CHARACTERIZATION OF THE WISTAR-KYOTO RAT MODEL OF DEPRESSION IN THE CONTEXT OF HIPPOCAMPAL SYNAPTIC PLASTICITY AND KETAMINE'S ANTIDEPRESSANT PROPERTIES

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Abstract

Accumulating evidence implicates dysfunction within the glutamatergic system and dysregulation of synaptic plasticity in the pathophysiology of depression, particularly in the hippocampus (HPC). Ketamine has rapid and sustained antidepressant activity in treatment-resistant depression and various animal models; however, its effects on synaptic plasticity, as well as their contribution to ketamine's antidepressant action, are still unclear. To address this, we utilized the Wistar-Kyoto (WKY) model of endogenous stress susceptibility and depression. Consistent with the literature, WKY rats exhibited various depressive-like phenotypes compared to Wistar controls. In addition, we revealed that while *in vivo* hippocampal long-term depression (LTD) at the Schaffer collateral–CA1 (SC-CA1) synapse was not facilitated in the WKY strain, both early and late long-term potentiation (LTP) were significantly impaired. Importantly, both ketamine (5mg/kg, ip), as well as its metabolite (2R,6R)-HNK (5mg/kg, ip), acutely rescued the LTP deficit in WKYs at 3.5h following injection. Consistent with a sustained LTP-like effect, ketamine also increased SC-CA1 basal synaptic transmission at 24h in these rats. Importantly, ketamine, but not (2R,6R)-HNK, was found to have rapid and sustained antidepressant effects in WKY rats in the FST, leading to a dissociation between FST antidepressant-like activity and dorsal HPC synaptic plasticity. However, consistent with the observed SC-CA1 L-LTP deficit and corresponding effects of drug treatment, WKY rats exhibited impaired hippocampal-dependent long-term spatial memory compared to Wistar controls (as measured by the novel object location recognition test at a delay of 24h), which was effectively restored by both ketamine and (2R,6R)-HNK. We propose that, in the WKY rat model, restoring dorsal HPC LTP does not underlie ketamine's antidepressant effects in FST, but may instead mediate reversal of hippocampal-dependent cognitive deficits, which are also key features of clinical depression.

This work supports the theory that ketamine may reverse the stress-induced loss of connectivity in key neural circuits by engaging synaptic plasticity processes to “reset the system”, and highlights the importance of deconstructing depression-like phenotypes and identifying the neural circuits that mediate them more precisely. Based on our results, the existing hypothesis that ketamine’s antidepressant effects are solely due to the actions of its metabolite (2R,6R)-HNK is effectively challenged.

Lay Summary

One theory of depression points to a problem in synaptic plasticity, the activity-dependent strengthening or weakening of neural connections (long-term potentiation or depression, LTP or LTD, respectively), which is thought to allow the brain to learn and remember information. Ketamine has recently shown great potential in the treatment of depression; however, we still do not understand its effects on synaptic plasticity or how they contribute to its therapeutic action. To address this, we used stress-prone Wistar-Kyoto rats, which showed depressive-like behaviours compared to normal rats. This strain also had a dramatic LTP impairment in the hippocampus (key brain region implicated in depression), and a single antidepressant injection of ketamine rescued this deficit. We found that by restoring hippocampal LTP, ketamine seems to rescue long-term memory for a new location in these depressive-like rats, consistent with the role of hippocampal synaptic plasticity in spatial memory and the cognitive symptoms of depression.

Preface

The studies in this thesis were designed by me (LRA) under the direction of my co-supervisors, Dr. Anthony G. Phillips (AGP) and Dr. Yu Tian Wang (YTW). I conducted all experiments, including behavioral studies, drug treatments and electrophysiological recordings. Dr. Peter Axerio-Cilies synthesized the ketamine metabolite (2R,6R)-HNK for experiments in Chapters 5 and 6. Data analysis was done by myself, with methods and approaches guided by AGP, YTW and Dr. Stan Floresco. I conducted literature reviews and prepared manuscripts for publication, with AGP and YTW critically revising subsequent drafts. All experimental protocols were approved by the Animal Care Committee (ACC), University of British Columbia (ACC certificate number: A15-0131) and conducted in compliance with guidelines provided by the Canadian Council on Animal Care (CCAC).

Portions of this dissertation have been published previously. **Aleksandrova** LR, Phillips AG, Wang YT. Antidepressant effects of ketamine and the roles of AMPA glutamate receptors and other mechanisms beyond NMDA receptor antagonism. *JPN* 2017; 42(4): 222–229. doi: 10.1503/jpn.16017; **Aleksandrova** LR, Wang YT, Phillips AG. Hydroxynorketamine: Implications for the NMDA Receptor Hypothesis of Ketamine's Antidepressant Action. *Chronic Stress* 2017; 1: 1–12. doi: 10.1177/2470547017743511. Parts of this work is currently in press. **Aleksandrova** LR, Wang YT, Phillips AG. Evaluation of the Wistar-Kyoto Rat Model of Depression and the Role of Synaptic Plasticity in Depression and Antidepressant Response. *Neurosci Biobehav Rev* 2019 (epub July 20). doi: 10.1016/j.neubiorev.2019.07.007. The results in Chapters 3-6 will be assembled into a manuscript for publication: **Aleksandrova** LR, Wang YT, Phillips AG. Ketamine and (2R,6R)-HNK Restore Hippocampal LTP and Long-Term Spatial Memory in the Wistar-Kyoto Rat Model of Depression. *Biol Psych* 2019 *(in preparation)*. All figures were reprinted with permission, with citations contained in figure legends.

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List of Abbreviations

5-HT Serotonin

5-HTT Serotonin transporter

8-OH-DPAT 8-hydroxy-2-(di-n-propylamino)tetralin

ANOVA Analysis of variance

ACTH Adrenocorticotropic hormone

AP Anterior-posterior

AMPAR α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor

BDNF Brain-derived neurotrophic factor

BLA Basolateral amygdala

BNST Bed nucleus of the stria terminalis

Ca Calcium

CMS Chronic mild stress

COMT Catechol-O-Methyltransferase

CORT Corticosterone

CRF Corticotrophin releasing factor

CSD Chronic social defeat

DA Dopamine

DAT Dopamine transporter

DBS Deep brain stimulation

DG Dentate gyrus

DOPAC 3,4-Dihydroxyphenylacetic acid

DRN Dorsal raphe nucleus

DTI Diffusion tensor imaging

DV Dorso-ventral

EA Electroacupuncture

eEF2 Eukaryotic elongation factor 2

EPM Elevated plus maze

fEPSP Field excitatory post-synaptic potential

FL Familiar location

fMRI Functional magnetic resonance imaging

FR Fixed ratio

FST Forced swim test

GABA Gamma-aminobutyric acid

GC Glucocorticoid

GluA1 AMPAR subunit

GluA2 AMPAR subunit

GR Glucocorticoid receptor

HFS High-frequency stimulation

HNK Hydroxynorketamine

HPA Hypothalamic-pituitary-adrenal

HPC Hippocampus

HPT Hypothalamic-pituitary-thyroid

IN Inhibitory interneurons

ip Intraperitoneally

ITI Inter-train interval

IV Intravenous

KET Ketamine

LC Locus coeruleus

LFS Low-frequency stimulation

LH Learned helplessness

LPS Lipopolysaccharide

LTD Long-term depression

LTM Long-term memory

LTP Long-term potentiation

MAO Monoamine oxidase

MAOI Monoamine oxidase inhibitor

MDD Major depressive disorder

Mg Magnesium

mGluR1 Metabotropic glutamate receptor subtype 1

mGluR2 Metabotropic glutamate receptor subtype 2

ML Medio-lateral

mPFC Medial prefrontal cortex

mPP Medial perforant path

mTOR Mammalian target of rapamycin

MWM Morris water maze

NAc Nucleus accumbens

nAchR Nicotinic acetylcholine receptor

NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline

NE Norepinephrine

NET Norepinephrine transporter

NL New location

NMDAR N-methyl-d-aspartate receptor

NR2A NMDAR subunit

NR2B NMDAR subunit

NSF Novelty-suppressed feeding

OFT Open field test

OLR Object location recognition

PET Positron emission tomography

PFC Prefrontal cortex

PN Pyramidal neurons

POMC Proopiomelanocortin

PR Progressive ratio

PS Population spike

PSD-95 Post-synaptic density protein 95

PTSD Post-traumatic stress disorder

PV Parvalbumin

REM Rapid eye movement

RM-ANOVA Repeated measures analysis of variance

SAL Saline

SC Schaffer collateral

SD Sprague-Dawley

SEM Standard error of the mean

SI Social interaction

SNRI Selective norepinephrine reuptake inhibitors

SPT Sucrose preference test

SSRI Selective serotonin reuptake inhibitors

STM Short-term memory

SVZ Subventricular zone

TCA Tricyclic antidepressant

TH Tyrosine hydroxylase

TMS Transcranial magnetic simulation

TPH2 Tryptophan hydroxylase 2

TRD Treatment-resistant depression

TSH Thyroid stimulating hormone

TST Tail suspension test

USV Ultrasonic vocalization

VGCC Voltage-gated calcium channel

Vs. Versus

VBM Voxel-based morphometry

vSub Ventral subiculum

VTA Ventral tegmental area

WIS Wistar

WKY Wistar-Kyoto

Acknowledgements

First, I would like to express my sincere gratitude to my co-supervisors Dr. Anthony G. Phillips and Dr. Yu tian Wang for their continuous support throughout my PhD studies, as well as their unique expertise and critical input into this research project. I would like to thank Tony for his unwavering enthusiasm, optimism, and moral support in the face of every achievement, obstacle and frustration encountered, as well as his far-reaching expertise and connections in the field of behavioural neuroscience. I would like to thank Yu tian for his positive outlook, his expertise in synaptic plasticity and electrophysiology, as well as his critical, often skeptical, eye when it came to data interpretation, particularly in relation to some behavioural assays. Most of all, I am grateful that over the years both Tony and Yu tian encouraged me to write and publish several comprehensive review papers on various topics related to this thesis, which not only familiarized me with the literature in the field, but also helped me keep the big picture in mind, as well as to further develop my scholarly writing and passion for science communication. Next, I would like to express the deepest appreciation to my supervisory committee members, Dr. Stan Floresco, Dr. Jeremy Seamans, Dr. Shernaz Bamji and Dr. Michael Krausz for their expert input and insightful comments. Stan was always available and willing to provide a critical eye, words of encouragement or technical support, as well as straight-forward answers to all my burning stats questions. His guidance was invaluable for some of the behavioural experiments and data analyses in this thesis. Jeremy provided important critiques of the project, which helped me develop my thinking and improved the way I present my research. I thank my colleagues in the Phillips and Wang labs, past and present, particularly Dr. Gemma Dalton, Dr. Donovan Ashby, Dr. Steven Connor, Dr. Peter Axerio-Cilies, Dr. Camille Potey, Dr. Lidong Liu and Dr. Jie Lu for their excellent training, advice and technical help. I would also like to thank the University of

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Chapter 1: Introduction

1.1 Depression and Classical Antidepressants

Major depressive disorder (MDD) is a highly prevalent and debilitating mental disorder that affects more than 300 million people globally and is associated with an extremely high personal and socioeconomic burden, especially in light of the major limitations of current antidepressant therapies (Gerhard et al., 2016; Schwartz et al., 2016). The rationale for traditional antidepressant medications, such as selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), is based on the concept that monoamine neurotransmitter systems (i.e. serotonin (5-HT), norepinephrine (NE) and dopamine (DA)) are hypoactive, especially in brain regions strongly implicated in MDD (Duman et al., 2016; Hindmarch, 2001; Reus et al., 2016). However, although classical antidepressants cause a rapid elevation in central monoamine levels, they are associated with a significant time lag of several weeks to months before therapeutic effects are observed. This not only calls into question the monoamine theory of depression, but also creates a major clinical concern given individuals with depression can be at a high risk of suicide (Abdallah et al., 2015; Duman et al., 2012). In addition, meta-analyses of clinical trials have reported that more than 60% of patients fail to obtain significant or sustained remission with any single traditional antidepressant, with approximately one-third of all depressed individuals failing two or more first-line antidepressant courses of treatment, consistent with the diagnosis of treatment-resistant depression (TRD) (Gerhard et al., 2016; Huynh et al., 2008).

It is now over 50 years since the introduction of antidepressants into clinical practice, and with very few exceptions, new medications in this class have been variants of existing drugs and

not surprisingly have similar limitations (i.e. delayed onset of action and low response rates) (Sanacora and Schatzberg, 2015; Willner et al., 2014). Disappointingly, little progress has been made in identifying truly novel antidepressant targets to improve clinical outcomes (Willner and Belzung, 2015). Thus, despite decades of research, the fundamental pathophysiological mechanisms underlying depression are still poorly understood, while as noted, current antidepressants still have important shortcomings, highlighting a clear unmet clinical need for novel antidepressants with higher response rates and a rapid onset of action.

1.2 Glutamate and Synaptic Plasticity in Depression

Despite its popularity, the monoamine hypothesis of depression cannot by itself provide a comprehensive understanding of the pathophysiology of MDD or the exact mechanism of action of antidepressant drugs. Given these shortcomings, attention has been directed towards other promising targets, and in particular, emerging evidence implicates dysfunctions within glutamatergic systems and a dysregulation of synaptic plasticity in the pathophysiology of depression (Aleksandrova et al., 2019; Duman et al., 2016; Gerhard et al., 2016; Lener et al., 2017b; Marsden, 2013; Pittenger and Duman, 2008; Wang et al., 2014). The two major forms of synaptic plasticity at the glutamatergic synapse, long-term potentiation (LTP) and long-term depression (LTD), which involve activity-dependent strengthening or weakening of synaptic connections, respectively, are thought to represent the cellular substrates of learning and memory in the brain (Citri and Malenka, 2008; Collingridge et al., 2004; Howland and Wang, 2008). Activation of glutamatergic N-methyl-D-aspartate receptors (NMDAR) is involved in the induction of LTP and LTD (e.g. in the hippocampus, HPC), triggering intracellular cascades that subsequently alter surface expression levels and/or function of α-amino-3-hydroxy-5-methyl-

isoxazole-4-propionic acid receptors (AMPARs), leading to either an increase or decrease in AMPAR-mediated synaptic transmission (Citri and Malenka, 2008; Collingridge et al., 2004; Howland and Wang, 2008).

In depressed patients, abnormalities in glutamate/glutamine cycling, as well as elevated glutamate levels in plasma, CSF and key brain areas including the HPC and prefrontal cortex (PFC) have been reported (Lener et al., 2017b; Musazzi et al., 2013; Zarate et al., 2003) (Lener et al., 2017; Musazzi et al., 2013; Zarate et al., 2003). Furthermore, abnormal NMDAR and AMPAR gene expression, density, and function are observed in MDD (Feyissa et al., 2009; Ghasemi et al., 2014; Machado-Vieira et al., 2009a). Preclinical studies have established that chronic stress, which in turn elevates glucocorticoid (GC) levels, is associated with pathological glutamate excitotoxicity and synaptic dysfunction, leading to reductions in dendritic branching and spine density of pyramidal neurons, and eventually neuronal atrophy, in areas implicated in MDD, particularly the HPC and PFC (Abdallah et al., 2015; Duman et al., 2016; Marsden, 2013; Wang et al., 2014; Wong et al., 2007; Zarate et al., 2003). Similar to findings in MDD and consistent with these disruptions in neuronal function and morphology, chronic stress in rodents lowers the expression of critical receptors and proteins involved in synaptic plasticity, including AMPAR (GluA1-3), NMDAR (GluN1, 2B) subunits and other synaptic proteins (e.g. synapsin 1 and post-synaptic density 95, PSD95) in these regions (Duman et al., 2016; Marsden, 2013).

Importantly, preclinical studies (mostly using hippocampal slice preparations) indicate that both acute and chronic stress, as well as exogenous glucocorticoids, can perturb the normal balance between LTP and LTD, by inhibiting LTP and/or facilitating LTD, particularly in the rodent HPC (Aleisa et al., 2006b, 2006a; Aleksandrova et al., 2019; Alfarez et al., 2003; Artola et al., 2006; Cao et al., 2004; Chattarji et al., 2015; Foy et al., 1987; Holderbach et al., 2007;

Howland and Wang, 2008; Kim et al., 1996; Kim and Diamond, 2002; Leuner and Shors, 2013; Licznerski and Duman, 2013; Luo et al., 2014; Maggio and Segal, 2011, 2009; Marsden, 2013; Myers et al., 2014; Pavlides et al., 2002, 1996; Riga et al., 2017; Ryan et al., 2010; Shors et al., 1989; Von Frijtag et al., 2001; M. Wang et al., 2006; Wong et al., 2007; Xiong et al., 2004, 2003; Xu et al., 1998, 1997; Yang et al., 2006, 2005, 2004, 2007). Accumulating evidence suggests that when prolonged (e.g. under chronic stress), such an imbalance between activity-dependent potentiation and weakening can lead to a propensity toward synaptic destabilization and neuronal atrophy in the HPC and PFC (Figure 1), paralleling or possibly mediating (or at least contributing to) the structural and functional findings in MDD (Aleksandrova et al., 2019; Arnsten, 2015; Marsden, 2013; Papp et al., 2017; Roiser and Sahakian, 2013).

In particular, human *postmortem* and imaging studies show significant grey matter volume reductions in the HPC, orbital, dorsolateral and medial PFC and ventral striatum, as well as regional reductions in the numbers and/or size of neurons and glia (Campbell and MacQueen, 2003; Licznerski and Duman, 2013; Manji et al., 2003). Consistent with these effects in diverse brain regions, MDD is also associated with various cognitive deficits, such as impairments in attention, episodic memory and executive function, in addition to the core symptoms of emotional dysregulation and anhedonia, all of which could be mediated by impaired synaptic plasticity processes and loss of connectivity between these key brain regions particularly vulnerable to stress (Abdallah et al., 2017; Aleksandrova et al., 2019; Campbell and MacQueen, 2003; Chattarji et al., 2015; Cornwell et al., 2010; Gass et al., 2018; Licznerski and Duman, 2013; Park et al., 2015; Pittenger and Duman, 2008; Rock et al., 2014; Russo and Nestler, 2013). Despite this, surprisingly, a comprehensive understanding of the role of synaptic plasticity in the pathophysiology of depression or in mediating antidepressant response is still lacking.

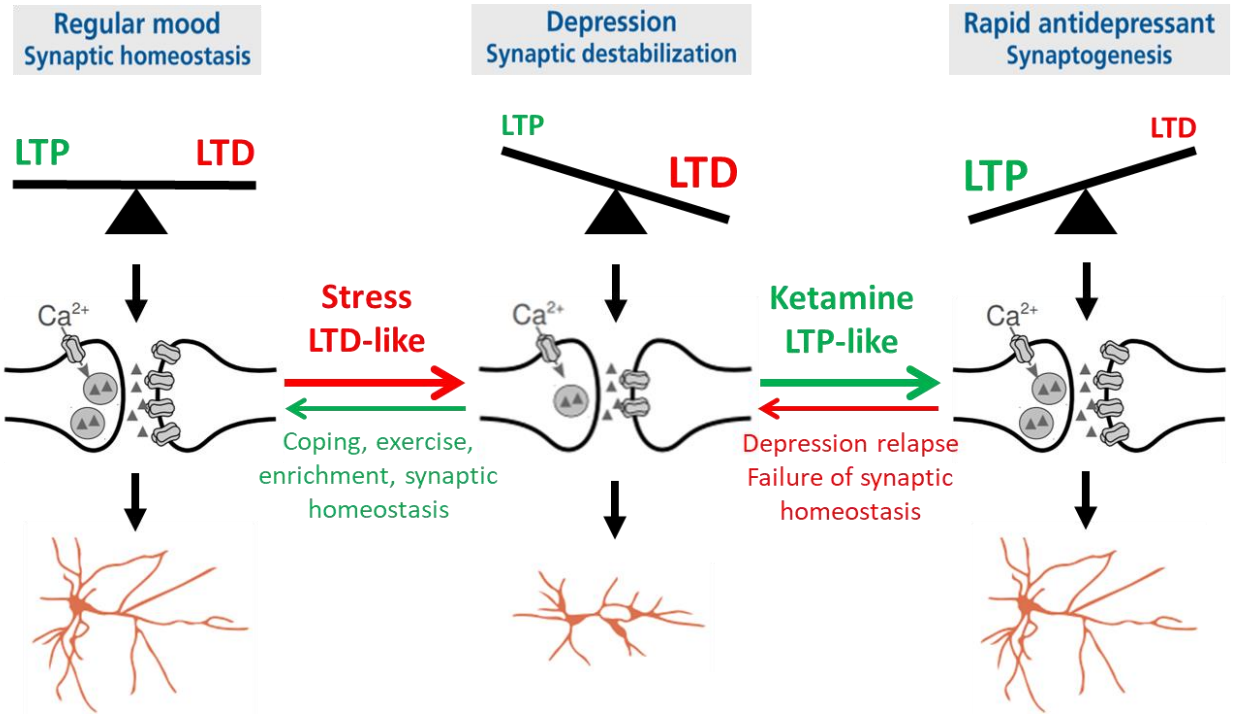


Figure 1. Role of synaptic plasticity in depression and antidepressant response.

Accumulating evidence implicates a dysregulation of synaptic plasticity in the pathophysiology of depression. Preclinical studies have indicated that stress can perturb the normal balance in synaptic plasticity, inhibiting LTP and/or facilitating LTD, leading to synaptic weakening and neuronal atrophy, particularly in the HPC and mPFC. Rapid antidepressants such as ketamine, on the other hand, are thought to cause a glutamate burst, which enhances AMPAR mediated transmission and causes rapid synaptogenesis by increasing BDNF and mTOR signaling in the HPC and mPFC, effectively resetting the system by engaging an LTP-like process. Despite this, surprisingly, a comprehensive understanding of the role of synaptic plasticity in the pathophysiology of depression or in mediating antidepressant response is still lacking. Figure reprinted from Aleksandrova et al. (2019) with permission.

In the last decade, neurotrophic factors, which are now known to regulate adult synaptic plasticity and neuronal survival, in addition to neural growth and differentiation during development, are strongly implicated as important mediators of antidepressant response (Duman, 2014a; Marsden, 2013; Nestler et al., 2002; Neto et al., 2011). The link between neurotrophins and stress/depression is supported by preclinical studies showing that chronic stress and/or elevations of GCs induce significant reductions in brain-derived neurotrophic factor (BDNF)

levels, which, at least in part, mediate the synaptic dysfunction and neuronal atrophy in vulnerable brain regions including the HPC and PFC (Björkholm and Monteggia, 2016; Neto et al., 2011). Consistent with this conclusion, plasticity-related changes such as hippocampal atrophy are associated with decreased expression or function of BDNF and/or its high-affinity receptor TrkB in depressed patients (Autry and Monteggia, 2012; Björkholm and Monteggia, 2016). Importantly, preclinical research has demonstrated that chronic (but not acute) administration of virtually all classes of antidepressants increases BDNF expression in the rodent HPC; BDNF signaling, in turn, promotes synaptic plasticity (LTP), synaptogenesis and hippocampal/cortical function (Autry and Monteggia, 2012; Björkholm and Monteggia, 2016; Panja and Bramham, 2014; Radecki et al., 2005). The fact that increases in neurotrophin levels occur downstream of changes in monoamine signaling could explain the delay in therapeutic onset with classical antidepressants (Duman, 2002; Nestler et al., 2002).

In support of the role of synaptic plasticity in the pathophysiology of depression and in mediating antidepressant response, accumulating evidence suggests that ketamine, an NMDAR antagonist with robust rapid and sustained antidepressant effects in TRD, may reverse the loss of normal connectivity between the HPC, PFC and associated regions by engaging synaptic plasticity and synaptogenesis to "reset the system" (Figure 1).

1.3 Ketamine as an Antidepressant

1.3.1 Antidepressant Efficacy and the NMDAR Hypothesis

Ketamine hydrochloride is a non-competitive, non-subtype selective NMDAR antagonist, which has been used primarily as an anesthetic agent (at doses in the range of 1-3mg/kg) since the 1960s (Reus et al., 2016). One of the most exciting discoveries in the field of depression

treatment came in 2000, when a seminal pilot clinical trial first reported a rapid and robust antidepressant effect following a single intravenous (IV) infusion of ketamine at a subanesthetic dose in individuals with treatment-resistant depression (TRD) (Berman et al., 2000). Following this initial finding, several additional placebo-controlled randomized clinical trials and meta-analyses have been conducted, supporting the utility of ketamine as a novel and superior antidepressant (Abdallah et al., 2016; Kishimoto et al., 2016; Newport et al., 2015). Most of these trials used a single 40min-long IV infusion of (R,S)-ketamine (racemic mixture) at a dose of 0.5 mg/kg, resulting in response rates of 50-70% in TRD populations (Gerhard et al., 2016; Kishimoto et al., 2016; Muller et al., 2016). Following a single infusion of ketamine, the majority of patients experienced significant symptomatic relief (i.e. reductions in depressed mood, anhedonia, and suicidal thoughts) with onset as early as 2 hours, and therapeutic effects peaking at 24 hours and lasting up to 1-2 weeks, after ketamine administration (Kishimoto et al., 2016; Park et al., 2015). Unlike classical antidepressants, ketamine infusions have also been consistently shown to be effective in treating major depressive episodes in patients with bipolar disorder (Kishimoto et al., 2016). Importantly, several studies have recently supported the idea that repeated infusions of ketamine can effectively extend treatment response in patients with TRD; however, more data are required to determine whether intermittent ketamine administration can sustain MDD remission (Kishimoto et al., 2016; Phillips et al., 2019; Shiroma et al., 2014). Therefore, ketamine's robust, rapid and sustained antidepressant effects, as well as its high response rates especially in TRD populations, are in stark contrast to the inadequacy of traditional antidepressants (Berman et al., 2000; Newport et al., 2015; Schwartz et al., 2016), and have inspired preclinical studies of ketamine for depression.

Numerous studies have successfully duplicated the positive effects of ketamine in rodent tests and/or models of depression, including the forced swim test (FST, the most commonly used preclinical screen for antidepressant activity) and the chronic mild stress paradigm (CMS, the most commonly used preclinical model of depression) (Abelaira et al., 2013; Autry et al., 2011; Koike et al., 2011; Li et al., 2011; Zanos et al., 2016; Zhou et al., 2014). A single systemic injection of ketamine at 10mg/kg (intraperitoneal, ip) produces a significant reduction in FST immobility as early as 30min after administration, which has been shown to persist for an average of 7 days in both rats and mice (Autry et al., 2011; Koike et al., 2011; Zanos et al., 2016; Zhou et al., 2014). In addition, while rodents exposed to CMS exhibit depressive-like behaviours including abnormal stress coping in the FST and anhedonia in the sucrose preference test (SPT), ketamine effectively reverses these stress-induced deficits (Li et al., 2011; Zanos et al., 2016). Ultimately, ketamine's unprecedented antidepressant activity not only provides support for an alternative to the monoamine deficiency hypothesis of depression, but also, has inspired tremendous interest in the identification of molecular mechanisms mediating the drug's clinical efficacy in TRD.

Despite its promise, it is important to note that even at subanesthetic doses, ketamine administration is associated with dissociative effects, neurocognitive and sensory-motor disturbances (although mild and transient), as well as short-lasting elevations in heart rate and blood pressure (Abdallah et al., 2015; Schwartz et al., 2016). In addition, ketamine is used as a recreational drug and thus holds the potential of being abused, while prolonged use may cause neurotoxic effects (Gerhard et al., 2016; Reus et al., 2016). The hope is that understanding ketamine's antidepressant mechanism of action will assist in the development of a new

generation of novel rapid-acting antidepressants lacking these unfavourable features that currently limit ketamine's wider clinical use.

The initial line of thought was that ketamine's effects were simply attributed to its ability to block the NMDA receptor; therefore, the NMDAR inhibition hypothesis of ketamine in depression prompted the evaluation of alternative NMDAR antagonists as novel, safer antidepressants. However, human clinical trials indicate that alternative NMDAR blockers, such memantine, AZD6765 and CP-101,606, generally lack ketamine's robust, rapid and/or sustained antidepressant effects (Newport et al., 2015; Yang et al., 2015; Zanos et al., 2016). Indeed, to date, ketamine is the only NMDAR antagonist to consistently demonstrate antidepressant efficacy in multiple trials (Newport et al., 2015; Yang et al., 2015; Zanos et al., 2016). Similarly, for example, animal studies indicate that treatment with the NMDAR antagonist MK-801, which binds to the same receptor site as ketamine, failed to produce sustained antidepressant effects in rodents (Maeng et al., 2008; Zanos et al., 2016). Importantly, the differential clinical action of NMDAR blockers can be attributed to differences in the nature of NMDAR inhibition (i.e. specificity in terms of receptor subunit and localization, the extent/nature of channel block (e.g. affinity, trapping), etc.), as well as the unique downstream effects of the drug-receptor interaction (Aleksandrova et al., 2017b). Therefore, while the absence of antidepressant properties of one NMDAR antagonist (i.e. MK-801, memantine, etc.) should not be taken as evidence against the NMDAR hypothesis of ketamine; it is becoming increasingly clear that it is crucial to consider not only ketamine's unique action on the NMDA receptor, but also the key downstream signaling events as a result of ketamine-induced NMDAR inhibition, which are not observed with alternative NMDAR antagonists lacking antidepressant properties.

1.3.2 Ketamine's Mechanism of Action: NMDAR and beyond

Given its short half-life (1-3h in plasma) (Li and Vlisides, 2016; Zarate et al., 2012; Zhao et al., 2012), ketamine's sustained antidepressant efficacy, which is maintained long after its complete metabolism and elimination (~1week), cannot be solely attributed to the drug's acute receptor effects (Aleksandrova et al., 2017a, 2017b; Machado-Vieira et al., 2015; Marsden, 2013). Instead, its therapeutic activity appears to be due to activation of crucial downstream signaling cascades as a secondary consequence of NMDAR inhibition, which results in long-lasting adaptations in key neural circuits (Aleksandrova et al., 2017a, 2017b; Machado-Vieira et al., 2015; Marsden, 2013). Recent advances in the field have been reflected in significant elaboration of the NMDAR hypothesis of ketamine action, as new evidence has emerged concerning events downstream of NMDAR inhibition (Figure 2). The NMDAR hypothesis of ketamine has given rise to two major, non-mutually exclusive, models to explain the antidepressant action of this compound, the "disinhibition" (2A) and the "direct inhibition" (2B) hypotheses (Aleksandrova et al., 2017b; Miller et al., 2016; Zorumski et al., 2016).

The first hypothesis (Figure 2A) proposes that low doses of ketamine selectively antagonize NMDARs on GABAergic inhibitory interneurons (INs) leading to disinhibition of excitatory pyramidal neurons (PNs), a burst of glutamate release and acute AMPAR activation in the PFC and HPC (Aleksandrova et al., 2017b; Miller et al., 2016; Zorumski et al., 2016). This, in turn, results in the activation of key downstream signaling pathways, particularly those involving BDNF release and activation of the mammalian target of rapamycin (mTOR) (Abdallah et al., 2016; Du et al., 2006; Maeng et al., 2008; Yang et al., 2013). Under the second hypothesis (Figure 2B), direct antagonism of NMDARs on PNs by ketamine at rest is thought to block tonic NMDAR activation by ambient or spontaneously released glutamate (which can

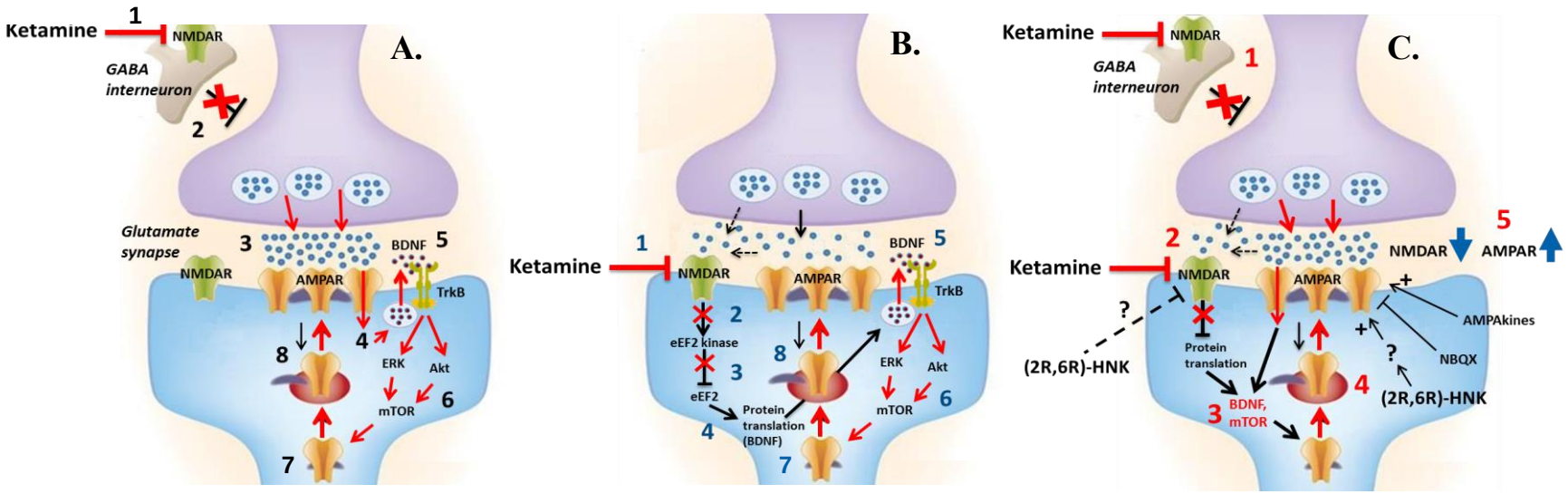


Figure 2. Schematic of three competing hypotheses of ketamine action in depression.

A. Disinhibition hypothesis: 1) At low doses ketamine preferentially inhibits NMDARs on inhibitory GABAergic interneurons (INs), 2) causing disinhibition of glutamatergic pyramidal neurons (PNs), 3) a burst of glutamate release, 4) AMPAR activation and Ca2+influx, which 5) triggers activity-dependent BDNF release and binding to TrkB, 6) to activate two major downstream signaling cascades (MEK-ERK & PI3K-Akt), which converge onto mTOR, 7) whose activation leads to disinhibition of synaptic protein translation (e.g. GluA1-2, PSD95, etc.), with 8) newly synthesized AMPARs and other synaptic components being inserted into the postsynaptic density. ***B. Direct inhibition hypothesis:*** 1) At rest, ketamine directly inhibits NMDARs on PNs, which can be tonically activated by ambient and spontaneously released glutamate (due to an incomplete Mg^{2+} block), 2) this prevents glutamate excitotoxicity and blocks activation of the eukaryotic elongation factor 2 (eEF2) kinase, 3) reducing suppression of eEF2 and 4) thus enhancing eEF2-mediated protein translation, particularly of BDNF, 5-8) (see A.): briefly, downstream signaling pathways involving BDNF and mTOR are recruited, leading to enhanced protein synthesis and surface AMPAR upregulation. ***C. Unifying model:*** The 1) disinhibition and 2) direct inhibition hypotheses are non-mutually exclusive, and likely complementary. A unifying theory postulates that ketamine has the unique ability to 3) recruit key intracellular signaling cascades (BDNF and mTOR) and 4) initiate an LTP-like process involving acute AMPAR activation and sustained enhancement of AMPAR-mediated transmission. 5) Ultimately, ketamine increases the ratio of AMPAR to NMDAR throughput via directly blocking NMDARs and indirectly enhancing AMPAR function, leading to synaptic protein synthesis, synaptogenesis and reversal of stress-induced synaptic dysfunction and neuronal atrophy in brain areas implicated in MDD (HPC, PFC). The active metabolite (2R,6R)-HNK may recapitulate aspects of ketamine action and facilitate AMPAR-mediated transmission; however, its exact mechanism of action remains unknown. Figure reprinted from Aleksandrova et al. (2017b) with permission.

occur due to an incomplete Mg2+ block) (Autry et al., 2011; Miller et al., 2016). This has been proposed to block glutamate excitotoxicity, reducing suppression of eukaryotic elongation factor 2 (eEF2)-mediated protein synthesis and recruiting the downstream signaling pathways mentioned above (e.g. BDNF, mTOR) (Autry et al., 2011; Miller et al., 2016). Consistent with a direct action on PNs, selective genetic deletion of the NMDAR subunit GluN2B from cortical PNs both mimics and occludes ketamine's antidepressant effects in mice, as well as enhancing protein synthesis and AMPAR-mediated transmission in the PFC (Miller et al., 2014). It has been proposed that low doses of ketamine selectively block GluN2B-containing NMDARs as they are thought to be 1) tonically activated by spontaneously released and/or ambient glutamate, and 2) mainly extra-synaptic and potentially more accessible to exogenous antagonism, although this continues to be debated (Collingridge et al., 2010; Kiss et al., 2012; Miller et al., 2016; Strasburger et al., 2017). At high doses, ketamine may gradually inhibit synaptic NMDARs, leading to dissociative effects and eventually anesthesia (Miller et al., 2016). This body of work has prompted the hypothesis that while ketamine is non-subunit specific, antagonism of GluN2B-containing NMDARs might be responsible for its antidepressant action (Miller et al., 2016, 2014; Strasburger et al., 2017). In partial support of this, the selective GluN2B antagonist, Ro25-698 possesses rapid antidepressant action in rodents, but effects are reported to be less robust and/or shorter-lasting compared to ketamine (Jimé Nez-Sánchez et al., 2014; Li et al., 2011; Lima-Ojeda et al., 2013; Maeng et al., 2008; Zanos et al., 2016).

Regardless of the exact trigger, ketamine initiates a long-term potentiation (LTP)-like process leading to synaptic protein synthesis, surface AMPAR subunit upregulation and synaptogenesis in the HPC & PFC (Figure 1) (Aleksandrova et al., 2019, 2017b; Björkholm and Monteggia, 2016; Li et al., 2010; Maeng et al., 2008). Thus, it is thought to induce an NMDAR

inhibition-dependent form of synaptic plasticity, reversing the stress-induced synaptic dysfunction and neuronal atrophy in brain areas implicated in MDD, effects believed to underlie the antidepressant response to ketamine (Aleksandrova et al., 2019, 2017a; Leuner and Shors, 2013; Licznerski and Duman, 2013). Together, these studies have significantly expanded on the simplified NMDAR inhibition hypothesis of ketamine action, supporting the crucial involvement of AMPARs and synaptogenic pathways in mediating ketamine's antidepressant effects (Abdallah et al., 2015; Aleksandrova et al., 2017a; Maeng et al., 2008). A unifying theory (Figure 2C) postulates that ketamine's mechanism of action in depression may depend on its ability to increase the ratio of AMPA to NMDA receptor throughput via directly blocking NMDARs and indirectly enhancing AMPAR function, which in turn appears to activate key signaling pathways, particularly BDNF and mammalian target of rapamycin (mTOR) (Aleksandrova et al., 2017b; Chan et al., 2016; Du et al., 2006; Duman, 2014a; Dwyer and Duman, 2013; Marsden, 2013; Miller et al., 2016).

Many studies show consistent increases in BDNF translation and secretion in the rodent HPC and PFC following ketamine (Schwartz et al., 2016; Yang et al., 2013; Zhou et al., 2014), although one study reports that ketamine's antidepressant efficacy is preserved in bdnf +/- heterozygous null mice (Lindholm et al., 2012). BDNF is also associated with antidepressant responses to classical antidepressants (e.g. SSRIs, TCAs), which lack efficacy in BDNF knockout mice (Björkholm and Monteggia, 2016; Duman et al., 2016; Schwartz et al., 2016). Although it takes several weeks for these traditional agents to trigger BDNF-mediated synaptic plasticity, such changes can occur within hours following ketamine administration (Duman et al., 2016; Schwartz et al., 2016). Therefore, while typical antidepressants may only indirectly modulate signaling pathways relevant to antidepressant response, ketamine has the unique ability

to trigger long-lasting adaptations within hours thus explaining its rapid and sustained antidepressant effects (Ampuero et al., 2010; Kavalali and Monteggia, 2015; Machado-Vieira et al., 2009b; Sanacora and Schatzberg, 2015; Wang et al., 2014). Another key downstream convergence pathway implicated in depression susceptibility and ketamine action is the mammalian target of rapamycin (mTOR) signaling pathway, which regulates activity-dependent synaptic plasticity and translation of synaptic proteins (Ignacio et al., 2015; Li et al., 2010). Consistent with this, both the antidepressant and synaptogenic effects of ketamine in rodents are blocked by pre-administration of rapamycin, a selective mTOR inhibitor (Li et al., 2010). Importantly, mTOR activation is not seen following chronic treatment with any class of traditional antidepressant drugs (Duman and Aghajanian, 2012), likely contributing to ketamine's superior antidepressant efficacy.

Importantly, a single systemic injection of ketamine (standard dose of 10mg/kg, ip) leads to synaptogenesis in the rodent HPC and mPFC, reversing the stress-induced synaptic destabilization and neuronal atrophy (Aleksandrova et al., 2017a; Autry et al., 2011; Fukumoto et al., 2015; Garcia et al., 2008; Kavalali and Monteggia, 2012; Li et al., 2011, 2010; Maeng et al., 2008; Marsden, 2013; Moda-Sava et al., 2019; Nosyreva et al., 2013; Tizabi et al., 2012; Yang et al., 2013; Zhou et al., 2014). Specifically, ketamine has been shown to cause a burst of glutamate release, increase surface expression of AMPAR subunits GluA1 and/or GluA2, glutamate transporters and other synaptic proteins (e.g. PSD95 and synapsin), enhance excitatory synaptic transmission, as well as increasing mushroom spines, dendritic branching, vascularization and glial coverage in the HPC and/or mPFC (Aleksandrova et al., 2017a; Autry et al., 2011; Fukumoto et al., 2015; Garcia et al., 2008; Kavalali and Monteggia, 2012; Li et al.,

2011, 2010; Maeng et al., 2008; Marsden, 2013; Moda-Sava et al., 2019; Nosyreva et al., 2013; Tizabi et al., 2012; Yang et al., 2013; Zanos et al., 2016; Zhou et al., 2014; Zhu et al., 2017).

In contrast to the ambivalent findings concerning NMDAR inhibition mentioned previously, accumulating evidence supports the crucial involvement of AMPA receptors in mediating the antidepressant effects of ketamine (Koike et al., 2011; Machado-Vieira et al., 2015; Maeng et al., 2008; Park et al., 2015). Preclinical studies indicate that ketamine produces a rapid (but transient) rise in glutamate release and cycling in the medial prefronal cortex (mPFC), which in turn leads to the acute activation of AMPA receptors, resulting in activation of synaptogenic pathways and long-lasting upregulation of AMPAR function, effects proposed to mediate the rapid and sustained antidepressant effects of ketamine, respectively (Chowdhury et al., 2016; Moghaddam et al., 1997). Consistent with this, quantitative electroencephalography (qEEG) performed in both humans and rodents within hours after ketamine administration has shown that this drug induces significant increases in gamma-band power, which depends on the activation of fast ionotropic excitatory receptors, mainly AMPARs (Zanos et al., 2016). Interestingly, both the ketamine-induced synaptic potentiation and behavioural antidepressant effects are absent in GluA2 knock-out mice (Nosyreva et al., 2013). In addition, several studies show that pre-treating rats or mice with the AMPAR antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) 10min before ketamine injection blocks its rapid and sustained antidepressant effect in the FST, without affecting other behaviours such as the ketamine-induced hyperlocomotion (Koike et al., 2011; Koike and Chaki, 2014; Maeng et al., 2008; Zanos et al., 2016). Furthermore, NBQX administration 23.5h after ketamine treatment blocked its antidepressant effects in the FST at 24h (Koike and Chaki, 2014; Zanos et al., 2016). Also consistent with the role of AMPARs in the actions of ketamine, the ketamine-induced

increases in mTOR, BDNF and GluA1 levels can be prevented by pre-treatment with the AMPA antagonist NBQX (Maeng et al., 2008; Zhou et al., 2014). Moreover, administration of AMPAkines appears to mimic these key molecular effects of ketamine and can be abolished by NBQX (Akinfiresoye and Tizabi, 2013; Karasawa et al., 2005; Koike and Chaki, 2014; Lindholm et al., 2012; Machado-Vieira et al., 2015; Schechter et al., 2005; Yang et al., 2013; Zhou et al., 2014). Taken together, these data indicate that activation of AMPA receptors is required for both the rapid and sustained antidepressant actions of ketamine. Interestingly, preclinical studies indicate that group II metabotropic glutamate (mGlu2/3) receptor antagonists possess ketamine-like antidepressant effects, which can also be blocked by NBQX pre-treatment and thus similarly involve AMPAR stimulation (Karasawa et al., 2005; Koike and Chaki, 2014). Thus, emerging evidence indicates that facilitation of AMPAR-mediated signaling might represent a downstream point of convergence in the antidepressant action of rapid antidepressants (Aleksandrova et al., 2017a, 2017b; Du et al., 2007; Freudenberg et al., 2015).

