmission (El Mansari *et al.*, 2005; Chernoloz *et al.*, 2012). As agents that block these transporters are currently first-line in treatment of mood

disorders, monoamine reuptake blocking properties could contribute to the clinical efficacy of brexpiprazole.

To complement and extend insight in its action on therapeutically relevant targets, the present study used electrophysiological techniques to determine the *in vivo* activity of brexpiprazole on 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and D_2 receptors, α_1- and α_2-adrenoreceptors, and on the SERT and NET.

Methods

Animals

Experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 275-325 g housed under standard laboratory conditions (12:12 light-dark cycle with food and water ad libitum). *In vivo* extracellular unitary recordings were carried out in chloral hydrate anaesthetized rats (400 mg/kg; i.p.) that were mounted in a stereotaxic apparatus. Body temperature was maintained at 37 °C throughout the experiment utilizing a thermistor-controlled heating pad. If applicable, prior to the electrophysiological recordings a catheter was inserted in a lateral tail vein for systemic intravenous (i.v.) injection of pharmacologic agents. At the end of experiments, animals were euthanized by a lethal dose of chloral hydrate (4 % solution, i.p). 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, Ontario, Canada).

Compounds

Brexpiprazole (Maeda, Sugino, *et al.*, 2014; 200 μg/kg), aripiprazole (200 μg/kg) and the DA agonist apomorphine (40 μg/kg) were dissolved in a 0.5 % lactic acid solution in distilled water; pH of the solution was adjusted to 4.5 by addition of NaOH. The preferential 5-HT_{2A} receptor agonist DOI (100 μg/kg), the α_2-adrenoreceptor agonist clonidine (10 and 400 μg/kg), and the 5-TH_{1A} receptor antagonist WAY 100.635 were dissolved in distilled water. The selective α_{1A}-adrenoreceptors antagonist (Wetzel *et al.*, 1995) SNAP 5089 (1 mg/kg) was dissolved in 20 % beta-cyclodextrin in distilled water. Brexpiprazole and aripiprazole were provided by Lundbeck A/S (Valby, Denmark); all other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada).

In vivo electrophysiological recordings

Extracellular recordings of neurons in the VTA, DRN and locus coeruleus (LC) were carried out with a single-barrel glass micropipette (Stoelting, Spencerville, MD) preloaded with 2 M NaCl and with impedance between 2-6 MΩ. Neurons in the CA3

region of the hippocampus and lateral geniculate nucleus (LGN) were recorded with a 5-barrel micropipette (impedances: central barrel 2-5 MΩ, side barrels 20-30 MΩ). The central barrel, used for unitary recordings, and one sidebarrel, used for automatic current balancing, were filled with 2 M NaCl; the other barrels were filled with brexpiprazole (1.2 mM in distilled water and 0.5% lactic acid, pH=4.5), 5-HT creatinine sulfate (10 mM in 0.2 M NaCl, pH=4), NE (10 mM in 0.2 M NaCl, pH=4) or quisqualalic acid (1.5 mM in 0.2 M NaCl, pH=4). 5-HT and NE were ejected as cations and retained with a negative current; quisqualate and brexpiprazole were ejected as anions (-3 to to +3 nA and -55 nA to-90 nA, respectively) and retained with a positive current.

Recording of 5-HT neurons

Putative 5-HT neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): anterior/posterior (AP) 1.0 to 1.2, mediolateral (ML) 0, dorsal/ventral (DV) 5.0 to 7.0. At these coordinates, only neurons with a bi- or triphasic extracellular waveform with a long-duration (0.8-1.2 ms) positive phase, and regular firing in the range of 0.8-2 Hz were recorded (Vandermaelen and Aghajanian, 1983).

Recording of LC NE neurons

NE neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP -1.0 to -1.2, ML 1.0 to 1.3, DV 5.0 to 7.0. NE neurons were identified using the following criteria: regular firing rate (1-3 Hz), a long duration (0.8-1.2 ms) of the rising phase of the action potential, and a brisk excitatory response followed by a short period of inhibition (~1 s) in reaction to a nociceptive pinch of the contralateral hind paw (Vandermaelen and Aghajanian, 1983). To test the effect of brexpiprazole on 5-HT_{2A} receptors, NE neurons were inhibited by the preferential 5-HT_{2A} receptor agonist DOI (100 μg/kg, i.v; Szabo and Blier, 2001). Following a 60-second inhibition period, cumulative doses of brexpiprazole (50 and 100 μg/kg, i.v.) were administered to reverse the inhibitory effect of DOI. The reversing effect of brexpiprazole was quantified relative to baseline firing activity.

Recording of VTA DA neurons

Putative DA neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP 3.2 to 3.6, ML 0.6 to 1.0, DV 7.0 to 9.0. At these coordinates, neurons with a long-duration (3-5 ms) triphasic action potential with a marked negative deflection, an inflection or "notch" on the rising phase, irregular spontaneous single firing pattern (3-6 Hz), and slow bursting activity with decrementing action potential amplitude were recorded (Grace and Bunney, 1983). The start of a burst was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an ISI>160 ms (Grace and Bunney, 1984). Firing and burst activity was quantified relative to baseline, and values were obtained from the second half of a 120 second period after injection of pharmacological agents. To test the effect of brexpiprazole on D_2 autoreceptors, DA neurons were inhibited by the DA agonist apomorphine (40 μg/kg, i.v.) which is known to produce a sustained suppression of firing on these neurons (Wang, 1981). Following a 60-second inhibition period, cumulative doses of brexpiprazole (25, 50 and 100 μg/kg, i.v.) were administered to reverse the inhibitory effect of apomorphine. The reversing effect of brexpiprazole on firing rate was quantified relative to baseline firing activity.

Recording of dorsal lateral geniculate nucleus (LGN) neurons

LGN neurons were recorded by positioning multibarrel micropipettes at the following coordinates (in mm from lambda): AP 3.8-4.2, ML 4.0-4.2, DV 4.5-5.5. They were characterized by flashing light in the eye of the rat, which causes a brisk excitatory response in these neurons (Curet and de Montigny, 1988). LGN neurons are known to be excited by iontophoretic application of NE via activation α_1-adrenergic receptors (Rogawski and Aghajanian, 1980, 1982; Menkes *et al.*, 1981). Since adrenoceptors of the 1B subtype are densely expressed in the dorsal LGN, whereas α_{1D}-adrenoceptors are absent and there is minimal presence of α_{1B}-adrenoceptors (Day *et al.*, 1997), the LGN was chosen to assess the effect of brexpiprazole on α_{1B}-adrenoceptors. Neuronal responsiveness to the iontophoretic application of NE before and after administration of brexpiprazole was assessed by determining the total number of spikes produced, corrected for baseline activity. Baseline activity was defined as the average firing rate 30-

seconds pre- and post ejection. For all data points, the average number of spikes excited was obtained by averaging the neural response to at least two consecutive ejections of NE separated by an interval of at least 100 seconds.

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 mm from lambda): AP: 3.8-4.2, L: 4.0-4.2, D 3.5-4.5. Since most CA3 pyramidal neurons are not spontaneously active in chloral hydrate anesthetized rats, a small ejection current (-1 to +1 nA) was applied to the quisqualate barrel in order to activate them within their physiological firing range (10 to 15 Hz; Ranck 1973). The current and duration of 5-HT and NE ejection was kept constant before and after each i.v. injection of brexpiprazole. Neuronal responsiveness to the iontophoretic application NE was assessed by determining the total number of spikes suppressed from start of ejection to recovery to 80% of baseline firing rate. Activity of the NET was assessed by determining the recovery time to 50% of the maximal inhibitory effect following a high current ejection of NE (10-20 nA), and was expressed at RT_{50} (de Montigny *et al.*, 1980). Neural responsiveness to 5-HT was assessed by determining the total number spikes inhibited during a 50-second ejection divided by the ejection current of 5-HT (2-5 nA). Activity of the SERT was assessed by determining the recovery time (in seconds) from the end of a 50-second ejection of 5-HT (20 nA) that fully inhibited the neuron, to 50% recovery of baseline firing and was expressed as RT_{50} (Piñeyro *et al.*, 1994). Partial or full agonism of brexpiprazole on 5-HT_{1A} receptors was assessed by comparing the inhibitory effect of ejection 5-HT alone to the inhibitory effect of concomitant ejection of 5-HT and brexpiprazole, following restoration of the firing rate to the same level as before ejecting brexpiprazole by increasing quisqualate ejection. In this paradigm, co-application of partial agonist reduces the inhibitory effect of 5-HT, whereas co-application of a full agonist does not change the inhibitory effect of 5-HT (Dong *et al.*, 1998; Ghanbari *et al.*, 2010). To test whether the inhibitory effect of 5-HT and brexpiprazole was mediated by 5-HT_{1A} receptors, the inhibitory effect of iontophoretic 5-HT and brexpiprazole application was compared before and after administration of the selective 5-HT_{1A} receptor antagonist WAY 100.635 (100 µg/kg, i.v.).

Electrical stimulation of afferent 5-HT projections to hippocampus

A bipolar electrode (NE-110, David Kopf, Tujanga, CA) was inserted at the following coordinates (in mm from lambda): AP +1.0, L0.0, D: 7.8-8.2, in order to electrically stimulate 5-HT afferents while recording a CA3 pyramidal neuron using a multibarrel pipette (see above). A stimulator (S8800, Grass instruments, Quincey, MA) was used to deliver 200 square pulses (0.5 ms, 1 or 5 Hz) at 300 μA. Duration of inhibition per stimulation was plotted in a peristimulus time histogram with a 2 ms bin size. The inhibitory effect of 5-HT fiber stimulation on CA3 neurons was expressed as duration of silence (DOS, in ms), defined as the period from the first bin showing a 50% reduction in the number of events per bin from the prestimulus value and the first subsequent bin attaining a 90% recovery of the number of events per bin from prestimulus values (Chaput *et al.*, 1986). Electrical stimulation of 5-HT afferents causes endogenous release of 5-HT, and briefly suppresses firing of CA3 pyramidal neurons by activating postsynaptic 5-HT_{1A} receptors, an effect previously shown to be independent of 5-HT reuptake inhibition (Chaput *et al.*, 1986) To determine the action of brexpiprazole on α_2-adrenoceptors on 5-HT terminals, we determined whether brexpiprazole (500 μg/kg, i.v.) could prevent and, in a subsequent experiment, reverse the decreasing effect on DOS of a high dose of the α_2-adrenoceptor agonist clonidine (400 μg/kg i.v). To validate this paradigm, a low dose of clonidine (10 μg/kg, i.v.) was administered before the high dose of clonidine. Indeed, it is well established that a low dose of clonidine primarily activates α_2-adrenergic autoreceptors on NE terminals and so decreases NE tone on α_2-adrenoceptors located on 5-HT terminals, resulting in an increased DOS. A subsequent high dose of clonidine activates α_2-adrenoceptors on 5-HT terminals, thereby producing a decrease in DOS (Mongeau *et al.*, 1994).

To determine the effect on the activity of terminal 5-HT_{1B} autoreceptors, the effect of 3 doses of brexpiprazole (500 μg/kg, i.v.) on the DOS following low and high frequency (1 and 5 Hz, respectively) stimulations in the same neuron was compared. Previous *in vivo* and *in vitro* studies showed that increasing the stimulation from 1 to 5 Hz results in greater activation of terminal 5-HT_{1B} receptors and consequently, decreased 5-HT release. Therefore, a longer DOS following 1 Hz compared to 5 Hz stimulations is

indicative for functional terminal 5-HT_{1B} autoreceptors (Chaput *et al.*, 1986; Blier *et al.*, 1989).

Data analysis / Statistics

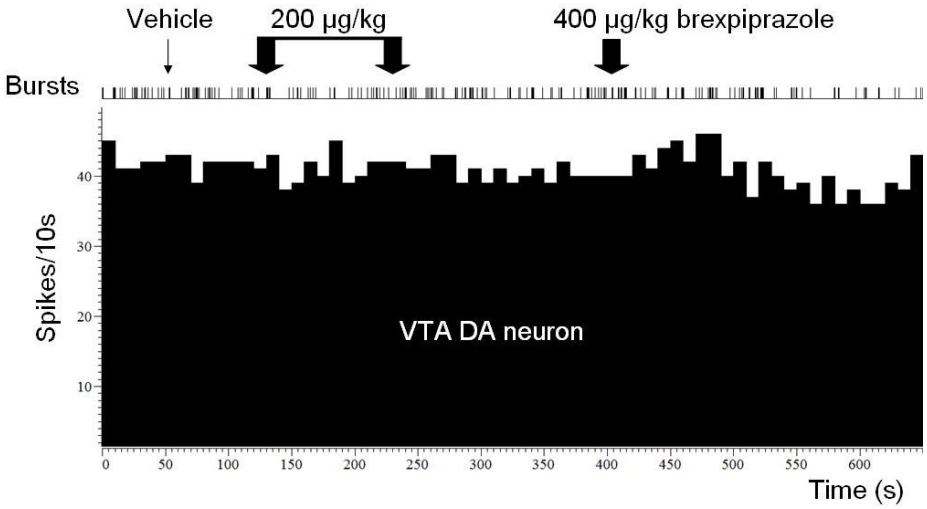
Electrophysiological recordings made, and filtered from artifacts by wavemark analysis, using Spike2 software version 6.17 (Cambridge Electronic Design, Cambridge, United Kingdom). Quantification of firing activity was performed using Spike2, with the exception of firing and burst analysis of VTA DA neurons for which burstiDAtor (Oosterhof and Oosterhof, 2013) software was used. In experiments when no competitive exogenous ligand was present, linear regression analysis was used to determine ED_{50} values, and to compare the slope and intercept when comparing the effect of aripiprazole and brexpiprazole. In presence of a competitive exogenous ligand, non-linear curve fitting was used to obtain ED_{50} values. Experiments with less than 3 observations within the same subject were analyzed with a paired t-test; experiments with more than 3 observations within the same subject were analyzed with repeated measurements analysis of variance followed by a Tukey *post-hoc* test. All data were analyzed with Graphpad Prism (Version 5.01, Graphpad software, La Jolla, CA). Data is presented as mean ± S.E.M.; a p-value <0.05 was considered significant.

Results

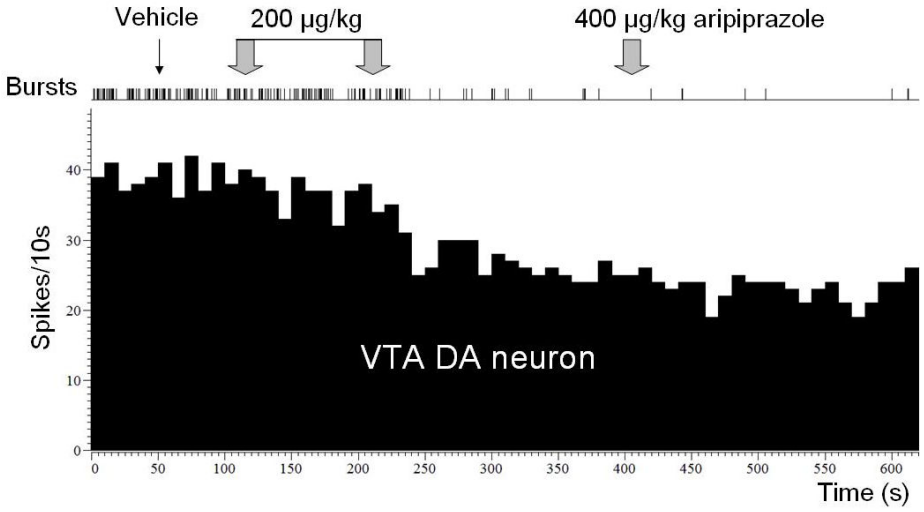
Effect of brexpiprazole and aripiprazole on VTA DA neurons

Brexpiprazole at cumulative doses of 200, 400 and 800 µg/kg did not significantly alter firing rate ($F_{1,39}$=3.15, n=11, p=0.083, figure 1C) or bursting activity ($F_{1,29}$=1.61, n=10, p=0.21 figures 1D) of VTA DA neurons from baseline activity. Aripiprazole, administered at these same doses, significantly decreased firing activity ($F_{1,22}$=11.93, n=6, p=0.0023, figures 1C) and bursting activity of VTA DA neurons ($F_{1,18}$=7.58, n=5, p=0.013, figures 1D). For an illustrative firing histogram of the effect of brexpiprazole and aripiprazole see figures 1A and 1B, respectively.

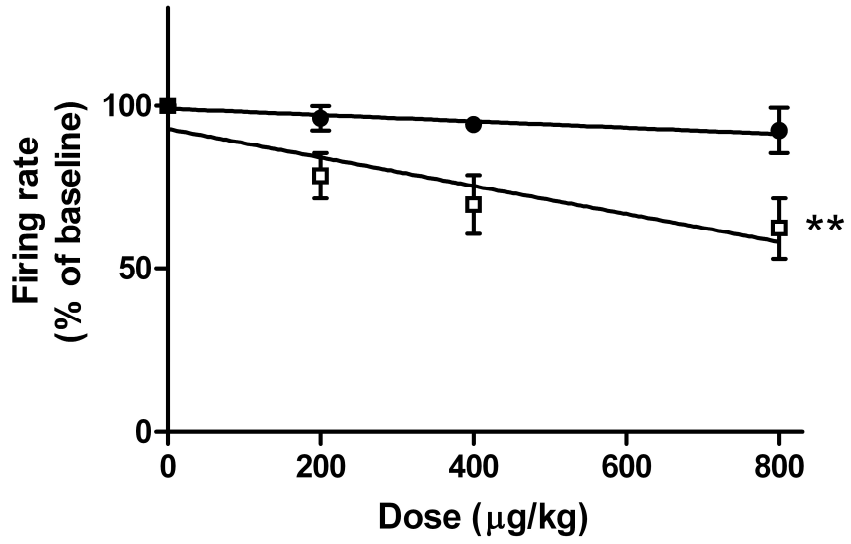
1A



1B



1C



1D

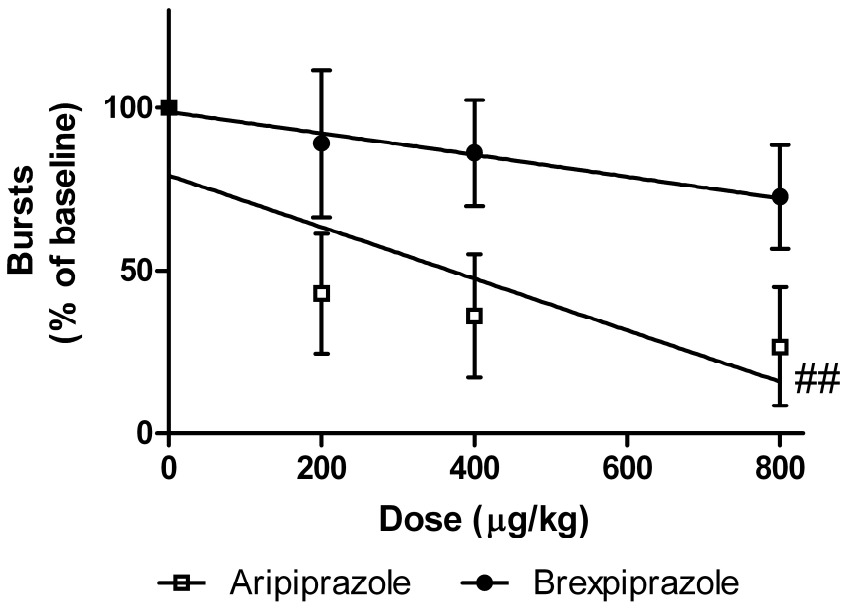


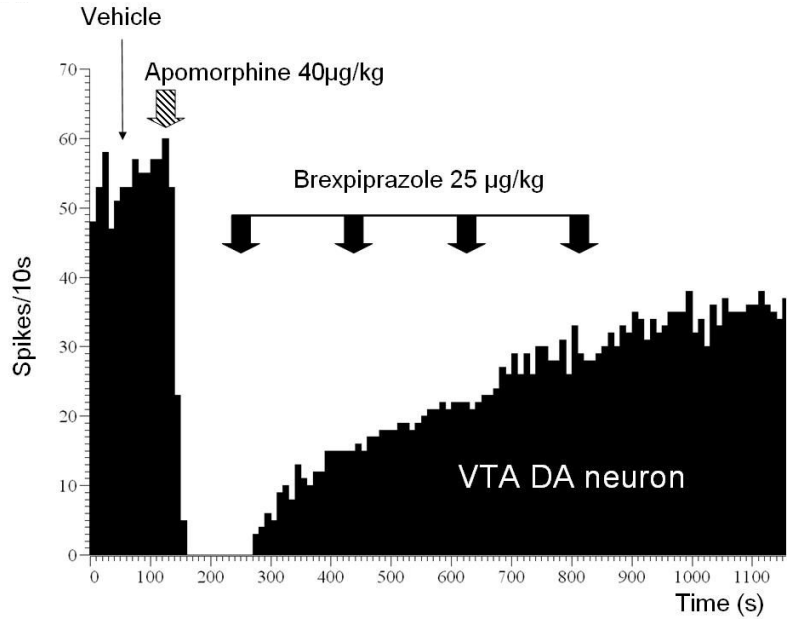
Figure 1: effect of systemic brexpiprazole and aripiprazole administration on VTA DA neurons. A, B: illustrative firing histogram of DA neurons and response to intravenous administration of brexpiprazole (black arrows, figure A) and aripiprazole (grey arrows, figure B). C, D: brexpiprazole (n=11) did not change firing activity of VTA DA neurons, whereas aripiprazole (n=6) significantly reduced both firing and bursting activity of these neurons. Data were analyzed using linear regression and are presented as mean ± S.E.M.

***Significant effect of brexpiprazole in comparison to saline administration, **slope: $p<0.01$, ##intercept: $p<0.01$.**

Effect of brexpiprazole on D_2 autoreceptors on VTA DA neurons

Following a 60-second inhibition period of putative DA neurons in the VTA by the DA receptor agonist apomorphine (40 µg/kg, i.v.), brexpiprazole administration (25-100 µg/kg), reversed the effect of apomorphine to approximately 65% of baseline firing. Sigmoidal curve fitting (n=9) yielded an ED_{50} value of 61 µg/kg for brexpiprazole on this effect (figure 2B; for an illustrative firing histogram see figure 2A).

2A



2B

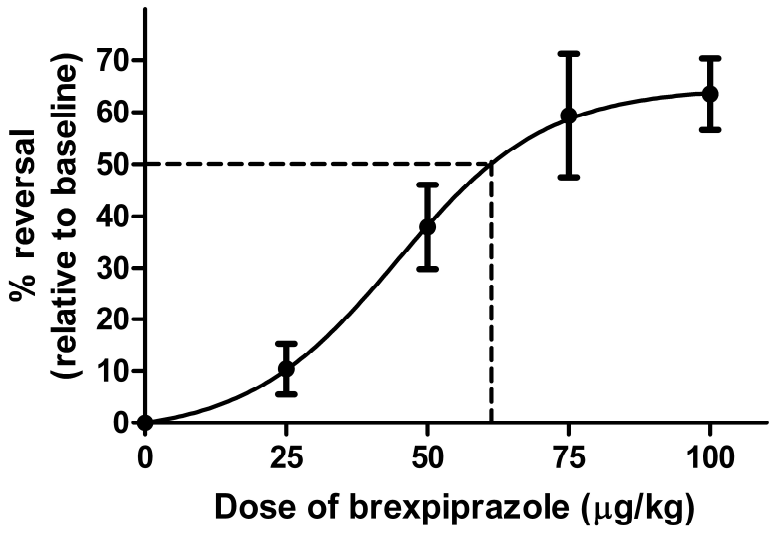
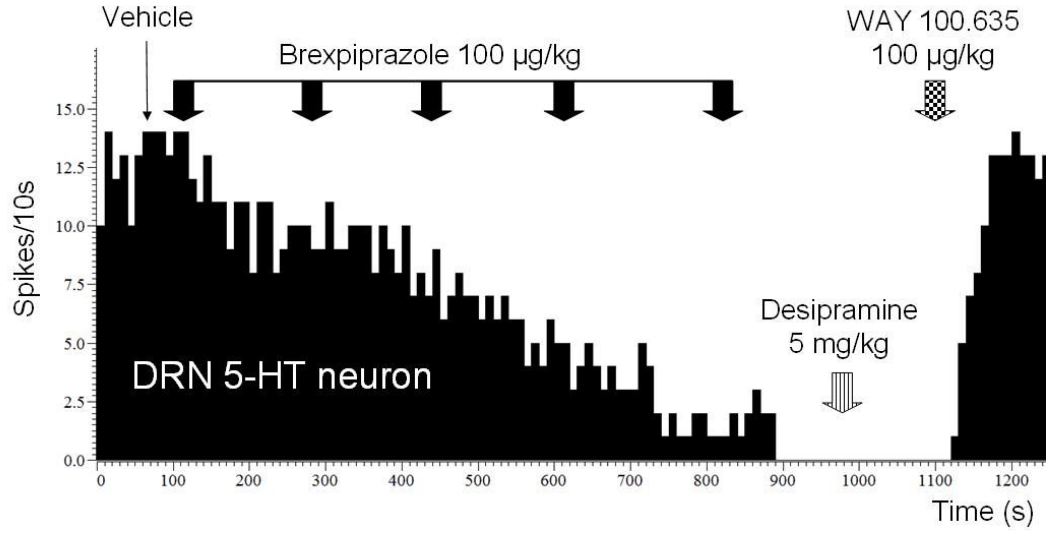


Figure 2: effect of systemic brexpiprazole administration on VTA DA neurons inhibited by the dopamine agonist apomorphine. A: illustrative firing histogram of a DA neurons and response to intravenous administration of apomorphine and brexpiprazole. B: brexpiprazole reversed the inhibitory effect of apomorphine (n=9). The ED_{50} value was obtained with a sigmoidal curve fit; data are presented as mean ± S.E.M.

Effect of brexpiprazole and aripiprazole on 5-HT$_{1A}$ autoreceptors

In the DRN, brexpiprazole at cumulative doses of 100 and 200 µg/kg inhibited the firing of 5-HT neurons (n=11), an effect not reversed by administration of the NE reuptake inhibitor desipramine (5 mg/kg) whereas the selective 5-HT$_{1A}$ antagonist WAY 100.635 reversed this inhibition (n=3, for an illustrative firing histogram see figure 3A). Similarly, an inhibitory effect of aripiprazole was observed on 5-HT neurons (n=15) that was reversed by the selective 5-HT$_{1A}$ antagonist WAY 100.635 but not desipramine (n=3). For aripiprazole, the experiment was initially conducted by administering cumulative doses of 200 µg/kg (n=6) based on a previous study (Dahan *et al.*, 2009). As the inhibitory effect of these injections did not fully inhibit 5-HT neurons up to a cumulative dose of 600 µg/kg, aripiprazole was administered at 500 µg/kg (n=9) in a subsequent experiment; data of these experiment were pooled, as statistical analysis demonstrated that the dose-responsiveness of DRN neurons to aripiprazole at increments of 200 µg/kg and 500 µg/kg were not different (slope: $F_{1,45}$=0.04, p>0.05, intercept: $F_{1,46}$=0.08, p>0.05). Statistical analysis demonstrated a significantly lower ED_{50} value for brexpiprazole than for aripiprazole (ED_{50} = 230 and 700 µg/kg, respectively, $F_{1,78}$=31.51, p<0.0001, figure 3B).

3A



3B

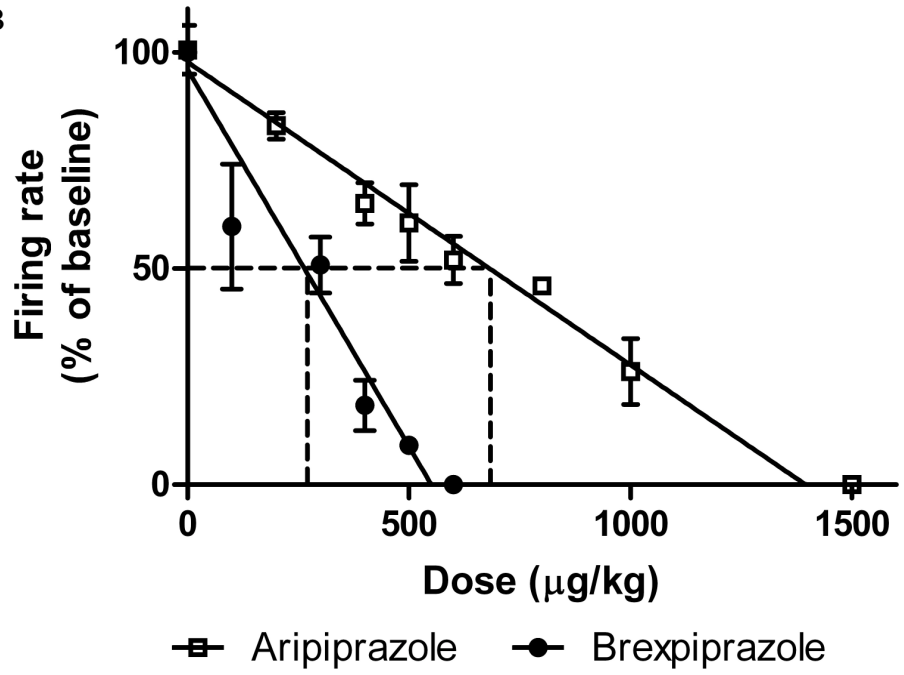


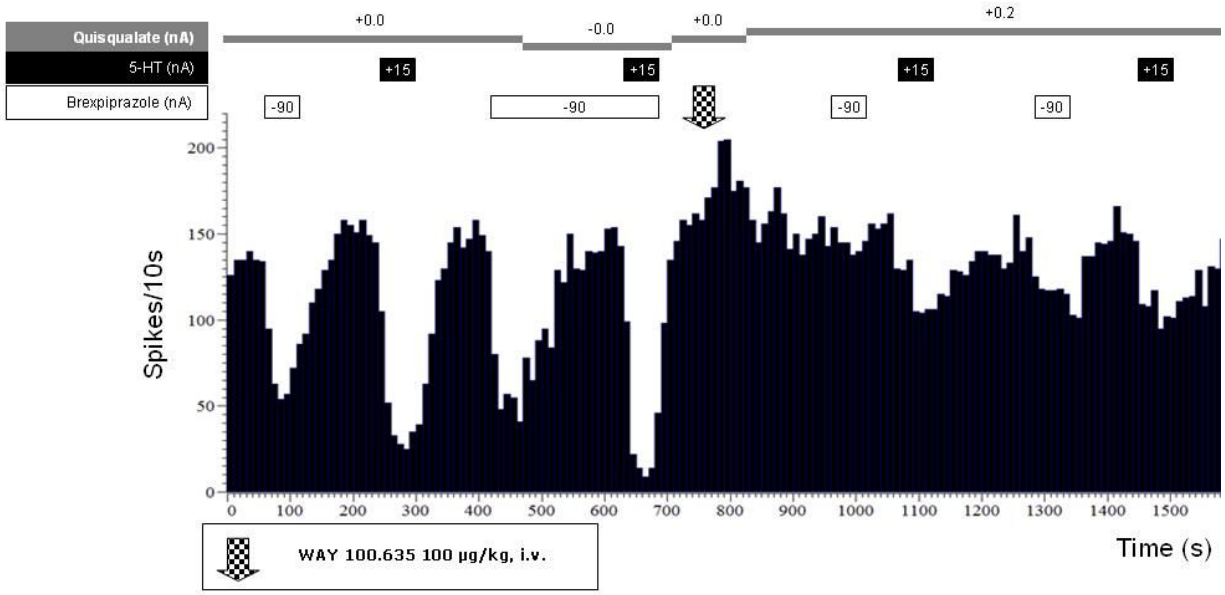
Figure 3: effect of systemic brexpiprazole and aripiprazole administration on 5-HT neurons in the DRN. A: illustrative trace of a 5-HT neuron and response to intravenous administration brexpiprazole. B: brexpiprazole (n=11) is a more potent 5-HT_{1A} agonist than aripiprazole (n=15). Data were analyzed using linear regression and are presented as mean ± S.E.M.

Effect of brexpiprazole on postsynaptic 5-HT$_{1A}$ receptors

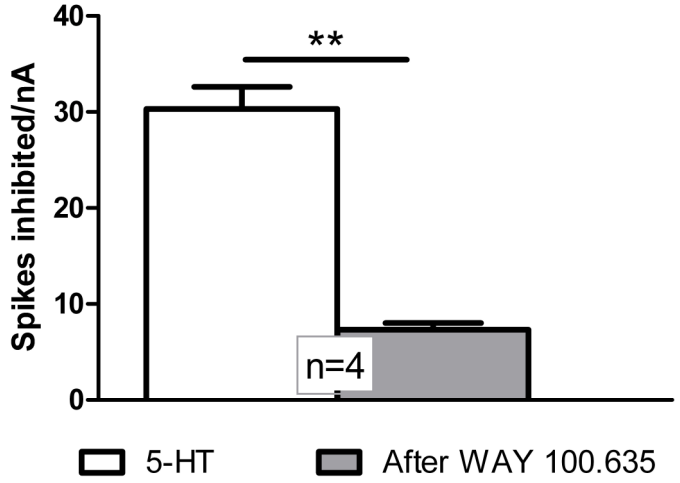
In the hippocampus, systemic administration of brexpiprazole up to a dose of 1500 µg/kg did not change the number of spikes suppressed/nA following iontophoretic application of 5-HT on CA3 pyramidal neurons ($F_{1,30}$=2.72, p>0.05; data not shown).

Iontophoretic application of both brexpiprazole and 5-HT had an inhibitory effect on CA3 pyramidal neurons (n=12, for an illustrative firing histogram see figure 4A). When brexpiprazole and 5-HT were ejected concomitantly, the neuronal inhibition of CA pyramidal neurons did not differ from when 5-HT was ejected alone (p>0.05, n=12, figure 4D). This inhibitory effect of both agents was significantly dampened after systemic administration of the 5-HT$_{1A}$ receptor antagonist WAY 100.635 (p<0.05 and p<0.01, respectively, figure 4B and 4C).

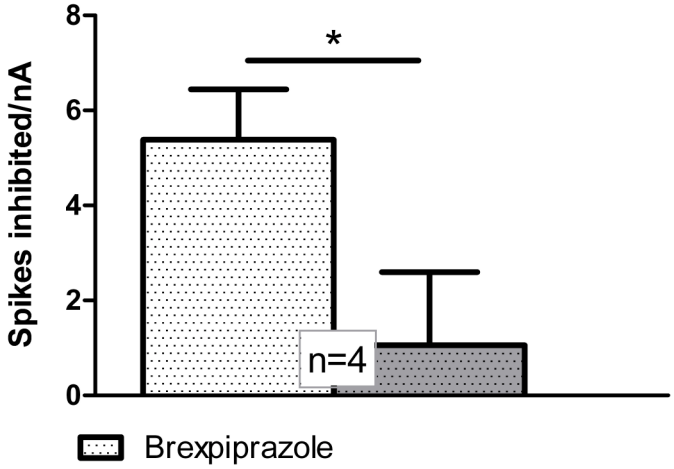
4A



4B



4C



4D

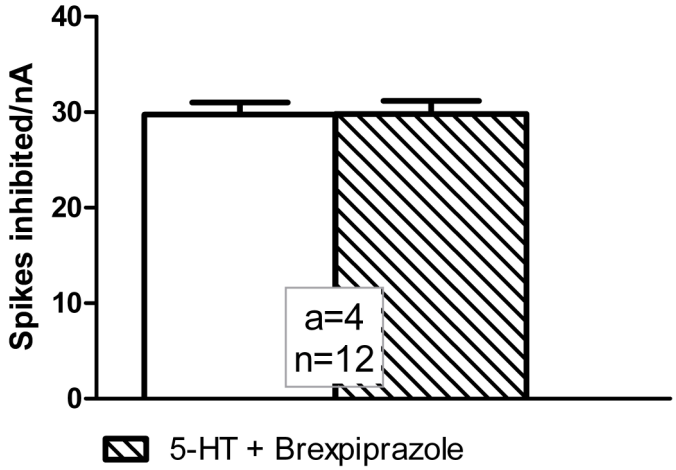


Figure 4: effect of iontophoretically applied brexpiprazole, 5-HT, and concomitant ejection of 5-HT and brexpiprazole on pyramidal neurons in the hippocampus CA3 region. A: illustrative firing histogram of discharge activity of a pyramidal neuron in the CA3 region of hippocampus, and effects of iontophoretically applied agents before and after systemic WAY 100.635 (WAY) administration. B, C: the inhibitory response to iontophoretic application of brexpiprazole and 5-HT is significantly reduced after WAY administration. D: There was no different inhibitory effect of co-ejection of brexpiprazole and 5-HT compared to 5-HT ejection alone. The number of neurons (n) and animals (a) is presented in histograms; data were analyzed with a paired t-test and are presented as mean ± S.E.M.
***Significant effect of WAY 100.635 administration on the inhibitory effect of brexpiprazole and 5-HT; *p<0.05, **P<0.01.**

Effect of brexpiprazole on the SERT

In the hippocampus, the RT_{50} value (a measure for SERT activity) following iontophoretic application of 5-HT on CA3 pyramidal neurons was not changed by administrations of brexpiprazole up to a dose of 1500 µg/kg ($F_{1,32}$=0.47, p>0.05; data not shown).

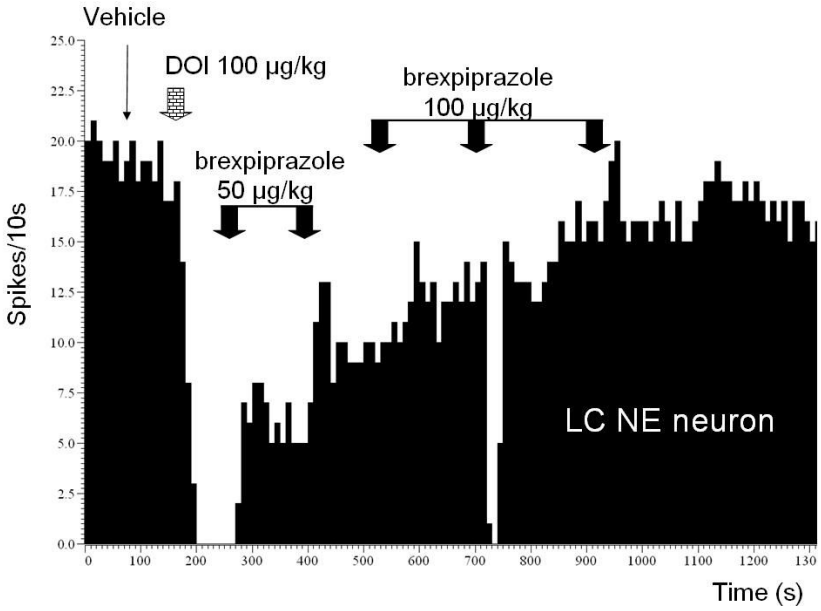
Effect of brexpiprazole on terminal 5-HT_{1B} autoreceptors

The DOS produced on the same neuron by electrical stimulation of 5-HT afferents with stimulation frequencies of 1 and 5 Hz was increased by brexpiprazole at doses of 500, 1000 and 1500 µg/kg (figure 7B). For 5 Hz stimulations, the corresponding DOS at these doses were 27 ± 2, 38 ± 3, 48 ± 5 and 49 ± 4. Statistical analysis demonstrated that under control conditions and after cumulative doses of brexpiprazole, the DOS after 1 Hz stimulation remained significantly greater than the DOS after 5 Hz (slope: $F_{1,36}$=0.91, p>0.05, intercept: $F_{1,37}$=13.96, p<0.001).

Effect of brexpiprazole on 5-HT_{2A} receptors in the LC

Following a 60-second inhibition period of NE neurons in the LC by the 5-HT_{2A} agonist DOI (100 µg/kg, i.v.), brexpiprazole administration (50-400 µg/kg), reversed NE neural firing to approximately 80% of baseline firing with an ED_{50} value estimated at 110 µg/kg (figure 5B; for an illustrative firing histogram see figure 5A).

5A



5B

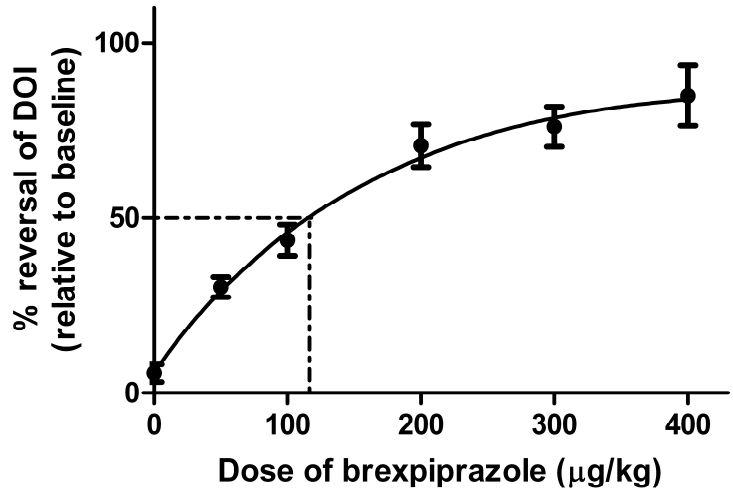
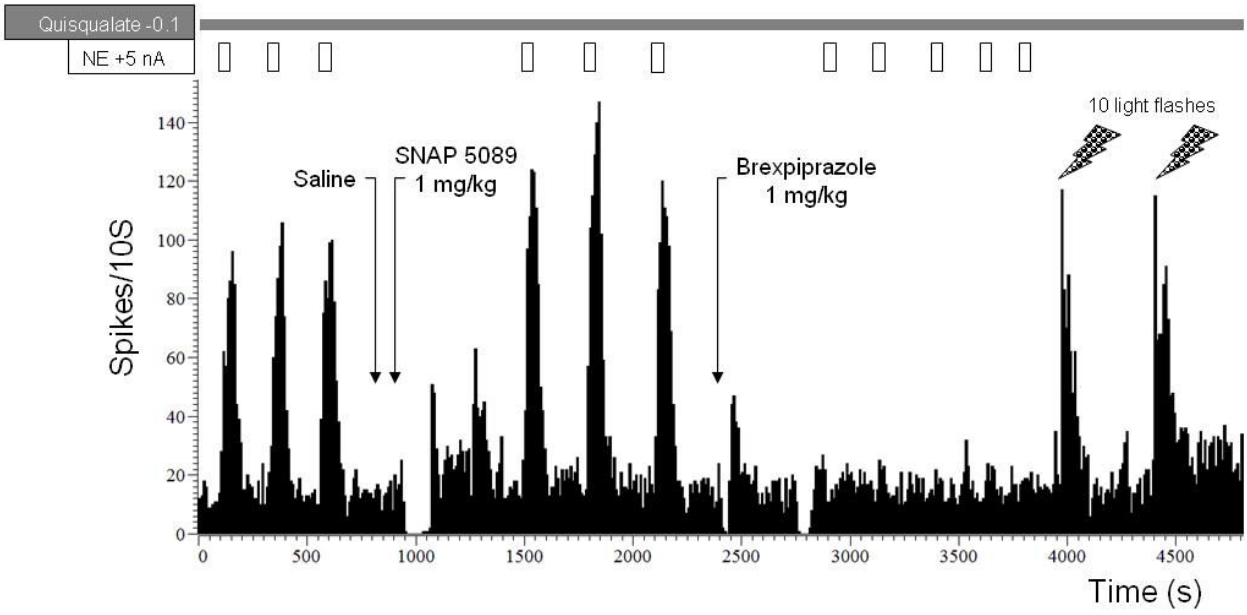


Figure 5: effect of systemic brexpiprazole on LC NE neurons inhibited by the preferential 5-HT_{2A} agonist DOI. A: illustrative trace of an NE neuron inhibited by intravenous administration of DOI and reversal of inhibition by brexpiprazole. Between 720-740 seconds the electrical signal of the neuron decreased below the level of the differential amplitude discriminator. Following this decrease, the electrode was quickly lowered by ~20 micrometer and the signal was restored to its prior amplitude. Wavemark analysis confirmed that the same neuron was recorded before and after this period. B: graphic presentation of the reversing effect of brexpiprazole on neurons inhibited by DOI (n=10). The ED_{50} value was obtained with simple non-linear regression; data are presented as mean ± S.E.M.

