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General anesthetics effects in behavioral modulation: the particular case of ketamine

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Índex

Abreviations and acronims

CMS chronic mild stress

DA Dopamine

D2R dopamine 2 receptor

NAc Nucleus accumbens

NMDA N-methyl-D-aspartate acid

PD Parkinson's Disease

PFC Prefrontal Cortex

SNRIs Serotonin–norepinephrine reuptake inhibitors

SSRIs Selective serotonin reuptake inhibitors

Str Striatum

VDS Variable Delay to Signal Test

5-HT Serotonine

A introdução da Anestesia na prática clínica permitiu a realização de tratamentos invasivos e procedimentos de diagnóstico. A generalização destas práticas levou a um aumento da exposição dos anestésicos gerais (AG) e consequentemente a uma maior preocupação sobre os seus efeitos nas funções cerebrais. Simultanemanente, os AG podem apresentar benefícios terapêuticos em condições clínicas particulares. Existe uma sobreposição entre os receptores com os quais os AGs interagem e os alvos farmacológicos no tratamento de vários distúrbios psiquiátricos. Estes fatos levaram a considerer a hipótese de que os AGs podem ter um papel na abordagem e na compreensão de patologias psiquiátricas.

A Cetamina é um anestésico dissociativo, que para além das suas propriedades anestésicas também possui ação analgésica e antidepressiva. É também um potente modulador dos neurotransmissores glutamato e dopamina. Partindo das caraterísticas específicas deste AG, nós estabelecemos como principal objetivo do nosso trabalho investigar os efeitos da cetamina como potencial agente terapêutico em patologias do foro psiquiátrico. Do leque de patologias psiquiátricas, privilegiamos a patologia depressiva e o estudo da impulsividade.

A patologia psiquiátrica, com maior relevo para a depressão major, é uma das doenças com maior prevalência na sociedade moderna. Durante as últimas décadas, o modelo de tratamento farmacológico da depressão foi construído com foco na reposição dos neurotransmissores monoaminérgicos, baseado fortemente na teoria monoaminérgica da depressão. Os fármacos inibidores seletivos de recaptação da serotonina e os inibidores seletivos de recaptação da serotonina-noradrenalina têm como principal efeito o aumento da disponibilidade destes neurotransmissores. No entanto os antidepressivos utilizados na atualidade têm várias limitações, tais como: o inicio de ação muito lento que pode demorar semanas ou mesmo meses; a incapacidade de evitar novos episódios clínicos de depressão ou até mesmo o desenvovimento de resistência ao efeito antidepressor dos fármacos. Esta ineficácia terapêutica é um problema não apenas para o indivíduo, mas também para a sociedade.

O sistema glutamatérgico foi recentemente identificado como um potencial alvo. A exposição ao stress ambiental em modelos animais provoca uma libertação de glutamato nas areas límbicas e corticais, induzindo alterações estruturais muito semelhantes às observadas em pacientes deprimidos (Musazzi *et al,* 2011). A cetamina, é um antagonista dos receptores de glutamato e, apresenta uma ação antidepressiva rápida.

Nesta tese é investigado o papel da cetamina como um tratamento co-adjuvante dos antidepressivos clássicos num contexto de depressão. Para testar tal hipótese, foi utilizado um modelo animal com comportamento semelhante ao depressivo, designado de "chronic mild stress". O nosso trabalho produziu evidência de que a cetamina é capaz de induzir uma recuperação mais rápida no fenótipo anedónico quando foi associada ao tratamento com fluoxetina. Verificamos também que a nível morfológico a adição de cetamina aos antidepressivos (imipramina e fluoxetina) traduziu-se na recuperação das alterações provocadas pelo modelo, nomeadamente ao nível da maturação de espinhas dendríticas. Também detetamos uma alteração dos níveis de glutamato ao nível da região cerebral do *nucleus accumbens*, o que pode sugerir uma maior importância desta área do cérebro no efeito específico de cetamina quando administrada em conjunto com antidepressivos.

Para além da ação glutamatérgica, a cetamina também aumenta agudamente os níveis de dopamina do cérebro. Existem vários distúrbios que são caracterizadas por um desequilíbrio da dopamina no cérebro e se traduzem em alterações comportamentais, nomeadamente do foro executivo e motivacional. Propusemo-nos então a investigar o efeito da cetamina num traço comportamental em particular: a impulsividade. Para esse efeito utilizamos um paradigma comportamental desenvolvido especificamente para avaliação de impulsividade em roedores: o Variable Delay to Signal Test. O nosso trabalho foi capaz de demonstrar que nos animais com exposição repetida a procedimentos anestésicos com cetamina ocorreu uma alteração do padrão de impulsividade. Além desse facto, desencadeou também alterações estruturais no *nucleus accumbens* assim como alterações ao nível da expressão dos recetores de dopamina no *striatum.*

Podemos afirmar que embora permaneçam por responder muitas questões relativamente ao potencial não-anestésico da cetamina, o nosso trabalho demonstrou uma potencial aplicação do fármaco como um potencial agente terapêutico em doenças psiquiátricas.

A b s t r a c t

The introduction of general anesthesia in clinical practice allowed invasive treatment and diagnostic procedures. The generalization of these practices leads to an increased exposure to general anesthetics (GA) with increasing preoccupation about the effects of GA on brain function. While potential detrimental GA may have therapeutic benefits in particular clinical conditions. The overlap between the molecular targets of GA and drugs used to treat psychiatric disorders such as anxiety, depression and obsessive-compulsive disorder suggests that GA might have a therapeutic use in these conditions.

Ketamine, in addition to its GA properties, is an analgesic, amnesic, and fast acting antidepressant, making it a very versatile molecule. The work presented in this thesis departs from this evidence to study Ketamine's role as a behavioral modulator particularly in mood and motivational disorders.

Mood disorders, such as major depression are highly prevalent in modern societies. During the last 50 years the pharmacological treatments of major depression has focused on the modulation of monoamine neurotransmission, including selective serotonin reuptake inhibitors (SSRIs) and serotonin–norepinephrine reuptake inhibitors (SNRIs). Despite a reasonable clinical efficacy these antidepressants have a slow onset and in some patients fail to respond at all. New targets and/or therapeutic molecules are therefore an urgent need. In this context, the glutamatergic system has recently been identified as a potentially interesting target. Exposure to environmental stressors in animal models enhance glutamate release in limbic and cortical areas and induce structural changes resembling those observed in depressed patients (Musazzi *et al,* 2011). Ketamine is an antagonist of glutamate receptors and possesses a fast antidepressant action. We investigated the potential role of ketamine as an adjuvant treatment to classical antidepressants in major depression. To test our hypothesis, we used an established animal depressive-like behavior animal model, the chronic mild stress (CMS). We observed that Ketamine was able to induce a faster recovery in the anhedonic phenotype when it was associated with treatment with fluoxetine. To clarify the neural substrates of such changes we conducted a detailed morphological analysis of the underlying

circuits. At a morphological level, the addition of Ketamine to antidepressants (imipramine and fluoxetine) translated in the recovery of the effects of the CMS model in dendritic spines maturation. We also detected a change in glutamate metabolism in the nucleus accumbens (NAc), which might suggest an essential role for this brain area in the specific effect of ketamine acting together with antidepressants.

Ketamine can also acutely increase brain dopamine (DA) levels in the brain. There are several disorders which are characterized by an imbalance in brain DA and that are associated with a change in motivational behavior. In this line of thought, we proposed to investigate one specific behavioral trait of motivational behavior influenced by dopaminergic metabolism: impulsivity; more specifically how exposure to Ketamine can affect impulsive behavior.

Through the novel behavioral paradigm developed in our laboratory, Variable Delay to Signal Test (VDS) paradigm, we were able to demonstrate that repeated exposure to ketamine anesthesia was able to change the impulsivity drive in animals after recovery from anesthesia. Moreover the exposure induced neuronal structural changes in the NAc and molecular changes in DA 2 receptor(DA2R) in the Striatum(Str).

This thesis provides evidence for a role of Ketamine as a potential therapeutic agent in psychiatric disorders.

General Anesthesia is a state of loss of both conscience and reactivity to noxic stimuli, produced by the action of general anesthetics (GA), characterized by the triad of analgesia, amnesia and immobility. The introduction of GA in clinical practice provided an invaluable contribution to the advance of healthcare, allowing the performance of complex and invasive surgical and diagnostic procedures. In fact, according to the World Health Organization (WHO, 2009) no single improvement in the care of surgical patients has had a profound impact as the advancement of safe practices in anesthesia, highlighting the importance of the study of GA.

Ancient cultures already used substances, such as alcohol and opioids, to induce states of lethargy. For instance, the Sumerians and ancient Egyptians were known to harvest *Papaver somniferum* (Bisset *et al*, 1994) to produce opium, and the Inca are also known for the use of coca leaves in traditional medicine.

The concept of GA applied surgical procedures appeared later in the 19th century. In 1886, William T. G. Morton was the first to report the accomplishment of an anesthetic procedure using ether (Greene *et al*, 1979).

Safety of using general anesthetics

Developments in both pharmacology knowledge and the establishment of standardized procedures transformed anesthesiology into a medical field driven by safety improvement (Botney *et al*, 2008). The first true attempt to access mortality numbers of anesthesia was the Beecher-Todd anesthesia death rate study (Beecher *et al*, 1954). Several others studies have since been reported (Bainbridge *et al*, 2012; Watters *et al*, 2015) showing that anesthesia procedures are one of the safest medical activities with very low intraoperative mortality rates due to anesthetic procedures (Li *et al*, 2009; Newland *et al*, 2002)

However, continuous focus on patient safety assessment shows us that there is always room for improvement. Recently, Glance and colleagues observed that patient outcomes may be

affected by different intraoperative anesthetic management strategies (Glance *et al*, 2015), highlighting the importance of tailored choices of anesthetic procedures.

Other focus is on professional exposure to general anesthetics, which mainly concerns inhalational agents. In order to prevent this issue, legislation on scavenging anesthetic gases and on air quality was implemented. Specifically, some agents considered to be hazardous to public health stop being used.

Despite the improvements there are several GA related issues that require immediate attention such as effects on patient's brain or misuse by health professionals. We will further develop these subjects on the following sections.

Evidence of general anesthetics effects in brain

The classical pharmacokinetic concept of a GA is that as long as the drug is eliminated, its effects will disappear, and normal brain function is restored.

Anesthesia is known to have effects in the connectivity between different brain regions. The effects on cortico-cortical connectivity is varied, depending on the anesthetic agent and the specific network examined; however, fronto-parietal connectivity is often reduced in anesthesia.

Most studies focus on the moment of loss of consciousness during general anesthesia and research suggests that the networks based on the posterior parietal-cingulate-precuneus region and the thalamus are candidates for the neural correlate of the state of consciousness (Alkire *et al,* 2008).

In light of increasing safety and reduced mortality with the use of GA, more recently, the discussion moved towards the effects of GA in brain function, in particular, the effects of GA in both the healthy and ageing brain.

Most studies on the effect of GA in diseased brain were performed in order to answer if exposure could trigger or be related to diseases such as Alzheimer's disease in elderly or attention deficit hyperactivity disorder in children (Culley *et al*, 2007; Sprung *et al*, 2010),

however there is no direct cause-effect relation between those pathologies and GA exposure (Planel *et al*,2007; Hansen *et al*; 2015).