It is important to note that, in addition to its glutamatergic effects discussed here, even at low sub-anesthetic doses, ketamine may have non-NMDAR targets (e.g. sigma receptors, mu opioid receptors, etc.), and is a powerful mobilizer of midbrain catecholamines (in addition to glutamate), and these properties may account for/contribute to some aspects of both the psychotomimetic and antidepressant properties of this drug (Sanacora and Schatzberg, 2015).

Although the key molecular and structural effects of ketamine reviewed here (e.g. on NMDAR and AMPA function, BDNF and mTOR signaling, synaptogenesis) are now well established, mechanisms underlying the drug's antidepressant effects on a "systems level" remain unclear. One theory is that ketamine may reverse the loss of normal connectivity between the HPC, PFC and associated regions by engaging synaptic plasticity and synaptogenesis to

"reset the system" (Figure 1, 2) (Aleksandrova et al., 2019; Chattarji et al., 2015; Gerhard et al., 2016; Licznerski and Duman, 2013; Manji et al., 2003; Marsden, 2013; Refsgaard et al., 2017; Schwartz et al., 2016). However, surprisingly, systematic studies of ketamine's effects on regional synaptic plasticity, especially *in vivo* and in the context of different animal model of depression are largely lacking. Incorporating synaptic plasticity into the current framework of ketamine antidepressant action may serve to bridge understanding of its molecular and cellular effects with those related to regional structural plasticity and neural circuit functioning.

1.3.3 Ketamine's Metabolite, (2R,6R)-HNK

In 2016, a pivotal paper published in Nature (Zanos et al., 2016) reported the discovery of an active ketamine metabolite, which exhibited similar antidepressant efficacy without key receptor binding properties or side effects of the parent compound in mice (Zhou et al., 2014). Ketamine is stereoselectively metabolized into a wide range of metabolites, many previously presumed to be clinically inactive due to the absence of anesthetic properties resulting from NMDAR antagonism (Moaddel et al., 2013). Importantly, in this study, one ketamine metabolite, (2R,6R)-hydroxynorketamine (HNK), was observed to have 3-fold higher brain concentrations in female versus male rats, and this was postulated to underlie the enhanced potency of ketamine observed in females (Zanos et al., 2016; Zhou et al., 2014). Furthermore, Zanos et al. showed that a metabolically inert form of ketamine, with apparently the same receptor binding properties but no metabolism into HNK, lacked the sustained antidepressant actions of ketamine in several rodent depression-relevant tasks (Zanos et al., 2016). Next, Zanos et al. demonstrated that the (2R,6R)-HNK metabolite (10mg/kg, ip) itself had robust rapid and sustained antidepressant action similar to ketamine in normal mice and several preclinical models of depression (Zanos et al., 2016). Similar to findings with ketamine, the AMPAR antagonist NBQX administered either

before HNK injection or at the time of behavioural testing, blocked the rapid and sustained antidepressant effects of the metabolite (Zanos et al., 2016). Intriguingly, unlike its parent compound (Ki for NMDAR = 465nM and 1340nM for (S)- and (R)-Ket, respectively), (2R,6R)-HNK does not functionally inhibit NMDARs (Ki>10μM) (Zanos et al., 2016). Instead it causes a dramatic increase in AMPAR-mediated synaptic transmission in hippocampal CA1 slices (at 10μM, bath applied), as well as rapid upregulation of GluA1 and GluA2 AMPAR subunits in hippocampal synaptosomes (within an hour) and elevated levels of hippocampal BDNF (at 24h) following systemic treatment (10mg/kg, ip) (Zanos et al., 2016). Finally, in contrast to ketamine, HNK lacked sensory-dissociation and hyperlocomotion effects, or reinforcing properties, and thus abuse potential in preclinical tests (Zanos et al., 2016).

Overall, these exciting findings prompted Zanos et al. to propose that this key metabolite of ketamine is necessary and sufficient in rats for antidepressant actions of the pro-drug, in addition to effectively challenging the NMDAR hypothesis of ketamine action (Zanos et al., 2016). Indeed, (2S,6S;2R,6R)-HNK is a major metabolite of ketamine, and thus, its potential contribution to ketamine's clinical effects is a very appropriate line of research. However, available data indicate that peak (2R,6R)-HNK concentrations in the brain following systemic administration of antidepressant doses of ketamine may be lower than those used by Zanos et al. (likely translating to a magnitude of ~0.1-1μM vs. 10μM used in their study), although considerable debate exists over this point due to the lack of human data and difficulties in translating data from rodents to humans (Strasburger et al., 2017; Yang et al., 2016; Zanos et al., 2016; Zarate et al., 2012). As (2S,6S;2R,6R)-HNK does not apparently inhibit NMDARs at therapeutically relevant concentrations, the molecular target(s) responsible for its behavioural and synaptic effects reported are still unclear. Importantly, one previous study reported that

(2R,6R)- and (2S;2R)-HNK inhibit α7-nicotinic acetylcholine receptor (nAchR) function at concentrations <1μM using path-clamp techniques (Moaddel et al., 2013). It is important to note that clinical studies have supported the utility of open-channel non-competitive α7-nAchR inhibitors for the treatment of depression (Moaddel et al., 2013). Another previous study reported significant effects of ketamine metabolites on D-serine concentrations *in vitro*, highlighting another possible mechanism of action for (2R,6R)-HNK (Singh et al., 2016). Although (2R,6R)-HNK does not directly inhibit NMDARs at clinically relevant concentrations, it is possible that its mechanism of action involves indirect NMDAR inhibition elicited by a reduction in D-serine co-agonist levels, a theory that awaits validation *in vivo* (Singh et al., 2016). Despite the significant gap in current understanding of (2R,6R)-HNK's mechanism of action, available data supports a hypothesis that like ketamine, (2R,6R)-HNK might be able to increase the AMPA to NMDA receptor throughput (Figure 2C), although future studies are required to define (2R,6R)-HNK's molecular target(s) and exact mechanism of action, and importantly, determine to what extent they contribute to ketamine's antidepressant action.

Not surprisingly, these breakthrough findings by Zanos et al. created great interest in the field, prompting a re-evaluation of the NMDAR hypothesis of ketamine, and highlighting (2R,6R)-HNK as a promising new, safer, rapid-acting antidepressant (Zanos et al., 2016). As may be expected, however, this discovery has come under close scrutiny (for a review on the debate surrounding (2R,6R)-HNK, see Aleksandrova et al., 2017b). Adding to the controversy surrounding (2R,6R)-HNK, Hashimoto and colleagues failed to replicate the antidepressant effects of the ketamine metabolite in two rodent models of depression, the lipopolysaccharide (LPS) injection and the chronic social defeat (CSD) stress models, which were also used in the Zanos et al. study (Yang et al., 2016). In this study, while a single dose of (R)- and (S)- ketamine

(10mg/kg, ip) had rapid and sustained antidepressant effects in the FST, tail suspension test (TST) and SPT, an equivalent dose of (2R,6R)-HNK failed to alleviate both the inflammation- and chronic stress- induced depression-like phenotypes (Yang et al., 2016). No apparent reason for the discrepancy in findings between the two groups can be identified. Consistent with this negative finding, several other groups subsequently failed to demonstrate any antidepressant activity of (2R,6R)-HNK in various animal models of depression (e.g. LPS, CSD and LH) (Shirayama and Hashimoto, 2018; Xiong et al., 2019; Yang et al., 2016; Zhang et al., 2018). Therefore, while the discovery of HNK represents important progress in the field, the claim that ketamine's clinical efficacy is exclusively due to this active metabolite, should be taken with caution. While important questions remain as to whether or not HNK contributes to ketamine's therapeutic actions, it may represent a new promising candidate antidepressant compound, with a more favourable side effects profile and the ability to modulate AMPAR function independent of NMDAR inhibition, which warrants further preclinical and clinical investigation.

1.4 Preclinical Models of Depression

Undoubtedly, over the past few decades, animal models of depression have contributed to our understanding of the neurobiological changes induced by stress, while providing valuable insight into the mechanisms of action of classical and novel antidepressants. However, the shortcomings of available preclinical models of depression have also been recognized as a major impediment to effective translation of basic research on novel antidepressant targets into clinical practice (Belzung, 2014; Willner and Belzung, 2015). As stress often precipitates the onset of depression, many animal models of this disorder employ acute or chronic stress procedures, such as chronic mild stress (CMS), chronic social defeat (CSD) and learned helplessness (LH), to

induce depressive-like phenotypes in normal animals (Chourbaji et al., 2005; Krishnan and Nestler, 2011; Overstreet, 2012; Vollmayr and Gass, 2013; Wiborg, 2013; Willner, 2017; Willner et al., 2018). However, it is becoming increasingly clear that these models largely ignore factors related to depression vulnerability (e.g. genetic, developmental or personality factors), as well as antidepressant resistance (Caldarone et al., 2015; Willner et al., 2014; Willner and Belzung, 2015; Willner and Mitchell, 2002). Thus, drugs that simply reverse the neurotoxic effects of stress in the otherwise normal brain may have limited efficacy and scope of antidepressant action, especially in vulnerable and treatment-resistant populations (Willner et al., 2014; Willner and Belzung, 2015).

Importantly, the association between exposure to high levels of stress and the onset of depression is weaker in individuals with a high genetic risk of depression, as well as in subsequent depression episodes throughout a patient's lifetime (Kendler et al., 2017; Monroe and Harkness, 2005). Moreover, earlier disease onset and increased number and length of depressive episodes increase susceptibility to subsequent episodes, precipitating an accelerating, neuro-progressive illness course and leading to poorer symptomatic, treatment and functional outcomes in such patients (Moylan et al., 2013). Indeed, from a clinical perspective, heightened vulnerability to MDD and resistance to antidepressant treatment are highly correlated, such that patients with recurrent depression are more treatment-resistant than those experiencing their first episode (Willner et al., 2014). As we seek to ensure that the next generation of antidepressants will be effective for vulnerable and treatment-resistant populations, animal models should encompass not only stress-induced phenotypic parallels to clinical depression, but also aspects of heightened stress susceptibility and resistance to conventional antidepressant drugs (Caldarone et al., 2015; Willner and Belzung, 2015; Willner and Mitchell, 2002). This diathesis-stress

perspective supports a shift away from purely stress-based models of depression and towards models of depression vulnerability and partial/full antidepressant resistance.

Current attempts to model depression draw a crucial distinction between specific animal models of depression (i.e. the various stress-based, genetic or surgical models, e.g. CMS, Wistar-Kyoto (WKY) rat or olfactory bulbectomy) and the behavioural readout(s) of depression or treatment response (i.e. the various preclinical tests of depressive-like behaviours/ screens of antidepressant activity, e.g. the forced swim test (FST), sucrose preference test (SPT) or novelty-suppressed feeding (NSF) test) (Overstreet, 2012). Since over-reliance on a single readout of depressive-like behaviour (e.g. FST immobility) is still common practice, in an effort to better characterize available animal models of depression and screen novel compounds in terms of their antidepressant activity, the need to utilize more comprehensive batteries of preclinical tests across various domains affected in MDD (e.g. stress/emotional reactivity, motivation, cognition, etc.) has been emphasized.

Furthermore, a reconceptualization of depression symptoms and their preclinical correlates in terms of circuit dysfunction bears careful consideration (Calcagno et al., 2016; Jay et al., 2004; Panksepp, 2016; Phillips et al., 2018; Wright and Panksepp, 2011). To this end, pioneering work by Panksepp, which supports the existence of at least seven primal emotional systems in the mammalian brain (seeking, rage, fear, lust, care, panic/grief and play), highlights the relevance of three of these systems in particular, to clinical depression. These include an overactive panic system that underlies the “psychic pain of separation distress” (excessive sadness and grief), along with diminished seeking and play systems, which mediate the reduced reward-seeking and play urges (anhedonia and social withdrawal) (Panksepp, 2010; Panksepp and Watt, 2011; Panksepp and Yovell, 2014; Wright and Panksepp, 2011). Thus, Panksepp

proposes that depression can be explained by the interplay between dysfunctions within these emotional circuits (involving key brain areas such as the HPC, PFC, amygdala, etc.) and emphasizes the need to reflect their incorporation into preclinical test batteries used to assess neural correlates of depression and antidepressant response (Panksepp, 2010; Panksepp and Watt, 2011; Panksepp and Yovell, 2014; Wright and Panksepp, 2011). Therefore, careful selection of the appropriate animal model of depression with a stress-diathesis approach in mind, the use of a comprehensive battery of preclinical tests spanning various domains affected in depression, as well as the careful dissection of relevant neural circuits mediating these various depressive-like phenotypes, are some important ways to increase the translational power of preclinical research related to depression (Aleksandrova et al., 2019).

1.5 The Wistar-Kyoto Rat Model of Depression

1.5.1 Behavioural Parallels to Depression

Following their initial use as a control strain in studies of hypertension, WKY rats were first recognized for their susceptibility to developing stress-induced ulcers (Paré, 1989; Paré and Kluczynski, 1997; Paré and Redei, 1993a; Tejani-Butt et al., 2003), which highlighted the fact that their physiology and behaviour are not "normal". Since then, an extensive body of literature (behavioural findings for the WKY model summarized in Table 1) (Aleksandrova et al., 2019), including the pioneering work by Paré, has demonstrated that when compared to control rats, the stress-prone WKY rat exhibits passive coping strategies and behavioural inhibition at baseline (Myers et al., 2014; Paré, 1994a; Paré and Kluczynski, 1997; Paré and Redei, 1993b; Redei et al., 1994; Tejani-Butt et al., 1994; Tizabi et al., 2012), as well as enhanced physiological and behavioural responses to stress (Morilak et al., 2005; Willner and Belzung, 2015). Together,

these traits meet many of the criteria for a valid animal model of depression (Aleksandrova et al., 2019; Overstreet, 2012; Willner and Belzung, 2015).

Table 1. Behavioural findings in the WKY rat compared to outbred controls.

Preclinical Test	**WKY behavioural phenotype**	**References** (*not a comprehensive list)
Body weight	Reduced body weight	Bjorkholm et al., 2015; De La Garza 2nd, 2005; Nagasawa et al., 2015; Paré, 1994b; Solberg et al., 2001
Open field test (OFT)	Hypolocomotion (general behavioural inhibition and psychomotor slowing), less time in the center of the arena (anxiety-like behaviour)	Berton et al., 1997; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993b; Tejani-Butt et al., 2003; Van Zyl et al., 2014
Forced swim test (FST)	Dramatic immobility even without a pre-test session, learned helplessness effect with 2-day FST procedure (abnormal stress-coping)	Lahmame et al., 1997b; Lopez-Rubalcava and Lucki, 2000; Marti and Armario, 1996; Nam et al., 2014; O'Mahony et al., 2011; Paré, 1992; Paré and Redei, 1993a; Tejani-Butt et al., 2003; Tizabi et al., 2012
Learned Helplessness (LH)	Enhanced freezing when pre-exposed to inescapable shock (general behavioural inhibition, abnormal stress-coping)	Belujon and Grace, 2014; Nam et al., 2014; Paré and Redei, 1993a
Active avoidance	Enhanced acquisition of avoidance learning with repeated testing, resistance to avoidance extinction	Cominski et al., 2014; Fragale et al., 2016; Fortress et al., 2018
Sucrose preference test (SPT)	Deficits in sucrose consumption (anhedonia), but conflicting results and sex-specific differences (no deficit in females)	D'Souza and Sadananda, 2017; De La Garza 2nd, 2005; Malkesman et al., 2005 Dommett and Rostron, 2013; Kin et al., 2017; Nam et al., 2014; Burke et al., 2016; Mileva and Bielajew, 2015
Motivated behaviour	Lower effort/motivation to earn sucrose reward under fixed and progressive ratio schedules of reinforcement, reduced nicotine self-administration (anhedonia)	De La Garza, 2005
Social interaction (SI)	Social avoidance at baseline and following stress, reduced aggression and social play in younger rats (withdrawal)	Berton et al., 1997; Malkesman et al., 2005; Nam et al., 2014; Pardon et al., 2002; Van Zyl et al., 2014

Preclinical Test	**WKY behavioural phenotype**	**References** (*not a comprehensive list)
Ultrasonic vocalizations (USVs)	Fewer USVs emitted in novel and social settings (deficient social communication, anhedonia)	Rao and Sadananda, 2015; Shetty and Sadananda, 2017; Van Zyl et al., 2014
Elevated plus maze (EPM)	Less entries made into and less time spent in the open arms (anxiety-like behaviour)	Berton et al., 1997; Nam et al., 2014; Pardon et al., 2002; Tejani-Butt et al., 2003; Van Zyl et al., 2014
Novelty-suppressed feeding (NSF) test	Longer latency to feed (anxiety-like behaviour)	Burke et al., 2016; Nam et al., 2014; Paré, 1994b
Morris water maze (MWM)	Longer latency to escape to the target platform, even with repeated training (impaired spatial learning and memory)	Diana et al., 1994; Grauer and Kapon, 1993; She et al., 2015
Novel object recognition (NOR)	Impaired object location recognition (impaired recognition memory)	Shoval et al., 2016
Sleep-wake cycle	Increased REM (rapid eye movement) sleep and sleep fragmentation (disturbed sleep), altered circadian rhythm of activity (reduced light sensitivity)	DaSilva et al., 2011; Dugovic et al., 2000; Solberg et al., 2001
Repeated stress (e.g. restraint)	Enhanced physiological responses, failure to adapt to repeated stress (two-hit model)	Morilak et al., 2005; Tejani-Butt et al., 1994; Zafar et al., 1997
Chronic mild stress (CMS)	Exacerbated depression- and anxiety- like phenotypes (two-hit model)	Tejani-Butt at al., 1994; Willner et al., 2018; Luo et al., 2015
Early-life stress (e.g. neonatal handling, maternal separation)	Do not generally exacerbate depression- and anxiety- like phenotypes (two-hit model)	Nam et al., 2014; Van Zyl et al., 2014; Zalsman et al., 2015, but Shetty et al., 2017
Males vs. Females	Females may exhibit more pronounced depressive- and anxiety- like phenotypes and higher physiological responses to stress; although others report the opposite	Courvoisier et al., 1996; Paré et al., 1999; Paré and Redei, 1993b; Servatius et al., 2015; Tizabi et al., 2012, 2010 but Burke et al., 2016; Will et al., 2003

Under baseline conditions, WKY rats consistently exhibit hypolocomotion in the open field test (OFT), indicative of general behavioural inhibition and psychomotor slowing (Berton et

al., 1997; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993b; Tejani-Butt et al., 2003; Van Zyl et al., 2014). This is paralleled by the presence of dramatic immobility in the forced swim test (FST) even without a pre-test session, which is thought to reflect abnormal stress-coping and is commonly used as a screen for antidepressant action (Lahmame et al., 1997b; Lopez-Rubalcava and Lucki, 2000; Marti and Armario, 1996; Nam et al., 2014; O'Mahony et al., 2011; Paré, 1992; Paré and Redei, 1993a; Tejani-Butt et al., 2003; Tizabi et al., 2012). WKYs also exhibit enhanced freezing when pre-exposed to inescapable shock in the LH paradigm (Belujon and Grace, 2014; Nam et al., 2014; Paré and Redei, 1993a). Consistent with enhanced fear conditioning, naïve WKY rats display increased acquisition of an active lever-press avoidance paradigm, as well as resistance to avoidance extinction when the shock is eliminated (Cominski et al., 2014; Fortress et al., 2018; Fragale et al., 2016).

In another canonical test of depressive-like behaviour, the sucrose preference test (SPT), WKY rats exhibit pronounced deficits in sucrose consumption indicative of anhedonia (D'Souza and Sadananda, 2017; De La Garza 2nd, 2005; Malkesman et al., 2005). However, it is important to note reports of similar (Dommett and Rostron, 2013; Kin et al., 2017) or even elevated (Nam et al., 2014) sucrose consumption in this strain, as well as possible sex-specific differences (i.e. SPT deficit in males but not females) (Burke et al., 2016; Mileva and Bielajew, 2015). Consistent with a possible deficit in sucrose preference, one study reports lower effort/ motivation to earn sucrose reward under both fixed and progressive ratio schedules of reinforcement, as well as reduced nicotine self-administration, in WKY rats as compared to control rats (De La Garza 2nd, 2005). Importantly, WKY rats display deficiencies in tests of social interaction, aggression and social play (younger rats), reflecting social avoidance at baseline and following stress (Berton et al., 1997; Malkesman et al., 2005; Nam et al., 2014;

Pardon et al., 2002; Van Zyl et al., 2014). Therefore, the literature points toward a general tendency for WKY rats to withdraw from both social and non-social (e.g. novelty, stress) challenges (Berton et al., 1997; Nam et al., 2014; Nestler and Carlezon, 2006; Pardon et al., 2002; Paré, 1994b, 1994a). Interestingly, WKY rats have also been reported to emit fewer ultrasonic vocalizations (USVs), which provide information on social context and status, as well as the emotional state of an animal (Burgdorf et al., 2011a; Rao and Sadananda, 2015; Shetty and Sadananda, 2017a; Van Zyl et al., 2014). Consistent with deficits in social interaction in this model, the reduction in USVs emitted in novel or social settings could be indicative of deficient social communication, or may also reflect anhedonia since novelty and social behaviours seem to be less rewarding (and even aversive/ stressful) in WKY compared to normal rats (Rao and Sadananda, 2015; Shetty and Sadananda, 2017a; Van Zyl et al., 2014).

Consistent with the high comorbidity between depression and anxiety disorders, WKY rats exhibit anxiety-like behaviours in the OFT where they spend less time in the center of the arena (Berton et al., 1997; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993b; Tejani-Butt et al., 2003; Van Zyl et al., 2014). Moreover, they make less entries into the open arms in the elevated plus maze (EPM) (Berton et al., 1997; Nam et al., 2014; Pardon et al., 2002; Tejani-Butt et al., 2003; Van Zyl et al., 2014), and have longer latencies to feed in the novelty-suppressed feeding (NSF) test (Burke et al., 2016; Nam et al., 2014; Paré, 1994b). Therefore, the endogenous depression-like phenotype of the WKY strain is unique in its consistency across virtually all relevant preclinical indices (although conflicting reports exist in the literature), unlike the often more limited and transient repertoire displayed by normal rats previously subjected to different types of stress.

Consistent with their hypersensitivity to stress under baseline conditions, WKY rats also show enhanced physiological responses and a failure to adapt to repeated stress (Morilak et al., 2005; Tejani-Butt et al., 1994; Zafar et al., 1997). In addition, subjecting WKY rats to CMS under a two-hit model exacerbates their depression- and anxiety- like phenotypes (Luo et al., 2015; Tejani-Butt et al., 1994; Willner et al., 2018). Interestingly, early-life stress, such as neonatal handling or maternal separation, do not generally enhance the WKY phenotype, indicating a strong genetic component (Nam et al., 2014; Van Zyl et al., 2014; Zalsman et al., 2015), although some exceptions exist in the literature (Shetty and Sadananda, 2017b).

Importantly, as in MDD, various cognitive deficits have been reported in WKY rats, which have been proposed as a model of cognitive dysfunction in depression (Grauer and Kapon, 1993). For example, despite some conflicting reports, these rats appear to exhibit a pronounced spatial learning impairment in the Morris water maze (MWM), where their latency to escape to the target platform is significantly longer than in controls, even with repeated training (Diana et al., 1994; Grauer and Kapon, 1993; She et al., 2015). Moreover, WKY rats have been reported to show a significant deficit in the novel object recognition (NOR) task, indicative of memory dysfunction (Shoval et al., 2016). Importantly, compared to control rats, WKY rats display other features often associated with depression in humans, such as reduced body weight (Bjorkholm et al., 2015; De La Garza 2nd, 2005; Nagasawa et al., 2015; Paré, 1994b; Solberg et al., 2001), disturbances in the sleep-wake cycle including increased REM (rapid eye movement) sleep and sleep fragmentation (DaSilva et al., 2011; Dugovic et al., 2000), as well as an altered circadian rhythm of activity suggesting decreased light sensitivity (Solberg et al., 2001).

Clinically, the prevalence of MDD in women is approximately two times higher than in men (Altemus et al., 2014). Despite this important sex difference, as is too frequently observed

in animal experiments, most studies of the WKY rat model utilize male subjects with a few important exceptions. The available data suggests that the WKY strain may have good etiological validity in this respect, as females have been reported to exhibit more pronounced depressive- and anxiety- like phenotypes, as well as higher physiological responses to stress (Courvoisier et al., 1996; Paré et al., 1999; Paré and Redei, 1993b; Servatius et al., 2015; Tizabi et al., 2012, 2010); although others have reported the opposite effects (Burke et al., 2016; Will et al., 2003). Therefore, while robust anxiety- and depressive- like behaviours are present in both sexes, there is currently no consensus as to whether they are more prominent in male or female WKY rats. Clearly, sex differences within this strain are complex and may depend on the testing condition, warranting further investigation.

1.5.2 Neurochemical and Endocrine Parallels to Depression

Although the exact genetic or molecular mechanisms underlying the depressive-like phenotype of WKY rats are still unresolved and published reports are not always consistent, various abnormalities in different neurotransmitter and endocrine systems have been demonstrated in WKY compared to control outbred rats (neurochemical and endocrine findings for the WKY model summarized in Table 2) (Aleksandrova et al., 2019).

Consistent with the monoamine theory of depression, there is strong evidence of abnormalities in monoamine systems (5-HT, DA and NE) in WKY rats (Bruzos-Cidon et al., 2014; Lemos et al., 2011; O'Mahony et al., 2011; Pardon et al., 2002). This strain exhibits lower levels of all three monoamines (5-HT, NE and DA) in the HPC, PFC, nucleus accumbens (NAc) shell and basolateral amygdala compared to Wistar (WIS) rats (Bruzos-Cidon et al., 2014; De La Garza 2nd and Mahoney 3rd, 2004; Getachew et al., 2010; Scholl et al., 2010). Moreover, the dorsal raphe nucleus (DRN) of WKY rats is characterized by significantly lower neuronal

Table 2. Neurochemical and endocrine findings in the WKY rat compared to outbred controls.

Neurochemical / Endocrine Findings by Brain System	References
Serotonin (5-HT) • Lower basal 5-HT levels in DRN and projection sites (HPC, PFC, NAc shell, BLA) • Lower neuronal excitability of DRN 5-HT neurons • More hyperpolarized resting membrane potential of DRN 5-HT neurons • Reduced burst activity of DRN 5-HT neurons • Decreased DRN TPH2 production • Reduced sensitivity of DRN 5-HT1A autoreceptors • Higher expression of post-synaptic 5-HT1A receptors (HPC and hypothalamus) • Lower abundance of serotonin transporter (5-HTT) sites (cortex, HPC) • Higher 5-HT turnover (striatum, NAc, PFC) and lower tissue 5-HT levels (PFC, striatum, NAc, amygdala) following acute stress	Getachew et al., 2010; Scholl et al., 2010; Bruzos-Cidon et al., 2014; De La Garza 2nd and Mahoney 3rd, 2004; Felten et al., 1984; Lemos et al., 2011; Yamada et al., 2013; Bruzos-Cidon et al., 2014; Paré et al., 1999; Paré and Tejani-Butt, 1996
Norepinephrine (NE) • Lower NE levels in LC and projection sites (HPC, PFC, NAc shell, BLA) • Increased reuptake due to higher density of NE transporter (NET) sites (HPC, amygdala) • Increased basal activity LC neurons due to less inhibitory action onto LC α2-adrenoautoreceptors • Altered NE turnover due to increased expression of genes encoding enzymes for NE synthesis and metabolism (TH, COMT and MAO) • Similar resting levels, but higher plasma NE and EPI following acute stress (exaggerated peripheral sympathoadrenal stress response) • Blunted stress-induced elevations in LC TH mRNA • Attenuated stress-induced NE release (BNST, striatum) • Blunted stress-induced c-Fos expression (LC, medial amygdala) • Lower β-adrenergic receptor and NET binding (HPC, amygdala) following CMS	Felten et al., 1984; Getachew et al., 2010; Scholl et al., 2010; De La Garza 2nd and Mahoney 3rd, 2004; Tejani-Butt et al., 1994; Bruzos-Cidon et al., 2014; Pearson et al., 2006; McCarty and Kopin, 1978; Morilak et al., 2005; Pardon et al., 2002; Sands et al., 200;0 Ma and Morilak, 2004
Dopamine (DA) • Attenuated tissue DA levels (HPC, PFC, NAc shell, BLA) • Differential patterns of expression of the DA transporter (DAT) and various dopamine receptors (D1, D2 and D3) • Altered density of DAT binding sites (lower in NAc, VTA, amygdala; higher in HPC, hypothalamus) • No strain differences within the nigrostriatal system • Reductions in tissue DA and DOPAC levels, elevations in DA turnover (PFC, striatum, NAc), effects exacerbated by acute stress	Getachew et al., 2010; Jiao et al., 2003; Novick et al., 2008; Scholl et al., 2010; Yaroslavsky et al., 2006; De La Garza 2nd and Mahoney 3rd, 2004
Acetylcholine • Hippocampal cholinergic hypofunction exacerbated by aging	Gilad et al., 1987; Grauer and Kapon, 1993

Neurochemical / Endocrine Findings by Brain System	References
Glutamate • Elevated basal glutamate levels (PFC and HPC) • Decreased evoked glutamate release and aberrant glutamate uptake (PFC, striatum) • Lower expression of NMDAR subunits (GluN1, GluN2A and GluN2B), PSD95 and mGluA1 (PFC) • Lower NMDA receptor binding (anterior cingulate cortex, caudate putamen, NAc, HPC CA1 only, substantia nigra pars reticulata) (males) • Similar ratio of HPC AMPA/NMDA receptor densities (whole HPC, females) • Increased NMDAR binding (PFC, NAc, caudate putamen) following chronic stress	Jastrzębska et al., 2015; Miller et al., 2014; Millard et al., 2019; Lei et al., 2009; Tizabi et al., 2012; X. Jiao et al., 2011; Lei and Tejani-Butt, 2010
GABA • Either similar or elevated basal GABA tissue levels • Higher GABA-A receptor binding (amygdala, caudate putamen, HPC CA2 and CA3, periaqueductal grey, substantia nigra) • Altered density of parvalbumin (PV) immunoreactive cells (lower in amygdala; higher in PFC) • Elevation in GABA levels (amygdala)	O'Mahony et al., 2011; Jastrzębska et al., 2015; Lei et al., 2009; Jiao et al., 2011a; Jiao et al., 2011b
Neurotrophic Factors and Structural Plasticity • Reports of either normal or attenuated basal serum BDNF levels • Lower HPC and PFC tissue BDNF levels • Significant reductions in hippocampal and cortical volume • Attenuated neurogenesis in HPC DG, but not in the subventricular zone (SVZ)	O'Mahony et al., 2011; Kyeremanteng et al., 2012; Malkesman and Weller, 2009; Vinod et al., 2012; Cominski et al., 2014; Gormley et al., 2016; Lemos et al., 2011; Kin et al., 2017
Hypothalamic-pituitary-adrenal (HPA) axis • Higher anterior pituitary mRNA levels of stress-related CRF receptor, ACTH and POMC • Increased basal plasma levels and prolonged diurnal secretory patterns of ACTH and CORT • Facilitated and prolonged ACTH and CORT responses to stress • Deficient glucocorticoid negative feedback control • Enlarged adrenal glands	Rittenhouse et al., 2002; Solberg et al., 2001 Johnson and Chang, 2002; Redei et al., 1994; Courvoisier et al., 1996; De La Garza 2nd and Mahoney 3rd, 2004; Durand et al., 2000; Gomez et al., 1996; Malkesman and Weller, 2009; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993b
Hypothalamic-pituitary-thyroid (HPT) axis • Elevated basal plasma TSH and T3 levels	Redei et al., 2001; Solberg et al., 2001

excitability (due to a more hyperpolarized resting membrane potential), reduced burst activity of 5-HT neurons, lower basal 5-HT levels (as low as 30% of those in Wistar rats) and decreased tryptophan hydroxylase 2 (TPH2) production (Bruzos-Cidon et al., 2014; De La Garza 2nd and Mahoney 3rd, 2004; Felten et al., 1984; Lemos et al., 2011; Yamada et al., 2013). In addition, the reduced sensitivity of DRN 5-HT1A autoreceptors, the higher expression of post-synaptic 5-HT1A receptors in the HPC and hypothalamus, and the lower abundance of serotonin transporter (5-HTT) sites in the cortex and HPC reported in WKY rats are thought to reflect compensatory mechanisms to a hypoactive 5-HT system in this strain (Bruzos-Cidon et al., 2014; Paré et al., 1999; Paré and Tejani-Butt, 1996). Furthermore, exposure to an acute stress challenge (swim stress) induces higher 5-HT turnover (striatum, NAc and PFC) and lower tissue 5-HT levels (PFC, striatum, NAc and amygdala) in WKY compared to WIS rats, possibly indicating blunted stress-reactivity (De La Garza 2nd and Mahoney 3rd, 2004).

As mentioned, WKY rats also have significantly lower NE levels in the locus coeruleus (LC) and many of its projection sites, including the HPC, PFC, NAc shell and basolateral amygdala (Felten et al., 1984; Getachew et al., 2010; Scholl et al., 2010). In addition, WKY rats exhibit significantly higher density of NE transporter (NET) sites in the HPC and amygdala than Sprague-Dawley (SD) controls, which may lead to increased reuptake and thus lower synaptic NE levels in limbic brain regions (De La Garza 2nd and Mahoney 3rd, 2004; Tejani-Butt et al., 1994). LC neurons from WKY rats have increased basal activity, which could be due to the lower NE levels in these rats exerting less inhibitory action onto LC α2-adrenoautoreceptors (Bruzos-Cidon et al., 2014). Increased expression of genes encoding enzymes for NE synthesis and metabolism (tyrosine hydroxylase, COMT and MAO) in WKY rats implies a dysfunction in NE turnover (Pearson et al., 2006). Similar to observations related to 5-HT function, the

available data suggests that stress may fail to properly engage the NE system in these rats, leading to abnormal monoamine adaptations to stress, contributing, in turn, to enhanced stress susceptibility in this genetic model of depression (Paré et al., 1999; Paré and Tejani-Butt, 1996; Sands et al., 2000; Tejani-Butt et al., 1994; Zafar et al., 1997). Interestingly, an early study indicates that although resting levels are similar between strains, the WKY strain displays significantly higher plasma NE and EPI following footshock stress compared to Wistar and SD rats, indicative of an exaggerated peripheral sympathoadrenal response to stress (McCarty and Kopin, 1978; Pardon et al., 2002). However, the central NE system of WKY rats may be less reactive to stress, given blunted stress-induced elevations in tyrosine hydroxylase (TH) mRNA in the LC, attenuated NE release in the lateral bed nucleus of the stria terminalis (BNST) and striatum (De La Garza 2nd and Mahoney 3rd, 2004; Morilak et al., 2005; Pardon et al., 2002; Sands et al., 2000), as well as lower β-adrenergic receptor and NET binding in the HPC and amygdala after CMS (Tejani-Butt et al., 1994). In addition, blunted stress-induced c-Fos expression is observed in the LC and medial amygdala of WKY compared to SD rats (Ma and Morilak, 2004). It is possible that the chronic state of hyperreactivity to stress may lead to desensitization of central noradrenergic stress responses in WKYs, which may in turn contribute to reduced arousal and a behavioural inhibition phenotype (Pardon et al., 2002; Sands et al., 2000; Tejani-Butt et al., 1994).

Finally, tissue DA levels are attenuated in the HPC, PFC, NAc shell and basolateral amygdala, along with differential patterns of expression of the DA transporter (DAT) and various dopamine receptors (D1, D2 and D3) in WKY rats, compared to control strains (Getachew et al., 2010; Jiao et al., 2003; Novick et al., 2008; Scholl et al., 2010; Yaroslavsky et al., 2006). The observation of significantly fewer DAT binding sites in the NAc, ventral

tegmental area (VTA) and amygdala, along with elevated DAT levels in the HPC and hypothalamus of WKY compared to SD and WIS rats, is consistent with altered modulation of synaptic DA levels within the mesolimbic dopaminergic system in the WKY model (Jiao et al., 2003). Interestingly, no strain differences are found within the nigrostriatal system, suggesting that the motor retardation seen in WKY rats is likely due to their stress-reactivity and behavioural inhibition phenotype, rather than alterations in DA innervation of motor control circuits (Jiao et al., 2003). Reductions in tissue DA and DOPAC levels and elevations in DA turnover are reported in the WKY PFC, striatum and/or NAc compared to WIS rats, effects which are exacerbated by acute stress (De La Garza 2nd and Mahoney 3rd, 2004; Jiao et al., 2003). Together, these results are generally consistent with deficient mesolimbic and mesocortical DA function in WKY rats, which may contribute to certain depressive-like behaviours exhibited by these rats including the reported anhedonia, social withdrawal and cognitive deficits.

In summary, while some discrepancies exist, monoamine hypofunction at baseline (i.e. decreased tissue monoamine levels, alterations in receptor and transporter sites, etc.), as well as abnormal adaptations of monoamine systems in response to acute or repeated stress, likely render WKY rats unable to initiate or regulate the stress response appropriately, thereby predisposing them to adopt passive coping strategies in the face of stress (Bruzos-Cidon et al., 2014; Jiao et al., 2003; Lemos et al., 2011; O'Mahony et al., 2011; Pardon et al., 2002; Tizabi et al., 2012). Such a maladaptive stress response appears to result in a relatively greater impact of stressful stimuli, making this strain more susceptible to developing depression- and anxiety-like phenotypes, which often present in the absence of explicit stress exposure (although even normal experimental manipulations are innately more stressful in WKY rats) (Felten et al., 1984; Pardon

et al., 2002; Paré et al., 1999; Paré and Tejani-Butt, 1996; Sands et al., 2000; Tejani-Butt et al., 2003, 1994). In addition, hypofunction within the brain's dopaminergic system in WKY rats likely contributes to motivational and hedonic deficits observed in this rat strain.

Available data indicate that molecular and cellular abnormalities in glutamate and gamma-aminobutyric acid (GABA) function are also present in WKY rats. Elevated basal levels of glutamate in the PFC and HPC of WKY compared to WIS rats have been reported (Jastrzębska et al., 2015), which is consistent with the clinical literature and the glutamate excitotoxicity observed following chronic stress in normal rodents (Duman et al., 2016; Lener et al., 2017b; Musazzi et al., 2013). Interestingly, one study found decreased evoked glutamate release and aberrant glutamate uptake in the PFC and striatum of WKY rats; with the caveat that SHR was the only reference strain used in this study (itself represents a disease model and thus cannot be used as a reliable control strain) (Miller et al., 2014). *Postmortem* analyses of brain tissue from individuals with MDD indicate significantly lower NMDAR (GluN2A and GluN2B subunit) expression (Feyissa et al., 2009; Fragale et al., 2016). Consistent with this, WKYs display reductions in the expression of NMDAR subunits (GluN1, GluN2A and GluN2B), as well as PSD95 and metabotropic glutamate receptor subtype 1 (mGluR1) in the PFC compared to SD rats (Millard et al., 2019). In addition, a quantitative autoradiographic study revealed lower NMDA receptor binding in the anterior cingulate cortex, caudate putamen, NAc, HPC (CA1 only) and substantia nigra pars reticulata of male WKY compared to WIS rats (Lei et al., 2009). However, one study reported a similar ratio of HPC AMPA/NMDA receptor densities in whole HPC tissue samples in female WKY and WIS rats (Tizabi et al., 2012). Interestingly, chronic stress induced an upregulation of NMDAR binding in the PFC, NAc and caudate putamen in

WKY but not WIS rats, leading to the hypothesis that NMDARs in these regions may be more sensitive to stress in this model (Jiao et al., 2011a; Lei and Tejani-Butt, 2010).

Although the data are more sparse, generally no differences in basal GABA tissue levels were found between WKY and WIS rats (O'Mahony et al., 2011), with the exception of one report of elevated GABA content in the WKY NAc (Jastrzębska et al., 2015). On the other hand, higher GABA-A receptor binding was demonstrated in the WKY amygdala, caudate putamen, HPC (CA2 and CA3), periaqueductal grey and substantia nigra (Lei et al., 2009). Moreover, the density of parvalbumin (PV) immunoreactive cells was lower in the amygdala and higher in PFC of WKYs compared to SD rats (Jiao et al., 2011a, 2011b). Interestingly, chronic stress induced an elevation in amygdala GABA in WKY rats only, which may lead to aberrant cortico-amygdalar connectivity (O'Mahony et al., 2011). Future studies of the WKY model should further investigate abnormalities within the glutamatergic and GABAergic systems, in order to elucidate their contributions to the depressive- and anxiety- like phenotype of WKY rats. With respect to other major neurochemical systems, notably, hippocampal cholinergic hypofunction is also observed in the WKY strain, which is exacerbated by aging and may contribute to the loss of hippocampal pyramidal neurons, as well as the reported memory deficits in these rats (Gilad et al., 1987; Grauer and Kapon, 1993).

Consistent with the role of neurotrophic factors in depression and antidepressant response, attenuated basal serum BDNF levels in WKY compared to WIS rats have also been reported (Kyeremanteng et al., 2012). However, this relationship remains uncertain as a separate study observed similar basal levels, with reductions in plasma BDNF in WKY but not SD rats only following chronic restraint stress (O'Mahony et al., 2011). Importantly, WKY rats exhibit lower HPC and PFC tissue BDNF levels compared to controls (Malkesman and Weller, 2009;

Vinod et al., 2012), which is also associated with significant reductions in hippocampal and cortical volume in this strain (Cominski et al., 2014; Gormley et al., 2016; Lemos et al., 2011), paralleling the lower BDNF levels and significant grey matter volume reductions in MDD (Duman, 2014b; Roiser and Sahakian, 2013). In addition, adult neurogenesis in the dentate gyrus (DG) of the hippocampus, but not in the subventricular zone (SVZ), is significantly attenuated in WKY compared to Wistar rats (Kin et al., 2017).

Importantly, WKY rats also exhibit hormonal alterations characteristic of depression, particularly dysregulation of the hypothalamic-pituitary-adrenal (HPA), as well as the hypothalamic-pituitary-thyroid (HPT) axes (Rittenhouse et al., 2002; Solberg et al., 2001). Although there are several distinct clinical endophenotypes characterized by either high or low stress hormone levels as in the case of typical and atypical depression, respectively, hypercortisolism remains one of the most robust biological markers associated with major depression (Krishnan and Nestler, 2008; Villanueva, 2013). Consistent with this, studies of WKY rats report higher anterior pituitary mRNA levels of stress-related corticotrophin releasing factor (CRF) receptor, adrenocorticotropic hormone (ACTH) and proopiomelanocortin (POMC) (Johnson and Chang, 2002; Redei et al., 1994), as well as increased basal plasma levels and prolonged diurnal secretory patterns of ACTH and corticosterone (CORT) (Rittenhouse et al., 2002; Solberg et al., 2001). In addition, endocrine responses under stress conditions are facilitated and prolonged in WKY rats compared to control rats (Courvoisier et al., 1996; De La Garza 2nd and Mahoney 3rd, 2004; Durand et al., 2000; Gomez et al., 1996; Malkesman and Weller, 2009; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993b; Rittenhouse et al., 2002). Furthermore, there is also evidence of deficient glucocorticoid negative feedback control of the HPA axis in this strain (Redei et al., 1994; Rittenhouse et al., 2002). In addition, WKY rats

have the largest adrenal glands when compared to several control strains (Nam et al., 2014). These findings support the idea that the WKY HPA axis of WKY rats is in a chronic state of activation or disinhibition, recapitulating what has been commonly reported in MDD (Villanueva, 2013). In fact, elevated cortisol levels may predict hippocampal atrophy and memory deficits in rodents as well as in MDD (Campbell and MacQueen, 2003; Lupien et al., 1998; Sapolsky, 2003). Finally, one study found that WKY rats have elevated basal plasma TSH and T3 levels, which may reflect reduced central sensitivity to thyroid hormones in WKY compared to Wistar rats, consistent with reports of hypothalamic-pituitary-thyroid (HPT) dysregulation in treatment-resistant depressed patients (Redei et al., 2001; Solberg et al., 2001).

1.5.3 Responsiveness to Antidepressants

Importantly, WKY rats are at least partially resistant to traditional antidepressants (studies of antidepressant response in WKY rats are summarized in Table 3) (Aleksandrova et al., 2019), establishing this strain as a putative model of treatment-resistant depression (TRD), which is the conclusion of a pivotal review by Willner and Belzung of potential animal models of TRD (Willner and Belzung, 2015).

Specifically, unlike outbred control strains (i.e. normal rats), WKY rats fail to respond to standard acute doses of various traditional antidepressants in the FST, including imipramine (TCA), desipramine (TCA, SNRI), fluoxetine (SSRI), citalopram (SSRI) and 8-hydroxy-2-(di-*n*-propylamine)tetralin (8-OH-DPAT, 5-HT1A agonist) (Griebel et al., 1999; Lahmame et al., 1997a; Lahmame and Armario, 1996; Lopez-Rubalcava and Lucki, 2000; Pollier et al., 2000; Will et al., 2003), which is consistent with the fact that these drugs are clinically ineffective when administered acutely (Abdallah et al., 2015; Duman et al., 2012). In addition, the antidepressant response to chronic imipramine administration in WKY rats is markedly reduced

Table 3. Findings related to antidepressant response in the WKY rat.