Effect of brexpiprazole on postsynaptic α_{1B}-adrenoceptors in the LGN

In the LGN, acute administrations of brexpiprazole at a dose of 500 and 1000 µg/kg significantly decreased the excitatory action of exogenous NE on LGN neurons by 44 % and 77 %, respectively ($F_{2,5}$=20.10, p=0.0034, p<0.05 and p<0.001 for these respective doses, figure 6B) with an ED_{50} value estimated at 630 µg/kg. The excitatory effect of NE ejection was not changed by prior administration of the α_{1A}-adrenoceptor antagonist SNAP 5089 (1 mg/kg, i.v), whereas brexpiprazole administration still decreased neuronal excitability in the LGN after SNAP 5089 administration (n=2, for illustration see figure 6A).

6A



6B

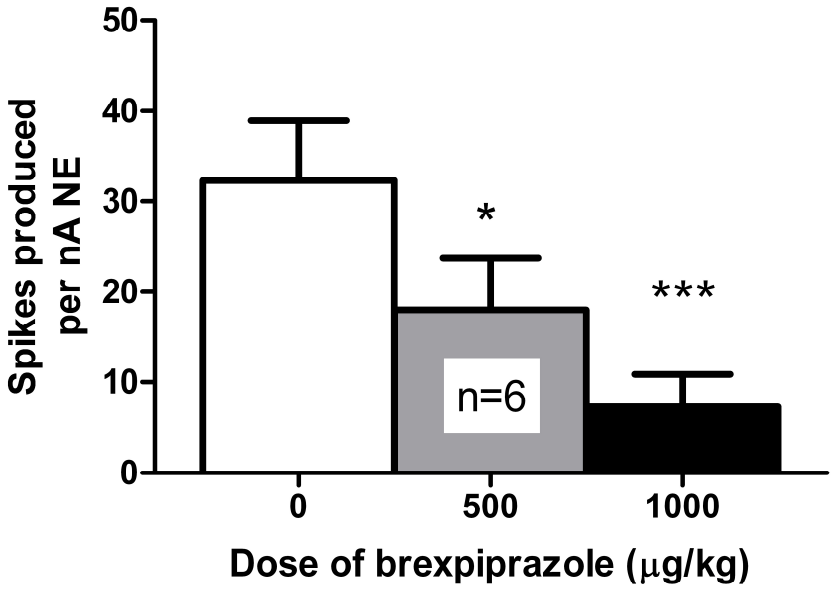


Figure 6: effect of brexpiprazole on the excitatory effect of iontophoretically applied NE on LGN neurons. A: illustrative trace of the electrical activity of an LGN neuron, its excitation by iontophoretically applied NE, ineffectiveness of the selective α_{1A}-adrenergic receptor antagonist SNAP 5089 to alter this excitation, blockade of this excitation by systemic brexpiprazole administration, and brisk excitatory response to light flashes, confirming proper recording electrode positioning. B: effect of brexpiprazole on the excitatory response of LGN neurons to iontophoretic application of NE. The number of neurons tested is presented in histograms; data were analyzed with repeated measures ANOVA and Tukey *post-hoc,* and are presented as mean + S.E.M.
***Significant effect of brexpiprazole administration; *p<0.05, ***P<0.001.**
N.B. Data from the neuron recorded in (A) were not included in (B) due to the injection of SNAP 5089 prior to brexpiprazole administration.

Effect of brexpiprazole on postsynaptic α_2-adrenoceptors

In the hippocampus CA3 region, systemic administration of brexpiprazole up to a dose of 1500 μg/kg did not change the number of spikes inhibited/nA following iontophoretic application of NE ($F_{1,28}$=0.95, p>0.05; data not shown).

Effect of brexpiprazole on the NET

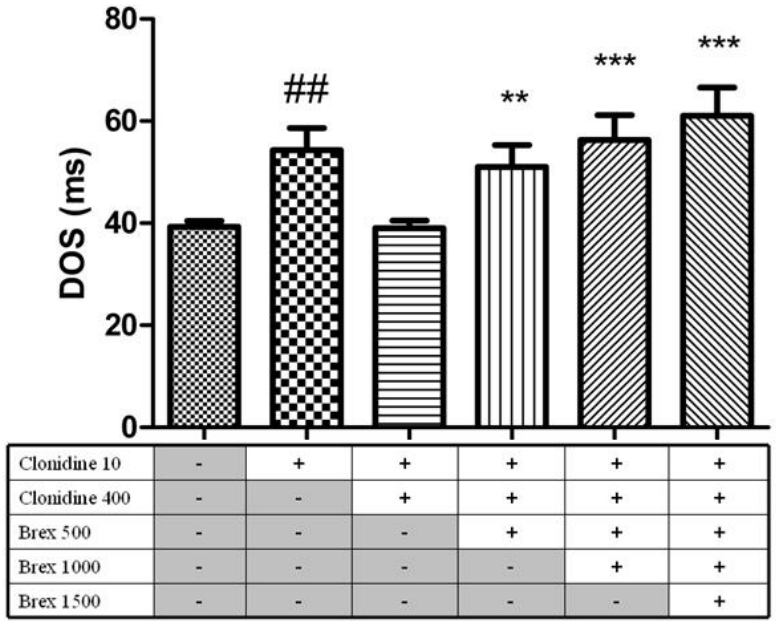
In the hippocampus CA3 region, the RT_{50} value following iontophoretic application of NE on CA3 pyramidal neurons was not changed by administration of brexpiprazole up to a dose of 1500 μg/kg ($F_{1,30}$=0.01, p>0.05; data not shown).

Effect of brexpiprazole on terminal α_2-adrenoceptors on 5-HT terminals

Clonidine at a dose of 10 μg/kg significantly increased the DOS value following 1 Hz stimulation compared to baseline, an effect completely reversed by a subsequent injection of clonidine at a dose of 400 μg/kg ($F_{3,5}$=13.63, n=6, p<0.0001, figure 7A). Following these doses of clonidine, the DOS increased with cumulative administrations of brexpiprazole (500, 1000 and 1500 μg/kg, i.v.) in a dose-dependent fashion (Tukey *post-hoc*, p<0.01, p<0.001 and p<0.001 for these doses, respectively).

Administration of brexpiprazole alone also increased the DOS value in a dose-dependent manner ($F_{3,4}$=4.16, n=5, p=0.02, figure 7B). Tukey *post-hoc* testing revealed a significant effect of brexpiprazole on DOS at a dose of 1000 and 1500 μg/kg (p<0.01 and p<0.01, respectively). Clonidine administration (10 and 400 μg/kg) had no altering effect on the DOS when it was administered after brexpiprazole (p>0.05, figure 7B).

7A



7B

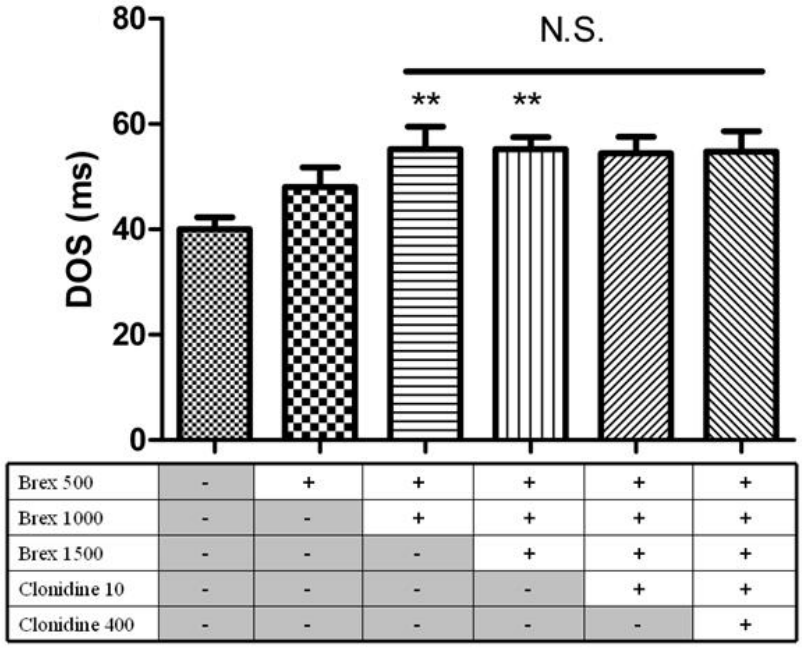


Figure 7: effect of brexpiprazole on α_2-heteroceptors located on 5-HT terminals. A: schematic presentation of the effect of three cumulative doses of brexpiprazole (500 µg/kg, i.v.) after clonidine (10 and 400 µg/kg, i.v.) on DOS (n=6). B: effect of three cumulative doses of brexpiprazole (3 x 500 µg/kg, i.v.) on DOS alone, and absence of effect of clonidine on DOS after brexpiprazole administration (n=5). Brex; brexpiprazole, Clo; clonidine, numbers after abbreviations indicate doses in µg/kg, numbers in brackets indicate first, second or third administration of brexpiprazole. Data were analyzed with repeated measures ANOVA followed by a Tukey *post-hoc* test, and are presented as mean + S.E.M.

***Significant effect of brexpiprazole administration on DOS relative to clonidine 400 µg/kg (A) and baseline values (B), respectively. #significant effect of clonidine administration on DOS compared to baseline. *p<0.05, **P<0.01, ***P<0.01; $^{\#\#}$P<0.01).**

Discussion

Brexpiprazole: effect on VTA DA neurons

Brexpiprazole reversed the inhibitory action of the DA agonist apomorphine on neural firing of VTA DA neurons, thus demonstrating antagonistic action at D_2 autoreceptors (figure 2). Interestingly, brexpiprazole by itself did not significantly change the firing rate or bursting activity of DA neurons (figure 1). In contrast, the classical D_2 receptor antagonist haloperidol is known to increase firing and burst activity of VTA DA neurons when administered acutely, an effect attributable to blockade of endogenous DA inhibitory tone on D_2 autoreceptors (Pucak and Grace, 1994). Since brexpiprazole did not alter firing and bursting activity of VTA DA neurons, this result demonstrates that *in vivo*, it neither acts as a pure antagonist, nor as an agonist with high intrinsic activity on D_2 autoreceptors. Aripiprazole, similar to brexpiprazole, was previously shown to reverse neuronal inhibition of VTA DA neurons by apomorphine, although less potently (Dahan *et al.*, 2009). In the present study, aripiprazole significantly reduced the firing and bursting activity of VTA DA neurons to a similar degree as reported previously (figure 1; Dahan et al. 2009). The difference in effects on DA neurons of aripiprazole and brexpiprazole is likely due to a lower intrinsic activity of the latter agent at D_2 receptors, in line with their *in vitro* profiles (Maeda, Sugino, *et al.*, 2014). Notably, the present study also demonstrated more potent *in vivo* agonism of brexpiprazole on 5-HT_{1A} receptors than aripiprazole (discussed below). Since activation of prefrontal 5-HT_{1A} receptors is known to excite VTA DA neurons (Arborelius *et al.*, 1993; Díaz-Mataix *et al.*, 2005; Gronier, 2008), a difference in degree of 5-HT_{1A} receptor agonism could contribute to the distinct effects of brexpiprazole and aripiprazole on the firing activity of DA neurons.

Brexpiprazole: effect on the 5-HT system

Brexpiprazole dose-dependently inhibited the firing rate of 5-HT neurons in the DRN (figure 3). This inhibitory effect was due to agonism at 5-HT_{1A} receptors on these neurons, as the selective 5-HT_{1A} receptor antagonist WAY 100.635, but not the NE reuptake inhibitor desipramine reversed the inhibitory action of brexpiprazole (figure

3A). Compared to aripiprazole, brexpiprazole was significantly more potent at activating 5-HT_{1A} autoreceptors (figure 3B), a finding in line with the *in vitro* affinity for 5-HT_{1A} receptors of these agents (Maeda, Sugino, *et al.*, 2014). To the best of our knowledge, two previous studies assessed the dose-dependent effect of aripiprazole to inhibit DRN 5-HT neurons; Dahan et al. (2009) reported an ED_{50} value of 540 μg/kg, in close similarity to the present findings, while the ED_{50} for aripiprazole in the work by Stark et al. (2007) is estimated at 200-250 μg/kg. The reason for this difference remains to be established, although linear vs. logarithmic dose-response estimation could play a role.

In the hippocampus, the activity of the SERT, assessed using the RT_{50} index, remained unaltered following brexpiprazole administration up to a dose of 1500 μg/kg, whereas drugs that occupy SERT to a significant extent *in vivo* are known to prolong the RT_{50} index (Piñeyro *et al.*, 1994; Ghanbari *et al.*, 2010). Microiontophoretic application of brexpiprazole and 5-HT both inhibited CA3 pyramidal neurons (figure 4A,C), an effect attributable to 5-HT_{1A} activation. Indeed, the inhibitory effect of these agents was blocked after systemic administration of the 5-HT_{1A} antagonist WAY 100.635 (figure 4B,C). Importantly, the inhibitory effect of 5-HT alone was unaltered when brexpiprazole was co-ejected (figure 4D). This absence of competitive action on 5-HT_{1A} receptors of brexpiprazole with 5-HT indicates that brexpiprazole acted as a full 5-HT_{1A} receptor agonist *in vivo*. Indeed, previous studies using the same paradigm showed full agonistic action of BAY 3702 (Dong *et al.*, 1998) and trazodone (Ghanbari *et al.*, 2010), whereas 8-OHDPAT (2-dipropylamino-8-hydroxy-1,2,3,4- tetrahydronaphthalene; Hadrava et al. 1996), gepirone (Hadrava *et al.*, 1995), and asenapine (Ghanbari *et al.*, 2009) acted as partial agonists at 5-HT_{1A} receptors in the hippocampus.

Brexpiprazole administration dose-dependently increased the DOS following electrical stimulations of 5-HT afferents at 1 Hz (figure 7B), an effect that could theoretically be attributable to terminal 5-HT_{1B} autoreceptors and/or α_2-adrenergic heteroceptor antagonism (Chaput *et al.*, 1986; Mongeau *et al.*, 1993). However, brexpiprazole administration did not change the difference in DOS following 1 and 5 Hz stimulations, indicating that terminal 5-HT_{1B} autoreceptors functionality was unaffected by brexpiprazole. In contrast, brexpiprazole acted as a potent α_2-adrenergic heteroceptor antagonist, as shown in two complementary experiments where brexpiprazole reversed

(figure 7A) and prevented (figure 7B) the typical decrease in DOS caused by a high dose of the α_2-adrenoceptor agonist clonidine (400 µg/kg, i.v.). Such effects have previously been observed following acute administration of the antidepressants mianserin and mirtazapine (Mongeau *et al.*, 1993; Haddjeri *et al.*, 1996).

Brexpiprazole: effect on the NE system.

In the LC, NE neurons are innervated by inhibitory GABA neurons that express excitatory 5-HT_{2A} receptors; activation of 5-HT_{2A} receptors enhances GABA neurotransmission and inhibits NE neurons (Szabo and Blier, 2001). Indeed, systemic administration of the preferential 5-HT_{2A} receptor agonist DOI strongly inhibited NE neurons. Since this effect was potently reversed by brexpiprazole, antagonistic action of this agent on 5-HT_{2A} receptors was clearly demonstrated (figure 5). In the present study, the ED_{50} value of brexpiprazole for 5-HT_{2A} receptors antagonism was approximately 2-fold higher than for D_2 receptor antagonism (110 vs. 61 µg/kg, respectively), which is qualitatively in line with its *in vitro* profile (Maeda, Sugino, *et al.*, 2014). Notably, caution should be taken when comparing *in vitro* and *in vivo* data of agents in their effectiveness to reverse the action of receptor agonists, since ED_{50} values are relative to the affinity, intrinsic activity, and dose of the used agonist. Additionally, the multiple receptor activity of brexpiprazole could affect more than one neuronal substrate *in vivo*. To illustrate this point, the *in vitro* affinity of asenapine is one order of magnitude greater for 5-HT_{2A} than D_2 receptors (Shahid *et al.*, 2009), whereas the *in vivo* ED_{50} value for D_2 receptors type was found to be approximately two-fold higher than for 5-HT_{2A} receptors (Ghanbari *et al.*, 2009).

In the hippocampus CA3 region, brexpiprazole did not modify the inhibitory response to iontophoretic application of NE or the RT_{50} index, indicating no action on postsynaptic α_2-adrenoceptors and the NET, respectively (de Montigny *et al.*, 1980; Curet and de Montigny, 1988). In the LGN, neurons are known to almost exclusively express α_1-adrenergic receptors of the 1B subtype (Day *et al.*, 1997). In this brain region, the antagonistic effect of brexpiprazole on these receptors was assessed by quantifying neuronal excitation following iontophoretically applied NE, a response known to be mediated by α_1-adrenoceptors (Rogawski and Aghajanian, 1980, 1982; Menkes *et al.*,

1981). Neuronal excitation decreased with brexpiprazole administration, strongly suggesting antagonistic action at α_1-adrenergic receptors (figure 6B). Since there is no expression of α_{1B}-adrenoreceptors yet minimal expression of α_{1A}-adrenoceptors, (Day *et al.*, 1997), the excitatory effect of NE after administration of the selective α_{1A}-adrenoreceptor antagonist SNAP 5089 was assessed (Wetzel *et al.*, 1995). The excitatory effect of iontophoretic application of NE on LGN neurons was not altered by SNAP 5089 administration, whereas consecutive administrations of brexpiprazole largely blocked this excitatory effect (figure 6A). Taken together, these result suggests antagonistic properties of brexpiprazole predominantly at α_{1B}-adrenoceptors, consistent with its 20-fold higher affinity for this receptor subtype over α_{1A}- and α_{1D}-adrenoceptors (Maeda, Sugino, *et al.*, 2014).

Conclusion

The present results show acute *in vivo* action of brexpiprazole at all three monoamine (5-HT, NE and DA) systems. Similar to aripiprazole, brexpiprazole acted as a partial D_2 receptor agonist *in vivo*, but is relatively less efficacious at this receptor type. Clinically, D_2 receptor partial agonism is thought to buffer fluctuations in DA transmission (Burris, 2002; Shapiro *et al.*, 2003), in line with the behavioural effects of brexpiprazole in animal models for schizophrenia (Maeda, Lerdrup, *et al.*, 2014). Furthermore, the potent *in vivo* agonistic action on 5-HT_{1A} receptors of brexpiprazole could be a relevant pharmacological feature in treatment of both mood disorders and schizophrenia (Blier and Ward, 2003; Newman-Tancredi, 2010). Acute brexpiprazole reduced inhibition on two important interaction nodes between the 5-HT and NE system; first it blocked 5-HT_{2A} receptors, a receptor type that dampens LC NE firing when 5-HT neurotransmission is enhanced (Dremencov *et al.*, 2007; Chernoloz *et al.*, 2009). Second, brexpiprazole blocked α_2-adrenoceptors on 5-HT terminals, a receptor type that dampens 5-HT release when NE neurotransmission is elevated (Mongeau *et al.*, 1993). As the therapeutic effect of potent 5-HT_{2A} receptor antagonism in combination with 5-HT reuptake inhibitors is well recognized (Nelson and Papakostas, 2009), the present data support the use of brexpiprazole as an augmentation strategy. This notion is strengthened by the recent demonstration of clinically efficaciousness of brexpiprazole as adjunct to

antidepressants in major depressive disorder (Thase *et al.*, 2014). In addition, combined but not separate administration of brexpiprazole and NE or 5-HT reuptake inhibitors had an antidepressant-like effect in rodents (Hirose *et al.*, 2014). Following this *in vivo* pharmacological characterization and since brexpiprazole will be administered on long-term basis in the clinic, it will be crucial to investigate the effect of sustained brexpiprazole administration on therapeutically relevant monoamine targets.

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Chapter III: Sustained brexpiprazole

Following the in vivo characterization of brexpiprazole following its acute administration (chapter II), the present study assessed the effect of subacute (two-day) and sustained (14-day) brexpiprazole administration. Based on pilot studies and pharmacodynamic data, all experiments were performed under a regimen of 1 mg/kg/day or vehicle. To facilitate a comparison with aripiprazole, the pharmacological mechanisms assessed in the present work are similar to a previously study on the effects of sustained aripiprazole (Chernoloz *et al.*, 2012). In addition, the effect on the status of D_2 autoreceptors, α_2-adrenergic autoreceptors, 5-HT_{2A} receptors, terminal α_2-adreneric heteroceptors and 5-HT_{1B} autoreceptors, the SERT, the NET, and tonic activation on postsynaptic 5-HT_{1A}, α_1-adrenergic, and α_2-adrenergic receptors was assessed to provide further insight in the sustained *in vivo* actions of brexpiprazole on therapeutically relevant targets.

The experimental design of this study was drafted by Dr. Blier, Dr. El Mansari, and the present author. All electrophysiological experiments and their analysis were conducted by the present author. All authors contributed to and approved of the final manuscript. Preliminary data of this study were presented at the annual meeting of the Society of Biological Psychiatry in May 2015, and the manuscript is in its final stage for submission to the International Journal of Neuropharmacology.

Brexpiprazole alters monoaminergic systems following sustained administration: an *in vivo* electrophysiological study

Running title: Sustained brexpiprazole and monoamine systems

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Abstract

Background: Brexpiprazole (OPC-34721) is currently under investigation for treatment of schizophrenia and as add-on treatment of depression. To complement results from a previous study in which its acute effects were characterized, the present study assessed the effect of sustained brexpiprazole administration on monoaminergic systems.

Methods: Brexpiprazole (1 mg/kg, subcutaneous) or vehicle was administered once daily for two and 14 days. Single-unit electrophysiological recordings from noradrenergic neurons in locus coeruleus (LC), serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN), dopaminergic (DA) neurons in the ventral tegmental area (VTA), and pyramidal neurons in the hippocampus CA3 region were obtained in adult male Sprague-Dawley rats under chloral hydrate anesthesia.

Results: Brexpiprazole blunted D_2 autoreceptor responsiveness, while firing activity of VTA DA neurons remained unaltered. Brexpiprazole increased the firing rate of LC noradrenergic neurons, and increased NE tone on α_2-adrenergic receptors in the hippocampus. Administration of brexpiprazole for two but not 14 days increased the firing rate of 5-HT neurons in the DRN. In the hippocampus, 5-HT_{1A} receptor blockade significantly disinhibited pyramidal neurons after two- and 14-day brexpiprazole administration. In contrast, no significant disinhibition occurred after 24 hour washout or acute brexpiprazole.

Conclusions: Compared to other atypical antipsychotics, sustained brexpiprazole administration had distinguishable effects on the DA system, presumably due to its partial agonistic action on D_2 autoreceptors. Brexpiprazole enhanced serotonergic and noradrenergic tone in the hippocampus, effects common to antidepressant agents. Together, these results provide novel mechanisms to explain therapeutic effects of brexpiprazole.

Introduction

Brexpiprazole (OPC-34721) was recently shown to be clinically efficacious in the treatment of schizophrenia (Kane *et al.*, 2015) and as an augmentation strategy in treatment of depression (Thase *et al.*, 2014). Similarly to aripiprazole, brexpiprazole is a partial dopamine (DA) D_2 receptor agonist, a pharmacological feature that distinguishes these agents from other antipsychotics, which are D_2 receptor antagonists (Stark *et al.*, 2007). Although D_2 receptor antagonism effectively reduces positive symptoms in schizophrenia (Seeman and Lee, 1975; Rao and Remington, 2013), blockade of the D_2-receptor mediated signal might be undesirable for management of negative symptoms and/or cognitive symptoms. Indeed, aripiprazole improves negative and cognitive symptoms in schizophrenia and schizoaffective disorder (Stip and Tourjman, 2010), effects partly explained by its combined D_2 receptor partial agonism, 5-HT_{1A} receptor agonism, and 5-HT_{2A} receptor antagonism (Hirose *et al.*, 2004).

The degree of D_2 receptor activation could be of crucial importance to the antipsychotic properties of partial D_2 receptor agonists. Indeed, the clinical failure of bifeprunox has, in part, been ascribed to its excessive agonism at D_2 receptors, hence limited effects on positive symptoms (Stahl, 2008). Compared to aripiprazole, brexpiprazole has a higher *in vitro* affinity yet lower intrinsic activity at D_2 receptors (Maeda, Sugino, *et al.*, 2014). Accordingly, acute *in vivo* administration of brexpiprazole reduced the firing activity of ventral tegmental (VTA) DA neurons to a lesser extent than aripiprazole, while it more potently reversed the inhibitory effect of D_2 autoreceptor agonism on these neurons (Dahan *et al.*, 2009; Oosterhof *et al.*, 2014). In animal models, brexpiprazole administration inhibited conditioned avoidance response, apomorphine- and d-amphetamine hyperlocomotion, and apomorphine-induced eyeblinking, confirming functional *in vivo* antagonism of D_2 receptors, thereby indicating antipsychotic potential of this agent (Maeda, Lerdrup, *et al.*, 2014). Accordingly, brexpiprazole was recently shown to be clinically effective in the treatment of acute schizophrenia (Kane *et al.*, 2015).

Brexpiprazole had potent antagonistic action on serotonin $(5\text{-HT})_{2A}$ receptors both *in vitro* and acute *in vivo* (Maeda, Sugino, *et al.*, 2014; Oosterhof *et al.*, 2014), a defining

pharmacological quality of atypical antipsychotics though to underlie a lower incidence of side-effects on motor function relative to typical antipsychotic (Kuroki *et al.*, 2008). Blockade of 5-HT_{2A} receptors is also known to prevent the dampening effect on the noradrenaline (NE) system of sustained selective serotonin reuptake inhibitors (SSRIs; Dremencov *et al.*, 2007a; b; Chernoloz *et al.*, 2009), providing a neural mechanism by which co-administration of low-dose atypical antipsychotics improves the therapeutic efficacy of antidepressants (Blier and Szabo, 2005). In addition to blocking inhibitory input of the 5-HT system on NE, the inhibitory effect on 5-HT release mediated by terminal α_2-adrenergic heteroceptor activation was potently reduced by brexpiprazole (Oosterhof *et al.*, 2014), an effect potentially preventing attenuated 5-HT release when NE neurotransmission is elevated (Mongeau *et al.*, 1993; Chernoloz *et al.*, 2012).

Both *in vitro* and *in vivo* studies demonstrated more potent 5-HT_{1A} receptor agonism by brexpiprazole compared to aripiprazole (Maeda, Sugino, *et al.*, 2014; Oosterhof *et al.*, 2014). Interestingly, acute brexpiprazole – but not aripiprazole – improved phencyclide-induced cognitive impairments in the novel object recognition test (Maeda, Sugino, *et al.*, 2014). Co-administration of the selective 5-HT_{1A} receptor antagonist WAY 100.635 prevented this behavioral effect of brexpiprazole, suggesting that 5-HT_{1A} receptor activation was involved in the restorative effects of brexpiprazole on cognitive-like measures (Maeda, Sugino, *et al.*, 2014; Yoshimi *et al.*, 2014). Furthermore, since all antidepressants enhance tonic activation of 5-HT_{1A} receptors, the potent *in vivo* agonism of brexpiprazole on these receptors could be of therapeutic relevance in the treatment of mood disorders (Blier and Ward, 2003; Blier and El Mansari, 2013).

The present study investigated the effects of two- and 14-day brexpiprazole administration (1 mg/kg/day, s.c.) on the discharge activity of VTA DA, LC NE, and DRN 5-HT neuron populations. In these brain regions, the functional status of D_2, α_2-adrenergic, 5-HT_{1A}, and 5-HT_{2A} receptors was assessed. In the CA3 region of the hippocampus, the effect of sustained brexpiprazole administration on the activity of the 5-HT and NE transporters (SERT and NET, respectively), the status of terminal 5-HT_{1B} auto- and α_2-adrenergic heteroceptors, sensitivity of postsynaptic 5-HT_{1A} and α_2-adrenergic receptors, and degree of tonic activation of 5-HT_{1A}, α_1- and α_2-adrenergic receptors was assessed using electrophysiological and pharmacological strategies.

Methods

Animals

Experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 275-450 g housed under standard laboratory conditions (12:12 light-dark cycle with food and water ad libitum). *In vivo* extracellular unitary recordings were carried out in chloral hydrate anaesthetized rats (400 mg/kg, i.p.) that were mounted in a stereotaxic apparatus. Body temperature was maintained at 37 °C throughout the experiment utilizing a thermistor-controlled heating pad. If applicable, prior to the electrophysiological recordings a catheter was inserted in a lateral tail vein for systemic intravenous (i.v.) injection of agents. At the end of experiments, animals were euthanized by a lethal dose of chloral hydrate (4 % solution, i.p). 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, Ontario, Canada).

Compounds

Brexpiprazole (Maeda, Sugino, *et al.*, 2014) was dissolved in a 0.5 % lactic acid solution in distilled water; pH of the solution was adjusted to 4.8 by addition of NaOH. Brexpiprazole (1 mg/kg, s.c.) or vehicle was administered for two or 14 days, with the last injection occurring at least 30 minutes prior to electrophysiological recordings. No experiments were performed >4 hours post brexpiprazole administration.

The D_2 receptor antagonist haloperidol (200 µg/kg) and the DA receptor agonist apomorphine (40 µg/kg) were dissolved in a 0.5 % lactic acid solution in distilled water; pH of the solution was adjusted to 4.5 by addition of NaOH. The 5-HT_{2A} receptor antagonist M100907 was dissolved in Tween 80 (0.2%) in distilled water. The 5-HT_{1A} receptor agonist flesinoxan (100 µg/kg), the 5-HT_{1A} receptor antagonist WAY 100.635 (100 µg/kg), the preferential 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI, 100 µg/kg), the α_1-adrenoreceptor antagonist prazosin (100µg/kg), the α_2-adrenoreceptor agonist clonidine (10 µg/kg), and the α_2-adrenoreceptor antagonist

idazoxan (1000 µg/kg) were dissolved in distilled water. Brexpiprazole was provided by Lundbeck A/S (Valby, Denmark); all other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada).

***In vivo* electrophysiological recordings**

Extracellular recordings of neurons in the VTA, dorsal raphe nucleus (DRN) and locus coeruleus (LC) were carried out with single-barrel glass micropipettes (Stoelting, Spencerville, MD) preloaded with 2 M NaCl, and with impedance between 2-6 MΩ. Neurons in the CA3 region of the hippocampus were recorded with 5-barrel micropipettes (impedances: central barrel 2-5 MΩ, side barrels 20-30 MΩ). The central barrel, used for unitary recordings, and one sidebarrel, used for automatic current balancing, was filled with 2 M NaCl; the other barrels were filled with 5-HT creatinine sulfate (10 mM in 0.2 M NaCl, pH 4), NE bitartrate (10 mM in 0.2 M NaCl, pH 4) or quisqualalic acid (1.5 mM in 0.2 M NaCl, pH 4). 5-HT and NE were ejected as cations (+2 to +20 nA) and retained with a negative current; quisqualate was ejected as an anion (-3 to +1 nA) and retained with a positive current.

Recording of VTA DA neurons

The electrical activity of putative DA neurons was recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP 3.2 to 3.6, ML 0.6 to 1.0, DV 7.0 to 9.0. At these coordinates, neurons with a long-duration (3-5 ms) triphasic action potential with a marked negative deflection, an inflection or "notch" on the rising phase, irregular spontaneous single firing pattern (1-10 Hz), and slow bursting activity with decrementing action potential amplitude were recorded (Grace and Bunney, 1983). The start of a burst was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an ISI>160 ms (Grace and Bunney, 1984).

The status of D_2 autoreceptors after brexpiprazole administration was assessed by determining the dose-response relation between the DA receptor agonist apomorphine (40 µg/kg, i.v.) and firing activity of VTA DA neurons. Firing and burst activity was quantified relative to baseline, and values were obtained from the second half of a 120

second period after injection of pharmacological agents, as described previously (Hand *et al.*, 1987).

Recording of LC NE neurons

The electrical activity of NE neurons was recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP -1.0 to -1.2, ML 1.0 to 1.3, DV 5.0 to 7.0. NE neurons were identified using the following criteria: regular firing rate (0.1-6 Hz), a long duration (0.8-1.2 ms) of the rising phase of the action potential, and a brisk excitatory response followed by a short period of inhibition (~1 s) in reaction to a nociceptive pinch of the contralateral hind paw (Vandermaelen and Aghajanian, 1983). ISI analysis with the same criteria as for DA neurons was used to determine the spontaneous burst activity of NE neurons (Chenu *et al.*, 2013).

To assess the acute effect of brexpiprazole on the firing activity of LC NE neurons, ~10 LC NE neurons before, and >30 minutes after brexpiprazole administration (1 mg/kg, s.c.) were recorded. In two- and 14-day vehicle or brexpiprazole administered animals, ~20 neurons were recorded per animal.

To assess the status of 5-HT_{2A} receptors after brexpiprazole administration, the dose-response relation between the preferential 5-HT_{2A} receptor agonist DOI (50 µg/kg, i.v.) and firing activity of LC NE neurons was determined. Similarly, the status of α_2-adrenergic autoreceptors was assessed by establishing the dose-response relation between the α_2 adrenoceptor agonist clonidine (5 µg/kg, i.v.) and firing rate of LC NE neurons, as described previously (Chernoloz *et al.*, 2012).

Recording of 5-HT neurons

The electrical activity of putative 5-HT neurons was recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): anterior/posterior (AP) 1.0 to 1.2, mediolateral (ML) 0, dorsal/ventral (DV) 5.0 to 7.0. At these coordinates, only neurons with a bi- or triphasic extracellular waveform with a long-duration (0.8-1.2 ms) positive phase, and regular firing in the range of 0.1-5 Hz were recorded (Vandermaelen and Aghajanian, 1983). Bursts were defined as the occurrence of spikes with an ISI < 20 ms (Hajós *et al.*, 2007).

To assess the status of 5-HT_{1A} autoreceptors after brexpiprazole administration, the dose-response relation between the selective 5-HT_{1A} receptor agonist flesinoxan (100 μg/kg, i.v.) and firing activity of DRN 5-HT neurons was assessed, as described previously (Hadrava *et al.*, 1995).

Recording of pyramidal neurons in the CA3 region of the hippocampus

The electrical activity of CA3 pyramidal neurons was recorded by positioning multibarrel micropipettes at the following coordinates (in mm from lambda): AP: 3.8-4.2, L: 4.0-4.2, D 3.5-4.5. Since most CA3 pyramidal neurons are not spontaneously active in chloral hydrate anesthetized rats, a small ejection current (-3 to +1 nA) was applied to the quisqualate barrel in order to activate them within their physiological firing range (10 to 15 Hz; Ranck 1973).

Neural responsiveness to local application of 5-HT, an effect mediated by postsynaptic 5-HT_{1A} receptors (de Montigny and Aghajanian, 1978), was assessed by determining the total number of spikes inhibited during a 50-second ejection period, divided by the current of 5-HT ejection (2 nA) producing a submaximal inhibitory effect on the firing activity of CA3 pyramidal neurons. Activity of SERT was assessed by determining the recovery time (in seconds) from the end of a 50-second ejection of 5-HT (20 nA) that fully inhibited the neuron, to 50% recovery of baseline firing and was expressed as RT_{50} (Piñeyro *et al.*, 1994).

To determine the degree of tonic activation of postsynaptic 5-HT_{1A} receptors, firing activity of CA3 pyramidal neurons was decreased to ~4 Hz and a physiological saline injection was administered (100 μL, i.v.) followed by four consecutive injections of the 5-HT_{1A} receptor antagonist WAY 100.635 (25 μg/kg at two-minute intervals). The average firing rate (spikes/s) of neurons during the second half of the 120 second period following each WAY 100.635 injection was expressed as percentage change from baseline. At the end of each experiment, 5-HT (20 nA) was ejected; reduced effectiveness of this ejection confirmed blockade of 5-HT_{1A} receptors by WAY 100.635.

In order to electrically stimulate 5-HT afferents while recording a CA3 pyramidal neuron, a bipolar electrode (NE-110, David Kopf, Tujanga, CA) was inserted at the following coordinates (in mm from lambda): AP +1.0, L0.0, D: 7.8-8.2. A stimulator

(S8800, Grass instruments, Quincey, MA) was used to deliver 200 square pulses (0.5 ms, 1 or 5 Hz) at 300 μA. Electrical stimulation of 5-HT afferents causes endogenous release of 5-HT, and briefly suppresses firing of CA3 pyramidal neurons by activating postsynaptic 5-HT_{1A} receptors, an effect independent of 5-HT reuptake inhibition (Chaput *et al.*, 1986). The inhibitory effect of 5-HT fiber stimulation on CA3 neurons was plotted in a peristimulus time histogram (PSTH) with 2 ms bin width. The effect of 200 stimulations was expressed as duration of silence (DOS, in ms), defined as the period from the first bin showing a 50% reduction in the number of events per bin from the prestimulus value and the first subsequent bin attaining a 90% recovery of the number of events per bin from prestimulus values (Chaput *et al.*, 1986). Previous *in vivo* and *in vitro* studies showed that increasing the stimulation from 1 to 5 Hz results in greater activation of terminal 5-HT_{1B} receptors and consequently, decreased 5-HT release. Therefore, a greater DOS following 1 Hz compared to 5 Hz stimulations is indicative for intact function of terminal 5-HT_{1B} autoreceptors (Chaput *et al.*, 1986; Blier *et al.*, 1989). In contrast to terminal 5-HT_{1B} receptors, the activity of α_2-adrenergic receptors is known to be frequency-independent (Blier *et al.*, 1989). A reduced DOS at 1 Hz is therefore indicative for enhanced activation of α_2-adrenergic receptors by its endogenous ligand (Mongeau *et al.*, 1993), while a normal or DOS in presence of enhanced NE neurotransmission demonstrates α_2-adrenergic heteroceptor blockade and/or desensitization (Chernoloz *et al.*, 2012).

Neuronal responsiveness to the iontophoretic application NE, an effect mediated by postsynaptic α_2-adrenergic receptors (Curet and de Montigny, 1988), was assessed by determining the total number of spikes suppressed from start of ejection to recovery to 80% of baseline firing rate. Activity of the NET was assessed by determining the recovery time (in seconds) to 50% of the maximal inhibitory effect following ejection of NE (10 nA), and was expressed at RT_{50} (de Montigny *et al.*, 1980).

To determine the degree of tonic activation of postsynaptic adrenoceptors, firing activity of hippocampal neurons was reduced to ~4 Hz. After obtaining a stable baseline firing rate, saline, the α_2-adrenoceptor antagonist idazoxan (1 mg/kg, i.v.), and the α_1-adrenoceptor antagonist prazosin (100 μg/kg) were administered. Percent change of the firing activity of CA3 pyramidal neurons was expressed relative to baseline activity, and

quantified during the last two minutes of a five minute period following administration of idazoxan or prazosin.

Data analysis / Statistics

Electrophysiological recordings were filtered from artifacts by wavemark analysis in Spike2 software version 6.17 (Cambridge Electronic Design, Cambridge, United Kingdom). Firing and burst analysis of DRN 5-HT, LC NE and VTA DA neurons was quantified using burstiDAtor software (Oosterhof and Oosterhof, 2013). The effect of acute brexpiprazole administration on the firing and burst activity of LC NE neurons was analyzed with one-way ANOVA. The effect of two- and 14-day brexpiprazole administration on the firing and burst activity of DRN 5-HT, LC NE and VTA DA neurons was analyzed using two-way ANOVA. The status of D_2, $5\text{-}HT_{1A,}$ $5\text{-}HT_{2A}$ and α_2-adrenergic receptor was assessed by determined using linear regression. When a plateau effect was obtained, data was plotted using non-linear regression; in this case, linear regression was performed on dose-response effects up to the ED_{100} in controls. Degree of tonic activation on α-adrenergic receptors was analyzed using two-way ANOVA. The effect of brexpiprazole on body and degree of activation on $5\text{-}HT_{1A}$ receptors was assessed using repeated measures ANOVA followed by Bonferroni post-hoc analysis. Effect of 5-HT afferent stimulation was analyzed with two-way ANOVA. All data were analyzed with Graphpad Prism (Version 5.01, Graphpad software, La Jolla, CA). Data are presented as mean, error bars represent S.E.M., and a p-value <0.05 was considered statistically significant.

Results

Results: Blood plasma levels

Brexpiprazole administration for two days produced in a blood plasma concentration of 62 ± 8 ng/mL, while its administration for 14 days resulted a concentration of 131 ± 11 ng/mL.

Results: Body weight

Administration of brexpiprazole for 14 days had no effect on body weight ($F_{13,43}$=1.8, p>0.05, figure 1)

Figure 1

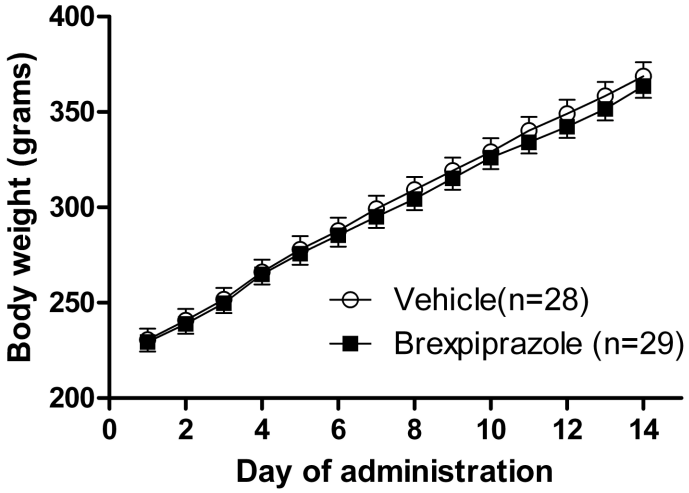

Figure 1: 14-day brexpiprazole administration had no significant effect on body weight. Data are presented as mean; error bars represent S.E.M.

Results: VTA DA neurons

Brexpiprazole administration for 2 and 14 days did not modify the firing activity of VTA DA neurons (figure 2A). In addition, no changes by brexpiprazole administration were observed on VTA DA neuron population or burst activity (table 1).

To assess the status of D_2 autoreceptors, the relationship between cumulative doses of the dopamine agonist apomorphine and firing rate of VTA DA neurons was determined. At baseline, the firing rate of these neurons did not differ between vehicle, two-day and 14-day brexpiprazole administered animals (4.3 ± 0.5, 4.3.0 ± 0.5, and 4.3 ± 0.5 Hz, respectively, $p>0.05$), and saline administration had no effect on their firing rate ($p>0.05$). In vehicle-administered animals, apomorphine (40 µg/kg, i.v.) completely inhibited the firing activity of all VTA DA neurons tested (figure 2E, for an illustrative firing histogram see figure 2B). Following two-day and 14-day brexpiprazole administration, 40 µg/kg of had a significantly smaller inhibitory effect in both 2- and 14-day brexpiprazole administered animals ($F_{2,15}=133$, $p<0.001$). Up to a dose of 80 µg/kg, apomorphine inhibited the firing of VTA DA neurons to approximately 70% of baseline firing rate, and not a single neuron was inhibited completely (figure 2E, for illustrative firing histograms see figure 2C and 2D). There was no statistical difference between the inhibitory effect of apomorphine after two- and 14-day brexpiprazole administration (linear regression, $F_{1,31}=0.2$, $p>0.05$).