Other field of research focus in the assessment if exposure of developing brain to GA could induce changes. A bulk of preclinical data suggests that exposure to commonly used anesthetic agents during key periods of brain development can lead to neurodegeneration, synaptic loss and learning and memory deficiencies that might persist as the organism matures (Jevtovic-Todorovic *et al*, 2003; Lunardi *et al*, 2010) In addition, neonatal anesthetic exposure in *in vivo* models was confirmed to change neurogenesis and synaptogenesis processes, indicating that GA might influence neuroplasticity as well (De Roo *et al*, 2009; Briner *et al*, 2010)

Throughout this thesis we will focus in a different concept, targeting adult brain and exploring long lasting effects in specific brain functions after GA exposure.

The influence of general anesthetics on mood disorders

The effect of GA on mood is a relatively new field of knowledge (Rasmussen *et al*, 2003; Poulsen *et al*, 2018). The overlap between GA targets in brain and the targets of drugs used to treat psychiatric disorders open the pathway to the hypothesis that GA exposure might somehow have an effect in patient's mood.

Anesthetics are thought to work by interacting with ion channels that regulate synaptic transmission and membrane potentials in key regions of the brain and spinal cord, such as neurotransmitter receptors. These ion-channel targets are differentially sensitive to various anesthetic agents. Anesthetics hyperpolarize neurons by increasing inhibition or decreasing excitation (Ries *et al*, 1999) and, consequently, alter neuronal activity: The sustained firing typical of the aroused brain changes to a bistable burst-pause pattern (Llinás *et al*, 2006) that is also observed in sleep.

The specific case of Ketamine

Ketamine (2-chlorphenyl-2-methylamino-cyclohexanone) was first synthesized by Calvin Stevens of the Parke-Davis pharmaceutical company in 1962 and formally described in 1965 (Domino *et al*, 1965).

Ketamine is recognized as a dissociative anesthetic (Domino *et al*, 1966), with additional analgesic, amnesic, and fast acting antidepressant properties (Berman *et al*, 2000; Zarate *et al*, 2006). Ketamine is used as a general anesthetic both in human and veterinary medicine (Green *et al,* 1981).

Ketamine's two enantiomers, (R)-ketamine and (S)-ketamine, have different pharmacological properties, as well as differential effects on psychiatric symptoms and cerebral metabolism in healthy volunteers (Oye *et al*, 1992; Vollenweider *et al*, 1997). (S)- ketamine has a higher affinity for the N-methyl-D-aspartate (NMDA) receptors PCP binding site than (R)-ketamine by a factor of 4–5:1. Whereas (R)-ketamine does not significantly alter scores on psychiatric assessments, (S)-ketamine produces symptoms of depersonalization, derealisation, visual disturbances, thought disorders, and apathy (Vollenweider *et al*, 1997).

The influence of ketamine on central nervous system as a GA has been investigated with various imaging techniques, including positron emission tomography and task-related functional magnetic resonance imaging, with several brain regions displaying ketamine-dependent activity changes, such as the prefrontal cortex (PFC), ventral tegmental area, substantia nigra in midbrain, posterior cingulate cortex, visual cortex, insular cortex and thalamus (Rogers *et al*; 2004; Schmidt *et al*, 2005; Nagels *et al*, 2011, Yu *et al*, 2012). Additionally, the effect of ketamine, specifically sub-anesthetic doses in brain connectivity is now being subject of study. Sub-anesthetic doses of ketamine were found to increase the connectivity in the cerebellum and visual cortex, and to induce a decrease in connectivity in the auditory and somatosensory network in relation to several regions, which included the amygdala, insula, and anterior cingulate cortex (Niesters *et al*, 2012). Acute ketamine treatment was also found to increase PFC metabolic activity while reducing metabolic activity in the dorsal reticular thalamic nucleus, in association with an abnormal functional

connectivity between the PFC and multiple thalamic nuclei, including the dorsal reticular thalamic nucleus, mediodorsal thalamus and anteroventral thalamus (Dawson *et al*, 2013). One recent study (Dawson *et al,* 2014) found that acute NMDA receptor blockade with ketamine promotes increased connectivity in functional brain networks, namely, that acute ketamine treatment enhances connectivity of PFC and thalamic brain regions in brain networks, with enhancement of PFC neuromodulatory subsystem connectivity in animals. Other work exploring the effects of different sub-anesthetic doses of ketamine showed dose- and exposure-dependent increases in functional connectivity within the PFC and in anterior–posterior connections between the posterior hippocampus and retrosplenial cortex, and PFC regions (Gass *et al*, 2004).

Curiously, while GA produce a substantial and global reductions in cerebral metabolic rate and in cerebral blood (Alkire *et al*, 1995; Alkire *at al*, 1997; Alkire *et al*, 1999), ketamine has the opposite effect increasing cerebral metabolic rate in most brain regions (Langsjo *et al*, 2005).

It is not completely clear if these characteristics are responsible for the antidepressant effect of ketamine.

Major Depression/Depressive disorders

Depression is a heterogeneous disorder with a variable course, an inconsistent response to current treatment and a pathophysiological mechanism that is still poorly understood. The diagnose of major depressive disorder requires a distinct change of mood, characterized by sadness or irritability and accompanied by at least several psychophysiological changes, such as disturbances in sleep, appetite, or sexual desire; loss of the ability to experience pleasure in work or with friends; crying; suicidal thoughts; and slowing of speech and action. These changes must last a minimum of 2 weeks and interfere considerably with work and family relations (American Psychiatric Association, 2013).

Depression is likely to be a multifactorial disease. Concerning genetic factors, most of the evidences comes from twin studies. Some studies show an estimated heritability of MD at 38% (Kendler *et al*, 2006). Environmental factors and genetics both seem to play a role in the etiology of MD (Saveanu *et al*, 2012). Several theories have been developed in the last years trying to explain the mechanism that underlies this complex disease.

The monoaminergic theory of depression

The monoamine theory was raised in 1960´s upon early empirical clinical observations and it postulates that a deficiency of monoamine (serotonin and norepinephrine) neurotransmission in the brain is the main cause for depression. This is corroborated by the observation that monoamine enhancing drugs (e.g. cocaine) quickly improve humor and monoamine depleting drugs (e.g. reserpine) lead to the rapid establishment of depressive humor (Krishnan *et al*, 2008). Monoaminergic neurotransmission is mediated by serotonin (5-hydroxytryptamine 1A and 5-hydroxytryptamine 1B) or norepinephrine (noradrenaline) released from presynaptic neurons. The synthetic pathway of serotonin starts with the catalytic action of tryptophan hydroxylase on tryptophan; norepinephrine is synthesized from tyrosine, with the first step being catalyzed by tyrosine hydroxylase. After synthesis, both monoamine transmitters are stored in vesicles in the presynaptic neuron and released into the synaptic cleft on demand. Upon release, monoamines act in receptors both in pre- and post-synaptic neurons. The activity of monoamines is terminated by local metabolism and reuptake to the pre-synaptic neuron through specific transporter proteins (NET for norepinephrine and SERT for serotonin). Additionally, the activity on pre-synaptic receptors works as a negative feedback system reducing the release of further neurotransmitter. Findings in patients with depression that support the monoamine-deficiency hypothesis include the relapse of depression with inhibition of tyrosine hydroxylase or depletion of dietary tryptophan, increased frequency of a mutation affecting the brain-specific form of tryptophan hydroxylase (TPH-2) in patients, increased specific ligand binding to MAO-A, subsensitive 5-HT1A receptors, malfunctioning 5-

HT1B receptors, decreased levels of p11, polymorphisms of the serotonin-reuptake transporter associated with depression, an inadequate response of G proteins to neurotransmitter signals, and reduced levels of cAMP, inositol, and CREB in postmortem brains (Nutt *et al*, 2002).

A strong point of the monoamine theory has been its predictive power. Almost every compound that has been synthesized for the purpose of inhibiting norepinephrine or serotonin reuptake has been proved to be a clinically effective antidepressant, demonstrated in the Forced Swim Test (a behavioral test specifically designed and validated to evaluate rodents learned helplessness) (Porsolt *et al*, 1978). A single prior injection of antidepressant increases the struggling time in the forced swimming test; results in this model have excellent predictive validity when applied to new antidepressants.

The effects of stimulants on mood indirectly support the monoamine-deficiency hypothesis of depression and show that mood can be altered rapidly. Cocaine and amphetamines are powerful releasers of monoamines into the synapse as well as inhibitors of reuptake (Sitte *et al*, 2015). Their mood-elevating effects are immediate, but in patients with severe depression they have often been reported to cause agitation rather than relief of depression (Post *et al*, 1978). This finding could reflect the additional ability of these stimulants to deplete the presynaptic monoamines and thus cause depression.

The role of DA deficiency in depression is suggested by the frequency of depression in patients with Parkinson's disease (PD) (which is characterize by a massive loss of dopaminergic neurons) and the effect of reserpine, which depletes serotonin, norepinephrine, and DA, causing a hypoactive state in animals (Santiago *et al*, 2014). The antidepressant agent buproprion inhibits the reuptake of DA reinforcing the clinical aspect of DA in depression (Stahl *et al*, 2004).

Current standard of major depressive disorders treatment

The standard of care in pharmacological treatment of major depression for the last 50 years has focused on monoamine neurotransmission modulation, including such treatments as

SSRIs and SNRIs. Both treatment and development of new drugs have been heavily based in the monoamine theory (Schildkraut *et al*, 1995; Racagni *et al*, 2008).

A major problem of the monoamine hypothesis is the lack of pharmacological treatment efficiency. Currently available antidepressants were developed in order to increase the availability of monoamines; however, treatment modalities suffer from drawbacks such as slow onset of clinical effects and limited efficacy in treatment-resistant cases as well as a considerable placebo effect. Approximately only two thirds of patients have a clinical response to these agents and one third have antidepressant response to placebo treatment.

The limited efficacy of treatment imposes substantial burdens on the affected individuals as well as on public health and society. Perhaps the monaminergic hypothesis cannot explain the mechanism of all depressive disorders. Further mechanisms must be explored with the objective to improve knowledge and therapeutic approach to major depression.

Glutamate: a new promise in depression treatment?

Glutamatergic synaptic transmission in the mammalian central nervous system was established in the 1950s and later described as the most important one in excitatory neurotransmission in brain (Orrego *et al*, 1993). Glutamate neurons and synapses outnumber by far most of the other neurotransmitter systems with the exception of GABA. In the early 1980s, NMDA receptors were shown to be involved in several central synaptic pathways, acting together with non-NMDA receptors under conditions where an excitatory postsynaptic potential was elicited in response to intense stimulation of presynaptic fibres (Watkins *et al*, 2006).

In the matter of neurotransmission, brain can be drawn mainly as a glutamatergic excitatory system in competition with a smaller GABAergic inhibitory system that is modulated by a smaller population of heterogeneous neurons releasing several other neurotransmitters, such as the example of neuroamines. The fast regulation of neurotransmission by monoamines is responsible for the modulation of several brain functions such as sleep or motivational

behavior. However, it is the equilibrium between excitatory and inhibitory transmission that mediates most brain functions.

Glutamatergic neurotransmission, present throughout the brain, is responsible for the establishment of a balanced and regular connectivity between different brain areas.