Antidepressant	Treatment Response	References
Acute classical antidepressants	• Acute imipramine (TCA), desipramine (TCA, SNRI), fluoxetine (SSRI), citalopram (SSRI) and 8-hydroxy-2-(di-n-propylamine) tetralin (8-OH-DPAT, 5-HT1A agonist) failed to increase FST immobility	Griebel et al., 1999; Lahmame et al., 1997a; Lahmame and Armario, 1996; Lopez-Rubalcava and Lucki, 2000; Pollier et al., 2000; Will et al., 2003
Chronic classical antidepressants	• Response to chronic imipramine (TCA) was markedly reduced (FST) • Chronic fluoxetine or paroxetine (SSRIs) failed to increase FST immobility • Chronic desipramine and imipramine (TCAs), as well as nomifensine (NE/DA reuptake blocker, although the latter also caused hyperactivity) decreased FST immobility • Both significant sensitivity and resistance to subacute desipramine (FST) reported by different groups • WKY rats subjected to CMS failed to respond to chronic imipramine (TCA), citalopram (SSRI) and venlafaxine (SNRI) (SPT, EPM, NOR)	Lahmame et al., 1997a; Lahmame and Armario, 1996; Chaouloff, 2002; Griebel et al., 1999; Lopez-Rubalcava and Lucki, 2000; Nagasawa et al., 2015; Paré, 1992; Tejani-Butt et al., 2003; Will et al., 2003; Willner et al., 2018
Ketamine	• Acute ketamine (2.5–5.0 mg/kg, ip) significantly reduced FST immobility in female WKY • Chronic ketamine (daily for 10days) was effective at dose as low as 0.5mg/kg in both males and females • Combination of AMPA* + ketamine reduced FST immobility and restored sucrose intake at very low doses (0.25mg/kg, ip each for 10 days) (FST, SPT) • *Chronic AMPA itself (0.5-1mg/kg, ip daily for 11 days) had antidepressant effects (FST, SPT) • Ketamine increased the ratio of AMPA to NMDA receptor density, as well as the levels of HPC BDNF, synapsin and mTOR in the WKY HPC • Acute and repeated ketamine (5mg/kg, ip) reversed failure to escape in helpless WKY rats (LH) • Acute ketamine (5mg/kg, ip) facilitated extinction of persistent avoidance responding when shock is eliminated in subset of WKY rats (responders) for 3+ weeks (active avoidance) • Subacute ketamine (10 mg/kg, ip, for 7days) had antidepressant effects in WKY rats subjected to CMS (SPT, EPM, NOR)	Tizabi et al., 2012; Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012; Belujon and Grace, 2014; Fortress et al., 2018; Willner et al., 2018
ECT	• ECT (daily for 5-7 days) reversed depressive-like phenotypes (SPT, FST, OFT, active avoidance, MWM) and increased HPC BDNF	Kyeremanteng et al., 2014; Luo et al., 2015

Antidepressant	Treatment Response	References
DBS	• DBS of the NAc (either intermittent or continuous stimulation over 2 weeks) increased exploratory activity and exerted anxiolytic effects (OFT) • NAc DBS also increased apical and basal dendrite length in mPFC pyramidal neurons • Unilateral DBS of the PFC (two 2h sessions) reversed the anhedonic, anxiogenic and dyscognitive effects of CMS in WKY rats previously resistant to classical antidepressants (SPT, EPM, MWM)	Falowski et al., 2011; Willner et al., 2018

compared to that in two control strains, which did not appear to reflect strain differences in pharmacokinetics or monoamine receptor adaptations (e.g. 5-HT or β-adrenoceptors) (Lahmame et al., 1997a). It is noteworthy that, chronic administration of desipramine and imipramine (TCAs), as well as nomifensine (NE/DA reuptake blocker, although the latter also caused hyperactivity), but not fluoxetine or paroxetine (SSRIs), decreased FST immobility time in WKY rats, suggesting this strain may exhibit some sensitivity to chronic noradrenergic and possibly dopaminergic, but not serotonergic agents (Chaouloff, 2002; Griebel et al., 1999; Lahmame et al., 1997a; Lopez-Rubalcava and Lucki, 2000; Nagasawa et al., 2015; Paré, 1992; Tejani-Butt et al., 2003). Interestingly, DRN neurons in the WKY rat may be more sensitive to the inhibitory effect of SSRIs even though DRN 5-HT1A autoreceptors are desensitized (Bruzos-Cidon et al., 2014), which may contribute to the apparent resistance of this strain to SSRIs. It is suggested that the lack of antidepressant responses to SSRIs in WKY rats may also involve 5-HT transporter–independent mechanisms, as one study found that acute citalopram elicited similar magnitudes of serotonin uptake blockade in the different strains tested (Pollier et al., 2000). Most recently, a pivotal study by Willner et al. (2018) provided another clear demonstration of the resistance of this model to classical antidepressants, as WKY rats subjected to CMS failed to respond to the

chronic administration (daily for 3 weeks) of three neurochemically different drugs, imipramine (TCA), citalopram (SSRI) and venlafaxine (SNRI), suggesting that chronic stress may render WKY rats resistant to a broader range of antidepressant classes (Willner et al., 2018).

Importantly, work by Tizabi and colleagues first reported that ketamine exerts rapid and sustained antidepressant effects in WKY rats, where a single low dose of ketamine (2.5–5.0 mg/kg, ip) significantly reduced FST immobility in female WKY rats without affecting general locomotor activity in this strain (Tizabi et al., 2012). These results suggest this strain may be more sensitive to the antidepressant effects of ketamine than other strains, as the standard ketamine antidepressant dose in the general literature is typically higher (10mg/kg, ip) (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). When administered chronically (daily for 10days) ketamine was effective at a dose as low as 0.5mg/kg in both male and female WKY rats (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). Interestingly, the same group demonstrated that chronic administration of AMPA itself (0.5-1mg/kg, ip daily for 11 days) has antidepressant effects in WKY males in the FST and SPT, and that the combination of AMPA and ketamine at low doses that were ineffective on their own (0.25mg/kg, ip each for 10 days), also significantly reduced FST immobility and restored sucrose intake in these rats (Akinfiresoye and Tizabi, 2013). Importantly, these studies with WKY rats also found that ketamine increased the ratio of AMPA to NMDA receptor density, as well as the levels of BDNF, synapsin and mTOR in the HPC, implicating neuroplasticity changes in the long-lasting antidepressant effects of ketamine in this model (Akinfiresoye and Tizabi, 2013). In the LH paradigm, both acute and repeated administration of ketamine (5mg/kg, ip) prior to avoidance training reversed the failure to escape in WKY rats rendered ‘helpless’ by pre-exposed to inescapable shock, without affecting locomotor activity or avoidance performance in control and ‘non-helpless’ rats (Belujon and

Grace, 2014). A single dose of ketamine (5mg/kg, ip) also facilitated the extinction of persistent avoidance responding in an active level-press paradigm (when the shock is eliminated) in a subset of WKY rat (responders) for up to three weeks following administration (Fortress et al., 2018). Finally, subacute administration of ketamine (10 mg/kg, ip, for 7days) normalized sucrose intake, anxiety-like behaviours and object recognition memory in WKY rats subjected to CMS (Willner et al., 2018). Taken together, these findings demonstrate the ability of ketamine to exert rapid and sustained antidepressant-like effects in the WKY model.

Interestingly, electroconvulsive therapy (ECT, daily for 7 days), another effective therapeutic approach for TRD, has been shown to reverse depressive-like phenotypes (SPT, FST, OFT, active avoidance, MWM) and increase HPC BDNF of WKY rats (Kyeremanteng et al., 2014; Luo et al., 2015). Interestingly, deep brain stimulation (DBS) of the NAc (either intermittent or continuous stimulation over 2 weeks) increased exploratory activity and exerted anxiolytic-like effects (OFT) in male WKY rats (Falowski et al., 2011). Notably, NAc DBS also increases apical and basal dendrite length in pyramidal neurons of the mPFC, suggesting its therapeutic effects may involve synaptogenesis and neuroplasticity changes downstream of its direct target (Falowski et al., 2011). Finally, unilateral DBS of the PFC (two 2h sessions) reversed the anhedonic (SPT), anxiogenic (NSF) and dyscognitive (MWM) effects of CMS in WKY rats (Willner et al., 2018). Importantly, in the latter study, DBS was effective in chronically stressed WKY rats previously demonstrated to be resistant to chronic treatment with three different classical antidepressant classes, further highlighting the robust antidepressant response to DBS in this model (Willner et al., 2018).

In summary, the finding that WKY rats are unresponsive to acute classical antidepressants may parallel the delay to efficacy observed in the clinic, thus increasing the

predictive validity of this model; whereas their suboptimal response to chronic antidepressant therapy (particularly SSRIs) supports the idea that WKY rats may be a good model for, at least partial, antidepressant resistance or TRD (Griebel et al., 1999; Lahmame et al., 1997a; Lopez-Rubalcava and Lucki, 2000; Nagasawa et al., 2015; Paré, 1992; Tejani-Butt et al., 2003; Willner et al., 2018; Willner and Belzung, 2015). In addition, combining the WKY rat with the well-validated CMS paradigm may represent a TRD model resistant to the administration of virtually all major classes of traditional antidepressant drugs (Willner et al., 2018). On the other hand and perhaps of more immediate relevance, WKY rats respond to novel, rapid-acting antidepressant therapies proven effective in TRD, including ketamine, ECT and DBS (Belujon and Grace, 2014; Falowski et al., 2011; Fortress et al., 2018; Kyeremanteng et al., 2014; Luo et al., 2015; Tizabi et al., 2012; Willner et al., 2018).

1.5.4 Evaluation of the Wistar-Kyoto Rat Model of Depression

As emphasized here, the search for novel antidepressant treatments with a wider therapeutic reach requires animal models that incorporate aspects of heightened stress responsiveness and vulnerability to depression, as well as partial or full resistance to classical antidepressants (Aleksandrova et al., 2019; Caldarone et al., 2015; Willner et al., 2014; Willner and Belzung, 2015). Accumulating preclinical research supports the WKY rat as a valid model of endogenous stress susceptibility and depression that satisfies these conditions and exhibits many specific behavioural, neurochemical and endocrine parallels to clinical depression. These include deficient monoamine (5-HT, NE, DA) and neurotrophin signaling, aberrant glutamatergic function, hyperactive HPA axis, as well as a pronounced hyper-susceptibility to stress and various depressive-like phenotypes including behavioural inhibition, psychomotor slowing, anhedonia, social withdrawal, comorbid anxiety, cognitive deficits and altered body weight and

sleep patterns (see sections 1.5.1-1.5.2 for specific references). This phenotype not only closely parallels the human condition, but in fact satisfies virtually all DSM-5 diagnostic criteria for depression with the exception of those that cannot be modelled or assessed in animals (i.e. depressed mood and suicidal ideation) (American Psychiatric Association, 2013). Therefore, because WKY rats exhibit various behavioural, neurochemical and endocrine parallels to clinical depression, the strain represents a valid animal model of depression, with several unique features that differentiate it from other animal models of the disorder. The combined phenotypic profile of WKY rats appears to resemble features reported for certain clinical endophenotypes, particularly the relatively severe, genetic or chronic/recurrent, melancholic (typical) subtypes of MDD, which are characterized by increased susceptibility to stress/depression, hypercortisolism and (at least partial) treatment resistance to classical antidepressants (Aleksandrova et al., 2019; Lahmame et al., 1997a; Willner and Belzung, 2015). The latter feature, in particular, might lend itself to studies of novel antidepressants such as ketamine (Aleksandrova et al., 2019). Interestingly, it has been proposed that the WKY phenotype corresponds to a known clinical risk factor for mood and anxiety disorders, namely the personality trait of neuroticism (Willner et al., 2014; Willner and Belzung, 2015; Willner and Mitchell, 2002). Moreover, prepubertal WKY rats have also been proposed as a putative model of childhood depression (Braw et al., 2006; Malkesman and Weller, 2009).

Despite its advantages over purely stress-based models of depression, the WKY rat model has several potential limitations, which should be carefully considered. Since the WKY strain is hyper-susceptible to stress and may react more to subtle environmental changes than other rat strains, it is crucial that housing and experimental procedures be kept as stable as possible (Will et al., 2003). In addition, although the WKY strain is theoretically inbred, it is

important to note that unlike other inbred strains, WKY rats exhibit considerable genetic and behavioural heterogeneity in terms of the severity of their depression-like phenotype and the extent of their antidepressant resistance, both between different commercial sources and within individual colonies (Paré and Kluczynski, 1997; Will et al., 2003; Zhang-James et al., 2013). For example, one study reported that despite decades of inbreeding, around 15% of genetic markers tested were found to be polymorphic between WKY rats from Charles River Laboratories and NIH (Deschepper et al., 1997), indicating that the phenotypic variability in this strain is in part due to a higher than usual genetic heterogeneity. Furthermore, one group exploited this heterogeneity through selective breeding, giving rise to "WKY most immobile" and "WKY least immobile" sub-strains that differed dramatically in the severity of the depressive-like phenotype and their responsiveness to various antidepressants (Will et al., 2003). In agreement with this, WKY rats from different commercial sources appear to harbour a wide range of responsiveness to antidepressants, for example with different groups reporting either significant sensitivity or resistance to sub-acute desipramine (Lahmame and Armario, 1996; Lopez-Rubalcava and Lucki, 2000; Will et al., 2003).

Finally, the endogenous nature of the depressive-like phenotype of WKY rats arguably represents the most major weakness of this model (Aleksandrova et al., 2019). In this respect, the WKY strain has lower etiological validity, as MDD is generally an episodic disease that comes and goes (Kanter et al., 2008; Willner and Belzung, 2015). On the other hand, in a congenital model like the WKY strain, some depression-like symptoms appear to be an innate characteristic of the strain (i.e. are endogenous and constant), a phenotype that may more closely resemble dysthymia (DSM-V diagnosis for a pervasive/chronic depressive disorder) (American Psychiatric Association, 2013). However, as pointed out in Willner and Belzung (2015), there is

little to distinguish the chronic depressive phenotype of dysthymia from MDD other than chronicity, and this feature may render the WKY model more suitable for the study of ketamine for highly resistant and persistent forms of depression (Willner and Belzung, 2015). In addition, the endogenous WKY phenotype can be worsened by acute or chronic stress in a more episodic fashion (Tejani-Butt et al., 1994; Willner et al., 2018; Zafar et al., 1997).

Overall, the WKY model exhibits a high face validity due to the close phenomenological similarity to a wide range of symptoms associated with MDD, as well as a high construct validity that reflects a diathesis-stress approach of combining endogenous stress susceptibility and environmental stress (where even without an explicit acute/chronic stress challenge, basic experimental procedures are often sufficient to induce depressive-like phenotypes in WKYs, as they are innately much more prone to stress) (Aleksandrova et al., 2019; Lahmame et al., 1997a; Willner and Belzung, 2015; Willner and Mitchell, 2002). Furthermore, the WKY strain has a high predictive validity as these rats fail to respond to standard acute doses of various traditional antidepressants in the FST, consistent with the fact that these drugs are clinically ineffective when administered acutely (Abdallah et al., 2015; Duman et al., 2012).

In addition, this strain has been established as a model of, at least partial, resistance to classical antidepressants, as in many cases these rats fail to respond to chronic administration of these agents, depending on the drug class (Griebel et al., 1999; Lahmame et al., 1997a; Lopez-Rubalcava and Lucki, 2000; Nagasawa et al., 2015; Paré, 1992; Tejani-Butt et al., 2003; Willner and Belzung, 2015). The available data suggests this strain may be sensitive to chronic administration of noradrenergic and possibly dopaminergic but not serotonergic agents, consistent with the clinical observation that tricyclic antidepressants may be more effective in severe, melancholic and treatment-resistant depression than SSRIs (Huynh et al., 2008;

Rittenhouse et al., 2002). However, WKY rats subjected to CMS have been reported to be resistant to chronic administration of virtually all major classes of traditional antidepressant drugs (Willner et al., 2018). Importantly, WKY rats seem to respond well to novel rapid acting antidepressant therapies proven effective in TRD, particularly ketamine, ECT and DBS (Akinfiresoye and Tizabi, 2013; Falowski et al., 2011; Fortress et al., 2018; Kyeremanteng et al., 2014; Luo et al., 2015; Tizabi et al., 2012; Willner et al., 2018), making this model particularly useful in assessment of novel antidepressants for TRD (Willner and Belzung, 2015).

1.6 Synaptic Plasticity in the Wistar-Kyoto Rat

As previously discussed, accumulating evidence from both animal and human studies supports an emerging theory of the etiology of depression that implicates dysfunction within the glutamatergic system and dysregulation of synaptic plasticity (Chattarji et al., 2015; Gerhard et al., 2016; Lener et al., 2017a; Licznerski and Duman, 2013; Marsden, 2013; Pittenger and Duman, 2008; Zhou et al., 2014). Despite this, surprisingly, a comprehensive understanding of the role of synaptic plasticity in the pathophysiology of depression or in mediating antidepressant response is still lacking. Nevertheless, the number of peer-reviewed studies that include direct assessments of synaptic plasticity processes (LTP, LTD) in brain areas implicated in MDD in the context of animal models of depression, at baseline or following antidepressant treatment (e.g. ketamine), is growing. In the last five years, a few important examples performed in WKY rats not only provide further support for this theory, but also highlight the utility of this model for the study of synaptic plasticity changes and how they may contribute to key depressive-like phenotypes and antidepressant responses (Aleksandrova et al., 2019; Belujon and Grace, 2014; Cominski et al., 2014; Fortress et al., 2018; Fragale et al., 2016; Han et al., 2018; Kanzari et al.,

2018; She et al., 2015). Because there was no available data characterizing synaptic plasticity processes in WKY rats prior to the beginning of this project, these studies are briefly summarized here in Table 4 and discussed in detail later (in sections 5.9 and 7.2).

Table 4. Findings related to synaptic plasticity in the WKY rat.

Synapse	WKY circuit dysfunction / behavioural phenotype	References
Medial Perforant Path – Dentate Gyrus	Impaired LTP following HFS, associated with impaired extinction of avoidance, reversed by ketamine (PS LTP, but not fEPSP LTP) at 24h in 'responders' only Successful LTP induction (e.g. following theta-like HFS protocol) may have FST antidepressant effects	Cominski et al, 2014 Fortress et al., 2018 Kanzari et al., 2018
Schaffer Collateral – CA1	Impaired LTP following HFS EA restored the impaired LTP and normalized FST, SPT, OFT & MWM performance	She et al., 2015 Han et al., 2018
Basolateral Amygdala – Prelimbic Prefrontal Cortex	Impaired LTP linked to impaired extinction of avoidance	Fragale et al., 2015
Ventral Subiculum – Nucleus Accumbens Shell	Impaired LTP (LTD instead of LTP) following HFS, associated with susceptibility to LH and lower VTA DA neuron activity Ketamine rescues the impaired LTP and lower VTA DA neuron activity and reverses helplessness	Belujon and Grace, 2014

General support for the presence of aberrant connectivity in the WKY model of depression is provided by one study characterizing the white matter integrity of four fibre tracts known to be altered in human mood disorders, which reports significantly reduced connectivity in WKY compared to Wistar control rats (Zalsman et al., 2016). Moreover, reduced resting-state frontal cortical perfusion is also observed in these rats (Gormley et al., 2016). Although LTP/LTD were not measured directly, the importance of hippocampal synaptic plasticity changes in the WKY model was highlighted early by the findings of Tizabi and colleagues. As

discussed previously, ketamine enhanced hippocampal BDNF, synapsin and mTOR levels, as well as increased the ratio of AMPA to NMDA receptor density in the HPC of both male and female WKY rats (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). These findings are consistent with the hypothesis that ketamine's antidepressant mechanism of action is associated with activation of key synaptogenic signaling cascades, facilitation of AMPAR-mediated transmission and the induction of long-lasting neuroplasticity changes within the HPC, consistent with an LTP-like effect (Aleksandrova et al., 2019, 2017a; Du et al., 2006; Duman, 2014b; Marsden, 2013; Wang et al., 2014).

1.7 Rationale, Aims and Hypotheses

Given the therapeutic limitations of current antidepressants, as well as the lack of strong, direct evidence to support the monoamine deficiency hypothesis of depression, understandably, there has been growing interest in new targets for antidepressant action (Abdallah et al., 2015; Gerhard et al., 2016; Park et al., 2015). Accumulating evidence implicates dysfunction within the glutamatergic system and dysregulation of synaptic plasticity in the pathophysiology of depression, particularly in the HPC (Aleksandrova et al., 2019). Compelling clinical and preclinical data support ketamine's utility in treating depression and inspire massive efforts to uncover the molecular mechanisms responsible for its unprecedented efficacy, which holds promise for a new generation of much needed, superior antidepressant agents. One theory is that ketamine may reverse the stress-induced loss of connectivity in neural pathways (HPC and beyond) implicated in MDD by engaging synaptic plasticity processes to "reset the system".

In light of this accumulating evidence, **we hypothesize that synaptic plasticity (LTP, LTD) in key circuits may play important roles in the pathogenesis of MDD and underlie certain antidepressant actions of ketamine and its metabolites**. This thesis utilized the Wistar-Kyoto (WKY) rat, a valid model of endogenous stress susceptibility and depression. **The overall aim of this thesis was to validate our overall hypothesis by investigating the role of dorsal HPC synaptic plasticity in depression and ketamine antidepressant response in the context of the WKY model using the following specific research aims:**

Aim #1: Behavioural characterization of the Wistar-Kyoto model of depression **(Chapter 3)**

Hypothesis: Compared to normal Wistar rats, stress-susceptible WKY rats exhibit depressive-like behaviours across a battery of preclinical tests assessing stress/emotional reactivity, motivation and cognition.

Aim #2: Synaptic characterization of the Wistar-Kyoto model of depression **(Chapter 4)**

Hypothesis: Compared to normal Wistar rats, stress-susceptible WKY rats exhibit an imbalance between LTP and LTD in the HPC, key brain area implicated in depression.

Aim #3: Characterization of ketamine's effects on synaptic plasticity and their contribution to its antidepressant activity in the WKY model **(Chapters 5 and 6)**

Hypothesis: Based on the idea that rapid antidepressants may restore normal connectivity in depression by engaging synaptic plasticity processes, ketamine and its metabolite (2R,6R)-HNK may effectively reverse any imbalances in HPC LTP/LTD in stress-susceptible WKY rats, which may contribute to their rapid or sustained antidepressant effects in this model.

This thesis emphasizes the importance of 1) using an animal model that incorporates not only various stress-induced behavioural, neurochemical and endocrine parallels to MDD but also aspects of heightened stress susceptibility and resistance to conventional drugs, 2) incorporating synaptic plasticity into the current framework of ketamine antidepressant action, which may serve to bridge understanding of an antidepressant drug's molecular and cellular effects with those related to regional structural plasticity and neural circuit functioning, and 3) deconstructing depression-like phenotypes and identifying the neural circuits that mediate them more precisely.

Chapter 2: Methods

2.1 Subjects

Stress-prone Wistar-Kyoto (WKY) rats and control Wistar (WIS) rats were used. Male subjects (age 10-12wks at arrival) were pair-housed under a reverse light/dark cycle, with food and water available ad libitum (except in the NSF and PR tests, see sections 2.3.3 and 2.3.4). Animal experiments were carried out in accordance with the Canadian Council of Animal Care and with the approval of the Animal Care Committee at the University of British Columbia (A15-0131).

2.2 Drugs

Ketamine HCl and its metabolite (2R,6R)-HNK (5mg/kg) were dissolved in saline (NaCl 0.9%) and administered intraperitoneally (ip) at a final volume of 1ml/kg. Ketamine HCl was purchased from Medisca Pharmaceuticals Inc. (St-Laurent, Quebec). The ketamine metabolite (2R,6R)-HNK was synthesized and verified in the lab, according to a previously published protocol (Zanos et al., 2016, see appendix). Control animals received the vehicle (saline) at a volume of 1ml/kg. Urethane for *in vivo* electrophysiology experiments was obtained from Sigma-Aldrich (St. Louis, USA), dissolved in distilled water and injected ip at a dose of 1.5g/kg.

2.3 Behavioural Assays

Upon arrival, animals were allowed to acclimatize to the housing facility for at least a week, with regular handling beginning on day 3. Thereafter, animals underwent behavioural testing on a set of validated rodent tests relevant to depression, which aim to determine the prevalence of depressive-like behaviours in Wistar-Kyoto rats and their non-stress prone control

strain, the Wistar rats. Different groups of animals were tested in each task, with behavioural testing performed during the animals' dark (active) phase (i.e. between 10am and 3pm).

2.3.1 Open Field Test (OFT)

The open field test (OFT) is used to assess an animal's exploration of a novel environment (an open-field arena) by measuring spontaneous locomotor activity (i.e. total distance traveled, velocity, number of rearings, time spent in center of the arena, etc.). Eight black plexiglass boxes measuring 41.25cm * 41.25cm * 41.25cm were used as arenas. Red LED lights placed above the boxes provided lighting and the room was otherwise held in darkness. OFT testing was performed without habituation to the testing apparatus. Locomotor activity within each arena was tracked by two overhead video cameras and scored with Ethovision XT (Noldus), which was set to track 10min of locomotor activity upon detection of movement within each arena zone. Following testing completion, the absence of tracking errors was verified using the stored video recording. In experiments involving drug treatments, saline, ketamine or HNK were injected either 30min or 24h before OFT testing.

2.3.2 Forced Swim Test (FST)

The forced swim test (FST) is commonly used to measure abnormal stress coping in rats and serves as a canonical screen for antidepressant drug action. Briefly, rats were individually placed into a clear plastic cylinder (20cm in diameter, 50cm tall) filled with room temperature water (25±0.5 °C) at a height of 30cm to ensure that animals could not touch the bottom of the container with their hind paws or tails, with water changed between every rat. Animals underwent a 15min pre-exposure FST session on day 1, followed by a 5min test session on day 2. FST test sessions were recorded using a video camera and manually scored later. After the

FST, rats were dried and placed in a recovery chamber under a heat lamp for 10min before being returned to their home cage. A time-sampling scoring technique was used, whereby the predominant behavior (immobility or swimming) in each 5s period of the 300s test was recorded. The definition of immobility in the FST is that the rat remained floating in the water without struggling and made only movements necessary to keep its head above the water. We included the climbing score with the swimming as an active behavior because very few such incidences were observed. Scoring was done by an experienced experimenter blinded to the experimental condition. In experiments involving drug treatments, saline, ketamine or HNK were injected either 30min or 24h before FST test on day2.

2.3.3 Novelty-Suppressed Feeding (NSF) Test

The NSF test is based on the phenomenon of hyponeophagia, or the inhibition of feeding behaviour caused by exposure to a novel environment due to an innate fear of novelty in rats. This phenomenon is thought to reflect a depression- / anxiety-like behaviour, and is also used to screen for drug antidepressant activity (Blasco-serra et al., 2017; Yan et al., 2010). As is always done in the literature, animals were food restricted to 85-90% of their free feeding weight for 3 days prior to testing to ensure they are hungry and motivated to perform this appetitive task (Blasco-serra et al., 2017; Yan et al., 2010). On the day before NSF resting, animals were habituated to a novel, highly palatable food (sucrose pellets) in their home cages. On the day of testing, animals are placed in an open field arena (novel environment) containing a dish with 10 sucrose pellets for 10min. The NSF session is recorded using a video camera and the latency to feeding, as well as the frequency of feeding/approach and amount eaten were determined by manual scoring of the video recordings, done by an experimenter who was blinded to the experimental condition. Latency to feed was also determined in the home cage.

2.3.4 Progressive Ratio (PR) Schedule of Reinforcement

Under the progressive ratio schedule, the number of lever presses to receive a single food reward increases in each trial, eventually reaching a "breaking" point when the animal stops lever-pressing. This task measures how much work/effort rodents are willing to put in to earn a reward (i.e. their motivation). Because the PR task is highly appetitively motivated, following acclimatization to the facility, animals were gradually food-restricted (~0.5-1g less of food/day) over one week to a final food intake equal to 5-6% of their body weight a day (12-16g/day). Animals were fed once a day, always shortly after training. Under this schedule rats maintain 85-90% of free feed weight, remain healthy, increase their body weight steadily and maintain stable levels of behavioural performance. Rats were maintained under this feeding schedule for the 6-week period of the PR experiment and were weighed each day with their weights compared to charts of expected free-feeding weights for age-matched animals. Rats were first habituated to the operant chamber for one day (lever retracted), then trained to press a retractable operant lever to receive a sucrose pellet delivered into reinforcement spout. After a lever press, the lever retracted, a food pellet was delivered and in 15s the lever was presented again. After each rat reached a minimum number of lever presses, which remained stable for at least 2 consecutive sessions, the reinforcement schedule changed to the next training schedule (FR1, FR3, FR5, PR2, PR3, PR exponential). Under the final exponential progressive ratio schedule, the number of lever presses to receive a single pellet increases exponentially in each trial, until animals reach the first failure, or the "breaking" point. Since we used 15min trials with 15sec inter-trial interval, it took about 45min until the break point. However, average duration of a test session depended on the training stage and level of proficiency of the rat. It took 10-15 sessions for an animal to be ready for the final PR schedule, and it took about 15 sessions on the exponential

scale to reach stable responding, for a total of ~5-6 weeks of testing (4-5 days/week) required to complete the PR experiment. For each testing session, the PR breakpoint, number of lever presses and latency to complete each PR level, which were the primary measures of interest, were averaged and compared for each week of PR testing (1-3).

2.3.5 Object Location Recognition (OLR) Test

The object location recognition test is a hippocampal-dependent task, which allows for measurement of both short-term (at a delay of 1h between training and testing) and long-term (at a delay of 24h) spatial memory (Vogel-Ciernia and Wood, 2014). In the OLR, animals were habituated to an open field arena (60 x 60 x 60cm) placed in a room with external cues, for 3 consecutive days (10min sessions). They were then given 2 training sessions on day 4 (T1 and T2, 10min each, morning and afternoon), where two identical objects (Lego figures mounted to the arena floor) were placed in the arena in a certain spatial configuration (i.e. in two opposite corners of the apparatus, 10cm from the sidewall, with starting locations counterbalanced between different experimental groups to reduce potential biases due to preferences for particular locations). Rats were placed in the middle of the apparatus and were left to explore these two identical objects for 10min. After T1, rats were put back in their home cages and an ITI of ~4h was given before subjecting them to a second training session (T2) as before (with objects in the same locations at in T1). During the testing session (T3, either 1h or 24h later), one of the objects remained at the familiar location (FL), while the other was moved to a new location (NL), and rats were again allowed to explore freely for 10min. The apparatus and the objects were thoroughly cleaned after each trial to avoid the presence of olfactory trails. Sessions T1-3 were recorded using a video camera and the time spent exploring each object location were determined only for the first 2min of each session by manual scoring of the video recordings,

done by an experienced experimenter blinded to the experimental condition. Object exploration was defined as the time when the rat's head was oriented toward the object within 45 degrees and was within 3cm of the object. An OLR preference of around/above 60% for the object that has been moved to the novel location on the test day (NL/NL+FL) indicates good spatial recognition memory (since animals are drawn to explore novelty). In experiments involving drug treatments, saline, ketamine or HNK were injected 3.5h before the second OLR training session on day 4, or 27.5h before OLR testing on day 5 (time point chosen based on our electrophysiology data).

2.4 *In vivo* Electrophysiology

In vivo single-unit extracellular recordings were used to measure and compare hippocampal basal synaptic transmission, as well as synaptic plasticity (LTP and LTD), between the two strains (WKY and WIS) and under the different treatment conditions (drug-free, saline, ketamine or HNK administered to different groups of rats). The *in vivo* electrophysiological recordings were conducted using techniques described previously (Wong et al., 2007). On the day of the experiment, rats were removed from the colony room, weighed and immediately anesthetized (using urethane, 1.5g/kg, ip, for non-recovery experiments). Where necessary, a supplemental dose of anesthesia (urethane in increments of 0.3ml at a time) was administered as appropriate. Once fully unconscious, they were placed in a stereotaxic frame, and gel tears applied to their eyes. The animals' body temperature was controlled with a regulated heating pad set at 36.8°C. The degree of anesthesia was monitored through respiratory rate and withdrawal reflex. Using sterile techniques, an incision was made along the dorsal surface of the skull to expose the landmark bregma. Two holes (1-2mm in diameter, for the recording and stimulating electrodes, respectively) were drilled with a stereotaxic drill above the brain structures of interest (in this case the hippocampus). One hole was drilled posterior and lateral to bregma for the

recording electrode ground wire, and one over the left frontal lobe for a single reference electrode. A bipolar stainless steel (0.0045" coated diameter, A-M Systems, Inc) stimulating electrode and a unipolar iridium-coated platinum (0.0080" coated diameter, supplier A-M Systems, Inc) recording electrode were individually lowered into their respective targets under stereotaxic control, with final stimulating electrode coordinates -3.3mm AP, +/-3.0mm ML, -2.6-3.2mm DV (Schaffer collateral) and recording electrode coordinates -3.3mm AP, +/- 2.0mm ML, -2.6-3.2mm DV (CA1 stratum radiatum) (Paxinos atlas). Electrode positions within the dorsal-ventral range were optimized to maximize the evoked field excitatory post-synaptic potential (fEPSP). Electrical stimulation of the SC-CA1 pathway was accomplished using computer generated pulses (duration 0.1ms, biphasic) and delivered by a stimulator at low intensities (200µA initially, adjusted later based on response). Evoked field potential responses (fEPSPs) were recorded with a standard differential amplifier and acquisition board, as well as passed through a notch filter, and recorded using computer software WinLTP. The peak amplitude and initial slope of the fEPSP was measured, but slope is reported throughout as it is considered to be a more reliable measure to quantify synaptic strength (Johnston and Wu, 1995). Baseline evoked responses were then tested at a range of stimulation intensities (0-400µA, in increments of 10µA to 200µA, and then of 20µA to 400µA) to obtain an input-output curve, and a stimulation magnitude evoking 50% of the maximal response was used for the remainder of the recording. Once the actual recording began, evoked field potential responses were sampled at a frequency of 0.033 Hz (1 pulse/30s), with pairs of pulses averaged to give one value per minute (for fEPSP amplitude and slope). In different groups, three stimulation protocols were applied to induce 1) LTD following low-frequency stimulation (LFS, 3Hz, 900 pulses, 5min), 2) early LTP (E-LTP) following one train of high-frequency stimulation (HFS, 100Hz, 1s), or 3) late LTP (L-LTP)

following 4 trains of HFS (100Hz, ITI 5min). In drug-free recordings, a stable baseline of at least 15-20minutes was obtained first, before delivery of the LTP/LTD stimulation protocols. The duration of the recording following the induction of LTP/LTD depended on the form of synaptic plasticity as follows: 30min for E-LTP and LTD, and 90min for L-LTP. In experiments involving drug treatments, saline, ketamine or HNK were injected systemically (ip) 30min, 3.5h or 24h before the induction of L-LTP. In the case of the two earlier timepoints, drugs were injected into anesthetized rats following a baseline fEPSP recording (20min), followed by the LTP protocol 30min or 3.5h later; for the 24h recording, awake animals were pre-treated with drug 24h before conducting the electrophysiological recording the next day (anesthesia, 20min baseline, followed by LTP protocol 30min later). Animals were euthanized at the end of the recording period by administration of an anesthetic overdose of urethane (4g/kg, ip), without ever regaining consciousness. The primary measure of interest, fEPSP slope (average of two values per minute), was normalized to average baseline fEPSP slope and expressed as a percent (%) of baseline. Data were analyzed by first averaging normalized fEPSP slope (%) into several 5min time bins depending on the recording: LTD (pre-LFS, 5min post-LFS and 30min post-LFS), E-LTP (pre-HFS, 5min post-HFS and 30min post-HFS) and L-LTP (pre-HFS, 5min post-HFS and 90min post-HFS), as well as an additional pre-drug bin for experiments involving drug treatments. Time bins for normalized fEPSP slope (%) were calculated as follows: pre-drug (average of 5min before drug injection), pre-LFS/HFS (average of 5min before LFS/HFS), 5min post-LFS/HFS (average of 5min after LFS/HFS), 30min/90min post-LFS/HFS (last 5 min of the recording, lasting 30min or 90min after the stimulation protocol), and compared between the different experimental groups.

2.5 Data Analysis

Data are presented throughout as mean ± SEM, where n is the number of rats (except in electrophysiology experiments in Chapter 4, where n may refer to the number of hemispheres (not rats, that were recorded from), as noted in the text. Throughout the study, most comparisons (e.g. all electrophysiological recordings and most drug treatment effects) were conducted by 1- or 2- way analysis of variance testing (ANOVA, repeated measures or not, as specified) with appropriate post-hoc tests (Tukey's or Sidak's, as detailed in the text), or on a few occasions by a two-tailed t test (for some simpler behaviours, e.g. OFT, FST, NSF). Results were analyzed and graphed using Prism 6.0 (GraphPad, San Diego, California, USA). Significance in all analyses was set at $\alpha = 0.05$, with multiplicity adjusted *p* values for each comparison reported throughout the text.

Chapter 3: Characterization of WKY Behaviours Relevant to Depression

3.1 Open Field Test (OFT)

First, WKYs exhibited dramatic hypo-locomotion in the open field test (OFT) compared to WIS controls. When rats were allowed to freely explore an empty arena for 10 minutes, the total distance travelled was significantly lower in WKY compared to WIS rats (2352.2 ±116.57cm vs. 4552.8 ±172.91cm, n=10/strain, two-tailed t test $p<0.0001$, Figure 3A), along with deficits on other measures of general locomotor activity (e.g. velocity and rearings) (data not shown). In addition, the total time spend in the center of the arena during the session was also dramatically reduced in the stress-prone strain compared to WIS controls (42.32 ±5.53s vs. 66.91 ±5.54, n=10/strain, two-tailed t test $p=0.0056$, Figure 3B), indicative of anxiety-like behavior.

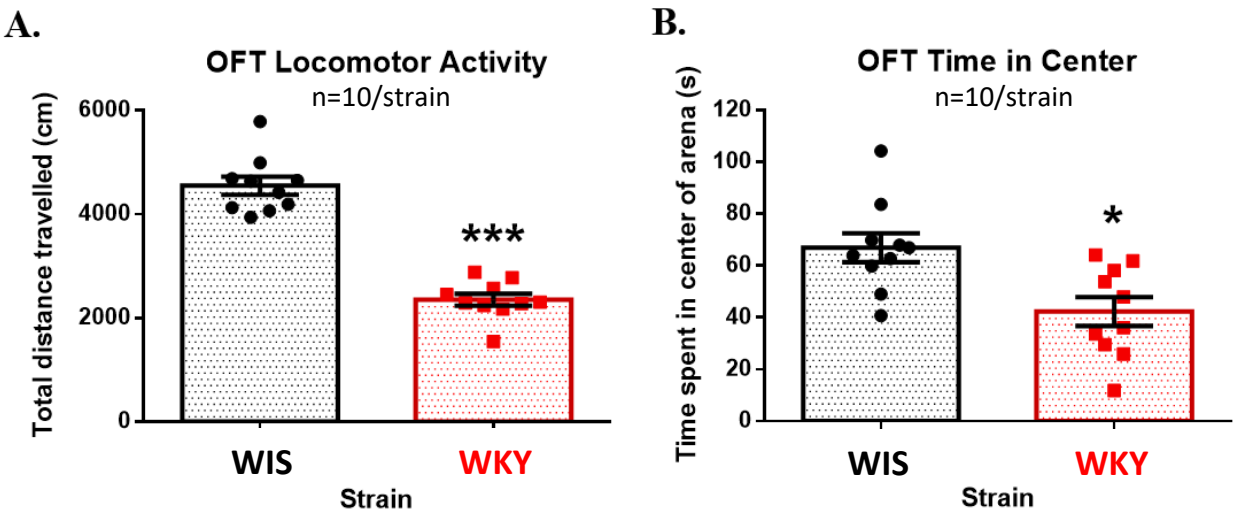


Figure 3. WKY rats display hypolocomotion and anxiety-like behavior in the open field test (OFT).
A. Average total distance travelled (cm) and B. average time spent in the center of the arena (s) during the 10min test session were significantly lower in WKY rats compared to WIS controls (two-tailed t test ***$p<0.0001$ and *$p=0.0056$, n=10 rats/strain).

3.2 Forced Swim Test (FST)

Next, we found that WKY rats are characterized by pronounced immobility in the forced swim test (FST) (Figure 14). On day 1 (15min pre-exposure), the average latency to immobility for normal Wistars was ~5min and the proportion of time spent immobile during each subsequent minute of the test increased steadily, eventually accounting for about half of the time at the end of the 15min session (averaging 34.38 ±2.86s during minute 15, Figure 4A). In contrast, from the beginning of the pre-exposure session, stress-prone WKYs were dramatically more immobile (averaging 16.88 ±3.76s during minute 1), with rats spending the last 10minutes of the session largely immobile (53.75 ±2.21s during minute 15). A 2-way repeated measures ANOVA (RM-ANOVA, with strain as the between subject factor and time (minute1-15) as the within subject factor) revealed significant main effects of strain and time ($F_{1,30}$=123.60, p<0.0001 and $F_{14,420}$=53.57, p<0.0001), as well as a significant strain x time interaction ($F_{14,420}$=7.30, p<0.0001). Subsequent analysis of these data indicated that FST immobility was significantly higher in WKY compared to WIS rats for every minute of the FST day1 session (Sidak's p<0.0004 for minutes 1-15). Following pre-exposure, these strain differences in the FST were even more pronounced on day 2 (5min test), where the average total immobility for the session was again significantly different between the strains (WKY: 240.17 ±7.89s, n=30 vs. WIS: 90.36 ±7.22s, n=28, two-tailed t test p<0.0001, Figure 4B), indicative of abnormal stress coping and serving as a canonical measure of depressive-like behavior in the WKY model.

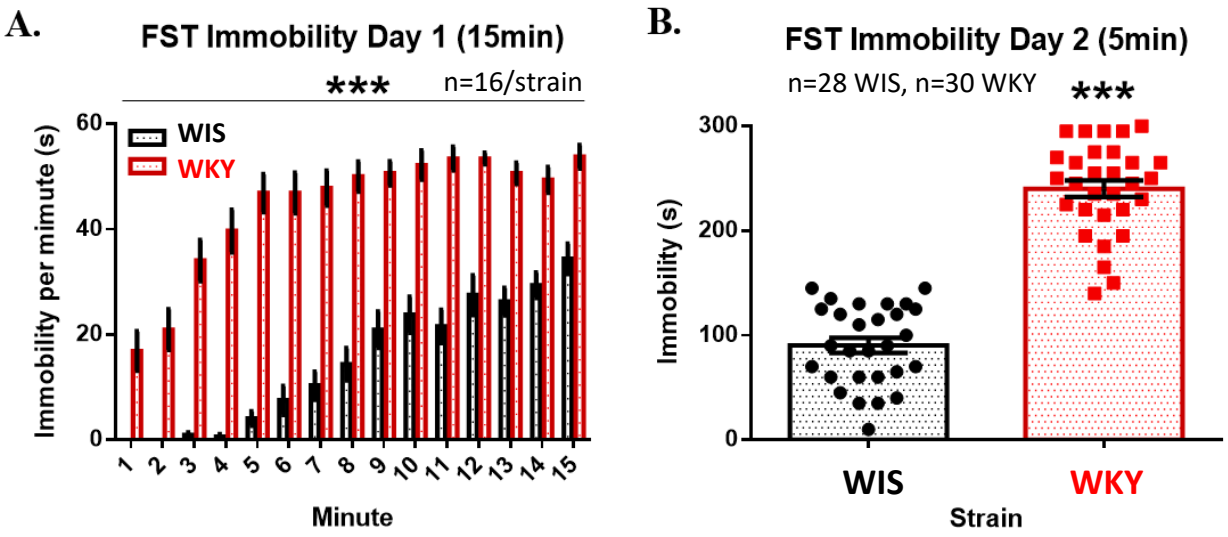


Figure 4. WKY rats display depressive-like behavior in the forced swim test (FST).

A. FST Day1 average immobility per minute (s) was significantly higher in WKY compared to WIS rats for every minute of the 15min pre-test session (Sidak's ***p<0.0004 for minutes 1-15, n=16/strain). B. FST Day2 (5min test) average total immobility was again significantly higher in WKY rats compared to WIS controls (two-tailed t test ***p<0.0001, WKY: n=30, WIS: n=28), indicating abnormal stress coping (canonical depressive-like behavior) in this strain, which is exacerbated by pre-exposure.

3.3 Novelty-Suppressed Feeding (NSF) Test

Next, performance in the novelty-suppressed feeding (NSF) test was also dramatically different between the two strains. Namely, following acute food restriction, the latency to feed in a novel environment was significantly longer in WKY compared to WIS rats (515.10 ±45.27s vs. 206.60 ±34.80s, n=10/strain, two-tailed t test p<0.0001, Figure 5A), while the latency to feed in the home cage was similar between the two strains (7.80 ±0.88s vs. 6.70 ±0.63s for WKY and WIS rats, respectively, two-tailed t test p=0.32, n.s., Figure 5B). In addition, 7/10 WKY rats failed to consume any of the highly palatable food during the 10min of the NSF test, while all controls did, with animals consuming an average of 8.20 ±0.85 and 2.50 ±0.79 out 10 pellets for WIS and WKY rats, respectively.