	Bursts/min	% spikes in burst	Spikes/ burst	ISI (ms)	% neurons bursting	Neurons per tract
2-day vehicle	26 ± 3	31 ± 4	3.1 ± 0.2	70 ± 2	90	0.9 ± 0.2
2-day brexpiprazole	23 ± 3	30 ± 4	3.0 ± 0.1	67 ± 2	86	1.1 ± 0.4
14-day vehicle	28 ± 3	35 ± 4	3.2 ± 0.2	71 ± 2	80	1.0 ± 0.1
14-day brexpiprazole	25 ± 3	33 ± 4	3.0 ± 0.2	72 ± 2	92	1.0 ± 0.2

Table 1: Burst parameters of VTA DA neurons after 2- and 14-day brexpiprazole administration. No significant differences were detected.

Figure 2A

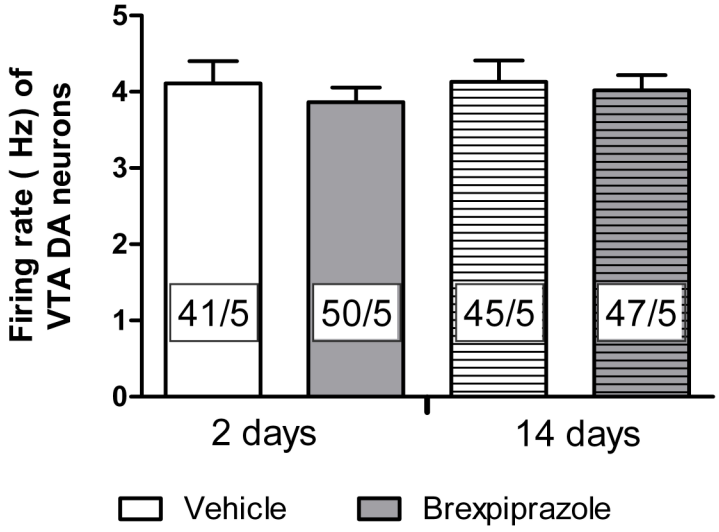

Figure 2B

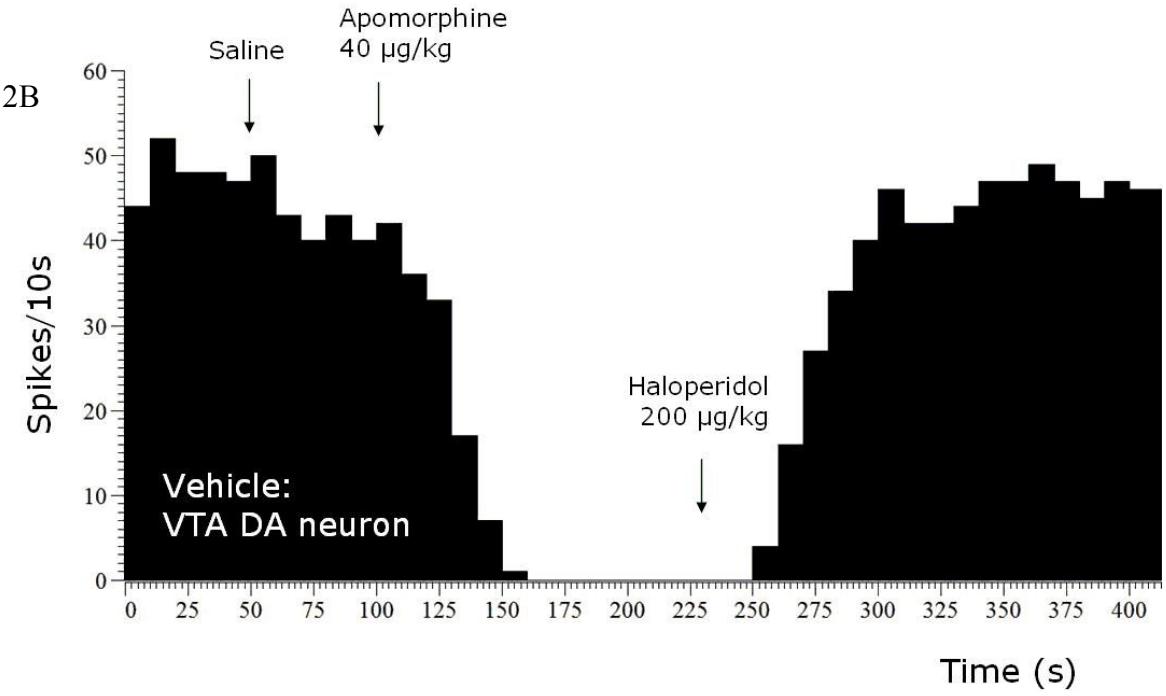

Figure 2C

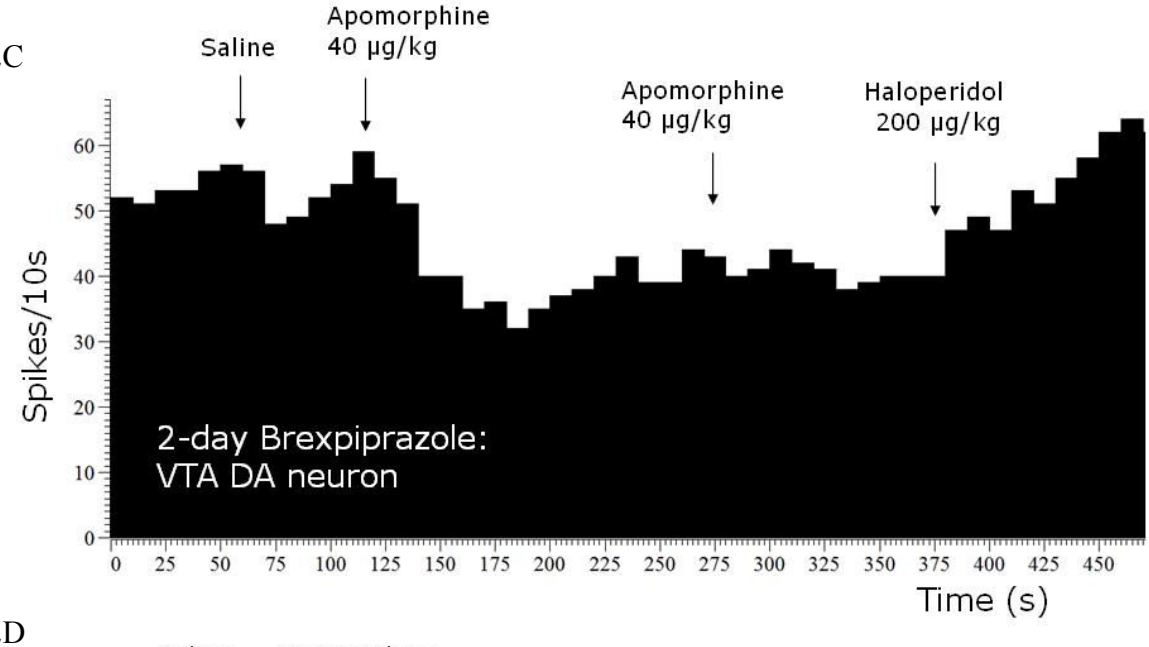

Figure 2D

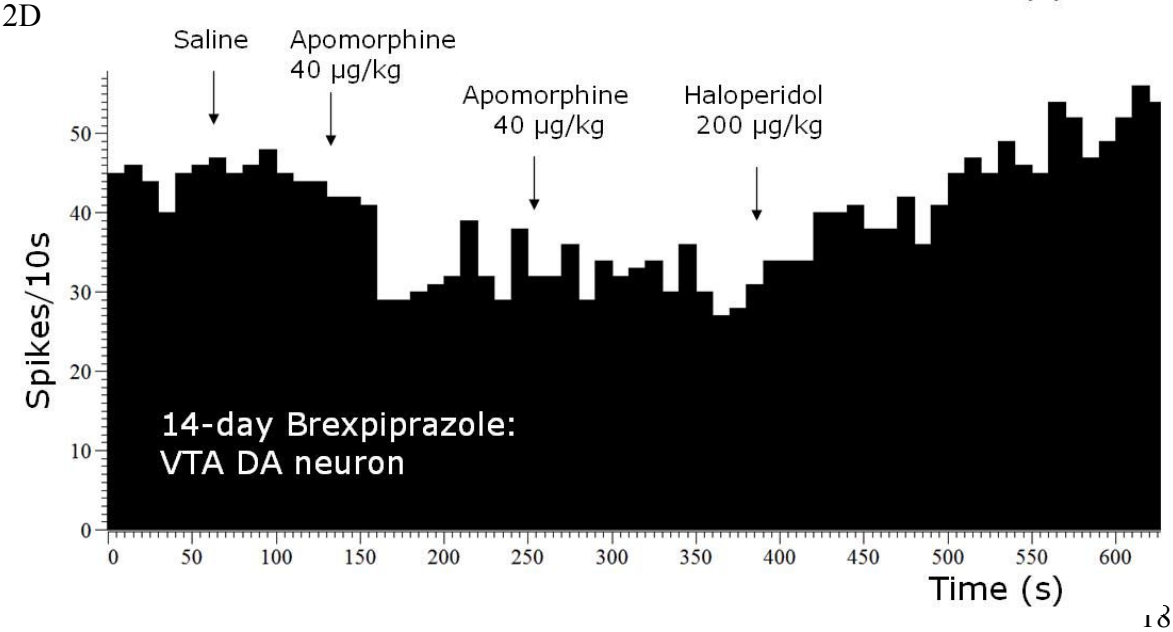

Figure 2E

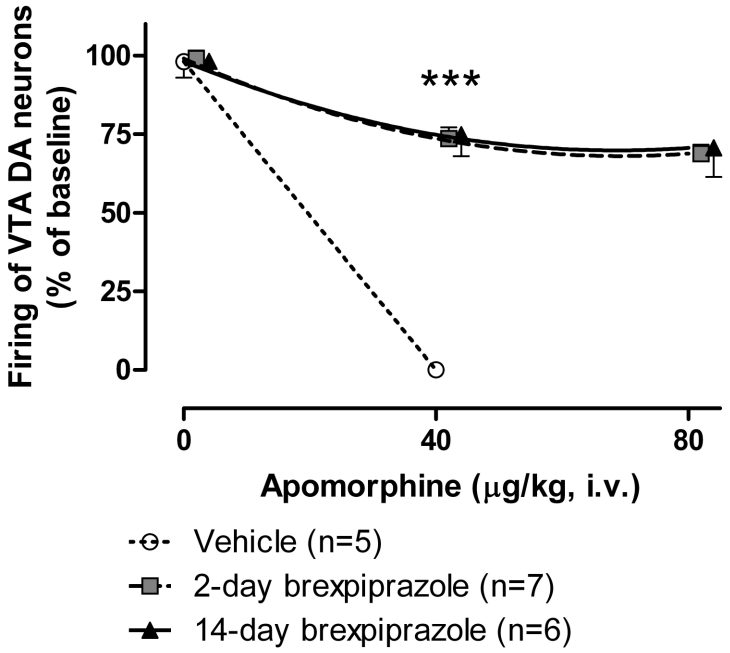

Figure 2: Effect of two and 14 day brexpiprazole administration on VTA DA neurons. A) Firing activity was unaltered by brexpiprazole administration. (B-D) illustrative firing histograms of the inhibitory effect of apomorphine in a vehicle (B), two-day brexpiprazole C) and 14-day brexpiprazole administered animal (D). E) Graphic presentation of the inhibitory effect of apomorphine in vehicle, two-day and 14-day brexpiprazole administered animals. Data were analyzed with two-way ANOVA (A) and linear regression (E); error bars represent S.E.M., numbers in histograms of (A) represent neurons recorded/animals used. In (E), data points were nudged to prevent overlap.
*** Significant effect of brexpiprazole administration; ***p<0.001**

Results: NE system

Acute brexpiprazole administration significantly increased the firing rate of LC NE neurons ($F_{1,96}$=9.9, p=0.002, figure 3A), while it did not alter burst activity of these neurons (p>0.05, data not shown). Brexpiprazole administration for two and 14 days significantly increased the firing activity of LC NE neurons ($F_{1,402}$=40.6, p<0.001, figure 3B). Furthermore, a significant interaction effect was found between brexpiprazole x duration of administration, showing increased firing activity after 14- compared to two-day brexpiprazole administration ($F_{1,402}$=8.2, p=0.004). Burst parameters of LC NE neurons were unchanged by brexpiprazole administration (p>0.05, data not shown).

To assess the status of 5-HT_{2A} receptors, the relationship between cumulative doses of the 5-HT_{2A} agonist DOI and firing rate of LC NE neurons was determined. At baseline, the firing rate of these neurons did not differ between vehicle, two-day and 14-day brexpiprazole administered animals (1.5 ± 0.2, 2.0 ± 0.9, and 2.2 ± 0.6 Hz, respectively, p>0.05). In vehicle-administered animals, DOI completely inhibited the firing activity of all LC NE neurons tested, with an ED_{100} estimated at 82 µg/kg (figure 3G, for an illustrative firing histogram see figure 3C). Following brexpiprazole administration, DOI up to a dose of 200 µg/kg inhibited the firing of LC NE neurons to approximately 50% of baseline firing rate, and complete inhibition was not observed in brexpiprazole-administered animals (figures 3D and 3E). Statistical analysis revealed a significantly smaller inhibitory effect of 50 µg/kg DOI in both brexpiprazole groups ($F_{2,16}$=130, p<0.001, figure 3G). Furthermore, the inhibitory effect of DOI reached a plateau in both two- and 14-day brexpiprazole groups after administration of 100 µg/kg (linear regression, test for slope ≠ 0, p>0.05 in both groups, figure 3G). After DOI administration, the α_2-adrenoceptor agonist clonidine (5 µg/kg, i.v.) inhibited all neurons tested (vehicle, n=3; two-day brexpiprazole, n=7; 14-day brexpiprazole, n=4; for illustrative firing histograms of the effects of clonidine see figures 3C-3E). To avoid possible drug-drug interactions, inhibitory effects of clonidine after DOI administration were not used for statistical assessment of the status of α_2-adrenergic autoreceptors, and serve for illustrative purposes only.

The status of α_2-adrenergic autoreceptors was based on the relationship between clonidine administration and firing activity of LC NE neurons. At baseline, there was no difference in firing activity of these neurons in vehicle, two- and 14-day brexpiprazole administered animals (1.7 ± 0.3, 1.7 ± 0.2, and 1.8 ± 0.1 Hz, respectively, $p>0.05$). Clonidine administration (5 µg/kg, i.v.) completely inhibited the firing activity of all neurons tested ($n\geq3$ per group, data not shown). Accordingly, brexpiprazole (500 µg/kg, i.v.) did not reverse the inhibitory effect of clonidine in two vehicle-administered animals (for an illustration of this effect see figure 3F).

In the hippocampus, the inhibitory effect of NE ejection did not differ between vehicle and 14-day brexpiprazole administered groups (39 ± 3 vs. 34 ± 3 spikes inhibited/nA, data from 30 and 23 neurons in 14 and 13 animals, respectively, $p>0.05$). Also, the activity of the NET was unaltered following this drug regimen (RT_{50}= 77 ± 7 vs. 73 ± 5 seconds, data from 23 and 23 neurons in 14 and 13 animals, respectively, $p>0.05$).

Prior to assessment of degree of α-adrenergic receptor activation, there was no difference in firing activity of CA3 pyramidal neurons between vehicle and 14-day brexpiprazole administered animals (3.5 ± 0.3 vs. 3.2 ± 0.3 Hz, respectively, t-test, $p>0.05$). Administration of saline, and blockade of α_1-adrenergic receptors with prazosin, did not significantly alter the firing activity of CA3 pyramidal neurons ($F_{1,10}$=1.1 and $F_{1,10}$=3.6, respectively, $p>0.05$). Blockade of α_2-adrenergic receptors with idazoxan had a significant disinhibitory effect on CA3 pyramidal neurons in 14-day brexpiprazole administered animals ($F_{1,10}$=10.3, $p=0.009$, figure 4C; for firing histograms illustrating these effects see figures 4A and 4B).

Figure 3A

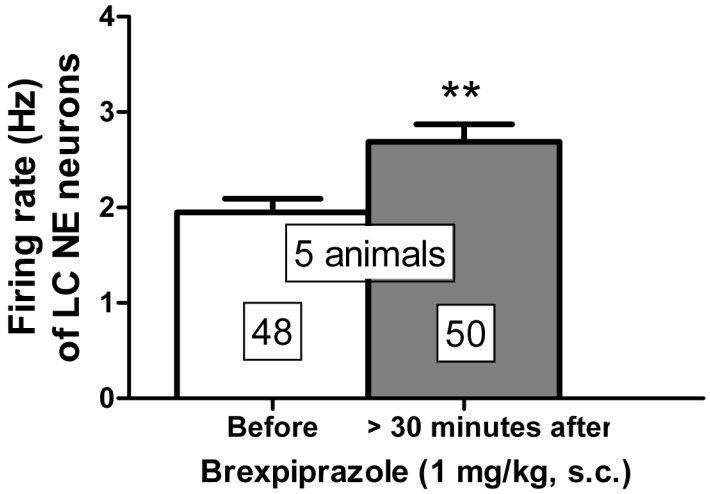

Figure 3B

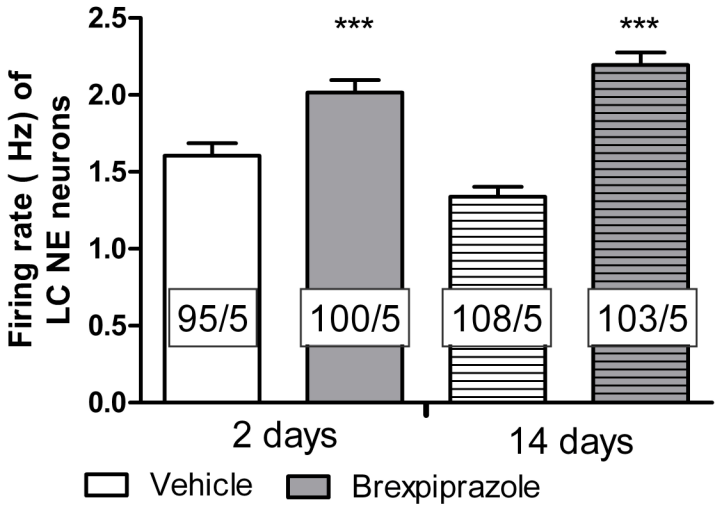

Figure 3C

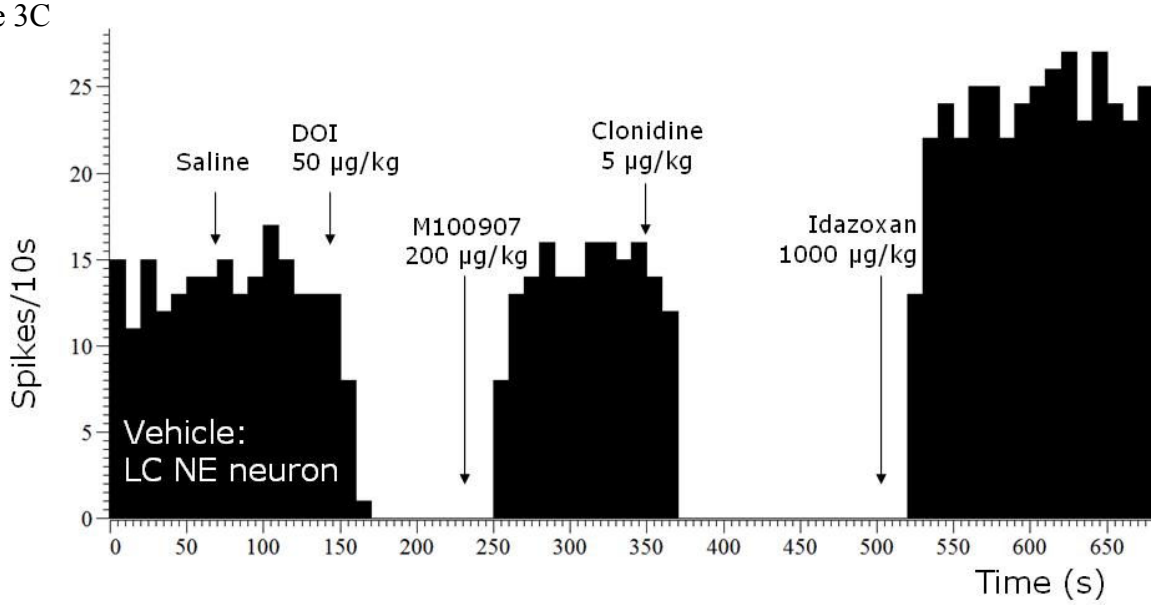

Figure 3D

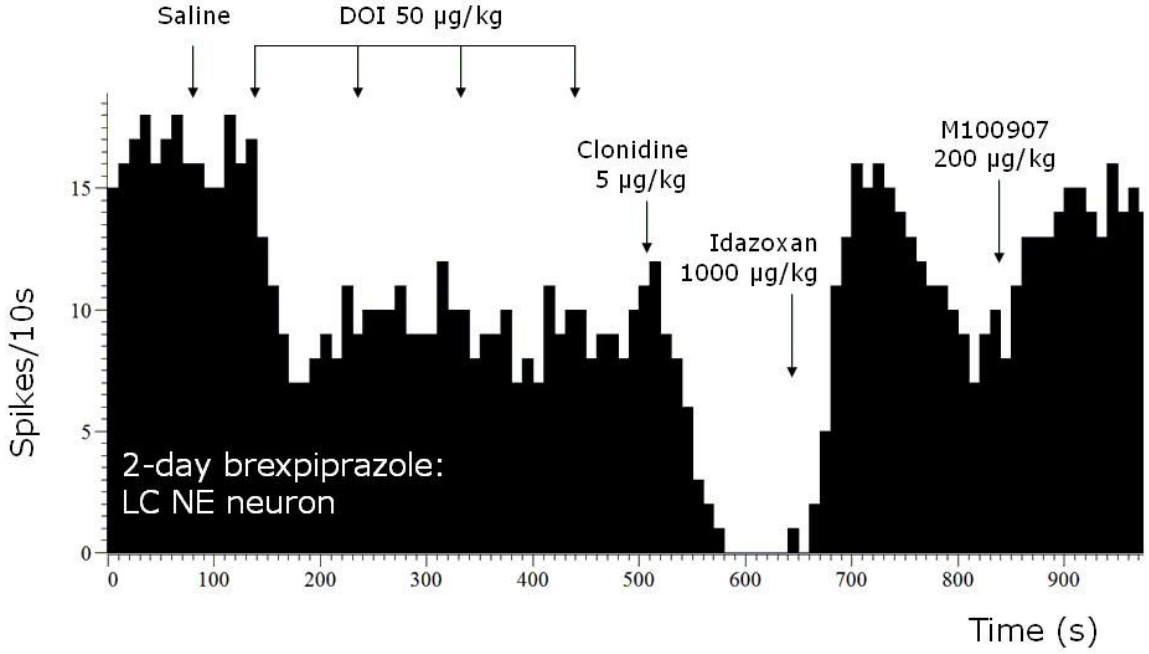

Figure 3E

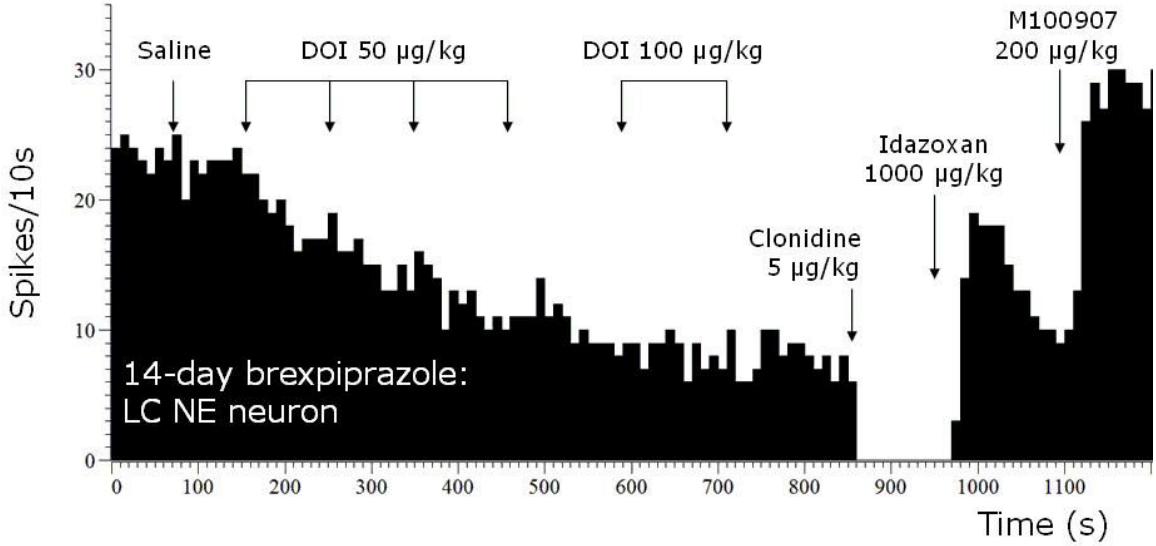

Figure 3F

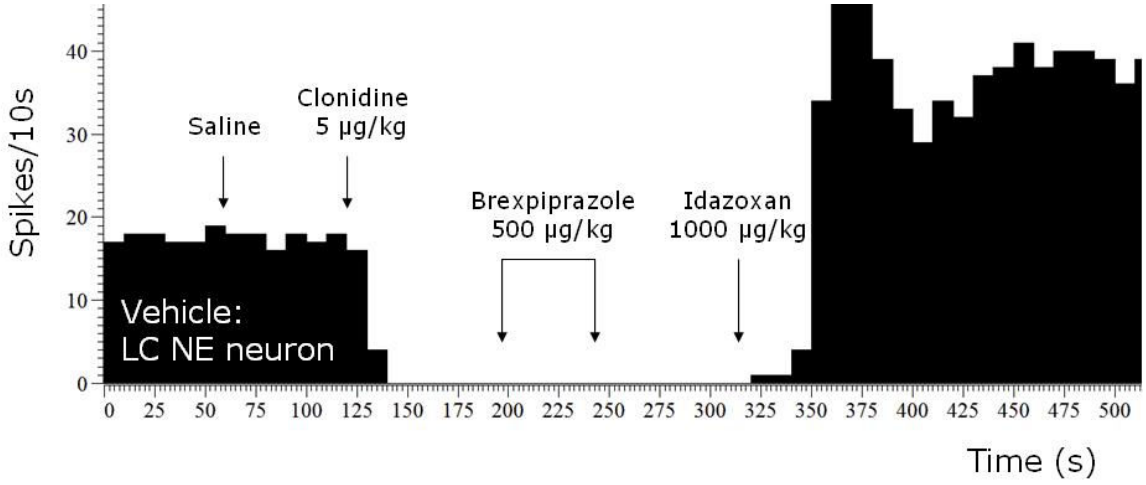

Figure 3G

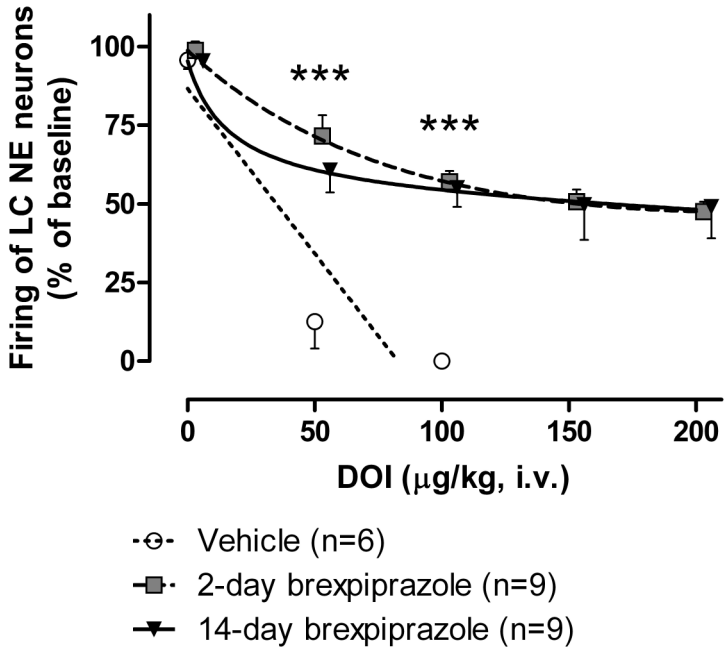

Figure 3: Effect of acute, 2 and 14 day brexpiprazole administration on LC NE neurons and status of 5-HT_{2A} receptors. (B) Firing activity after 2 and 14 days of brexpiprazole administration. (C-E) illustrative firing histograms of the inhibitory effect of the 5-HT_{2A} receptor agonist DOI after vehicle (C), two-day brexpiprazole (D) and 14-day brexpiprazole administration (E). Note the unaltered response to acute administration of the α_2-adrenoceptor agonist clonidine after brexpiprazole administration. (F) Firing histogram illustrating no reversal by brexpiprazole of neural inhibition by clonidine in a vehicle-administered animal. (G) Graphic presentation of the inhibitory effect of DOI in control, 2-day and 14-day brexpiprazole administration. Data were analyzed with one-way (A) or two-way ANOVA (B); error bars represent S.E.M; numbers in histograms of (A) and (B) represent neurons recorded/animals used. *Significant effect of brexpiprazole administration, ***p<0.001

Figure 4A

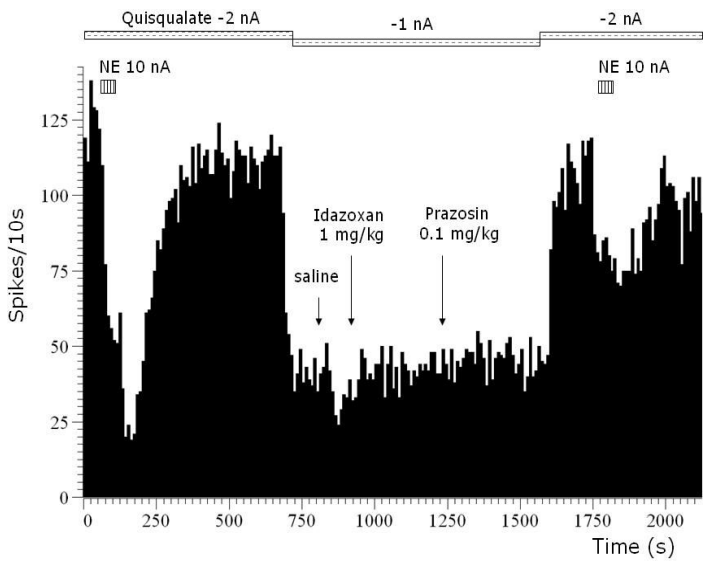

Figure 4B

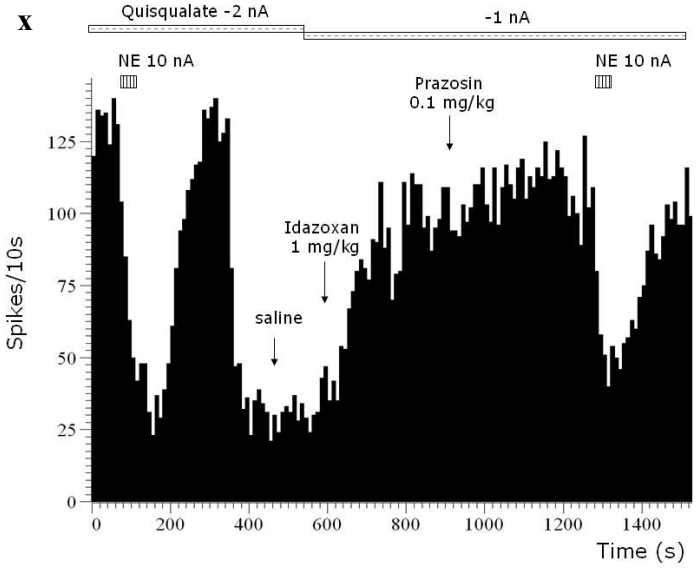

Figure 4C

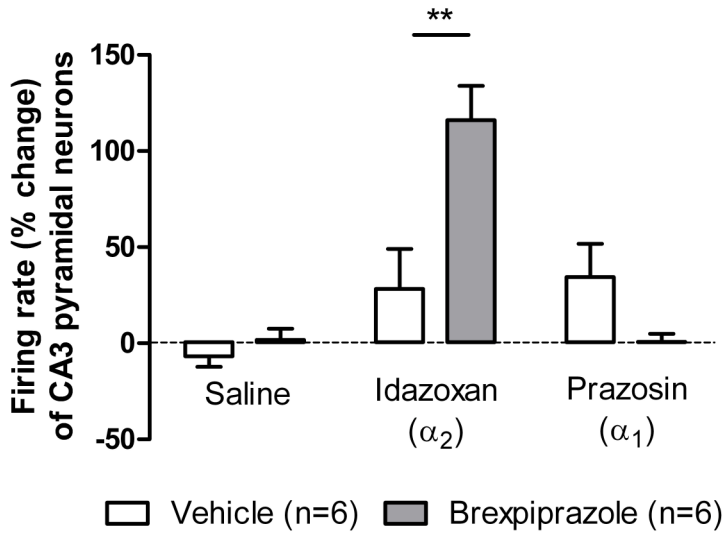

Figure 4: Effect of 14-day brexpiprazole administration on the status of α-adrenoceptors in the hippocampus CA3 region. (A, B) Firing histograms illustrating the effect of acute administration of the α_2-adrenoceptor antagonist idazoxan and the α_1-adrenoceptor antagonist prazosin administration on the firing activity of a CA3 pyramidal neuron in a 14-day vehicle (A) and 14-day brexpiprazole administered animal (B). C) Effect of the α_2-adrenoceptor antagonist idazoxan (1 mg/kg, i.v.) and prazosin (0.1 mg/kg, i.v.) on firing rate of CA3 pyramidal neurons after 14-day vehicle or brexpiprazole administration. Data were analyzed with two-way ANOVA; error bars represent S.E.M.

***Significant effect of brexpiprazole administration; **p<0.01**

Results: 5-HT system

Brexpiprazole administration for two, but not 14 days increased the firing activity of 5-HT neurons in the DRN ($F_{1,346}$=9.8, p=0.002, figure 5A). No effect of brexpiprazole was detected on burst parameters (p>0.05, data not shown). At baseline, the firing rate of 5-HT neurons used for determining 5-HT_{1A} autoreceptor sensitivity did not differ between vehicle, two-day and 14-day brexpiprazole administered animals (1.2 ± 0.3, 1.4 ± 0.4, and 1.2 ± 0.3 Hz, respectively). Administration of saline did not alter firing activity relative to baseline (p>0.05), and responsiveness to flesinoxan was unaltered after two- and 14-day brexpiprazole administration ($F_{2,16}$=0.18, p>0.05, figure 5B).

In hippocampus, baseline firing activity of CA3 pyramidal neurons before assessment of degree of tonic 5-HT_{1A} receptor activation did not differ between 14-day vehicle, acute, two-day brexpiprazole + 24 hour washout, two-day brexpiprazole, and 14-day brexpiprazole administered animals (3.6 ± 0.3, 3.6 ± 1.2, 4.0 ± 0.5, 3.9 ± 0.2 and 3.3 ± 0.2 Hz, respectively, $F_{2,15}$=1.4, p>0.05). Blockade of 5-HT_{1A} receptors by WAY 100.635 at a dose of 25 µg/kg had a significant overall disinhibiting effect only in 14-day brexpiprazole administered animals (RM ANOVA with Bonferroni post-hoc, p<0.05, figure 6D). At doses of 50, 75 and 100 µg/kg, WAY 100.635 at caused a significant disinhibition in two- and 14-day brexpiprazole administered animals (RM ANOVA with Bonferroni post-hoc, p<0.001 for 50, 75 and 100 µg/kg, figure 6D; for illustrative firing histograms of a neuron in a vehicle, 2- and 14-day brexpiprazole administered animal see figure 6A-6C, respectively). No significant disinhibition was produced by acute brexpiprazole administration, or by two-day brexpiprazole administration following a 24 hour washout period.

The inhibitory effect of 5-HT ejection on hippocampal neurons did not differ between vehicle and 14-day brexpiprazole administered animals (189 ± 11 vs. 181 ± 14 spikes inhibited/nA, data from 24 and 19 neurons in groups of 8 and 7 animals, respectively, p>0.05). Similarly, the activity of the SERT was unaltered in vehicle and brexpiprazole-administered animals (RT_{50}= 33 ± 3 vs. 38 ± 3 seconds, data from 24 and 19 neurons in 8 and 7 animals respectively, p>0.05). Electrical stimulation of 5-HT afferents at 5 Hz resulted in a significant decrease in DOS compared to 1 Hz in both

groups ($F_{1,44}$=62.0, $p<0.001$, figure 6E). Both at 1 and 5 Hz stimulations, there was no difference in DOS after 14 day vehicle and brexpiprazole administration ($p>0.05$).

Figure 5A

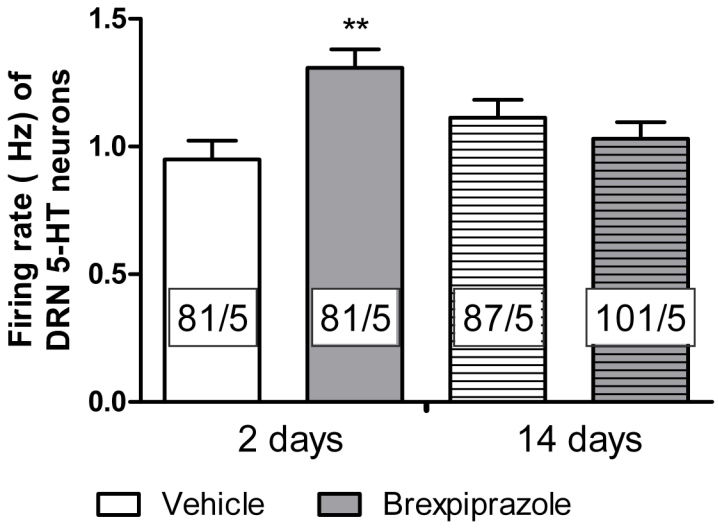

Figure 5B

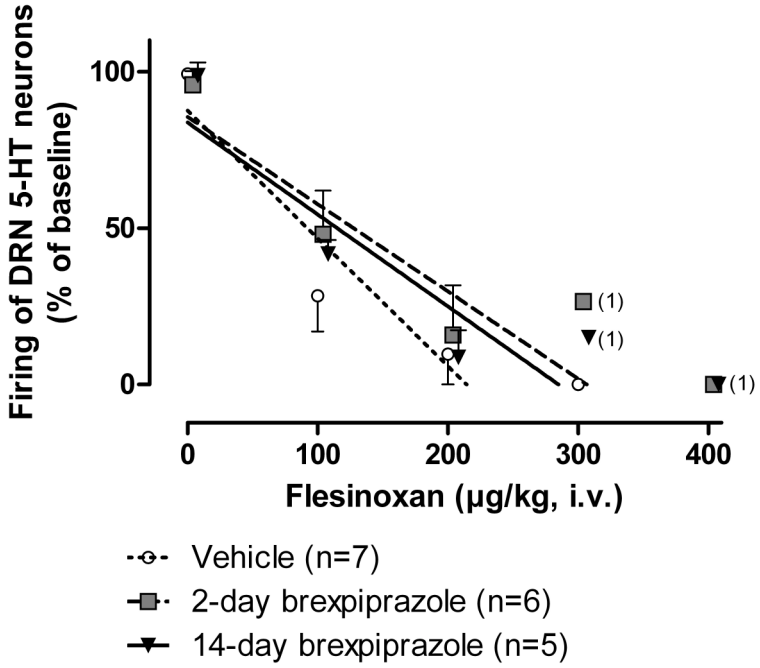

Figure 5: Effect of two- and 14 day brexpiprazole administration on DRN 5-HT neurons firing activity and autoreceptor status. A) Firing activity was increased after two, but not 14 days of brexpiprazole administration. B) The dose-response relationship between DRN 5-HT neuron firing activity and administrations of flesinoxan in vehicle, two- and 14-day brexpiprazole administered animals. Data were analyzed with two-way ANOVA (A) or linear regression (B), are presented as mean, error bars represent S.E.M, numbers in histograms represent neurons recorded/animals used in (A) or neurons per group in (B). In (B), data points were nudged to prevent overlap.
***Significant effect of brexpiprazole administration, **p<0.01**

Figure 6A

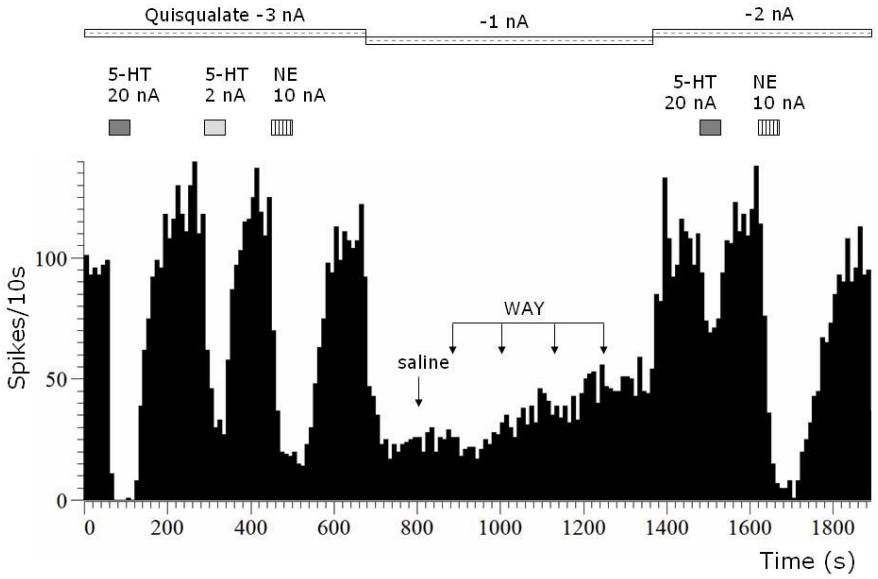

Figure 6B

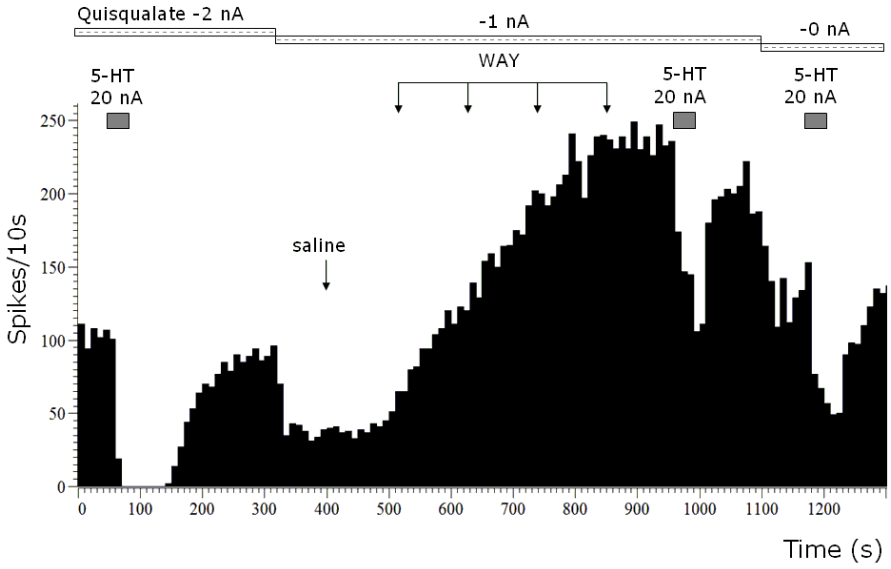

Figure 6C

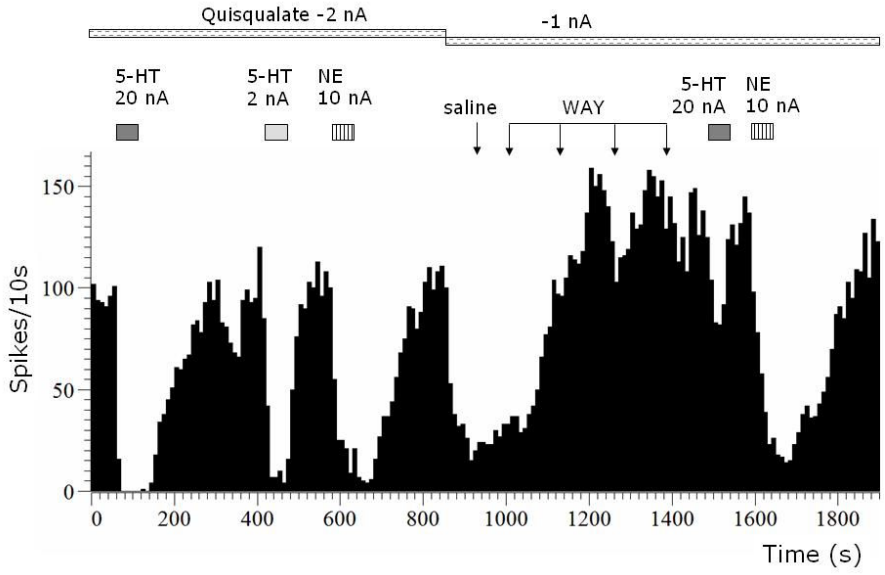

Figure 6D

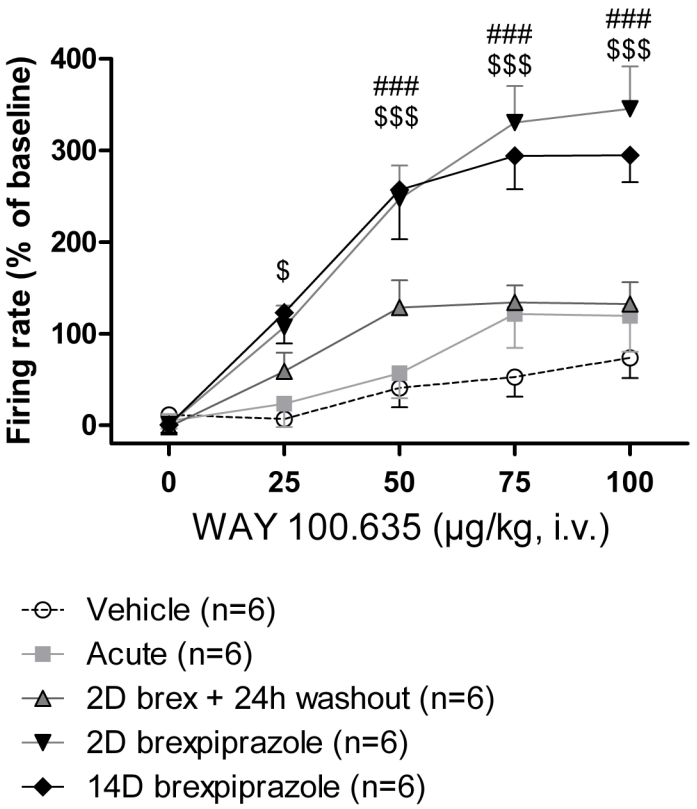

Figure 6E

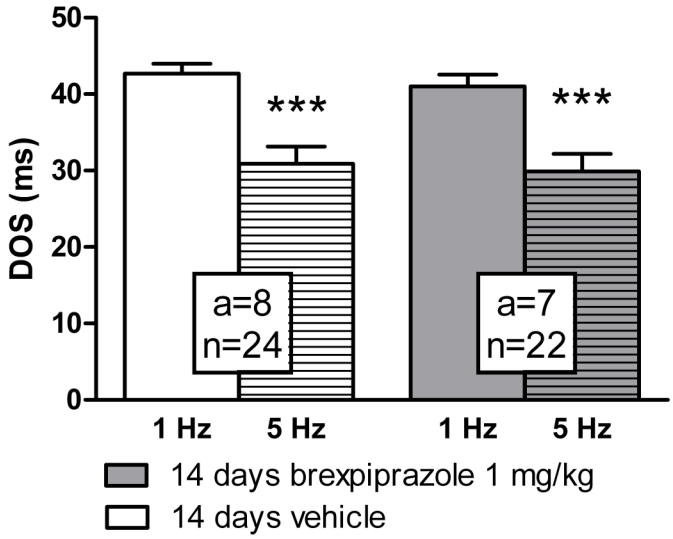

Figure 6: Effect of 14-day brexpiprazole administration on the status of 5-HT$_1$ receptors in the CA3 region of the hippocampus. A-C) Illustrative firing histograms on the effect of cumulative WAY 100.635 (WAY) administrations on the firing activity of a CA3 pyramidal neuron in a 14-day vehicle (A), a two-day brexpiprazole (B) and 14-day brexpiprazole administered animal (C). D) Quantification of the effect of WAY 100.635 on basal firing rate in rats administered with vehicle or brexpiprazole days. E) Comparison of DOS on CA3 pyramidal neurons produced by 5-HT fiber bundle stimulation in vehicle and 14-day brexpiprazole administered animals. Data were analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis (D), or a two-way ANOVA (D). The number of animals (a) and neurons (n) is provided within the histograms (D); error bars represent S.E.M.
#significant effect of 2-day brexpiprazole administration compared to controls; #p<0.05, ###p<0.001
$significant effect of 14-day brexpiprazole administration compared to controls; $p<0.05, $$$p<0.001
***significant effect of stimulation frequency; ***p<0.001**

Discussion

After two and 14 days of brexpiprazole administration, blood plasma levels were in the clinical range observed in patients taking 1-4 mg/day. Blood levels of brexpiprazole in rats still doubled after 14 day administration compared to two-day administration, demonstrating accumulation of this agent. Because the half-life of brexpiprazole in humans is of approximately 65 hours, it requires two weeks to reach steady state in patients. Thus, the present results might be directly relevant to the clinical effects of brexpiprazole.