Depressed patients are known to have an imbalance in glutamatergic system, and the antidepressant response is also accompanied by changes in glutamatergic system. Measurements of glutamate and its metabolites in depressed patients revealed changes in several body compartments: there seems to be a trend for increase in glutamate and decrease in glutamine/glutamate ratio in the plasma of patients with depressive disorders when compared with healthy patients (Kim *et al*, 1982; Mauri *et al*, 1998; Mitani *et al*, 2006; Kuçukibrahimoglu *et al*, 2009); there are reports of reduced glutamate in Cerebral Spinal Fluid (*CSF)* in individuals with major depressive and bipolar disorder (Frye *et al*, 2007); and post mortem histopathology and magnetic resonance imaging analysis of studies of depressed patients are in line with the information above (Campbell *et al*, 2006; Lorenzetti *et al*, 2009; Hashimoto *et al*, 2007).

The use of NMDA receptor antagonists in major depression treatment has been defended since 1990s, when early reports suggested that NMDA receptor antagonists showed antidepressant-like action (Trullas *et al*, 1990). Nowadays, none of the approved antidepressants administrated directly targets specifically the glutamatergic system but novel compounds are being explored with that purpose (Agbo *et a*l, 2017; Sanacora *et al*, 2017). Extensive preclinical characterization of the effects of ketamine has partially clarified its mechanism of action, though how ketamine's activity is linked to known pathophysiological changes in depression has just begun to be understood. The glutamate hypothesis is expected to complement the monoamine hypothesis of mood disorders.

Morphologic correlates of depressive models

Analysis of brain volume areas in neuroimaging studies revealed changes in brains of patients with psychiatric disorders, mood, anxiety and depression (Konarski *et al*, 2006; Lorenzetti *et al*, 2009; Koolschijn *et al*, 2009). An imbalance in neural activity within large-scale networks appears to be an important pathophysiological aspect of depression. Major depressive disorder has been associated with abnormal resting-state functional connectivity, especially in cognitive processing and emotional regulation networks.

The default mode network (Collin *et al*, 2014, Stevens *et al*, 2010), a key network activated during rest and thought to reflect self-directed thinking, has frequently been found to show increases in regional cerebral blood flow (Lui *et al*, 2011), cerebral glucose metabolism (Mah *et al*, 2007), and functional connectivity (Sheline *et al*, 2010; Zhu *et al*, 2012; Li *et al*, 2013) in depressed patients. These increases of default mode network connectivity suggest their strengthened roles in coordinating information transfer in brain networks, which may reflect pathologic adaptations.

Using resting-state functional brain imaging techniques, researchers have shown that major depression is associated with hypoactivity in cognitive control brain regions, especially pregenual anterior cingulate, posterior cingulate, and middle frontal gyri and affective control cortical regions such as insula (Sawaya *et al*, 2015). Regions considered overactive in major depression include deeper brain structures such as the thalamus and caudate, among others (Fitzgerald *et al*., 2008). Investigators have also observed increases in functional connectivity of the posterior cingulate cortex with the orbitomedial pre-frontal cortex and dorsolateral pre-frontal cortex in acute depression (Zhou *et al*., 2010) and of the posterior cingular cortex with the medial temporal lobe in remitted depression (Wu *et al.,* 2013). Findings of reduced functional connectivity are most frequently found between cortical and subcortical regions, for example between the anterior cingulate cortex, medial thalamus and pallidostriatum (Anand *et al.,* 2005), the ventromedial pre-frontal cortex, and the cerebellum (Liu *et al*, 2012), and the middle frontal gyrus with the hippocampus (Cao *et al*, 2012). Together, these R-fMRI studies suggest topological disorganization of brain functional networks in depression. Given some

inconsistent findings among studies there is need for further validation of all these findings. However functional dysconnectivity has been reported when depressed patients perform specific emotional or cognitive tasks (Frodl *et al,* 2010; Versace *et al,* 2010). All these works show us that system-level disruption of functional brain networks underlies the mood and cognitive impairments associated with depression, and the regulation of connectivity might be a target to improve treatment.

Depressed patients seem to have a decrease in brain volumes in frontal regions such as the PFC, orbitofrontal cortex, the anterior cingulate, the hippocampus and Str (Grieve *et al*, 2013). Additionally, the structural changes in neuron dendrites are widely studied in preclinical models of stress models which induce depressive like behavior animal models: several models of stress have been shown to induce atrophy, retraction and consistently reproduced remodeling of dendrites in pyramidal neurons of CA3 hippocampal region, in medial PFC and orbitofrontal cortex (Michelsen *et al*, 2007; Cerqueira *et a*l, 2007). The dendritic remodeling induced by stress was reversible after cessation of the exposure to stressors, or after pharmacological treatment (Bessa *et al,* 2009). In other areas, such as the amygdala and the NAc, stress induced an increase in dendritic length (Vyas *et a*l, 2002; Pego *et al*, 2006; Bessa *et al*, 2013). Evidences in rodent studies suggest that stress is a powerful factor in remodeling neuronal architecture, synaptic connections and circuits.

In summary, volumetric alterations are known to take place in brain regions associated to functional changes in depressed patients. The common feature in all these structural changes is occurrence of an overlap between brain areas with volumetric changes and regions where there is a decrease in glial cell population and neuronal structural changes in depressed patients (Rajkowska *et al*, 2002). These data seem to support that changes in glutamate metabolism and synaptic function might be correlated and might be responsible for the dendritic remodeling and tissue morphological changes found in depressive disorders. The use of animal models of stress-induced depressive-like behavior has shown that environmental stress enhances glutamate release and reduce glutamate cycling. The exposure of animals to acute stressors such as tail-pinch or restraint, or the administration of corticosterone produced a rapid increase in the levels of extracellular glutamate measured by

in vivo microdialysis in different brain areas, such as the hippocampus, amygdala or PFC (Moghaddam *et al*, 1993; Reznikov *et al*, 2007).

Impulsivity

Impulsivity can be defined as "a predisposition towards rapid unplanned reactions to internal or external stimuli with reduced regard to the negative consequences of these reactions to the individual or to others" (Moeller *et al*, 2001). Impulsivity is not a unitary construct and it can manifest itself as several behaviors, which include the tendency to act without thinking, on the urge of the moment, the inability to delay gratification, distractibility or the difficulty in the inhibition of incorrect or inappropriate responses. Changes in impulsive behavior have been observed in several mental illnesses. These phenomena can lead to harmful behaviors, including violence, and thus represent a serious public health concern.

Impulsive behavior has been grouped in two subclasses: 1) decision impulsivity, when the actions are initiated without due deliberation of other possible options or outcomes and associated with an increased preference for (smaller) immediate rewards over more beneficial delayed rewards; and

2) response impulsivity when actions are premature and difficult to suppress or control, often related to the inability to inhibit an initiated response.

Impulsivity measurement

The gold standard for measuring decision impulsivity is the delay discounting (Evenden *et al*, 1996), and for response impulsivity is the 5-choice serial reaction time task (Carli *et al*, 1983). Both paradigms are laborious to establish with very long learning curves that lend them unsuitable for testing acute effects of drugs. A new experimental paradigm, designated Variable Delay to Signal (DVS) paradigm, was developed to measure impulsive behaviour in

rodents which provides an effective assessment for both response and decision impulsivity (Leite-Almeida *et al*, 2013). In this task, animals are required to detect and respond to light stimuli presented in one single hole. The animal then indicates the perception of the stimuli by nose-poking the hole. Correct responses are awarded with a food pellet in a dispenser, while incorrect responses cause a time out period. Failure of the animal to wait for the stimuli to respond produces premature responses. In the VDS paradigm, during the test phase the animal is exposed to a period of variable delays to the stimuli, providing a fast-multidimensional assessment of impulsive behaviour with fewer burdens to the animals (Chapter 3).

Neural subtracts of impulsive behaviour

Significant research has been developed in the last years regarding the identification of the brain areas that could be specifically relevant for the impulsivity behavior. In this line of thought, it was identified that brain areas including PFC, Str and NAc (McClure *et al*, 2004; Tamm *et al*, 2004) were involved in impulsivity behavior. The PFC was determined to be involved in the performance of tasks that require behavioral inhibition and in tasks implying variation in delay to perform an action (McClure *et al*, 2004). Beyond frontostriatal circuits, two other circuits were suggested to be particularly important to impulsivity, namely, a reward-discounting circuit where a ventral striatal loop involves the ventral medial PFC, the subgenual cingulated cortex, and the NAc/ventral Str, and a motor control circuit including the ventrolateral PFC, the anterior cingulate, and the presupplementary motor and their link to the caudate nucleus and putamen (Kim *et al*, 2013).

In healthy individuals, stronger ventral Str-seeded connectivity predicted reduced impulsivity in everyday life (Gordon *et al*, 2015). In juvenile subjects, impulsive-irresponsible traits were associated with altered connectivity patterns in the fronto-parietal cognitive control networks (Cohn *et al*, 2015). Many studies have implicated dysfunctional frontotemporal circuitry in impulsivity and aggression in schizophrenia. In patients with cocaine dependence there is a

correlation between impulsivity and cortical connectivity of Orbitofrontal–subgenual cingulate cortex (Contreras-Rodríguez *et al*, 2015), however, the association between network changes and impulsivity is not exclusive to pathological subjects. All these works also show us that impulsive trait, in healthy or diseased subjects, is related with changes in brain connectivity.

Neurotransmitters in impulsivity

Wide ranges of disorders, such as attention deficit/hyperactivity, substance abuse or pathological gambling are characterized by changes in impulsivity. These pathologies also have in common DA changes (Buckholtz *et al*, 2010). To reinforce this observation, Catalan and colleagues later reported that patients of DA related-medical conditions, such as PD, showed impulsive behavioural changes after being treated with DA agonists (Catalan *et al*, 2013).

Evidence for the involvement of the dopaminergic system in impulsivity was initially suggested following the identification of receptor gene variants in attention deficit hyperactivity disorder (Gadow *et al*, 2008). More specifically it was observed that DA plays a paramount role in the development and function of frontostriatal circuits and for value based decisions-making (Volkow *et al*, 2009). Runia and colleagues have demonstrated that DA is also important for attention processes (Rubia *et al*, 2009).

DA neurotransmission is also affected by GA and it may lend subjects more prone to impulsive/risky behavior. This subject is further explored in chapter 1.

Serotonine (5-HT) was also observed to be important in impulsive behavior. Further investigation of attention deficit hyperactivity disorder, Oades and colleagues were able to establish an inverse correlation between the role of this neurotransmitter levels and the impulsive traits in different spectrum of the disease (Oades *et al*,2008).

The study of potential long-term effects of exposure to the GA focused on determining the role of GAs exposure to the deterioration of brain functions. Following the above, Ketamine is an interesting study topic both in the perspective of GAs as well as antidepressants.

The main action of ketamine is at the level of the NMDA receptors as a noncompetitive antagonist, but it also interacts with DA and serotonin receptors. For clinical application, the ketamine is a drug with a broad spectrum of effects: at low doses has analgesic and antidepressant action, at higher doses induced psychotic effects, and at high doses induces anesthesia. The versatility of ketamine makes it a drug with great potential in the study and design of new therapeutic approaches in several pathologies. The potential as an antidepressant as well as the effect of ketamine in modulating circuits important for motivational behavior were the reasons that led us to develop the experimental work leading to the preparation of this thesis.

Our laboratory has a long tradition in the study of the effects of stress. The study developed in our laboratory on the effects of stress is multidisciplinary and involves not only the study of the changes triggered by stress (Pego *et al*, 2008, Cerqueira *et al*,2007) but also the evaluation of potential therapies (Bessa *et al*, 2009). Previously the morphological changes induced by chronic stress models were identified (Cerqueira *et al*, 2007), as were also evaluated the effects of drugs in reversing them (Bessa *et al*, 2009; Bessa *et al*, 2013). There is also an extensive work in developing new paradigms for the evaluation of animal behavior. (Leite-Almeida *et al*, 2013; Morgado *et al*, 2014).