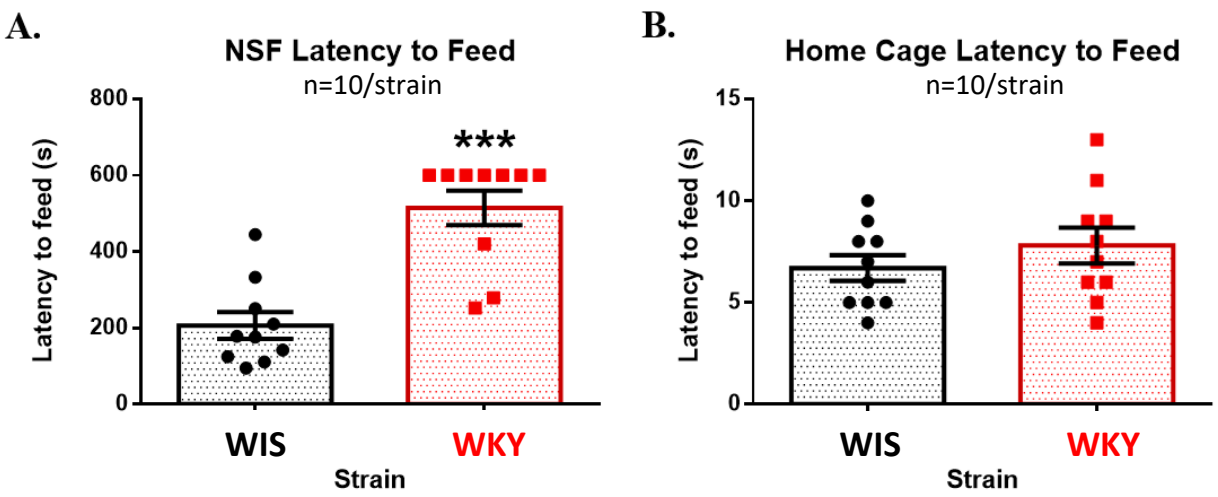


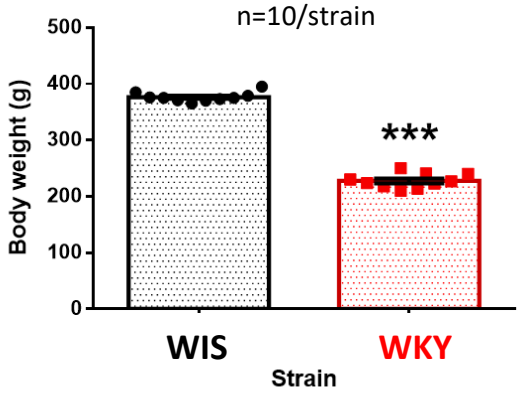
Figure 5. WKY rats display depressive-like behaviour in the novelty-suppressed feeding (NSF) test. A. Average latency to feed in a novel environment was significantly higher in WKY compared to WIS rats (two-tailed t test ***$p<0.0001$, n=10/strain). In total, 7/10 WKY rats failed to consume any food during the 10min of the NSF test, while all controls did. B. Average latency to feed in the home cage, on the other hand, was similar between the two strains (two-tailed t test $p=0.32$, n.s., n=10/strain).

3.4 Progressive ratio (PR) Schedule of Reinforcement

In order to assess motivated behaviour, we trained rats of both strains to press a lever to earn food reward under a progressive ratio (PR) schedule of reinforcement, where each subsequent pellet requires exponentially more lever presses, until animals are no longer willing to work and reach a breaking point. It is important to note that, as widely reported in the literature, body weight differed significantly by strain, with WKY rats consistently weighing less at arrival than their age-matched control counterparts (227.70 ±4.03g vs. 376.40 ±2.67g, n=10, two tailed t test $p<0.0001$, Figure 6A). To compensate for this baseline difference, animals of each strain were food restricted and fed 5-6% of their corresponding body weight each day (always after testing) for the duration of the experiment. Following habituation to the testing apparatus, rats completed several training phases (FR1, FR3, FR5, PR2, PR3) before reaching the exponential PR schedule (Figure 6B). The two strains acquired the task at a similar pace and

significant differences in performance at the final PR schedule were observed only following repeated testing. The number of pellets earned at break point was averaged for each week of testing (week1-3, Figure 6C) and analyzed using a 2-way RM-ANOVA (with strain as the between subject factor and time (week1-3) as the within subject factor), which indicated significant time and strain x time effects ($F_{2,32}$=4.44, p=0.020 and $F_{2,32}$=4.52, p=0.019), as well as a trend toward a significant main effect of strain ($F_{1,16}$=3.33, p=0.087, n.s.). Subsequent analysis revealed that while PR break point was initially similar between strains (week 1, WKY: 10.20 ±0.37 pellets vs. WIS: 10.69 ±0.51 pellets, n=9/strain, Sidak's p=0.87, n.s.) and performance in WIS rats remained stable with repeated testing (week 2: 10.24 ±0.66 pellets, week 3: 10.59 ±0.44 pellets; Tukey's p>0.49), it gradually decreased over time in WKY rats, reaching significance at week 3 (8.37 ±0.44 pellets, vs. WKY week1 Tukey's p=0.001; vs. WIS week3 Sidak's p=0.009, Figure 6C, D). Accordingly, the average total number of lever presses per session was also significantly lower in WKY compared to WIS rats at week 3 (200.11 ±24.96 vs. 363.00 ±35.82, two-tailed t test p<0.0018, Figure 6E). Furthermore, a 2-way RM-ANOVA of the latencies to complete each PR level at week 3 (with strain as the between subject factor and PR level as the within subject factor (1-8 only, as higher PR levels were not completed by all animals and were thus excluded from the analysis)) revealed a significant main effect of PR level and a trend towards a significant strain effect ($F_{7,112}$=32.41, p<0.0001 and $F_{1,16}$=3.01, p=0.10, n.s.), with no significant interaction ($F_{7,112}$=1.31, p=0.25, n.s.). This indicated that in addition to each subsequent PR level being more time consuming (e.g. 3-6s for PR1 vs. 60-65s for PR8), as expected from the increased effort required for completion, WKYs consistently tended to take longer to complete each PR level (post-hoc was only detected between strains at PR level 6, Sidak's p=0.012, Figure 6F), consistent with a general psychomotor slowing effect.

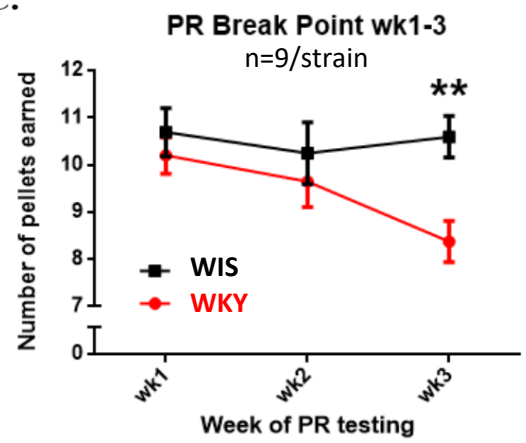
A.



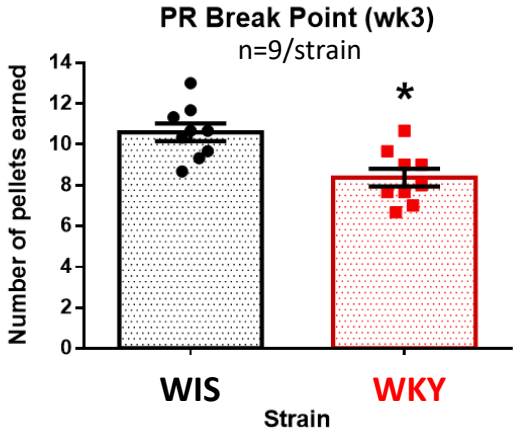
B.

PR Level	Total Presses	Cumulative Presses
1	3	3
2	6	9
3	10	19
4	15	34
5	20	54
6	25	79
7	32	111
8	40	151
9	50	201
10	62	263
11	77	340
12	95	435
13	118	553
14	145	698

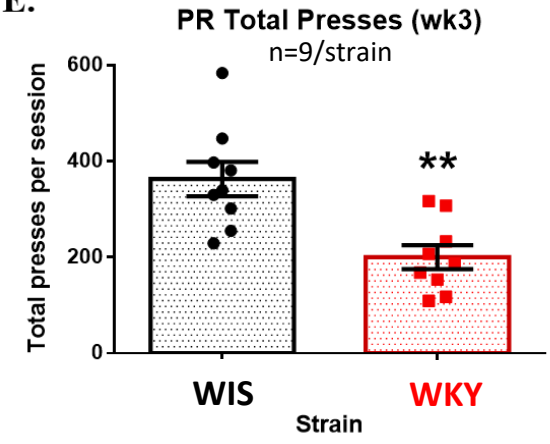
C.



D.



E.



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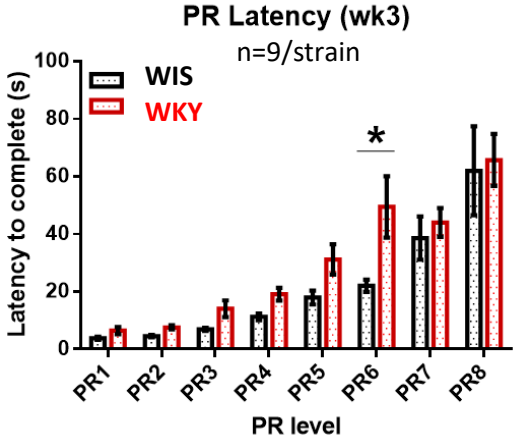

Figure 6. WKY rats display a motivational deficit under a progressive ratio (PR) schedule of reinforcement, which develops with repeated testing.

A. Body weight by strain, with WKY rats weighing significantly less at arrival than age-matched WIS controls (two tailed t test ***$p<0.0001$, n=10/strain). B. Final exponential PR schedule, where each subsequent pellet requires exponentially more lever presses. C. Number of pellets earned at break point averaged for each week of testing (week1-3) started put equivalent between the two strain, but gradually decreased in WKYs, reaching significance at week 3 of PR testing (vs. WKY week1 Tukey's **$p=0.001$, n=9/strain). D. Average PR break point at week 3 (Sidak's *$p=0.009$) and E. total number of lever presses per session at week 3 (two-tailed t test **$p<0.0018$) were significantly lower in WKY compared to WIS rats. F. Average latencies to complete each PR level at week 3 indicated a significant main effect of PR level as expected ($p<0.0001$) and a trend towards a significant main effect of strain ($p=0.10$, n.s.), being consistently higher in WKY rats (with post-hoc significance at PR level 6 only, Sidak's *$p=0.012$).

3.5 Summary and Discussion

In this thesis, we emphasize that the search for novel antidepressant treatments with a wider therapeutic reach requires animal models that incorporate aspects of heightened stress responsiveness and vulnerability to depression, as well as partial or full resistance to classical antidepressants (Aleksandrova et al., 2019; Willner et al., 2014; Willner and Belzung, 2015). Accumulating preclinical research supports the WKY rat as a valid model of endogenous stress susceptibility and depression that satisfies these conditions and exhibits many specific behavioural, neurochemical and endocrine parallels to clinical depression.

The key findings of our behavioural characterization of this model are that WKY rats exhibit various depressive and anxiety -like behaviours compared to WIS controls, as has been previously demonstrated in the literature. Consistent with our results, WKY rats have been widely reported to exhibit hypo-locomotion in OFT, indicative of general behavioural inhibition and psychomotor slowing, as well as to spend less time in the center of the OFT arena, reflecting an anxiety-like behavior (Berton et al., 1997; Nam et al., 2014; Pardon et al., 2002; Paré and Redei, 1993a; Tejani-Butt et al., 2003; Van Zyl et al., 2014). Abnormal stress-coping in the FST,

evident by the presence of dramatic immobility (even in naïve rats, but worsened by pre-exposure in the 2-day version of the FST), has been consistently demonstrated in the WKY rat and was replicated here (Lahmame et al., 1997a; Lopez-Rubalcava and Lucki, 2000; Marti and Armario, 1996; Nam et al., 2014; O'Mahony et al., 2011; Paré, 1992; Paré and Redei, 1993a; Tejani-Butt et al., 2003; Tizabi et al., 2012). Moreover, we found that WKY rats have longer latencies to feed in the NSF test (but not in the home cage), similar to previous reports (Burke et al., 2016; Nam et al., 2014; Paré, 1994b). Compared to age-matched control rats, stress-prone WKYs display reduced body weight, which has been noted in the literature and may be a part of their depressive-like phenotype (Bjorkholm et al., 2015; De La Garza 2nd, 2005; Nagasawa et al., 2015; Paré, 1994b; Solberg et al., 2001).

A single study previously demonstrated that WKY rats exhibit lower effort/motivation to earn sucrose reward under both fixed and progressive ratio schedules of reinforcement compared to control rats (De La Garza 2nd, 2005). Here we found that although PR performance was comparable between strains at the start of the experiment, with repeated testing WKYs developed a deficit in the task as indicated by a significant reduction in PR break point by week 3, in addition to generally taking longer than controls to complete each PR requirement.

Therefore, we replicated many of the pronounced behavioural phenotypes of stress-prone WKY rats, confirming they display various depressive and anxiety -like behaviours compared to WIS controls at baseline. Based on the extensive literature available on this model, the endogenous depression-like phenotype of the WKY strain is unique in its consistency across virtually all relevant preclinical indices, including stress coping, motivation and cognition. However, as noted previously (section 1.5.4), WKY rats are known to exhibit considerable genetic and behavioural heterogeneity especially for an inbred strain of rats, which is reflected in

the substantial variability within the WKY strain in the performance of the tasks used here (OFT, FST, NSF and PR test), where some WKY rats show extreme deficits while others behave more similarly to control rats in each of these behavioural paradigms. Overall, given the robust depressive-like phenotype of the WKY strain, the next question of interest was whether the normal balance between hippocampal LTP and LTD is perturbed in this model.

Chapter 4: Characterization of WKY Hippocampal Synaptic Plasticity

4.1 SC-CA1 Basal Synaptic Transmission

We were interested in characterizing the WKY model in terms of hippocampal synaptic plasticity, and to this end we performed *in vivo* single-unit extracellular recordings at the SC-CA1 synapse in anesthetized rats (Figure 7A). Basal evoked fEPSP signals were comparable in WIS (n=42, 21 rats x 2 hemispheres) and WKY (n=38, 19 rats x 2 hemispheres), with similar input-output curves observed in the two strains (Figure 7B). Responses were very similar between the left and right hemispheres in both strains ($F<0.08$, $p>0.77$, n.s.), so data was combined. A 2-way RM-ANOVA of average absolute fEPSP slope (with strain as the between subject factor and current intensity (0-400µA) as the within subject factor) indicated a significant main effect of current intensity as expected ($F_{25,1950}=393.50$, $p<0.0001$) but not of strain ($F_{1,78}=0.08$, $p=0.78$, n.s.), as well as a significant strain x current intensity interaction ($F_{25,1950}=4.12$, $p<0.0001$). Subsequent analyses revealed no significant differences in average fEPSP between the two strains at any current intensity (Sidak's $p>0.66$, n.s., Figure 7B), indicating comparable basal synaptic transmission at this synapse. At the highest current intensities (300-400µA), fEPSP slope was consistently slightly lower in WKY compared to WIS rats (maximum fEPSP slope at 400µA: 1.28 ±0.09 vs. 1.46 ±0.09 units/ms, respectively, Figure 7B). Because of the higher WIS maximum as well as the slightly steeper WKY I/O curve slope at this range, the stimulation magnitude evoking ~50% of the maximal response was also slightly higher in WIS compared to WKY rats (~100 vs. 80µA).

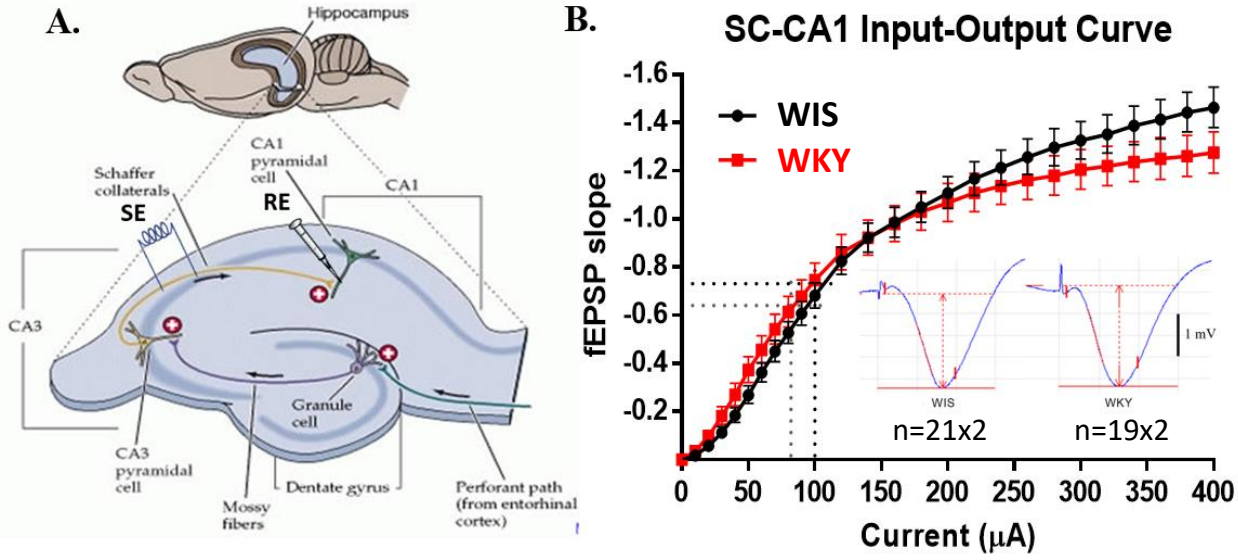

Figure 7. SC - CA1 basal synaptic transmission is unaffected in WKY rats.

A. We performed *in vivo* single-unit extracellular recordings at the SC-CA1 synapse in anesthetized rats (SE: stimulating electrode, RE: recording electrode). B. Representative basal evoked fEPSP signals and average input-output curves (absolute fEPSP slope by current intensity) for each strain (WIS: n=42, 21 rats x 2 hemispheres; WKY: n=38, 19x2). Despite a significant ANOVA main effect of current intensity and a significant strain x current intensity interaction ($p<0.0001$), post-hoc analyses revealed no significant differences in average fEPSP between the two strains at any current intensity (Sidak's $p>0.66$, n.s.), indicating comparable basal synaptic transmission at this synapse.

4.2 SC-CA1 Long-Term Depression (LTD)

Although LTD is generally hard to obtain *in vivo*, we utilized a low-frequency stimulation protocol (3Hz, 900 pulses, 5min) previously reported to have some success, particularly following stress. However, we failed to detect any robust LTD in either WKY or WIS rats (Figure 8A-C). A 2-way RM-ANOVA of average fEPSP slope (with strain as the between subject factor and time point (pre-LFS, 5min post-LFS and 30min post- LFS) as the within subject factor) indicated a significant main effect of time ($F_{2,42}=5.50, p=0.0076$) but no strain or strain x time interaction effects ($F_{1,21}=0.53, p=0.48$, n.s. and $F_{2,42}=0.12, p=0.89$, n.s.). Subsequent analyses revealed that average fEPSP slope was transiently reduced immediately

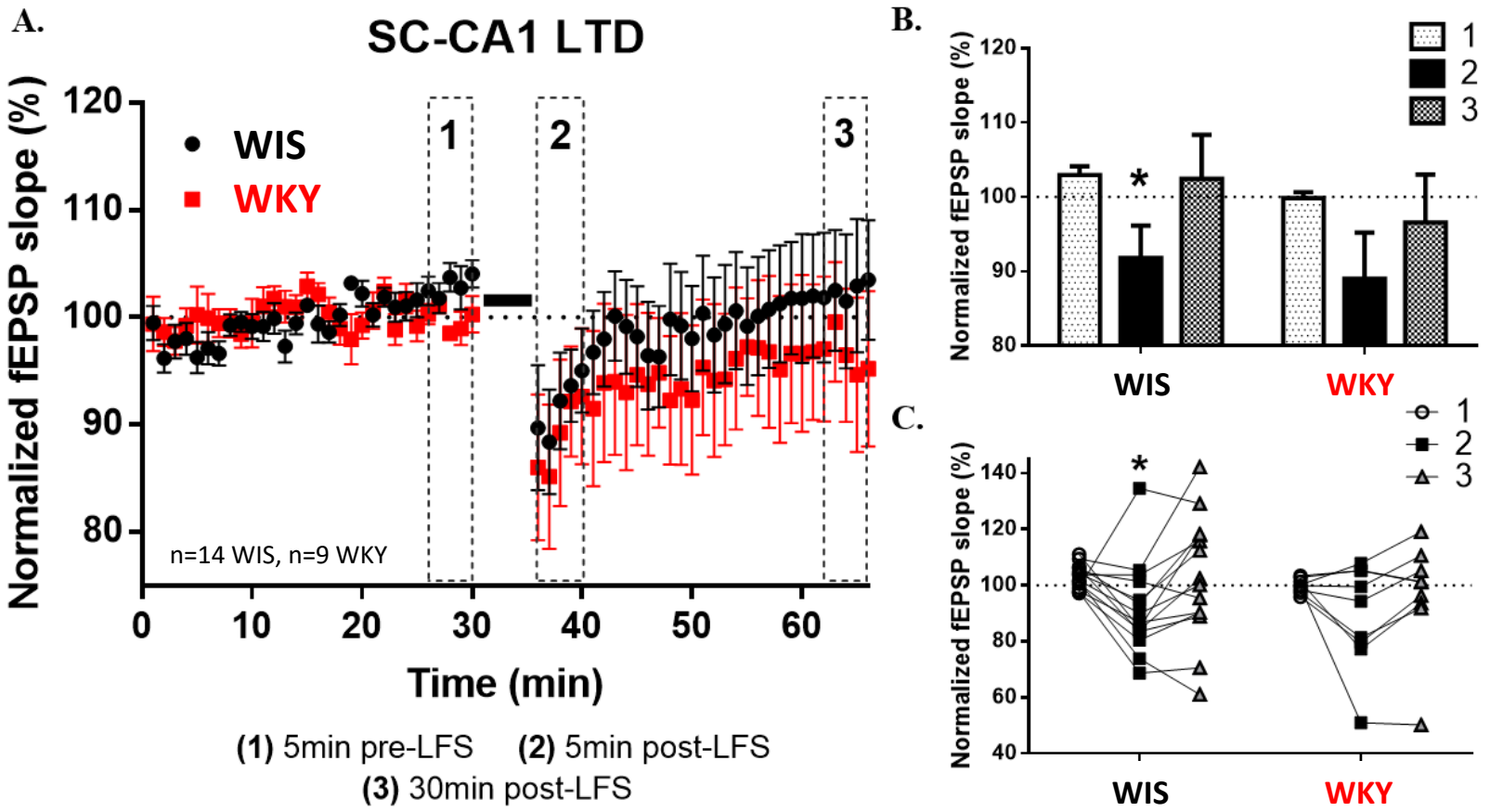

Figure 8. No facilitation of SC-CA1 long-term depression (LTD) in WKY rats following LFS (3Hz, 5min).

Average normalized fEPSP slope (%) A. by strain (WIS: n=14, WKY n=9) for the duration of the recording (30min post-LFS; ▬ = LFS protocol) B. by time bin (5min pre-LFS, 5min post-LFS, 30min-LFS). C. by time bin for each individual animal. ANOVA indicated a significant main effect of time (p=0.0076). Although average fEPSP slope was transiently reduced 5min post-LFS compared to baseline in both strains (Tukey's *p=0.041 and p=0.14, n.s., for WIS and WKY rats; WIS vs. WKY, Sidak's p= 0.97, n.s.), average fEPSP slope at 30min post-LFS was not statistically different from pre-LFS baseline or between the two strains (vs. pre-LFS Tukey's p>0.83, n.s. for WKY and WIS rats; WIS vs. WKY, Sidak's p=0.77, n.s.). A total of 2/14 (14%) of control WIS rats and 1/9 (11%) of WKY rats still expressed robust LTD at 30min post-LFS, indicating no facilitation of LTD in WKY rats at this synapse.

after the LTD protocol in both strains (5min post-LFS, WKY: 89.34 ±6.18%, n=9, pre-LFS Tukey's p=0.14, n.s.; WIS: 91.78 ±4.35%, n=14, vs. pre-LFS Tukey's p=0.041; 5min post-LFS WIS vs. WKY, Sidak's p= 0.97, n.s., Figure 8A,B). However, responses fully recovered within 7min in WIS rats (30min post-LFS: 102.45 ±5.89%, vs. pre-LFS Tukey's p=0.99, n.s.), and although fEPSP slope in WKYs remained slightly below baseline levels, near full recovery of responses was observed within the 30min of the recording (30min post-LFS: 96.57 ±6.46%, vs. pre-LFS Tukey's p=0.83, n.s.). Accordingly, average fEPSP slope at 30min post-LFS was not statistically different between the two strains (Sidak's p=0.77, n.s., Figure 8A,B). Consistent with this, at the end of the recording, a total of 2/14 (14%) of control WIS rats and 1/9 (11%) of WKY rats still expressed robust LTD (defined as 20% or more reduction in fEPSP slope at 30min post-LFS) (Figure 8C). Therefore, we found no evidence of significant facilitation of LTD in the stress-prone WKY strain compared to normal WIS controls.

4.3 SC-CA1 Early Long-Term Potentiation (E-LTP)

Importantly, in contrast to findings on LTD, both early and late LTP (induced by either 1x or 4x trains of HFS: 100Hz, 1s, 5min inter-train interval) at the SC-CA1 synapse were significantly impaired in stress-prone WKYs compared to control WIS rats (E-LTP: Figures 9 and 10). In the case of E-LTP (Figure 9A-C), a 2-way RM-ANOVA of average fEPSP slope (with strain as the between subject factor and time point (pre-HFS, 5min post-HFS and 30min post-HFS) as the within subject factor) revealed a significant main effects of time and strain ($F_{2,90}$=39.76, p<0.0001 and $F_{1,45}$=12.36, p=0.001), as well as a significant strain x time interaction effect ($F_{2,90}$=6.58, p=0.0022). As expected, average fEPSP slope was significantly increased immediately after the E-LTP protocol in both strains (5min post-HFS, WKY: 115.68

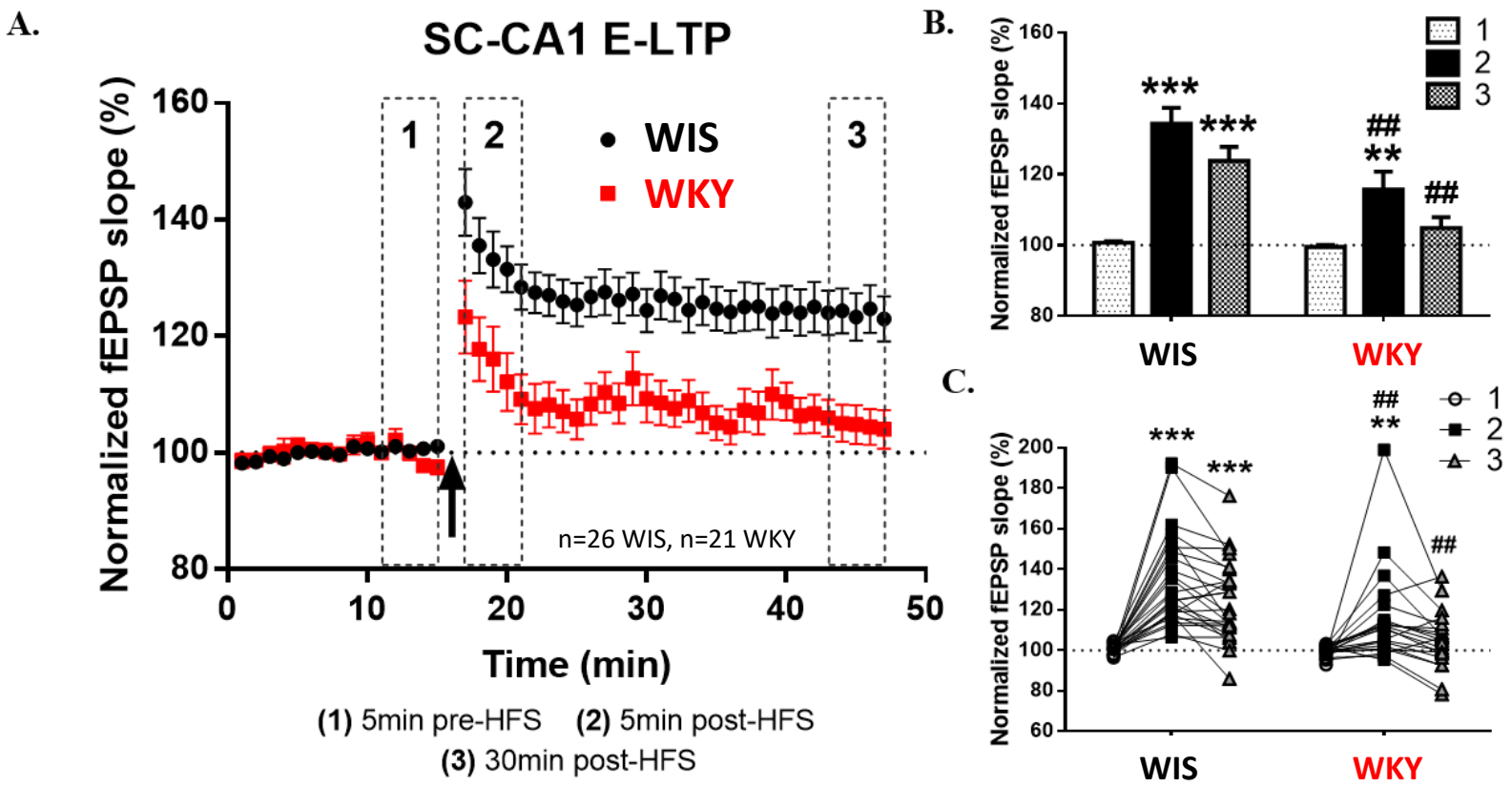

Figure 9. Significant impairment of SC-CA1 early long-term potentiation (E-LTP) in WKY rats following HFS (100Hz, 1s).

Average normalized fEPSP slope (%) A. by strain (WIS: n=26, WKY n=21) for the duration of the recording (30min post-HFS, ↑ = HFS protocol). B. by time bin (5min pre-HFS, 5min post-HFS, 30min-HFS). C. by time bin for each individual animal. ANOVA revealed significant main effects of time and strain (p<0.0001 and p=0.001) and a significant strain x time interaction (p=0.0022). While fEPSP slope increased immediately following HFS in both strains (5min post-HFS vs. pre-HFS, Tukey's WIS: ***p<0.0001 and WKY: **p=0.0006), E-LTP induction was significantly compromised in WKY compared to WIS rats (5min post-HFS Sidak's ##p=0.0007). Significant LTP was still observed 30min later in control WIS but not WKY rats (30min post-HFS vs. pre-HFS Tukey's ***p<0.0001 and p=0.41, n.s.; WIS vs. WKY Sidak's ###p=0.0005). At the end of the recording, 13/26 (50%) of WIS and 2/21 (9.5%) of WKY rats still expressed robust LTP, indicating significant deficits in E-LTP induction and/or maintenance in WKY rats at this synapse.

±5.06% of baseline, n=22 and WIS: 134.33 ±4.50% of baseline, n=26, compared to pre-HFS, Tukey's $p<0.0001$ and $p=0.0006$ for WIS and WKY rats, Figure 9A,B). However, subsequent analyses revealed that this increase was significantly less pronounced in WKY compared to WIS rats (5min post-HFS Sidak's $p=0.0007$, Figure 9A,B), indicating a deficit in E-LTP induction. In addition, despite some decay, significant LTP was still observed at 30min after induction in the control WIS group, whereas post-HFS responses in the WKY strain almost fully recovered within 10min (30min post-HFS WIS: 123.87 ±3.88%, Tukey's $p<0.0001$; WKY: 104.86 ±3.03%, Tukey's $p=0.41$, n.s. compared to pre-HFS baseline; 30min post-HFS WIS vs. WKY, Sidak's $p=0.0005$, Figure 9A,B). Consistent with this, at the end of the recording, responses from 13/26 (50%) control WIS rats still expressed robust LTP (defined as 20% or more increase in fEPSP slope at 30min post-HFS), with only 2/21 (9.5%) corresponding WKY rats (Figure 9C). Therefore, we found strong evidence of deficits of E-LTP in induction and/or maintenance at the SC-CA1 synapse of stress-prone WKY rats compared to normal WIS controls.

4.4 SC-CA1 Late Long-Term Potentiation (L-LTP)

It is possible that the threshold for LTP induction may be shifted in WKY rats, so that while a weak protocol (E-LTP, 1 train of HFS) may not be sufficient, a stronger protocol (L-LTP, 4 trains of HFS) could push synapses to express comparable levels of LTP to those in control rats. In the case of L-LTP (Figure 10A-C), a 2-way RM-ANOVA of average fEPSP slope (with strain as the between subject factor and time point (pre-HFS, 5min post-HFS and 90min post-HFS) as the within subject factor) revealed a significant main effects of time and strain ($F_{2,48}=32.36$, $p<0.0001$ and $F_{1,24}=7.27$, $p=0.01$), as well as a significant strain x time interaction effect ($F_{2,48}=4.00$, $p=0.025$). Subsequent analyses revealed that, as expected, average fEPSP

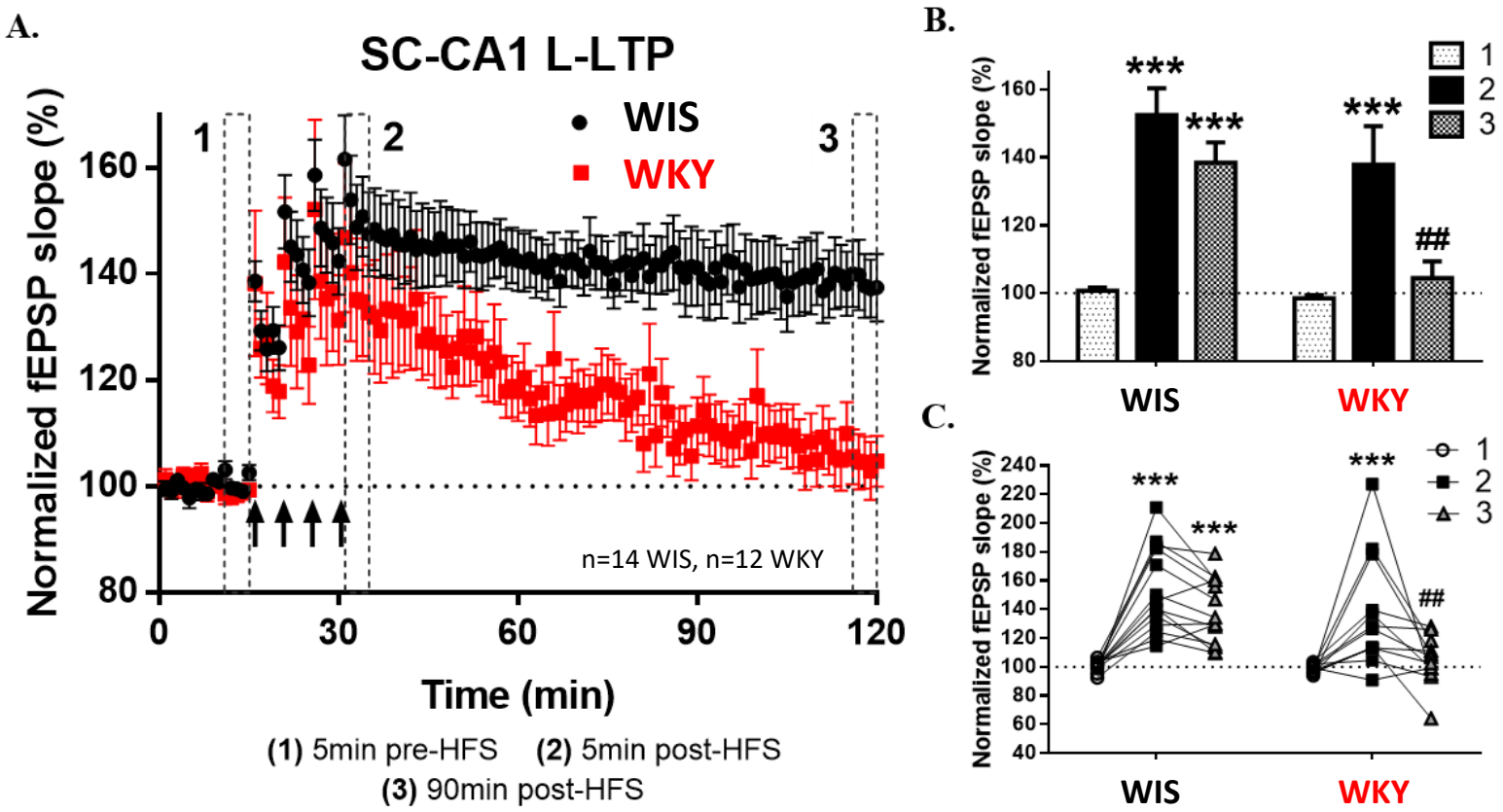

Figure 10. Significant impairment of SC-CA1 late long-term potentiation (L-LTP) in WKY rats following HFS (4 x 100Hz).

Average normalized fEPSP slope (%) A. by strain (WIS: n=14, WKY n=12) for the duration of the recording (90min post-HFS, ↑↑↑↑ = HFS protocol). B. by time bin (5min pre-HFS, 5min post-HFS, 90min-HFS). C. by time bin for each individual animal. ANOVA revealed significant main effects of time and strain (p<0.001 and p=0.01) and a significant strain x time interaction (p=0.025). fEPSP slope increased immediately following HFS in both strains (5min post-HFS vs. pre-HFS, Tukey's ***p<0.0001) and L-LTP induction was comparable between the two strains (although slightly lower in WKY rats, 5min post-HFS, Sidak's p=0.30, n.s.). Importantly, significant LTP was still observed 90min later in control WIS but not WKY rats (90min post-HFS vs. pre-HFS Tukey's WIS: ***p<0.0001, WKY: p=0.76, n.s., WIS vs. WKY, Sidak's ##p=0.0011). At the end of the recording, 10/14 (71%) of WIS and 2/12 (17%) of WKY rats still expressed robust LTP, indicating a pronounced deficit in L-LTP maintenance in WKY rats at this synapse.

slope was significantly increased immediately after the L-LTP protocol in both strains (5min post-HFS, WKY: 137.90 ±11.32% of baseline, n=12 and WIS: 152.49 ±7.88% of baseline, n=14; vs. pre-HFS, Tukey's $p<0.0001$ for WIS and WKY rats, Figure 10A,B). Although this increase tended to be less pronounced in WKY compared to WIS rats, differences in L-LTP induction between the two strains did not reach statistical significance (5min post-HFS, Sidak's $p=0.30$, n.s., Figure 10A,B). Therefore, the ability to induce SC-CA1 LTP was mostly rescued in WKY rats by utilizing a stronger LTP protocol. Importantly, however, despite some decay, significant LTP was still observed at 30min after induction in control WIS rats (90min post-HFS: 138.40 ±6.00%, vs. pre-HFS Tukey's $p<0.0001$), whereas post-HFS responses in the WKY group almost fully recovered within 60-90min (90min post-HFS: 104.44 ±4.91%, vs. pre-HFS Tukey's $p=0.76$, n.s.; 90min post-HFS WIS vs. WKY, Sidak's $p=0.0011$, Figure 10A,B). Consistent with this, at the end of the recording, responses from 10/14 (71%) control WIS rats still expressed robust LTP (defined as 20% or more increase in fEPSP slope at 90min post-HFS), with only 2/12 (17%) corresponding WKY rats (Figure 10C). Therefore, while the magnitude and duration of LTP were enhanced by using a stronger induction protocol, we found strong evidence of a significant deficit in the maintenance of L-LTP at the SC-CA1 synapse of stress-prone WKY rats compared to normal WIS controls.

4.5 Summary and Discussion

Accumulating evidence from both animal and human studies supports an emerging theory of the etiology of depression that implicates dysfunction within the glutamatergic system and dysregulation of synaptic plasticity (Aleksandrova et al., 2019). As discussed in section 1.2, preclinical studies mostly performed in hippocampal slice preparations indicate that both acute

and chronic stress, as well as exogenous glucocorticoids, perturb the normal balance between LTP and LTD, inhibiting LTP and/or facilitating LTD, particularly in the rodent HPC (Aleksandrova et al., 2019). Despite this, a comprehensive understanding of changes in synaptic plasticity in animal models of depression or in mediating antidepressant response is lacking, and the number of peer-reviewed studies that include direct assessments of LTP and LTD in brain areas implicated in MDD in the context of animal models of depression, at baseline and following antidepressant treatment (e.g. ketamine) is limited. At the beginning of the current study, no data were available characterizing synaptic plasticity processes in the WKY model, so we elected to measure and compare SC-CA1 LTP and LTD in WIS and WKY rats by performing *in vivo* single-unit extracellular recordings in anesthetized animals.

In this study, we found that basal evoked fEPSP signals at this synapse were comparable between the two strains, with similar input-output curves observed, indicating no significant differences in basal synaptic transmission at this synapse. Next, we utilized a LFS protocol (3Hz, 900 pulses, 5min), which transiently reduced fEPSP responses in both strains; however we failed to detect any robust LTD in either WKY or WIS rats. Therefore, we found no evidence of significant facilitation of LTD in the stress-prone WKY strain compared to normal WIS controls. On the other hand, LTP can be temporally and mechanistically divided into two phases, early LTP (E-LTP) and late LTP (L-LTP) (Hardt et al., 2013). E-LTP is often induced with a weaker induction protocol (e.g. 1x 100Hz), is short-lived (one to a few hours), and does not involve new protein synthesis. Conversely, L-LTP can be induced with stronger stimulation protocols (e.g. 4x 100Hz), lasts at least several hours and requires new protein synthesis (Hardt et al., 2013). Importantly, in contrast to our findings on LTD, both early and late LTP (induced by either 1x or 4x trains of HFS, 100Hz, 1s, 5min ITI) at the SC-CA1 synapse were significantly impaired in

WKY compared to WIS rats. Importantly, while the magnitude and duration of LTP in WKY rats were facilitated by using a stronger induction protocol, we found strong evidence of a pronounced deficit in the maintenance of SC-CA1 L-LTP of stress-prone WKYs compared to control WIS rats, which is not simply due to a shift in the threshold for LTP induction.

Several important studies have been published recently characterizing synaptic plasticity processes in various neural circuits relevant to depression in the WKY model (summarized in Table 4, pg. 43), which not only provide support for the hippocampal LTP deficit we observed here, but also further highlight the utility of this strain for the study of synaptic plasticity changes and how they may contribute to key depressive-like phenotypes and antidepressant responses (Belujon and Grace, 2014; Cominski et al., 2014; Fortress et al., 2018; Fragale et al., 2016; Han et al., 2018; Kanzari et al., 2018; She et al., 2015). Work by Pang and colleagues first described abnormalities in hippocampal synaptic plasticity in the WKY strain using field recordings in anesthetized rats (Cominski et al., 2014; Fortress et al., 2018). Importantly, Cominski et al. (2014) reported that in addition to the behavioural deficits and significant reductions in hippocampal and cortical volume in WKY rats, this strain is also characterized by impaired hippocampal LTP *in vivo* compared to SD rats (Cominski et al., 2014; Fortress et al., 2018). First, and consistent with our findings, basal synaptic transmission in this study was comparable between the two strains, as indicated by similar input-output curves at the medial perforant pathway (mPP) to dentate gyrus (DG) synapse (Cominski et al., 2014; Fortress et al., 2018). Importantly, a HFS protocol (3 sets of 4 trains given 5min apart, each train consisting of 8 pulses at 400Hz, ITI 10s) failed to induce any mPP-DG LTP (beyond a small transient fEPSP increase at 15min post-HFS) in WKY rats (Cominski et al., 2014; Fortress et al., 2018). In this study, WKYs also had pronounced resistance to extinction of avoidance in an active lever-press

avoidance paradigm (Cominski et al., 2014; Jiao et al., 2011b; Servatius et al., 2008). Since the mPP-DG pathway represents a major cortical input to the HPC implicated in learning the significance of conditioned stimuli signaling including safety, the authors proposed that the LTP deficit observed in WKY rats at this synapse may contribute to this persistent avoidance phenotype (Cominski et al., 2014). In direct support of the link between hippocampal integrity and the pronounced avoidance behaviour, hippocampal lesions in control SD rats mimicked the impaired extinction of avoidance responding phenotype of WKYs (Cominski et al., 2014). This study focused on the abnormally persistent avoidance responding in the WKY strain as the behavioural phenotype of interest, and since excessive avoidance is a core feature of anxiety disorders and post-traumatic stress disorder (PTSD), these authors emphasize the role of hippocampal dysfunction in anxiety vulnerability (Cominski et al., 2014; Fortress et al., 2018). However, these findings are also clearly relevant in the context of depression, since mood and anxiety disorders are highly comorbid and hippocampal dysfunction is implicated in both (Calcagno et al., 2016; Leuner and Shors, 2013; Nestler et al., 2002). The role of HPC LTP in the context of the depressive-like phenotype of WKY rats was not addressed in this study.