VTA DA neurons

Brexpiprazole administration for two and 14 days did not alter the firing, bursting, and population activity of VTA DA neurons (figure 2A, table 1), similarly to the effects of subacute and sustained aripiprazole administration (Chernoloz *et al.*, 2009). Interestingly, administration of the dopamine agonist apomorphine (40 µg/kg, i.v.; corresponding to the ED_{100} in controls) reduced the firing activity of VTA DA neurons in two- and 14-day brexpiprazole administered animals but only to ~70% of baseline activity, and a second injection of apomorphine had no further effect (figures 2C-2E). These effects are similar to acute intravenous brexpiprazole administration, which did not alter the firing and burst activity of VTA DA up to a dose of 800 µg/kg while it reversed the inhibitory effect of apomorphine to ~70% of baseline firing at a fraction of that dose (ED_{50}=61 µg/kg; Oosterhof *et al.*, 2014).

These data support and extend insight in different dynamics of agents with antagonistic vs. partial agonistic action on D_2 receptors on the activity of VTA DA neurons. Acutely, D_2 receptor antagonists robustly increase the firing activity of VTA DA neurons by blocking the D_2 receptor-mediated autoinhibitory signal of dopamine (Chiodo and Bunney, 1983; Ghanbari *et al.*, 2009). Depending on their degree of intrinsic activity, partial D_2 receptor agonists acutely either decrease (e.g. aripiprazole, bifeprunox) or do not alter (brexpiprazole) the firing activity of DA neurons (Dahan *et al.*, 2009; Oosterhof *et al.*, 2014). Chronic administration of D_2 receptor antagonists causes D_2 autoreceptors to become more sensitive, and dose-dependently alter the population activity of VTA DA neurons (Vogelsang and Piercey, 1985; Skarsfeldt, 1995). Of particular interest,

asenapine partially blocked the inhibitory effect of apomorphine on DA neurons after two days of administration – similarly to the effect of two-day brexpiprazole administration. However, after 21 days of asenapine administration, apomorphine fully inhibited VTA DA neurons, indicating D_2 receptor sensitization (Oosterhof *et al.*, 2015). In contrast, the response of VTA DA neurons to apomorphine was indistinguishably dampened following two- and 14-day brexpiprazole administration, demonstrating unaltered D_2 receptor sensitivity following these drug regimens (figures 2C-2E).

NE system

Acute administration of brexpiprazole increased the firing activity of LC NE neurons (figure 3A). This firing activity remained elevated after sustained administration (figure 3B), similarly to of the effect of sustained asenapine, clozapine, quetiapine and olanzapine administration (Ramirez and Wang, 1986; Chernoloz *et al.*, 2012; Oosterhof *et al.*, 2015). Interestingly, two- and 14-day aripiprazole administration did not alter the firing activity of LC neurons (Chernoloz *et al.*, 2009), demonstrating distinct effects of these piperazines on the NE system. Brexpiprazole had no detectable action on α_2-adrenergic autoreceptors: clonidine (5 µg/kg, i.v.) indistinguishably inhibited LC NE neurons in vehicle, two- and 14-day brexpiprazole administered animals (see figures 3C-3F for illustrations). In addition, brexpiprazole did not reverse the inhibitory effect of the α_2-adrenergic agonist clonidine on NE neurons in vehicle-administered animals (figure 3F), suggesting little antagonistic action on α_2-adrenergic autoreceptors.

Activation of 5-HT_{2A} receptors located on terminals arising from the hypoglossal nucleus stimulates GABA release on NE neurons, providing a pharmacological node by which the 5-HT system modulates the activity of LC NE neurons (Szabo and Blier, 2001). Indeed, acute administration of the 5-HT_{2A} receptor agonist DOI inhibited all LC NE neurons in control animals (ED_{100}=83 µg/kg), an effect fully reversed by the selective 5-HT_{2A} receptor antagonist M100907 (figures 3C & 3G). In accordance with its acute behavioral and *in vivo* electrophysiological effects (Maeda, Sugino, *et al.*, 2014; Oosterhof *et al.*, 2014), brexpiprazole administration for two and 14 days potently reduced the inhibitory effect of DOI, thus demonstrating antagonistic action on 5-HT_{2A} receptors (figures 3D, 3E, 3G). Using the same methodology, antagonistic effects on 5-

HT_{2A} receptors were previously demonstrated following sustained asenapine and quetiapine + norquetiapine administration (Chernoloz *et al.*, 2012; Oosterhof *et al.*, 2015). Notably, the in vitro affinity for 5-HT_{2A} receptor of asenapine (Ki=0.06 nM; Shahid *et al.*, 2009)), brexpiprazole (Ki=0.47 nM; Maeda, Sugino, *et al.*, 2014)) and quetiapine + norquetiapine (quetiapine Ki=101 nM [Kroeze *et al.*, 2003]; norquetiapine Ki=58 nM [Jensen *et al.*, 2008]) is reflected in their respective complete, partial (figure 3G), and slight blockade of DOI in vivo (Chernoloz *et al.*, 2012; Oosterhof *et al.*, 2015).

Previous studies showed that sustained antidepressant administration increases 5-HT neurotransmission, causing tonic activation of 5-HT_{2A} receptors and consequently, decreased NE firing. Under these conditions, 5-HT_{2A} receptor blockade normalizes NE firing (Dremencov *et al.*, 2007a; b; Chernoloz *et al.*, 2009, 2012), providing a rationale for the augmenting effects of co-administrating low-dose atypical antipsychotics an antidepressant regimen (Blier and Szabo, 2005). In accordance, the present demonstration of 5-HT_{2A} receptor blockade provides one mechanism for the clinical effectiveness of brexpiprazole when administered in adjunct to antidepressants (Thase *et al.*, 2014).

In the hippocampus, brexpiprazole increased the tonic activation of α_2- but not α_1-adrenergic receptors on CA3 pyramidal neurons (figure 4C). Since the activity of the NET, and the sensitivity of postsynaptic α_2-adrenergic receptors remained unaltered in this brain region, increased hippocampal NE transmission was likely due to the excitatory effect of brexpiprazole on LC NE neurons - an effect previously observed following sustained trazodone and quetiapine administration (Chernoloz *et al.*, 2012; Ghanbari *et al.*, 2012). In further resemblance to these agents, brexpiprazole has antagonistic action on α_1-adrenergic receptors *in vivo* (Oosterhof *et al.*, 2014), providing a mechanism by which it prevented tonic activation of these receptors. Importantly, brexpiprazole did not increase hippocampal or cortical NE levels following its acute administration (Maeda, Sugino, *et al.*, 2014), in contrast to other atypical antipsychotics (Westerink *et al.*, 1998), suggesting that its enhancing effect on NE neurotransmission requires delayed neural adaptations.

5-HT system

Brexpiprazole administration for two days significantly increased the firing activity of DRN 5-HT neurons (figure 5A), similarly to the effect of two-day aripiprazole administration (Chernoloz *et al.*, 2009). However, the firing activity of 5-HT neurons returned to control levels after 14-day brexpiprazole administration, while this remained elevated after 14-day aripiprazole administration (Chernoloz *et al.*, 2009). In further contrast to aripiprazole, two- and 14-day brexpiprazole administration did not alter the responsiveness of 5-HT neurons to acute 5-HT_{1A} receptor agonist administration, demonstrating that 5-HT_{1A} autoreceptor desensitization did not occur (figure 5B). This finding might be surprising, especially since brexpiprazole was previously shown to act as a full 5-HT_{1A} receptor agonist in the hippocampus (Oosterhof *et al.*, 2014). Indeed, the full 5-HT_{1A} receptor agents BAY x 3072 and trazodone desensitized 5-HT_{1A} autoreceptors after long-term administration (Dong *et al.*, 1998; Ghanbari *et al.*, 2010). On the other hand, these agents decreased the firing activity of 5-HT neurons following two-day administration, in contrast to the effect of brexpiprazole (figure 5A). The DRN receives excitatory NE projections from the LC (Svensson *et al.*, 1975), while excitatory D_2 receptors are located on DRN 5-HT neurons (Haj-Dahmane, 2001). Possibly, a combination of these excitatory inputs prevented DRN 5-HT neurons to decrease their firing. Indeed, the excitatory effect of aripiprazole on DRN 5-HT neurons was to a significant degree mediated by partial D_2 receptor agonism (Chernoloz *et al.*, 2009). In addition, since brexpiprazole plasma concentrations were significantly higher after 14 compared to two days, it is conceivable that this caused a greater activation of 5-HT_{1A} autoreceptors, preventing firing of DRN 5-HT neurons to remain elevated after the longer regimen.

In the hippocampus CA3 region, brexpiprazole increased the tonic activation of 5-HT_{1A} receptors on pyramidal neurons after 14 days of administration (figure 6D), an effect common to all antidepressant agents tested in this paradigm (Blier and El Mansari, 2013). Since brexpiprazole did not alter the firing activity of 5-HT neurons, activity of the SERT, and status of 5-HT_{1B} terminal autoreceptors, it was deemed critical to assess whether this enhanced tonic activation was due to adaptive changes, or attributable to the full agonistic action of brexpiprazole on postsynaptic 5-HT_{1A} receptors (Oosterhof *et al.*, 2014). Interestingly, two-day brexpiprazole administration caused a comparable tonic

activation on 5-HT_{1A} receptors as the 14-day regimen. In contrast, two-day bupropion, mirtazapine, paroxetine administration all produced a lesser degree of postsynaptic 5-HT_{1A} receptor activation presumably due to adaptive changes following sustained administration (Besson *et al.*, 2000; Ghanbari *et al.*, 2011). Importantly however, acute brexpiprazole or the two-day regimen followed by 24 hour washout did not lead to enhanced 5-HT_{1A} receptor activation. Possibly, acute administration resulted in lower brexpiprazole levels and less tonic activation of 5-HT_{1A} receptors compared to the two- and 14-day regimens, where brexpiprazole accumulated with repeated injections. Alternatively, it is possible that adaptive changes occurred in the presence of brexpiprazole and contributed to the enhanced tonic activation of postsynaptic 5-HT_{1A} receptors.

Conclusion

Sustained administration of brexpiprazole had profound effects on the activity of the DA, NE and 5-HT monoamine systems, both at the cell body level an in projection areas. Brexpiprazole produced a significant occupation of D_2 autoreceptors while the firing activity of VTA DA neurons remained unchanged. These results support a stabilizing effect of partial D_2 receptor agonism on DA neurotransmission by brexpiprazole that could be important in the treatment of schizophrenia and mood disorders (Kane *et al.*, 2015). Sustained brexpiprazole administration caused a blockade of two receptor populations mediating inhibitory crosstalk between the 5-HT and NE systems. First, it blocked 5-HT_{2A} receptors, a pharmacological target important in the lower incidence of motor side-effects produced by second-generation antipsychotics in the treatment of schizophrenia (Tarazi and Stahl, 2012) and in adjunct to serotonin reuptake inhibitors in the treatment of depression (Blier and Szabo, 2005). Secondly, it blocked α_2-adrenergic heteroceptors on 5-HT terminals, thereby normalizing 5-HT release despite enhanced NE neurotransmission. Finally, accumulation of brexpiprazole and/or adaptive changes produced enhanced tonic activation of postsynaptic 5-HT_{1A} receptors, a common effect of all agents with antidepressant action (Blier and El Mansari, 2013). Together, these findings provide further insight into the effects of brexpiprazole on monoamine systems with relevance to therapeutic domains.

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Chapter IV: Prenatal stress

Because assessing the effects of antidepressant-like challenges on monoamine system activities in naïve animals might have limited face validity, the present study aimed to characterize whether these activities were altered in depressive-like animals. Accordingly, a study was designed to assess the effects of prenatal stress (PNS), a commonly used an animal model for depression, on monoamine systems. To mimic a major stressful event, which is a risk factor for depression in humans, animals were exposed to the forced swim stress in adulthood. This experimental design concomitantly allowed assessment of depressive-like behavior, which was previously shown to be induced by PNS. Ultimately, this study aimed to determine if monoamine systems in depressive-like animals respond differently to antidepressant administration and if so, to characterize the underlying neural mechanisms.

The experimental design of this study was drafted by Dr. Blier, Dr. El Mansari, and the present author. All experiments and their analysis were conducted by the present author. All authors contributed to and approved of the manuscript. Preliminary data of this study were presented at the annual meeting of the Society of Biological Psychiatry in May 2012 and the Canadian Depression Research Intervention Network in February 2015. The manuscript is in its final stage for submission to the Journal of Brain Research.

Altered monoamine system activities after prenatal and adult stress: a role for stress resilience?

Running title: PNS and monoamine systems

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Abstract

Introduction: Prenatal stress (PNS) and stress in adulthood are risk factors for development of major depressive disorder. The present study aimed to 1) confirm previous neuroendocrine and behavioral changes induced by PNS, and 2) to characterize the effect of early- and late life stress on the *in vivo* activity of monoamine systems.

Methods: Gestational dams were restrained thrice daily under bright illumination from gestational day (GD)11-20. On the first and last day of restraint, the corticosterone (CORT) response to restraint stress was assessed. Birth weight of offspring was determined on postnatal day 1. In adulthood (postnatal day 60-90), behavioral and CORT responses in the forced swim test (FST) were assessed. Dorsal raphe nucleus serotonin, ventral tegmental area dopamine and locus coeruleus norepinephrine neurons were recorded at baseline and 24 hours after the FST, using established electrophysiological methods.

Results: Gestational dams did not habituate to chronic restraint stress, and PNS reduced the birth weight of offspring. In adulthood, swim stress elevated CORT levels longer in PNS animals, while it had no effect on swim behaviors. Firing activity of serotonin neurons was decreased in PNS animals, while PNS and swim stress had interdependent effects on the firing activity of norepinephrine and dopamine neurons.

Conclusion: The present data confirm previously established effects on neuroendocrine and physiological measures, and demonstrate an altering effect of PNS and stress on monoamine system activities in adulthood. Since PNS did not result in a depressive-like phenotype, these central changes following PNS might play reflect adaptive changes contributing to stress resilience in adulthood.

Introduction

A growing body of evidence indicates a close relationship between excessive allostatic load (stress) and development of major depressive disorder (MDD). For example, stressful life events are one of the most important triggers to a depressive episode (Kendler *et al.*, 1999). Conversely, hyperactivity and –reactivity of the hypothamalo-pituitary-adrenal (HPA) axis has been proposed as a biomarker for MDD, illustrating high prevalence in MDD (Heuser *et al.*, 1994; Stetler and Miller, 2011; Lok *et al.*, 2012). While important in its development, the role of HPA axis activity in recovery from MDD is more elusive. For example, pharmacological reduction of HPA axis activity has produced mixed results (for review see Kling, Coleman, and Schulkin 2009), non-pharmacological reduction of HPA axis activity is not necessarily followed by clinical improvement, and clinical improvement can occur in absence of HPA axis normalization (Zobel *et al.*, 1999, 2001; Schüle, 2007). Furthermore, antidepressant medications, which clinical efficacy is through to be primarily attributable to normalization of brain monoamine systems, does not necessarily normalize HPA axis activity under basal (Lok *et al.*, 2012; Keating *et al.*, 2013) or activated conditions (Zobel *et al.*, 2001; Sarubin *et al.*, 2014). Clearly, the interaction between the neuroendocrine HPA axis, monoamine systems and depressive symptoms requires further exploration.

Human and animals studies suggests that early life stress can predispose to both HPA axis hyperreactivity and development of psychopathologies in adulthood (Maccari *et al.*, 1995; Brown *et al.*, 2000; Rice *et al.*, 2007; Entringer *et al.*, 2009; Heim *et al.*, 2010). Maternal restraint stress, first described by Ward & Weisz in 1984, is amongst the

most commonly used animal models to study the effects of prenatal stress (PNS), and has been shown to predispose to impairments in behaviors related to learning and memory (Lemaire *et al.*, 2000), anxiety (Van den Hove *et al.*, 2014), and depression (Maccari *et al.*, 1995; Sun *et al.*, 2013). Especially, exposure to high corticosterone (CORT) levels during the last week of gestation appears to be a critical window to induce long-lasting behavioral impairments, perhaps because this period encompasses development of the fetal HPA axis. Indeed, hippocampal mineralocorticoid and glucocorticoids receptors (MR and GR, respectively), which provide substantial negative feedback to the HPA axis, are expressed in the fetal brain from GD13 onwards (Diaz *et al.*, 1998). Exposure to excessive levels of glucocorticoids has been shown to decrease hippocampal MR levels and as a consequence, longer HPA axis activation following stress exposure in adulthood (Maccari *et al.*, 1995; Barbazanges *et al.*, 1996). In turn, HPA axis activation was shown to positively correlate with depressive-like behavior (Morley-Fletcher *et al.*, 2003), providing a mechanism by which early life stress could predispose to a depressive-like phenotype in adulthood.

Notably, the kinetic properties of HPA axis activation make it unlikely that behavioral impairments are solely mediated by CORT, since peak CORT levels are observed ~20 minutes after stressor onset. Indeed, a growing body of literature suggests that interactions between the HPA axis and other neurotransmitter systems regulate behavioural output (for reviews see Tasker *et al.*, 2006; Joëls *et al.*, 2011). This notion is supported by several PNS studies, showing that antidepressant administration can normalize central, neuroendocrine and/or depressive-like behavioral measures (Morley-Fletcher *et al.*, 2004; Szymańska *et al.*, 2009; Rayen *et al.*, 2011; Nagano *et al.*, 2012).

Notably, all antidepressants activate forebrain 5-HT_{1A} receptors (Haddjeri *et al.*, 1998), PNS decreases forebrain 5-HT_{1A} receptor expression (Van den Hove *et al.*, 2006; Nagano *et al.*, 2012), and blockade of these receptors reduces HPA axis activation (Vicentic *et al.*, 1998; Goel *et al.*, 2014), suggesting that these receptors could play a pivotal role in the behavioral, neuroendocrine, and central changes induced by PNS.

Previous PNS studies have shown changes in brain monoamine content at baseline (Ward and Weisz, 1984; Peters, 1988; Gerardin *et al.*, 2005) or following stress in adulthood (Takahashi *et al.*, 1998; Bowman *et al.*, 2004). However, the *in vivo* activity of monoamine systems in PNS animals, both at baseline and after a stressor, remains largely unexplored. Accordingly, the aims of this study were threefold. First, to validate the PNS procedure by assessing the maternal CORT response to restraint stress, birth weight of offspring, and HPA axis reactivity of PNS animals in adulthood. Second, to determine whether PNS alters the *in vivo* activity of 5-HT, DA and NE monoamine systems at baseline and/or after stress exposure. Third, to assess whether PNS produces a depressive-like phenotype in adulthood that could be linked to changes in monoaminergic neuronal firing. Based on the "cumulative stress" or "two-hit" concept (Nederhof and Schmidt, 2012), it was hypothesized that PNS by itself, or combined with a second stressor, could induce changes in monoamine systems that might be related to depressive-like measures.

Methods

Animal and procedures

All experiments were carried out in accordance with the Canadian Council on Animal Care (CCAC) and the local Animal Care Committee (University of Ottawa, Institute of Mental Health Research, Ottawa, Ontario, Canada). Female Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) gestational upon arrival, and their male offspring were used in the present study. Animals were housed individuals under standard laboratory conditions (12:12 light-dark cycle with lights on at 7:00 am, and food and water *ad libitum*). Restraint stress was performed from GD 11-20. Gestational female rats were immobilized three times daily at 9:00 AM, 12:00 AM, and 5:00 PM for 45 minutes under bright illumination (Verilux Happylight, Waitsfield, VT) as described previously (Ward and Weisz, 1984), with the exception that instead of cylinders, cone-shaped transparent polyester bags (12" Disposable Decorating Bags, Wilton, Woodrigde, IL) closed around the tail with adhesive tape were used to restrain animals. Arguably, cone-shaped bags better approximate the body shape of rats than cylinders, and the flexibility of polyester reduces the chance of insult. Control animals were left undisturbed in their cages unless stated otherwise. The body weight of stressed dams in the first batch of animals was daily recorded throughout the restrain procedure (from GD 11 – GD 20 at 9:00 AM). Since the effect of the restraint procedure on body weight was obvious, dams in subsequent groups were not weighed to minimize stress and handling. On GD11 and GD20, blood samples were collected in 15 control and 15 PNS dams before (t0), during (t25 and t45) and after (t90) the restraint with a tail-nick (described below); samples were

used for subsequent corticosterone analysis. On postnatal day (PND)1, body weight of offspring was recorded in our first batch of animals. Litters were culled to 4 males within the first three postnatal days. On PND21 the weaning period ended and offspring was socially housed (2 per cage). Subsequent experiments were performed on male adult offspring at PND60-90. For all experiments, a maximum of 2 males per litter was used.

Drugs and compounds

WAY 100.635 (WAY; Sigma-Aldrich, Oakville, ON) was dissolved in distilled water at a concentration of and 60 μg/mL for subcutaneous (s.c.) administration.

Electrophysiological recordings

In vivo extracellular unitary recordings were carried out in chloral hydrate (400 mg/kg; i.p.) anaesthetized rats that were mounted in a stereotaxic apparatus. Body temperature was maintained at 37 °C throughout the experiment utilizing a thermistor-controlled heating pad. Extracellular recordings of neurons in the VTA, DRN and LC were carried out with a single-barrel glass micropipette (Stoelting, Spencerville, MD) with impedance between 2-6 MΩ mounted and preloaded with 2 M NaCl.

Recording of 5-HT neurons

Putative 5-HT neurons were recorded by positioning glass micropipettes at the following coordinates (in mm from lambda): anterior/posterior (AP) 1.0 to 1.2, mediolateral (ML) 0, dorsal/ventral (DV) 5.0 to 7.0. At these coordinates, neurons with a bi- or triphasic extracellular waveform with a long-duration (0.8-1.2 ms) positive phase,

and regular firing in the range of 0.8-5 Hz were recorded (Vandermaelen and Aghajanian, 1983). The start of a burst for DRN 5-HT neurons was defined as the occurrence of 2 spikes within 20 ms; end of a burst was defined as an ISI>20 ms (Hajós *et al.*, 2007). The following burst parameters were studied: spikes/minute, percent spikes in burst, spikes/burst, and interspike interval (ISI) in bursts.

Recording of LC NE neurons

NE neurons were recorded by positioning glass micropipettes at the following coordinates (in mm from lambda): AP -1.0 to -1.2, ML 1.0 to 1.3, DV 5.0 to 7.0. NE neurons were identified using the following criteria: regular firing rate (0.1-5 Hz) and a long duration (0.8-1.2 ms) in the rising phase of the action potential (Vandermaelen and Aghajanian, 1983), and a brisk excitatory response followed by a short period of inhibition (~1 s) in response to a nociceptive pinch of the contralateral hind paw, administrated with forceps. The start of a burst for LC NE neurons was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an ISI>160 ms (Chenu *et al.*, 2013). The following burst parameters were studied: spikes/minute, percent spikes in burst, spikes/burst, and ISI in bursts.

Recording of VTA DA neurons

Putative DA neurons were recorded by positioning glass micropipettes at the following coordinates (in mm from lambda): AP 3.2 to 3.6, ML 0.6 to 1.0, DV 7.0 to 9.0. At these coordinates, neurons with a long duration (3-5 ms) triphasic action potential with a marked negative deflection, often with an inflection or “notch” on the rising phase,

irregular spontaneous single firing pattern (1-10 Hz) and slow bursting activity with decrementing action potential amplitude were recorded (Grace and Bunney, 1983). The start of a burst for VTA DA neurons was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an ISI>160 ms (Grace and Bunney, 1984). The following burst parameters were studied: spikes/minute, percent spikes in burst, spikes/burst, and ISI in bursts. To quantify of the number of active DA neurons per rat, 9 tracts spaced by 200 μm were performed in standardized grid. The total number of DA neurons recorded divided by the number of tracts was used as a measure for dopamine population activity (Chenu *et al.*, 2013).

"Tail-nick" blood sample collection and corticosterone analysis

Blood samples were by obtained by a "tail-nick", a method with modest invasiveness (Milot *et al.*, 2012). Briefly, the extremity of the tail was punctured with a virgin 26 gauge needle, followed by application of gentle manual pressure on the lateral tail veins while moving fingers from the middle of the tail to the tip until a blood was collected on a protein saver card (Whatman, Westborough, MA). Corticosterone samples were obtained from a paper punch of the protein saver card, and were analyzed with an immunoradioactive assay.

Forced swim test

The FST apparatus consisted of a vertical cylindrical Plexiglas tank (height 60 cm; diameter 20 cm) filled to a depth of 40 cm with tap water of 23.0 ± 0.1 ºC. Animals were exposed to a 15 minute pretest 24 hours prior to the 5 minute test (Detke *et al.*, 1995).

After the pretest, animals were quickly removed from the cylinder, dried with a towel, and single housed in a fresh cage. On the testing day, animals were transported to the test room 30 minutes prior to testing. If applicable, a subcutaneous injection of the 5-HT_{1A} antagonist WAY 100.635 was administered briefly after arrival in the test room. All tests were performed between 11-12 AM.

To assess the CORT response to forced swimming, blood samples were collected at t0, t5, t20, t35, t65 and t125, with t0 the time point directly before the animal was introduced in the FST cylinder. In animals receiving WAY 100.635, an additional blood sample was obtained before injection (t-30) to assess a possible effect of WAY 100.635 on circulating CORT levels.

To assess depressive-like behavior in the FST, the test sessions were recorded with a digital video camera (Sony CX7 Handycam, New York, NY) and used for subsequent behavioral analysis. Videos were randomized (by MEM) and scored blind (by CAO). Behaviors were defined as follows: (1) immobility – floating with the absence of paw movement, (2) swimming – movements necessary to move through the swimming chamber and (3) climbing – upward directed movements all limbs are moving and the head is in an upright position. The average of 3 scorings per video was used to quantify behavior in the FST.

Data analysis / Statistics

Recordings of 5-HT, NE and DA neurons were filtered from noise using post-recording waveform analysis, then exported as text files and analyzed using The BurstiDAtor software (Oosterhof and Oosterhof, 2013) to obtain firing and burst

parameters. Body weight gain of female dams, CORT responses of dams during restraint, and CORT responses in the FST were analyzed with repeated measures (RM) ANOVA, followed by Bonferroni *post-hoc* testing if applicable. Body weight on PND1, firing activity of monoamine neurons, and swim behavior was analyzed with two-way ANOVA, followed by a Bonferroni *post-hoc* test if applicable. Data analysis was performed with Graphpad Prism software version 5.01. Data is presented as mean, and error bars represent S.E.M. A p-value <0.05 was considered significant.

Results

Dams and pups: body weight

Restraint stress significantly reduced the body weight gain of gestational dams (RM ANOVA, n=5 per group, $F_{1,8}$=31.3, p<0.0001, figure 1A). Bonferroni *post-hoc* testing revealed a significant decreasing effect of restraint on body weight gain on all time points relative to the first day of restraint.

On PND 1, there was a significant effect of main effect of PNS ($F_{1,117}$=17.9, p<0.0001) and sex ($F_{1,117}$=9.3, p=0.003) on body weight of pups, demonstrating reduced body weight in PNS animals and females (figure 1B).

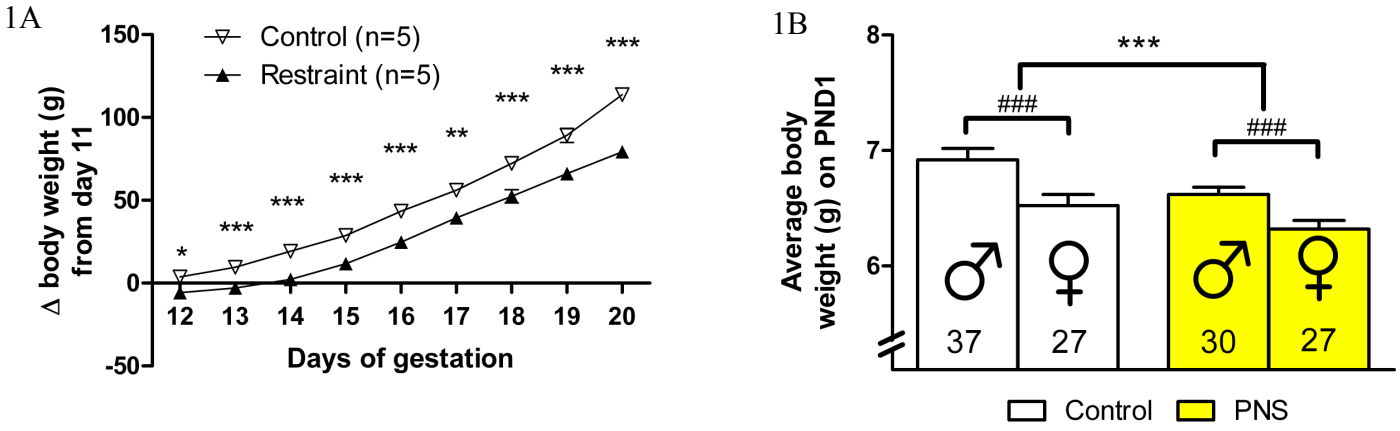

Figure 1: Effect of restraint stress on body weight gain in gestational dams (1A) and on weight of offspring on PND1 (figure 1B). Data were analyzed with repeated measures ANOVA followed by a Bonferroni *post-hoc* test (1A), or with two-way ANOVA (1B). Dare presented as mean, error bars represent S.E.M. and numbers in histograms represent number of animals per group.
***effect of restraint stress; *p<0.05, ***p<0.001**
#effect of sex; ###p<0.001

Restraint during gestation: blood corticosterone levels

Blood samples were collected from gestational control and stressed dams directly before restraint (t0), during restraint (t25 and 45) and 45 minutes after restraint (t90) on the first and last day of restraint (GD 11 and GD 20; see figure 2A and 2B, respectively). There was a significant main effect of restraint stress and a stress x day interaction effect on maternal CORT levels (restraint stress: $F_{3,54}$=12.1, p<0.001; stress x day: $F_{3,54}$=3.9, p<0.05). Post-hoc testing revealed a significant elevation by restraint stress on t0, t25 and t45 ($F_{1,56}$=11.2, p<0.001, $F_{1,56}$=29.8, p<0.001 and $F_{1,56}$=214.8, p<0.001, respectively). Furthermore, CORT levels before restraint stress (t0) were significantly elevated on GD20 compared to GD11 ($F_{1,56}$=9.9, p=0.003).

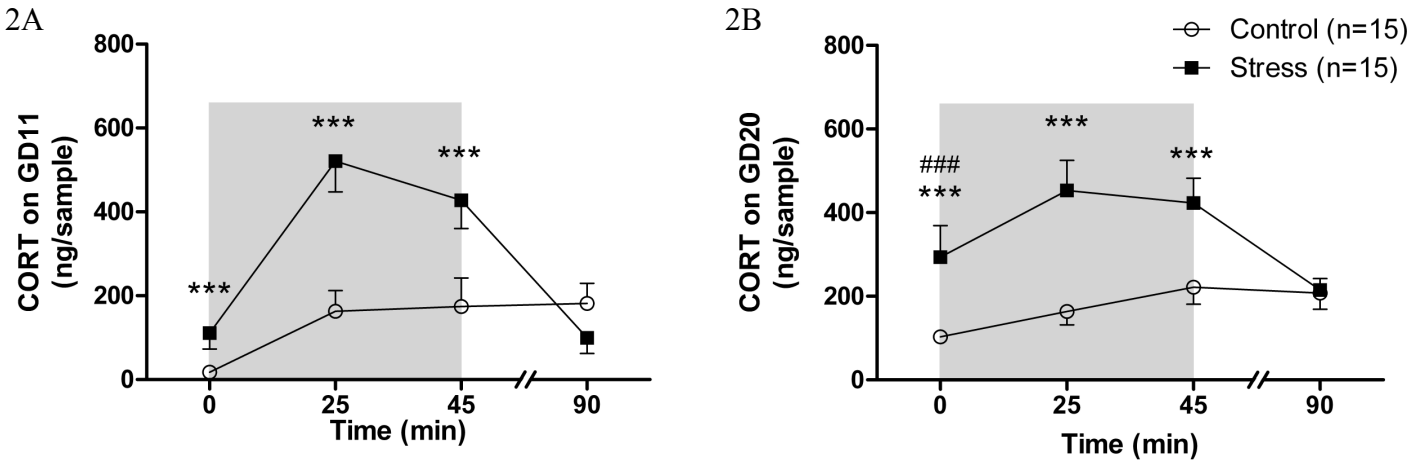

Figure 2: corticosterone response in control (open circles) and restraint (black squares) gestational dams on the first (2A) and last (2B) day of restraint. Blood samples were collected during the first restraint session of the day (starting at 9.00 AM). The grey box represents the restraint period. Data were analyzed with repeated measures ANOVA followed by a Bonferroni *post-hoc* test, are presented as mean with error bars representing S.E.M.
***effect of restraint stress; ***p<0.001**
#significant day effect; ###p<0.001

Forced swim stress: corticosterone response

To determine blood CORT levels in response to swim stress, blood samples were collected before and after the FST. In a subset of animals, the 5-HT_{1A} receptor antagonist WAY 100.635 was administered 30 minutes prior to the FST to assess whether the role of 5-HT_{1A} receptors in the CORT response was changed by PNS. Both PNS and WAY 100.635 had a significant main effect on CORT levels after FST ($F_{5,36}$=3.2, $p<0.05$ and $F_{5,36}$=28.4, $p<0.0001$). Further statistical testing revealed a significant elevation of CORT levels in PNS animals at t65 ($F_{1,40}$=10.0, $p>0.004$, figure 3A and 3B), but not at any other time point. Compared to the experiment without WAY 100.635, CORT levels were increased in the WAY experiment at t0, t5 t20 and t120 in both control and PNS animals (RM ANOVA, $F_{1,40}$=45, $p<0.001$, $F_{1,40}$=85, $p<0.001$ and $F_{1,40}$=13, $p<0.001$). Possibly, the blood sample collection, subcutaneous injection, and/or inter-assay variability might have played a role in this quantitative difference. Most importantly however, WAY 100.635 administration did not change baseline CORT levels (paired t-test, $p>0.05$), and the CORT responses to WAY was qualitatively similar to that in absence of 5-HT_{1A} blockade in controls and PNS animals.

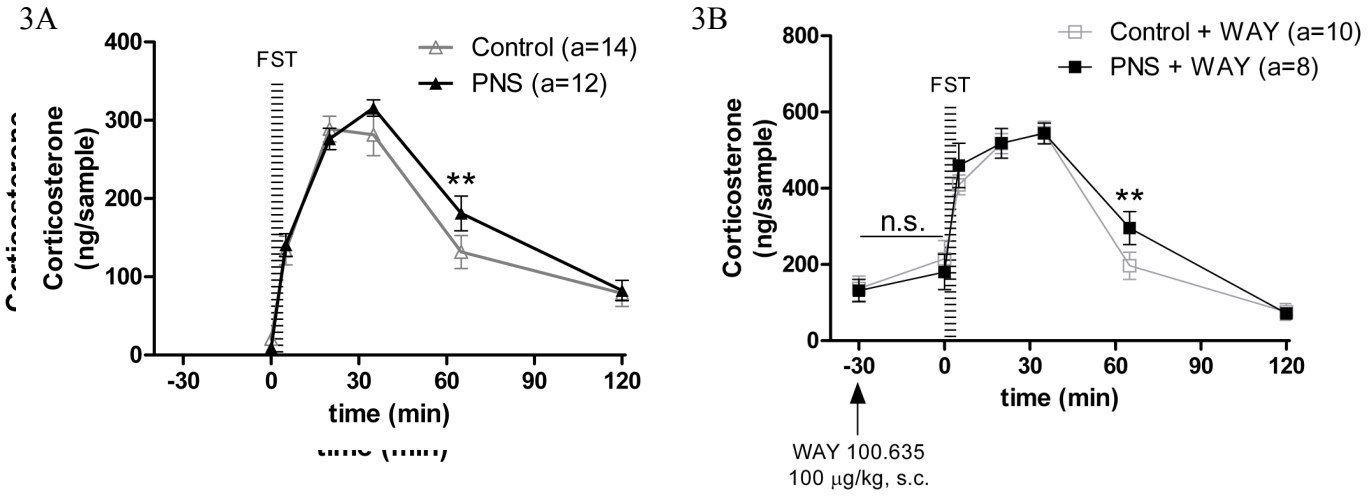

Figure 3: corticosterone response to forced swim stress in PNS and control animals without a pharmacological challenge (3A) and following a subcutaneous administration of the selective 5-HT_{1A} receptor antagonist WAY 100.635 (100 µg/kg, s.c., 3B). Data are presented mean ± S.E.M., and were analyzed with repeated measures ANOVA followed by Bonferroni *post-hoc* testing. The "a" correspond to the number of animals per group, WAY indicates pretreatment with WAY 100.635.
***significant effect of PNS; **p<0.01.**

Firing activity of LC NE neurons: effect of PNS and FST

PNS and the FST alone had no effect on the firing rate of LC NE neurons ($p>0.05$), however, a significantly PNS x FST interaction effect was detected ($F_{1,516}=17.4$, $p<0.001$, figure 4). Post-hoc testing demonstrated that prior to the FST, the firing rate of LC NE neurons was increased in PNS animals compared to controls ($p=0.009$). After the FST, the firing rate of these neurons was significantly lower in PNS animals compared to controls ($p=0.001$). Exposure to FST normalized the firing rate of NE neurons in PNS animals ($p=0.002$), while it increased this firing activity in naïve animals ($p=0.005$). The FST and PNS had no effect on burst activity of NE neurons ($p> 0.05$, data not shown).