In order to combine the experience of the working group with the study of a drug that looks promising, the main objectives we set in the development of this thesis were:

- Identify the effect of ketamine as an adjuvant to the classical antidepressants in the response to treatment in an animal model of induced depressive like behavior.
- Evaluate the effects of ketamine on the reversal of neuronal morphological changes induced in the animal model of depressive like behavior

- Assess the effects of ketamine on glutamatergic dynamic in key areas affected by animal model of depressive like behavior.
- Assess the long-lasting effects of ketamine in impulsive behavior when used in anesthetic doses and in repeated GA procedures.

CHAPTER 1

A. Melo; I. Tavares, N. Sousa; J.M Pêgo

"Can the dopaminergic-related effects of general anesthetics be linked to mechanisms involved in drug abuse and addiction?"

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Can the dopaminergic-related effects of general anesthetics be linked to mechanisms involved in drug abuse and addiction?

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General anesthetics (GA) are well known for the ability to induce a state of reversible loss of consciousness and unresponsiveness to painful stimuli. However, evidence from animal models and clinical studies show that GA exposure may induce behavioral changes beyond acute effects. Most research and concerns are focused on changes in cognition and memory. We will look at effects of GA on behavior that is mediated by the dopaminergic system. Pharmacological resemblance of GA with drugs of abuse, and the complexity and importance of dopaminergic systems in both reward seeking and addictive illnesses make us believe that it deserves an overview about what is already known and what matters to us as healthcare workers and specifically as anesthesiologists. A review of available evidence strongly suggests that there may be a link between the effects of GA on the brain and substance abuse, partly explained by their influence on the dopaminergic system.

Editorial comment: what this article tells us
This review article points out that general anesthesia may induce behavioral changes mediated via the dopaminergic system, and that some of the same mechanisms may be involved in substance abuse and reward seeking behavior.

Development of new drugs and technics in anesthesia was of paramount importance to the revolution of modern medicine. The pursuit of the ultimate perioperative homeostatic balance and increased awareness of safety issues allowed us to achieve lower levels of morbidity and mortality as standard of care.[1]

We have used GA regularly since 1846, but we have not been able to build a complete theory that unifies both the molecular effects and the behavioral response of loss and gain of consciousness. The first theory (Meyer–Overton correlation) stated that lipid solubility of GA was responsible for their anesthetic effects.[2] When

such a generalistic statement failed to explain how GA work, then the paradigm moved looking for specific molecular targets. The discovery that GA could interact with the firefly luciferase enzyme directed research toward proteic targets.[3] Today, we know that GA target neurotransmitter receptors in the whole brain, inducing fast modulation of membrane potentials and neuronal cell firing, as well as slower modulation of second messenger cascades and protein synthesis compounds that are responsible for fast behavioral changes involving arousal, memory, nociception and fear. Additionally, research has shown us that exposure to GA can also be responsible for changes that are not so short-lived.

Reports of temporary cognitive impairment and long-term neurodevelopmental impairment in animal models of anesthetic exposure[4–6] as well as clinical reports of decline in cognitive performance after surgery/anesthesia in humans[7,8] brought an old question to the spotlight: can GA exposure change the way we subsequently behave? Now we know that factors such as surgical procedure together with individual factors influence patient cognitive outcome[9,10] and that exposure to GA alone has not been proven to be responsible for cognitive impairment in humans, even in groups thought to be particularly susceptible such as children.[11] Most research is focused on cognitive processes, but one particular clinical study, the ISPOCD, reported both higher risk of prematurely leaving labor market and of dependence on social transfer payments in patients who developed postoperative cognitive dysfunction,[12] suggesting that impairment may extend beyond cognition. Do GA exert a more subtle influence on us, not necessarily through a decline in cognition? To answer this particular questions, we will focus our discussion on other dimensions of behavior. The molecular resemblance of GA with drugs of abuse and the misuse of GA for recreational purposes[13] raise concerns about the possible role of GA as agents that induce changes in motivational behavior. From all the neurotransmitters that are targeted by GA, dopamine (DA) is the most important in motivational and reward circuitries, with a strong role in conditioning behaviors. In this review, we will focus on the possible link between the effects of GA and drug abuse and how these mechanisms may help explain some of the potential effects of GA on the brain.

Understanding the role of dopamine

DA is a catecholaminergic neurotransmitter present both in the central nervous system and in several other tissues such as the cardiovascular and digestive systems. DA is synthesized by the hydroxylation of the amino acid L-tyrosine to L-DOPA by tyrosine hydroxylase (TH) which is further converted to DA by DOPA decarboxylase (or aromatic L-amino acid decarboxylase). DA is stored in vesicles in the presynaptic terminal by the action of vesicular monoamine transporter. DA release from dopaminergic neurons into the synaptic cleft is achieved either through a calcium-dependent exocytic process similar to other neurotransmitters or through membrane DA transporter (DAT). Once in the synaptic cleft, DA binds to and activates DA receptors (DAR). According to their biochemical and pharmacological properties, the receptors can be divided into two subtype families: D1-like receptor subfamily that includes the D1 and D5 receptors, and the D2-like receptor subfamily comprising the D2, D3, and D4 receptors.[14] The turnover of extracellular DA involves both degradation by two main enzymes: monoamine oxidase and catechol-O-methyltransferase and reuptake by DAT, all critical elements in DA homeostasis.[15,16]

Dopaminergic neurotransmission plays a critical role in processes such as learning, memory, motivation, reward, risk assessment and locomotion.[17–19] Conditions that challenge DA balance may impair these functions. In the brain, we can find higher content in production areas like *pars compacta* of the *substantia nigra* (SN) and the ventral tegmental area (VTA). From these, dopaminergic pathways project to the nucleus accumbens (NAc), the frontal cortex (FC), and the striatum (Str).

Parkinson's disease (PD) is a DA-related pathology in which there is a state of low DA levels in SN, characterized by several motor coordination and involuntary movement disorders. PD treatment is based on the use of DA precursors such as Levodopa (L-DA) and DA

agonists. Importantly, several reports show that the prolonged use of these drugs in PD patients is related to an increase in compulsive gambling/shopping/eating behavior, hypersexuality, and hyperphagia disorders.[20]

Changes in brain DA content induce behavioral modifications, but how does this knowledge correlate with the anesthesia field?

Occupational addiction in anesthesia

Substance abuse in health professionals[21] is a known problem. The literature about this subject shows that occupational hazards do not translate in an increase in mortality of anesthesiologists compared either with other specialties or general population[22,23]; however, there seem to be an increased risk of substance abuse and suicide.[24,25]

The pharmacokinetic of short-acting drugs such as propofol, remifentanil, and volatile anesthetics make them virtually impossible to trace in routine testing, and unless the health worker is caught consuming or stealing, only testing all health workers for drugs of abuse would give us the real picture.

The anesthesiologist faces professional challenges such as exposure to stressful situations and work overload that can lead to isolation, burnout,[26] and depression.[27] Physicians under these conditions may, therefore, develop maladaptative strategies that lead to substance abuse.[28] Stress is a known trigger of changes in brain reward circuits[29] that may enhance the reinforcing properties of drugs. GA have pharmacological similarities to drugs of abuse: reports show characteristics of high psychological dependence such as relapse, strong cravings, and continuous auto-administration irrespective of negative consequences.[30] On the top of the most misused drugs, we can find opioids and intravenous anesthetics, benzodiazepines, and lastly volatile anesthetics. There is also speculation that environmental exposure to GA can induce changes that in a certain way could lead to the development of addictive traits.[31] The fact that healthcare professionals exposed to stressful environments also have easy access to drugs with abuse and misuse potential turn this issue not an institutional problem but a public health one.

May general anesthetics be involved in development of addiction?

Several drugs used during anesthetic procedures have a direct effect on the dopaminergic system. The most well-known and studied substances that induce DA changes and addiction are opioids, but we will focus specifically on GA. Acute exposure to most GA produces a mixture of sensations described as feeling drunk, confusion, sedation, and loss of concentration capacity. It can also induce psychadelic-like effects such as dissociation, hallucinations, and distortions in perception of reality. Volatile anesthetics are chemically similar to solvent agents often used as recreational drugs and produce similar behavioral effects.[32,33] It is impossible to talk about anesthetics and DA without recalling the origins of anesthesia: the first two substances used as anesthetics, nitrous oxide and ether, were used recreationally even before being introduced in medical practice as stated in historical reports describing "laughing gas parties".[34] In human studies,[35–38] subanesthetic doses of sevoflurane, nitrous oxide, propofol, and ketamine all correlated with liking and were rated as something the subject "will try again"; they also produced dose-related reinforcement and abuse-related subjective effects. Ketamine is a well-known club drug, and users display riskier behavior.[39] The effects of GA exposure in behavior of animal models have also been studied and correlates with behavioral changes similar to drugs of abuse such as anxiety and craving. Nitrous oxide is known to induce anxiolysis in animal models, and the effect is reversed by the benzodiazepine antagonist flumazenil.[40] These reports suggest that exposure to GA can induce addictive behaviors both in animal models and in humans.

The impact of general anesthetics on brain dopamine

As above mentioned, GA act in the whole brain: they modify neuronal system, the release and reuptake of neurotransmitters, and the way neurons respond to them. The sum of all these effects represents the behavioral endpoint of GA action: loss of consciousness, immobility, and amnesia. DA is believed to contribute to GA

effects as the amount of dopaminergic activity influences the amount of GA needed to induce anesthesia.[41] On the other end, the depletion of brain DA can induce a state of immobility.[42]

We will now make considerations on the modulation of DA in brain by different anesthetic agents. Most of the data are based in microdialysis studies where samples of brain interstitial fluid are sampled during exposure to GA alone or with DA modulators in translational research using rodent and primates.

Halotane

Exposure to halothane in high doses increases extracellular DA levels in Str[43–45] and potentiate the dopaminergic action of other drugs.[46,47] The level of dopaminergic metabolites is also increased indicating a higher turnover. The use of lower anesthetic doses fail to increase DA levels; however, DA metabolites still increase. So, there seems to be a complex dose-related response, but there is always some effect. In the NAc, there is also an increase in DA.[48] So evidence show that halothane seems to induce DA availability in areas that play an important role in DA driven behavior.

Isoflurane

Isoflurane anesthesia also induces a dose-dependent increase in Str DA[49–51] with lower doses failing to show changes in brain DA but producing changes in metabolites.