In addition to findings of impaired mPP-DG LTP, and directly confirming what we observed here, a similar LTP deficit was reported a year later at the canonical SC-CA1 synapse in hippocampal slices obtained from WKY compared to WIS rats (She et al., 2015). First, no differences in basal synaptic transmission were mentioned between the two strains (She et al., 2015). Importantly, however, while HFS (100Hz, 1s) induced a transient increase in fEPSP responses in both strains (although to a lesser degree in WKYs), this synaptic potentiation decayed completely within ~45min in WKY rats (She et al., 2015), closely paralleling our own findings with E-LTP. As WKY rats were compared to Wistar rats in our and this latter study, as

opposed to SD rats used in the studies by Pang and colleagues above, it appears that the hippocampal insufficiency can be detected in WKY rats regardless of the control strain (Cominski et al., 2014; Fortress et al., 2018; She et al., 2015). In addition, the reductions in total hippocampal volume and the failure to obtain LTP at the mPP-DG and SC-CA1 synapses in WKY rats observed by us and others, appear to reflect a relatively global impairment of hippocampal synaptic plasticity and function in this model.

Overall, accumulating evidence supports the pronounced deficit in SC-CA1 LTP we observed in the WKY rat; however, to our knowledge, we are the first to study hippocampal synaptic plasticity processes (LTD, E-LTP and L-LTP) more systematically in this model of depression. Namely, while enhancement of LTD was not detected at this synapse *in vivo*, LTP decay was significantly facilitated in stress-prone WKY rats compared to WIS controls, which is not just due to a shift in the threshold for LTP induction and cannot be overcome by simply using a stronger HFS protocol. Therefore, consistent with previous findings with hippocampal slices obtained from stressed normal rats, as well as these recent findings with the WKY rat, the balance between LTP and LTD in the HPC is innately perturbed in the WKY model of depression. Moreover, this lack of activity-dependent potentiation in the WKY HPC may lead to a propensity toward synaptic destabilization, loss of connectivity and eventually, neuronal atrophy in this key neuronal circuit implicated in MDD, possibly mediating or at least contributing to the structural and functional findings in the WKY strain (Arnsten, 2015; Campbell and MacQueen, 2003; Duman, 2014b; Marsden, 2013; Roiser and Sahakian, 2013). Next, we were interested in testing whether this altered hippocampal synaptic plasticity is modulated by ketamine, as well as whether such changes may contribute to the antidepressant effects of ketamine in the WKY model.

Chapter 5: Effects of Ketamine and (2R,6R)-HNK on Synaptic Plasticity

5.1 Effects of Ketamine on WKY SC-CA1 Basal Synaptic Transmission (acute)

Given the pronounced hippocampal LTP deficit we observed in the WKY model, we aimed to test the effects of ketamine on the impaired L-LTP in this strain. First, we evaluated ketamine's effects on basal synaptic transmission at the SC-CA1 synapse (Figure 11). Once a stable fEPSP baseline was obtained, saline (1ml/kg) or ketamine (5mg/kg/ml) were administered systemically (ip) to WKY rats (SAL: n=19 and KET: n=18) and the recording was continued for 3.5h thereafter. Although ketamine appeared to cause a small run-up (~10%) in the first 15min following injection, this increase was transient as fEPSP slope quickly returned to baseline (Figure 11) and was not always observed (Figure 12). Overall, ketamine administration did not have any major effects on SC-CA1 basal synaptic transmission at the 5mg/kg dose in WKY rats.

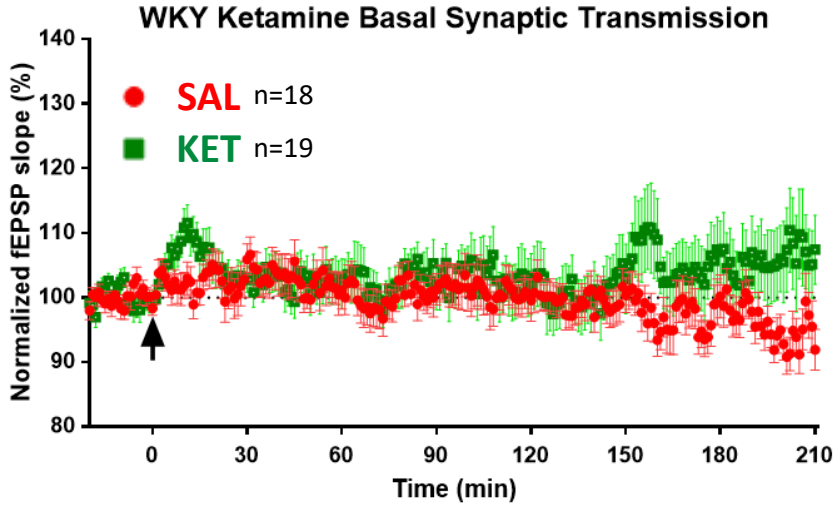

Figure 11. Ketamine has no acute effects on SC-CA1 basal synaptic transmission in WKY rats.
Once a stable fEPSP baseline (20min) was obtained, saline (1ml/kg) or ketamine (5mg/kg) were administered systemically at time point 0 (↑ = ip injection) to WKY rats (SAL: n=19, KET: n=18) and the recording was continued for 3.5h thereafter. Overall, ketamine administration did not have any major effects on SC-CA1 basal synaptic transmission at this dose in WKY rats.

5.2 Effects of Ketamine on WKY SC-CA1 L-LTP (30min)

Next, we tested the effects of ketamine (5mg/kg, ip) on the induction and maintenance of SC-CA1 L-LTP in the WKY rat, where the HFS protocol (4 trains of 100Hz, 5min ITI) was delivered at three different time points following ketamine (or saline) administration (30min, 3.5h and 24h). In the case of the early time point (30min post-ketamine, Figure 12A,B), a 2-way RM-ANOVA of average fEPSP slope (with drug as the between subject factor and time point (pre-HFS, 5min post-HFS and 90min post-HFS) as the within subject factor) only revealed a significant main effect of time ($F_{3,45}=8.21$, $p=0.0002$), but not of drug treatment or the drug x time interaction ($F_{1,15}=0.45$, $p=0.51$, n.s. and $F_{3,45}=0.58$, $p=0.63$, n.s.). Subsequent post-hoc analyses indicated there were no significant differences in fEPSP slope between saline (n=12) and ketamine (n=5) treated rats at any time point (Sidak's $p>0.51$, n.s.). Although fEPSP responses in both groups were potentiated immediately post-HFS (SAL: 137.90 ±11.32% and KET: 122.83 ±11.34% of baseline), this effect was more pronounced, only reaching statistical significance, in the saline group while only a trend was observed in ketamine treated rats, likely due to the lower n (pre- vs. post-HFS, SAL: Tukey's $p<0.0001$, KET: Tukey's $p=0.14$, n.s., Figure 12A,B). In both groups, however, the observed L-LTP undergoes nearly complete decay by the end of the recording (90min post-HFS SAL:104.44 ±4.91% and KET:107.80 ±6.95%, vs. pre-HFS Tukey's $p>0.76$, n.s.), as in drug-naïve WKY rats. Therefore, overall, when the HFS protocol was given 30min after injection, there were no significant effects of ketamine treatment on the SC-CA1 L-LTP observed in WKY rats.

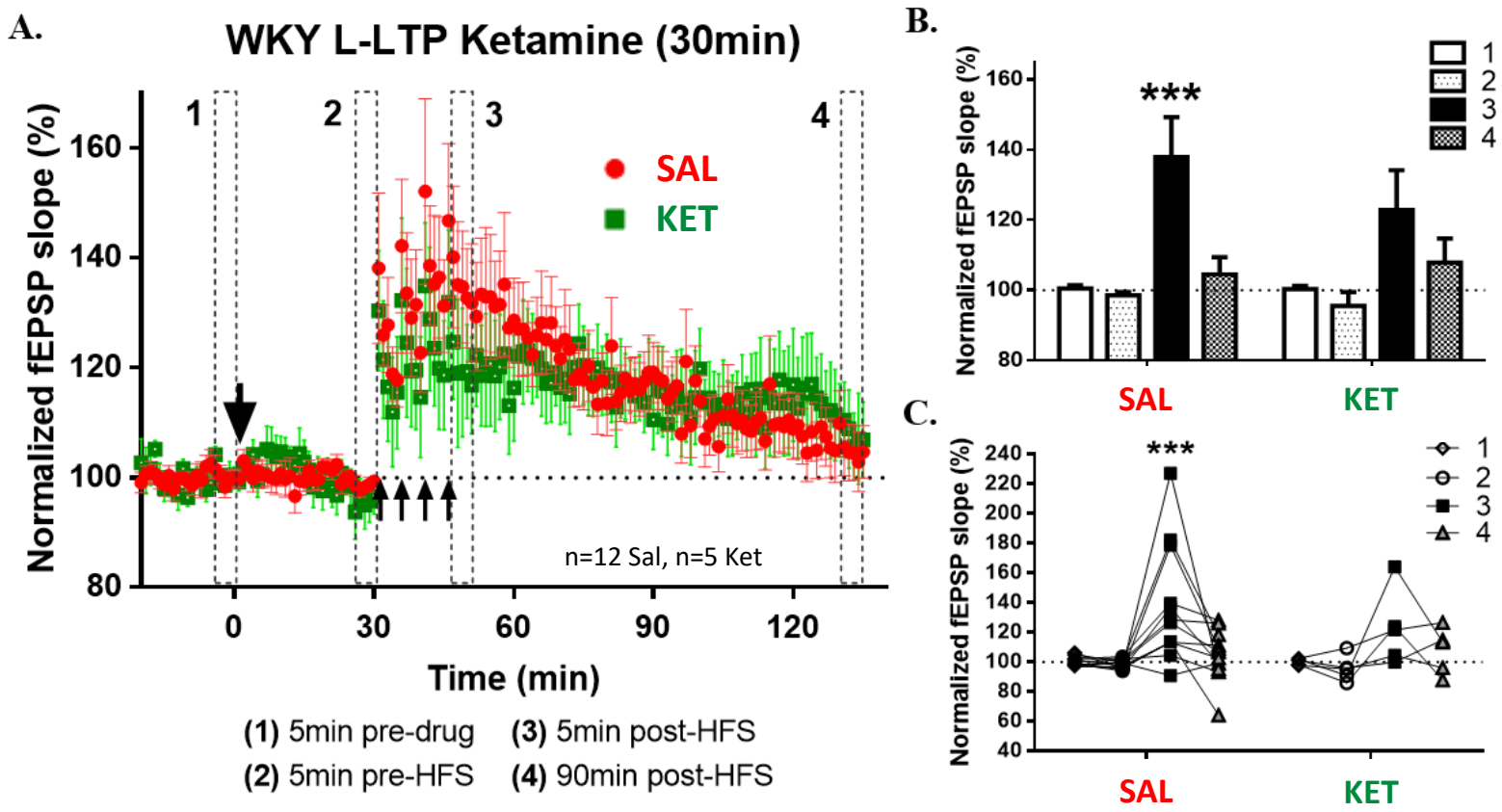

Figure 12. Ketamine has no significant effects on the impaired SC-CA1 L-LTP in WKY rats at 30min.

Once a stable fEPSP baseline (20min) was obtained, saline or ketamine (5mg/kg) were administered systemically (ip) at time point 0 to WKY rats (SAL: n=12, KET: n=5), followed by the HFS protocol 30min later. Average normalized fEPSP slope (%) A. by strain for the duration of the recording (90min-HFS). B. by time bin (5min pre-drug, 5min pre-HFS, 5min post-HFS, 90min-HFS). C. by time bin for each individual animal. ANOVA only revealed a significant main effect of time (p=0.0002). Although L-LTP induction was more pronounced in saline compared to ketamine treated rats (pre- vs. 5min post-HFS, Tukey's SAL: ***p<0.0001, KET: p=0.14, n.s.), there were no significant differences in fEPSP slope between saline and ketamine at any time point (Sidak's p>0.51, n.s.). In both groups, L-LTP undergoes nearly complete decay by the end of the recording (90min post-HFS vs. pre-HFS Tukey's p>0.76, n.s.), indicating no significant effects of ketamine on the WKY L-LTP deficit at this dose when the HFS protocol was given 30min after injection.

5.3 Effects of Ketamine on WKY SC-CA1 L-LTP (3.5h)

Next, we tested the effects of ketamine on SC-CA1 L-LTP at 3.5h after injection. Saline and ketamine (3.5h) data from this experiment, as well as saline and (2R,6R)-HNK (3.5h) data from a later experiment (section 5.6) were analyzed together in a RM-ANOVA with drug (saline, ketamine or HNK) as the between subject factor and time point as the within subject factor. These data were combined since recordings from saline-treated groups in the two cohorts were not statistically different ($F_{3,126}$= 0.67, p=0.53, n.s.). In the case of the intermediate time point (3.5h post-ketamine, Figure 13A,B), a 2-way RM-ANOVA (drug by time point) of average fEPSP slope revealed significant main effects of time and drug treatment ($F_{3,126}$=110.2, p<0.0001 and $F_{2,42}$=5.31, p=0.0088), as well as a significant drug x time interaction ($F_{6,126}$=6.53, p<0.0001). Subsequent analyses indicated that, as expected, fEPSP responses in both groups were potentiated immediately post-HFS (5min post-HFS, SAL: 159.52 ±9.47% of baseline, n=19; KET: 167.20 ±10.29% of baseline, n=18, vs. pre-HFS Tukey's p<0.0001 for SAL and KET, Figure 13A,B). Interestingly, regardless of the treatment group, the L-LTP observed in WKY rats was more robust when the HFS protocol was given 3.5h compared to 30min after injection (e.g. SAL fEPSP slope at post-HFS: 159.52% vs. 137.90%, respectively; Figures 10B and 11B), indicating that simply allowing the synapses to recover over a few hours facilitated the induction of LTP in this strain. However, while L-LTP in saline-treated WKY rats again completely decayed within 90min of its induction as previously observed (90min post-HFS SAL: 102.69 ±4.37% vs. pre-HFS Tukey's p=0.75, n.s.), robust L- LTP was still present at 90min in the ketamine treated group (90min post-HFS, KET: 141.90 ±7.34%, vs. pre-HFS Tukey's p<0.0001, Figure 13A,B). Thus, importantly, while L-LTP induction was not affected (5min post-HFS Sal vs. KET, Sidak's p=0.99, n.s.), ketamine effectively restored normal SC-CA1 L-

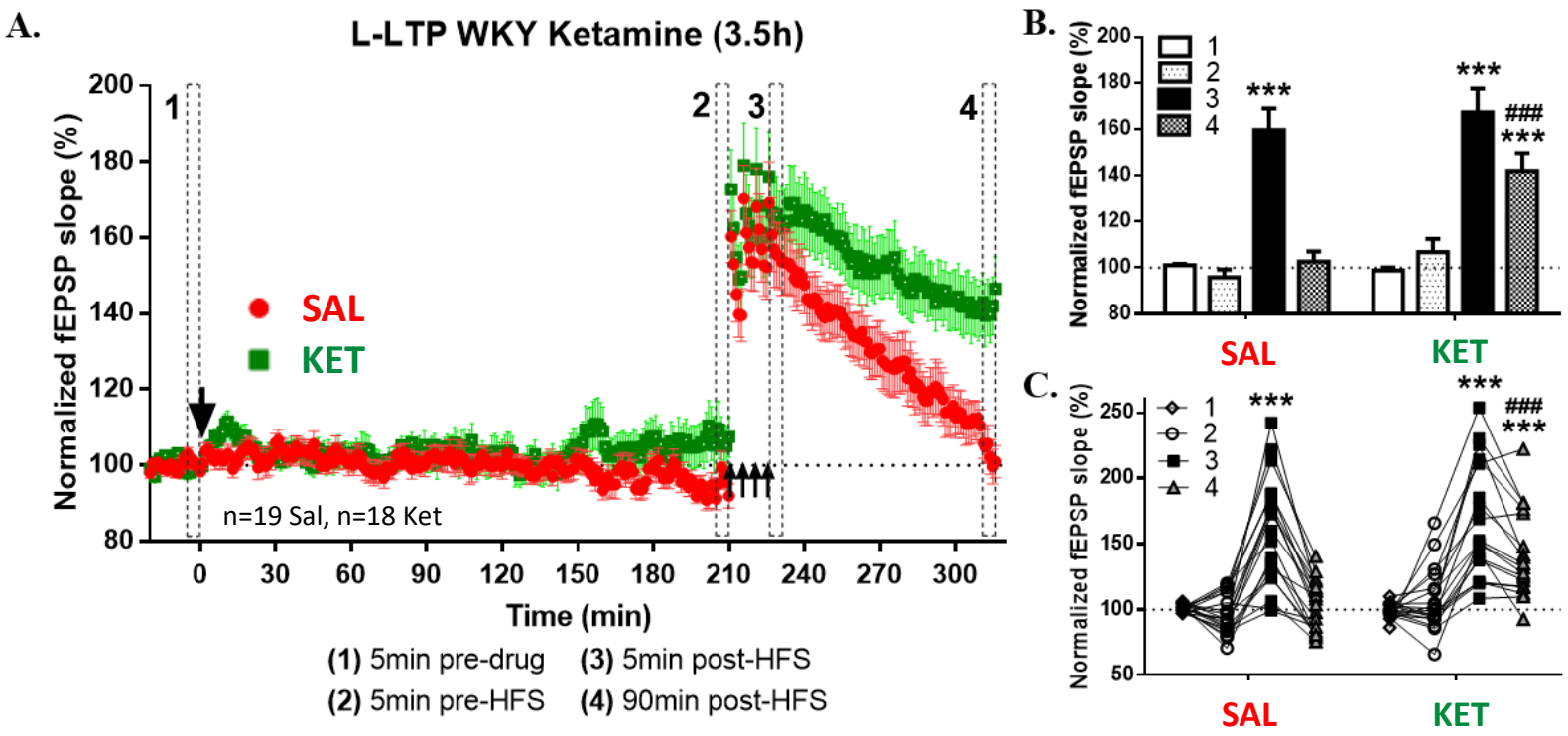

Figure 13. Ketamine significantly rescues the impaired SC-CA1 L-LTP in WKY rats at 3.5h.

Once a stable fEPSP baseline was obtained, saline or ketamine (5mg/kg, ip) were administered to WKY rats (SAL: n=19, KET: n=18), followed by the HFS protocol 3.5h later. Average normalized fEPSP slope (%) A. by drug treatment for the duration of the recording (90min-HFS). B. by time bin (5min pre-drug, 5min pre-HFS, 5min post-HFS, 90min-HFS). C. by time bin for each individual animal. ANOVA revealed significant main effects of time and drug treatment, and a significant interaction ($p<0.0001$, $p=0.0088$ and $p<0.0001$). Significant LTP was induced in both strains (5min post-HFS vs. pre-HFS Tukey's ***$p<0.0001$); however, while it completely decayed within 90min in saline-treated WKY rats, robust L-LTP was still present in the ketamine group (90min post-HFS vs. pre-HFS Tukey's $p=0.75$, n.s. and ***$p<0.0001$, respectively). Thus, while L-LTP induction was not affected (5min post-HFS SAL vs. KET, Sidak's $p=0.99$, n.s.), ketamine significantly facilitated L-LTP maintenance (90min post-HFS Sal vs. KET, Sidak's ###$p=0.0003$), with potentiation at 90min being comparable to that in normal WIS rats (~140%). At the end of the recording, 13/18 (72%) of ketamine-treated WKY rats (vs. 71% of control WIS rats) and 4/19 (21%) of saline treated WKY rats still expressed robust LTP at 90min post-HFS. Therefore, ketamine (5mg/kg) completely eliminated the SC-CA1 L-LTP deficit in WKY rats when the HFS protocol was given 3.5h after injection, indicating a pronounced facilitatory effect on L-LTP maintenance.

LTP in WKY rats by significantly facilitating its maintenance (90min post-HFS Sal vs. KET, Sidak's p=0.0003, Figure 13A,B), with the magnitude of synaptic potentiation at 90min in ketamine-treated WKY rats being comparable to that observed in control WIS rats (141.90% and 138.40%, respectively; Figures 8B and 11B). Consistent with this, at the end of the recording, responses from 13/18 (72%) ketamine-treated WKY rats (vs. 71% of control WIS rats) still expressed robust LTP (defined as 20% or more increase in fEPSP slope at 90min post-HFS), with only 4/19 (21%) corresponding saline treated WKY rats (Figure 13C). Therefore, when the HFS protocol was given 3.5h after injection, ketamine (5mg/kg, ip) completely eliminated the SC-CA1 L-LTP deficit in WKY rats, reflecting a robust positive effect on the maintenance of LTP in this strain.

5.4 Effects of Ketamine on WKY SC-CA1 L-LTP (24h)

In the case of the late time point (24h post-ketamine, Figure 14A,B), a 2-way RM-ANOVA (drug by time point) of average fEPSP slope only revealed a significant main effect of time ($F_{2,40}$=17.22, p<0.0001), but not of drug treatment or the drug x time interaction ($F_{1,20}$=0.51, p=0.23, n.s. and $F_{2,40}$=0. 82, p=0.45, n.s.). Subsequent post-hoc analyses indicated that there were no significant differences in fEPSP slope between saline (n=12) and ketamine (n=10) treated rats at any time point (Sidak's p>0.22, n.s.). As expected, fEPSP responses in both groups were potentiated immediately post-HFS (5min post-HFS, SAL: 137.90 ±11.32% vs. pre-HFS Tukey's p=0.0004, KET: 143.20 ±12.28% vs. pre-HFS Tukey's p=0.0009, Figure 14A,B). However, while L-LTP in saline-treated WKY rats again completely decayed within 90min of its induction (90min post-HFS SAL:104.44 ±4.91% vs. pre-HFS Tukey's p=0.80, n.s., vs. 5min post-HFS Tukey's p=0.0024), only partial decay was observed in the ketamine treated group, so

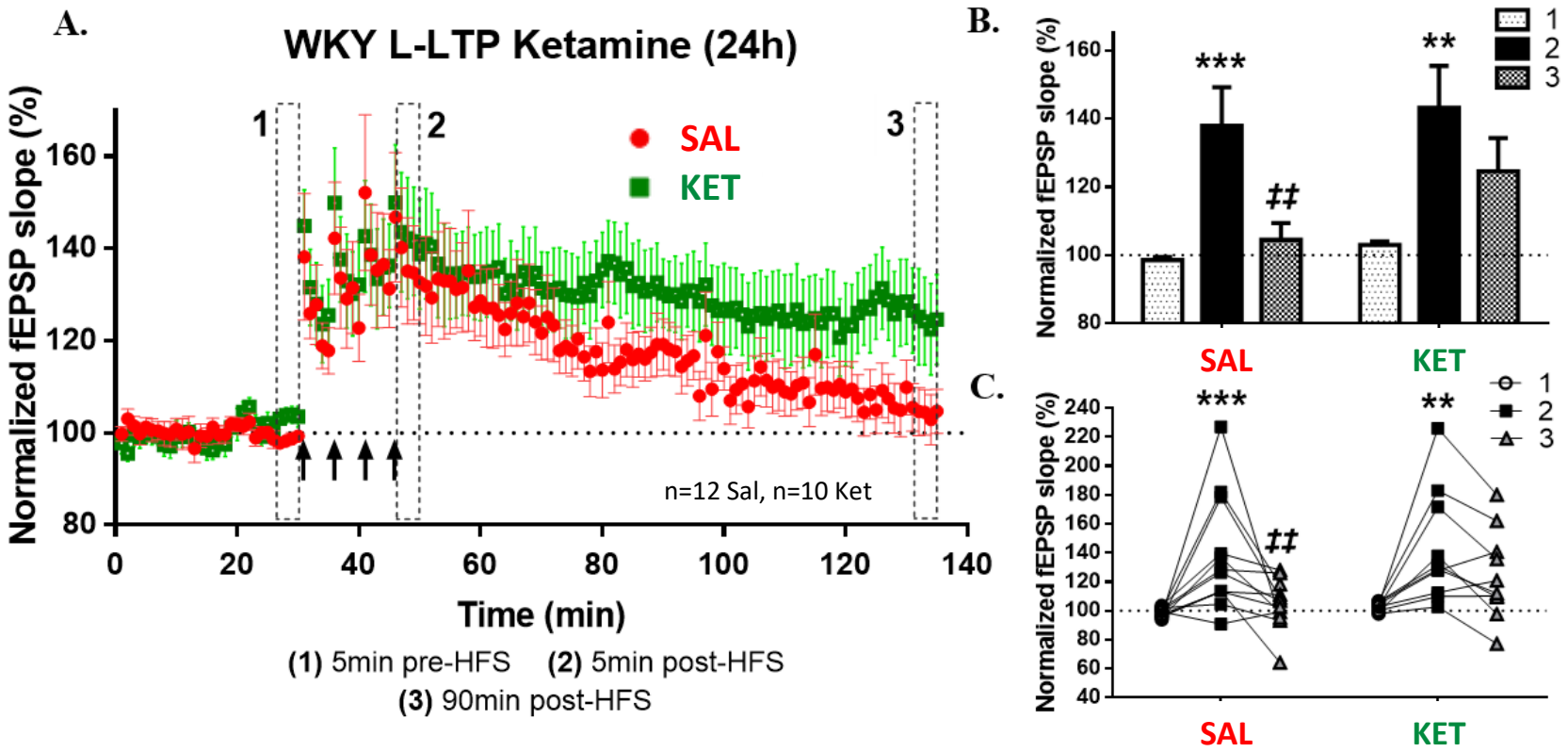

Figure 14. Ketamine partially rescues the impaired SC-CA1 L-LTP in WKY rats at 24h.

WKY rats (SAL: n=12, KET: n=10) were pre-treated with saline or ketamine (5mg/kg, ip) and anesthetized 24h later when a stable fEPSP baseline was obtained, followed by the HFS protocol 30min later. Average normalized fEPSP slope (%) A. by drug treatment for the duration of the recording (90min-HFS). B. by time bin (5min pre-drug, 5min pre-HFS, 5min post-HFS, 90min-HFS). C. by time bin for each individual animal. ANOVA only revealed a significant main effect of time ($p<0.0001$), with no significant differences in fEPSP slope between saline and ketamine treated rats at any time point (Sidak's $p>0.22$, n.s.). Significant LTP was induced in both groups (5min post-HFS vs. pre-HFS Tukey's SAL: ***$p=0.0004$, KET: **$p=0.0009$); however, while L-LTP in saline-treated WKY rats completely decayed within 90min (90min post-HFS vs. pre-HFS Tukey's $p=0.80$, n.s., vs. 5min post-HFS Tukey's ‡‡p=0.0024), only partial decay was observed in the ketamine treated group, so that some L-LTP was still present 90min later (90min post-HFS vs. pre-HFS Tukey's $p=0.17$, n.s., 90min post-HFS SAL vs. KET: p=0.22, n.s.). At the end of the recording, 5/10 (50%) of ketamine treated and 2/12 (17%) of saline treated rats still expressed robust LTP, indicating that when the HFS protocol was given 24h after injection, there was some residual facilitatory effect of ketamine (5mg/kg, ip) on SC-CA1 L-LTP in WKY rats.

that some L-LTP was still present at 90min (90min post-HFS KET: 124.61 ±9.68%, vs. pre-HFS Tukey's p=0.097, n.s., vs. 5min post-HFS Tukey's p=0.17, n.s., 90min post-HFS SAL vs. KET: p=0.22, n.s., Figure 14A,B). Consistent with this, at the end of the recording, responses from 5/10 (50%) ketamine treated WKY rats still expressed robust LTP (fEPSP increase of 20% or more at 90min post-HFS), and only 2/12 (17%) corresponding saline-treated rats (Figure 14C). Therefore, when the HFS protocol was given 24h after injection, there was some residual facilitatory effect of ketamine (5mg/kg, ip) on SC-CA1 L-LTP in WKY rats.

5.5 Effects of Ketamine on WKY SC-CA1 Basal Synaptic Transmission (24h)

Unlike experiments where L-LTP was induced 30min or 3.5h after injection (i.e. ketamine and saline were administered to anesthetized rats after an input-output curve and a baseline recording were already obtained under drug-free conditions, followed by HFS 30min or 3.5h later); for the 24h time point, awake animals were pre-treated with drug 24h before conducting the electrophysiological recording the next day (since anesthetized rats cannot be kept alive for longer than 6-8h). This unique design of the 24h experiment allowed comparing the input-output curves for saline and ketamine treated WKY rats (SAL: n=12, KET: n=10) at 24h post-injection (Figure 15). A 2-way RM-ANOVA of average absolute fEPSP slope (with drug treatment as the between subject factor and current intensity (0-400μA) as the within subject factor) indicated a significant main effect of current intensity ($F_{25,1250}$=174.20, p<0.0001, as expected), as well as a significant drug x current intensity interaction ($F_{25,1250}$=1.92, p=0.004), and a trend towards a significant drug treatment effect ($F_{1,50}$=2.28, p=0.14, n.s.). Subsequent analyses revealed no significant differences in average fEPSP between saline and ketamine at any current intensity (Sidak's p>0.31, n.s., Figure 15). However, average fEPSP slope was

consistently higher in ketamine compared to saline treated WKY rats over a wide range of current intensities, where the stimulation magnitude evoking ~50% of the maximal response (i.e. slope of ~0.7) was effectively shifted leftward, from 100µA to 60µA in rats pre-treated with saline compared to ketamine, respectively (Figure 15). Thus, although its facilitatory effects on L-LTP maintenance at 24h were not as pronounced as at 3.5h, ketamine (5mg/kg, ip) additionally caused a dramatic leftward shift of the WKY SC-CA1 input-output curve 24h later, which likely reflects an enhancement of basal synaptic transmission as a result of sustained ketamine-induced potentiation of WKY SC-CA1 synapses at this later time point.

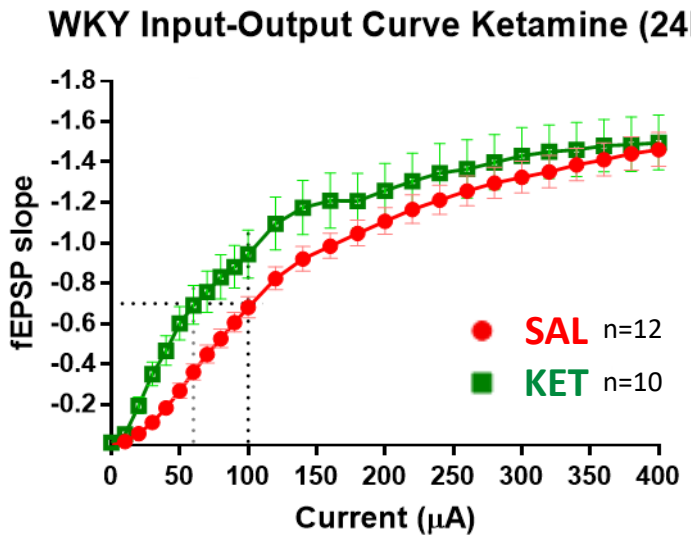

Figure 15. Ketamine enhances SC-CA1 basal synaptic transmission in WKY rats at 24h.

Average input-output curves for WKY rats (SAL: n=12, KET: n=10) pre-treated with saline or ketamine (5mg/kg, ip) 24h earlier. ANOVA revealed a significant main effect of current intensity ($p<0.0001$, as expected), a significant drug x current interaction ($p<0.004$), and a trend towards a significant drug treatment main effect ($p=0.14$, n.s.), with no post-hoc significance between saline and ketamine at any current intensity (Sidak's $p>0.31$, n.s.). However, average fEPSP slope was consistently higher in ketamine treated WKY rats over a wide range of current intensities, where the stimulation magnitude evoking ~50% of the maximal response (i.e. slope of ~0.7) was effectively shifted leftward (from 100µA to 60µA) in rats pre-treated with ketamine compared to saline. Thus, ketamine (5mg/kg, ip) caused a dramatic leftward shift of the WKY SC-CA1 input-output curve at 24h after injection, which likely reflects a potentiation of WKY synapses at this later time point.

5.6 Effects of (2R,6R)-HNK on WKY SC-CA1 Basal Synaptic Transmission (acute)

Given the recent discovery of an active ketamine metabolite and ketamine's striking effects on WKY SC-CA1 L-LTP observed here, we were interested in testing the effects of (2R,6R)-HNK in our model. First, we evaluated (2R,6R)-HNK's effects on basal synaptic transmission at the SC-CA1 synapse (Figure 16). Once a stable fEPSP baseline was obtained, saline or (2R,6R)-HNK (5mg/kg, ip) were administered to WKY rats (n=19 and n=8, respectively) and the recording was continued for 3.5h thereafter. Although (2R,6R)-HNK increased the variability in fEPSP slope, overall, the metabolite did not have any major effects on SC-CA1 basal synaptic transmission at the 5mg/kg dose in WKY rats (Figure 16).

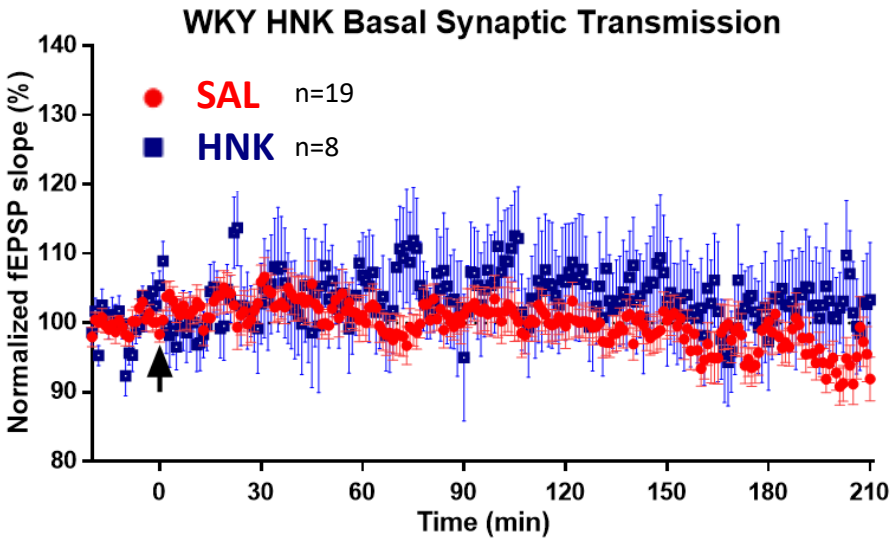

Figure 16. (2R,6R)-HNK has no acute effects on SC-CA1 basal synaptic transmission in WKY rats. Once a stable fEPSP baseline (20min) was obtained, saline (1ml/kg) or (2R,6R)-HNK (5mg/kg) were administered systemically (ip) at time point 0 to WKY rats (SAL: n=19, HNK: n=8) and the recording was continued for 3.5h thereafter. Although (2R,6R)-HNK increased the variability in fEPSP slope, overall, administration of the metabolite did not have any major effects on SC-CA1 basal synaptic transmission at this dose in WKY rats.

5.7 Effects of (2R,6R)-HNK on WKY SC-CA1 L-LTP (3.5h)

Next, we were interested in directly comparing ketamine and its metabolite in terms of their effects on the SC-CA1 L-LTP deficit observed in WKY rats, and found that similar to its parent drug, (2R,6R)-HNK had pronounced facilitatory effects at 3.5h post-injection (Figure 17A,B). A 2-way (drug (saline, ketamine or HNK) by time point) RM-ANOVA of average fEPSP slope revealed significant main effects of time and drug treatment ($F_{3,126}$=110.2, p<0.0001 and $F_{2,42}$=5.31, p=0.0088), as well as a significant drug x time interaction ($F_{6,126}$=6.53, p<0.0001, as reported previously in section 5.3). As expected, fEPSP responses in both groups were potentiated immediately post-HFS (5min post-HFS, SAL: 159.52 SAL:102.69% vs. pre-HFS Tukey's p=0.75, n.s.; 90min post-HFS SAL vs. HNK Sidak's 9.47%, n=19; HNK: 205.14±16.86%, n=8, vs. pre-HFS Tukey's p<0.0001 for SAL and HNK, Figure 16A,B). However, L-LTP induction was significantly facilitated in HNK treated rats compared to those that received saline but also ketamine (5min post-HFS HNK vs. SAL, Sidak's p=0.0015, vs. KET, Sidak's p=0.017; Figures 11B and 14B). In addition, robust L-LTP was still present 90min later in the HNK treated group (HNK 90min post-HFS:157.02 ±11.53% vs. pre-HFS, Tukey's p<0.0001, Figure 16A,B), which was similar in magnitude to that following ketamine (90min post-HFS KET vs. HNK Sidak's p=0.93), while no L-LTP was observed in the saline group as before (90min post-HFS SAL: 102.69 ±4.37% vs. pre-HFS Tukey's p=0.75, n.s.; 90min post-HFS SAL vs. HNK Sidak's p<0.0001, Figure 17A,B). At the end of the recording, responses from 7/8 (88%) HNK-treated WKY rats still expressed robust LTP (defined as 20% or more increase in fEPSP slope at 90min post-HFS), compared to 72% for ketamine and 21% for saline (Figure 17C). Despite the slightly higher frequency of sustained L-LTP following HNK, average fEPSP slope at 90min post-HSF was not significantly different between HNK and ketamine

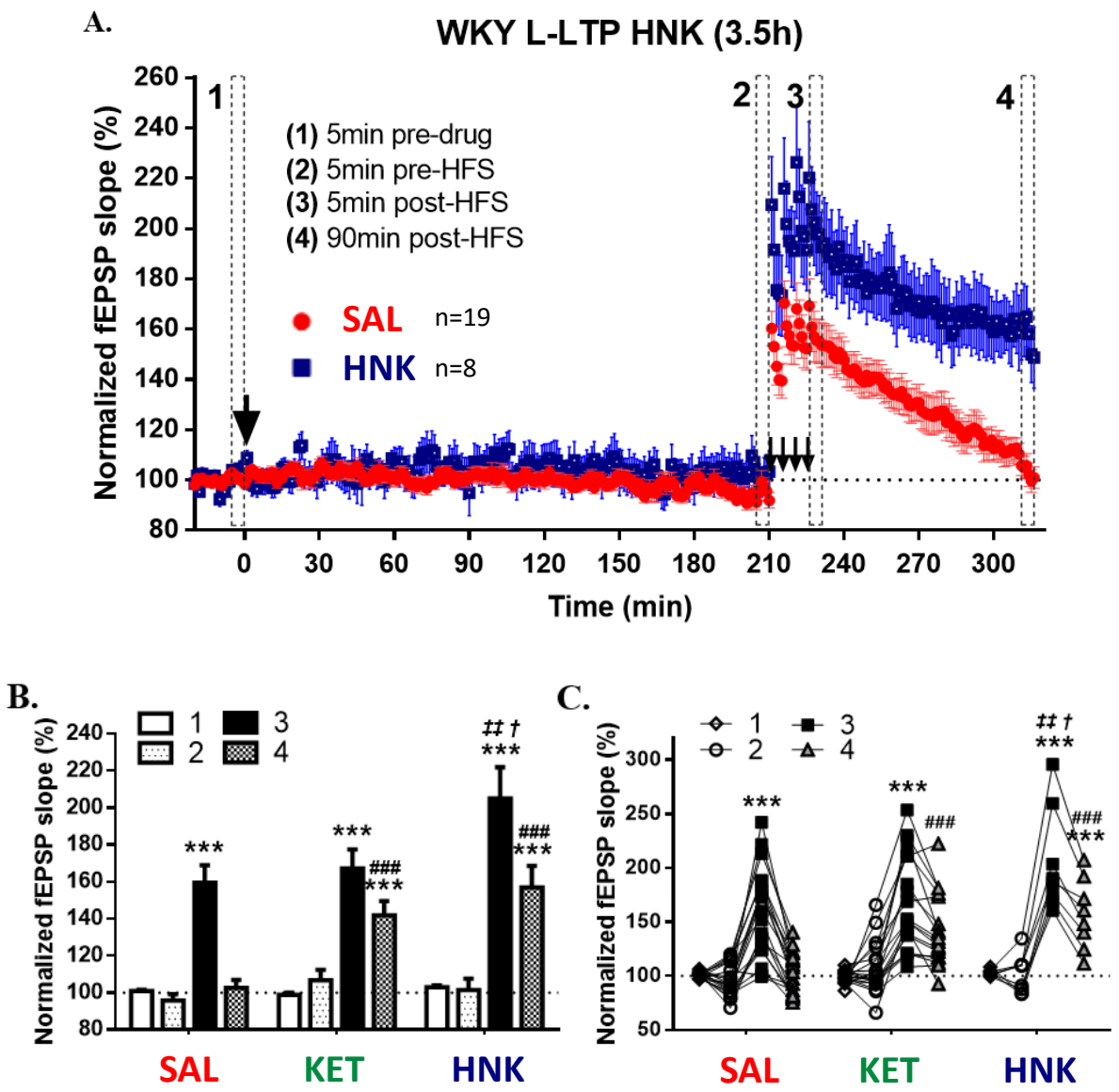

Figure 17. (2R,6R)-HNK significantly rescues the impaired SC-CA1 L-LTP in WKY rats at 3.5h, similar to ketamine.

Once a stable fEPSP baseline was obtained, saline or (2R,6R)-HNK (5mg/kg, ip) were administered to WKY rats (SAL: n=19, KET: n=8), followed by the HFS protocol 3.5h later. Average normalized fEPSP slope (%) A. by drug treatment for the duration of the recording (90min-HFS). B. by time bin (5min pre-HFS, 5min post-HFS, 90min-HFS). C. by time bin for each individual animal. ANOVA revealed significant main effects of time and drug treatment ($p<0.0001$ and $p=0.0088$) and a significant drug x time interaction ($p<0.0001$). Significant LTP was induced in all groups (5min post-HFS vs. pre-HFS Tukey's ***$p<0.0001$); however, L-LTP induction was significantly facilitated in HNK compared to saline, but also ketamine, treated rats (5min post-HFS HNK vs. SAL, Sidak's ‡‡$p=0.0015$, vs. KET, †$p=0.017$). In addition, robust LTP was still present 90min later in the HNK treated group (90min post-HFS vs. pre-HFS, Tukey's ***$p<0.0001$), which was similar in magnitude to that following ketamine (90min post-HFS KET vs. HNK Sidak's $p=0.93$), while no L-LTP was observed in the saline group

(90min post-HFS vs. pre-HFS Tukey's p=0.75, n.s.; SAL vs. HNK Sidak's ###p<0.0001). At the end of the recording, 7/8 (88%) of HNK-treated WKY rats still expressed robust LTP, compared to 72% for ketamine and 21% for saline. Despite the slightly higher proportion for HNK, LTP at 90min post-HSF was not significantly different between HNK and ketamine (Sidak's p=0.93, n.s.). Therefore, (2R,6R)-HNK (5mg/kg) also completely eliminated the SC-CA1 L-LTP deficit in WKY rats when the HFS protocol was given 3.5h after injection, with a magnitude of synaptic potentiation ~15-20% higher than that for following an equivalent dose of (R,S)-ketamine.

treated animals (157.02 ±11.53% vs. 141.91 ±7.74%, Sidak's p=0.93, n.s.). Therefore, when the HFS protocol was given 3.5h after injection, (2R,6R)-HNK (5mg/kg) also effectively restored SC-CA1 L-LTP in WKY rats by significantly facilitating its induction and maintenance, where the magnitude of synaptic potentiation was ~15-20% higher than that observed following an equivalent dose of (R,S)-ketamine.

5.8 Effects of Ketamine on WIS SC-CA1 L-LTP (3.5h)

Finally, we tested whether ketamine's robust facilitatory effect on L-LTP under this experimental design is specific to stress-prone WKY rats, which display a pronounced deficit in LTP maintenance. As in WKY rats, ketamine had no major effects on basal synaptic transmission (0-3.5h, Figure 18A) in WIS rats. For L-LTP at 3.5h post-ketamine (Figure 18A,B), a 2-way (drug by time point) RM-ANOVA of average fEPSP slope only revealed a significant main effect of time ($F_{3,21}$=29.74, p<0.0001), but not of drug treatment or the drug x time interaction ($F_{1,7}$=0.35, p=0.57, n.s. and $F_{3,21}$=0.17, p=0.92, n.s.). As expected, average fEPSP responses in all WIS rats were potentiated immediately post-HFS (5min post-HFS, SAL: 161.27 ±6.46%, n=3 vs. pre-HFS Tukey's p=0.0003; KET: 168.43 ±12.96%, n=6 vs. pre-HFS Tukey's p<0.0001, Figure 18A,B). In addition, independent of the treatment group, robust L-LTP was still present 90min later (90min post-HFS, SAL: 134.39 ±2.71% vs. pre-HFS Tukey's p=0.028;

KET: 136.86 ±12.00% vs. pre-HFS Tukey's p=0.013, Figure 18A,B). Post-hoc analyses also indicated that there were no significant differences in fEPSP slope between saline and ketamine treated WIS rats at any time point (Sidak's p>0.88, n.s. Figure 18A,B). Therefore, when the HFS protocol was given 3.5h after injection, ketamine (5mg/kg, ip) had no effect on SC-CA1 L-LTP in control WIS rats at this dose.