Fig. 4

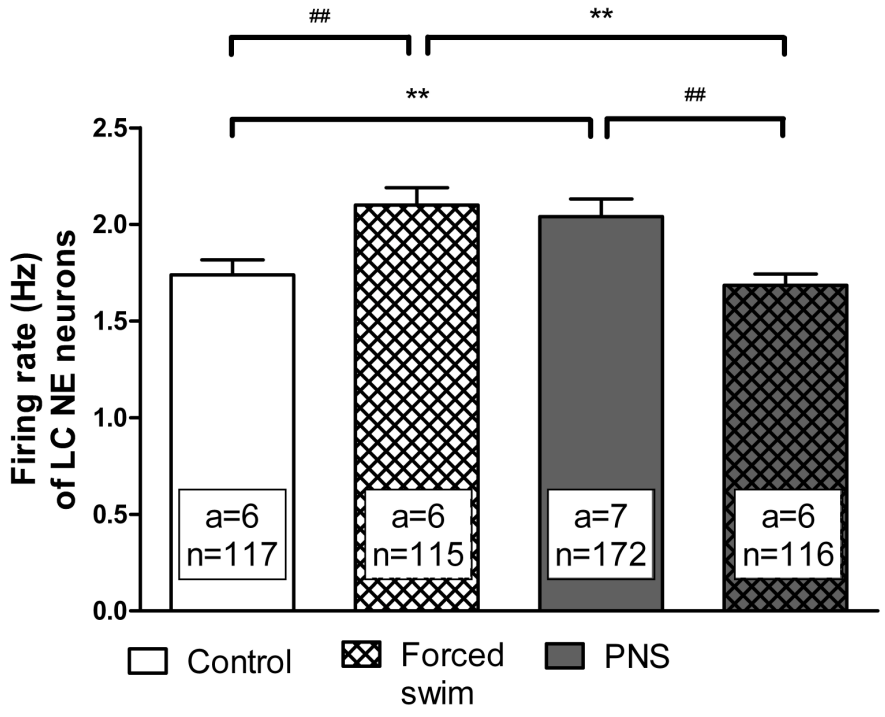

Figure 4: firing rate of LC NE neurons at baseline and one day after forced swim stress. Data are presented as mean + S.E.M., and were analyzed with two-way ANOVA and Bonferroni *post-hoc* test. The "a" and "n" in histograms correspond to the number of animals used and neurons recorded, respectively.
*** significant PNS effect; **p<0.01**
[#]significant FST effect; [###]p<0.001

Firing activity of VTA DA neurons: effect of PNS and FST

PNS and the FST alone had no effect on the firing rate of VTA DA neurons ($p>0.05$), however, a PNS x FST interaction effect was detected ($F_{1,332}=5.5$, $p<0.05$, figure 5). Post-hoc testing demonstrated that in PNS animals, the firing rate of VTA DA neurons was significantly elevated compared to controls ($p=0.02$), while this parameter normalized after the FST ($p=0.19$).

There was no effect of PNS or FST on VTA population activity ($p>0.05$, data not shown). PNS had no effect on burst parameters in the subpopulation of VTA DA neurons that displayed burst activity ($p>0.05$), however, a significant effect was detected of the FST ($F_{4,277}=4.1$, $p=0.001$), as well as a FST x PNS interaction effect ($F_{4,277}=2.4$, $p<0.05$). Post-hoc testing revealed that FST significantly decreased the ISI in bursts ($F_{1,280}=5.2$, $p<0.05$, table 1). Furthermore, FST decreased the number of bursts per minute, percent of spikes in burst, and spikes per burst in PNS animals, whereas it had the opposite effect in controls (PNS x FST interaction, burst/min, $F_{1,280}=7.6$, $p<0.01$; percent spikes in burst, $F_{1,280}=8.5$, $p<0.01$; spikes/burst, $F_{1,280}=5.3$, $p<0.05$, table 1).

	Bursts per minute$^{\#\#}$	% spikes in burst$^{\#\#}$	Spikes/ burst	ISI$^{\$}$	n/a	% neurons bursting
Control	17 ± 2	20 ± 2	2.8 ± 0.1	68 ± 2	95/6	74
Control + FST	26 ± 2	34 ± 3	2.9 ± 0.1	66 ± 1	93/6	92
PNS	28 ± 3	35 ± 3	3.3 ± 0.2	70 ± 2	64/5	89
PNS + FST	23 ± 2	31 ± 3	2.8 ± 0.1	65 ± 2	89/5	84

Table 1: VTA neuron burst activity recorded at baseline and one day after forced swim test (FST). ISI; inter-spike interval of spikes in burst, n/a; neurons/animals recorded, % n bursting; percentage of neurons displaying burst activity.
$^{\#}$PNS x FST interaction effect, $^{\#}p<0.05$, $^{\#\#}p<0.01$
$^{\$}$FST effect, $^{\$}p<0.05$

Fig. 5

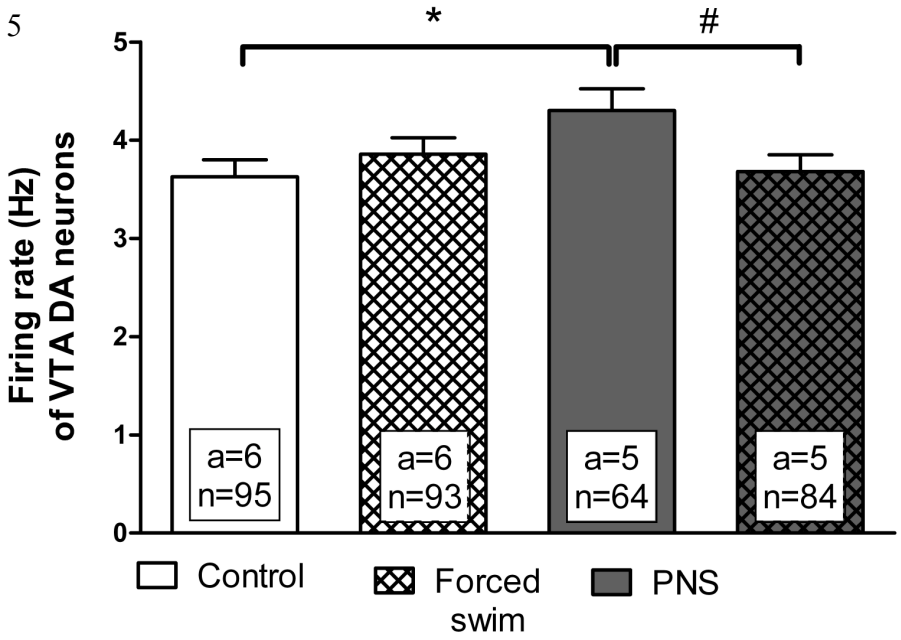

Figure 5: firing rate of VTA DA neurons at baseline and one day after forced swim stress. Data are presented as mean + S.E.M., and were analyzed with two-way ANOVA and a Bonferroni *post-hoc* test. The "a" and "n" in histograms correspond to the number of animals used and neurons recorded, respectively.
***Significant effect of PNS; *p<0.05**
#significant effect of the FST; #p<0.05

DRN 5-HT neurons: effect of PNS, FST and chronic restraint stress

DRN 5-HT neurons were recorded at baseline, or one day after the FST. In PNS animals, the firing rate of DRN 5-HT neurons was significantly lower compared to controls (two-way ANOVA, $F_{1,362}$=8.2, p<0.01, figure 6), while no effect of the FST alone, or a PNS x FST interaction effect was detected (p<0.05).

In the subpopulation of 5-HT neurons displaying burst activity (control: 24%, PNS: 25%) PNS significantly elevated the percentage of spikes in burst (19 ± 4 in control vs 36 ± 4 in PNS animals, $F_{1,89}$=6.7, p<0.05), while FST exposure had no effect on this measure. No effect on other burst parameters was detected (data not shown).

Fig. 6

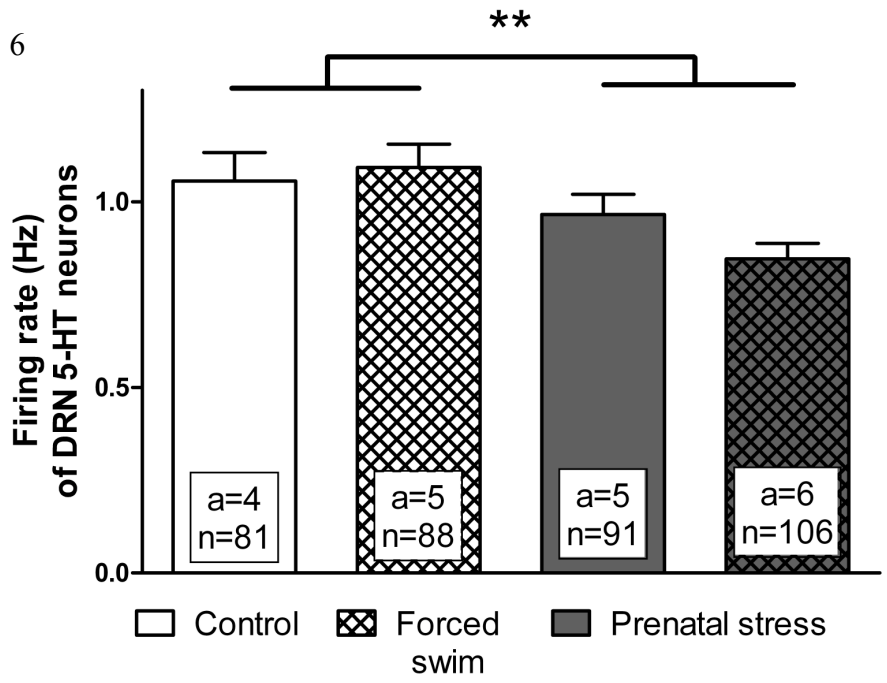

Figure 6: firing rate of DRN 5-HT neurons at baseline and one day after forced swim stress. Data are presented as mean + S.E.M., and were analyzed with two-way ANOVA.. The "a" and "n" in histograms correspond to the number of animals used and neurons recorded, respectively. *significant effect of PNS; **p<0.01

Effect of PNS and 5-HT_{1A} receptor blockade on forced swim behavior

During the 5-minute FST, there was no difference between PNS animals on immobility, swimming and climbing scores ($p>0.05$, figure 7). Pretreatment with WAY 100.635 (100 µg/kg, s.c.) administered half an hour prior to the FST slightly but significantly increased climbing behavior in both groups (ANOVA, $F_{1,54}=6.1$, $p>0.05$, figure 7) but not other behavioral measures.

Fig. 7

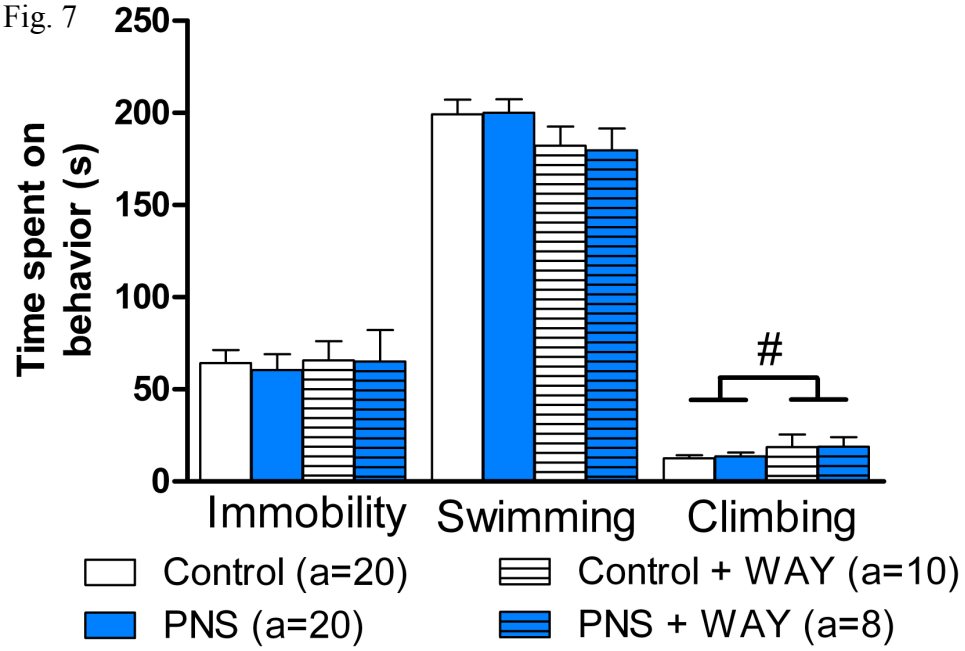

Figure 7: behavior during the FST in absence, or half an hour after WAY 100.635 administration (100 µg/kg, s.c.). Data are presented mean + S.E.M. and were analyzed with a two-way ANOVA. The "a" correspond to the number of animals per group, "WAY" indicates pretreatment with WAY 100.635. #significant effect of WAY 100.635; #p<0.05.

Discussion

In the present study, a widely applied chronic predictable stress paradigm (Ward and Weisz, 1984) was used to activate the maternal HPA axis during gestation. This activation causes fetal exposure to excessive levels various stress mediators, in particular glucocorticoids, which is thought to be a causal factor of maladaptive neuroendocrine and behavioral responses in adulthood (Maccari *et al.*, 1995; Barbazanges *et al.*, 1996). As suggested previously (Shalev, 2001), a possible culprit in the use of predictable stressors to induce PNS is that animals could habituate to the procedure, hence diminish the maternal CORT response over time (Pitman *et al.*, 1988; Ottenweller *et al.*, 1992). To test whether habituation to gestational stress occurred, maternal CORT responses were compared on the first and last day of restraint (GD11 vs. GD20, figure 2). Importantly, the magnitude of the CORT response was not different between GD11 and GD20, indicating that habituation to restraint stress did not occur. Rather, gestational dams seemed to anticipate to restraint stress, as CORT levels stress were already significantly elevated on GD20 prior to restraint stress (figure 2B).

Restraint stress reduced maternal weight gain from gestational day GD11 to GD20 (figure 1A), confirming and extending findings from previous studies (Igosheva *et al.*, 2007; Van den Hove *et al.*, 2014). Furthermore, restraint stress significantly reduced the birth weight of the offspring (figure 1B), a common effect of gestational stress related to a broad range of physiological and psychological disorders (Van Den Hove *et al.*, 2005; Zagron and Weinstock, 2006; Hausknecht *et al.*, 2013; Sun *et al.*, 2013; Modir *et al.*, 2014; Van den Hove *et al.*, 2014).

In adult PNS offspring, baseline CORT levels did not differ between control and PNS animals. However, CORT levels remained significantly elevated 60 minutes after swim stress (figure 3), likely reflecting decreased negative feedback on the HPA axis activity, an effect commonly observed after PNS (Barbazanges *et al.*, 1996; Morley-Fletcher *et al.*, 2003; Viltart *et al.*, 2006; Szymańska *et al.*, 2009) that has been related to depression in humans (Heuser *et al.*, 1994; Stetler and Miller, 2011; Lok *et al.*, 2012). Similar effects have previously been related to diminished GR and MR signaling and consequently, a reduction in blunted negative feedback on the HPA axis (Maccari *et al.*, 1995; Barbazanges *et al.*, 1996; Welberg *et al.*, 2001; Brunton and Russell, 2010).

Intensive prenatal glucocorticoid exposure, decreased birth weight, and blunted HPA axis feedback in adulthood are implicated as risk factors for depressive-like behavior. Indeed, PNS was shown to cause a despair-like phenotype, as measures by increased immobility in the FST (Stöhr *et al.*, 1998; Drago *et al.*, 1999; Morley-Fletcher *et al.*, 2004, 2011; Yang *et al.*, 2006; Szymańska *et al.*, 2009; Borges *et al.*, 2013). Clearly, these studies support the ”two-hit” stress hypothesis, whereby early life stress causes a maladaptive response to stress exposure in adulthood. Importantly however, immobility in the FST was normal in the present (figure 7) as well as previous PNS studies (Alonso *et al.*, 1991; Frye and Wawrzycki, 2003; Hauser *et al.*, 2009; Van den Hove *et al.*, 2013, 2014). In addition, others reported decreased immobility by PNS (Welberg *et al.*, 2001; Rayen *et al.*, 2011). Clearly, these studies support the “mismatch” hypothesis, suggesting that PNS induced adaptive changes that lead to normal or positive behavioral output in response to stress exposure in adulthood. Accordingly, the observed

changes in monoamine system activities by PNS and FST exposure are interpreted as adaptive changes that might have contributed to stress resilience.

NE system

The behavioral response to acute stress exposure is, in part, mediated by firing activity of LC NE neurons (Rasmussen *et al.*, 1986; Abercrombie and Jacobs, 1987a). Prior to the FST, a lower firing activity of LC NE neurons has been correlated to increased immobility in the FST (Simson and Weiss, 1988). LC perfusion with the α_2-adrenoceptor agonist clonidine, which attenuates LC NE firing activity (Cedarbaum and Aghajanian, 1977), increased immobility in the FST (Simson *et al.*, 1986; Weiss *et al.*, 1986) while conversely, LC perfusion with the α_2-adrenoceptor antagonist idazoxan reduced immobility in the FST (Simson *et al.*, 1986; Weiss *et al.*, 1986). FST exposure caused a short-term depletion of LC NE content which is thought to facilitate NE system activation upon subsequent stressor exposure (Weiss *et al.*, 1981; Simson *et al.*, 1986). Accordingly, the increased firing activity of LC NE neurons in control animals after FST exposure might reflect a "normal" response to stress (figure 4).

The firing activity of LC NE neurons was significantly elevated by PNS (figure 4). Since PNS was previously shown to reduce the level of tyrosine hydroxylase in the LC (Green *et al.*, 2011; Bingham *et al.*, 2013), enhanced firing could have stemmed from less α_2-adrenergic autoreceptor mediated feedback. LC hyperactivity could be a maladaptive effect of chronic stress; indeed, LC NE neurons were hyperactive in depressive-like animals (Simson *et al.*, 1988; Mana and Grace, 1997), while clonidine perfusion of the LC normalized such behavioral impairments (Simson *et al.*, 1986).

Interestingly, the NE response became blunted in animals successfully adapting to repeated stress exposure, (Abercrombie and Jacobs, 1987b), possibly due to sensitization of α_2-adrenergic autoreceptors on LC NE neurons (Pavcovich *et al.*, 1990). Taken together with increased LC NE turnover after stress exposure in PNS animals (Takahashi *et al.*, 1992), it is possible that enhanced α_2-adrenergic autoreceptor activation after FST exposure contributed to normalized firing of LC NE neurons and behavioral output in the present work (figure 4 & 7).

DA system

In control animals, FST exposure increased bursting, but not firing and population activity of VTA DA neurons (table 1, figure 5). Previously, acute stressor exposure was shown to increase in VTA DA population activity (Valenti *et al.*, 2011, 2012). Although population activity of VTA DA neurons remained unaltered, the detected increase in bursting activity (table 1) might reflect an activating effect of acute stress exposure on the DA system (Deutch *et al.*, 1990; Doherty and Gratton, 1996). Possibly, stressor nature, duration, and interval between stressor and electrophysiological experiments could underlie variation in mechanisms by which VTA DA neurons are activated by stress.

In PNS animals, baseline firing and bursting activity of VTA DA neurons was significantly elevated (table 1, figure 5). These results are in line with a previous microdialysis study demonstrating elevated baseline DA levels in the NAcc of PNS animals (Silvagni *et al.*, 2008). Interestingly, VTA activity was normalized by FST exposure in PNS animals (table 1, figure 5). Similarly, chronic stress was previously shown to decrease the population activity of VTA DA neurons, while subsequent acute

stress exposure normalized the activity of DA neurons (Valenti *et al.*, 2012). Hypothetically, the normalizing effect of FST exposure might reflect adaptive changes leading to DA homeostasis by PNS, which could have contributed to normal behavior in the FST. In support of this notion, depressive-like behavior in the FST was previously related to attenuated DA firing activity and neurotransmission (Friedman *et al.*, 2008). Furthermore, decreased activity of VTA DA neurons in PNS animals has been related to addiction risk (Hausknecht *et al.*, 2013) and anhedonic-like behavior (Chang and Grace, 2014). Taken together, diminished VTA DA activity might be related to behavioral impairments, while normal and/or enhanced DA output might be involved in counteract such behavioral effects.

5-HT system

In control and PNS animals, FST exposure had no effect on the firing activity of DRN 5-HT neurons (figure 6). Interestingly however, PNS significantly decreased the firing activity while it increased bursting activity of DRN 5-HT neurons (figure 6). Interestingly, a previous study where PNS had no effect immobility on in the FST demonstrated increased 5-HT immunoreactivity in the DRN (Van den Hove *et al.*, 2014). Based on this result, enhanced 5-HT_{1A} autoreceptor activation possibly attenuated the firing activity of 5-HT neurons in the present study. Notably, depressive-like behavior induced by unpredictable stress (Bambico *et al.*, 2009) and olfactory bulbectomy (El Mansari *et al.*, 2014) produced a much greater attenuation in firing of 5-HT neurons than PNS. Thus possibly, the reduction in firing of 5-HT neurons after PNS was insufficient to

produce abnormalities in the FST. In addition, PNS enhanced burst activity of DRN 5-HT neurons, which might reflect compensation for reduced single spike activity.

Previous PNS studies reported altered 5-HT_{1A} receptor expression levels at baseline (Van den Hove *et al.*, 2006) and after stress (Morley-Fletcher *et al.*, 2004). Interestingly, agonism of 5-HT_{1A} receptors was previously shown to activate the HPA axis, while their blockade reduced the CORT response to restraint stress (Vicentic *et al.*, 1998; Goel *et al.*, 2014). Furthermore, behavioral impairments produced by PNS were normalized by imipramine, fluoxetine, mirtazapine, aripiprazole and agomelatine administration (Morley-Fletcher *et al.*, 2004, 2011; Szymańska *et al.*, 2009; Ratajczak *et al.*, 2013) all of which mediate their effects at least in part by enhancing neurotransmission through 5-HT_{1A} receptors (Blier and El Mansari, 2013). Since in the present study no behavioral impairment was detected, it was hypothesized that PNS caused endogenous enhancement of 5-HT_{1A} receptor activation, leading to a normal behavioral phenotype. Furthermore, enhanced 5-HT_{1A} receptor activation could have contributed to elevated CORT response in PNS animals following swim stress. Following this hypothesis, 5-HT_{1A} receptor blockade by WAY 100.635 prior to the FST should have resulted in behavioral impairments and a normal CORT response. Clearly, this was not the case (figure 3B & 7), demonstrating unaltered function of 5-HT_{1A} receptors under the present experimental conditions.

Conclusion

The present data confirm and extend a significant impact of chronic maternal restraint stress on physiological and neural measures in adult male offspring. To the best

of knowledge, the current results are the first to demonstrate no habituation of gestational dams to chronic predictable restraint stress. This demonstration is important, since excessive exposure to glucocorticoids in early life is a constituent element of the fetal programming hypothesis (Zagron and Weinstock, 2006). Stress exposure resulted in decreased maternal growth during gestation, as well as reduced offspring birth weight, supporting detrimental effects of the intensive PNS procedure as applied in the present work. In line with previous studies, a decreased negative feedback on the HPA axis in animals exposed to PNS was demonstrated, an effect thought to contribute to behavioral deficits in both animals and humans (Heuser *et al.*, 1994; Maccari *et al.*, 1995). Together, all of these results support a successful application of maternal restraint stress. Although in some studies, these effects were linked to depressive-like behavior, not all PNS studies found maladaptive effect of PNS on behavioral. Together with the latter studies, the normal behavior of PNS animals in the present work indicates that physiological alterations induced by PNS should not be interpreted as maladaptive *per se*. Although the reason for variable effects of PNS remains to be fully elucidates, factors such as placental 11ß-hydroxy steroid dehydrogenase content (Diaz *et al.*, 1998), maternal care (Maccari *et al.*, 1995), and housing conditions (Morley-Fletcher *et al.*, 2003; Murthy *et al.*, 2013) might have contributed herein. Finally, the activity of all three monoamine systems (5-HT, NE, DA) was changed by PNS alone and FST exposure in directions that might hold relevance to stress resilience after PNS exposure.

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4) General discussion

Although an imbalance in monoamine neurotransmission may be implicated in depression, the heterogeneity of neurochemical changes presumably leading to clinical symptoms cause a major complication in the treatment of MDD. Deficiency of one monoamine system can be sufficient to produce depressive symptoms, and might be selective for subgroups of patients. Indeed, 5-HT but not catecholamine depletion causes relapse in patients responding to a 5-HT agent (Shopsin, 1976; Smith *et al.*, 1997), while depletion of catecholamines but not 5-HT worsens depressive symptoms in patients responding to a NE agent (Brodie *et al.*, 1979; Miller *et al.*, 1996b). Furthermore, both insufficient and excessive neurotransmission, particularly of catecholamines, can contribute to depressive symptoms (Blier and Briley, 2011). It might therefore be unsurprising that only one-third of patients achieve remission to a first-line antidepressant, suggesting that monoamine neurotransmission is not adequately corrected in partial- and non-responders. Currently however, there are limited tools to predict the effectiveness of an antidepressant *a priori*, in part due to an incomplete understanding why depression occurs. These observations call for a better understanding of the etiology of depression at the neurobiological level. Furthermore, characterizing mechanisms by which antidepressants exert their action could provide insight into the neural changes contributing to the therapeutic response, and facilitate the design of antidepressant strategies when the clinical response is suboptimal.

4.1 Prenatal stress

In an attempt to gain more insight in the etiology of depression, monoamine systems activities were characterized following prenatal and adult stress in chapter IV. Major questions in this study were how monoamine system activities were altered in animals predisposed to depressive-like behavior, and if antidepressants would normalize these systems. Indeed, PNS did change the activity of all three monoamine systems assessed, both at baseline and after stress exposure. However, in absence of a depressive-like phenotype, these changes in monoamine system activities might reflect resilience – rather than contribute – to a depressive-like phenotype. Because the effect of antidepressants in animals behaving normally would be of little relevance to the human situation, it was instead decided to assess whether enhanced neurotransmission through 5-HT_{1A} receptors contributed to antidepressant-like adaptation to PNS. However, 5-HT_{1A} receptor blockade did not alter the behavioral or neuroendocrine response to the FST, indicating that 5-HT_{1A} receptor function was normal under these conditions.

Although PNS failed to induce depressive-like behavior, the results from this study are not without implications to the field of depression research. Indeed, although predictable restraint stress is the most commonly applied method for fetal exposure to high levels of glucocorticoids and other deleterious conditions, the CORT response of gestational dams throughout the restraint period was not characterized previously. Thus, a novel finding of this study is a similar CORT response on the first and last day of restraint stress. These data indicate that adaptation to restraint stress did not occur, despite the stressor being predictable. Furthermore, fetal exposure to high CORT levels

during the fetal life stage resulted in slower HPA axis termination after the FST in adulthood, in line with previous studies.

HPA axis hyperactivity and low birth weight are common effects of PNS (Drago *et al.*, 1999; Van Den Hove *et al.*, 2005; Hausknecht *et al.*, 2013), and are thought to represent risk factors for MDD in humans (Holsboer *et al.*, 1982; Thompson *et al.*, 2001; Beydoun and Saftlas, 2008). Despite detecting these physiological effects, they did not coincide with depressive-like behavior in the FST. Therefore, the increased firing activities of LC NE and VTA DA neurons, as well as attenuated firing of DRN 5-HT neurons might play a counteracting role in otherwise detrimental effects of early life stress exposure, eventually resulting in normal behavioral output. Interestingly, firing activity of monoamine neurons was not only distinguishable between PNS and control animals at baseline, but also changed considerably after FST exposure. In control animals, FST exposure enhanced NE firing, in line with the effect of acute stress exposure (Weiss *et al.*, 1981, 1986). In PNS animals, however, FST exposure normalized the firing of VTA DA and NE neurons, suggesting that the catecholamine response to stress in adulthood was modulated by environmental conditions in early life.

Notably, previous PNS studies reported considerable variation in the FST, showing increased immobility (Stöhr *et al.*, 1998; Drago *et al.*, 1999; Morley-Fletcher *et al.*, 2004, 2011; Yang *et al.*, 2006; Szymańska *et al.*, 2009; Borges *et al.*, 2013), no effect (Alonso *et al.*, 1991; Frye and Wawrzycki, 2003; Hauser *et al.*, 2009; Van den Hove *et al.*, 2013, 2014), and decreases in this depressive-like measure (Welberg *et al.*, 2001; Rayen *et al.*, 2011). Thus, PNS appears to cause a maladaptive response to stress under some conditions, in line with the "two-hit" hypothesis. Following this logic, normal

behavior indicates that adaptation to early life stress occurred, in line with the "mismatch" hypothesis. Given these bidirectional effects, a behavioral measure is crucial to determine whether the impact of PNS was detrimental or adaptive. Although this might be an obvious point, some PNS studies related central changes to detrimental effects and/or human psychopathologies in absence of behavioral correlates (e.g. Barbazanges *et al.*, 1996; Takahashi *et al.*, 1998; Bhatnagar *et al.*, 2005; Viltart *et al.*, 2006; Igosheva *et al.*, 2007; Silvagni *et al.*, 2008; Mastorci *et al.*, 2009; Fumagalli *et al.*, 2009; Katunar *et al.*, 2009; Carboni *et al.*, 2010; Neeley *et al.*, 2011; Bock *et al.*, 2011; Muhammad *et al.*, 2012; Suenaga *et al.*, 2012; Pallarés *et al.*, 2013; Tavassoli *et al.*, 2013; Amugongo and Hlusko, 2014; Baier *et al.*, 2014; Szewczyk *et al.*, 2014; Luoni *et al.*, 2014). Thus, by emphasizing the importance of behavioral measures, the present work should encourage future studies to further characterize the significance of physiological changes following PNS to the behavioral domain.

4.2 Asenapine & brexpiprazole

In chapters I-III , the *in vivo* effects of the atypical antipsychotics asenapine and brexpiprazole on monoamine systems were characterized. Although it might be counterintuitive to discuss effects of antipsychotics in the context of MDD, it needs emphasis that their add-on administration at a sub-antipsychotic dosage can be an effective antidepressant strategy (McIntyre *et al.*, 2007; Keitner *et al.*, 2009; Nelson and Papakostas, 2009). Indeed, brexpiprazole was recently shown to augment the clinical efficacy of an existing antidepressant regimen (Thase *et al.*, 2014). Furthermore,

depressive symptoms commonly overlap in schizophrenia, and both asenapine and brexpiprazole were shown to improve such negative symptoms (Szegedi *et al.*, 2011; Buchanan *et al.*, 2012; Kane *et al.*, 2015). Clearly, classifying agents as "antipsychotic" or "antidepressant" can be misleading – a recognized problem that might be circumvented in the near future using neuroscience-based nomenclature (Zohar *et al.*, 2014). To gain more insight in their mode of action, the present pharmacological characterization of brexpiprazole and asenapine on the DA, NE and 5-HT systems were primarily aimed to provide a neural mechanism for their therapeutic effects.

4.2.1 The DA system

In the field of electrophysiology, substantial attention has been attributed to the effect of antipsychotic agents on the number of active DA neurons per tract – or population activity – in the VTA. Under chloral hydrate anesthesia, approximately 50% of neurons are inactive, in part due to basal D_2 autoreceptor activation (Chiodo and Bunney, 1983) and inhibitory GABA innervation from the ventral pallidum (Chang and Grace, 2014). Accordingly, increased VTA DA population activity after acute haloperidol, clozapine, and olanzapine is presumably due to their D_2 receptor antagonism, causing previously inactive neurons to depolarize (Chiodo and Bunney, 1983; Stockton and Rasmussen, 1996). Importantly, sustained administration of these agents, as well as quetiapine and risperidone, caused a pronounced decrease in the population activity of VTA DA neurons, which is due to excessive membrane depolarization, resulting in a depolarizing block (Chiodo and Bunney, 1983; Sorensen *et al.*, 1993; Skarsfeldt, 1995;

Jones-Humble *et al.*, 1996; Stockton and Rasmussen, 1996). Given this ubiquitous effect of antipsychotics, it has been hypothesized that a depolarizing block could mediate the therapeutic effect of antipsychotics (Grace *et al.*, 1997). Clearly, the effects of the D_2 receptor antagonist asenapine and the partial D_2 receptor agonist brexpiprazole are discordant with this hypothesis.

Indeed, study I showed that asenapine caused an approximate doubling of the population activity of VTA DA neurons after both two and 21 days of administration, despite equipotent *in vitro* D_2 receptor antagonism as haloperidol (pKi =8.9 and 8.8, respectively; Shahid *et al.*, 2009). Possibly, the differential effect of asenapine (0.1 mg/kg/day) was due to a lower D_2 receptor occupancy compared to those obtained with haloperidol, which is routinely administered at a dose of 0.5 mg/kg/day (Chiodo and Bunney, 1983; Di Giovanni *et al.*, 1998). In support of this, inhibition VTA DA neurons by apomorphine after two- and 21-day asenapine administration was fully reversed by a subsequent intravenous administration of asenapine (0.5 mg/kg), demonstrating submaximal D_2 receptor occupancy by this regimen. Thus, these data are not discordant with the idea that D_2 receptor antagonists cause a depolarizing block, but rather, indicate a dose-dependent biphasic effect on the population activity of VTA DA neurons. Despite this lower dosage, this regimen resulted in blood plasma levels comparable to those where asenapine was clinically effective in the treatment of schizophrenia (Chapel 2009). Thus, these data indicate that a depolarizing block might not be fundamental to the therapeutic effects of antipsychotic agents. In further support of this, sustained brexpiprazole did not alter the population activity of VTA DA neurons despite its clinical efficacy in the treatment of schizophrenia (Kane *et al.*, 2015).

Because both asenapine and brexpiprazole did not produce a depolarizing block, it was possible to assess the *in vivo* D_2 receptor status in presence of these agents. Indeed, although sustained antipsychotics increased D_2 receptor expression and -binding *ex vivo* (Schettini *et al.*, 1992; Geurts *et al.*, 1999), the *in vivo* demonstration of D_2 receptor sensitization required a washout period due to limited discharge activity resulting from a depolarizing block (Vogelsang and Piercey, 1985). Interestingly, two-day asenapine administration partially blocked the inhibitory effect of apomorphine, while apomorphine fully inhibited DA neurons after 21-day asenapine administration, demonstrating a sensitization of D_2 autoreceptors. In contrast, acute, two-day and 14-day brexpiprazole administration caused a similar blockade of apomorphine, suggesting that the status of D_2 autoreceptors remained unchanged. Together, these data suggest that D_2 receptor antagonism causes a sensitization of D_2 autoreceptors, while partial agonism of D_2 receptors has no effect on this measure.

Although asenapine and brexpiprazole had distinguishable actions on the population activity and D_2 autoreceptors of VTA DA neurons, they did not alter the firing rate of these neurons. Also, bursting activity of DA neurons remained unchanged by sustained administration of these agents. Taken together, the data suggest that sustained asenapine might increase DA release in some, but not all VTA projection areas. Indeed, although acute asenapine elevated DA levels in both PFC and NAc, only the latter effect was prevented by VTA TTX pretreatment (Frånberg *et al.*, 2009). Thus, increased VTA DA activity might in particular enhance DA neurotransmission in the mesolimbic pathway. Given the important role of this pathway in reward and hedonic processing (Corbit *et al.*, 2001; Schoenbaum and Setlow, 2003; Grace *et al.*, 2007), enhancement of

DA might contribute to the therapeutic effect of asenapine on negative symptoms. Interestingly, the antidepressant agomelatine, which - similarly to asenapine - increased VTA DA neuron population activity after sustained administration (Chenu *et al.*, 2013), has distinguishable positive effect in the hedonic domain of depressed patients (Martinotti *et al.*, 2012; Corruble *et al.*, 2013; Di Giannantonio *et al.*, 2014). However, since the effects of asenapine on the DA system were not reported previously in the class of antipsychotics, more clinical data are required to interpret their therapeutic significance.

The therapeutic effects of brexpiprazole might be explained in part by its partial agonism on D_2 receptors. This pharmacological quality was compellingly demonstrated in chapter II, where acute brexpiprazole reversed the inhibitory effect of apomorphine on DA neurons while it did not increase the firing activity of VTA DA neurons, the latter effect unlike that of D_2 receptor antagonists (Bunney *et al.*, 1973; Gessa *et al.*, 2000; Díaz-Mataix *et al.*, 2005; Ghanbari *et al.*, 2009). Similarly to its acute effect, sustained brexpiprazole did not alter the discharge parameters of VTA DA neurons while D_2 receptors were considerably occupied. As attenuated DA neurotransmission has been linked to depression, brexpiprazole could be therapeutically beneficial by acting as a D_2 receptor agonist when endogenous DA levels are low (Dunlop and Nemeroff, 2007). Conversely, in schizophrenia where striatal DA levels are presumably excessive (Abi-Dargham *et al.*, 1998; Howes *et al.*, 2013), brexpiprazole could dampen the DA signal by acting as an antagonist.

Although the effects of antipsychotics on the population activity of VTA DA neurons has been extensively reported, their effect on firing rate has only been reported following sustained aripiprazole, haloperidol and olanzapine administration (Vogelsang and Piercey, 1985; Stockton and Rasmussen, 1996; Chernoloz *et al.*, 2009). Similarly to the effects of asenapine and brexpiprazole, these agents did not alter the firing rate of DA neurons despite a substantial decrease in population activity by olanzapine and haloperidol. Unfortunately, the firing frequency of DA neurons after sustained quetiapine, clozapine, risperidone and ziprasidone administration was not reported (Chiodo and Bunney, 1983; Sorensen *et al.*, 1993; Skarsfeldt, 1995; Jones-Humble *et al.*, 1996). Furthermore, the effect of sustained antipsychotics on bursting activity of VTA DA neurons has not been reported, despite an important effect of burst architecture on DA neurotransmission (Grace and Bunney, 1984; Chiodo, 1988; Floresco *et al.*, 2003). Since the analysis of firing and burst activity can be time consuming, the open-source software "BurstiDAtor: a light-weight discharge analysis program for neural extracellular single unit recordings" (Oosterhof and Oosterhof, 2013) was developed in collaboration with my brother, a computer programming expert. Using existing burst criteria (Grace and Bunney, 1984; Hajós *et al.*, 2007), this program provides a novel method to quickly analyze firing and bursting activity of monoamine neurons. Accordingly, BurstiDAtor is now routinely used in our laboratory, and contributed to several peer-reviewed publications (Oosterhof *et al.*, 2014, 2015; El Iskandrani *et al.*, 2015). Hopefully, BurstiDAtor will facilitate analyzing discharge parameters of monoamine neurons in future studies, and save researchers valuable time.

4.2.2 The NE system

Sustained asenapine and brexpiprazole increased the firing activity of LC NE neurons, similarly to the effects of other atypical antipsychotics (Ramirez and Wang, 1986; Chernoloz, El Mansari, *et al.*, 2012). This increased firing was not due to blockade of α_2-adrenergic receptors, since dose-responsiveness of clonidine was unaltered after asenapine and brexpiprazole. This result was surprising for asenapine, as its acute administration increased the firing activity of LC NE neurons, and reversed the inhibitory effect of clonidine, demonstrating antagonistic action at α_2-adrenergic autoreceptors. Intriguingly, mirtazapine also produced acute α_2-adrenergic autoreceptor blockade, while this receptor population was normosensitive after sustained mirtazapine (Haddjeri *et al.*, 1998a). Perhaps, these dynamic effects on α_2-adrenergic autoreceptors are specific to tetracyclic agents, although additional research would need to confirm this.

Similarly to other atypical antipsychotics, asenapine and brexpiprazole potently blocked 5-HT_{2A} receptors, a pharmacological quality likely contributing to the superior side effect of atypical antipsychotics on motor function in schizophrenia (Tarsy *et al.*, 2002; Tarazi and Stahl, 2012). Furthermore, 5-HT_{2A} receptors mediate attenuated firing of LC NE neurons when 5-HT neurotransmission is enhanced. Indeed, sustained SSRI administration profoundly decreases the firing rate of NE neurons, while blockade of 5-HT_{2A} receptors by addition of the atypical antipsychotics aripiprazole, hquetiapine and risperidone restored firing of NE neurons (Dremencov *et al.*, 2007a; b; Chernoloz *et al.*, 2009; Chernoloz, El Mansari, *et al.*, 2012). In line with the clinical effects of these agents when co-administered with SSRIs (section 2.5.12), the therapeutic effect of add-on brexpiprazole in treatment-resistant MDD might be in part due to 5-HT_{2A} receptor

blockade (Thase *et al.*, 2014). In addition, these data imply that asenapine augmentation might be an efficacious antidepressant strategy.

Sustained brexpiprazole enhanced NE tone on α_2-adrenoceptors in the hippocampus, similarly to the effect of trazodone and hquetiapine (Ghanbari *et al.*, 2011; Chernoloz, El Mansari, *et al.*, 2012). Furthermore, antidepressants with NET blocking properties, such as reboxetine and desipramine, should produce the same effect (Linnér *et al.*, 1999; Sacchetti *et al.*, 1999). Together, these findings suggest that brexpiprazole might be therapeutically beneficial in MDD patients when NE neurotransmission is inadequate. In contrast, both acute and sustained asenapine administration caused a significant blockade on α_2-adrenoceptors on hippocampal neurons. Although the prototypical α_2-adrenoceptor antagonist mirtazapine shared this acute effect (Haddjeri *et al.*, 1996), asenapine is the first agent to produce a blockade of this receptor population after sustained administration. Since NE tone on α_2-adrenergic receptor was not enhanced, asenapine likely had an overall dampening effect on NE neurotransmission. Accordingly, asenapine might be therapeutically beneficial, particularly in patients where NE neurotransmission is excessive.

4.2.3 The 5-HT system

Asenapine and brexpiprazole had distinguishable effects on the firing activity of DRN 5-HT neurons. Asenapine, with relatively low partial agonism on 5-HT_{1A} autoreceptors of asenapine, did not prevent or reverse the inhibitory effect of 8-OHDPAT (Ghanbari *et al.*, 2009). Accordingly, sustained asenapine administration at a low dose

had limited effects on the firing activity of DRN 5-HT neurons. In contrast, acute brexpiprazole and aripiprazole caused full inhibition of DRN 5-HT neurons by 5-HT_{1A} receptor agonism. Interestingly, firing of 5-HT neurons was increased after two-day administration of both agents, although by different mechanisms. Indeed, aripiprazole caused a desensitization of 5-HT_{1A} autoreceptors, which combined with D_2 receptor agonism elevated the firing of 5-HT neurons (Chernoloz *et al.*, 2009). Excitation of 5-HT neurons by brexpiprazole presumably resulted from enhanced NE input to the DRN and activation of D_2 receptors, while 5-HT_{1A} autoreceptors remained normosensitive.

Despite their distinguishable effects on firing of DRN 5-HT neurons, sustained asenapine and brexpiprazole enhanced the tonic activation of 5-HT_{1A} receptors on hippocampal neurons. Interestingly, asenapine had this effect, by acting as a partial agonist on terminal 5-HT_{1B} autoreceptors and postsynaptic 5-HT_{1A} receptors, providing a novel mechanism by which 5-HT neurotransmission was enhanced. The enhanced tonic activation was quantitatively similar after two and 14 days of administration. In contrast, delayed adaptive changes following bupropion, mirtazapine and paroxetine resulted in a submaximal enhancement of 5-HT neurotransmission after a two-day administration (Besson *et al.*, 2000; Ghanbari *et al.*, 2011). Since acute brexpiprazole acted as a full agonist on postsynaptic 5-HT_{1A} receptors (chapter II), marked tonic activation of this receptor population after two- and 14-day brexpiprazole likely resulted from a concert of its accumulation and adaptive changes that occurred within two days. Importantly, both asenapine and brexpiprazole did not attenuate but rather enhanced neurotransmission through postsynaptic 5-HT_{1A} receptors. Since all pharmacological and non-pharmacological antidepressant strategies share this effect, enhanced 5-HT

neurotransmission by add-on asenapine or brexpiprazole to an antidepressant regime might be a relevant effect for treatment of mood disorders.