Nitrous oxide

The use of this volatile NMDA antagonist induced a slight DA increase in NAc and a decrease or no effect in Str.[52,53]

Xenon

Use of xenon failed to change DA levels in NAc. There are no works regarding other brain areas.[54]

Ketamine

In animal models, the NMDA antagonist seems to have almost no effect in DA levels when used in low dosages, but higher subanesthetic and anesthetic dosages increase DA in Str, NAc and FC.[55–58] This effect is also seen in human in vivo imaging studies that report an increase in striatal DA release after an acute challenge with ketamine.[59] But when the exposure is repeated, there is a reduction in FC dopaminergic function with impairment in working memory and executive functions.[60]

Pentobarbital

Pentobarbital induces decreases DA in the NAc, producing a state of ataxia in rodents. It also inhibits the effect of L-DOPA in extracellular DA increase.[50] Like other GAs, when given in lower doses does not change DA levels.[58]

Propofol

Propofol at lower subanesthetic dosages decreases DA NAc content while more clinically relevant higher subanesthetic and anesthetic dosages of propofol increase NAc DA levels.[61] Propofol also has the ability to induce expression of DeltaFosB in NAc, a protein whose expression is also increased by drugs of abuse.[62] Additionally, propofol exposure decreases DA levels in Str and in FC.[63]

Measurements of dopaminergic activity either in DA production, degradation, and reuptake can be used to assess dopaminergic pathways. DA is degraded into 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). In rodents, DOPAC is the major metabolite and DOPAC accumulation provides an indicator of dopaminergic neurons activity, while the DOPAC:DA ratio is an indication of DA turnover. In Str, DA metabolite levels are increased by halothane, isoflurane, sevoflurane, and propofol exposure.[44–46,64] In the NAc, they are increased after exposure to isoflurane,[65] sevoflurane, and propofol. In addition, DAT seems to be inhibited by most GAs. In fact, studies show that halothane, isoflurane, propofol, ketamine, ethomidate, and thiopental inhibit specific synaptosomal uptake of DA in a concentration-dependent manner in rat brain.[41,66,67] The overall effect of exposure to GA is a dose-dependent increase in DA and its metabolites during acute exposure.

What are the implications of changed DA levels induced by GA?

We will now focus the effect of GA in brain areas relevant to DA-driven behavior. Striatal influence of GA seems to be "agent" and "dose-specific", but there is no doubt that GA have an impact on Str DA release. The Str serves as the entry point for cortical and thalamic inputs into basal ganglia circuitry. The release of DA in Str during reward learning tasks is known to be an important modulator of acquisition of habit or goal-directed tasks. Disorders that affect DA such as PD, Huntington's disease, and substance abuse produce impairments in these processes. Exposure to GA specifically halothane, isoflurane, and ketamine have the potential to impair those functions through changes in Str DA.[43–47,49–51,55]

The NAc is believed to participate in many functions that have been shown to be important in reward learning tasks.[68] Most drugs of abuse are known to produce an increase in DA levels at the NAc, in a manner similar to propofol, ketamine, and halothane.[48,52,57]

Dopaminergic activity in the prefrontal cortex (PFC) plays an important role in cognitive functions. DA depletion in PFC impairs working memory performance tasks in primates[69,70] and the use of DA agonists improves performance in animals with poor working memory.[71,72] Both human and animal studies suggest that repeated exposure to noncompetitive NMDA antagonists reduces PFC dopaminergic function with impairment in working memory and executive function.[73,74] We can speculate that while acute exposure to GA with NMDA antagonist activity induces increase in PFC DA, continuous exposure is prone to decrease PFC DA and impair working memory and executive function performance which is the pattern found in chronic users. Chronic exposure to GA, such as repeated anesthetic procedures, theoretically can induce the same changes. Such as stated earlier, there are concerns of a similar mechanism responsible for development of addiction in susceptible individuals subjected to environmental exposure.

To summarize, increase in DA metabolites suggests that most GA induce higher DA levels and turnover in several brain regions, especially in the Str and in the NAc. Activation of these particular areas is a hallmark pattern of several drugs that induce addiction and impair DA driven behavior.

Conclusion

Review of the literature suggests that general anesthesia modulates the dopaminergic pathways. Behavioral data both in human and animal models support the possible development of an addictive trait in subjects exposed to GA. Some of the molecular features of drugs of abuse concerning DA are also found in GA such as DA release and availability in areas such as NAc and Str. It is likely that all behavior functions that rely on dopaminergic transmission can be potentially impaired after GA exposure. Changes in reward system and memory formation potentially may impair cognitive abilities such as reasoning, language comprehension, planning, and spatial processing. Several clinical trials show that surgery and anesthesia may cause "postoperative cognitive dysfunction" and changes in dopaminergic brain systems may contribute to this phenomenon. However, we still do not know how much it impacts on our behavior. The potential to play with reward mechanism, decision-making processes and cognitive performance impose a need for judicious use of GA. Further research is needed to answer all these questions and provide both even better standard of care to our patients and less occupational hazards to healthcare workers.

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C H A P T E R 2

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“The positive effect on ketamine as a priming adjuvant in antidepressant treatment”

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ORIGINAL ARTICLE

The positive effect on ketamine as a priming adjuvant in antidepressant treatment

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Ketamine is an anesthetic with antidepressant properties. The rapid and lasting effect of ketamine observed in preclinical and clinical research makes it a promising therapeutic to improve current major depression (MD) treatment. Our work intended to evaluate whether the combined use of classic antidepressants (imipramine or fluoxetine) and ketamine would improve the antidepressant response. Using an animal model of depressive-like behavior, we show that the addition of ketamine to antidepressants anticipates the behavioral response and accelerates the neuroplastic events when compared with the use of antidepressants alone. In conclusion, our results suggest the need for a reappraisal of the current pharmacological treatment of MD.

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INTRODUCTION

Major depression (MD) is a highly incident and prevalent multifactorial disease responsible for great personal and socio-economic burden.[1] Currently used therapeutic regimens have important limitations such as long-time lag for clinical response, induction of resistance or even response failure.[2,3] Several different factors seem to have a role in MD: imbalances in neurotransmitters[4] and cytokine production,[5,6] hypothalamic–pituitary–adrenal axis dysregulation[7] and impaired neuroplasticity.[8,9]

Currently used antidepressants target the metabolism/transport of monoaminergic neurotransmitters, such as serotonine and noradrenaline. However studies show that glutamate (GLU) deregulation is also implicated in MD.[10,11] The use of NMDA (*N*-methyl-D-aspartate) receptor antagonists as antidepressants is a relatively new concept.[12] Ketamine (KET), an NMDA antagonist, was observed to induce a rapid antidepressant action in MD patients,[13] even in those considered treatment resistant.[14,15] The rapid antidepressant effect of KET has also been described in animal models of depressive-like behavior.[16,17] Such an effective drug promises to be an important therapeutic tool to improve MD treatment efficacy but, surprisingly, studies where classic antidepressants are used in combination with NMDA antagonists are scarce. Therefore, herein we assessed whether the use of an acute KET treatment could improve the efficacy and/or anticipate the clinical effect of both tricyclic antidepressants and selective serotonin reuptake inhibitor agents in a rat model of depression.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River Laboratories, Barcelona, Spain), aged 3 months were housed (three per cage) under standard laboratory conditions (12 h light: 12 h dark cycle, at 22 °C, relative humidity of 55%; free access to food and water). Eighty-four animals were used. Animals initially performed the determination of the baseline value of sucrose consumption test (SCT) and then were randomly assigned to the following experimental groups—a control group without stress exposure and six groups exposed to unpredictable chronic mild stress (uCMS). After 4 weeks used to establish a depressive-like behavior phenotype, uCMS-exposed animals were randomly assigned to six different groups: one untreated group (i) uCMS, and the other five experimental groups according to the assigned treatment: (ii) imipramine, (iii) fluoxetine, (iv) KET alone for the first 3 days and saline for the remainder period (uCMS-KET), or treated with KET daily for 3 days and either (v) FLX (KET-FLX) or (vi) IMP (KET-IMP) for the remaining treatment period ($n = 12$ per group; Figure 1).

Behavioral tests were conducted during the diurnal phase, between 1000 and 1800 h. Animals were euthanized 24 h after performing the last behavioral evaluation and brains processed for neurochemical and structural analysis. All the procedures were carried out in accordance with the Animal Ethics Committee of the Portuguese National Veterinary Directorate and with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament.

Unpredictable chronic mild stress

A modified version of an uCMS protocol[18] was used to establish the animal model of depressive-like behavior. It consisted of chronic exposure to unpredictable mild stressors (confinement to a restricted space for 1 h, placement in a tilted cage (30 °) for 3 h, housing on damp bedding for 8 h, overnight illumination, 18 h food deprivation followed by exposure to inaccessible food for 1 h, water deprivation for 18 h followed by exposure to an empty bottle for 1 h, and reversed light/dark cycle for 48 h every 7 days) until establishment of the model phenotype and then through the duration of behavioral evaluation.

Antidepressant treatment

After establishment of depressive-like behavior, uCMS animals were treated according to the assigned group with daily intraperitoneal injections of saline, FLX (10 mg kg^{-1}, Kemprotec, Cumbria, UK), IMP (10 mg kg^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and KET (10 mg kg^{-1}, Pfizer) with 0.9% saline as vehicle for 14 days and then performed behavioral evaluation. Treatment and administration scheme were chosen on the basis of their therapeutic effects in previous studies.[8,19]

KET is a general anesthetic with psychomimetic characteristics and has been used to induce psychotic trait in animal models of schizophrenia. To rule out that the dosage-induced phenotypic changes related no animal

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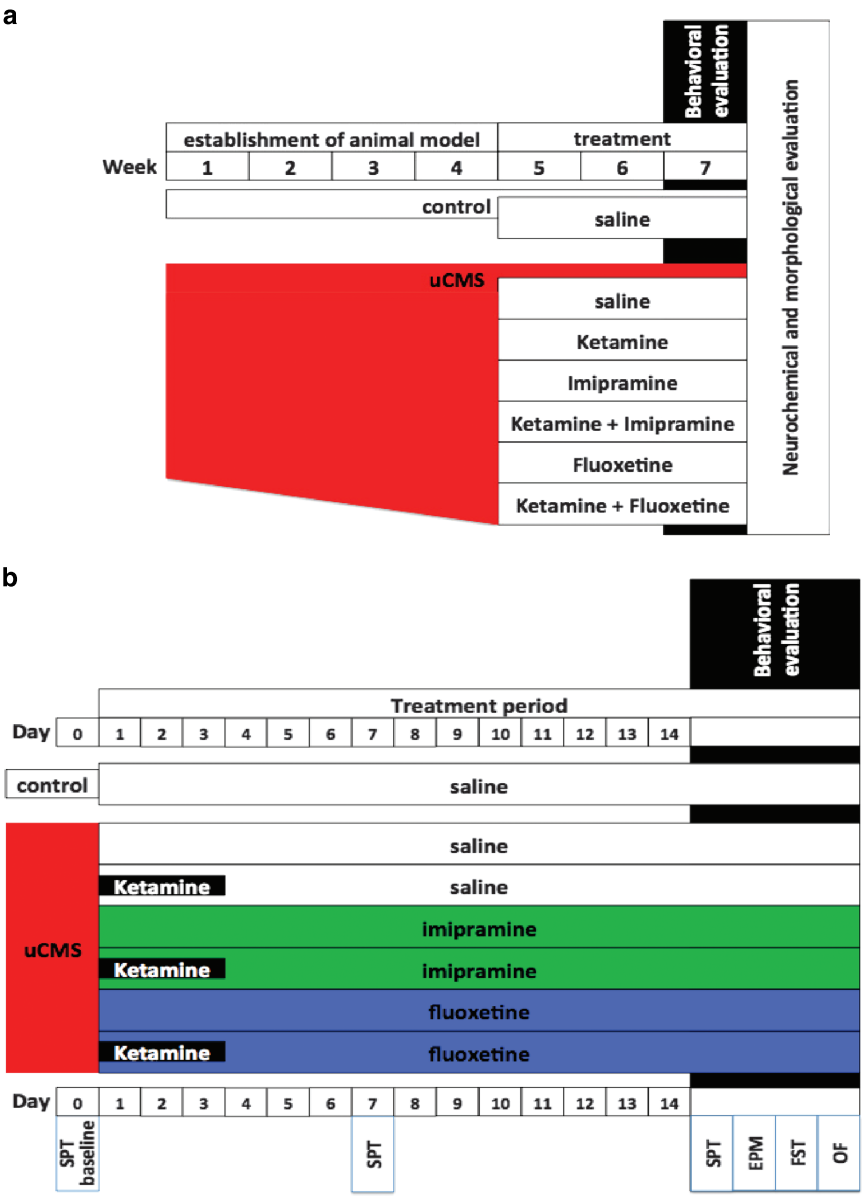


Figure 1. Diagram with experiment protocol. Complete experiment protocol (**a**); treatment period and behavioral evaluation (**b**). EPM, elevated-plus maze; FST, forced-swim test; OF, open field; SPT, sucrose preference test; uCMS, unpredictable chronic mild stress.

models of schizophrenia, we assessed locomotor activity in the open field and sensorimotor gating in the PPI.