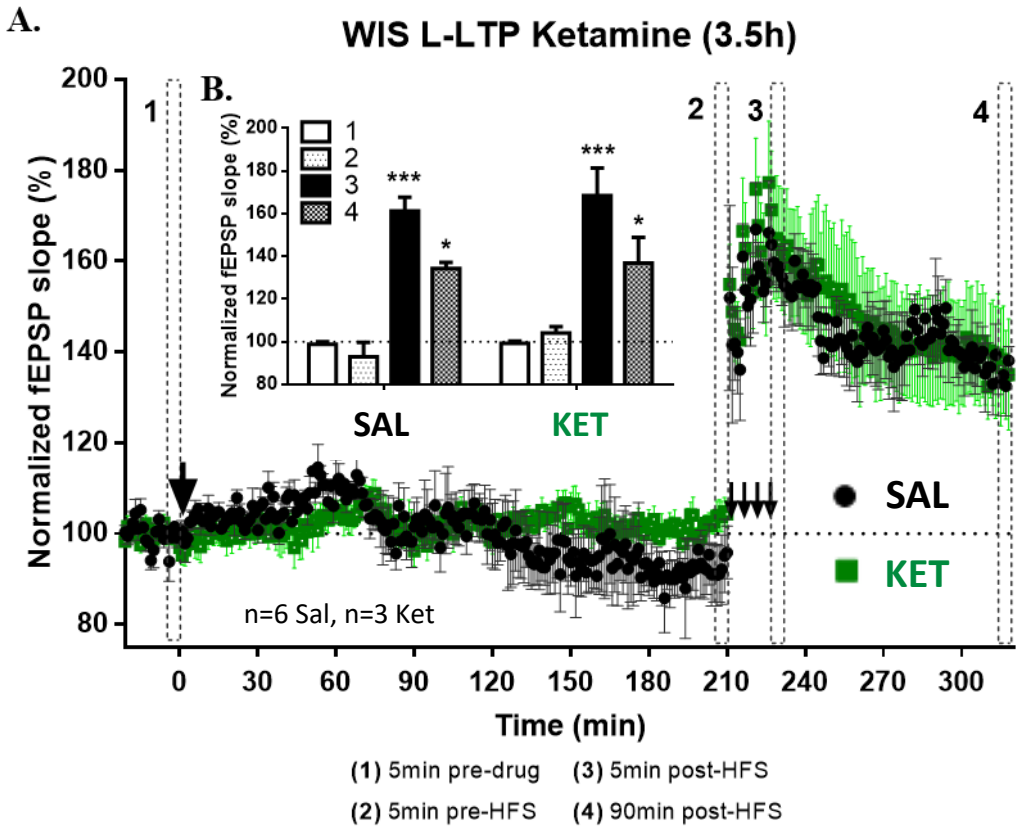

Figure 18. Ketamine has no significant effects on SC-CA1 L-LTP in control WIS rats at 3.5h.

Once a stable fEPSP baseline was obtained, saline or ketamine (5mg/kg, ip) were administered to normal WIS rats (SAL: n=6, KET: n=3), followed by the HFS protocol 30min later. A. Average normalized fEPSP slope (%) A. by drug treatment for the duration of the recording (90min-HFS). B. by time bin (5min pre-drug, 5min pre-HFS, 5min post-HFS, 90min-HFS). Ketamine had no major effects on basal synaptic transmission in WIS rats. For L-LTP, ANOVA only revealed a significant main effect of time (p<0.0001). Significant LTP was induced in both groups (5min post-HFS vs. pre-HFS Tukey's SAL: ***p=0.0003, KET: ***p<0.0001), and independent of the treatment group, robust L-LTP was still present 90min later (90min post-HFS vs. pre-HFS Tukey's, SAL: *p=0.028; KET: *p=0.013, Figure 18A,B). There were no significant differences in fEPSP slope between saline and ketamine at any time point (Sidak's p>0.88, n.s.), indicating no effects of ketamine on WIS L-LTP at 3.5h at this dose.

5.9 Summary and Discussion

Given the accumulating evidence supporting a role of synaptic plasticity in depression and antidepressant response, it is surprising that synaptic plasticity processes (LTP, LTD) in key neural circuits implicated in MDD remain understudied in the context of animal models of depression or the antidepressant action of rapid antidepressants, especially *in vivo*. Ketamine has demonstrated rapid and sustained antidepressant activity in TRD and various animal models including the WKY rat; however, its effects on synaptic plasticity, as well as their direct contribution to ketamine's antidepressant action, are still unclear. As reviewed in section 1.3.2, it is now well known that ketamine's mechanism of action involves acute direct NMDAR antagonism and indirect AMPAR activation, rapid induction of key synaptogenic signaling pathways (mTOR, BDNF) and sustained enhancement of AMPAR function, particularly in the HPC and PFC (Aleksandrova et al., 2017a). Based on this, and knowledge of the unique roles of NMDA and AMPA receptors in the induction and expression of synaptic plasticity processes, it is crucial to consider the temporal effects of ketamine treatment on hippocampal LTP. Therefore, we tested the effects of ketamine (5mg/kg, ip) on the WKY SC-CA1 LTP deficit at various time points after administration (30min, 3.5h and 24h).

Overall, we found that ketamine administration did not have any major acute effects on SC-CA1 basal synaptic transmission at the 5mg/kg dose in either strain. Moreover, when the HFS protocol was given 30min after injection, there were no significant effects of ketamine treatment on the SC-CA1 L-LTP deficit observed in WKY rats, indicating that around the time when drug concentrations in the brain are peaking, LTP at this synapse is unaffected. On the other hand, when the HFS protocol was given 3.5h after injection, when ketamine has been cleared from the system but has engaged key downstream events, the SC-CA1 L-LTP deficit in

WKY rats is completely eliminated, reflecting a robust positive effect of ketamine on the maintenance of LTP in this strain at the intermediate time point. Interestingly, although its facilitatory effects on L-LTP maintenance are no longer as pronounced at 24h after injection, ketamine pre-treatment caused a leftward shift of the WKY SC-CA1 input-output curve at 24h, which likely reflects an enhancement of basal synaptic transmission as a result of sustained ketamine-induced potentiation of WKY SC-CA1 synapses at this later time point.

Until very recently, the effects of ketamine on hippocampal synaptic plasticity in the WKY model had never been addressed. Last year, Pang et al. extended their initial findings of a mPP-DG LTP deficit in WKY rats (discussed previously in section 4.5), by demonstrating that an antidepressant dose of ketamine (5mg/kg, ip) not only facilitated the extinction of avoidance in a subset of WKY rats (responders) for up to three weeks following administration, but the authors attributed this effect to ketamine's ability to restore of mPP-DG LTP at 24h (Fortress et al., 2018). Similar to these findings, not all depressed human patients experience a clinically significant response to ketamine (response rates of 50-70% in patients with TRD), so the neurobiological factors differentiating 'responders' and 'non-responders' are of great interest (Gerhard et al., 2016; Kishimoto et al., 2016; Muller et al., 2016). By showing that ketamine exerts its long-lasting behavioural effects only in subjects that experience an enhancement of mPP-DG LTP at 24h after injection, these findings seemingly emphasize the role of changes in hippocampal synaptic plasticity in mediating the persistent avoidance phenotype in WKY rats and the antidepressant response to ketamine (Fortress et al., 2018). However, an important caveat to this interpretation presented by Fortress et al., is the fact that ketamine only restored LTP of the population spike (PS) but not of the fEPSP at the mPP-DG synapse in WKY responders (Fortress et al., 2018). It is important to note that while fEPSP LTP represents classical synaptic

plasticity, PS LTP alone likely reflects an enhancement of the excitability of the post-synaptic granule cell, while synaptic efficacy is not actually facilitated (Fortress et al., 2018; Taube and Schwartzkroin, 1988). Therefore, the ketamine-induced potentiation of EPSP-spike coupling reported by Fortress et al. can more likely be attributed to granule cell disinhibition resulting from reduced local GABAergic interneuron firing (i.e. the disinhibition hypothesis of ketamine action), rather than an increase in LTP per se (Fortress et al., 2018). Considering this important distinction between PS and fEPSP LTP, Pang and colleagues actually observed no effect of ketamine on WKY mPP-DG LTP as is classically defined (fEPSP LTP) at 24h after administration (Fortress et al., 2018). While this is somewhat contrary to our finding of a small but significant residual effect of ketamine on WKY LTP at the 24h time point, it is important to consider two key factors, which could lead to this apparent discrepancy. First, the different synapse under investigation (mPP-DG in the latter study vs. SC-CA1 here) gives rise to the possibility that ketamine may modulate synaptic plasticity processes in a region-specific manner, restoring LTP at the SC-CA1, but not the mPP-DG, synapse in WKY rats. A second, and probably more likely, possibility is that the robust facilitatory effect of ketamine on WKY LTP maintenance we reported at 3.5h post-injection, may have been missed by Fortress et al. since LTP was only measured only at 24h following ketamine administration, when drug effects on LTP observed in our study were significantly diminished. On the other hand, we found that ketamine caused an enhancement of WKY SC-CA1 basal synaptic transmission at 24h post-injection (as indicated by a leftward shift of the input-output curve), likely as a result of sustained ketamine-induced potentiation of WKY SC-CA1 synapses at this later time point, an effect which was not studied by Fortress et al (Fortress et al., 2018).

In contrast to our findings in the WKY strain, ketamine administration had no effect on SC-CA1 L-LTP in control WIS rats when the HFS protocol was given 3.5h after injection. Importantly, it is not surprising that ketamine did not facilitate L-LTP in normal WIS controls, since the L-LTP protocol utilized here (4 trains, 100Hz) is thought to produce maximum levels of LTP in normal rats (i.e. stronger stimulation protocols do not cause further facilitation), indicating a ceiling effect for L-LTP under our experimental design. Therefore, this was just a control experiment and our study was not designed to detect any facilitatory effects of ketamine on LTP in normal rats. The question remains as to whether ketamine administration would facilitate E-LTP or L-LTP following sub-maximal protocols in WIS rats, which would determine if its effects are specific to the stress-susceptible WKY rat or are an innate feature of ketamine action. Interestingly, accumulating evidence in the field suggests that the ability of ketamine to modulate synaptic plasticity processes may not be limited to the WKY model of depression.

Consistent with what we observed in control WIS rats, antidepressant concentrations of ketamine (either <10μM applied to brain slices or 10mg/kg, ip) do not generally affect hippocampal basal transmission in normal outbred rats (Izumi and Zorumski, 2014; Michaëlsson et al., 2018; Ribeiro et al., 2014). Instead, emerging evidence indicates ketamine may selectively modulate synaptic plasticity processes, particularly in the rodent HPC; however, a lot of conflicting reports currently exist in the literature. Namely, two previous studies reported that ketamine (3-10mg/kg, IV or 30 mg/kg, ip) enhances SC-CA1 fEPSP LTP (following sub-maximal HFS) in hippocampal slices obtained from SD rats at 24h post-injection (Burgdorf et al., 2013; Graef et al., 2015), similar to what we observed in the WKY strain (although at a lower dose of 5mg/kg, ip). However, importantly, other studies have also reported no change or even a decrease in LTP (Fortress et al., 2018; Izumi and Zorumski, 2014; Michaëlsson et al., 2018;

Ribeiro et al., 2014), and/or a block of LTD (Huang et al., 2016; Ribeiro et al., 2014) in the HPC of outbred rats following ketamine administration. Likely contributing to the conflicting results, collectively, these studies suffer from important limitations, such as the fact that they utilize electrophysiological recordings performed *in vitro* (in hippocampal brain slices), use different doses and routes of ketamine administration (including bath application of excessive concentrations onto slices), different (often single) time points after ketamine administration, different LTP/LTD protocols and strains of outbred rats, leading to an abundance of possible reasons for the discrepancies seen in the literature (further discussed in section 7.4). Therefore, our study highlights the need for systematic investigations of ketamine's effects on *in vivo* hippocampal synaptic plasticity at therapeutically relevant doses, as well as at different time points after injection and in the context of valid preclinical models of depression.

Following the exciting claim that an active metabolite is necessary and sufficient for ketamine's antidepressant effects in mice (Zanos et al., 2016) and given the pronounced facilitatory effect of the parent drug we observed in our model, we were interested in testing the effects of (2R,6R)-HNK on the WKY LTP deficit. First, we found that like ketamine, (2R,6R)-HNK (5mg/kg, ip) had no effect on basal synaptic transmission in WKY rats. Interestingly, in their original paper, Zanos et al. reported that bath application of 10μM (2R,6R)-HNK to hippocampal slices from SD rats caused a dramatic run-up of CA1 basal synaptic transmission (fEPSP slope increased by ~600%) within an hour, which persisted after wash-out (Zanos et al., 2016). This is in stark contrast to what was observed here following systemic administration of (2R,6R)-HNK (5mg/kg, ip) in rats (Zanos et al., 2016). Zanos et al. determined that the maximum drug concentration reached in the mouse brain following a systemic injection of (2R,6R)-HNK (10mg/kg, ip) was 10.69μM, which is similar to the concentration used in their

electrophysiology experiments (Zanos et al., 2016), but is substantially higher than what would be observed following the 5mg/kg, ip dose we used here. More importantly, bath application of (2R,6R)-HNK onto hippocampal slices has crucial limitations, such as lack of intact brain circuitry, poor spatial and temporal resolution when applying compounds, difficulty in mimicking physiological conditions, such as normal drug distribution, metabolism and elimination, all of which likely give rise to this dramatic discrepancy in the reported effects of (2R,6R)-HNK on basal synaptic transmission. Indeed, it is extremely unlikely that a rapid, 600% increase in hippocampal basal synaptic transmission would be left unchecked *in vivo*, and if observed, would presumably cause severe adverse effects such as seizures, contrary to (2R,6R)-HNK being very well tolerated *in vivo* both in our and the Zanos et al. study. Clearly, mimicking the spatial and temporal pattern of drug exposure following systemic HNK administration cannot be reliably accomplished by continuously bath applying the maximum concentration of HNK observed following systemic administration *in vivo* to mouse brain slices *in vitro*. Therefore, the fact that Zanos et al. bath applied high concentrations of HNK onto hippocampal slices from normal SD rats, whereas we tested the effects of systemic administration of HNK in intact stress-prone WKY rats likely contributes to the discrepancy in effects on basal synaptic transmission.

Next we found that, similar to its parent drug, (2R,6R)-HNK (5mg/kg, ip) also effectively restored normal SC-CA1 L-LTP in WKY rats when the HFS protocol was given 3.5h after injection, by significantly facilitating LTP induction and maintenance, with the magnitude of synaptic potentiation after HNK being slightly higher than that observed following an equivalent dose of (R,S)-ketamine. Importantly, the effects of (2R,6R)-HNK on synaptic plasticity have yet to be addressed outside of our study. Similar to ketamine, Zanos et al. previously reported that a single dose of (2R,6R)-HNK (10mg/kg, ip) in mice caused a significant upregulation of surface

GluA1 and GluA2 AMPAR subunits, a decrease in eEF2 phosphorylation, as well as an increase in BDNF expression in HPC (but not PFC) synaptosomes 24h after treatment (Zanos et al., 2016). Furthermore, systemic injection of both ketamine and (2R,6R)-HNK induced a selective increase in gamma band power *in vivo* as measured by qEEG, consistent with increased activation of fast ionotrophic excitatory receptors (including AMPAR) (Zanos et al., 2016). Therefore, seemingly through an NMDAR inhibition-independent mechanism, HNK has been shown to lead to the rapid and sustained enhancement of AMPAR function, however, we found that both ketamine and its metabolite may do so in an activity-dependent manner (i.e. selectively affecting metaplasticity processes over basal synaptic transmission), consistent with an LTP-like effect in the WKY model. Although (2R,6R)-HNK's molecular target(s) and exact mechanism of action have yet to be defined, it is crucial to determine to what extent HNK and its effects on hippocampal synaptic plasticity contribute to ketamine's antidepressant action.

Therefore, our results provide compelling evidence for the role of synaptic plasticity in the antidepressant actions of ketamine and its metabolite, and emphasize hippocampal LTP as potentially key to understanding the mechanisms mediating the development and reversal of WKY depressive-like phenotypes. Given the pronounced facilitatory effects of ketamine and its metabolite on LTP we observed in the WKY model, next we were interested in characterizing the antidepressant responses of the WKY rat to ketamine and (2R,6R)-HNK, and importantly, to test whether the ability to rescue SC-CA1 L-LTP in these rats mediates/ contributes to their mechanism of action as an antidepressant.

Chapter 6: Effects of Ketamine and (2R,6R)-HNK on Behaviours Relevant to Depression

6.1 Effects of Ketamine on Depressive-Like Behaviour in WKY rats

Based on the robust depressive-like phenotype observed in the WKY model (Figure 4B), day2 FST immobility was used initially to screen for drug antidepressant activity. Following pre-exposure, WKY rats underwent a 5min FST test session on day 2, with saline or ketamine (5mg/kg, ip) administered 30min or 24h before (SAL: n=16 and n=14, KET: n=16 and n=15 for 30min and 24h, respectively). A 2-way ANOVA (drug treatment x time point) of day2 FST immobility scores indicated only a significant main effect of drug treatment ($F_{1,57}$=63.05, $p<0.0001$) but no time or drug x time interaction effects ($F_{1,57}$=0.17, p=0.68, n.s. and $F_{1,57}$=0.29, p=0.59, n.s.). Subsequent analyses revealed that ketamine significantly decreased WKY FST immobility compared to that of saline treated WKYs at both time points (30min: from 258.75 ±6.65s to 193.13 ±11.10s; 24h: from 267.14 ±4.56s to 192.00 ±10.71s, Sidak's p<0.0001 for both, Figure 19A). The magnitude of the ketamine effect was comparable at 30min and 24h post-injection (Sidak's p>0.99, n.s.). Importantly, ketamine treatment (5mg/kg, ip) had no effect on general locomotor activity of WKY rats at these time points (n=6/group, 2 way drug x time point ANOVA of OFT total distance travelled revealed only significant main effect of time point ($F_{1,20}$=6.02, p=0.024), with no post-hoc pairwise significance, Sidak's p>0.82, n.s., Figure 19B).

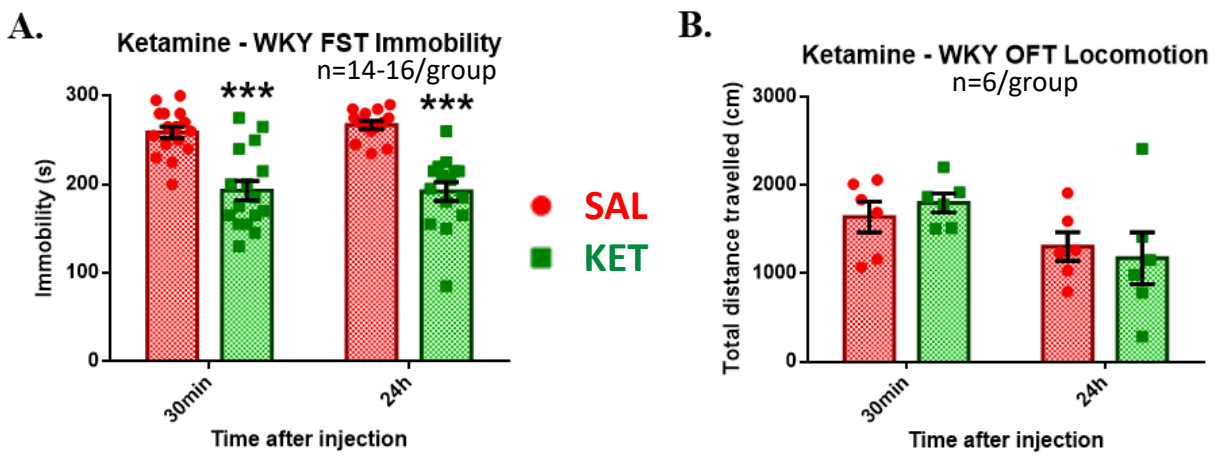

Figure 19. Ketamine has significant rapid (30min) and sustained (24h) FST antidepressant effects in WKY rats, without any locomotor effects.

A. Average Day2 FST immobility for saline and ketamine treated WKY rats at 30min and 24h post-injection. Following pre-exposure, WKY rats underwent a 5min FST test session, with saline or ketamine (5mg/kg, ip) being administered 30min or 24h before (SAL: n=16 and n=14, KET: n=16 and n=15 for 30min and 24h, respectively). ANOVA revealed only a significant main effect of drug treatment ($p<0.0001$). Ketamine significantly decreased WKY FST immobility compared to that of saline treated WKYs at both time points (Sidak's ***$p<0.0001$), with effects being comparable at 30min and 24h post-injection (Sidak's $p>0.99$, n.s.). B. Average total distance travelled for saline and ketamine treated WKY rats at 30min and 24h post-injection. Animals (n=6/group) underwent a 10min OFT session, with saline or ketamine (5mg/kg, ip) administered 30min or 24h before. Importantly, ketamine had no effect on general locomotor activity of WKY rats at these time points (ANOVA revealed only significant main effect of time point ($p=0.024$), with no post-hoc pairwise significance Sidak's $p>0.82$, n.s.).

6.2 Effects of Ketamine on Depressive-Like Behaviour in WIS rats

For WIS rats (SAL: n=6 and n=6, KET: n=7 and n=7 for 30min and 24h), a 2-way ANOVA (drug treatment x time point) of day2 FST immobility scores indicated significant main effects of drug treatment and time point ($F_{1,22}=27.94$, $p<0.0001$ and $F_{1,22}=6.60$, $p=0.0175$) but no significant drug x time interaction ($F_{1,22}=0.0018$, $p=0.67$, n.s.). Subsequent analyses revealed that ketamine significantly decreased WIS FST immobility compared to that of saline treated WIS rats at both time points (30min: from 148.33 ±9.28s to 96.43 ±8.07s; 24h: from 174.17 ±14.69s to 121.43 ±7.13s, Sidak's $p=0.0073$ and $p=0.0063$, Figure 20A). The magnitude of the

ketamine effect was again comparable at 30min and 24h post-injection (Sidak's p=0.38, n.s.). Importantly, ketamine treatment (5mg/kg, ip) had no effect on general locomotor activity of WIS rats at these time points (n=6/group, 2-way drug x time point ANOVA of OFT total distance travelled revealed no significant effects ($F<1.25$, $p>0.28$, n.s., Figure 20B). Therefore, ketamine (5mg/kg, ip) had significant rapid (30min) and sustained (24h) antidepressant effects in both WIS and WKY rats, without affecting general locomotion in either strain.

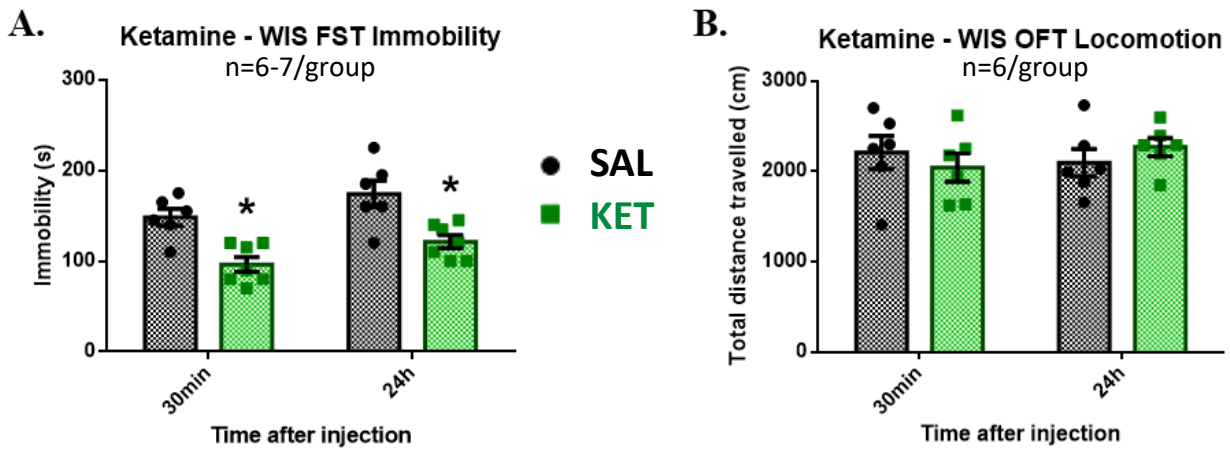

Figure 20. Ketamine has significant rapid (30min) and sustained (24h) FST antidepressant effects in WIS rats, without any locomotor effects.

A. Average Day2 FST immobility for saline and ketamine treated WIS rats at 30min and 24h post-injection. Following pre-exposure, animals (SAL: n=6/group, KET: n=7/group for 30min and 24h) underwent a 5min FST test session, with saline or ketamine (5mg/kg, ip) being administered 30min or 24h before. ANOVA revealed significant main effects of drug treatment and time point ($p<0.0001$ and $p=0.0175$). Ketamine significantly decreased WKY FST immobility compared to that of saline treated WKYs at both time points (Sidak's *$p=0.0073$ and *$p=0.0063$), with effects being comparable at 30min and 24h post-injection (Sidak's $p=0.38$, n.s.). B. Average total distance travelled for saline and ketamine treated WIS rats at 30min and 24h post-injection. Animals (n=6/group) underwent a 10min OFT session, with saline or ketamine (5mg/kg, ip) being administered 30min or 24h before the test. Importantly, ketamine treatment had no effect on general locomotor activity of WIS rats at these time points (ANOVA revealed no significant effects, $p<0.28$, n.s.).

6.3 Effects of (2R,6R)-HNK on Depressive-Like Behaviour in WKY rats

Next, we tested the effects of (2R,6R)-HNK (5mg/kg, ip) in WKY rats (SAL: n=7/group, HNK: n=9/group for 30min and 24h) in the FST (Figure 21A). A 2-way ANOVA (drug treatment x time point) of day2 FST immobility scores indicated no significant main or interaction effects ($F<0.67$, $p>0.42$, n.s.). In fact, day2 FST immobility was virtually identical between saline and (2R,6R)-HNK treated WKY rats at both 30min (254.29 ±5.71s and 255.00 ±5.20s) and 24h (260.00 ±5.00s and 258.33 ±5.71s) after injection. Therefore, we failed to detect any antidepressant activity of (2R,6R)-HNK (5mg/kg, ip) in this model; nor were effects observed on general locomotor activity (n=6/group, 2-way drug x time point ANOVA of OFT total distance travelled indicated no significant effects ($F<0.59$, $p>0.45$, n.s., Figure 21B).

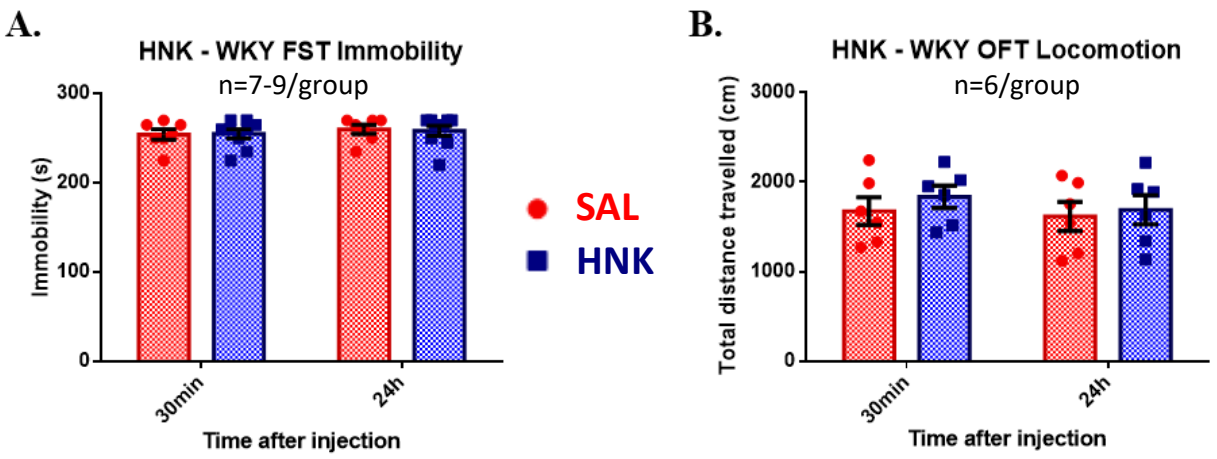

Figure 21. (2R,6R)-HNK has no FST antidepressant or OFT locomotor effects in WKY rats.

A. Average day2 FST immobility for saline and (2R,6R)-HNK treated WKY rats at 30min and 24h post-injection. Following pre-exposure, animals (SAL: n=7/group, HNK: n=9/group for 30min and 24h) underwent a 5min FST test session, with saline or (2R,6R)-HNK (5mg/kg, ip) being administered 30min or 24h before the test session. ANOVA revealed no significant main or interaction effects ($p>0.42$, n.s.). In fact, day2 FST immobility was virtually identical between saline and HNK treated WKY rats at both time points. B. Average total distance travelled for saline and (2R,6R)-HNK treated WKY rats at 30min and 24h post-injection. Animals (n=6/group) underwent a 10min OFT session, with saline or (2R,6R)-HNK (5mg/kg, ip) being administered 30min or 24h before the test. HNK treatment also had no locomotor effects in WKY rats (no significant ANOVA effects, $p<0.28$, n.s.).

6.4 Object Location Recognition (OLR) Task

Only ketamine, but not (2R,6R)-HNK, possessed antidepressant activity as assessed by the FST in WKY rats, while both compounds completely eliminated the WKY SC-CA1 L-LTP deficit. Accordingly, there was a clear dissociation between hippocampal synaptic plasticity and antidepressant response in the FST in the context of the WKY model. Therefore, we sought evidence for a functional correlate between SC-CA1 LTP and cognitive deficits on a test of spatial memory mediated by activity in the dorsal hippocampus in these rats. Accordingly, we chose a hippocampal-dependent task to further study the effects of ketamine and its metabolite. To this end, we first compared performance of WKY and WIS rats on the object location recognition task (OLR, Figure 22), a hippocampal-dependent spatial memory task.

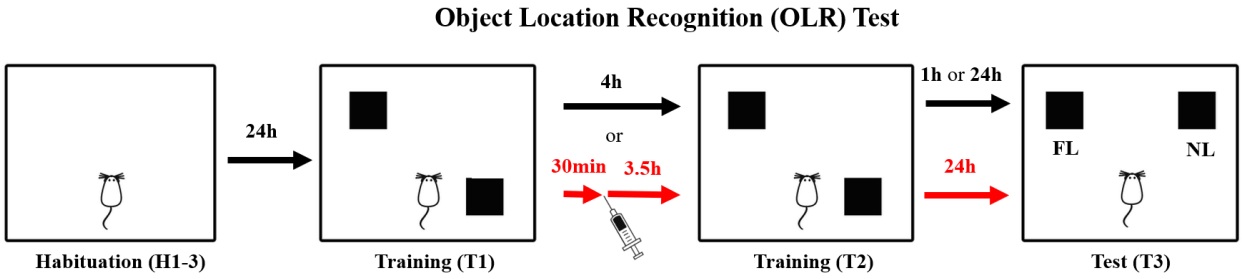

Figure 22. Schematic of the object location recognition (OLR) test, a hippocampal-dependent spatial memory task.

Briefly, following 3 days of habituation (H1-3), rats received 2 training sessions (T1 and T2, 10min each, AM and PM), where two identical objects were placed in two opposing corners of the arena. During the testing session (T3, either 1h or 24h later for short or long -term memory), one of the objects stays at the familiar location (FL), while the other was moved to a new location (NL). In experiments involving drug treatment (in red), saline, ketamine or (2R,6R)-HNK were injected 3.5h before the second OLR training session (T2), i.e. 27.5h before the 24h testing session (T3).

Following habituation, rats received training sessions (T1 and T2, 10min each), where two identical objects were placed in two opposing corners of the arena. During the testing session (T3, either 1h or 24h later for short or long -term memory), one of the objects stayed at the familiar location (FL), while the other was moved to a new location (NL) and an NL preference of around/above 60% on the test day indicates good location recognition memory. In the OLR task, 2-way strain x time delay ANOVA of total exploration time during the test session (NL+FL) indicated only a significant main effect of strain ($F_{1,35}$=7.38, p=0.010), with no post-hoc pairwise significance between the two strains at either time point (Sidak's p>0.77, n.s., Figure 23A). Therefore, total exploration time in the task was comparable between the two

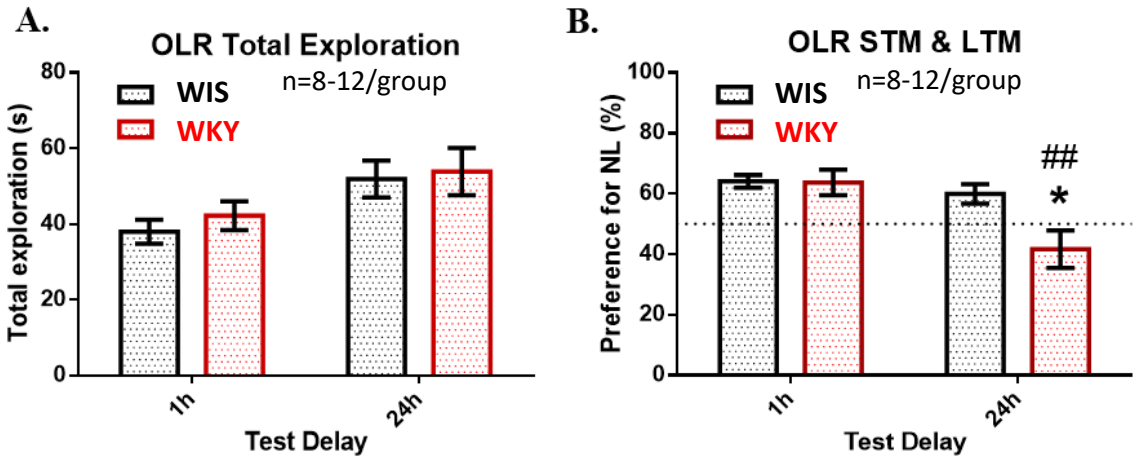

Figure 23. Long-term (LTM at 24h delay), but not short-term (STM at 1h delay) OLR memory is significantly impaired in WKY rats.

A. Average total exploration time during the OLR test session (NL+FL). ANOVA only revealed a significant main effect of strain (p=0.010), with no post-hoc pairwise significance (Sidak's p>0.77, n.s.). Total exploration time was comparable between the two strains (WIS: n=8 and n=10, WKY: n=12 and n=9 for 1h and 24h) although it was generally slightly higher in WKY rats. B. Average NL preference (%, NL/NL+FL) for the 24h OLR test session (for the same rats as in A.). ANOVA revealed significant main effects of strain and test delay and a significant interaction (p=0.039, p=0.0049 and p=0.047). While short-term memory was equivalent between the two strains (1h WKY vs. WIS, Tukey's p>0.99, n.s.), long-term spatial memory was significantly impaired in WKYs (24h WKY vs. WIS, Tukey's *p=0.027; WKY 24h vs. 1h, Tukey's ##p=0.0037).

strains although it was slightly higher in WKY rats at both the 1h and 24h delay (1h: 38.00 ±3.19s (n=8) and 42.25 ±3.79s (n=12), 24h: 51.90 ±4.84s (n=10) and 53.66 ±6.24s (n=9) for WIS and WKY, respectively). A 2-way strain x test delay ANOVA of average NL preference (%, NL/NL+FL) for the test session indicated significant main effects of strain and test delay, as well as a significant strain x test delay interaction ($F_{1,35}$=4.58, p=0.039, $F_{1,35}$=9.02, p=0.0049 and $F_{1,35}$=4.24, p=0.047*)*. Subsequent analyses revealed that, as we hypothesized, while short-term location recognition memory was equivalent between the two strains (1h OLR NL preference, WKY: 63.74 ±4.22% vs. WIS: 64.09 ±2.16%, Tukey's p>0.99, n.s., Figure 23B), long-term memory was significantly impaired in WKYs (24h OLR NL preference, WKY: 41.65 ±6.23% vs. WIS: 59.97 ±3.24%, Tukey's p=0.027; WKY 24h vs. 1h OLR NL preference, Tukey's p=0.0037, Figure 23B), consistent with the SC-CA1 LTP deficit in these rats. Consistent with this, one-sample t tests revealed that OLR performance (% NL preference) was significantly different from chance (50%) in WIS rats at 1h and 24h (p=0.0003 and p=0.013) and in WKY rats at 1h (p=0.008), indicating significant object recognition memory, while this was not true for WKY rats at 24h (p=0.22, n.s.).

6.5 Effects of Ketamine and (2R,6R)-HNK on Location Recognition Memory

Next, we tested the effects of ketamine and (2R,6R)-HNK (5mg/kg, ip) on the OLR long-term (24h) memory deficit in WKY rats. Saline (n=25), ketamine (n=25) or (2R,6R)-HNK (n=15) were injected 3.5h before the second OLR training session (T2), i.e. 27.5h before the 24h testing session (Figure 22, time point was chosen based on our L-LTP results). A 1-way ANOVA of average OLR NL preference (%) for the 24h test session with drug treatment as the between subject factor indicated a significant effect of drug treatment *(*F=7.75, p=0.001*)*.

Subsequent analyses revealed that both ketamine and (2R, 6R)-HNK significantly restored long-term object recognition memory at 24h in WKY rats (SAL: 41.21 ±4.71% vs. KET: 62.22 ±3.36% and HNK: 61.02 ±5.33%, Tukey's p=0.0017 and p=0.012, respectively, KET vs HNK: p=0.98, n.s., Figure 24) to levels comparable to those in control WIS rats at 24h (~60%, Figure 21B), consistent with the positive effects of drug treatment on SC-CA1 LTP. Consistent with this, one-sample t tests revealed that while OLR performance (% NL preference) at 24h was not significantly different from chance (50%) in saline-treated WKY rats (p=0.19, n.s.), significant object recognition memory was observed in ketamine-treated rats (p=0.018), falling just short of significance in the HNK-treated group (p=0.058, n.s.).

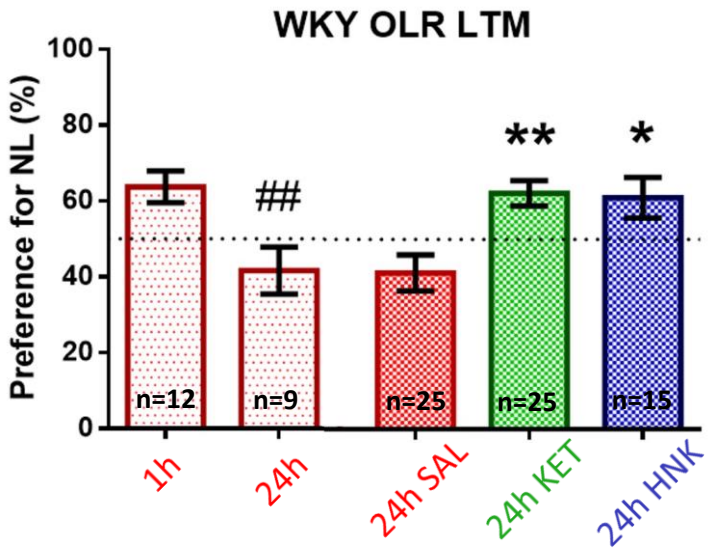

Figure 24. Ketamine and (2R,6R)-HNK both significantly restore OLR long-term memory (LTM at delay of 24h) in WKY rats.

Average NL preference (%, NL/NL+FL) for drug-free WKY rats at 1h (n=12) and (n=9) (same as in Figure 23B), as well as for the 24h OLR test session, with saline (n=25), ketamine (n=25) or (2R,6R)-HNK (n=15) injected 3.5h before OLR T2. While long-term spatial memory was significantly impaired in WKYs (1h vs. 24h, Tukey's ##p=0.0037), ANOVA (of SAL, KET, HNK groups only) revealed a significant effect of drug treatment *(p=0.001)*, where both ketamine and (2R, 6R)-HNK (5mg/kg, ip) significantly restored OLR memory at 24h in WKY rats (vs. SAL Tukey's **p=0.0017 and *p=0.012, respectively) to control WIS levels at 24h (~60%).

In contrast, ketamine (5mg/kg, ip) had no effect on long-term location recognition memory in control WIS rats, as OLR NL preference for the 24h test session did not differ between saline and ketamine treated WIS rats (SAL: 58.47 ±3.61% vs. KET: 59.20 ±9.66%, n=10/group, two-tailed t test p=0.88, n.s., Figure 25). As in drug-free WIS rats, one-sample t tests revealed significant OLR memory compared to chance (50%) (p<0.05).

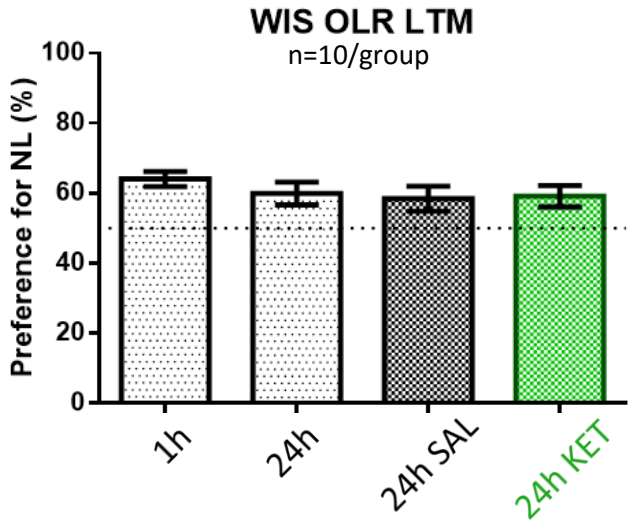

Figure 25. Ketamine has no significant effects on OLR long-term memory (LTM at delay of 24h) in WIS rats.

Average NL preference (%, NL/NL+FL) for the 24h OLR test session, with saline or ketamine (5mg/kg, ip) injected 3.5h before OLR T2. 24h OLR NL preference did not differ between saline and ketamine treated WIS rats (n=10/group, two-tailed t test p=0.88, n.s.).

6.6 Summary and Discussion

Importantly, we found that a single systemic injection of ketamine (5mg/kg, ip), which fully rescues the WKY SC-CA1 L-LTP deficit at 3.5h, also produces significant rapid (30min) and sustained (24h) antidepressant effects in the FST. Numerous studies have successfully

demonstrated the positive effects of ketamine in rodent tests or models of depression (see section 1.3.1) (Abelaira et al., 2013; Autry et al., 2011; Koike et al., 2011; Li et al., 2011; Zanos et al., 2016; Zhou et al., 2014). In most studies using normal rats or mice, a single systemic injection of ketamine (standard dose of 10mg/kg, ip) produces a significant reduction in FST immobility shortly (30min) after administration (without effects on locomotor activity), which has been shown to persist for an average of 7 days in rodents (Autry et al., 2011; Koike et al., 2011; Zanos et al., 2016; Zhou et al., 2014). Interestingly, there are reports of significant FST antidepressant effects in male WIS rats at 5mg/kg (Yang et al., 2013), as we demonstrated here; while others have found no FST effects at this dose in female WIS rats (Tizabi et al., 2012).

Similar to what we found here, work by Tizabi and colleagues first reported that ketamine exerts rapid and sustained antidepressant effects in WKY rats, where a single low dose of ketamine (2.5–5.0 mg/kg, ip) significantly reduced FST immobility in female WKYs without affecting general locomotor activity (Tizabi et al., 2012). When administered chronically (daily for 10days) ketamine was effective at a dose as low as 0.5mg/kg in both male and female WKY rats (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). It has been suggested that this strain may be more sensitive to the antidepressant effects of ketamine than outbred strains, as the standard ketamine antidepressant dose in the general literature is typically higher (2.5 vs. 10mg/kg, ip) (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). The antidepressant-like effects of ketamine in the WKY model are not limited to the FST, as a 5mg/kg, ip dose has also been reported to facilitate the extinction of persistent avoidance responding when the shock is eliminated in a subset of WKY rat (responders) for up to three weeks following administration (Fortress et al., 2018). Finally, subacute administration of ketamine (10 mg/kg, ip, for 7days) normalized sucrose intake, anxiety-like behaviours and object recognition memory in WKY rats

subjected to CMS (Willner et al., 2018). Although more studies are clearly warranted, taken together, accumulating evidence supports the ability of ketamine to exert rapid and sustained antidepressant-like effects in the WKY model of depression, as we observed here in the FST.

Therefore, we found that a single dose of ketamine (5mg/kg, ip) significantly reduced FST immobility at 30min and 24h in WKY rats, as well rescuing the impaired SC-CA1 LTP at 3.5h after administration. Importantly, while ketamine's effects on hippocampal synaptic plasticity clearly do not underlie its rapid antidepressant effects (at 30min), they may mediate or at least contribute to its sustained antidepressant action (at 24h). As previously mentioned, it was recently proposed that in the WKY model, rescuing hippocampal LTP may underlie ketamine's antidepressant effects in the FST (Fortress et al., 2018). However, as discussed (see section 5.9), since this study actually failed to demonstrate a true rescue of hippocampal synaptic plasticity (i.e. fEPSP LTP), our results are the first to demonstrate a correlation between ketamine's antidepressant effects in the FST and its ability to restore hippocampal LTP in the WKY rat.