4.3 Conclusion

The clinical effect of antidepressants is at least in part attributable to a restoration of monoamine neurotransmission. Due to dynamic changes occurring within and complex interactions between these monoamine systems, characterizing the effects of antidepressants on therapeutically relevant targets needs to be performed in the intact brain, and at doses relevant for their clinical effect. Accordingly, the present work described the effects of asenapine and brexpiprazole, two antipsychotic agents with antidepressant properties, on monoamine systems at a therapeutically relevant dose. Data from these studies provided novel neural mechanisms by which these agents might exert their clinical effects. In an attempt to explore the opposite side of the spectrum, the activity of monoamine systems was characterized in an animal model for depression. Since behavioral adaptation occurred, this study unexpectedly provided insight in the activity of monoamine systems in animals resilient to depressogenic-like conditions. Together, these studies provide neural mechanisms by which pharmacological and environmental challenges alter 5-HT, NE and DA system activities, which might be relevant for the antidepressant response.

4.3.1 Limitations

Due to the possibility of species differences, caution should be taken when translating the present data to the clinical situation. Indeed, important differences can exist in drug metabolism and receptor binding between rat and humans. For example, the absent metabolite norquetiapine (section 2.5.11) and limited 5-HT_{1A} binding of vortioxetine in rats (section 2.5.7) clearly illustrate such differences. Although for asenapine and brexpiprazole, striking inter-species differences have not been reported, data from basic research should be interpreted with this limitation in mind.

All electrophysiological experiments in the present work were performed in chloral hydrate anaesthetized animals. Hence, it could be argued that the reported changes in monoamine system activities by PNS, asenapine and brexpiprazole might be of limited physiological relevance to the awake, freely moving situation. Indeed, electrophysiological single-unit recordings reported differences between the awake and anesthetized state. For example, 5-HT neurons exhibited less regular firing patterns (Montagne-Clavel *et al.*, 1995), DA neurons fired more in more bursts (Freeman *et al.*, 1985), and the firing frequency of LC NE neurons was higher in awake animals (Aston-Jones and Bloom, 1981). However, since monoamine systems are involved in processing of internal and external stimuli, experimental conditions can also be a possible confound when studying these systems in awake, freely moving animals. The limited sensory input in anaesthetized rat overcomes this problem, and contributes to a stable experimental condition. Indeed, the baseline activity of neuron populations and responses to pharmacological challenges has been consistent between studies across research

institutions over decades. In addition, the invasiveness of the surgery required to lower electrodes in the brain makes working in anesthetized animals more acceptable from an ethical point of view. Thus although certain limitations apply, the chloral hydrate anesthetized rat is a justifiable and useful model to study monoamine systems *in vivo*.

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Appendix

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Long-term administration of the antidepressant vilazodone modulates rat brain monoaminergic systems

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ABSTRACT

Vilazodone has high affinity for the human 5-hydroxytryptamine$_{1A}$ (h5-HT$_{1A}$) receptor and for the serotonin transporter (5-HTT). A previous in vivo microdialysis experiment showed that a single administration of vilazodone, dose-dependently increases extracellular 5-HT but not norepinephrine (NE) or dopamine (DA) levels in rat medial prefrontal cortex and ventral hippocampus. The effects of vilazodone on monoaminergic systems were assessed using single-unit extracellular recordings and microiontophoresis in the rat brain. Following depletion of 5-HT with para-chlorophenylalanine methyl-ester hydrochloride (PCPA), vilazodone still suppressed neuronal firing of dorsal raphe nucleus (DRN) 5-HT neurons to a similar extent than controls, indicating that this inhibition is via 5-HT$_{1A}$ receptors activation. Following 2-day intraperitoneal administration of vilazodone (5 mg/kg/day), there was a significant decrease in 5-HT neuronal firing which recovered to baseline levels by day 14 of administration, likely due to 5-HT$_{1A}$ autoreceptor desensitization. Two- and 14-day administration of vilazodone decreased the mean firing and bursting activities of ventral tegmental area (VTA) DA neurons, while only its repeated administration significantly dampened the mean firing rate of locus coeruleus (LC) NE neurons. Vilazodone acted as an agonist at 5-HT$_{1A}$ receptors, while showing a 5-HTT blocking capacity when injected acutely. After repeated vilazodone regimen, while there was no change in sensitivity of 5-HT$_{1A}$ receptors, the enhancement in 5-HT transmission yielded an increase in the tonic activation of these receptors located in the hippocampus.

1. Introduction

The serotonin (5-HT) transporter (5-HTT) and the 5-hydroxytrypramine (5-HT)$_{1A}$ receptor can be important targets for an antidepressant and/or an anxiolytic response. When 5-HTT is inhibited, it increases extracellular 5-HT level at the cell body of 5-HT neurons in the raphe nuclei, which activates the 5-HT$_{1A}$ autoreceptors, thereby diminishing the firing rate of 5-HT neurons (Blier and El Mansari, 2013). This negative feedback action limits the degree of enhancement of 5-HT levels that can be achieved in postsynaptic areas because 5-HT release is intimately controlled by the firing of 5-HT neurons. With prolonged 5-HTT blockade, the desensitization of this 5-HT$_{1A}$ autoreceptor allows firing to normalize leading to sustained enhancement of 5-HT levels in projection areas (Bel and Artigas, 1993). In turn, this increased 5-HT transmission leads to a diminished norepinephrine (NE) tone as a result of enhanced activation of excitatory 5-HT$_{2A}$ receptors located on γ-aminobutyric acid (GABA) neurons that inhibit locus coeruleus (LC) NE neuronal firing (Szabo and Blier, 2001a, b). Consistent with these results, long-term 5-HTT inhibition decreases NE levels in the amygdala, as assessed using microdialysis in the rat brain (Kawahara et al., 2007). Similarly, there is a diminished dopamine (DA) tone resulting from enhanced activation of excitatory 5-HT$_{2C}$ receptors located on GABA neurons that inhibit DA neuronal firing in ventral tegmental area (VTA; Di Matteo et al., 2001; Dremencov et al., 2009; Chernoloz et al., 2009).

As is the case with selective serotonin reuptake inhibitors (SSRIs), acute administration of 5-HT$_{1A}$ agonists initially suppresses the firing rate of 5-HT neurons because of 5-HT$_{1A}$ autoreceptor activation. This activation limits the degree of enhancement of postsynaptic 5-HT$_{1A}$ receptors by the exogenous 5-HT$_{1A}$ agonists because 5-HT release is diminished as a result of the suppression of firing of 5-HT neurons. The 5-HT$_{1A}$ autoreceptors also desensitize

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after two weeks of 5-HT_{1A} agonist administration allowing a recovery of firing of 5-HT neurons (Hadrava et al., 1995; Blier and de Montigny, 1987). At this point in time, there is a net increase in the tonic activation of postsynaptic 5-HT_{1A} receptors resulting from enhanced 5-HT release combined with the exogenous 5-HT_{1A} agonist acting on normosensitive 5-HT_{1A} receptors (Haddjeri et al., 1998). In contrast to SSRIs, however, acute administration of 5-HT_{1A} agonists increases NE tone because of a diminished activation of excitatory 5-HT_{2A} receptors on GABA neurons that inhibit locus coeruleus NE neuronal firing (Szabo and Blier, 2001a, b). Similarly, DA tone is enhanced by 5-HT_{1A} agonists (Lejeune and Millan, 1998; Gronier, 2008) resulting from decreased activation of excitatory 5-HT_{2C} receptors on GABA neurons that inhibit VTA DA neuronal firing (Di Matteo et al., 2001; Di Giovanni et al., 2008). Accordingly, 5-HT_{1A} agonists increase microdialysate levels of DA in the frontal cortex (Gobert and Millan, 1999; Ichikawa et al., 2002).

Vilazodone is an antidepressant with high affinity for the human 5-HTT and for the 5-HT_{1A} receptor (K_i: 0.5 and 0.2 nM, respectively; Heinrich et al., 2004; Dawson and Watson, 2009). In the GTPγS assay, it displays high potency for 5-HT_{1A} receptors and near complete agonism (EC_{50}: 1.4 nM; E_{max}: 87%) when compared to the endogenous ligand 5-HT (EC_{50}: 40 nM; E_{max}: 100%). In both the rat hippocampus and cortex, acute vilazodone administration resulted in 50% occupancy of 5-HTT with 1–3 mg/kg and 100% occupancy with 10 mg/kg using the 5-HTT ligand [^{3}H]DASB (Hughes et al., 2005). Consequently, vilazodone dose-dependently increased extracellular 5-HT levels in the rat medial prefrontal cortex and ventral hippocampus (Page et al., 2002). In a similar study, vilazodone increased 5-HT levels by two-fold in the dorsolateral frontal cortex without altering NE and DA levels (Hughes et al., 2005).

The main goal of the present electrophysiological study was to investigate in vivo the subacute and repeated effects of vilazodone as a 5-HTT inhibitor and a 5-HT_{1A} agonist on the 5-HT system. In addition, given the important functional connectivity between 5-HT, NE and DA systems (Guiard et al., 2008), the effects of repeated administration of vilazodone were assessed on the firing activity of NE and DA neurons.

2. Materials and methods

2.1. Experimental preparations

The in vivo electrophysiological experiments were carried out in male Sprague–Dawley rats (Charles River, St. Constant, QC, Canada) weighing between 280 and 320 g at the time of recordings. The animals were socially housed (2 per cage), under standard laboratory conditions (12:12 light–dark cycle with access to food and water *ad libitum*). The rats were allowed to acclimatize to their new environment for one week prior to start of drug administration or experiment. All the experiments were approved by the local Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care, for the care and use of laboratory animals.

Vilazodone (EMD68843) was first administered via subcutaneous minipump at a dosage of 10 mg/kg/day for 2 days. However, during drug preparation, it was noted that the compound was difficult to dissolve at a high concentration which is required for the usage of minipumps. However, repeated intraperitoneal (i.p.) administration at this dose caused rats to lose weight and to become lethargic. This indicated that the drug may possibly be toxic when administered at 10 mg/kg/day. Because of these observations, a dose of 5 mg/kg/day (i.p.) of vilazodone was selected for 2 and 14 days administration and recordings were carried 24 h after the last dose. At this dose, rats did not experience any negative effects from the drug. For acute studies, vilazodone was intravenously (i.v.) administered to rats through a lateral tail vein.

Prior to the electrophysiological recordings, rats were anesthetized with chloral hydrate (400 mg/kg, i.p. and injected in a 3 ml volume for a 300 g rat) and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Supplemental doses of the anaesthetic (100 mg/kg, i.p.) were given to maintain constant anesthesia and to prevent any nociceptive reaction to pinching of the hind paws. Body temperature was maintained at 37 °C throughout the experiment. Prior to the electrophysiological experiments, a catheter was inserted in a lateral tail vein for intravenous injection of pharmacological agents.

2.2. Electrophysiological recording of dorsal raphe nucleus (DRN) 5-HT neurons

In order to record 5-HT neurons, single-barrel microelectrodes were positioned 0.9–1.2 mm anterior to lambda (Paxinos and Watson, 1986) on the midline and lowered into the DRN. The putative 5-HT neurons were encountered over a distance of 1 mm immediately below the ventral border of the Sylvius aqueduct, and identified by their slow (0.5–2.5 Hz), regular firing rate and long duration (~2 ms) positive action potential (VanderMaelen and Aghajanian, 1983).

To assess whether vilazodone inhibition of firing activity of 5-HT neurons is due to its 5-HT_{1A} receptor agonism or because its blockade of 5-HTT and the accompanying enhancement of 5-HT levels, hence, its effect was determined in rats that were depleted from 5-HT using para-chlorophenylalanine methyl-ester hydrochloride (PCPA). PCPA was dissolved in saline and injected for three consecutive days (300 mg/kg/day, i.p., dissolved in water and injected in a 3 ml volume for a 300 g rat).

2.3. Electrophysiological recording of VTA DA neurons

Recordings of putative DA neurons were obtained with single-barrel micropipettes lowered at 3.0–3.6 mm anterior to lambda and 0.6–1.0 to the midline suture. These neurons were encountered at the depth of 6.0–8.5 mm from the surface of brain. The DA neurons were identified by the established electrophysiological criteria; a regular firing rate (2–9 Hz), an irregular single spiking pattern, a long duration (>2.5 ms) often with a notch on the rising phase, and a slow bursting activity with the amplitude of action potentials progressively decreasing in a given burst and low pitch sound on the audiometer (Ungless and Grace, 2012). To define the number of DA neurons per track, the method previously described by Valenti et al. (2011) was utilized. Briefly, tracks were performed on a grid of 400 μm × 400 μm: AP: 3.2–3.6 mm, L: 0.6–1 mm, and number of neurons per track per rat was determined.

2.4. Electrophysiological recording of LC NE neurons

NE neurons were recorded with single-barrel micropipettes positioned at 0.9–1.2 mm posterior to lambda and 0.9–1.3 to the midline suture. These neurons were encountered at the depth of 4.5–6.0 mm from the surface of the brain. The NE neurons were identified by their regular firing rate (0.5–5 Hz), a biphasic action potential of long duration (~2 ms), and a characteristic volley of spikes discharge followed by a quiescent period in response to a nociceptive pinch of the contralateral hind paw (Marwaha and Aghajanian, 1982).

2.5. Burst analysis

The firing patterns of the monoaminergic neurons were analyzed by interspike interval (ISI) burst analysis. The onset of a

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burst was signified by the occurrence of two spikes with ISI <0.08 s for NE and DA neurons, and ISI <0.01 s for 5-HT neurons. The termination of a burst was defined as an ISI >0.16 s for NE and DA neurons (Grace and Bunney, 1984; Dawe et al., 2001) and ISI > 0.01 s for 5-HT neurons (Hajos and Sharp, 1996). Burstidator software was used for burst analysis (www.github.com/nno/burstidator/releases).

2.6. Extracellular recording and microiontophoresis of dorsal hippocampus CA3 pyramidal neurons

Extracellular recording of and microiontophoresis on CA3 pyramidal neurons was performed with five-barrel glass micropipettes. The impedance of these electrodes ranged from 2 to 4 MΩ. The central barrel used for the unitary recording was filled with a 2 M NaCl solution, and the four side barrels were filled with the following solutions: 5-HT creatinine sulfate (15 mM in 200 mM NaCl, pH 4), quisqualic acid (1.5 mM in 200 mM NaCl, pH 8), and a 2 M NaCl solution used for automatic current balancing. The electrodes were lowered into the dorsal CA3 region of the hippocampus using the following coordinates: 4–4.2 mm anterior to lambda and 4.2 mm lateral. Pyramidal neurons were encountered at a depth of 4.0 ± 0.5 mm below the surface of the brain. Because these pyramidal neurons do not discharge spontaneously in chloral hydrate anesthetized rats, a small current of quisqualate was ejected to activate them within their physiological firing range (10–15 Hz; Ranck, 1975). Pyramidal neurons were identified by their large amplitude (0.5–1.2 mV) and long-duration simple action potentials, alternating with complex spike discharges (Kandel and Spencer, 1961).

2.7. In vivo determination of the sensitivity of 5-HT$_{1A}$ receptors

The responsiveness of pyramidal neurons to the microiontophoretic application of 5-HT (20 nA) in rats that received vehicle or vilazodone was assessed by determining the number of spikes suppressed per nA for the 50 s ejection period (total number of spikes suppressed divided by the ejection current).

2.8. In vivo determination of 5-HT uptake

In order to assess the relative degree to which vilazodone blocked 5-HTT, the RT50 values were determined after microiontophoretic application of 5-HT (20 nA for 50 s) on pyramidal neurons. The RT50 values correspond to the time (s) elapsed from the cessation of microiontophoretic application of 5-HT to 50% recovery of the initial firing rate (Piñeyro et al., 1994). It is a reliable index of the 5-HT reuptake process in vivo. Neurons were inhibited at about 80–100% but only neurons responding with complete inhibition were used for RT50 analysis. Previous experiments showed that administration of the SSRIs escitalopram and paroxetine (both 10 mg/kg/day) increased by 3-fold the RT50 values (El Mansari et al., 2005; Piñeyro et al., 1994). Furthermore, the same degree of prolongation of the RT50 value was also observed in rats after lesion of 5-HT neurons, thereby eliminating 5-HTT (Piñeyro et al., 1994).

2.9. Determination of the tonic activation of 5-HT$_{1A}$ receptor in the hippocampus

In order to assess the degree of activation of the 5-HT$_{1A}$ receptors exerting an inhibitory influence on the firing activity of pyramidal neurons, the selective 5-HT$_{1A}$ receptor antagonist WAY 100,635 was administered intravenously to disinhibit these neurons resulting in an increase of their firing activity (Haddjeri et al., 1998). To prevent ceiling effect, their firing rate was decreased by reducing the ejection current of quisqualate. Following stable baseline activity, WAY 100,635 (25–100 μg/kg) was injected intravenously. It can be assumed that any increment in the firing activity of hippocampus pyramidal neurons would reflect an increased level in the tonic activation of the 5-HT$_{1A}$ receptors and the degree to which WAY 100,635 disinhibit this firing would be a measure of the level of tonic activation of these receptors by extracellular 5-HT. The percentage change of firing activity was assessed by calculating the mean firing rate of neurons from about 2 min prior to and after i.v. administration of the antagonist, and quantified as percentage change relative to baseline activity.

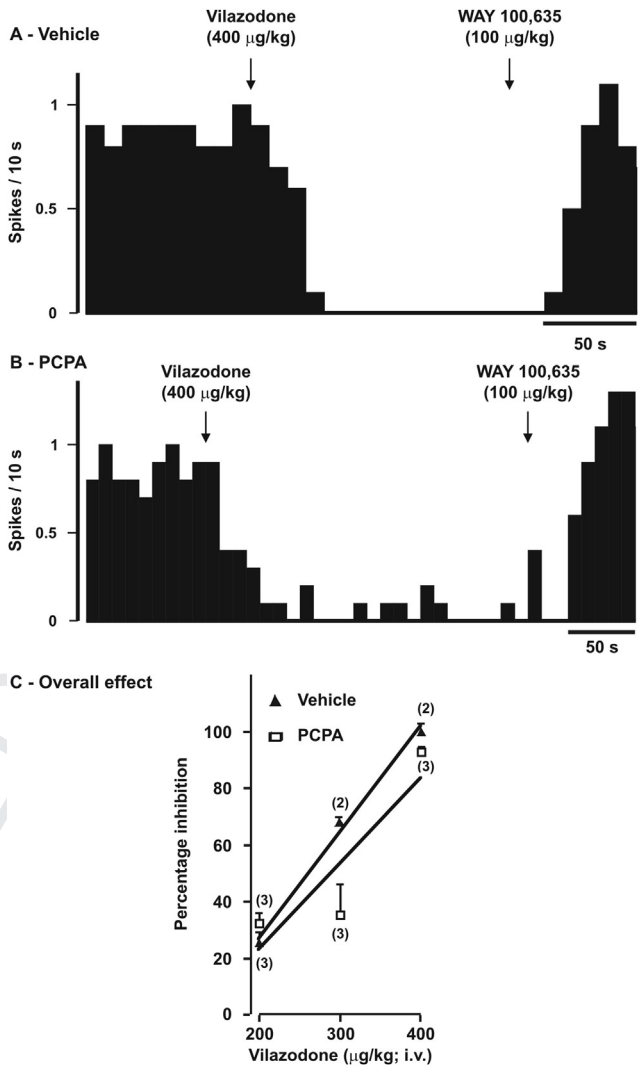

Fig. 1. Illustration of the acute effect of vilazodone (400 μg/kg; i.v.) on firing activity of 5-HT neurons in vehicle-rat (A) and rat administered with PCPA (B). (C) Percentage inhibition of 5-HT neuronal firing shown in response to 200, 300 and 400 μg/kg doses of vilazodone. Linear regression analysis indicates there is no significant difference between 5-HT neurons response to vilazodone following PCPA administration when compared to controls. For each dose, the number of used rats is indicated in brackets. Only one neuron was tested per rat.

2.10. Drugs

Vilazodone hydrochloride was provided by Actavis (Jersey City, NJ, USA). WAY 100,635(N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl] ethyl]-N-2 pyridinylcyclohexanecarboxamide maleate salt), 8-OH-

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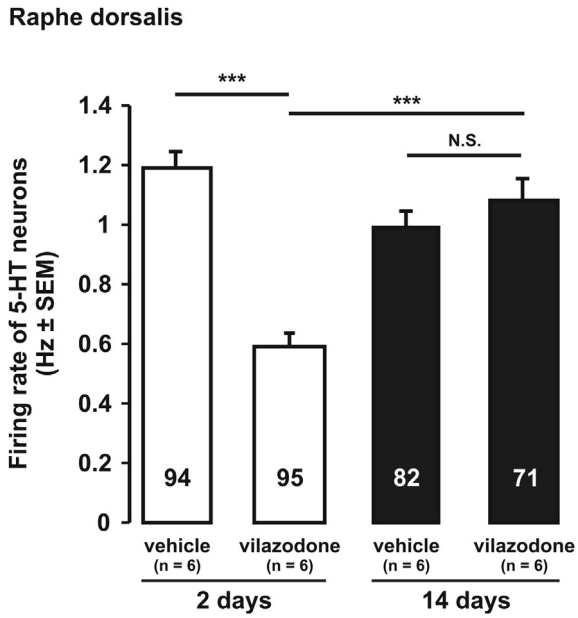

Fig. 2. Mean (±S.E.M.) firing rate of 5-HT neurons in vehicle- and vilazodone-administered rats (5 mg/kg/day; i.p.) for 2 and 14 days. Number of neurons is indicated at the bottom of histograms. Number of rats is indicated in brackets. ***P < 0.001; N.S., non-significant difference.

DPAT ((±)-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide), 5-HT creatinine sulfate (3-[2-Aminoethyl]-5-hydroxyindole creatinine sulfate complex), quisqualic acid (β-(3,5-Dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine, 3-(3,5-Dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine) and PCPA (para-chlorophenylalanine methyl-ester hydrochloride) were purchased from Sigma (St. Louis, MO, USA). Vilazodone was dissolved in 2-hydroxy-beta-cyclodextrin (40%) and injected i.p. in a 3 ml volume for a 300 g rat. 8-OH-DPAT and WAY 100,635 was dissolved in water and doses were not corrected for the salts and given i.v. in 0.1 ml for a 300 g rat.

2.11. Statistical analysis

The data are presented as mean values ± S.E.M. Paired t-test and Mann–Whitney U test were used when a parameter was studied in control and treated rats. Statistical comparisons were carried out using one-way analysis of variance (treatment as the main factor) followed by the Tukey *posthoc* analysis. Dose-response curves were compared using linear regression analysis. For the tonic activation, statistical comparisons were carried out using a Two-way analysis of variance with repeated measures (treatment as the main factor) followed by the Tukey posthoc analysis. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) and SigmaPlot 12 softwares were used. Statistical significance was taken as $p < 0.05$.

3. Results

3.1. Effects of vilazodone on DRN 5-HT neurons

3.1.1. Acute administration

Intravenous administration of vilazodone produced a dose-dependent decrease in 5-HT neuron firing with complete inhibition attained at a dose of 400 μg/kg (Fig. 1). This inhibition was reversed by injection of 100 μg/kg of the selective 5-HT_{1A} receptor antagonist WAY 100,635. To determine if vilazodone inhibited firing activity of 5-HT neurons by acting directly on 5-HT_{1A} autoreceptors or by increasing 5-HT levels in the DRN following 5-HTT

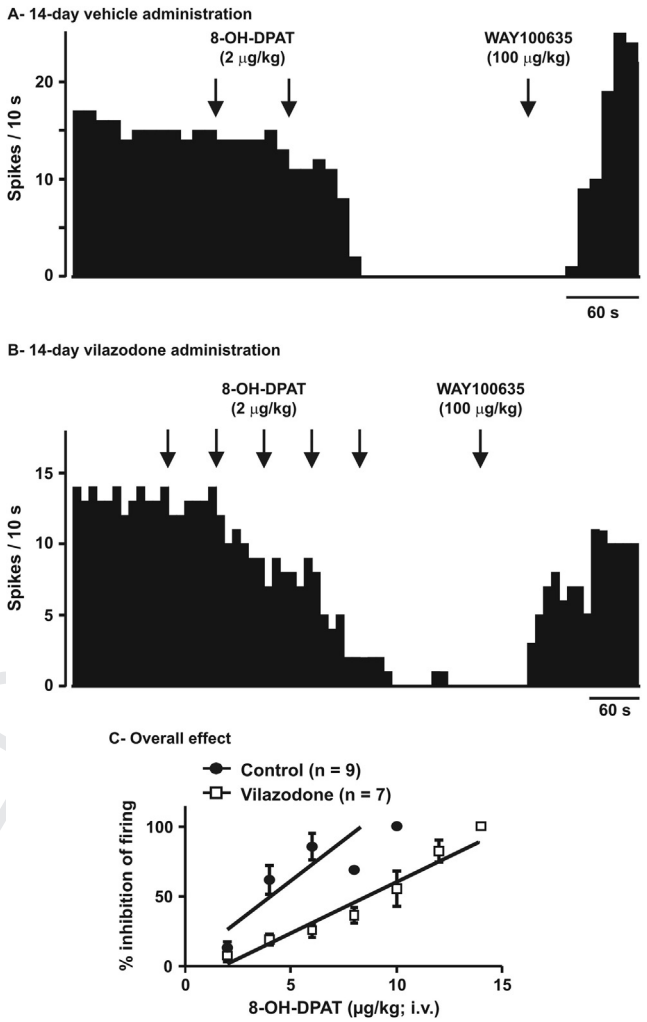

Fig. 3. Representative integrated firing rate histograms of DRN 5-HT neurons illustrating the effect of intravenous administration of the 5-HT autoreceptor agonist 8-OH-DPAT in suppressing neuronal activity in rats administered with vehicle (A) and vilazodone (5 mg/kg/day; B) for 14 days. C, Relationship between the percentage of inhibition of 5-HT neuronal firing activity and doses of 8-OH-DPAT administered intravenously in rats that received vehicle and vilazodone for 14 days. Data are presented as mean ± S.E.M.

blockade, its effect was studied in rats that received PCPA. Hence, the inhibitory effect of vilazodone on 5-HT neuronal firing was not significantly different in 5-HT-depleted rats compared to controls (ED50 for vehicle: 267 μg/kg and 294 μg/kg following PCPA pretreatment; slope: $F[1,12] = 0.67$, $p > 0.05$; Fig. 1).

3.1.2. Repeated administration

3.1.2.1. Effects of 2- and 14-day administration on the firing activity of DRN 5-HT neurons. While 2-day administration of vilazodone (5 mg/kg/day; i.p.) significantly decreased firing activity of 5-HT neurons (50%; $p < 0.001$; One-Way ANOVA followed by Tukey posthoc), there was no longer a significant difference after 14 days of administration when compared to controls (Fig. 2). There was no significant change in bursting activity or in the number of 5-HT neurons per track following 2- and 14-day administration of vilazodone.

The 5-HT autoreceptor agonist 8-OH-DPAT was used as a probe to assess responsiveness of 5-HT_{1A} autoreceptors following repeated administration of vilazodone. A dose-dependent suppression of 5-HT neurons firing was observed with the intravenous

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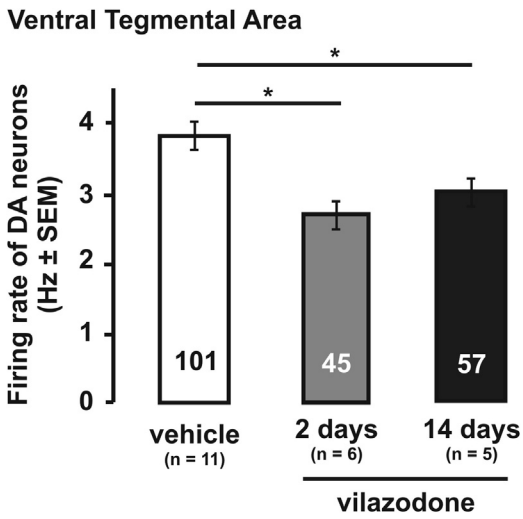

Fig. 4. Mean (±S.E.M.) firing rate of VTA DA neurons in vehicle- and vilazodone-administered rats (5 mg/kg/day; i.p.) for 2 and 14 days. Number of neurons is indicated at the bottom of histograms. Number of rats is indicated in brackets. *P < 0.05.

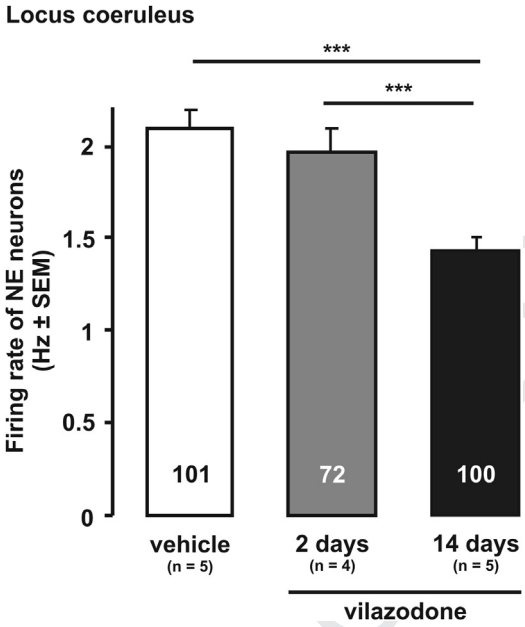

Fig. 5. Mean (±S.E.M.) firing rate of LC NE neurons in vehicle- and vilazodone-administered rats (5 mg/kg/day; i.p.) for 2 and 14 days. Number of neurons is indicated at the bottom of histograms. Number of rats is indicated in brackets. ***P < 0.001.

administration of 8-OH-DPAT in the range of 2–14 μg/kg. In control rats, 8-OH-DPAT completely suppressed the firing activity of 5-HT neurons with an ED50 of 4.1 μg/kg (n = 9), whereas in rats that received vilazodone for 14 days, the dose required for complete inhibition was 8.2 μg/kg (n = 7; Fig. 3). In addition to the altered ED50 values, the slopes of the two dose–response curves were significantly different (Linear regression; F[1,65] = 5.4, p = 0.02; Fig. 3), indicating that the long-term vilazodone regimen resulted in 5-HT_{1A} autoreceptor desensitization.

3.2. Effects of 2- and 14-day administration of vilazodone on the firing activity of VTA DA neurons

There was no significant difference in firing activity of DA neurons in 2- and 14-day vehicle administered rats (2 days administration: 4 ± 0.2 Hz [n = 56] versus 3.7 ± 0.2 Hz [n = 45] following 14 days). These 2 groups were consequently pooled.

Administration of vilazodone (5 mg/kg/day; i.p.) for 2 days significantly decreased firing activity of DA neurons by 28% in comparison to control animals (p < 0.05; One-Way ANOVA followed by Tukey posthoc test; Fig. 4). Following administration of vilazodone for 14 days, the firing rate was still significantly decreased by 21% compared to controls (p < 0.05; One-Way ANOVA followed by Tukey posthoc test; Fig. 5), but not different from that observed after 2-day administration.

As shown in Table 1, significant decreases were also observed in bursts per minute, spikes per burst and percentage in burst of DA neurons following 2- and 14-day administration of vilazodone when compared to controls.

There was no change in the number of DA neurons found per track following 2 and 14 days administration of vilazodone (data not shown).

3.3. Effects of 2- and 14-day administration on the firing activity of LC NE neurons

In rats dosed with vehicle for 2 and 14 days, there was no significant difference in firing activity of NE neurons (2 days administration: 2.1 ± 0.1 Hz [n = 36] versus 2 ± 0.1 Hz [n = 65] after 14 days), therefore these 2 groups were pooled.

The 2-day administration of vilazodone (5 mg/kg/day; i.p.) caused no significant change in NE neurons firing (Fig. 5) in comparison to control animals. However, following 14-day vilazodone regimen, there was a significant decrease in firing activity when compared to control (31%) or 2-day administered rats (27%; p < 0.001; One-Way ANOVA followed by Tukey posthoc test; Fig. 5).

No significant change was observed in number of neurons per track and in bursting activity parameters following both 2- and 14-day administration of vilazodone when compared to controls (data not shown).

3.4. Effect of prolonged administration of vilazodone on the tonic activation of the 5-HT_{1A} receptor on CA3 pyramidal neurons

The effect of WAY 100,635 on firing activity of pyramidal neurons was assessed in controls and in rats that received vilazodone for 14 days (Fig. 6). A two-Way ANOVA (followed by Tukey posthoc test) on tonic activation of 5-HT_{1A} receptors revealed a significant effect of treatment with vilazodone ([F1, 27] = 13.3, p < 0.01), WAY 100,635 (F[1, 27] = 8.5, p < 0.001]) and a significant interaction between both variables (F[1, 27] = 4.9, p < 0.001]. WAY 100,635 did

Table 1
Bursting activity of VTA DA neurons (mean ± S.E.M.). Q3

	Burst/min	Spikes/burst	% Spikes in burst	Number of neurons/track
Vehicle (n = 79)	27 ± 2	3.2 ± 0.1	35 ± 3	1.8 ± 0.1 (11 rats)
2-day vilazodone (n = 34)	7 ± 2***	2.4 ± 0.1***	11 ± 3***	1.5 ± 0.1 (6 rats)
14-day vilazodone (n = 41)	12 ± 2***	2.5 ± 0.1***	15 ± 3**	1.6 ± 0.07 (5 rats)

***p < 0.001, compared to vehicle; n indicates the number of neurons.

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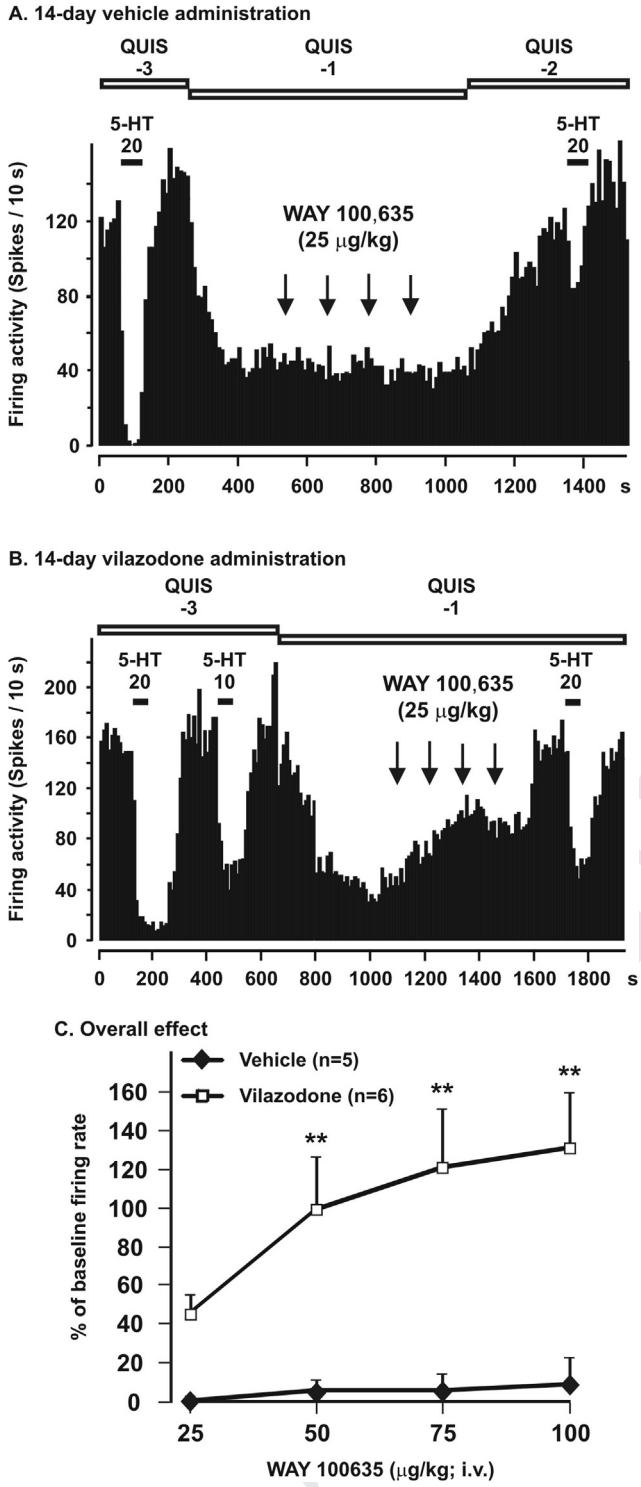

Fig. 6. Integrated firing rate histograms of CA3 pyramidal neuron showing its responsiveness to the microiontophoretic application of 5-HT and i.v. injection of WAY 100,635 in vehicle-administered rats (A; n = 5) and rats administered for 14 days with vilazodone (B; 5 mg/kg/day; i.p.; n = 6). Note the increase in firing activity of pyramidal neurons after the injection of WAY 100,635, after which the inhibitory effect of 5-HT was blocked. (C) Degree (% ± S.E.M.) of increase of the firing activity of pyramidal neurons after the administration of WAY 100,635 in vehicle rats and those administered with vilazodone for 14 days. In each rat only one neuron was tested. ***$p < 0.001$.

not significantly alter firing activity of pyramidal neurons in control rats (n = 5). In rats administered with vilazodone for 14 days (n = 6), WAY 100,635 induced a significant increase of the mean

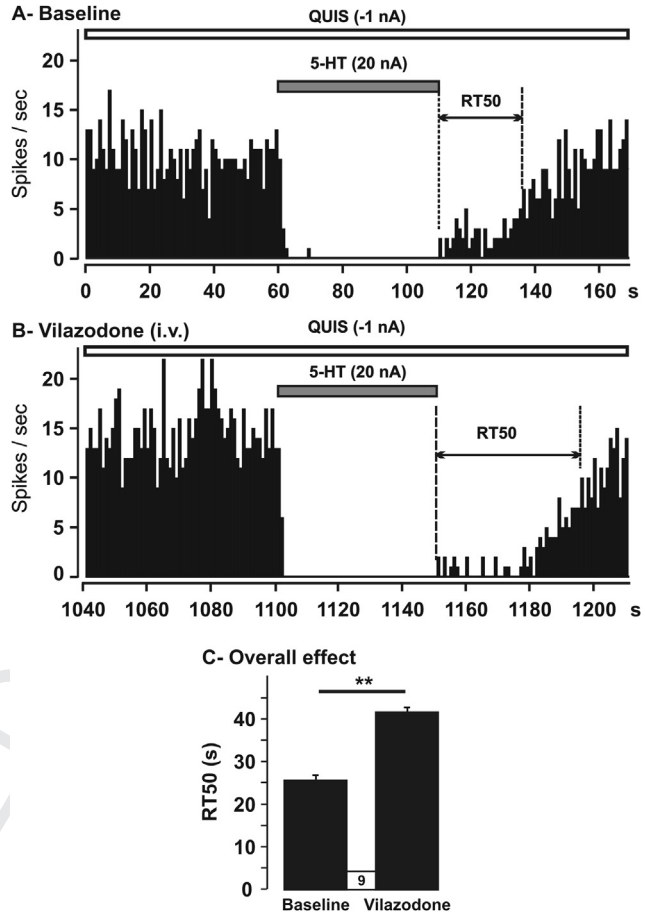

Fig. 7. Integrated firing rate histograms of a pyramidal neuron illustrating time (s) elapsed from the cessation of microiontophoretic application of 5-HT to 50% recovery of the initial firing rate (RT50) before (A) and at least 10 min following acute vilazodone administration (5 mg/kg; i.p.; B). (C) Overall effect showing a significant increase in RT50 values. **$P < 0.01$.

firing activity of pyramidal neurons, when compared to control. This significant enhancement was dose-dependent at 25 and 50 μg/kg, reaching a plateau starting at dose of 75 μg/kg and attaining 131% at 100 μg/kg.

3.5. Sensitivity of 5-HT_{1A} receptors on CA3 pyramidal neurons following 14-day administration of vilazodone

The next step was to determine if there was a change in the responsiveness of 5-HT_{1A} receptors on pyramidal neurons in rats that received vilazodone. Number of spikes suppressed by nA of 5-HT were determined as a measure of sensitivity of 5-HT_{1A} receptors. There was no alteration in sensitivity of the postsynaptic 5-HT_{1A} receptors, when 5-HT was microiontophoretically applied at currents of 20 nA (number of spikes suppressed by nA in control: 31 ± 2; n = 17, 14-day vilazodone: 36 ± 2; n = 16; $p < 0.05$; Mann–Whitney Rank Sum Test). This result show that sensitivity of the postsynaptic 5-HT_{1A} receptors was not changed after repeated administration of vilazodone.

3.6. In vivo 5-HTT blockade properties of vilazodone

Ejection of 5-HT at a current of 20 nA for 50 s was kept constant and produced inhibition of CA3 pyramidal neurons firing at about 80–100%. Only neurons that were completely inhibited were considered for RT50 analysis, as the RT50 value is better

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determined under this condition.

In order to assess the degree of in vivo reuptake inhibition by vilazodone in the hippocampus, the time elapsed from the cessation of microiontophoretic application of 5-HT to 50% recovery of the initial firing rate (RT50) was determined. Hence, the mean RT50 for control rats administered with vehicle was 29 ± 3 s (n = 15) and did not change following 14-day administration of vilazodone (33 ± 3 s; n = 15; $p > 0.05$).

This result was unexpected given that vilazodone has a subnanomolar affinity for 5-HTT. In order to ascertain that the apparent lack of blockade of 5-HTT was due to a regimen producing a subthreshold effect using this approach, we determined the effect of an acute dose of vilazodone on 5-HTT. As shown in Fig. 7, a single dose of vilazodone (5 mg/kg; i.p.) significantly increased the RT50 value by 62% (control: 26 ± 2 s; vilazodone: 42 ± 4 s, n = 9; $p < 0.01$; Paired t-test), confirming that vilazodone has the capacity to block 5-HTT in the hippocampus.

4. Discussion

The present in vivo electrophysiological experiments confirmed in vitro studies showing that vilazodone has a dual action at 5-HT_{1A} receptors because it inhibited 5-HT neurons firing in 5-HT depleted rats and on 5-HTT because it prolonged the effect of exogenously-applied 5-HT. Repeated administration of vilazodone (5 mg/kg/day) failed, however, to show blockade of 5-HTT in the hippocampus due may be to suboptimal effect on 5-HTT. Prolonged administration of vilazodone resulted in 5-HT neurons firing at normal level after 5-HT_{1A} autoreceptors desensitization. While the sensitivity of the postsynaptic 5-HT_{1A} receptors did not change, their tonic activation was enhanced, an effect similar to that observed with all SSRIs and 5-HT receptor agonists so far tested (Blier and El Mansari, 2013).

Acute injection of vilazodone resulted in an inhibition of 5-HT neurons firing activity, stemming from an activation of the 5-HT_{1A} autoreceptors. Such an effect could have resulted from either enhanced synaptic levels of 5-HT following inhibition of 5-HTT, or from direct activation of 5-HT_{1A} autoreceptor by vilazodone, as the latter is a 5-HT_{1A} receptor agonist. To examine these two possibilities, rats were pre-treated with the 5-HT synthesis inhibitor PCPA (300 mg/kg for 3 days). Indeed, it was already reported that after depletion of endogenous stores of 5-HT with PCPA, 5-HT reuptake and MAO inhibition no longer inhibit 5-HT neuronal firing (Aghajanian et al., 1970; Trulson and Crisp, 1986; Cunningham and Lakoski, 1990). Similarly, in markedly depleted levels of 5-HT after PCPA, vilazodone was still effective in suppressing the firing activity of 5-HT neurons. This indicated that inhibition of 5-HT neurons firing after acute administration of vilazodone is mostly due to its activation of 5-HT_{1A} autoreceptors. Interestingly, using the same rationale, it was shown that the effect of acute injection of VN2222, which is also a 5-HT_{1A} receptor agonist and 5-HTT blocker, is mainly related to a direct action on 5-HT_{1A} autoreceptors (Romero et al., 2003).