Behavioral analysis

All animals (controls and uCMS-exposed) were submitted to a series of behavioral testing. Sucrose preference tests were performed weekly over the 6 weeks of exposure to uCMS. The remainder tests (elevated-plus maze, forced-swim test and open field) were performed after 14 days of treatment (Figure 1).

Sucrose consumption test

Anhedonia was assessed weekly during exposure to uCMS using the SCT. Briefly, animals were allowed to habituate to the sucrose solution for 1 week before the uCMS protocol to establish baseline preference levels. To test sucrose preference, animals that were food- and water-deprived for 18 h were presented with two pre-weighed bottles containing 1% sucrose solution or tap water for a period of 1 h. Sucrose preference was calculated according to the formula: sucrose preference = (sucrose intake/(sucrose intake+water intake)) × 100, as previously described.[8] Anhedonia was defined as a reduction in sucrose preference.

Elevated-plus maze

To assess anxiety-like behavior, animals were tested on an elevated-plus maze (MED-NIRPMNR; Med Associates, St Albans, VT, USA) as previously described.[20] Animals were placed in the central junction facing an open arm, and allowed to explore for 5 min. Entry was defined as all four paws being positioned within one arm. The test was recorded and the ratio between time in open arms and time in closed arms was measured. Activity in the open arms was calculated as open arm entries percentage (entries into the open arms/total entries into all arms) and time spent in open arm percentage (time spent in the open arms/total time spent in all arms). The degree of anxiety was indirectly related to the time spent in the open arms and the number of open arm entries.

Forced-swim test

Learned helplessness was evaluated in the forced-swim test on the last day of exposure to uCMS. Twenty-four hours after a pre-test session (10 min), rats were placed in cylinders filled with water (25 °C; depth 30 cm) for a period of 5 min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint, Lyon, France); the system automatically calculated immobility time and latency to immobility. Learned

helplessness behavior was defined as an increase in time of immobility and a decrease in latency to immobility.

Open field

To assess locomotor activity, rats were placed in an open-field apparatus (43.2 (length) × 43.2 (width) × 30.5 (height) cm, transparent acrylic walls and white floor, Med Associates) in a room illuminated by white light. Instant position was monitored over a period of 5 min by an array of two 16 beam infrared arrays. Total distance and average speed was used as a measure of locomotor activity.

Pre-pulse inhibition

An additional setting of animals was used to evaluate KET safety regarding induction of pre-pulse inhibition (PPI) impairment. Startle reflexes were measured in two identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA), each consisting of a non-restrictive Plexiglas cylinder (8.8 (internal diameter) cm, 22.2 (length) cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system (San Diego Instruments) was used to ensure comparable startle magnitudes across the two devices. Startle stimuli were presented through a high frequency speaker located 33 cm above the startle chambers. Startle magnitudes were sampled each millisecond (ms) during a period of 200 ms beginning at the onset of the startle stimulus. A startle response is defined as the peak response during this 200-ms period. Animals were habituated to the apparatus 5 min daily 2 days before the testing period. After the habituation period, rats were taken from their home cage and placed in the test chamber. The chamber was then sealed and each animal allowed to acclimatize to the startle chamber for a period of 5 min. In addition, background white noise, with an intensity of 63 dB, was maintained to minimize the impact of acoustic stimuli outside the chamber environment. Following five introductory 120-dB startle trials (noise lasting 40 ms), a total of 35 test trials were pseudo-randomly delivered as follows: (a) five trials with background noise only, (b) 10 startle trials of 120 dB and (c) five pre-pulses of each of four different intensities preceding a startle trial. Pre-pulse intensities of 2, 4, 8 and 16 dB above the background noise level lasted 20 ms and preceded the 120-dB startle presentation in 100 ms. Inter-trial intervals ranged from 10 to 20 s. The average startle response (AVG) was assessed in the 100-ms period following the onset of the startle stimulus presentation.

Neurochemical analysis

Monoamines levels were measured using high performance liquid chromatography (HPLC), combined with electrochemical detection. From each experimental group, seven rats were killed by decapitation. After decapitation, the animal heads were snap-frozen in liquid nitrogen to prevent tissue degradation. The brains were quickly extracted and placed in an icy surface; under a stereomicroscope the prefrontal cortex (PFC), dorsal hippocampus (DH), ventral hippocampus (VH) and nucleus accumbens (NAc) were dissected. The dissected tissues were weighed and then homogenized and deproteinized in 100 µl of 0.2 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $Na_2S_2O_5$ and 91.3 mM Na_2-EDTA (Riedel-de Haën AG, Seelze, Germany), centrifuged at 15 000 r.p.m. for 45 min at 4 °C and the supernatant stored at − 80 °C until analysis.

The analytical measurements were performed using a GBC LC1150 (GBC, Braeside, VIC, Australia) HPLC pump coupled with a BAS LC4C (Bioanalytical Systems, West Lafayette, IN, USA) electrochemical detector and pre-column derivatization as described previously.[21] The working electrode was glassy carbon, the reference electrode was Ag/AgCl and the columns used were ODS Hypersil, 250 mm × 4.6 mm, 5 µm (Thermo Fisher Scientific, Waltham, MA, USA). The voltage of the working electrode was set at +800 mV in the LC4C amperometric detector and the flow rate of the LC1150 HPLC pump was set at 1.0 ml min^{-1}. The mobile phase consisted of an acetonitrile (Merck, New York City, NY, USA): 100 mM phosphate buffer (5:95) pH 4.9, containing 50 µM Na_2-EDTA (Riedel-de Haën). Samples were initially diluted 1:5 with ddH_2O, then further diluted 1:1 with 0.1 M Borax buffer (Sigma-Aldrich), pH 9.6. o-Phthalaldehyde (Sigma-Aldrich) was subsequently added and left to react at room temperature for 10 min before injection. Quantification of glutamate and aspartate was done by comparison of the area under the curve with that of reference external standards using HPLC software (Clarity, Data-Apex, Prague, Czech Republic), as previously described.[22,23]

Histological procedures

After the end of behavioral evaluation, five rats from each group were perfused transcardially with saline (NaCl 0.9%) under deep pentobarbital anesthesia. Brains were removed and kept in Golgi-Cox solution for 15 days and then transferred to a 30% sucrose solution for 5 days. Sections (200 µm) were obtained using a vibratome and collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene-cleared before coverslipping. Dendritic arborization and spine numbers were analyzed in layer II/III of infralimbic area of PFC, and dentate gyrus and CA3 region of hippocampus. Selected neurons had every branch of the dendritic tree reconstructed at × 1000 (oil) magnification using a motorized microscope (Axioplan 2; Carl Zeiss, Jena, Germany) and Neurolucida software (Microbrightfield, Williston, VT, USA) and three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). For each animal, 20 neurons were studied and measurements from individual neurons from each animal were averaged. The following dendritic morphology parameters were examined: dendritic length and the number of primary dendrites and dendritic branching points were compared across experimental groups; dendritic spines were assessed according to their morphology mushroom-shaped, thin, thick and ramified spines and dendritic spine density (number of spines/dendritic length) and the proportion of spines in each category was calculated for each neuron in branches that were either parallel or at acute angles to the coronal surface of the section. In dentate granule cells, proximal and distal branches were analyzed for each neuron; basal branches and proximal and distal apical branches in pyramidal neurons in the CA3 region and infralimbic area of the PFC were analyzed. Neuronal reconstructions were masked to the observer.

Statistical analysis

Sample size was determined considering a medium effect size ($f = 0.25$), a type I error $\alpha = 0.05$ and a statistical power (1—type II error) of 0.8, and also the performance of HPLC and golgi analysis after behavioral analysis, with the need to establish two diferent groups of animals. In practice, we ended up using a total of 84 animals, with $n = 12$ to each experimental group.

During the experiment, we performed three moments of randomization: the first one after the establishment of a baseline in SCT, the second one after establishment of the uCMS phenotype, and the last one after behavioral evaluation to establish two separate groups to perform either HPLC or neurostructural analysis.

We used masking of the experimental group assignment to all animal/samples during performance and analysis of behavior tests, HPLC and structural Golgi analysis.

Data obtained in experiment were analyzed applying analysis of variance. Quantitative data obtained for parameters analyzed regarding elevated-plus maze, forced-swim test, open field, PPI, neuronal dendritic architecture and HPLC measurements were analyzed using one-way analysis of variance, and whenever appropriate, *post hoc* comparisons between experimental groups were performed using Bonferroni's test. For data obtained in the SCT, we used repeated measures analysis of variance. Sphericity assumption and homogeneity of group variances were verified and statistical analyses were made accordingly. During statistical analysis, we did not perform any data transformation. All data are presented as means ± s.e.m. In all cases, statistical significance was set at $P \leqslant 0.05$ (two-sided). Statistical analysis was performed using IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Behavioral data

To evaluate hedonic behavior, the SCT was performed weekly during the establishment of the depressive-like behavior and during the treatment period. After 4 weeks of exposure to uCMS, animals developed a significant difference in sucrose preference compared with control animals ($P < 0.05$). On the seventh day of treatment, animals treated with KET ($P < 0.05$), FLX-KET ($P < 0.05$) and IMP-KET ($P < 0.05$) animals ($F_{6,77} = 4.179$, $P < 0.01$), but not the