Supporting evidence for the role of restoring normal hippocampal LTP in mediating antidepressant response in WKY rats comes from work by She et al. (2015). As mentioned previously (section 4.5), this study represents the only published report of a WKY SC-CA1 LTP deficit to date, but it also evaluated the antidepressant effects of electroaccupuncture (EA), an alternative and effective therapeutic approach for psychiatric disorders including depression, using the WKY model (She et al., 2015). Interestingly, EA (delivered over 3 weeks) significantly reduced FST immobility, restored sucrose intake in the SPT, increased the time spent in the center of the arena in the OFT and restored normal performance in the Morris water maze (MWM) in WKY rats, consistent with antidepressant and anxiolytic-like effects, as well as a rescue of spatial learning and memory (Han et al., 2018; She et al., 2015). Importantly, both this

and a later study by the same group demonstrated that EA rescued the ability to induce LTP at the SC-CA1 synapse, suggesting that EA may exert its antidepressant actions in the WKY model in a wide range of preclinical tests by restoring hippocampal synaptic plasticity (Han et al., 2018; She et al., 2015), similar to what we hypothesized here for ketamine.

Although the synaptic plasticity theory of depression has been the focus of several research projects, a recent study, which utilized both SD and WKY rats, claims to be the first demonstration that direct induction of LTP within the HPC has antidepressant action (Kanzari et al., 2018). Specifically, this study found that induction of mPP-DG LTP using a unique 'theta burst' HFS protocol *in vivo* is sufficient to induce a transient antidepressant effect in the FST (at 2 days post-HFS), without affecting general locomotor activity in both SD and WKY rats (Kanzari et al., 2018). While consistent with a role of synaptic plasticity in depression, the findings reported in the WKY arm of this study are somewhat puzzling given previous reports of a failure to induce LTP at this synapse (and in the HPC in general) (Cominski et al., 2014; Fortress et al., 2018; Han et al., 2018; She et al., 2015). One possible explanation for this discrepancy could lie in the unique theta-like HFS protocol used in this study by Kanzari et al. (Kanzari et al., 2018). However, it is important to note that electrophysiology data showing the induction of LTP at the PP-DG pathway in WKY rats are not presented in the paper (Kanzari et al., 2018). Furthermore, the WKY arm of the study involved a relatively small sample size (n=7/group, sham or HFS) and only a single behavioural readout (FST immobility), with the treatment effect being present only at a single time point (2 days post-HFS) (Kanzari et al., 2018). In addition, basal FST immobility scores for the WKY group in this study were very low compared to values consistently reported here in the literature (60s vs. >100-200s) and were similar to the control SD group (~60s). However, unlike SD controls, WKY rats did not undergo

an FST pre-exposure on day 1, thus confounding a direct comparison between strains (Kanzari et al., 2018). Therefore, successful induction of hippocampal LTP using a theta-like HFS protocol may be have FST antidepressant properties in the WKY strain; however, given the limitations of this study, the conclusions should be taken with caution.

Overall, the limited literature supports a hypothesis that novel antidepressant treatments (e.g. ketamine, electroacupuncture or even certain protocols of electrical stimulation) may rescue the ability to induce LTP in the HPC, thereby restoring normal hippocampal-dependent function and reversing key behavioural deficits in WKY rats (Aleksandrova et al., 2019; Fortress et al., 2018; Han et al., 2018; Kanzari et al., 2018; She et al., 2015). Based on this hypothesis and the findings of Zanos et al., we expected that (2R,6R)-HNK, which similar to ketamine restores SC-CA1 LTP in WKY rats, would also show rapid and sustained antidepressant effects in the FST in this model. Somewhat surprisingly, we found that the ketamine metabolite (2R,6R)-HNK (5mg/kg, ip) did not decrease FST immobility in WKY rats at either 30min or 24h after administration, despite restoring SC-CA1 LTP in these rats. The seminal study by Zanos et al. first reported that in addition to possibly contributing to ketamine's actions, the (2R,6R)-HNK metabolite itself (although at a higher dose of 10mg/kg, ip) has robust rapid and sustained antidepressant action in normal mice in multiple tests including the FST, as well as reversing social deficits induced by prior chronic social defeat stress (Zanos et al., 2016). However, following these encouraging initial findings, several reports failed to replicate the antidepressant effects of (2R,6R)-HNK (10mg/kg, ip) in several animal models of depression (e.g. LPS, CSD and LH, including the same ones used by Zanos et al.), giving rise to some unexplained discrepancy in the efficacy of HNK as an antidepressant (Shirayama and Hashimoto, 2018; Xiong et al., 2019; Yang et al., 2016; Zhang et al., 2018). Generally, as discussed, the WKY

model not only represents a relatively severe depressive-like phenotype, but this strain is generally more resistant to antidepressant treatment than control rats (Aleksandrova et al., 2019), which may also explain its apparent resistance to (2R,6R)-HNK. In addition, it is possible that (2R,6R)-HNK has antidepressant effects in this model at higher doses and/or in other preclinical tests beyond the FST. Overall, since unlike ketamine, (2R,6R)-HNK failed to decrease FST immobility in WKY rats despite restoring SC-CA1 LTP, this gives rise to a dissociation between rescuing LTP at this synapse and antidepressant effects in the FST in this model.

Therefore, we sought a different functional correlate of SC-CA1 synaptic plasticity in the WKY rat to determine whether rescuing LTP at this synapse contributes to ketamine's therapeutic effects beyond the FST. Previous work done in collaboration with our lab supports the critical role of dorsal hippocampal LTP in long-term spatial memory persistence, where AMPAR synaptic removal underpins the time-dependent memory decay (Hardt et al., 2013; Migues et al., 2010, 2016). Namely, these studies found that maintenance of LTP and associated long-term spatial memory in the HPC depends on the postsynaptic expression of GluA2-containing AMPA receptors (Hardt et al., 2013; Migues et al., 2010, 2016). The molecular processes involved in establishing LTP have been characterized well, but mechanisms underlying the decay of early and late LTP are poorly understood. The emerging literature implicates a decay-like forgetting process that involves loss of synaptic potentiation and the synaptic removal of GluA2-containing AMPARs, which actively erases consolidated long-term memories in the HPC and other brain structures over time (Hardt et al., 2013; Migues et al., 2010, 2016). This forgetting process, which is normally tightly regulated and contributes to establishing adaptive behavior, may be dysregulated in the context of neuropsychiatric disorders including depression, promoting the decline of memory and cognition. It has been shown that the

atypical protein kinase C isoform, protein kinase Mζ (PKMζ) is involved in long-term memory maintenance in various behavioral tasks and brain regions, including allocentric spatial memory and object location in the HPC (Hardt et al., 2013; Migues et al., 2010, 2016). Namely, studies found that maintenance of long-term memory in the HPC (as well as neocortex and amygdala) required the persistent action of PKMζ that regulates the trafficking of GluA2-containing AMPA receptors, which in turn directly correlates with the retention of object location recognition memory (Hardt et al., 2013; Migues et al., 2010, 2016). Although the underlying mechanisms are still under investigation, PKMζ seems to stabilize membrane AMPARs by persistently inhibiting their removal from postsynaptic sites, which effectively sustains synaptic potentiation and location memory (Hardt et al., 2013; Migues et al., 2010, 2016).

This led us to the hypothesis that the impaired SC-CA1 LTP in WKYs may lead to accelerated forgetting of long-term spatial memory, which should be rescued by ketamine and its metabolite. It is important to note that spatial tasks, such as Morris water maze (MWM) and contextual fear conditioning, are dependent upon the encoding and retrieval of emotionally aversive and inherently stressful training events (Vogel-Ciernia and Wood, 2014), which can obscure selective assessment of spatial memory processes, especially in stress-prone WKY rats. In addition, the psychomotor slowing and increased vulnerability to stress both lead to an increased tendency of WKY rats to float in water (e.g. FST), which likely complicates the interpretation of any deficits observed in the MWM. Furthermore, other spatial tasks, such as the T or Y maze, mainly involve food restriction and positive reinforcement (i.e. arms of the maze are baited with food reward) to motivate rodents to explore the maze (Vorhees and Williams, 2014), leading to possible confounds to their use in the WKY model due to the decreased body weight, motivational deficits and psychomotor slowing characteristic of this strain. In order to

avoid such possible confounding factors, we chose to assess hippocampal-dependent spatial memory using the object location recognition (OLR) test, an emotionally neutral task that involves unrestricted exploration in the absence of any external reinforcement or punishment, which instead exploits rodents' natural curiosity and innate preference for novelty (Migues et al., 2010; Vogel-Ciernia and Wood, 2014). This task is quite similar to procedures used in humans and should therefore have good predictive validity (Pitsikas et al., 2008).

Therefore, we utilized the OLR task, a hippocampal-dependent test of spatial memory to further study the effects of ketamine and its metabolite. First, we compared performance of WKY and WIS rats on location recognition memory at a delay of 1h or 24h, and found that as we hypothesized, while short-term OLR memory (at 1h) was equivalent between the two strains, long-term OLR memory (at 24h) was significantly impaired in WKYs, consistent with the hippocampal deficit in these rats. Moreover, consistent with the positive effects of drug treatment on WKY SC-CA1 LTP, ketamine and its metabolite (2R, 6R)-HNK both significantly restored long-term object recognition memory at 24h in WKY rats, to levels comparable to those in control WIS rats at 24h (~60%). In contrast, ketamine (5mg/kg, ip) had no effect on long-term location recognition memory in control WIS rats, consistent with the lack of effects on L-LTP in this strain. These results suggest that by facilitating LTP maintenance in WKY rats, ketamine and its metabolite prevented the accelerated forgetting of established, long-term object location memories in this model, highlighting the functional relevance of our findings.

Interestingly, although not significantly different from chance ($p>0.05$, n.s. vs. 50%), average OLR NL preference at 24h for the WKY strain at baseline was ~41%, instead of being closer to the 50% mark that indicates no discrimination between the objects at the novel and familiar locations and thus, no location recognition memory (i.e. chance performance). This

seems to be due to a small number of WKY rats, which exhibited a selective preference for the object at the FL (e.g. 4/25 SAL WKY rats with NL preference of 0%) and thus skew the group mean away from 50%. These results suggest that while most WKY rats display no significant long-term location recognition memory at a 24h delay (NL preference of around 50%), long-term OLR discrimination seems to be intact in a small subset of WKY rats, which in turn exhibit aversion towards novelty, consistent with the neophobia-like phenotype of this strain in other tasks (e.g. OFT, NSF, SI). Since ketamine and (2R,6R)-HNK both increase the WKY average NL preference at 24h to ~60%, as well as effectively eliminate this subpopulation of WKY rats displaying severe neophobia, it is possible that drug effects in the task involve a combination of eliminating a tendency toward novelty aversion in a small subset WKY rats and facilitating long-term memory retention in most WKY rats, which present with a spatial memory deficit.

Although systematic assessments of cognitive function have not been performed, several studies have reported various cognitive deficits in the WKY rat, highlighting this strain as a potential animal model for memory dysfunction in depression (Grauer and Kapon, 1993). As is the case of clinical depression, WKY rats have been reported to display various cognitive deficits, including impairments in spatial memory as we observed here. For example, although conflicting reports exist, these rats exhibit a pronounced learning impairment in the Morris water maze (MWM), where their latency to escape to the target platform is significantly longer than in controls (Diana et al., 1994; Grauer and Kapon, 1993; She et al., 2015). In addition, WKY rats fail to improve their performance in the MWM with repeated training, showing almost no retention of spatial information from trial to trial within the same day, indicating impaired memory capabilities compared to control rats (Diana et al., 1994; Grauer and Kapon, 1993; She et al., 2015). WKY rats have also been shown to have impaired performance in a T-maze using

non-matching-to-sample task, or in a Hebb-William's maze; however these studies used SHR rats as the reference strain (which represent a disease model themselves, while no proper outbred controls were included), and authors noted these differences could be attributed mainly to patterns of WKY behaviour that were incompatible with the required maze performance (Grauer and Kapon, 1993), echoing the potential confounds we considered in our choice of the OLR test. Moreover, WKY rats display a significant deficit in the novel object recognition task also indicative of memory dysfunction (Shoval et al., 2016). Given the limited literature and the multiple forms of learning and memory, we recommend that a battery of cognitive behavioral paradigms, which allows investigation of different memory systems, be applied to the WKY model, in order to further study the complex interactions between cognition, depression and regional abnormalities in synaptic and structural plasticity (e.g. in the HPC and PFC).

Outside of the WKY model, acute and chronic stress have been shown to impair hippocampal LTP, resulting in various working memory impairments rodents. Consistent with our findings with the WKY rat, exposure of normal rodents to learned helplessness (LH) chronic mild stress (CMS), chronic social defeat (CSD), as well as to exogenous chronic CORT administration, all of which represent established preclinical models of depression, has been shown to lead to cognitive deficits including an attenuation of spatial memory performance, in addition to a wide range of depressive-like behaviours (Darcet et al., 2014; Orsetti et al., 2007; Riaz et al., 2015; Riga et al., 2017; Song et al., 2006). In addition, such stress-induced impairments in cognitive performance have been linked to impaired LTP in pyramidal neurons of the dorsal HPC (e.g. in the case of the CMS and CSD paradigms) (Luo et al., 2014; Pavlides et al., 2002; Riga et al., 2017), paralleling what we observed in the WKY model. Interestingly, aging has been shown to cause a progressive decline in synaptic function and significant

impairments in the ability to express LTP, which correlate with the impaired ability to process and store information (Arias-Cavieres et al., 2017). For example, aged outbred rats, which display deficits in hippocampal LTP, also exhibit impairments in long-term spatial memory (including object location recognition). Therefore, the literature suggests that dysregulation of hippocampal synaptic plasticity and associated memory processes, which normally occurs with stress or aging, may be an innate characteristic of the WKY model (Arias-Cavieres et al., 2017).

Interestingly, acute high-dose or subchronic ketamine (e.g. 30-100mg/kg, ip , once; or 5-30mg/kg, ip, twice daily for 7-14 days) has been shown to impair various aspects of cognition including location recognition memory in rodents (Duan et al., 2013; Lin et al., 2016; Pitsikas et al., 2008; Pitsikas and Boultadakis, 2009; Sabbagh et al., 2012; Schumacher et al., 2016; Venâncio et al., 2011). However, ketamine's effects on cognitive function such as spatial learning and memory at low sub-anesthetic doses (5-10mg/kg, ip) in the context of depression have not been extensively studied (Refsgaard et al., 2017). In addition, previous studies indicate that while pre-training administration of ketamine may disrupt rodent acquisition of various memory paradigms, drug effects on post-training memory processes (consolidation and/or retrieval) are not well understood (Pitsikas et al., 2008). Overall, the limited preclinical literature has reported positive, negative, as well as no effects of ketamine (<30mg/kg, ip) on performance of various spatial memory tasks (Ribeiro et al., 2013; Valentim et al., 2013; J. H. Wang et al., 2006). Conflicting findings likely arise from differences in experimental design, such as the rodent model (rats vs. mice), dose regimen (1-30mg/kg, ip; once or repeated), timing (after ketamine injection and relative to training/testing), and the behavioral task investigated (e.g. from passive avoidance to object or location recognition memory), making it difficult to draw any conclusions.

Contrary to our findings of no effects of ketamine on OLR performance in WIS rats, one previous study reported that both pre- and post-training administration of ketamine at low doses (1-3mg/kg, ip) disrupted spatial and non-spatial recognition memory in WIS rats in a dose-dependent manner (Pitsikas et al., 2008). Interestingly, in this study ketamine impaired recognition memory at very low doses (1-3 mg/kg) compared with those normally reported to disrupt spatial working memory in rodents (15–30 mg/kg) (Boultadakis and Pitsikas, 2010; Moosavi et al., 2012; Pitsikas et al., 2008). Importantly, in this experiment, ketamine was administered right after the OLR training session and the test session was performed at a delay of 20min, ensuring that ketamine concentrations in the brain were peaking at the time of testing, when the memory disruption was observed (Pitsikas et al., 2008). As we observed, ketamine has no major effects on hippocampal synaptic plasticity at 30min after injection (at least in WKY rats); however, in this acute phase, non-specific effects (e.g. attentional and sensory-motor side effects, etc.) may have influenced the animals' performance, thus obscured effects any specific effects on spatial memory (Pitsikas et al., 2008). Therefore, it is possible that even acute, low-dose ketamine may worsen location recognition in rats when given immediately pre- training or testing (i.e. drug was on board during task performance) (Boultadakis and Pitsikas, 2010; Moosavi et al., 2012; Pitsikas et al., 2008). On the other hand, in our study, ketamine was not present during either the training and testing phases; instead, it was injected 3.5h before the second training session, at which point it has no effects in WIS rats in terms of either LTP or OLR performance, but seems to prevent the accelerated decay of training-induced hippocampal LTP in WKY rats and sustain the associated location recognition memory in this strain when tested 24h later (27.5h after ketamine injection).

Therefore, it is reasonable to conclude that at high doses and/or repeated schedules of administration, especially when cognitive training/testing is carried out with drug on board, ketamine is likely to have negative effects on hippocampal transmission and function (e.g. 30mg/kg, ip causes robust SC-CA1 synaptic depression for 4h *in vivo* (Duan et al., 2013)), and thus, to also impair spatial learning and memory (Duan et al., 2013; Lin et al., 2016; Pitsikas et al., 2008; Pitsikas and Boultadakis, 2009; Sabbagh et al., 2012; Schumacher et al., 2016; Venâncio et al., 2011). Indeed, high sub-anesthetic doses also lose their antidepressant efficacy (e.g. at 15 and 30 mg/kg, ip ketamine), due to possible onset of non-specific, sedative or cognitive (eventually psychotic-like) effects (Papp et al., 2017). However, at doses/time points after injection when ketamine has positive effects on hippocampal synaptic plasticity and function, especially in the context of in models of hippocampal dysfunction (e.g. the WKY rat, stressed or aged outbred rats), we would also expect to see pro-cognitive effects of ketamine treatment (e.g. restoration of spatial memory). Since data supporting this idea in the context of ketamine is still limited outside of our study, further investigation is clearly warranted.

Importantly, a pro-cognitive effect of ketamine, as observed in our study, is not unprecedented. Importantly, one recent study found that ketamine (10 mg/kg, ip for 5-7days) reversed the anhedonic (SPT), anxiogenic (EPM) and dyscognitive (NOR) effects of CMS in WKY rats (Willner et al., 2018). Another study, which exposed WIS rats to CMS followed by subacute (3-5 days) or chronic (5 weeks) treatment with ketamine (10 mg/kg, ip), found that in addition to sustained antidepressant-like effects, ketamine also eliminating the stress-induced loss of discrimination in the novel object recognition test, also supporting its pro-cognitive effects in the context of depression (Papp et al., 2017). Similarly, others have demonstrated positive effects of ketamine on cognition in the CMS model and following repeated restraint

stress using an attentional set-shifting task (Jett et al., 2015; Papp et al., 2017; Patton et al., 2017). Importantly, one study found that ketamine (5mg/kg, ip) reversed both the stress-induced impairments of SC-CA1 LTP *in vitro* and the associated deficits in spatial working memory and contextual fear memory at 24h post-injection in mice subjected to CSD (Yang et al., 2018).

Interestingly, as mentioned previously, electroacupuncture (EA), which was reported to exert antidepressant-like activity in WKY rats the FST, also rescued the impaired SC-CA1 LTP *in vitro* and restored HPC-dependent spatial memory in the MWM in these rats (She et al., 2015), similar to our observations with ketamine, SC-CA1 LTP and location recognition memory in this strain. Furthermore, one study found that aged outbred rats showed a robust age-related deficit in spatial learning in the MWM, as well as a selective impairment in the magnitude of SC-CA1 LTP, both of which were rescued by GLYX-13 (rapastinel), a novel compound with ketamine-like antidepressant activity which acts as an NMDAR partial agonist (Burgdorf et al., 2011b). In addition, chronic exposure of normal rats to stress has been shown to impair both hippocampal CA1 LTP and associated MWM performance, while pharmacological or environmental interventions (e.g. environmental enrichment) may reverse these deficits, further supporting a link between hippocampal synaptic plasticity and spatial memory in the context of stress/depression (Alfarez et al., 2003; Conrad et al., 1996; Luo et al., 2014; Pavlides et al., 2002; Yang et al., 2007).

Overall, consistent with the SC-CA1 LTP deficit and corresponding effects of drug treatment, WKYs exhibited accelerated forgetting of HPC-dependent long-term spatial memory, which was effectively restored by both ketamine and (2R,6R)-HNK, highlighting their potential pro-cognitive action in the context of depression, as well as the role of hippocampal synaptic plasticity in the development and reversal of certain depressive-like phenotypes.

Chapter 7: General Discussion

7.1 Overview of Findings

Accumulating evidence implicates dysregulation of synaptic plasticity, particularly in the hippocampus (HPC), in the pathophysiology of depression. Ketamine's efficacy in treating TRD holds promise for a new generation of much needed, superior antidepressant agents. One theory is that ketamine may reverse the stress-induced loss of connectivity in key neural circuits by engaging synaptic plasticity processes to "reset the system". However, its effects on *in vivo* synaptic plasticity in the HPC and beyond, as well as their direct contribution to ketamine's antidepressant action, are still unclear. The Wistar-Kyoto (WKY) rat is an established preclinical model of stress susceptibility and depression, which exhibits various behavioural, neurochemical and endocrine parallels to the clinical condition, including an endogenous depression-like phenotype spanning across a wide range of domains affected in MDD. In **Chapter 3**, we replicated key depressive and anxiety -like phenotypes of the WKY rat using the open field test (OFT), forced swim test (FST), novelty suppressed feeding (NSF) test, and progressive ratio (PR) schedule of reinforcement. We found that WKY rats exhibit pronounced impairments across all behavioural tests compared to normal Wistar rats, including hypolocomotion and less time spend in the center of the arena in OFT, dramatic immobility in the FST, longer latencies to feed in the NSF test, as well as lower break point and psychomotor slowing in the PR test (which develop with repeated testing). In **Chapter 4**, WKY rats were further characterized in terms of SC-CA1 synaptic plasticity using *in vivo* extracellular field recordings. First, we found that basal synaptic transmission at this synapse was comparable between the two strains. Moreover, LFS (3Hz, 900 pulses, 5min) failed to induce LTD in either WKY or WIS rats *in vivo*, indicating no significant facilitation of LTD in the stress-prone WKY strain. In contrast, both early and late

LTP (induced by either 1x or 4x trains of HFS, 100Hz, 1s, 5min ITI) at the SC-CA1 synapse were significantly impaired in WKY rats compared to controls due to a pronounced deficit in LTP maintenance in this model. In **Chapter 5**, we tested the effects of ketamine and its metabolite (2R,6R)-HNK on the SC-CA1 L-LTP deficit observed in WKY rats. We found that ketamine and (2R,6R)-HNK (5mg/kg, ip) did not have any acute effects on SC-CA1 basal synaptic transmission. Importantly, ketamine acutely restored the impaired SC-CA1 LTP in WKY rats (at 3.5h but not 30min after injection, with residual effects at 24h), leading to a subsequent increase in basal synaptic transmission at 24h, indicating potentiated WKY SC-CA1 synapses at this later time point. Similar to its parent drug, (2R,6R)-HNK also effectively restored normal SC-CA1 L-LTP in WKY rats when the HFS protocol was given 3.5h after injection. Therefore, at this dose, ketamine and its metabolite seem to facilitate AMPAR-mediated excitatory synaptic transmission in the WKY model in an activity-dependent manner (i.e. selectively modulating metaplasticity processes but not basal synaptic transmission *in vivo*). In **Chapter 6**, we aimed to understand whether rescuing SC-CA1 L-LTP in this model mediates, or at least contributes to, ketamine's mechanism of action as an antidepressant. First, we successfully replicated ketamine's significant rapid (30min) and sustained (24h) FST antidepressant effects in both WKY and WIS rats. However, (2R,6R)-HNK had no effect on FST immobility in the WKY model, leading to a dissociation between FST antidepressant-like activity and effects on hippocampal synaptic plasticity. Importantly, however, consistent with the observed SC-CA1 LTP deficit and corresponding effects of drug treatment, WKYs exhibited accelerated forgetting of hippocampal-dependent long-term spatial memories (as measured by the OLR test at a delay of 24h), which was effectively restored by pre-treatment with both ketamine and (2R,6R)-HNK. Based on these findings, we propose that, in the WKY model of

depression, restoring the impaired SC-CA1 LTP does not underlie ketamine's antidepressant effects in the FST (considered a "canonical" measure of antidepressant activity), but may more specifically mediate reversal of hippocampal-dependent cognitive deficits (including spatial memory), which are also key features of clinical depression.

7.2 Synaptic Plasticity in the WKY Model

Although the literature is still limited, several pivotal studies report various deficits related to synaptic plasticity in the WKY rat (also discussed in section 5.9), highlighting the utility of this model for the study of synaptic plasticity processes and how they contribute to the development or reversal of different depressive-like phenotypes (Aleksandrova et al., 2019; Belujon and Grace, 2014; Cominski et al., 2014; Fortress et al., 2018; Fragale et al., 2016; Han et al., 2018; Kanzari et al., 2018; She et al., 2015). The available data indicates deficits in long-term potentiation (LTP) in the WKY rat within several key neural circuits implicated in depression, namely intra-hippocampal pathways, as well as afferent projections from the amygdala to the prefrontal cortex and from the hippocampus to the nucleus accumbens (Figure 26).

Consistent with our results, the consensus is that WKY rats exhibit a pronounced deficit in hippocampal synaptic plasticity and function (Cominski et al., 2014; Fortress et al., 2018; Han et al., 2018; She et al., 2015). In addition to significant reductions in hippocampal volume, WKY rats are characterized by impaired hippocampal LTP, at both mPP-DG and SC-CA1 synapses (Cominski et al., 2014; Fortress et al., 2018; Han et al., 2018; She et al., 2015). On the other hand, it is possible that novel antidepressant treatment (e.g. ketamine, electroacupuncture or even certain protocols of electrical stimulation) may rescue the ability to induce LTP in these circuits, thereby restoring normal hippocampal-dependent function and reversing the associated

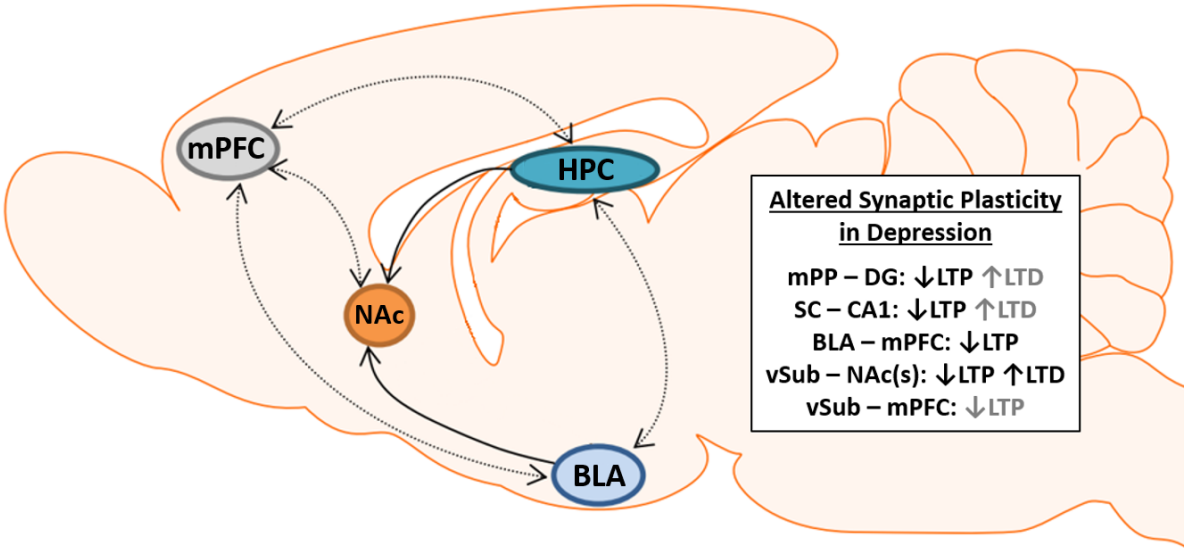

Figure 26. Neural circuitry in depression and corresponding synaptic plasticity changes in the WKY rat and other models.

Sagittal slice of the rat brain showing the hippocampus (HPC), medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and nucleus accumbens (NAc) as well as their direct connections. Altered synaptic plasticity within key projections has been demonstrated in the WKY rat (in black) and stress-based models (in grey). Circuit-specific dysfunctions in synaptic plasticity are thought to contribute to the loss of normal connectivity between key brain regions, and to the development and/or reversal of depressive- and anxiety-related behaviors. Figure reprinted from Aleksandrova et al. (2019) with permission.

behavioural deficits in WKY rats (Cominski et al., 2014; Fortress et al., 2018; Han et al., 2018; She et al., 2015). Therefore, the role of hippocampal synaptic plasticity in mediating specific depressive-like phenotypes and antidepressant responses of WKY rats is highlighted and warrants further systematic investigation. In addition, the limited data suggests that circuit dysfunctions in the WKY model are not limited to intra-hippocampal projections but extend to other key neural pathways implicated in depression. Interestingly, one study found a significant LTP deficit at the BLA-PL cortex projection in WKY rats, and lesions of the PL cortex in SD rats recapitulated the extinction-resistant avoidance phenotype of WKY rats, supporting the hypothesis that dysfunction of amygdala-prefrontal circuits underlies at least some aspects of the

WKY phenotype (Fragale et al., 2016). Another pivotal study reported that learned helplessness in WKY rats is associated with decreased VTA DA neuron population activity, as well as LTD (instead of LTP) in the vSub-shell NAc projection following HFS (Belujon and Grace, 2014). Importantly, ketamine reversed these effects and rescued escape behaviour in 'helpless 'WKY rats, suggesting that its antidepressant effects may be mediated at least in part, via synaptic plasticity within the mesolimbic DA system (Belujon and Grace, 2014). Finally, as mentioned, previous studies report significantly reduced connectivity in white matter fibre tracts known to be altered in MDD (Zalsman et al., 2016), as well as reduced resting-state frontal cortical perfusion in WKY compared to normal rats (Gormley et al., 2016). Therefore, our results and the existing literature support the idea that the complex depression-like phenotype of the WKY rat may result from complex dysregulation of regional synaptic plasticity leading to a breakdown of communication between key brain areas implicated in MDD, as well as highlighting key neural circuits where data is still lacking in the context of the WKY model (Figure 26).

7.3 Synaptic Plasticity in Depression

7.3.1 Roles of LTP and LTD

Synaptic plasticity underlies the fundamental ability of the brain to sense, evaluate and store complex information and to make appropriate, adaptive responses in an ever-changing environment (Duman et al., 2016). As described previously, accumulating preclinical evidence suggests that stress can perturb the normal balance between LTP and LTD, inhibiting LTP and/or facilitating LTD in key vulnerable areas of the forebrain (e.g. HPC, PFC) (see section 1.2 for specific references). Based on the available preclinical literature, stress seems to exert modulatory effects on synaptic plasticity by altering the induction threshold for LTP and LTD,

reflecting metaplastic changes in the ability to induce different forms of synaptic plasticity as a result of prior neuronal activity (Howland and Wang, 2008). In turn, since LTP facilitates spine formation and enlargement, whereas LTD is associated with spine shrinkage or retraction, chronic stress may lead to synaptic weakening and neuronal atrophy by more directly modulating synaptic plasticity processes (Duman, 2014b; Leuner and Shors, 2013; Marsden, 2013). Thus, prolonged stress-induced circuit-level changes in synaptic plasticity may underlie, or at least contribute to, the profound structural plasticity such as the significant grey matter volume reductions observed in MDD and following stress (Aleksandrova et al., 2019; Chattarji et al., 2015; Duman, 2014b; Marsden, 2013).

It is important to note that while studies using hippocampal slices from normal animals subjected to stress or treated with exogenous stress hormones have demonstrated both an inhibition of LTP and a facilitation of LTD in parallel, most studies performed *in vivo* in the context of valid models of depression (e.g. in the WKY rat) have reported selective impairments of LTP within key neural pathways of interest (Aleksandrova et al., 2019). This apparent discrepancy seems to be due to the fact that, as observed for the WKY model, LTP is more commonly studied than LTD since it is easier to investigate *in vivo* and has more clear functional/behavioural correlates, thus creating a bias for results involving LTP. As mentioned, homosynaptic LTD is reported to occur in the CA1 of hippocampal slices of young animals or adult rodents subjected to acute or chronic stress; unfortunately, however, many studies report a failure to induce *de novo* homosynaptic LTD in the intact brain in response to prolonged single-pulse stimulation at low frequency (Staubli and Scafidi, 1997). Therefore, hippocampal LTD has proven difficult to obtain reliably *in vivo*, and LFS protocols are generally associated with low success rates of inducing LTD in awake rats, as we found here regardless of the strain.

Moreover, it is questionable whether stimulation protocols represent patterns of activity likely to occur during behavior (Staubli and Scafidi, 1997). Indeed, traditional forms of plasticity are induced by protocols based on stimulation frequencies that are often far from physiological and thus unlikely to occur *in vivo* (Mateos-Aparicio and Rodríguez-Moreno, 2019). In addition, based on accumulating evidence indicating that LTP decay is not only an active process but is mechanistically similar to *de novo* LTD, LTP 'depotentiation' may serve as a proxy to studying the role of LTD in depression *in vivo*, which itself is complicated by technical and biological challenges (Collingridge et al., 2010; Staubli and Scafidi, 1997).

7.3.2 Role of Hippocampal Dysfunction

Clinical studies supporting the role of hippocampal dysfunction in the etiology of depression report that HPC volume loss predicts illness duration and severity and is associated with poorer clinical outcomes and well-documented cognitive deficits (Campbell and MacQueen, 2003; Duman et al., 2016). Since the hippocampus is a key hub within the limbic system crucial for processing of contextual information, a pronounced hippocampal deficit, as seems to be the case for both WKY rats and patients suffering from mood and/or anxiety disorders, can result in impairments in various aspects of hippocampal-dependent learning (Campbell and MacQueen, 2003; Chaudhury et al., 2015; Cornwell et al., 2010; Duman et al., 2016; Kim and Diamond, 2002; Pittenger and Duman, 2008). For example, hippocampal dysfunction can lead to disruption of context-dependent memories, resulting in an inability to distinguish between positive, negative or neutral contexts (e.g. safety vs. threat), which would explain the extinction-resistant avoidance phenotype of hippocampal-deficient WKY rats (Fortress et al., 2018). Given the role of the HPC in binding contextual and affective elements of

experience, such a failure to adequately process environmental cues required to facilitate adaptive behaviour may render an individual more vulnerable to developing symptoms of depression and/or anxiety, as well as contributing to the complex cognitive deficits observed in MDD (Campbell and MacQueen, 2003; Chaudhury et al., 2015; Cornwell et al., 2010; Duman et al., 2016; Fanselow and Dong, 2010; Fortress et al., 2018; Kim and Diamond, 2002; Pittenger and Duman, 2008). On the other hand, by promoting hippocampal synaptic plasticity, synaptogenesis and general function, antidepressant therapies, particularly ketamine, may have the ability to restore normal hippocampal connectivity, thereby normalizing depressive- and anxiety- like symptoms in animal models and human patients (Abdallah et al., 2017; Fortress et al., 2018). Although promising, the available data emphasizes the need for more direct, systematic evaluations of hippocampal synaptic plasticity changes at baseline and following antidepressant treatment in WKY rats, as well as other animal models of depression.

7.3.3 Role of Prefrontal Dysfunction

Similar to observations within the HPC, reductions in cortical volume and aberrant fronto-limbic brain circuitry have been reported in both the WKY model and clinical depression (Abdallah et al., 2017; Cominski et al., 2014; Duman et al., 2016; Licznerski and Duman, 2013; Nestler et al., 2002). The mPFC is a central hub underlying various executive and cognitive functions including attention, working memory, cognitive flexibility, decision making, inhibitory control, emotional regulation, long-term memory and habit formation, many of which are affected in MDD and may underlie core depression symptoms and/or worsen clinical outcomes (Licznerski and Duman, 2013; Riga et al., 2014; Rock et al., 2014). Therefore, since the mPFC is a key coordinator of autonomic, neuroendocrine and behavioural responses to stress,

dysfunctions in mPFC circuitry including its reciprocal projections with key limbic regions (e.g. amygdala and HPC) are linked, at least in part, to the emotional dysregulation and cognitive impairments associated with depression (Arnsten, 2015; Licznerski and Duman, 2013; McKlveen et al., 2015; Riga et al., 2014).

7.3.4 Role of Amygdala-Prefrontal Dysfunction

Paralleling findings within the HPC, abnormal synaptic plasticity (i.e. impaired LTP) in the amygdala-PFC pathway, which appears to be an innate characteristic of WKY rats and depressed patients, contributes to an increased vulnerability to developing depression- and/or anxiety- like symptoms (Dannlowski et al., 2009; Fragale et al., 2016; Maroun and Richter-Levin, 2003). Consistent with the one study reporting a significant LTP deficit at the BLA-PL cortex projection in WKY rats (Fragale et al., 2016), preclinical studies indicate that not only is LTP at the BLA-PL cortex pathway sensitive to modulation by stress, it also may participate in top-down control of emotional learning and memory (Fragale et al., 2016; Maroun and Richter-Levin, 2003). Indeed, impaired LTP at this synapse appears to block the executive functioning of the PR cortex, which in turn sends reciprocal connections back to the amygdala (Maroun and Richter-Levin, 2003). Normal cortical modulation of amygdala circuitry is important for aversive learning and when compromised, may heighten the response of the amygdala to stressors, as in the case for the extinction-resistant avoidance behaviour in WKY rats (Fragale et al., 2016). Importantly, direct support for a disruption in amygdala–prefrontal emotion regulation circuitry in depression is provided by functional magnetic resonance imaging (fMRI) studies showing significantly reduced amygdala–prefrontal connectivity in depressed patients, which correlated with disease severity (Dannlowski et al., 2009). Importantly, in contrast to the stress-induced

atrophy in the HPC and PFC, chronic stress leads to synaptogenesis and hypertrophy of amygdala neurons, which combined with the impaired top-down inhibitory control, results in increased amygdala activity, contributing to the altered mood, emotion, and anxiety in MDD (Chattarji et al., 2015; Licznerski and Duman, 2013).

7.3.5 Role of Hippocampal-Prefrontal Dysfunction

Another pathway strongly implicated in MDD and antidepressant response is the HPC-mPFC projection, which mediates hippocampal modulation of the ability of the mPFC to exert top-down executive control over the processing of appetitive and aversive stimuli (Godsil et al., 2013). In fact, the functional integrity and bidirectional flow of information (e.g. via correlated firing activity) between the hippocampus and mPFC is critically important for optimal mPFC functioning, processes that are particularly susceptible to stress (Godsil et al., 2013; Jay et al., 2004). Furthermore, it is possible that by inducing hippocampal synaptic dysfunction and neuronal atrophy, stress in turn, reduces afferent activity to the PFC, which could lead to fewer PFC dendritic spines (Leuner and Shors, 2013). In addition, Jay and colleagues have shown that LTP at the HPC-mPFC synapse is driven by the levels of mesocortical DA, and importantly, that stress leads to an LTP impairment, which may in turn underlie further decreases in PFC DA tone (Jay et al., 2004; Mailliet et al., 2008). Importantly, one fMRI study of MDD patients reports motor memory consolidation deficits associated with decreased HPC-PFC connectivity (Genzel et al., 2015). Thus, both preclinical and human studies support a role for structural and functional abnormalities in hippocampal-prefrontal connectivity in depression and the effects of stress (Genzel et al., 2015; Godsil et al., 2013; Jay et al., 2004). Rodent studies indicate the involvement of the ventral HPC (vSub) – mPFC projection in the mechanism of action of rapid

antidepressants, as indicated by the finding that activation of this pathway is both necessary and sufficient for the antidepressant effects of ketamine (Carreno et al., 2016; Jett et al., 2015; Li et al., 2010). Given the potential importance of the hippocampal–prefrontal circuit in depression and ketamine antidepressant response, future studies of synaptic plasticity at this synapse in the context of WKY rats, as well as other models of depression, are of great interest.

7.3.6 Role of Hippocampal-Nucleus Accumbens Dysfunction

In addition to contributing to aspects of cognition, executive function and emotional regulation via its projections to the mPFC, the ventral HPC (vSub) also serves an important role in the modulation of the DAergic system via its projection to the NAc (Herman and Mueller, 2006; Taepavarapruk et al., 2008). Although the monoamine hypothesis is no longer in vogue as the unifying theory of depression, dysfunction within the brain's reward/motivation circuitry, particularly the mesolimbic pathway from the VTA to the NAc, is thought to underlie aberrant reward-associated perception, learning and memory and the core symptom of anhedonia in MDD (Belujon and Grace, 2014; Russo and Nestler, 2013; Sanchis-Segura et al., 2005). Interestingly, learned helplessness in WKY rats was associated with decreased VTA DA neuron population activity, as well as LTD (instead of LTP) in the vSub-shell NAc projection following HFS, effects which were reversed by ketamine (5mg/kg, ip) (Belujon and Grace, 2014). Given that glutamatergic afferents from the vSub to the NAc exert a potent excitatory effect on VTA DA neurons, dysfunctions in hippocampal circuitries may contribute to the hypoexcitable state of the DA system, and thereby to the hedonic and amotivational deficits observed in depression and the WKY model (Floresco et al., 2001; Herman and Mueller, 2006; Russo and Nestler, 2013), which may be effectively eliminated by ketamine, a research avenue that warrants further investigation.

Taken together, several lines of evidence discussed here implicate the vSub in depression, particularly because of its role as the major output region of the HPC projecting to key areas such as the mPFC and NAc, as well as exerting inhibitory influence over the HPA axis (see below), thereby contributing to top-down executive control, context-dependent regulation of reward circuitry and stress integration (Floresco et al., 2001; Godsil et al., 2013; Herman and Mueller, 2006; Jay et al., 2004).

7.3.7 Role of Hypothalamic–Pituitary–Adrenal (HPA) Axis Dysfunction

Finally, precise regulatory control over the HPA axis and associated stress responses is clearly disrupted in patients suffering from mood and anxiety disorders, as well as in WKY rats (Krishnan and Nestler, 2008; Nestler et al., 2002; Rittenhouse et al., 2002; Solberg et al., 2001; Villanueva, 2013). The HPA stress response is primarily driven by corticotrophin releasing hormone (CRH) released from the paraventricular nucleus (PVN) of the hypothalamus. In turn, the PVN receives several major regulatory inputs, with the prelimbic cortex and hippocampal vSub exerting an inhibitory influence, whereas the amygdala and infralimbic cortex facilitate HPA axis activation (Herman et al., 2005; Jankord and Herman, 2008; Levy and Tasker, 2012; Nestler et al., 2002). Accordingly, the balance between these regulatory inputs under different conditions is crucial for proper stress integration and an adaptive stress response (Jankord and Herman, 2008; Kerr and Blanpied, 2012). As the HPC has the highest density of glucocorticoid receptors (GRs) in the brain, it is particularly vulnerable to the neurotoxic effects of stress, and prolonged exposure to GCs can lead to a loss of hippocampal GRs, reduced negative feedback and disinhibition of the HPA axis (Jankord and Herman, 2008; Willner et al., 2014). On the other hand, stress-induced hypertrophy and reduced cortical inhibition of the amygdala likely also

contribute to the pathological HPA hyperactivity commonly observed in patients with MDD (Chattarji et al., 2015; Licznerski and Duman, 2013). Although data are still lacking, differential effects on synaptic plasticity in the various PVN regulatory pathways may contribute to the increased vulnerability to stress, the chronic state of HPA disinhibition and the development and reversal of depression- and anxiety- like symptoms, a research avenue that warrants further investigation in animal models of depression including the WKY rat.

7.3.8 Summary of Synaptic Plasticity Theory of Depression

An emerging theory proposes that the wide range of clinical symptoms associated with depression may be mediated by impaired synaptic plasticity and reduced connectivity between key brain regions particularly susceptible to stress (Figure 27) (Aleksandrova et al., 2019). Given that LTP facilitates spine formation and enlargement, whereas LTD is associated with spine shrinkage or retraction, stress-induced circuit level changes in synaptic and structural plasticity

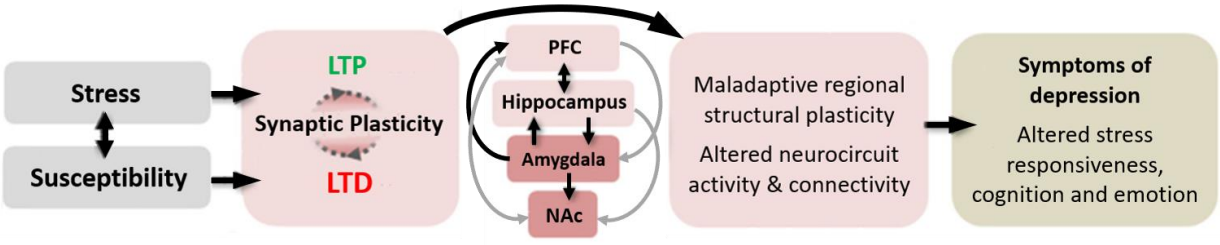

Figure 27. Simplified Hierarchical Diagram for the Pathogenesis of Depression.