Although vilazodone inhibited firing activity of 5-HT neurons following 2-day administration, this activity recovered after repeated administration, indicating that the 5-HT_{1A} autoreceptor had desensitized since the inhibitory effect of 8-OH-DPAT on 5-HT neurons firing activity was attenuated in vilazodone-administered rats. Interestingly, sustained administration of VN2222, which possesses similar pharmacological properties as vilazodone also resulted in desensitization of 5-HT_{1A} autoreceptors (Romero et al., 2003). This same adaptation occurs following sustained 5-HTT blockade by SSRIs or activation of 5-HT_{1A} receptors by 5-HT_{1A} receptor agonists (e.g. gepirone) allowing a restoration of 5-HT neuron firing to control level (Hadrava et al., 1995; Czachura and Rasmussen, 2000; Blier and De Montigny, 1983; El Mansari et al., 2005; Blier and El Mansari, 2013).

Despite evidence showing that vilazodone is a 5-HT_{1A} receptor agonist (Page et al., 2002; Heinrich et al., 2004; see also Dawson and Watson, 2009), the fact that it produces an elevation of 5-HT concentrations in the frontal cortex that is comparable to that obtained with a combination of the SSRI paroxetine and WAY 100,635 has led to the suggestion that vilazodone may act as a 5-HT_{1A} autoreceptor antagonist (Hughes et al., 2005). In support of this, the vilazodone-induced increase of 5-HT levels was attenuated by the addition of 8-OH-DPAT in the ventral hippocampus even though to a lesser extent in the frontal cortex (Page et al., 2002). Should vilazodone have been a 5-HT_{1A} autoreceptor antagonist, it would have been expected to prevent dampening of the firing activity of 5-HT neurons seen with 2-day regimen of vilazodone in the present study (Fig. 2). This is strengthened by the fact that previous study showing that blockade of 5-HTT by paroxetine decreases firing activity of 5-HT neurons after 2-day administration, but failed to do so after its combination with the 5-HT_{1A} autoreceptor antagonist/partial agonist pindolol (Romero et al., 1996). These observations, together with results showing an inhibition of 5-HT neuronal firing with vilazodone suggest it is acting more as an agonist for 5-HT_{1A} autoreceptors. It is worth noting that a drug endowed with 5-HT_{1A} receptor agonism may act as partial or full agonist at 5-HT_{1A} and may yield differential effect on 5-HT elements, as reported in Page et al. (2002). For instance, flibanserin, a 5-HT_{1A} receptor agonist/5-HT_{2A} receptor antagonist is a partial agonist in the hippocampus but a full agonist in the frontal cortex (Rueter et al., 1998).

In the hippocampus, the current study showed that sustained administration of vilazodone did not change the sensitivity of 5-HT_{1A} receptors located on pyramidal neurons, in line with studies showing that 5-HT_{1A} receptor agonists (Haddjeri et al., 1998) or SSRIs do not alter this parameter following sustained administration (e.g. escitalopram; El Mansari et al., 2005; Blier and El Mansari, 2013). Nevertheless, long-term administration of the monoamine oxidase inhibitor (MAOI) clorgyline and electroconvulsive shocks, respectively, decrease and increase sensitivity of these receptors (de Montigny, 1984; Blier et al., 1986).

Several lines of evidence confirmed the capacity of vilazodone to inhibit 5-HT reuptake in cells expressing human 5-HTT (Dawson and Watson, 2009) and ex vivo studies using [^{3}H]DASB ligand (Hughes et al., 2005). The present data show, however, that prolonged administration of vilazodone did not induce an alteration of the RT50 values (which reflects blockade of 5-HTT), even though studies from our laboratory have previously shown that RT50 values triple following SSRIs administration (Piñeyro et al., 1994; El Mansari et al., 2005). It is possible that the dose used in this study (5 mg/kg/day) was insufficient to induce a strong occupation of 5-HTT sites and a detectable 5-HTT inhibition by this method that uses exogenous application of 5-HT. Indeed, a prior ex vivo study showed that vilazodone at a dose of 3 mg/kg/day (p.o.) produces only 60% of 5-HTT occupation (Hughes et al., 2005). Interestingly, further experiments in the present study confirmed that higher bioavailability of vilazodone following acute administration (5 mg/kg; i.p.) did significantly increase RT50 values, strengthening the assumption that the lack of effect of repeated vilazodone on RT50 was may be due to lower drug bioavailability and consequently suboptimal occupation of 5-HTT. Thus, it appears that suboptimal occupation of 5-HTT is more easily detected through paradigms using endogenous 5-HT. With the SSRI paroxetine, for instance, a dose that completely suppresses DRN 5-HT neurons firing only marginally prolongs the RT50 in the hippocampus (Piñeyro et al., 1994). Therefore, with the repeated administration regimen used in the present study the effect induced by vilazodone is most likely due to an activation of 5-HT_{1A} receptors, although its action on 5-

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HTT may also contribute (see below discussion on the effects of vilazodone on DA and NE neurons).

All antidepressant treatments studied so far showed an enhancement of tonic activation of the 5-HT_{1A} receptors located on CA3 pyramidal neurons (Blier and El Mansari, 2013). Particularly, it was shown that prolonged administration of 5-HT_{1A} receptor agonists and SSRIs significantly increase tonic activation of the 5-HT_{1A} receptors in projection areas (Haddjeri et al., 1998; El Mansari et al., 2005; Blier and El Mansari, 2013). Accordingly, the present data showed that prolonged administration of vilazodone significantly increased tonic activation of the 5-HT_{1A} receptors following enhancement of 5-HT levels (Hughes et al., 2005; Dawson and Watson, 2009).

Several studies have shown that acute and sustained administration of SSRIs produced a decrease in firing and bursting activity of VTA DA neurons (Prisco and Esposito, 1995; Di Mascio et al., 1998; Dremencov et al., 2009; Chernoloz et al., 2009). Similarly, the current work demonstrated that two- and 14-day administration of vilazodone attenuated the firing and burst activity of DA neurons. It is likely that after 5-HT neurons regained their normal level of firing following 14-day vilazodone administration, the subsequent increase in 5-HT levels as showed by Hughes et al. (2005) may explain, at least in part, the observed decrease of DA neurons activity. Indeed, since 5-HT neurons exert a tonic inhibitory effect on DA neurons (Guiard et al., 2008; for review see Di Giovanni et al., 2008) and that selective lesion of 5-HT neurons with **5,7**-dihydroxytryptamine completely prevents the increase in the firing rate of DA neurons induced by 8-OH-DPAT (Prisco et al., 1994), it is postulated that the effect of vilazodone on DA neurons firing is through the 5-HT system. On the other hand, systemic administration of selective 5-HT_{1A} receptor agonists has been shown to enhance the firing and burst activity of the majority of midbrain DA neurons (Arborelius et al., 1993; Prisco et al., 1994; Lejeune and Millan, 1998). Possibly, the smaller attenuation of DA neurons firing and burst activities by repeated administration of vilazodone (28%) compared to that following SSRIs (40–50%; Chernoloz et al., 2009; Dremencov et al., 2009), resulted from the combination of inhibitory 5-HT_{2C} receptors activation by endogenous 5-HT and excitatory 5-HT_{1A} receptor agonism by vilazodone.

The lack of effect of the 2-day vilazodone regimen on LC NE neurons firing activity is consistent with a microdialysis study showing no alteration in levels of NE in the frontal cortex (Hughes et al., 2005). However, the inhibitory effect of 14-day administration on the firing activity of NE neurons could be due to 5-HTT blockade, despite not being optimal. Albeit 2- and 14-day administration of vilazodone did not change bursting activity of NE neurons, the present study showed that repeated regimen of vilazodone decreased their firing activity (33%), although to lesser extent than with SSRIs (50%; Szabo et al., 2000; Ghanbari et al., 2010; Chernoloz et al., 2009). It is indeed documented that an enhancement of 5-HT tone following 5-HTT blockade in the LC region results in the activation of postsynaptic 5-HT_{2A} receptors that are inhibitory on NE neurons (Haddjeri et al., 1997; Szabo and Blier, 2001b). On the other hand, previous electrophysiological experiments also showed that 5-HT_{1A} receptor agonists (e.g. gepirone, buspirone, ipsapirone and 8-OH-DPAT) increase firing activity of NE neurons (Piercey et al., 1994; Szabo and Blier, 2001b), an action reversed by systemic injection of WAY 100,635 (Szabo and Blier, 2001b). It is interesting that 14-day administration of vilazodone resulted in a decrease in firing rate of NE neurons in the present study, despite the fact that the latter drug is a 5-HT_{1A} receptor agonist. However, it is possible that desensitization of the 5-HT_{1A} receptor controlling NE neurons is responsible for this lack of activation under vilazodone, as was previously demonstrated with the SSRI citalopram (Szabo et al., 2000). Similarly to its effect on DA neurons, the relatively weaker inhibitory effect of vilazodone compared to SSRIs on NE neurons is likely due to a concomitant enhancement of inhibitory 5-HT neurotransmission and still a certain degree of activation by 5-HT_{1A} receptors.

Finally, the dose of vilazodone used in the current experiments (5 mg/kg, i.p.) was within the therapeutic range thus giving clinical relevance to our observations. Indeed, a dose of 4 mg/kg of vilazodone given by oral gavage to rats has been shown to result in peak plasma concentrations of ~30 ng/ml (Sui et al., 2014) which is the peak plasma concentration obtained after administration of a clinically effective dose of 20 mg in humans (Boinpally et al., 2013).

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Research Article

RESEARCH ARTICLE

Restoration of Serotonin Neuronal Firing Following Long-Term Administration of Bupropion but Not Paroxetine in Olfactory Bulbectomized Rats

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Abstract

Background: Olfactory bulbectomized rats generally manifest many of the neurochemical, physiological, and behavioral features of major depressive disorder in humans. Another interesting feature of this model is that it responds to chronic but not acute antidepressant treatments, including selective serotonin reuptake inhibitors. The purpose of the present study was first to characterize the firing activity of dorsal raphe serotonin neurons in olfactory bulbectomized rats and then examine the effects of 2 antidepressants, bupropion and paroxetine.
Methods: Olfactory bulbectomy was performed by aspirating olfactory bulbs in anesthetized rats. Vehicle and drugs were delivered for 2 and 14 days via subcutaneously implanted minipumps. In vivo electrophysiological recordings were carried out in male anesthetized Sprague-Dawley rats.
Results: Following ablation of olfactory bulbs, the firing rate of serotonin neurons was decreased by 36%, leaving those of norepinephrine and dopamine neurons unchanged. In olfactory bulbectomized rats, bupropion (30 mg/kg/d) restored the firing rate of serotonin neurons to the control level following 2- and 14-day administration and also induced an increase in the tonic activation of serotonin$_{1A}$ receptors; paroxetine (10 mg/kg/d) did not result in a return to normal of the attenuated firing of serotonin neurons in olfactory bulbectomized rats. In the hippocampus, although at a higher dose of WAY 100635 than that required in bupropion-treated animals, paroxetine administration also resulted in an increase in the tonic activation of serotonin$_{1A}$ receptors.
Conclusions: The present results indicate that unlike paroxetine, bupropion administration normalized serotonin neuronal activity and increased tonic activation of the serotonin$_{1A}$ receptors in hippocampus.

Keywords: electrophysiology, serotonin, bupropion, paroxetine, olfactory bulbectomy

Introduction

Several behavioral, physiological, and neurochemical adaptations taking place following bilateral olfactory bulbectomy (OBX) in rats can be found in patients with major depressive disorder (Jesberger and Richardson, 1986; Song and Leonard, 2005). These effects of OBX include hyperactivity to mild stress (Baumbach and Sieck, 1977; Kelly et al., 1997), cognitive impairment (van Rijzingen et al., 1995; Yamamoto et al., 1997), circadian rhythm and sleep disruptions (Lumia et al., 1992), perturbations of eating

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habits (Meguid et al., 1993), and enhanced levels of corticosteroids (Cairncross et al., 1979; Jesberger and Richardson, 1986).

The most replicated behavioral characteristic of this model is the increase in locomotor activity seen, in the open field, following olfactory bulb ablation (van Riezen et al., 1977; Cryan et al., 1999; Breuer et al., 2008). It is also well established that chronic but not acute administration of various drugs, such as the selective serotonin (5-HT) reuptake inhibitors (SSRIs) fluoxetine (Mato et al., 2010), paroxetine (Cryan et al., 1999), and escitalopram (for review, see Song and Leonard, 2005; Breuer et al., 2007), can reverse behavioral hyperactivity observed in OBX rats in the open field. Furthermore, in the latter study, reversal of hyperactivity was no longer present 10 weeks after cessation of drug administration (Breuer et al., 2007).

Removal of the olfactory bulbs in rats also results in alterations of 5-HT, norepinephrine (NE), and dopamine (DA) systems (Tonnaer et al., 1980; Masini et al., 2004; Slotkin et al., 2005; Song and Leonard, 2005). Particularly, changes in 5-HT synthesis rate, 5-HT levels, 5-HT transporters (5-HTTs), and 5-HT receptor densities are reported in different brain structures following OBX (Zhou et al., 1998; Watanabe et al., 2003; Hasegawa et al., 2005; van der Stelt et al., 2005; Saitoh et al., 2007; Sato et al., 2010).

Preclinical studies show that antidepressant treatments increase 5-HT neurotransmission with a time course that is consistent with their delayed therapeutic effect. This enhancement would, however, be mediated through different mechanisms. Whereas bupropion increases discharge frequency of 5-HT neurons after 2 and 14 days of administration in naïve rats (El Mansari et al., 2008; Ghanbari et al., 2010), SSRIs, including paroxetine, decrease 5-HT neuronal activity after 2 days of administration that recovers to the control level only after 2 to 3 weeks of administration (Pineyro and Blier, 1999; Czachura and Rasmussen 2000; El Mansari et al., 2005). Hence, long-term administration of SSRIs increases 5-HT levels in the projecting area following recovery of firing of 5-HT neurons to control level and desensitization of the terminal 5-HT_{1B} autoreceptor (Pineyro and Blier, 1999). An increase of 5-HT transmission was also obtained by short- and long-term administration of bupropion but also in a time-dependent fashion (Ghanbari et al., 2010, 2011). In contrast, sustained bupropion administration (2 days) increases the firing activity of 5-HT neurons above normal, partly because it desensitizes the 5-HT_{1A} autoreceptor through an indirect noradrenergic mechanism. In addition, prolonged administration (14 days) of bupropion desensitizes the α_2-adrenergic heteroreceptors on 5-HT terminals (Ghanbari et al., 2011), thereby delineating their primary site of action on the NE system.

The aim of this study was to determine how 2 antidepressant drugs with different mechanisms of action, the SSRIs paroxetine and bupropion, act on in vivo characteristics of the 5-HT system in an OBX rat model, where several 5-HT alterations have taken place. The choice of these 2 antidepressant drugs stemmed mainly from their different mechanism of action on 5-HT neurons.

Methods

Animals

The in vivo electrophysiological experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing between 300 and 500 g at the time of recordings. Upon arrival, the rats were allowed to acclimatize to their new environment for 1 week prior to start of any drug administration or experiment. The animals were kept, 2 per cage, under standard laboratory conditions (12:12-hour–light/–dark cycle with access to food and water ad libitum). Following surgery, control and OBX rats were kept individually in cages. All the experiments were approved by the local Animal Care Committee (Institute of Mental Health Research) and conducted in accordance with the Canadian Council on Animal Care) for the care and use of laboratory animals.

OBX Surgery

Bilateral OBX was performed in rats anesthetised with equitezine (Marcilhac et al., 1999). For olfactory bulb ablation, rats were mounted in a stereotaxic frame. A medline incision was made extending at least 1 mm to bregma. A burr hole was drilled at 7 mm anterior to bregma and 2.5 mm either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. After a localized electrical coagulation of the dura and sagittal sinus, olfactory bulbs were removed by suction with a vacuum pump attached to a Pasteur pipette. The burr hole was filled with haemostatic sponges and the wound was closed with sterile sutures. Sham-operated rats were treated similarly, with the exception that olfactory bulbs were not removed. After surgery, animals were injected 5 mL intraperitoneally (i.p.) with 5% glucose and temgesic for postsurgical analgesia. The animals were allowed to recover for 14 days following surgery, at which time behavioral changes occur (Lucas et al., 2007). On the 14th to 18th days following surgery, the rats were recorded or implanted with minipumps for drug administration. Only rats that showed complete ablation of the olfactory bulbs and no damage to the frontal cortex were included for data analysis.

Assessment of Hyperlocomotion in OBX Rats

The open field chamber consisted of a square plastic box (85 × 85 × 70 cm). Since conditions of very high brightness are crucial to observe the hyperactivity in OBX rats (Mar et al., 2000; Song and Leonard, 2005), the inner faces of the walls were covered with aluminium foil and illumination was provided by a 100-watt bulb. Each rat was placed into the center of the open field, and exploratory activity was monitored by a video camera fixed above the arena and relayed to a computer system that calculates the walked distance during the first 5 minutes (Viewpoint Life Sciences, Lyon, France). All experiments were performed between 8:30 and 10:30 AM.

Minipump Implantation and Electrophysiological Experiments

The rats were anesthetized with isoflurane to implant, subcutaneously, the osmotic Alzet minipumps (Alza, Palo Alto, CA) to ensure slow and steady release of bupropion (30 mg/kg/d; Dong and Blier, 2001), paroxetine (10 mg/kg/d; Besson et al., 2000), or vehicle (water for bupropion, 50% water + 50% ethanol for paroxetine) for 2, 14, and 28 days. The electrophysiological experiments were carried out with the minipump in place. Prior to the electrophysiological recordings, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Supplemental doses of the anesthetic (100 mg/kg, i.p.) were given to maintain constant anesthesia and prevent any nociceptive reaction to pinching of the hind paws. Body temperature was maintained at 37°C throughout the experiment with a heating pad. Prior to the electrophysiological experiments, a catheter was inserted in a lateral tail vein for systemic intravenous injection of pharmacological agents.

Electrophysiological Recordings of Dorsal Raphe Nucleus (DRN) 5-HT Neurons

In vivo extracellular recordings of presumptive 5-HT neurons were obtained using single-barrel glass micropipettes. The impedance of the electrodes was between 4 and 6 MΩ. The microelectrodes were positioned 0.9 to 1.2 mm anterior to lambda on the midline and lowered into the DRN. The 5-HT neurons were encountered over a distance of 1 mm starting immediately below the ventral border of the Sylvius aqueduct. These neurons were identified by their slow (0.5–2.5 Hz), regular firing rate and a long duration (0.8–1.2 ms) positive action potential (VanderMaelen and Aghajanian, 1983).

Electrophysiological Recordings of Locus Coeruleus NE Neurons

NE neurons were reliably recorded at the following coordinates (in millimeters from lambda): anterior-posterior (AP), -1.0 to -1.2; median-lateral (ML), 1.0 to 1.3; dorsal-ventral (DV), 5.0 to 7.0. They were identified by their regular firing rate (1–5 Hz) and their positive action-potential duration, which is between 0.8 and 1.2 milliseconds. They can also be identified by a brief neuronal excitation to a nociceptive pinch of the contralateral hind paw followed by a short period of inhibition (see Szabo et al., 2000).

Electrophysiological Recordings of Ventral Tegmental Area (VTA) DA Neurons

DA neurons were reliably recorded at the following coordinates (in millimeters from lambda): AP, 3.2 to 3.6; ML, 0.9 to 1.1; DV, 7.0 to 9.0. They were identified by their established electrophysiological criteria (Ungless and Grace, 2012), including: (1) spontaneous firing rate between 2 and 90 spikes/s, exhibiting bursting activity or irregular firing; (2) biphasic or triphasic waveforms, with an initial positive deflection (usually notched followed by prominent negative phase; (3) long duration potential (2–4 milliseconds); and (4) low pitch sound when monitored by an audioamplifier. A duration >1.1 milliseconds from the start of the action potential to the center of the negative trough was also used to determine the DA neuron identity (Ungless and Grace, 2012).

Bursts Analysis

The firing patterns of the monoaminergic neurons were analyzed by interspike interval (ISI) burst analysis. The onset of a burst was signified by the occurrence of 2 spikes with ISI <0.08 seconds for NE and DA and ISI <0.01 seconds for 5-HT. The termination of a burst was defined as an ISI >0.16 seconds for NE and DA and ISI >0.01 seconds for 5-HT (Grace and Bunney, 1983; Hajos and Sharp, 1996; Dawe et al., 2001).

Extracellular Recording and Microiontophoresis of Dorsal Hippocampus CA3 Pyramidal Neurons

Extracellular recording and microiontophoresis of CA3 pyramidal neurons were performed with 5-barreled glass micropipettes. The impedance of the electrodes ranged from 2 to 4 MΩ. The central barrel used for the unitary recording was filled with a 2-M NaCl solution, and the 4 side barrels were filled with the following solutions: 5-HT creatinine sulfate (10 mM in 200 mM NaCl, pH 4), quisqualic acid (1.5 mM in 200 mM NaCl, pH 8), and a 2-M NaCl solution used for automatic current balancing. The electrodes were lowered into the dorsal CA3 region of the hippocampus using the following coordinates: 4 mm anterior to lambda and 4.2 mm lateral. Pyramidal neurons were encountered at a depth of 4.0 ± 0.5 mm below the surface of the brain. Because these neurons do not spontaneously discharge in chloral hydrate anesthetized rats, they were activated within their physiological firing range (10–15 Hz; Kandel and Spencer, 1961) by a small current of quisqualate in nanoamperes (nAs; using Harvard Apparatus module, Montreal, Canada). The CA3 pyramidal neurons were identified by their large amplitude (0.5–1.2 mV) and long-duration simple action potentials, alternating with complex spike discharges.

5-HT_{1A} Receptor Sensitivity

Neuronal responsiveness of the CA3 pyramidal neurons after 5-HT_{1A} receptors activation was determined by microiontophoretically applying 5-HT (in nA) for 50 seconds and measuring the number of spikes suppressed per nA. The more spikes are suppressed per nA, the more sensitive are the receptors.

Determination of 5-HT Reuptake Inhibition

To determine the degree to which the 5-HTTs were blocked by paroxetine, the 50% recovery time (RT50) was measured by determining the time (in seconds) following a 50-second ejection period of 5-HT to recover 50% of the initial firing rate (Pineyro et al., 1994).

Statistics

The data are presented as mean values ± SEM. Statistical comparisons were performed using the Mann-Whitney U test or Wilcoxon signed rank test when a parameter was studied in control and treated rats. For DRN experiments, statistical comparisons were carried out using a 1-way analysis of variance (ANOVA) on ranks followed by Dunn's method for all pairwise multiple comparison procedures. For the tonic activation, statistical comparisons were carried out using a 2-way analysis of variance with repeated measures (treatment as the main factor) followed by the Tukey posthoc analysis. Statistical significance was taken as $P < .05$.

Drugs

Bupropion and paroxetine (Sequoia Research Products, Pangbourne, UK). WAY 100635, 5-HT creatinine sulfate, and chloral hydrate were purchased from Sigma (St. Louis, MO). Quisqualic acid was purchased from Tocris (Ellisville, MO).

Results

Open Field

Following ablation of their olfactory bulbs, rats were significantly more active compared with shams in the open field (total distance traveled: prebulbectomy, 1357 ± 222 cm [12 rats], postbulbectomy: 2809 ± 406 cm [12 rats]; $P < .01$; Mann-Whitney U test). Rats tested in the behavioral paradigm were not used for electrophysiology experiments.

DRN 5-HT Neurons Activity in OBX Rats

Sham rats from different treatment groups were pooled together, because there was no significant difference in their 5-HT neuron

firing activity. Fourteen to 18 days following OBX, the average firing rate of 5-HT neurons was significantly decreased by 36% compared with control (Figure 1; $P<.001$, 1-way ANOVA). However, the average firing activity returned to control levels 4 weeks after the olfactory ablation (Figure 1; $P>.05$, 1-way ANOVA).

The mean number of neurons found per track was not changed in OBX rats compared with controls (control group: 4±0.2, N=17 rats; OBX group: 4.4±0.5, N=9 rats; $P>.05$; Mann-Whitney U test).

Locus Coeruleus NE Neurons Activity in OBX Rats

OBX resulted in no change in the neuronal firing rate of NE neurons. However, although the number of neurons displaying burst activities was increased (22% in control [90 neurons in 5 rats] vs 42% in OBX rats [115 neurons in 7 rats], Mann-Whitney U, $P=.003$), the percentage of spikes in bursts was not changed (control: 5±1 [20 neurons]; OBX: 7±0.9 [48 neurons], t test, $P>.05$).

VTA DA Neurons Activity in OBX Rats

There was no change in firing rate of VTA DA neurons following olfactory bulb ablation (sham: 5±0.2 Hz, 56 neurons in 5 rats; OBX: 5±0.3 Hz, 64 neurons in 6 rats). Moreover, there was no change in bursting activity of VTA DA neurons (percentage of spikes in bursts, control: 29±3 [in 5 rats]; OBX: 34±4 [in 6 rats]).

Effect of Paroxetine and Bupropion on the Firing Activity of 5-HT Neurons in OBX Rats

Because the firing activity of NE and DA neurons was largely unaffected by OBX, the effects of 2 antidepressants with distinct mechanisms of action were examined only on the 5-HT system.

After 14 to 18 days following OBX, rats were administered either bupropion (30 mg/kg/d) or paroxetine (10 mg/kg/d) for short and long periods. Two days of bupropion administration to OBX rats resulted in recovery to normal of the dampened firing activity of 5-HT neurons compared with OBX rats receiving vehicle (Figure 1; $P < .01$, 1-way ANOVA). Following a 14-day administration of bupropion, the firing rate of 5-HT neurons in OBX rats remained at control levels (Figure 1).

In OBX rats administered with paroxetine for 2, 14, and 28 days, the discharge activity of 5-HT neurons remained significantly inhibited by 58%, 58%, and 42%, respectively, compared with rats treated with vehicle (Figure 1; $P < .01$, 1-way ANOVA). The inhibition of 5-HT neurons firing seen in OBX rats that received paroxetine (for 2 and 14 days) was reversed to control discharge level after the injection of 100 µg of the selective 5-HT_{1A} receptor antagonist WAY 100635 (control group: 1.2±0.1 Hz; 2-day treated group: 1.1±0.1 [23 neurons in 2 rats]; 14-day treated group: 1.1±0.1 [39 neurons in 2 rats]).

Effect of Bupropion and Paroxetine on the 5-HT Elements in the Hippocampus of OBX Rats

For all CA3 pyramidal neurons, iontophoretic application of 5-HT induced an inhibition of their firing activity (Figure 2). To determine the sensitivity of the 5-HT_{1A} receptors located on pyramidal neurons, the number of action potentials suppressed by 5-HT ejection (nA) was calculated. Short- and long-term administration of bupropion did not modify the suppressant effect of 5-HT on firing activity of CA3 pyramidal neurons. The mean number of spikes suppressed per nA was not significantly different in OBX rats administered paroxetine and bupropion for 14 days compared with OBX rats treated with vehicle (spikes suppressed per nA; vehicle group: 8±1 [n=7]; paroxetine group: 9±1 [n=8] and 7±1 [n=6] in the bupropion group; $P>.05$; 1-way ANOVA).

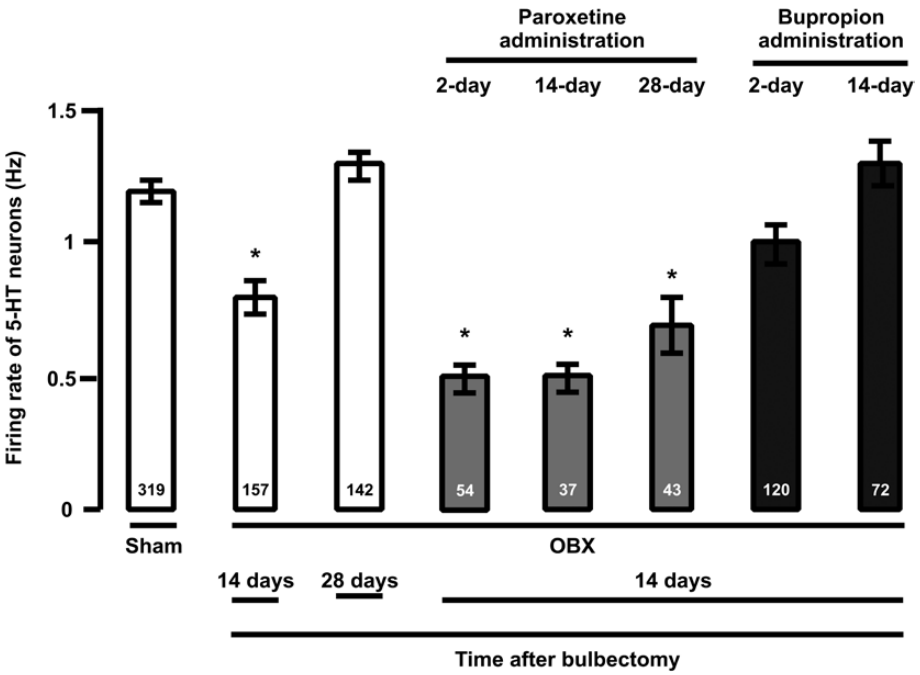

Figure 1. Mean (±SEM) of the firing rate of dorsal raphe nucleus (DRN) serotonin (5-HT) neurons in control and olfactory bulbectomy (OBX) rats that received vehicle, paroxetine (10 mg/kg/d) or bupropion (30 mg/kg/d), for 2, 14, and 28 days. The number at the bottom indicates the total number of neurons recorded in each group. **$P<.01$ and *$P<.05$.

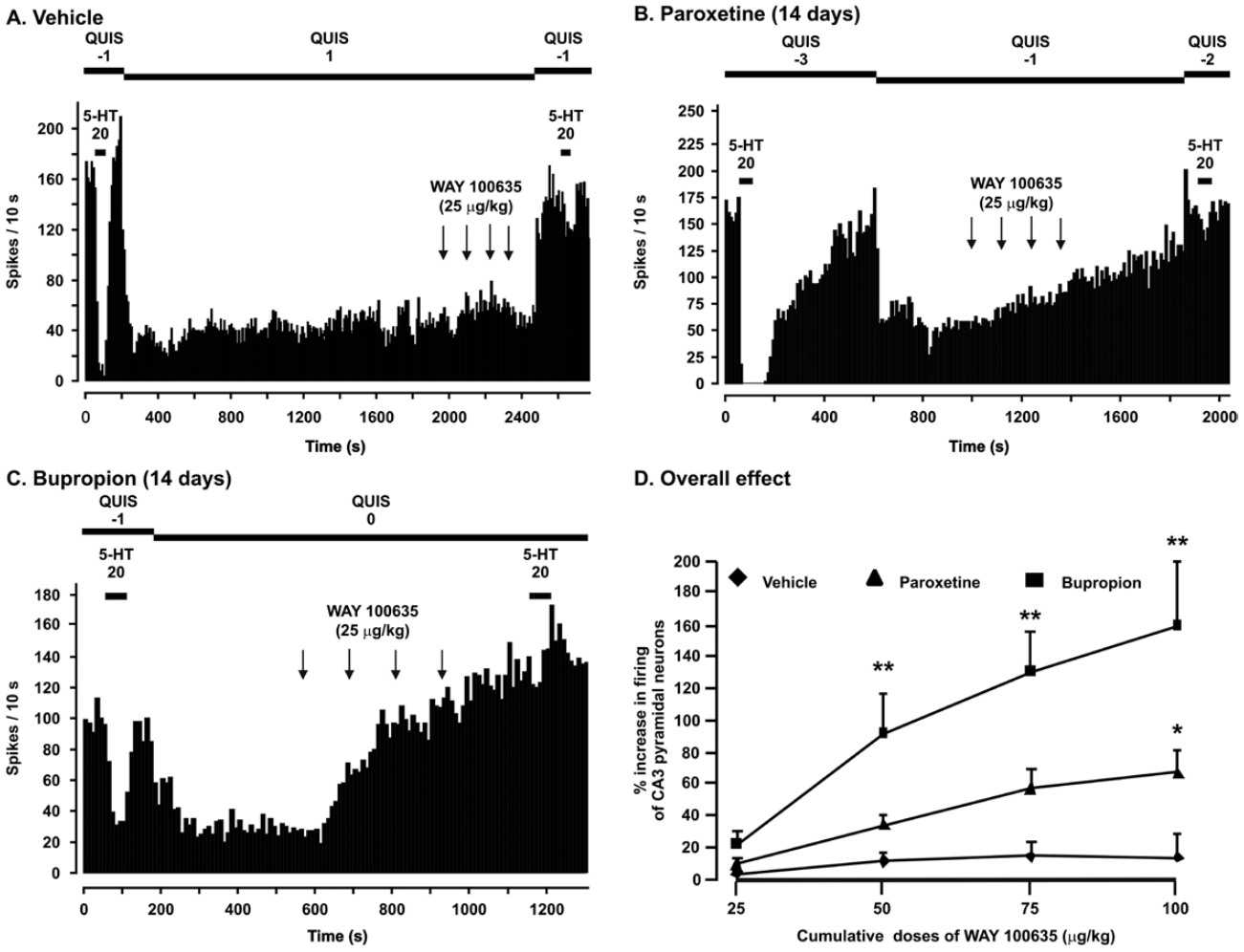

Figure 2. Integrated firing rate histograms of dorsal hippocampus CA3 pyramidal neurons showing their responsiveness to the microiontophoretic application of serotonin (5-HT) and intravenous injection of WAY 100635 in vehicle-treated rats (A) and a rat administered with either paroxetine (B; 10 mg/kg/d) or bupropion (C; 30 mg/kg/d). Note the increase in firing activity of pyramidal neurons after the injection of WAY 100635. (D) Degree (percentage ± SEM) of increase of the firing activity of pyramidal neurons in control rats and those treated with paroxetine and bupropion for 14 days after the administration of WAY 100635. * $P < .05$, ** $P < .01$.

It is established that 5-HT reuptake blockers increase RT50 values (Pineyro et al., 1994). Indeed, the mean RT50 value was significantly increased in OBX rats administered with paroxetine for 14 days (RT50; paroxetine group: 89 ± 15 s [n = 8] compared with vehicle group 24 ± 2 s [n = 7]; $P < .001$; Mann-Whitney U test).

As an indication that iontophoretic application of 5-HT was exerting its inhibitory effect via 5-HT_{1A} receptors, the latter action was nearly abolished by WAY 100635 administration (Figure 2; $P < .05$, Wilcoxon signed-rank test).

Should there be a significant tonic activation of the 5-HT_{1A} receptors, their blockade will result in significant enhancement of the firing activity of pyramidal neurons. In OBX rats that received vehicle, the overall effect of WAY 100635 was not significant on the firing activity of CA3 pyramidal neurons (Figure 2A, D). However, after 2 weeks administration of paroxetine, although the overall effect was not statistically significant, the tonic activation of 5-HT_{1A} receptors was significantly increased when the dose of WAY 100635 reached 100 µg/kg ($P < .05$). However, in rats that received bupropion for 2 weeks, blockade of 5-HT_{1A} receptors induced a significant enhancement in the mean firing activity of pyramidal neurons starting at 50 µg/kg (Figure 2; $P < .001$, $F(2, 81) = 8.7$, 2-way ANOVA for repeated measures, followed by Tukey post hoc test; vehicle group: $n = 8$, paroxetine group: $n = 8$, bupropion group: $n = 6$). This shows that the effectiveness of bupropion on tonic activation of 5-HT_{1A} receptors is higher than bupropion in this model.

Discussion

An increase in locomotor activity in a novel environment such as the open field has been reported in many studies following olfactory bulb removal (Song and Leonard, 2005; van der Stelt et al., 2005). It is noteworthy that this alteration seen after ablation was reported not to be due to anosmia proper, as peripherally anosmic rats do perform as sham rats in the open field paradigm (van Riezen et al., 1977; Cairncross et al., 1979; for review, see Song and Leonard, 2005). In keeping with these previous findings (Cryan et al., 1999; Song and Leonard, 2005; Lucas et al., 2007; Breuer et al., 2008), OBX rats in the present study showed a significant increase in locomotion in the open field. Interestingly, the hyperactivity phenomenon was reported to be a durable characteristic of the OBX rats, as it is still present 20 weeks after surgery (van der Stelt et al., 2005). It has also been reported to be reversed by chronic but not acute administration with several antidepressants, including SSRIs (Cryan et al., 1998, 1999; Song and Leonard, 2005).

The present study showed that the firing activity of DRN 5-HT neurons was significantly decreased 2 weeks after OBX. Because the firing activity of NE and DA neurons was unchanged, as shown in the present study, these results cannot be explained by a diminution of the excitatory effect of NE and DA neurons through an action on α_1-adrenoceptors and D_2 receptors, respectively (Katz et al., 2010).

This decrease in firing of 5-HT neurons was unlikely to have been due to an overactivation of 5-HT_{1A} autoreceptors, which would not have been possible in the presence of a significant decrease in 5-HT synthesis and a probable diminution of 5-HT concentrations in dorsal and medial raphe nuclei of OBX-rats (Barton and Hutson, 1999; Watanabe et al., 2003; Hasegawa et al., 2005). Even though the mechanism underlying this decrease in firing is not clear, data point to an alteration in 5-HT elements of DRN. Indeed, Saitoh et al. (2007) reported a significant decrease in tryptophan hydroxylase-positive cells in the rat DRN following OBX. However, in the present study, although 5-HT neurons firing was decreased, no reduction in the number of neurons encountered per electrode trajectory was observed, suggesting no change at least in the number of presumptive 5-HT neurons recorded in DRN of OBX rats. Consistently with the present results, it was reported that there was no general loss of neuronal function, because there was a similar increase in 5-HT accumulation following tryptophan loading in OBX rats compared with controls (van der Stelt et al., 2005).

Unlike behavioral adaptations that lasted weeks after OBX, the diminished firing rate of 5-HT in OBX rats at 14-day postsurgery recovered to control levels after 28 days. It is worth noting that previous studies have shown that some adaptations, such as hyperactivity, found in OBX rats are durable whereas others are reversible. Indeed, although it was shown that the hyperactivity seen in OBX-rats lasted for 5 months postbulbectomy (van der Stelt et al., 2005), at least one study showed, for instance, that brain reward function, as measured by intra-cranial self-stimulation, was deficient for 8 days after OBX before returning to baseline levels (Slattery et al., 2007). The exact mechanisms underlying the presence or absence of such adaptations are unclear and need to be examined in future studies.

Despite recovery of the activity of 5-HT neurons after 1 month of ablation, it remained inhibited following paroxetine administration for 14 and 28 days, at which time neurons usually return to control levels in naïve rats under the same regimen (Besson et al., 2000). It is possible that the lack of recovery of 5-HT neurons firing stems from the inability of paroxetine to increase 5-HT levels in DRN, which consequently would have resulted in 5-HT_{1A} autoreceptor desensitization, allowing a return to the control level of the firing. Indeed, 14-day administration of the SSRI citalopram in OBX rats did not reverse the decrease of DRN 5-HT synthesis and release (Barton and Hutson, 1999; Hasegawa et al., 2005). The lack of increase in 5-HT cannot be explained by an augmentation in 5-HTT density in OBX rats as was previously postulated, since there was no change in 5-HTT density in the DRN of these rats (Sato et al., 2010).

In the hippocampus, even though the density of 5-HT_{1A} receptors was increased in OBX rats (Sato et al., 2008), this did not result in an enhancement of the responsiveness of these receptors after 14-day paroxetine administration (Figure 2). This indicates that 5-HT_{1A} receptors remained normosensitive in the hippocampus, similarly to naïve rats that received the same regimen (Besson et al., 2000). However, an increase in the tonic activation of the 5-HT_{1A} receptors was shown here, although only following injection of a high dose of WAY 100635 (100 µg/kg) in paroxetine-treated OBX rats but to a lesser extent than that obtained in naïve rats that received this SSRI (Besson et al., 2000). Accordingly, a dampened enhancement of extracellular levels of 5-HT in the hippocampus was already reported, since local application of the SSRI fluvoxamine significantly increased extracellular 5-HT in this region and to a greater extent in sham than OBX rats (Watanabe et al., 2003; Hasegawa et al., 2005; van der Stelt et al., 2005; Prins et al., 2011). It is not clear if the increase in 5-HTT density in OBX rat hippocampus is the cause for this dampening in 5-HT levels, as was proposed, since conflicting results were reported (no change vs an increase; Zhou et al., 1998; Licht et al., 2010; Sato et al., 2010). Furthermore, 5-HTT blockade as measured by the RT50 (a direct measure of 5-HTT reuptake; Pineyro et al., 1994) was shown in the current work to be increased to the same degree in paroxetine-treated OBX rats than in naïve rats (Besson et al., 2000). Altogether, these results showed that in the hippocampus of OBX rats, paroxetine induced a relatively modest increase in tonic activation of 5-HT_{1A} receptor, 5-HT synthesis (Hasegawa et al., 2005), and 5-HT release (van der Stelt et al., 2005) compared with bupropion. It is worth noting, however, that paroxetine attenuated OBX-induced hyperactivity (Cryan et al., 1998, 1999).

An enhancement of tonic activation of the 5-HT_{1A} receptors was obtained in the hippocampus of OBX rats following a 14-day regimen with bupropion using smaller doses of WAY 100635 than those used to induce it in rats that received paroxetine (Figure 2). This increase is similar to that obtained from previous studies using bupropion in naïve rats (Ghanbari et al., 2011). Altogether, these results indicate that inasmuch as 5-HT_{1A} receptor sensitivity did not change following bupropion regimen, an increase was observed in its net effect of bupropion administration on 5-HT neurotransmission in OBX rats that was comparable with that obtained in naïve rats (Ghanbari et al., 2011). This increase likely resulted from attenuated function of α_2-adrenoceptors located on 5-HT terminals (Ghanbari et al., 2011) combined with normalized firing of 5-HT neurons after 14-day administration of bupropion, as reported here. However, the fact that 5-HT neuron firing was not higher in OBX rats that received bupropion than that in naïve rats (El Mansari et al., 2008; Ghanbari et al., 2010) may stem from the fact that basal firing of 5-HT neurons was much lower in OBX rats compared with naïve animals.

Clinically, it is well known that certain patients with major depressive disorder may respond to some classes of antidepressants but not to others. The biological basis for this remains elusive. The present results showed that 5-HT transmission was not enhanced to the same degree using a SSRI in rats with a presynaptic anomaly of 5-HT neurons (ie, a diminished firing activity) than in rats receiving bupropion, an antidepressant with primary sites of action on catecholamine systems, which appeared to be largely unaltered in this model. The therapeutic implications of these findings are that patients with olfactory bulb atrophy may have a preferential response to bupropion than to a SSRI. Indeed, in patients with major depressive disorder, there is a correlation between the severity of depressive symptoms and atrophy of the olfactory bulbs (Negoias et al, 2010). It would thus be interesting to examine the antidepressant response using the 2 drugs studied herein in a clinical setting in relation to bulb atrophy.

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Statement of Interest

P. Blier has financial involvements with: Astra-Zeneca, Bristol Myers, Eli Lilly, Janssen, Labopharm, Lundbeck, Merck, Pfizer, Servier, Takeda, and Valeant. M. El Mansari, S. Manta, C. Oosterhof, K. El Iskandrani, F. Chenu, and S. Shim declare no conflicts of interest.