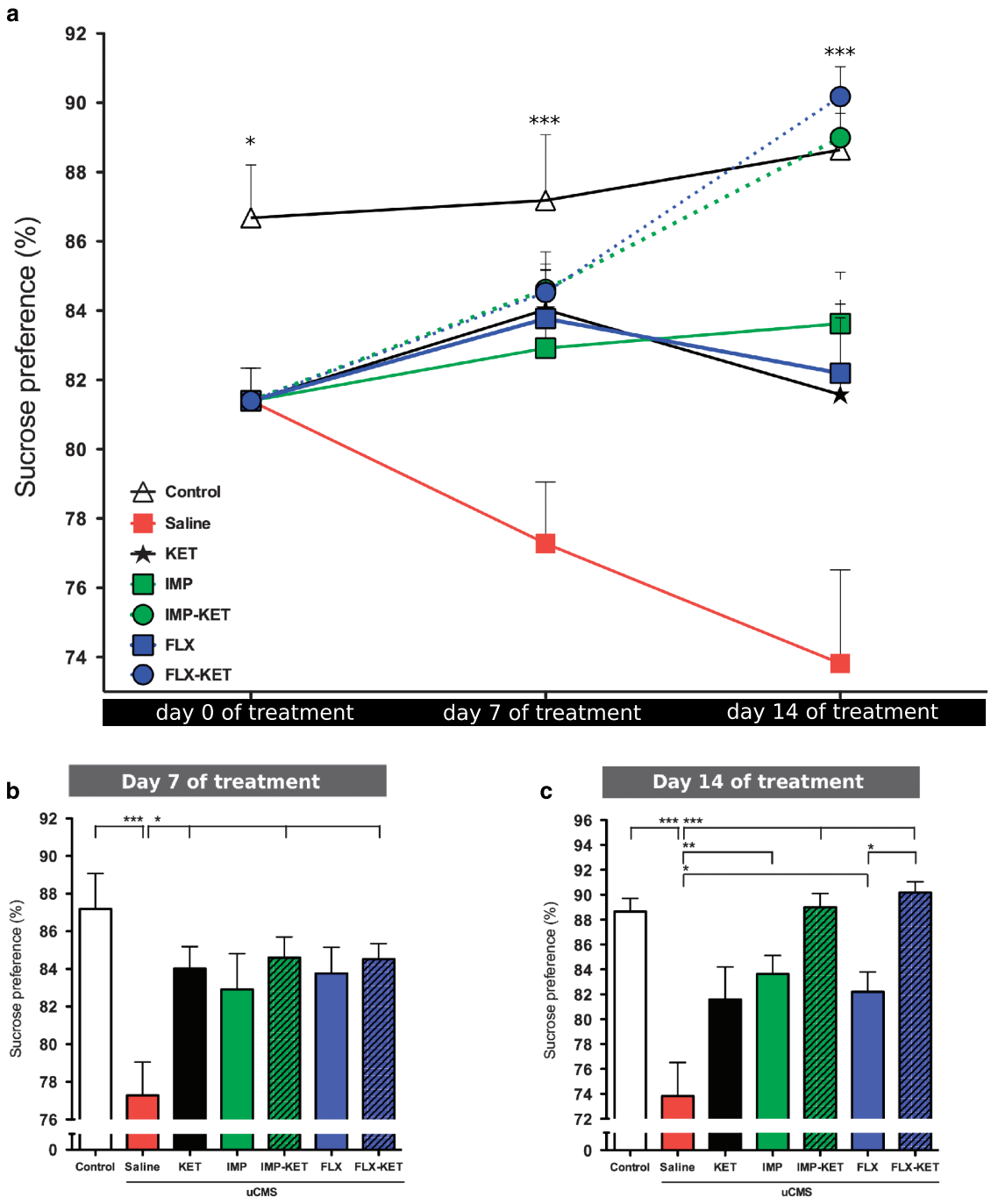


Figure 2. Ketamine effect in the reversion of anhedonic phenotype. Effect of uCMS in sucrose preference (**a**). Reversion of anhedonic phenotype at the end of first week of treatment in KET, FLX-KET and IMP-KET ($P < 0.05$) (**b**); Rescue of anhedonic behavior by the end of second week of treatment in all animals treated with antidepressant and the addition of KET produces a significantly higher sucrose preference by the end of the second week when comparing the group FLX-KET with FLX ($P < 0.05$) (**c**). uCMS, umpredictable chronic mild stress; FLX, fluoxetine; IMP, imipramine; KET, ketamine; (mean ± s.e.m., $n = 12$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

remaining treated groups had reversion of anhedonic phenotype (Figure 2b). On the 14th day, all animals treated with antidepressant display behavioral rescuing of anhedonic behavior (IMP ($P < 0.01$) and FLX ($P < 0.05$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$)), except for those given only KET that did not display any longer the behavioral rescuing effect detected 1 week before ($F_{6,77} = 10.50$, $P < 0.001$; Figure 2c). In animals given FLX, when KET was added (FLX-KET) there was a significantly higher sucrose preference at 14th day when compared with animals given FLX alone (Figures 2a and c).

In the forced-swim test, uCMS animals showed decreased latency to immobility time ($F_{6,77} = 7.912$, $P < 0.001$) and increased immobility time ($F_{6,77} = 11.00$, $P < 0.01$) when compared with control animals. Comparing with uCMS, all treated animals showed a significantly increased latency to immobility: FLX, KET ($P < 0.05$), IMP ($P < 0.01$) and IMP-KET, FLX-KET ($P < 0.001$; Figure 3a); and also a significant decrease in immobility time: IMP, FLX ($P < 0.05$) and IMP-KET, FLX-KET ($P < 0.01$; Figure 3b). In latency to immobility time, the addition of KET to those animals treated as well with FLX induced a significant beneficial effect.

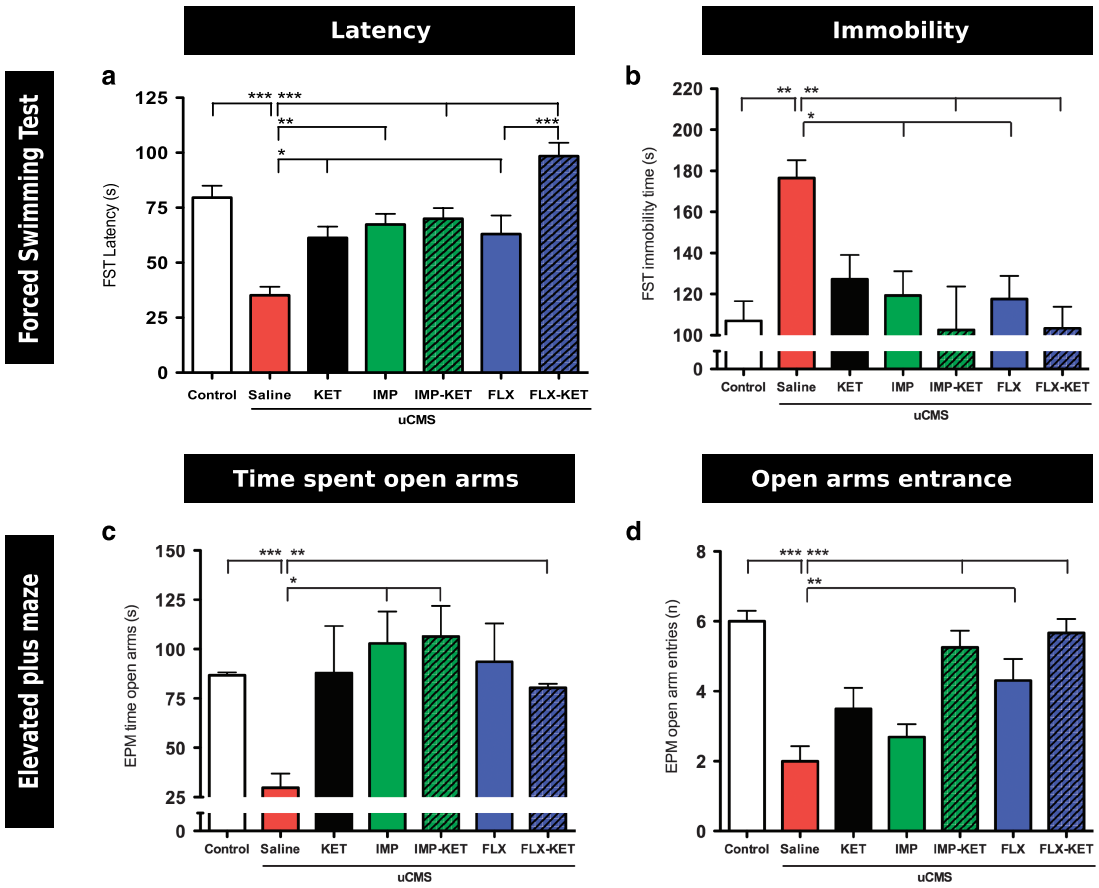


Figure 3. Ketamine treatment effect in anxiety and learned helplessness. All treated animals showed a significantly increased latency to immobility: FLX, KET($P < 0.05$), IMP, ($P < 0.01$) and IMP-KET, FLX-KET ($P < 0.001$). The addition of KET caused a significant increase in latency to immobility time in FLX-KET against KET (**a**); significant decrease in immobility time: IMP, FLX ($P < 0.05$) and IMP-KET, FLX-KET ($P < 0.01$) (**b**); IMP-KET ($P < 0.05$) and FLX-KET ($P < 0.01$) groups with significant increase in time spent in open arms when compared with uCMS animals (**c**); FLX ($P < 0.005$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) with significant higher entries in open arms (**d**). FLX, fluoxetine; IMP, imipramine; KET, ketamine; uCMS, unpredictable chronic mild stress; (mean ± s.e.m., $n = 12$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

In the elevated-plus maze test, uCMS animals displayed an anxious phenotype, spending significantly less time exploring open arms than control animals ($F_{6,77} = 3.111$, $P < 0.001$); both IMP-KET ($P < 0.05$) and FLX-KET ($P < 0.01$) groups showed a significant increase in time spent in open arms when compared with uCMS animals; no other significant differences were found (Figure 3c). Regarding the number of entries in open arms, control animals showed a significantly higher number than untreated uCMS group ($F_{6,77} = 11.05$, $P < 0.001$) and both FLX ($P < 0.01$) and IMP-KET, FLX-KET (both $P < 0.001$) also have significantly higher entries in open arms (Figure 3d).

No significant effects of uCMS or pharmacologic treatment were found concerning locomotory activity, as evaluated in the open-field test (data not shown). Treatment with KET, in the dosage used in our experimental protocol, failed to show PPI disruption (data not shown).

Neurotransmitter profile

In MD, therapy is still largely based on the 'monoaminergic hypothesis', which is fundamentally associated with alterations in the level of neurotransmitters. According to this and as an attempt to investigate the possible mechanism underlying the effect of giving KET together with antidepressants, the levels of neurotransmitters, more specifically GLU and aspartate (ASP) were analyzed by HPLC. For that, important key areas for MD such as PFC and hippocampus were selected. In addition, we have investigated the levels of these neurotransmitters in additional brain areas, which are related to the behavioral profile of recovery, such as the NAc. (Figure 4).

Taking into consideration the GLU levels, NAc was the only stress-induced brain region that displayed a reduction in its levels ($F_{6,55} = 2.254$, $P < 0.01$). Treatment with antidepressants produced an increase in GLU levels, but only IMP-KET and FLX-KET displayed a significant increase in GLU levels compared with untreated animals (both $P < 0.01$; Figure 4a).

Measurement of ASP levels showed no overall effect in PFC ($F_{6,55} = 0.672$, $P = 0.673$; Figure 4f), DH ($F_{6,55} = 1.397$, $P = 0.234$; Figure 4g) and VH ($F_{6,55} = 1.143$, $P = 0.325$; Figure 4h). Contrarily, in the NAc brain area, there was an overall group effect ($F_{6,55} = 2.554$, $P = 0.031$); *post hoc* analysis showed control animals have higher ASP than uCMS untreated animals ($F_{6,55} = 2.261$, $P < 0.05$) and that uCMS-exposed animals treated with IMP-KET and FLX-KET have significantly higher ASP levels than untreated uCMS animals ($F_{6,55} = 2.261$, $P < 0.05$; Figure 4f).

Neuronal structural analysis

Given the neuroplastic action of antidepressants, we thought it to be interesting to analyze dendritic architecture of neurons in these areas. There is evidence that KET can induce rapid changes in dendritic spine morphology, which may be relevant to the antidepressant effect shown in the behavioral analysis. We focus our attention in neurons from layers II/III of infralimbic (IL) cortex in PFC, pyramidal neurons from CA3 and granule neurons from the hippocampus and medium spiny neurons from NAc.