Complex interplay between environmental stressors and susceptibility to stress/depression (e.g. genetic, developmental or personality factors) may lead to dysregulation of synaptic plasticity processes in key neural circuits. Perturbing the normal balance between LTP and LTD may lead, or at least contribute, to the loss of connectivity between brain regions implicated in MDD, the hippocampus (HPC), medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and nucleus accumbens (NAc). Resulting maladaptive neurocircuit activity is, in turn, hypothesized to lead to a persistent shift in stress responsiveness, cognition and emotion, thus underlying the various clinical symptoms of depression. Figure reprinted from Aleksandrova et al. (2019) with permission.

are likely to be interdependent (Aleksandrova et al., 2019; Leuner and Shors, 2013). Thus, disruption of the normal balance between LTP and LTD by chronic stress may eventually result in destabilization and loss of synaptic connections in various key circuits, which together mediate the reduced top-down control, aberrant context-dependent emotional regulation, abnormalities in the brain's motivation/reward systems, maladaptive stress integration, as well as the various cognitive deficits observed in depressed patients (Aleksandrova et al., 2019; Chattarji et al., 2015; Cornwell et al., 2010; Duman et al., 2016; Dwyer and Duman, 2013; Gerhard et al., 2016; Lener et al., 2017b; Marsden, 2013; Pittenger and Duman, 2008; Roiser and Sahakian, 2013; Sapolsky, 2015). Overall, the stress-induced regional modulation of synaptic and structural plasticity appears to weaken hippocampal networks involved in processing of contextual information, stress integration and modulation of cortical function and reward circuits, as well as compromising the function prefrontal networks mediating higher cognition and top-down inhibitory control over limbic systems, while strengthening the amygdala, primary sensory cortices and the striatum (Arnsten, 2015; Chattarji et al., 2015; Licznerski and Duman, 2013; Marsden, 2013). These patterns of activity switch the brain from 'reflective to reflexive' control of behavior, and are exaggerated by chronic stress exposure or in stress susceptible individuals, eventually leading to maladaptive structural plasticity and persistent loss of normal connectivity in these key neural circuits implicated in MDD (Aleksandrova et al., 2019; Arnsten, 2015; Chattarji et al., 2015; Licznerski and Duman, 2013; Marsden, 2013).

7.4 Synaptic Plasticity in Ketamine's Mechanism of Action

7.4.1 Effects of Ketamine on Synaptic Plasticity

As discussed in section 5.9, emerging evidence indicates ketamine may selectively modulate synaptic plasticity processes, particularly in the rodent HPC; however, a lot of conflicting reports currently exist in the literature. Namely, as mentioned, two previous studies reported that ketamine (3-10mg/kg, IV or 30 mg/kg, ip) enhances SC-CA1 fEPSP LTP (following sub-maximal HFS) in hippocampal slices obtained from SD rats at 24h post-injection (Burgdorf et al., 2013; Graef et al., 2015), similar to what we observed in the WKY strain (although at a lower dose of 5mg/kg, ip). Interestingly, one study found that chronic social defeat stress applied to mice impaired LTP *in vitro* at the SC-CA1 synapse, which was reversed by administration of ketamine (5mg/kg, ip) at 24h post-injection (Yang et al., 2018). However, importantly, other studies have also reported no change or even a decrease in LTP (Fortress et al., 2018; Izumi and Zorumski, 2014; Michaëlsson et al., 2018; Ribeiro et al., 2014), and/or a block of LTD (Huang et al., 2016; Ribeiro et al., 2014) in the HPC of outbred rats following ketamine administration. For example, one study found no effect of ketamine (10mg/kg, ip) on synaptic efficacy or LTP induction at the SC-CA1 synapse at 24h post-injection in adolescent and adult depressive-like WIS rats treated with exogenous glucocorticoids (Michaëlsson et al., 2018). As discussed, likely contributing to the conflicting results, collectively, these studies suffer from important limitations, such as the fact that they use different doses and routes of ketamine administration (in several cases even bath application onto slices). In addition, these electrophysiology experiments are conducted at different (often single) time points after ketamine administration, as well as use different LTP/LTD protocols and strains of outbred rats (naïve or not), leading to an abundance of possible reasons for the discrepancy seen in the

literature. Moreover, most of the limited direct data comes from electrophysiological recordings performed in hippocampal brain slices (i.e. lack of intact brain circuitry, harder to mimic physiological conditions, etc.), often obtained from normal rodents (not in a stress-susceptible or depressed-like state) (Burgdorf et al., 2013; Izumi and Zorumski, 2014; Michaëlsson et al., 2018; Nosyreva et al., 2013; Ribeiro et al., 2014; Yang et al., 2018). It is important to note, the concentrations used in most electrophysiological studies that bath apply ketamine (e.g. 20μM ketamine for 1h) to study its effects of on synaptic transmission and plasticity far exceed peak therapeutic ketamine concentrations in depression (<1μM in humans following 0.5mg/kg, IV; ~5uM in mice after 10mg/kg, ip), putting the physiological relevance of such results into question (Zanos et al., 2018a, 2016).

As previously discussed, indirect preclinical evidence supports an antidepressant mechanism of action of ketamine that involves an LTP-like potentiation of excitatory synaptic transmission in the rodent HPC and mPFC. Namely, a single antidepressant dose of ketamine increases activation of key signaling pathways (BDNF and mTOR) and enhances excitatory synaptic transmission, ultimately leading to synaptogenesis and reversal of stress-induced synaptic destabilization and neuronal atrophy (Aleksandrova et al., 2019, 2017a). At the level of neural circuits, both animal and human imaging studies suggest that ketamine is be able to effectively reverse the loss of connectivity between the HPC, PFC and other limbic structures (e.g. amygdala, NAc, etc.) in depressed individuals. Although further research is clearly needed, through these actions, ketamine appears to reinstate normal hippocampal and prefrontal function, restoring appropriate top-down inhibitory control of amygdala and HPA axis reactivity (Chattarji et al., 2015; Licznerski and Duman, 2013). Importantly, fMRI studies of TRD patients found that antidepressant doses of ketamine normalized the significant reductions in prefrontal global brain

connectivity (Abdallah et al., 2017), as well as induced synaptic potentiation in the somatosensory cortex of responders but not non-responders (Cornwell et al., 2012), further supporting a relationship between synaptic plasticity and antidepressant response. Similarly, in rodent fMRI studies, ketamine administration (10mg/kg, ip) increases connectivity between regions implicated in MDD that mediate reward and cognitive aspects of emotional processing, including the ventromedial prefrontal cortex, nucleus accumbens and septal nuclei (Gass et al., 2018). As mentioned, one study performed in WKY rats demonstrated that ketamine reversed synaptic plasticity deficits within the vSub-NAc projection, which in turn may normalize VTA DA neuron population activity, suggesting that its antidepressant effects may be mediated at least in part, via synaptic plasticity within the mesolimbic DA system (Belujon and Grace, 2014). Accordingly, ketamine treatment may exert its action by engaging synaptic plasticity processes to "reset" key dysfunctional systems (Aleksandrova et al., 2019). Future research will shed more light ketamine's effects on *in vivo* synaptic plasticity (at therapeutically relevant doses and at different time points after injection) within different circuits implicated in MDD, as well as the direct contributions of such changes to ketamine's antidepressant effects in WKY rats and other animal models of depression.

7.4.2 Role of the Dorsal Versus Ventral Hippocampus, and Beyond

As mentioned, there is a clear functional and anatomical division within the HPC along the dorsal-ventral axis in the rodent brain. Lesions of the dorsal HPC in rats result in impairments of spatial memory, whereas lesions of the ventral subiculum, appear to remove the negative feedback inhibition on the HPA axis, and resulting in elevated glucocorticoid levels and abnormal stress responses (Campbell and MacQueen, 2003; Fanselow and Dong, 2010). In

addition, genes expressed in the dorsal HPC correlate with cortical regions involved in information processing, while gene expression in the ventral HPC correlates with regions involved in emotion and stress (amygdala and hypothalamus) (Fanselow and Dong, 2010). Anatomical studies further indicate that the input and output projections of the dorsal and ventral HPC are distinct. The dorsal HPC and its cortical projections form a critical network that mediates cognitive process such as navigation, and exploration, learning and memory (e.g. explicit, spatial, etc). (Campbell and MacQueen, 2003; Fanselow and Dong, 2010; Gold et al., 2015). On the other hand, connectivity of the VH, which sends key projections to the mPFC, NAc and hypothalamus, supports its crucial role in stress integration and regulation of motivated and emotional behavior (Campbell and MacQueen, 2003; Fanselow and Dong, 2010). Therefore, since the dorsal HPC mediates primarily cognitive functions and the ventral HPC (vSub) is mainly associated with stress integration, motivation and affect (Fanselow and Dong, 2010), parallel studies of synaptic plasticity changes within these different hippocampal circuits in the context of the WKY and other models of depression, as well as following antidepressant treatment (e.g. ketamine) are of great interest.

Importantly, given the functions mediated by the dorsal HPC, it is highly unlikely that the effects of ketamine within this region solely mediate the wide range of antidepressant effects achieved by ketamine. On the other hand, synaptic dysfunctions within the ventral HPC are more likely to contribute to the motivational and emotional disturbances in depression, a research avenue which warrants further investigation. As mentioned, the ventral HPC (vSub) – mPFC projection has been recently implicated in the mechanism of action of ketamine. Using dissociation and optogenetic approaches, several studies found that activation of this pathway of this pathway is both necessary and sufficient for the antidepressant effects of ketamine (Carreno

et al., 2016; Jett et al., 2015; Li et al., 2010). For example, the sustained antidepressant-like effects of ketamine in the FST were reported to require vSub activity both at the time of injection and testing, while optogenetic as well as pharmacogenetic activation of the vHipp–mPFC pathway mimicked the effects of ketamine in the FST (Carreno et al., 2016; Jett et al., 2015). In addition, HFS of the vHipp replicated ketamine's sustained antidepressant-like effects on cognitive flexibility and FST immobility in rats subjected to chronic stress for up to 7days following treatment (Jett et al., 2015). Therefore, accumulating evidence supports an essential role for the vSub-mPFC pathway in the sustained effects of ketamine in the FST. Importantly, other behavioural correlates of MDD such as the cognitive deficits, anhedonia and emotional dysregulation observed in animal models of depression likely have different neural substrates (e.g. dorsal HPC, PFC, mesolimbic system, amygdala, HPA axis). Therefore, the HPC (dorsal or ventral) is almost certainly not solely responsible for the wide range of symptoms associated with depression; however, the role of this highly plastic, stress-sensitive region may confer vulnerability to stress-associated disorders and may prove central to understanding some aspects of depression etiology and antidepressant response (Campbell and MacQueen, 2003).

Based on the dissociation between drug effects on SC-CA1 LTP and antidepressant-like effects in the FST immobility in our study, it is clear that synaptic plasticity in the dorsal HPC is not the sole mechanism underlying the behavioral response to ketamine. Additional studies are required to delineate the circuitry associated with ketamine's antidepressant-like effects in other behavioral paradigms, such as the FST, SPT, etc. Therefore, synaptic plasticity in the SC-CA1 pathway may represent a neural substrate for some of the antidepressant-like behavioral effects of ketamine, including those on more cognitive, hippocampal-dependent functions such as spatial memory, but other circuits (including between the vSub, mPFC, amygdala, NAc, etc.)

likely mediate the effects of ketamine on stress coping, anhedonia, emotional dysregulation, etc. Therefore, the various antidepressant, anxiolytic and pro-cognitive effects of ketamine may all rely on different circuitries, which should be dissected more precisely in future studies. Elucidating the mechanisms underlying different aspects of ketamine's therapeutic effects at the circuit level will enhance our understanding of the pathology underlying various symptoms of depression, and may provide novel strategies for enhancing antidepressant efficacy, as well as to more selectively target different symptoms/ endophenotypes of MDD.

7.4.3 Possible Mechanisms of Ketamine's Effects on Synaptic Plasticity

Clearly, the specific mechanisms underlying ketamine's effects on synaptic plasticity are not well understood and may involve a wide range of factors due to ketamine's unique abilities to modulate the release of glutamate and monoamines, the function of specific NMDA and AMPA receptor subtypes, and the expression of key pre-synaptic and post-synaptic proteins. In addition, changes to synaptic plasticity in stress and depression, as well as following antidepressant treatment, may correlate with key downstream signal transduction pathways implicated in the field (e.g. NOS-NO, cAMP-PKA, Ras-ERK, PI3K-Akt, GSK-3, BDNF-TrkB, mTOR and CREB) (Marsden, 2013).

While the question of how ketamine affects synaptic plasticity in the WKY rats and in depression is beyond the scope of this discussion, we highlight one example of a possible mechanism that mediates, or at least contributes to its ability to facilitate LTP. Namely, PKMζ, which as mentioned has been found to play an essential role in LTP maintenance and memory retention in various brain regions, including the HPC. Previous studies have revealed the regulatory effects of PKMζ on the expression and localization of synaptic proteins, especially

AMPARs, which ultimately counteract the active decay of LTP and maintain synaptic potentiation and memory retention (Yan et al., 2017). Importantly, one recent study reported that CMS decreased the expression of PKMζ in the mPFC and HPC, and that CMS-induced synaptic deficits and depressive-like behaviours were reversed or mimicked by mPFC PKMζ overexpression or inhibition, respectively (Yan et al., 2017). In addition, the antidepressants fluoxetine, desipramine and ketamine increased mPFC PKMζ expression, and its activity was necessary for the antidepressant effects of ketamine (Yan et al., 2017). Indeed, PKMζ synthesis is regulated by multiple kinases, including mTOR, which has been implicated in the actions of classical antidepressants and ketamine. These findings identify PKMζ as a potential critical mediator of depressive-like behavior and antidepressant response, providing a possible mechanism for the effects of ketamine on synaptic plasticity across different brain regions (Yan et al., 2017).

7.5 Cognition in Depression and Antidepressant Response

It is now clear that LTP maintenance and decay (depotentiation) are competing active mechanisms, which may underlie memory retention and decay. It has been postulated that, because of the limited storage capacity of memory systems such as the HPC, depotentation (a form of LTD) provides a mechanism by which one memory representation is replaced by another, preventing saturation of the system (Diamond et al., 2005). Normally, this depotentiation-induced memory decay can be seen as adaptive; however, when this process is dysregulated, as may be the case for the WKY rat, other preclinical models of depression (e.g. CMS), as well as humans suffering from MDD, it may underlie the observed impairments in learning and memory (Diamond et al., 2005).

Cognitive impairment is one of the core symptoms of MDD and represents a key diagnostic criterium ('diminished ability to think or concentrate') (American Psychiatric Association, 2013), with a large subset of patients reporting cognitive difficulties during every-day tasks (Hammar, 2009). Specifically, the characteristic cognitive profile in MDD includes impairments in attention, processing speed, executive function and various types of memory (working, episodic, visuo-spatial, pattern recognition, etc.) (Darcet et al., 2014; Hammar, 2009; Rock et al., 2014). Generally, cognitive deficits in MDD present in around two thirds of patients, and have been attributed to two major domains, which likely correspond to different underlying brain circuit dysfunctions (Pittenger and Duman, 2008; Rock et al., 2014). Namely, the available data in MDD implicates an impairment of concentration and attention, which has been attributed to well-documented abnormalities of dorsolateral PFC function in depressed patients, as well as a pronounced deficit in explicit memory, a cognitive capacity known to depend on the HPC and the medial temporal lobe (Pittenger and Duman, 2008). It is important to note, however, that some conflicting reports exist on the nature of cognitive impairment in depression, and the majority of trials are limited by small sample sizes, absence of placebo controls, lack of primary outcomes related to cognition, confounds related to illness phase and medication status, as well as high levels of heterogeneity of sample populations and methods for cognitive testing (Gould et al., 2007; Rosenblat et al., 2015). However, the consistent deficits in explicit/declarative, recollection and spatial memory reported in patients with MDD clearly support a role for hippocampus-mediated dysfunction in depression, since the HPC is involved in the learning and consolidation of explicit memory (or the ability to consciously recognize and recall events/places), as well as implicit memory of spatial context (Campbell and MacQueen, 2003; Darcet et al., 2014; Gould et al., 2007; Lamy et al., 2008; Nissen et al., 2010; Porter et al., 2003).

While there is some evidence that cognitive deficits may improve following effective antidepressant therapy, impairments have been shown to persist following remission of the depressive episode in up to half of patients (Hammar, 2009; Rock et al., 2014). Classical antidepressants have been shown to have pro-cognitive effects in some animal models as well as humans (Chen et al., 2018; Li et al., 2015; Song et al., 2006). Although clinical data is limited, drugs within this class, including SSRIs and SNRIs, seem to be associated with some degree of improvement in cognitive function compared with placebo; however, only in a subset of patients and following months of drug intake (Chen et al., 2018). Similar to findings with ketamine in rodents, while mild and transient cognitive deficits and psychotomimetic side effects are commonly observed during and shortly after infusion in the clinic, the sustained antidepressant effects of ketamine in MDD may be accompanied by significant pro-cognitive effects (Chen et al., 2018; Lara et al., 2013; Permoda-Osip et al., 2015). Importantly, recent clinical trials have reported that ketamine appears to cause improvements in cognitive function in patients with TRD, both 72h following a single infusion of ketamine (Chen et al., 2018; Permoda-Osip et al., 2015) and in patients treated intermittently over several weeks (Lara et al., 2013), independent of its antidepressant effects. Given the limited, but encouraging preclinical and clinical findings of pro-cognitive effects of ketamine in the context of depression, cognitive function in animal models of depression and patients with TRD before and after ketamine infusion should be more closely investigated, especially at later time points when subjects are not under the acute influence of ketamine.

Importantly, accumulating evidence supports the idea that cognitive impairments may contribute to the development, maintenance and treatment of depression, and often persist beyond recovery from mood disturbances (Hales et al., 2014; Hammar, 2009). In addition,

marked cognitive impairment may predict poor response to antidepressant medication independent of mood symptom severity, and may serve also as a predisposing factor for depression (Roiser and Sahakian, 2013). Therefore, since a substantial part of the burden associated with depression has been attributed to the cognitive impairments associated with the disorder, effective antidepressant treatment strategies that also target the cognitive symptoms of MDD are clearly needed to improve long-term outcomes, particularly functional recovery (Hammar, 2009).

7.6 Role of the (2R,6R)-HNK Metabolite

As discussed, the seminal study by Zanos et al. claimed that an active metabolite without NMDAR binding properties or key side effects of its parent compound, is both necessary and sufficient for ketamine's antidepressant effects in rodents (Zanos et al., 2016). Importantly, following these encouraging initial findings, however, our group and others have failed to replicate the antidepressant effects of (2R,6R)-HNK in various animal models of depression (e.g. LPS, CDS, LH and WKY rat) (Shirayama and Hashimoto, 2018; Xiong et al., 2019; Yang et al., 2016; Zhang et al., 2018), while others question the metabolite's contribution to ketamine's therapeutic effects and/or argue against rejecting the NMDAR hypothesis of ketamine action (Aleksandrova et al., 2017b).

As mentioned, the major concern regarding the contribution of (2R,6R)-HNK to ketamine's clinical effects is whether clinically relevant doses of ketamine could achieve the brain levels of (2R,6R)-HNK required for its reported antidepressant, molecular and synaptic effects (Aleksandrova et al., 2017b). Indeed, the (2R,6R)-HNK concentration used by Zanos et al. in their electrophysiology experiment (10μM), as well as the maximum concentration reached

following a systemic injection of antidepressant doses of (2R,6R)-HNK (10mg/kg, ip, 10.69μM) (Zanos et al., 2016), are ~10-100 times higher than peak plasma metabolite levels obtained following administration of clinically relevant doses of ketamine in mice (~1μM) and in humans (~0.1μM), although more direct human clinical data is needed (Aleksandrova et al., 2017b). Another general limitation prompting the field to question the relevance of preclinical findings with (2R,6R)-HNK to clinical situations is the difficulty in translating findings from rodents to humans given the different routes of administration involved. In rodents, ketamine is administered via an ip injection, and that will undoubtedly exaggerate the contribution of metabolites to its antidepressant effects due to extensive first-pass metabolism, which would not occur following an IV infusion in humans, giving rise to a different profile of parent/metabolite concentrations in the brain (Aleksandrova et al., 2017b). In addition, strong evidence against a role for metabolites in ketamine's antidepressant effects comes from the finding that a single, bilateral microinfusion of (R)-ketamine directly into the mPFC or HPC mimicked the effects of systemic administration, indicating clearly that ketamine itself can exert antidepressant effects (Shirayama and Hashimoto, 2017). In addition, the clinical efficacy of (S)-ketamine (esketamine) for depression, which does not involve metabolism into (2R,6R)-HNK, further indicates that ketamine does not acts exclusively via conversion to this particular metabolite (Rotroff et al., 2016). Therefore, despite the unreconciled finding that 6,6-dideuteroketamine (which cannot be metabolized into (2S,6S;2R,6R)-HNK but with all other pharmacokinetic and receptor properties apparently unaltered) lacks ketamine's sustained antidepressant effects (Zanos et al., 2016), a growing consensus suggests that (2R,6R)-HNK cannot be solely responsible for ketamine's clinical effects (Aleksandrova et al., 2017b).

Findings with (2R,6R)-HNK do, however, represent important progress in the field and are the latest piece of the ketamine puzzle. The possibility that a key metabolite previously deemed inactive could mediate, or at least contribute to, some aspects of the antidepressant activity of ketamine has revised the way we think about and probe ketamine's mechanism of action in depression. It is clear that (2R,6R)-HNK represents a major plasma metabolite with a considerably longer half-life, which could very well contribute to the remarkably long-lasting antidepressant effects following a single ketamine infusion (Aleksandrova et al., 2017b). Although the molecular target(s) of (2R,6R)-HNK have not been defined, studies have implicated increased AMPAR-mediated synaptic transmission, BDNF and protein synthesis, which seem to represent points of convergence with ketamine (Aleksandrova et al., 2017b; Pham et al., 2018; Zanos et al., 2018b, 2018a). It is also important to consider other unique effects of (2S,6S;2R,6R)-HNK such as its reported α7-nAchR antagonism (Moaddel et al., 2013), which may decrease glutamate excitotoxicity, and its possible attenuation of D-serine levels (Singh et al., 2016), which could lead to an indirect inhibition of NMDAR function (Zarate et al., 2012). More recent work by Zanos et al. has identified several potential mechanisms of action of the (2R,6R)-HNK metabolite, although again, the exact molecular target of this metabolite has remained elusive (Riggs et al., 2019; Zanos et al., 2019, 2018b, 2018a). Namely, one study found that (2R,6R)-HNK exerts antidepressant-like actions via a mechanism converging onto metabotropic glutamate receptor subtype 2 (mGluR2) signaling (Zanos et al., 2019), echoing the previously reported antidepressant action of mGluR2 antagonists in various animal models of depression (Fukumoto et al., 2015; Sanacora et al., 2008). Consistent with the role of pre-synaptic mGluR2 receptors in inhibiting glutamate release, another study by this group recently found that (2R,6R)-HNK's previously reported ability to enhance excitatory synaptic

transmission following bath application in hippocampal slices seems to be due to a concentration-dependent, NMDAR-independent and synapse-selective increase in glutamate release probability with no direct actions on AMPAR function (Riggs et al., 2019). However, as mentioned previously, while both ketamine and its metabolite may increase pre-synaptic glutamate release, leading to an indirect enhancement of AMPAR signaling, it is still unclear whether under therapeutically relevant conditions these compounds actually affect *in vivo* hippocampal basal synaptic transmission acutely, and/or perhaps more importantly, modulate synaptic plasticity (LTP, LTD) and metaplasticity processes, as well as downstream synaptogenesis. Overall, (2R,6R)-HNK could contribute to ketamine's unique ability to increase the AMPA to NMDA receptor throughput by indirectly enhancing AMPAR function and possibly inhibiting NMDARs, and converging onto downstream synaptogenic signaling pathways (Figure 2C). Moreover, when given at high enough doses, (2R,6R)-HNK may mimic some of the molecular, synaptic and behavioural effects of its parent drug. Based on our findings and those of others, however, it appears that (2R,6R)-HNK recapitulates some, but not all, aspects of ketamine's molecular and antidepressant effects, allowing for the more precise dissection of mechanistic points of overlap and divergence in the actions of ketamine and this key active metabolite. Thus, the contribution of metabolites to ketamine's therapeutic effects remains a distinct possibility and direct comparisons with (2R,6R)-HNK may hold the key to unlocking the mechanisms underlying ketamine's unique clinical efficacy.

7.7 General Limitations and Future Directions

We chose the WKY model of depression because as discussed, it incorporates aspects of heightened stress responsiveness and vulnerability to depression, resistance to classical

antidepressants, as well as various behavioural, neurochemical and endocrine parallels to MDD. Despite these advantages, some important limitations of this model should be noted (discussed in more detail in section 1.5.4), including its heightened reactivity to changes in housing and experimental procedures, and the considerable genetic and behavioural heterogeneity (between and within individual colonies, as well as within this study) in terms of the severity of their depression-like phenotype and the extent of their antidepressant resistance. In addition, the endogenous nature of the WKY depressive-like phenotype fails to recapitulate the episodic nature of MDD, leading to lower etiological validity of this model (Lahmame and Armario, 1996; Lopez-Rubalcava and Lucki, 2000; Paré and Kluczynski, 1997; Will et al., 2003; Willner and Belzung, 2015). Nonetheless, since ketamine is clinically indicated for patients with more severe, often recurrent, forms of treatment-resistant depression, the WKY rat seems particularly well suited for studying the antidepressant actions of ketamine. Further research is needed to determine whether circuit dysfunctions in key neural circuits observed in the WKY model can be replicated in other established preclinical models of depression (e.g. CMS, CSD, etc.), as well as whether insights gained about the mechanisms underlying depression and antidepressant response in the context of the WKY model can be generalized to the human condition.

In this study, we characterized WKY rats on a small battery of behavioural tests related to aspects of stress reactivity, motivation and cognition. However, despite the fact that antidepressant response is clearly not a simple, unitary measure and as is done far too frequently in the field, we relied on a single behavioural readout of antidepressant-like activity (besides our spatial memory task). Namely, we utilized the FST, which while considered a “canonical” test of stress coping and an antidepressant screen with good predictive validity, is crude and suffers from crippling limitations, such as low face and construct validity. Importantly, FST immobility

has been classically interpreted as “behavioural despair” and/or a “depressive-like behavior,” explanations which suffer from a degree of anthropomorphism and are somewhat counter-intuitive as immobility in the face of inescapable swim stress would promote conservation of the energy needed to prolong survival (de Kloet and Molendijk, 2016). Therefore, in recent years many have emphasized the more careful interpretation of the test, where the FST can actually measure abnormal coping (i.e. adopting a passive coping strategy) to an acute inescapable stress, in order to avoid misrepresenting both the utility and limitations of the FST (Commons et al., 2017). Therefore, given these limitations of the FST, better characterization of the WKY model in terms of the complex range of functional domains that define clinical depression and how each of these phenotypes responds to antidepressant treatment (particularly ketamine) is clearly warranted. Thus, in an effort to characterize available animal models of depression, redefine clinical symptoms of MDD in terms of brain circuit dysfunctions and screen candidate compounds for antidepressant activity more effectively, we emphasize the need to utilize more comprehensive batteries of preclinical tests across various domains affected in MDD, such as stress/emotional reactivity, motivation and cognition.

Despite the fact that the prevalence of MDD in women is roughly two times higher than that seen in men (Altemus et al., 2014), as is too frequently observed in animal research, we exclusively utilized male rats in our studies. While robust anxiety- and depressive- like behaviours are present in both sexes, there is currently no consensus as to whether they are more prominent in male or female WKY rats. Clearly, sex differences within this strain are complex and may depend on the testing conditions (see section 1.5.4). In addition, preclinical research investigating the mechanisms underlying ketamine’s antidepressant effects has been conducted almost exclusively in male rodents. Interestingly, studies that include both sexes report that

ketamine produces more robust antidepressant responses in female compared to male rodents in the FST (Aleksandrova et al., 2017b; Zanos et al., 2016). The higher potency of ketamine in females has been attributed to factors such as the higher brain levels of (2S,6S;2R,6R)-HNK, as well as the contribution of gonadal hormones, which have been shown to potentiate ketamine's antidepressant effects (Aleksandrova et al., 2017b). On the other hand, there is also emerging evidence that repeated ketamine treatment effectively sustains the antidepressant response in male mice, whereas it may actually worsen depression- and anxiety- related phenotypes in their female counterparts (Thelen et al., 2016). Therefore, in order to address outstanding questions in the field and the fact that females are underrepresented in preclinical research, as well as to gain insight into the sex-specific mechanisms in the pathophysiology and treatment of depression, it is crucial that both sexes be included in future studies of the WKY rat and other models of depression, and of antidepressant compounds, particularly ketamine and (2R,6R)-HNK. Interestingly, a recent human clinical trial reported no differences in ketamine response between men and women, and between pre- and post-menopausal women; however, the impact of sex on treatment outcomes warrants further clinical investigation (Freeman et al., 2019).

Generally, the lack of specific inhibitors for different types of LTP or LTD has hindered progress in determining the specific roles of synaptic plasticity in various forms of learning and memory in health and disease (Howland and Wang, 2008). Several peptides have been developed to modulate different aspects of LTP/LTD by our group and others, which could be useful in pinpointing the molecular mechanisms underlying the effects of ketamine on synaptic plasticity (Hardt et al., 2013; Migues et al., 2010, 2016). For example, future studies should test whether inactivating PKMζ in the HPC of WKY rats treated with ketamine (using the ZIP peptide) would abolish the pro-cognitive effects of ketamine in the object location recognition

test. In addition, testing whether blocking LTP decay by inhibiting the GluA2-dependent removal of postsynaptic AMPARs (using the $GluR2_{3Y}$ peptide) would abolish the deficits in LTP and long-term memory observed in the WKY strain, confirming the association between hippocampal LTP maintenance, postsynaptic GluA2 expression and long-term spatial memory retention in this model, and further implicating synaptic plasticity processes in the antidepressant action of ketamine.

As mentioned repeatedly, even though glutamatergic receptors modulated by ketamine (NMDAR and AMPARs) clearly play integral roles in the induction and expression of LTP/LTD and despite the growing argument for the role of synaptic plasticity in depression and antidepressant response, the effects of antidepressant doses of ketamine on regional, bidirectional synaptic plasticity *in vivo* or in a depression-like state remain to be adequately addressed. Therefore, systematic investigations of ketamine's effects on *in vivo* synaptic plasticity within different circuits implicated in MDD, as well as of the direct contributions of synaptic plasticity changes to ketamine's antidepressant effects in WKY rats and other animal models of depression, are clearly warranted. Importantly, future studies of synaptic plasticity in depression and antidepressant response should look beyond the canonical intra-hippocampal synapses (e.g. SC-CA1) and instead examine specific reciprocal projection pathways between key brain areas in MDD, including those discussed here (i.e. HPC, PFC, amygdala and NAc). Such experiments will address an apparent lack of understanding of the effects of ketamine on synaptic plasticity processes, effects which could provide a far-reaching mechanism to explain how ketamine's molecular and cellular effects translate into region-specific changes in structural plasticity and neural circuit functioning.

In light of the breakthrough, but controversial, findings of Zanos et al., as well as our own results, questions remain as to how (2R,6R)-HNK contributes to ketamine's antidepressant effects, and whether it holds potential as a novel, safer antidepressant compound. Further research is clearly required to replicate its antidepressant effects in animal models, to determine its molecular targets and exact mechanism of action, and to evaluate its potential utility as a novel antidepressant in the clinic. In order to settle the controversy currently surrounding the behavioural effects of this metabolite in rodents, it is crucial that (2R,6R)-HNK be systematically evaluated (at different doses and schedules of administration) in established animal models of depression with high predictive, face and construct validity, particularly the WKY rats and the CMS paradigm. As (2S,6S;2R,6R)-HNK does not apparently inhibit NMDARs at therapeutically relevant concentrations, the molecular target(s) responsible for its behavioural and synaptic effects are of great interest and are currently under active investigation. Finally, even though it is unlikely that conversion into (2R,6R)-HNK mediates all the clinical effects of ketamine in TRD, future research will determine the overlapping and divergent actions of the parent drug and its metabolite, in terms of mechanism of action, therapeutic and side effect profiles. Because of the significant gap in current understanding of (2R,6R)-HNK's mechanism of action, contribution to ketamine's antidepressant effects, as well as potential utility as a novel antidepressant or pro-cognitive agent, future studies are required to address these outstanding questions.

Finally, future research will determine whether more direct modulation of synaptic plasticity processes represents a viable novel antidepressant strategy that would improve treatment response rates and minimize the delay to onset of therapeutic effects in the clinic. Clearly, translating findings regarding the role of synaptic plasticity in depression from rodents to human patients is challenging. However, currently, several methods can be utilized in humans

to examine neural plasticity, including safe and non-invasive brain stimulation methods (transcranial magnetic simulation (TMS) and repetitive sensory stimulation), as well as functional (i.e. fMRI, positron emission tomography (PET) and auditory and visual evoked potentials) and structural (diffusion tensor imaging (DTI) and voxel-based morphometry (VBM)) imaging techniques (Mateos-Aparicio and Rodríguez-Moreno, 2019; Nathan et al., 2011). In the future, such approached should be systematically applied to study the role of synaptic plasticity processes within various circuits in depression and antidepressant response in humans.

7.8 Conclusions and Significance

Based on our findings, we propose that, in the WKY model of depression, restoring the impaired SC-CA1 LTP does not underlie ketamine's antidepressant effects in the FST (considered a "canonical" measure of antidepressant activity), but may more specifically mediate reversal of hippocampal-dependent cognitive deficits (including spatial memory), which are also key features of clinical depression. In addition, the existing hypothesis that ketamine's antidepressant effects are solely due to the actions of its metabolite (2R,6R)-HNK is effectively challenged here, since HNK exerted pro-cognitive, but no FST antidepressant-like, effects in the WKY model. Our results support an accumulating body of evidence indicating that ketamine may reverse the stress-induced loss of connectivity in key neural circuits by engaging synaptic plasticity processes to "reset the system". Restoring hippocampal-dependent function by modulating synaptic plasticity processes likely contributes to the therapeutic effects of ketamine; however, it is clear that this drug has unique effects in different brain regions that contribute to different aspects of its antidepressant activity. Importantly, while many preclinical studies postulate that stress and antidepressant drugs act primarily in the (dorsal) HPC and thus

exclusively focus their investigations there, it is becoming increasingly clear that depression and antidepressant response result from long-lasting adaptations within larger associated networks implicated in MDD that likely include the dorsal and ventral hippocampus, medial prefrontal cortex, amygdala, the mesolimbic system, HPA axis, etc., and their reciprocal connections (Aleksandrova et al., 2019). Therefore, in order to specify the relative contributions of individual systems to the effects of novel antidepressant drugs, this thesis emphasizes the importance of deconstructing depression-like phenotypes and identifying the circuits that mediate them more precisely. Accordingly, we recommend that future studies utilize more comprehensive batteries of preclinical tests relevant to depression (including measures of stress reactivity, motivation and cognition), as well as more carefully consider the neural circuits mediating the development and reversal of specific behavioural phenotypes of interest, in a continued effort to redefine clinical symptoms of MDD in terms of brain circuit dysfunctions. Undoubtedly, the unique antidepressant efficacy of ketamine lies in its heuristic influence and the fact that it likely works through a wide range of different mechanisms involving various molecular and anatomical targets. Indeed, great strides are being made towards uncovering the molecular mechanisms underlying the therapeutic effects of ketamine and (2R,6R)-HNK, hopefully will aid the development of novel and more efficacious antidepressant agents so urgently needed to address a major public health concern.

In conclusion, the Wistar-Kyoto rat represents a valid model of endogenous stress susceptibility and depression, which might be particularly useful in revealing the synaptic plasticity dysfunctions (within the HPC and beyond) associated with various depression-like phenotypes, as well as the ability of antidepressant treatment to reverse the loss of connectivity between key brain areas implicated in MDD. Given the accumulating evidence that synaptic

plasticity plays an important role in the pathophysiology and treatment of depression, further research into potential circuit dysfunctions in WKY rats and other animal models of depression is warranted. Such studies should include a fuller explanation of how novel antidepressant treatments including ketamine influence synaptic plasticity in key neural circuits. Incorporation of synaptic plasticity into the current framework of antidepressant action may serve to bridge the understanding of the molecular and cellular effects of antidepressant drug action with those on regional structural plasticity and neural circuit functioning. Ultimately, such research will determine whether more direct modulation of synaptic plasticity processes represents a viable novel antidepressant target that would improve treatment response rates and minimize the delay to onset of therapeutic effects in the clinic.

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Appendix

Synthesis and Verification of (2R,6R)-HNK:

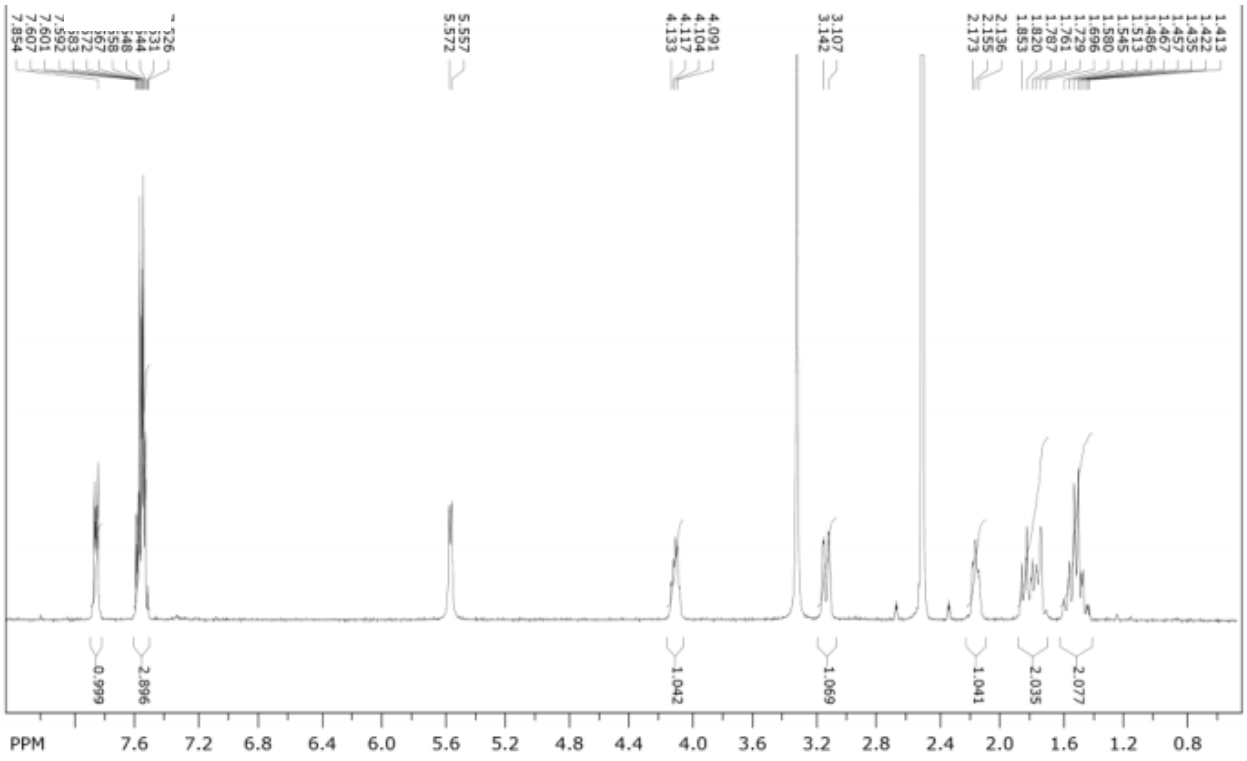
(2R,6R)-(-)-2-amino-2-(2-chlorophenyl)-6-hydroxycyclohexanone hydrochloride *aka* (2R,6R)-(-)-hydroxynorketamine hydrochloride

(2R,6R)-HNK was previously reported and synthesized by Zanos et al. (2016).

To a solution of tert-butyl ((1R,3R)-1-(2-chlorophenyl)-3-hydroxy-2-oxocyclohexyl)carbamate (14.3 mmol) in dichloromethane (10 mL) was added trifluoroacetic acid (TFA, 143 mmol, 10 eq.). The reaction was stirred at room temperature for 1 hour. The solvent TFA were then removed by rotary evaporation. The resulting TFA salt was dissolved in water, washed with a 1:1 mixture of saturated aqueous sodium bicarbonate and saturated aqueous potassium carbonate solution, and extracted with ethyl acetate (2X) to give the free base. The ethyl acetate was removed by rotary evaporation. Ethyl acetate (4 mL) was added and HCl in dioxane (4.0 M, 6.0 mL). The suspension was agitated for 30 seconds and then the solid was filtered off and dried under vacuum to give the desired final product (60 mg, 65% yield) (Zanos et al., 2016).

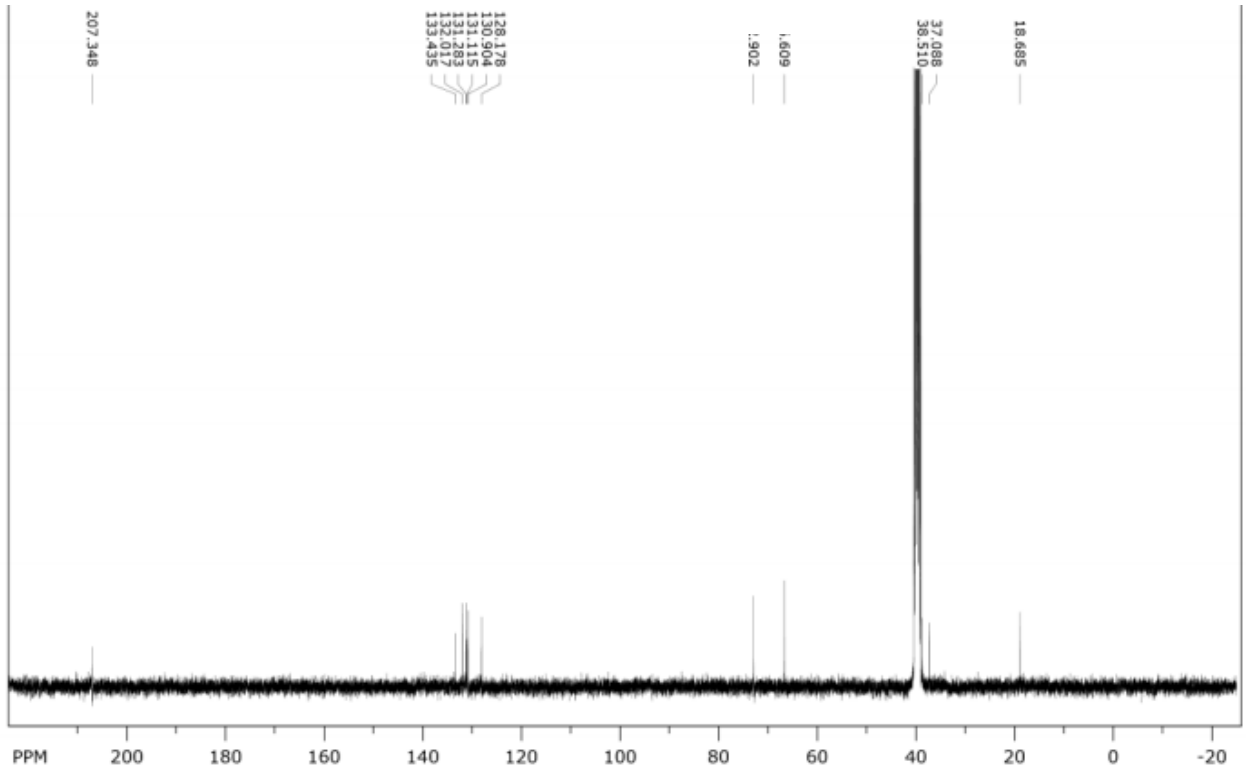
Chiral HPLC: 98.1% ee (Chiralpak AD column, 60% ethanol in hexanes, 1.0 mL/min), HRMS (ESI+): Expected 262.0605 [M+Na] ($C_{12}H_{14}ClNO_2Na$). Observed 262.0605 $[\alpha]_D^{20}$: -92°.

[1]H NMR spectra of (2R,6R)-(-)-2-amino-2-(2-chlorophenyl)-6-hydroxycyclohexanone hydrochloride



[1]H NMR (400 MHz, DMSO-d6): δ 7.89-7.85 (m, 1H), 7.61-7.53 (m, 3H), 4.10 (dd, J=11.6, 6.7 Hz, 1H), 3.15 (dd, J=14.0, 3.0 Hz, 1H), 2.16 (dddd, J=12.2, 6.6, 4.1, 2.3 Hz, 1H), 1.86-1.70 (m, 2H), 1.59-1.41 (m, 2H) ppm.

^{13}C NMR spectra of (2R,6R)-(-)-2-amino-2-(2-chlorophenyl)-6-hydroxycyclohexanone hydrochloride



^{13}C NMR (100 MHz, DMSO-d6): δ 207.4, 133.4, 132.0, 131.3, 131.1, 130.9, 128.2, 72.9, 66.6, 38.5, 37.1, 18.7 ppm.