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Original Paper

Impact of subanesthetic doses of ketamine on AMPA-mediated responses in rats: An *in vivo* electrophysiological study on monoaminergic and glutamatergic neurons

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Abstract

The rapid antidepressant action of a subanesthetic dose of ketamine in treatment-resistant patients represents the most striking recent breakthrough in the understanding of the antidepressant response. Evidence demonstrates tight interactions between the glutamatergic and monoaminergic systems. It is thus hypothesized that monoamine systems may play a role in the immediate/rapid effects of ketamine. *In vivo* electrophysiological recordings were carried in male rats following ketamine administration (10 and 25 mg/kg, i.p.) to first assess its effects on monoaminergic neuron firing. In a second series of experiments, the effects of ketamine administration on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and N-methyl-D-aspartate receptor (NMDA)-evoked responses in hippocampus CA3 pyramidal neurons were also investigated using micro-iontophoretic applications. Although acute (~2 hours) ketamine administration did not affect the mean firing activity of dorsal raphe serotonin and ventral tegmental area dopamine neurons, it did increase that of locus coeruleus norepinephrine neurons. In the latter brain region, while ketamine also enhanced bursting activity, it did increase population activity of dopamine neurons in the ventral tegmental area. These effects of ketamine were prevented by the prior administration of the AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide. An increase in AMPA-evoked response of CA3 pyramidal neurons was also observed 30 minutes following acute ketamine administration. The present findings suggest that acute ketamine administration produces a rapid enhancement of catecholaminergic neurons firing activity through an amplification of AMPA transmission. These effects may play a crucial role in the antidepressant effects of ketamine observed shortly following its infusion in depressed patients.

Keywords

Ketamine, major depressive disorder, monoamines, glutamate, AMPA, NMDA

Introduction

Several clinical studies have demonstrated the rapid but transient antidepressant effects of subanesthetic doses of the non-competitive NMDA receptor antagonist ketamine (Ki = 0.5 μM; Kapur and Seeman, 2002) in patients with treatment-resistant major depressive disorder (MDD). Significant improvement in depressive symptoms occurred within 72 hours following infusion of ketamine in seven subjects in the first placebo-controlled double-blind study (Berman et al., 2000). This was replicated in a larger double-blind study (Zarate et al., 2006). A larger two-site trial using the benzodiazepine midazolam to control for the psychotropic effects of ketamine confirmed a rapid onset of antidepressant effects (Murrough et al., 2013a). Moreover, repeated infusions of ketamine produced a more durable antidepressant response when compared to a single infusion (Murrough et al., 2013b).

Although studies have reported rapid antidepressant effects of ketamine 24 hours following an infusion, an immediate therapeutic effect of ketamine can manifest as early as an hour or two following intravenous infusions, once its psychotomimetic effects have subsided (Blier et al., 2012; Maeng and Zarate, 2007; Murrough et al., 2013b). The rapidity as well as the efficacy of the antidepressant response produced by ketamine in treatment-resistant patients is its most valuable asset. Understanding the mechanism behind these effects should bring the field a step closer to understanding and ultimately developing rapid, highly effective and mechanistically novel antidepressant treatments, leading to improved patient outcomes.

In preclinical studies, an increase in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to N-methyl-D-aspartate receptor (NMDA) throughput is the predominant hypothesis posited to explain the rapid antidepressant effect of

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ketamine. Specifically, ketamine, by directly blocking NMDA receptors on gamma-aminobutyric acid (GABA) interneurons increases glutamate and leads to an increase in AMPA receptors activation (Abdallah et al., 2014; Homayoun and Moghaddam, 2007). It was also postulated that the sustained antidepressant effect of ketamine is likely achieved through an initiation of a number of downstream signaling pathways, including activation of the mammalian target of rapamycin (mTOR) pathway (Li et al., 2010, 2011) which results in an increase in translation of the brain-derived neurotrophic factor (BDNF) protein (Naughton et al., 2014; Zhou et al., 2013), ultimately leading to enhanced synaptic plasticity and neurotrophic changes (Li et al., 2011). However, changes in BDNF and mTOR expression and activation are only seen 24 hours following ketamine administration (Li et al., 2010). Furthermore, while Li et al. (2010) showed that pre-administration of rapamycin blocked the ketamine-mediated decrease in immobility time in the forced swim test (FST), investigations by Autry et al. (2011) revealed that rapamycin failed to reverse such an effect. Hence, while enhanced neurotrophic factors might be involved in the sustained effects of ketamine observed 24 hours following its administration, the mechanisms behind the immediate effects remain elusive, suggesting that additional mechanisms might be involved.

All antidepressants currently in clinical use target one or more monoamine systems (El Mansari et al., 2010; Blier and El Mansari, 2013). Interactions between glutamate and monoamines are well documented and give rise to the interesting possibility that ketamine can produce, at least in part, its antidepressant effect by acting on monoamines neurotransmission (Paul and Skolnick, 2003; Millan 2006). The effect of NMDA receptor blockade on monoamines was already reported with phencyclidine (1-(1-phenylcyclohexyl)piperidine (PCP); Ki = 2 μM for NMDA receptor), a close congener of ketamine that is clinically not an antidepressant. Intravenous injection of PCP decreases firing rate of locus coeruleus (LC) norepinephrine (NE) neurons (Raja and Guyenet, 1980), while not modifying the firing activity of 5-HT neurons (Aghajanian et al., 1970; Raja and Guyenet, 1980). Another study demonstrated that intravenous ketamine increases firing of ventral tegmental area (VTA) dopamine (DA) neurons with potency 19 times weaker than the NMDA antagonist MK-801 (French and Ceci, 1990). Although some interactions between glutamate and monoamines have been documented, their exact functional connectivity remains to be investigated.

The present electrophysiological study was aimed at investigating whether a single dose of ketamine alters monoaminergic neuronal firing within a time frame whereby a therapeutic action can be observed, and whether any such changes are AMPA-dependent. Due to its pivotal role in neurocircuitry involved in major depression (see Drevets et al. 2008) and in light of studies showing neuronal plasticity following ketamine administration (see Kavalali and Monteggia, 2012), the hippocampus was selected to assess whether the responsiveness of CA3 pyramidal neurons to ketamine could as well promptly change following such a treatment. The results from this study could provide important insights in how the monoamine and glutamate systems interact, leading to better understanding of the mechanism by which ketamine produces its rapid antidepressant effect.

Methods and materials

Animals

The experiments were carried out in male Sprague–Dawley rats (Charles River, St Constant, Canada), weighing between 270 and 330 g at the time of the experiment. Rats were housed in groups of two per cage, under standard laboratory conditions (12:12 h light–dark cycle with access to food and water *ad libitum*). Body temperature was kept at 37°C during electrophysiological experiments. Animals were handled according to the guidelines of the Canadian Council on Animal Care (CCAC) and the local Animal Care Committee (Institute of Mental Health Research, Ottawa, Canada) approved protocols.

Drug administration

Ketamine hydrochloride was dissolved in 0.9% aqueous saline solution. For acute experiments, ketamine was administered at a dose of 10 mg/kg and 25 mg/kg intraperitoneally (i.p.) at least 30 minutes prior to the electrophysiological experiments, as previously described (Li et al., 2010; Gigliucci et al., 2013). In the two-day administration paradigm, ketamine was administered at a dose of 10 mg/kg/day for two days, and an additional third injection was administered on day 3, at least 30 minutes prior to the electrophysiological experiments. Control rats on the other hand received the vehicle (0.9% aqueous saline solution). In the case where 10 mg/kg (Li et al., 2010) did not induce any effect, 25 mg/kg dose of ketamine was used as in Gigliucci et al. (2013); at this dose, ketamine is still subanesthetic and produces a significant effect in the FST model (Li et al., 2010; Gigliucci et al., 2013). The AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [*f*]quinoxaline-7-sulfonamide (NBQX) was dissolved in 0.9% aqueous saline solution, and administered i.p. at a dose of 10 mg/kg for VTA experiments, and 3 mg/kg for LC experiments, 10 minutes prior to ketamine administration.

In vivo electrophysiological experiments

Rats were anesthetized with chloral hydrate (400 mg/kg i.p) and mounted in a stereotaxic apparatus (David Kopf; Tujunga, CA, USA). Supplemental doses of the anesthetic (100 mg/kg, i.p.) were given to maintain constant anesthesia and prevent any nociceptive reaction to pinching of the hind paws. Extracellular recordings in the dorsal raphe nucleus (DRN) serotonin (5-HT), VTA DA and LC NE neurons were performed using single-barrel glass micropipettes (Stoelting, Wood Dale, IL, USA), pulled on a pipette puller (Narishige, Japan), and filled with 2 M NaCl solution and an impedance range of 2–4 MΩ. A burr hole was drilled at the stereotaxic coordinates corresponding to the brain structure of interest (Paxinos and Watson, 2007). The shape, duration of spikes, as well as the frequency of firing was used to identify neurons of interest, and recorded in real-time using the Spike2 program (Cambridge Electronic Design, Cambridge, UK).

For the recording of monoaminergic neurons, several electrode descents were made and neurons encountered were

recorded in each brain structure to determine the effects of ketamine on the spontaneous firing rate of these neurons. Duration of recordings from the time of drug injection to the last neuron recorded varied between 100 and 140 minutes. Firing rate and percentage of burst firing were averaged to obtain the average firing rate and average burst activity of neurons in each rat.

Recording of DRN 5-HT neurons

Electrodes were positioned 0.9–1.1 mm anterior to lambda on the midline and lowered into the DRN. Presumed DRN 5-HT neurons were encountered over a distance of 1 mm starting immediately below the ventral border of the Sylvius aqueduct. 5-HT neurons are then identified according to the following criteria: a slow (0.5–2.5 Hz), regular firing pattern, long duration and a positive action potential (VanderMaelen and Aghajanian, 1983). Investigations of the effects of ketamine on 5-HT neuron firing activity were conducted using two methods. In the first, firing of 5-HT neurons was assessed preceding and following ketamine administration in the same rat. The use of this method eliminated any observed variability that could occur between rats. In a second method, the firing activity of 5-HT neurons was assessed in rats that received either vehicle or ketamine.

Recording of VTA DA neurons

Single-barrel glass micropipettes were positioned using the following coordinates (in mm from lambda): AP, +3.0 to +3.8; L, 1–0.6; V, 6.5–9. The presumed DA neurons were identified according to the well-established electrophysiological properties in vivo: a typical triphasic action potential with a marked negative deflection; a characteristic long duration (>2.5 ms) often with an inflection or 'notch' on the rising phase; a slow spontaneous firing rate (0.5–9 Hz) with an irregular single spiking pattern with burst activity (Ungless and Grace, 2012). The electrode was passed through the VTA in several tracks and spontaneously firing DA neurons were recorded. Population activity was determined as the number of neurons encountered in each rat divided by the number of tracks carried out (Grace and Bunney, 1983).

Recording of LC NE neurons

Single-barrel glass micropipettes were positioned at 0.9–1.2 mm posterior to lambda and 0.9–1.3 to the midline suture. NE neurons were encountered at a depth of 5.5–7 mm from the surface of the brain. They are identified by their regular firing rate (0.5–5 Hz), a biphasic action potential of long duration (~2 ms), and a characteristic volley of spikes followed by a quiescent period in response to a nociceptive pinch of the contralateral hind paw (Cedarbaum and Aghajanian, 1977).

Bursts analysis

The firing patterns of the monoaminergic neurons were analyzed by interspike interval (ISI) burst analysis. The onset of a burst was signified by the occurrence of two spikes with ISI < 0.08 s for NE and DA, and ISI < 0.01 s for 5-HT. The termination of a burst was defined as an ISI > 0.16 s for NE and DA (Dawe et al., 2001; Grace and Bunney, 1983) and ISI > 0.01 s for 5-HT (Hajos and Sharp, 1996).

Microiontophoresis and extracellular recording of dorsal hippocampus CA3 pyramidal neurons

Extracellular recording and microiontophoresis of glutamatergic CA3 pyramidal neurons were carried out using five-barreled glass micropipettes with a tip broken back to 10–12 μm. The central barrel used for the unitary recording was filled with a 2 M NaCl solution, and the impedance of these electrodes ranged from 2 to 4 MΩ. The side barrels were filled with the following solutions: AMPA hydrobromide (5 mM in 200 mM NaCl, pH 8), NMDA (10 mM in 200 mM NaCl, pH 8) and 2 M NaCl solution for automatic current balancing. The micropipettes were lowered into the dorsal CA3 region of the hippocampus using the following coordinates: 4.0 mm anterior to lambda and 4.2 mm lateral. CA3 pyramidal neurons were found at a depth of 4.0 ± 0.5 mm below the surface of the brain. Since pyramidal neurons do not discharge spontaneously in chloral hydrate anesthetized rats, a small current of AMPA (-2 to -5 nA) was constantly applied to locate those neurons, and activate them within their physiological firing range (10–15 Hz; Rank, 1975). When AMPA and NMDA were not ejected, a retention current of +15 nA was applied to prevent leakage from the barrels. Pyramidal neurons were identified by their large amplitude (0.5–1.2 mV) and long-duration (0.8–1.2 ms) simple action potentials, alternating with complex spike discharges (Kandel and Spencer, 1961). The duration of microiontophoretic ejections of NMDA and AMPA was constant at 60 s. During these experiments, both the duration and current of NMDA and AMPA for microiontophoresis ejection remained the same before and after the i.p. injection of ketamine or saline. Drug effect was assessed by measuring the degree of excitation of pyramidal neurons (measured as number of spikes generated for 60 s ejection) induced by NMDA and AMPA applications following the acute administration of ketamine or saline. Results are expressed as overall changes in the percentage of baseline firing rate of dorsal hippocampus CA3 pyramidal neurons following administration of ketamine or vehicle.

Statistical analysis

Data are expressed as means ± SEM. In DRN, LC and VTA, comparisons between controls and treated groups were carried out using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Analysis of data from microiontophoresis was carried out using the two-way ANOVA with repeated measures and the Bonferroni post-hoc analysis was conducted when significant ANOVA results were obtained. These comparisons were statistically analyzed and graphed using the software Graphpad (Prism software Inc, La Jolla, CA). In all data analysis, statistical significance was taken as $p<0.05$. Burst activity of DA and NE neurons was analyzed with burstiDAtor software (www.github.com/nno/burstidator/releases).

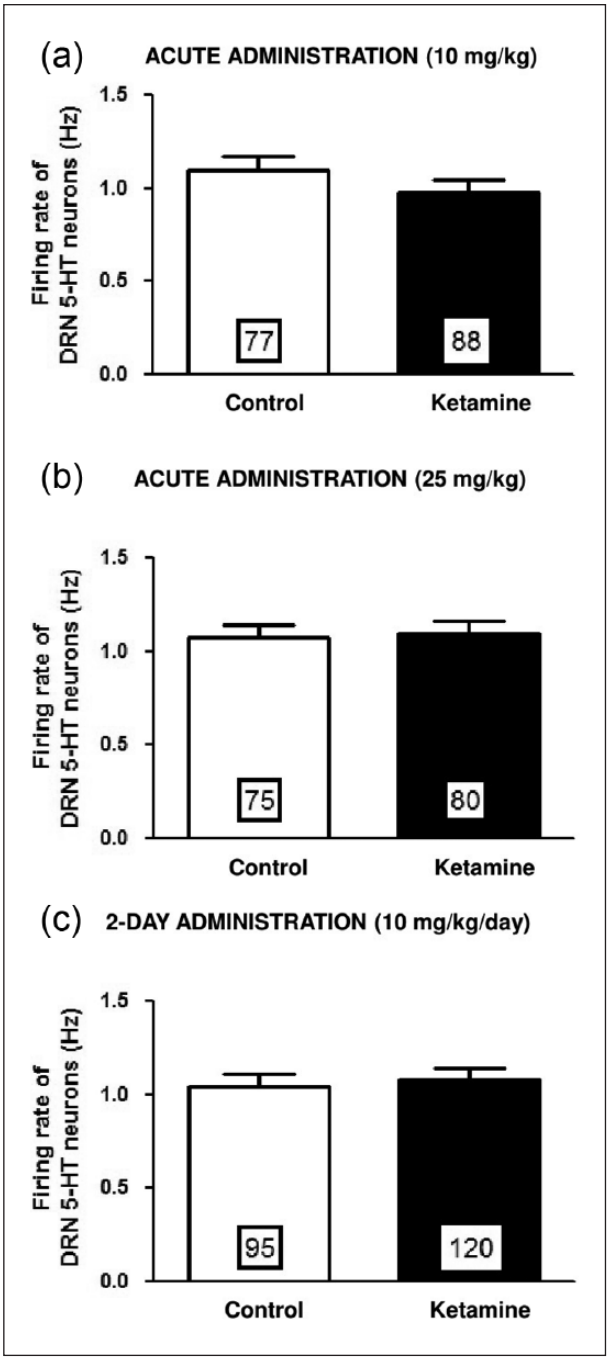

Figure 1. Effects of acute and two-day administration of ketamine on DRN 5-HT neuron firing. Mean (± SEM) of the firing rate of 5-HT neurons following acute (a) and (b) and two-day (c) administration of vehicle or ketamine at a dose of 10 mg/kg/day (a) and (c) and 25 mg/kg/day (b). Numbers in the histograms correspond to the number of neurons recorded (5–6 rats tested per group).

Drugs

Ketamine hydrochloride was purchased from ERFA Canada Inc. (Montreal, QC, Canada). AMPA hydrobromide, NMDA and NBQX were both purchased from Tocris Biosciences (Ellisville, MO, USA). Chloral hydrate was purchased from Sigma-Aldrich Canada Co. (Oakville, ON, Canada).

Results

Effects of acute and two-day administration of ketamine on the firing activity of DRN 5-HT neurons

Acute administration of ketamine (10 mg/kg; i.p.) yielded no significant change in the average firing activity of 5-HT neurons in the DRN when the paradigm in which firing was assessed prior to and following ketamine administration in the same rat. Similarly there was no significant alteration in 5-HT neurons firing in the paradigm in which vehicle and ketamine administered rats were used (Figure 1(a)).

A previous study showed that 25 mg/kg but not 10 mg/kg of ketamine elicited and antidepressant-like effect in the FST model (Gigliucci et al., 2013; Li et al., 2010). Therefore, to rule out the possibility that an insufficient dose of ketamine was used, a higher dose of 25 mg/kg was tested, but still did not produce any alteration of the firing activity of 5-HT neurons (Figure 1(b)).

In addition, no significant change in the proportion of neurons exhibiting burst activity was observed with either dose (vehicle: 22%; ketamine 10 mg/kg: 28%, and 25 mg/kg: 33%). Hence the firing activity of 5-HT neurons remained unaltered following acute administration of ketamine.

A two-day regimen of ketamine also yielded no change both on firing rate (Figure 1(c)) and burst activity (33%) of 5-HT neurons, compared to two-day vehicle-administered animals (22%).

Effects of acute and two-day administration of ketamine on the firing activity of VTA DA neurons

After both acute and two-day administration of ketamine, the firing rate of DA neurons was unaltered compared to rats administered with vehicle (Figure 2(a), (c)). Moreover, no alteration in the burst activity of these neurons was observed (data not shown).

Despite an absence of effect of acute administration of ketamine on the rate of DA neuronal firing and bursting, the number of neurons encountered per electrode descent (population activity) increased by 113% (one-way ANOVA; $F(3, 16)=5.4$; $p<0.01$; Tukey post hoc test; $n=5$; Figure 2(b)) compared to vehicle-treated rats. The administration of the AMPA receptor antagonist NBQX (10 mg/kg) alone had no effect on DA neurons firing; in animals pretreated with NBQX, the increase of population activity after ketamine administration was no longer present (Figure 2(b)).

Similarly, following two-day ketamine regimen, the increase in population activity that was seen following its acute injection was no longer observed (Figure 2(d)).

Effects of acute and two-day administration of ketamine on the firing activity of LC NE neurons

Acute administration of ketamine resulted in a significant elevation of the average rate of firing activity of NE neurons in the LC by 21% when compared to the vehicle-administered group (one-way ANOVA; $F(3, 375)=4.5$; $p<0.05$; Tukey post hoc test; $n=6$; Figure 3(a)). However, this ketamine-induced increase in firing rate was not present in groups that were pretreated with NBQX (Figure 3(a)).

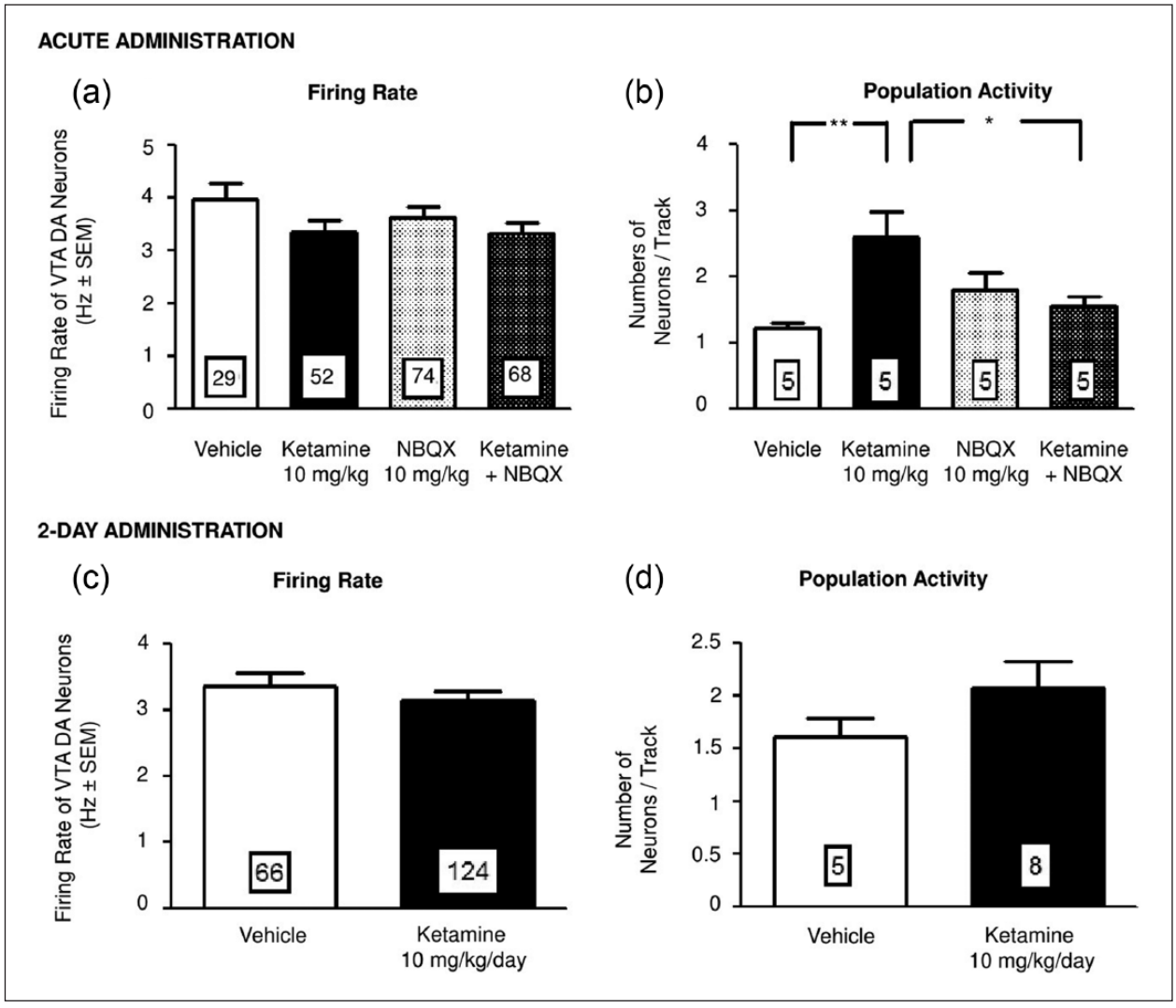

Figure 2. The effects of acute and two-day ketamine administration on VTA DA neuron firing. Mean (± SEM) of the firing rate and population activity of DA neurons following acute (a) and (b) and two-day (c) and (d) administration of vehicle or ketamine (10 mg/kg). The numbers in the histograms correspond to either the number of neurons recorded (a) and (c) or the number of rats tested per group (b) and (d). $^{**}p < 0.01$; $^{*}p < 0.05$.

After a two-day administration regimen of ketamine, the enhancement in firing rate of NE neurons was significantly maintained to a similar degree (23%; Mann–Whitney Rank sum; $p<0.01$; $n=6$; Figure 3(c)).

In addition, acute administration of ketamine nearly doubled the proportion of NE neurons displaying burst activity (control: 17% versus ketamine: 30%; one-way ANOVA; $p<0.01$; $F(3, 20)=9$; Bonferroni post hoc test; $n=6$; Figure 3(b)). Since NBQX by itself had an effect on the NE neurons firing when injected at 10 mg/kg, an alternative dose of 3 mg/kg that has no effect was used. At this dose, pre-treatment with NBQX resulted in a dampening in the increase of the number of neurons with burst activity induced by acute ketamine administration (Figure 3(b)). Following a two-day administration regimen, the ketamine-induced increase in burst activity was no longer present (Figure 3(d)).

Effects of acute ketamine administration on the responsiveness of hippocampus pyramidal neurons

There was no significant overall main effect of ketamine (10 and 25 mg/kg) on the number of spikes generated per nA in response to iontophoretically-applied AMPA on pyramidal neurons. However, a significant time interaction was obtained 30 minutes following ketamine administration (using 10 or 25 mg/kg, showing a 64% increase in AMPA-induced firing activity of pyramidal neurons compared to control rat that received saline (two-way ANOVA with repeated measures followed by Bonferroni post hoc test; $F(2, 48)=6$; $P<0.05$; Figure 4(a), (c)). However, there was no alteration in responsiveness of these neurons to iontophoresed NMDA, as indicated by a lack of significant change in pyramidal neuron firing activity to iontophoretically-applied

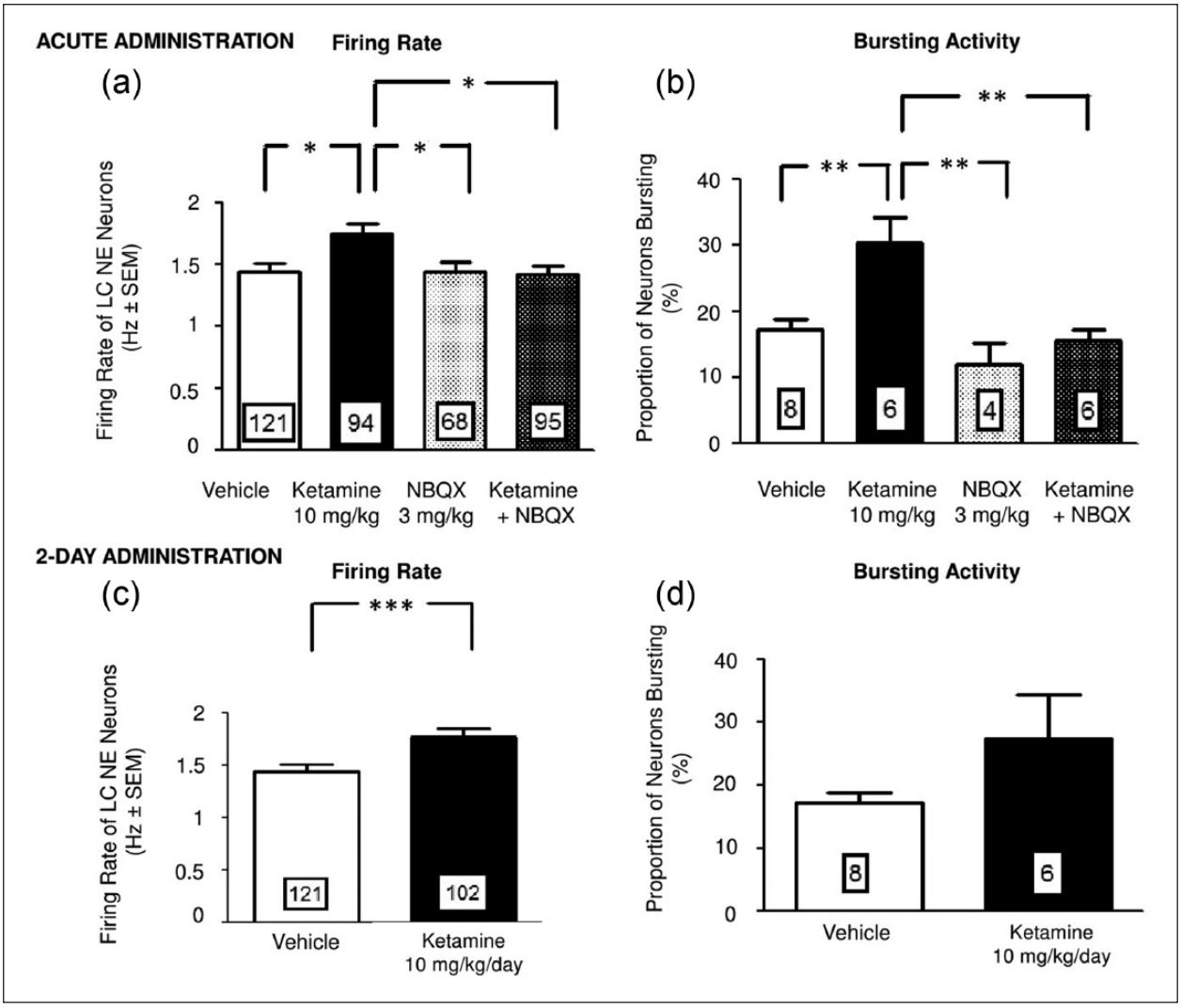

Figure 3. The effects of acute and two-day ketamine administration on LC NE neuron firing. Mean (± SEM) of the firing rate and burst activity of NE neurons following acute (a) and (b) and two-day (c) and (d) administration of vehicle or ketamine (10 mg/kg). The numbers in the histograms correspond to either the number of neurons recorded (a) and (c) or the number of rats tested per group (b) and (d). $^{**}p < 0.01$; $^{*}p < 0.05$.

NMDA when rats receiving ketamine and vehicle were compared (Figure 4(a), (d)).

As a 10 mg/kg dose of ketamine did not change NMDA-induced firing activity of pyramidal neurons, a higher yet still sub-anesthetic dose was used (25 mg/kg; Figure 4(b)). As was the case with the lower dose, a 25 mg/kg dose of ketamine produced no overall effect on NMDA-evoked firing activity when compared to the vehicle treated group (Figure 4(b), (d)). However, a significant increase in AMPA-evoked firing was observed 30 minutes following the higher ketamine administration compared to control rats that received saline (55% increase; two-way ANOVA followed by Bonferroni post hoc test; $p<0.05$; Figure 4(b), (c)).

Discussion

Several studies have already reported the involvement of the 5-HT system in the effect of ketamine. Indeed, ketamine administration reversed the 5-HT-induced reduction of excitatory postsynaptic current (EPSC) amplitude and frequency, observed in layer V of the prefrontal cortex after chronic unpredictable stress (Li et al., 2011). In addition, although the antidepressant-like effects of ketamine in the FST was elicited with 25 mg/kg but not 10 mg/kg (see Li et al., 2010), they were abolished following p-chlorophenylalanine administration that lowers 5-HT levels (Gigliucci et al., 2013). In the present study, however, the lack of effect on the firing activity of 5-HT neurons after both acute and two-day administration cannot be attributed to using an inadequate dose of ketamine, since a higher dose (25 mg/kg) was also without effect, whereas significant change were obtained on the firing activity of NE, DA and pyramidal neurons when using the low dose. The modulation exerted on 5-HT neuronal firing was previously shown to be through AMPA and NMDA receptors, since iontophoretic applications of AMPA and NMDA increased their firing activity that was blocked by glutamate receptor antagonists (Gartside et al., 2007). Consequently, the increase in glutamate release following NMDA blockade by

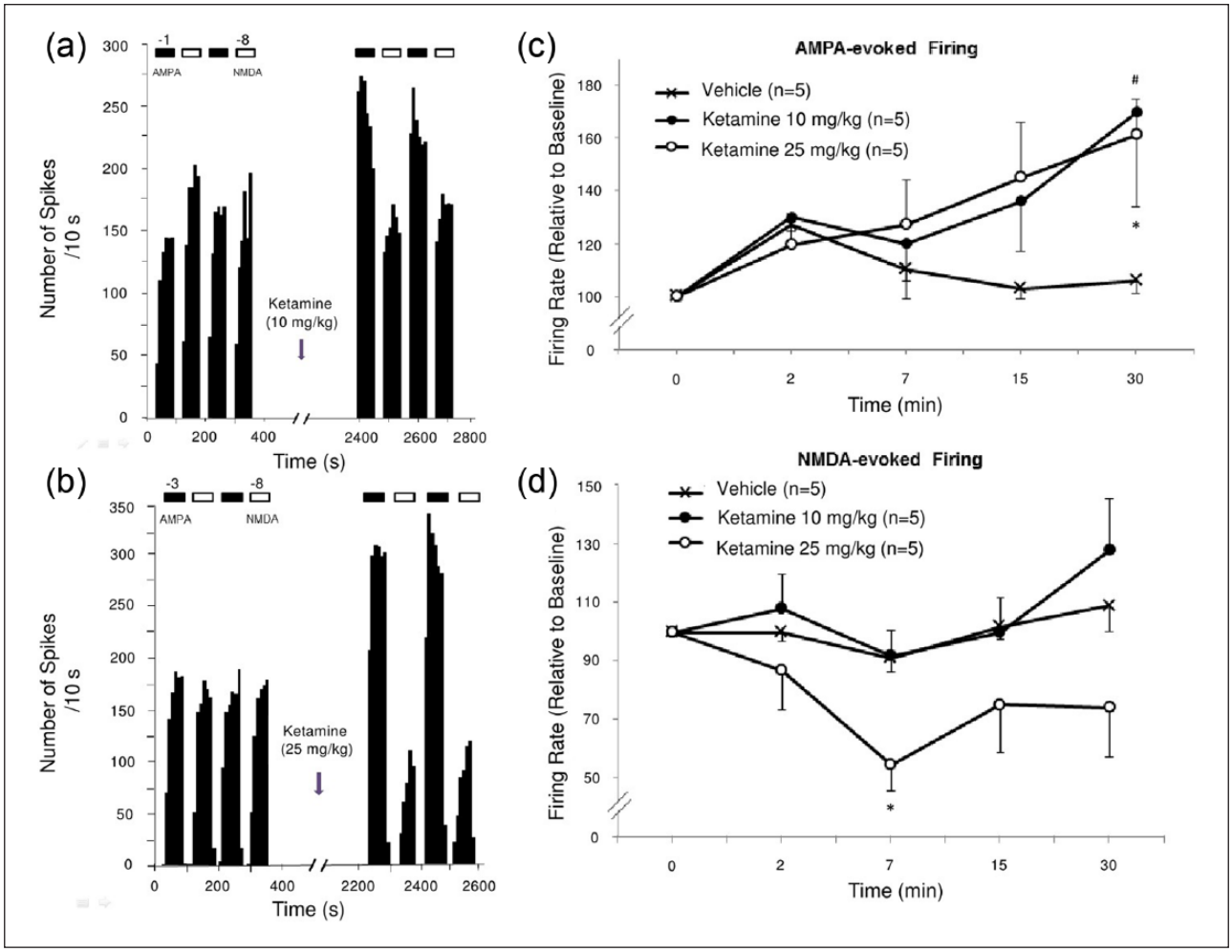

Figure 4. The effects of acute ketamine administration on the responsiveness of AMPA and NMDA receptors. Integrated firing rate histograms of dorsal hippocampus CA3 pyramidal neurons showing their responsiveness to ketamine administration (indicated by arrows) at a dose of 10 mg/kg (a), and 25 mg/kg (b). Horizontal bars indicate the duration of iontophoretic applications of AMPA (black) or NMDA (white). Ejection currents of -1 nA for AMPA and -8 nA for NMDA were used in this example. In (c) and (d), results are expressed as overall changes in the percent of baseline firing rate of dorsal hippocampus CA3 pyramidal neurons following administration of ketamine or vehicle; *p<0.05.

ketamine (Lorrain et al., 2003a; Moghaddam et al., 1997) would have increased firing activity of 5-HT neurons through AMPA receptors. However, it was previously shown that the AMPA response was a direct effect on 5-HT neurons rather than resulting from local release of glutamate (Gartside et al., 2007). Therefore, since ketamine acts through the latter mechanism, this may explain its lack of effect on 5-HT neurons. Despite unchanged 5-HT neuronal activity, other studies showed that acute administration of ketamine (25 mg/kg) induced a transient increase in the 5-HT efflux in medial prefrontal cortex in awake rats (López-Gil et al., 2006; Lorrain et al., 2003b). In order to determine whether this effect was due to stimulation of 5-HT neurons or local action in the prefrontal cortex, c-Fos experiments were carried out (Lopez-Gil et al., 2006). These results revealed that the increase in the number of c-Fos-positive cells following administration of the NMDA antagonist MK-801 was altered by application of tetrodotoxin in the medial prefrontal cortex (mPFC), but not the in DRN. While this result indicates an involvement of the mPFC, it did not show a greater activation in DRN following NMDA receptor blockade (Lopez-Gil et al., 2011), which is concordant with results obtained herein on the firing activity of 5-HT neurons. Altogether, these data show that although there was no change in firing activity of 5-HT neurons, an increase of 5-HT neurotransmission can be observed in projection areas following acute ketamine administration. Future experiments will be undertaken to determine electrophysiological changes in 5-HT transmission in projection areas such as the hippocampus and the frontal cortex.

A previous study (French and Ceci, 1990) showed that i.v. injection of ketamine increased the firing rate of DA neurons with only a single neuron tested per rat. However, the present study showed no change in this rate in a sample of neurons recorded in several electrode tracks. The discrepancy may stem from the use of different methods (a single neuron versus a sample of neurons) in addition to the dose of ketamine used in the aforementioned study, which was twice the one used in the present experiments. However, the current study showed an enhancement in population activity of DA neurons, yielding possibly an increased response in the DA

phasic bursts, therefore amplifying the salience signal (Lodge and Grace, 2006). This increase is concordant with the recently reported data showing that ketamine reversed a decrease in DA neurons population activity in Wistar–Kyoto rats exposed to inescapable and uncontrollable footshocks (Belujon and Grace, 2014). Furthermore, the present study also showed that the increase in population activity following ketamine in naïve rats was prevented by administration of the AMPA receptor antagonist NBQX, indicating that this effect is mediated by AMPA receptors. The involvement of AMPA receptors in the VTA is supported by data showing that local application of AMPA on DA neurons stimulates their neuronal firing (Tong et al., 1996; Zhang et al., 1997). Interestingly, it was also shown that a subanesthetic dose of ketamine increased the release of DA in the prefrontal cortex of conscious rats, through an action on AMPA receptors, because intra-PFC application of the AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), blocked this effect (Moghaddam et al., 1997).

This effect of ketamine on population activity of DA neurons coincides with previous studies revealing the major role glutamatergic afferents play in modulating DA population activity. Indeed, activation of glutamatergic afferents originating in the hippocampus stimulate the ventral subiculum, thus resulting in an inhibition of the GABAergic neurons of the ventral pallidum, thereby relieving its tonic inhibition of the VTA DA neurons (Floresco et al., 2001). Although the role of NMDA receptors was reported (Floresco et al., 2001), the present study showed an increase in AMPA receptor activation in the dorsal hippocampus following ketamine administration, providing a possible additional mechanism involved in the elevation of population activity in the VTA. However, following a two-day regimen, the ketamine-induced increase in population activity was no longer present, indicating that it is unlikely to contribute to the sustained effect of this drug. While an increase in DA population activity may alone be insufficient in mediating the antidepressant effects of ketamine, this enhancement may contribute to its immediate therapeutic effects, given the rapid increase resulting from acute administration, as was observed here.

Data from this study showed that acute administration of ketamine resulted in a significant increase in the firing rate of NE neurons and a doubling in the number of neurons exhibiting burst activity. Interestingly, when the LC was stimulated with burst pulses, it was previously shown that it enhances prefrontal cortex NE levels significantly higher than when tonic stimulation was applied (Florin-Lechner et al., 1996). Indeed, using microdialysis paradigm, Lorrain et al., (2003b) revealed that upon acute challenge, ketamine (12.5–50 mg/kg) increases hippocampal NE release that was blocked by the administration of AMPA/kainate receptor antagonist CNQX. This increased release is congruent with the enhancement in firing and burst activities of NE neurons observed in the present work. Furthermore, the latter was reversed by the selective AMPA receptor antagonist NBQX, indicating that this increased activity in LC is a AMPA-dependent effect. Interestingly, it was previously shown that the effect of glutamate upon NE neurons is largely of excitatory nature as demonstrated, for instance, by the fact that the AMPA antagonist LY293558 concentration-dependently blocked that effect (Rasmussen et al., 1996; see Jodo and Aston-Jones, 1997). The present result suggests that the increase in burst activity of NE neurons, which is mediated by AMPA receptors, might be contributory to the early antidepressant effects of ketamine. However, since the latter effect is not sustained, it may explain the lack of lasting antidepressant effects of this drug. Further studies are required in order to determine whether ketamine exerts a direct effect on LC NE neurons, or indirectly through the PFC or other structures.

In the present study ketamine administration resulted in an enhancement of AMPA- but not NMDA-evoked response in the hippocampus, suggesting that ketamine may exert a direct effect on AMPA receptors. Interestingly, chronic administration of ketamine resulted in a significant increase in AMPA but not NMDA receptor density in Wistar–Kyoto rats (a model for depression), whereas basal densities of these receptors were not significantly different when compared to Wistar rats (Tizabi et al., 2012). A synaptic potentiation of AMPA-mediated evoked neurotransmission was also shown in CA1 hippocampal slices. Furthermore, this was coupled with an increase in surface expression of both GluA1 and GluA2 subunits of AMPA receptors, which was inhibited by an AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX; Nosyreva et al., 2013). In addition, following acute ketamine application, an enhancement in synaptic efficacy was also observed at rest condition (Autry et al., 2011). In the present study, however, the lack of blockade of NMDA-evoked firing of glutamatergic pyramidal neurons by ketamine may appear puzzling, but it could stem from the fact that ketamine exerts its effect through GABA interneurons. Indeed, blockade by MK-801 of NMDA receptors located on GABA neurons leads to decreased inhibition (disinhibition) following a surge in glutamate, thus resulting in an enhancement of AMPA activation (Abdallah et al., 2014; Homayoun and Moghaddam, 2007). Future studies on the effect of ketamine on GABA interneurons in the hippocampus are needed to confirm this issue.

The current experiments were carried out in rats that were under chloral hydrate anesthesia, which is known to affect glutamatergic transmission. Therefore, under such conditions the absolute changes produced by ketamine may be different from those occurring in conscious freely-moving rats. However, several studies were able to detect positive effects when measuring glutamatergic transmission under chloral hydrate anesthesia (see Christophersen and Meltzer, 1995; McCardle and Gartside, 2012). Moreover, since chloral hydrate inhibits different types of glutamatergic receptors, it is possible that effects studied under this anesthetic are rather underestimated.

In summary, ketamine administration resulted in an increase in catecholamine activity, and this increase was AMPA-dependent. It is noteworthy that the time-course of the effects of ketamine on AMPA receptors in the hippocampus was consistent with the time-course in which the increase in catecholaminergic firing activity began to occur. These effects could be a consequence of a direct effect of ketamine on NE and DA neurons, or indirectly due to an effect of ketamine on their glutamatergic afferents which have previously been shown to control monoaminergic neuron firing (Carlsson et al., 2001; Millan, 2006; Paul and Skolnick, 2003). Moreover, this study suggests that the antidepressant effect of ketamine could be occurring directly on the glutamatergic system, as shown by the increase in AMPA receptors responsiveness in the hippocampus (see Papp and Moryl, 1994). Whether the immediate effect of ketamine is dependent on glutamate, monoamines or their interaction remains to be elucidated.

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