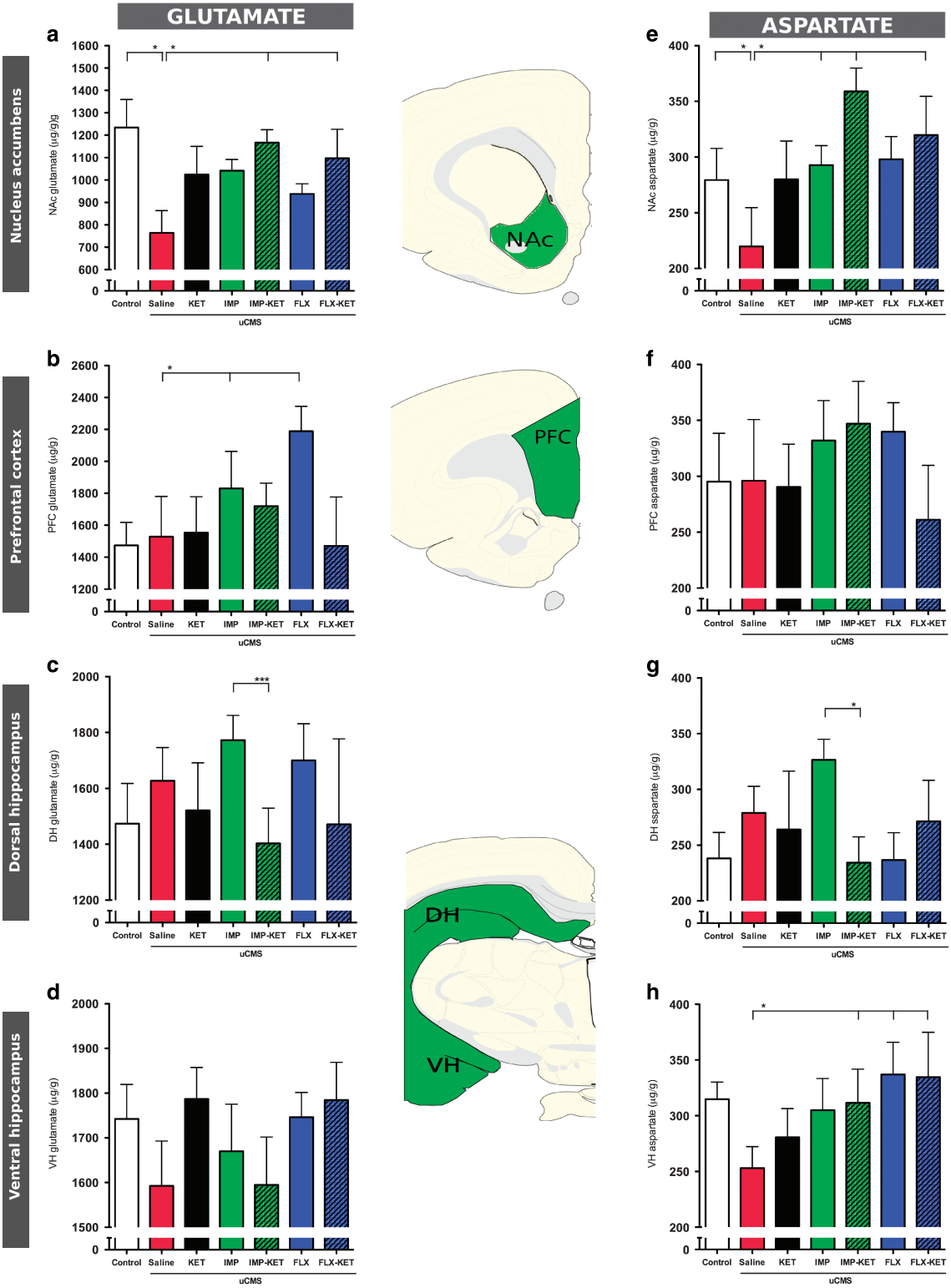
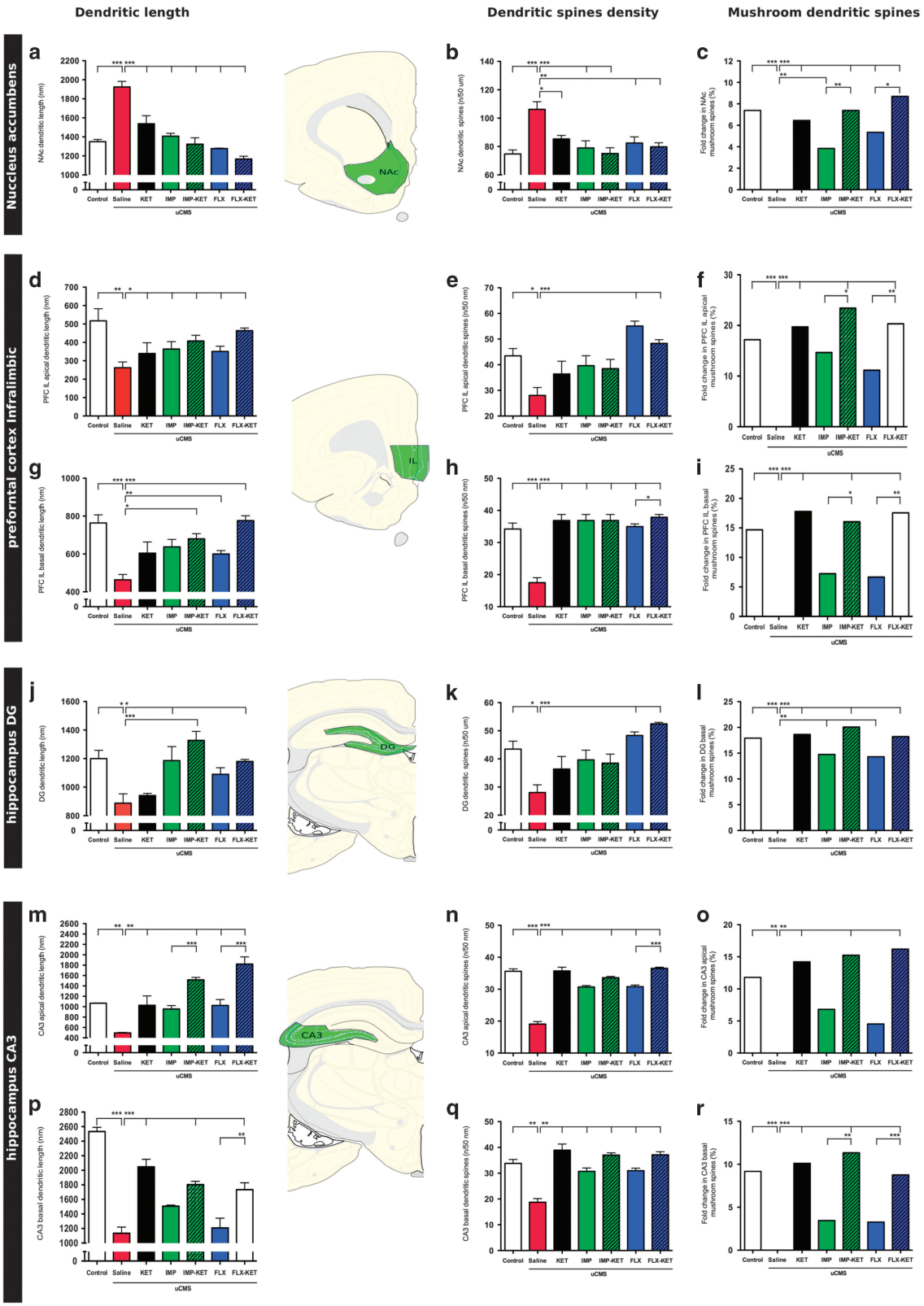


Figure 4. Ketamine treatment induces changes in HPLC measured levels of (**a**–**d**) glutamate (GLU) and (**e**–**h**) aspartate. IMP-KET and FLX-KET increase in GLU levels compared with untreated animals in NAc (both $P < 0.01$) (**a**). Treatment in IMP-KET and FLX-KET increases aspartate levels in PFC ($P < 0.05$) (**f**). FLX, fluoxetine; IMP, imipramine; KET, ketamine; NAc, nucleus accumbens; PFC, prefrontal cortex; (mean ± s.e.m., $n = 7$, $^{*}P < 0.05$; $^{***}P < 0.001$).

In the pyramidal neurons of the IL, uCMS induced atrophy in basal and apical dendrites ($F_{6,28} = 8.563$, $P < 0.001$; $F_{6,28} = 4.074$, $P < 0.01$). In basal dendrites, treatment with IMP-KET ($P < 0.01$), FLX ($P < 0.05$) and FLX-KET ($P < 0.001$) induced a reversion of dendritic shortening. In the apical dendrites, all treated animals ($P < 0.05$) had a significant recovery (Figures 5g and d).

In dentate gyrus granule cell dendrites, data confirm a significant stress-induced atrophy ($F_{6,28} = 7.103$, $P < 0.05$), which



was recovered by the treatment with IMP-KET ($P < 0.001$), IMP and FLX-KET (both $P < 0.05$). Animals treated with KET alone and FLX alone did not show significant changes in dendritic length (Figure 5j).

Hippocampal CA3 pyramidal neurons basal ($F_{6,28} = 33.28$, $P < 0.001$) and apical ($F_{6,28} = 17.93$, $P < 0.01$) dendrites show a significant decrease in their length in animals that were exposed to uCMS. Treatment with KET ($P < 0.001$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) recovered basal dendritic length, whereas

Figure 5. Addiction of ketamine induces changes in neuronal dendritic morphology. In NAc medium spiny neurons: all treatment groups restored decreased dendritic length (all $P < 0.01$) (**a**); increase in SD in IMP, IMP-KET, FLX-KET ($P < 0.01$) and FLX ($P < 0.05$) treatment groups (**b**); increase of MS population (IMP-KET and FLX-KET) compared with the animals given only antidepressant (IMP and KET) (**c**). IL apical dendrites recover from decreased dendritic length (**d**). In IL apical dendrites, FLX ($P < 0.001$) and FLX-KET ($P < 0.001$) increased (**e**). IL basal dendrites, treatment with IMP-KET ($P < 0.01$), IMP ($P < 0,05$) and FLX-KET ($P < 0,001$) induced a reversion of dendritic shortening (**g**). In IL basal dendrites, FLX-KET increased SD over FLX alone (**h**). Both IMP-KET and FLX-KET have increase in MS population in apical and basal dendrites when compared with IMP ($P < 0.05$) and FLX ($P < 0.01$) alone (**f** and **i**). In DG hippocampal neurons: IMP-KET ($P < 0.001$), IMP and FLX-KET (both $P < 0.05$) reverse the shortening in DG dendritic length (**j**); FLX and FLX-KET ($P < 0.001$) increased SD (**k**); KET ($P < 0.001$), IMP ($P < 0.01$), IMP-KET ($P < 0.001$), FLX ($P < 0.01$) and FLX-KET ($P < 0.001$) restored MS (**l**). In hippocampal CA3 pyramidal neurons: KET ($P < 0.001$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) recovered basal dendritic length and FLX-KET produced increase in basal dendritic length compared with FLX alone ($P < 0.01$) (**p**); KET ($P < 0.05$), IMP-KET ($P < 0.001$), FLX ($P < 0.05$) and FLX-KET ($P < 0.001$) induced recovery in apical dendrites, both IMP-KET and FLX-KET promoted a higher regrowth than IMP ($P > 0.001$) and FLX ($P > 0.001$) (**m**). Addition of KET to FLX treatment increases SD ($P < 0.001$) in apical dendritic tree (**n**) and KET ($P < 0.01$), IMP-KET ($P < 0.01$) and FLX-KET ($P < 0.01$) increased the amount of MS (**o**); In CA3 basal dendrites all treated groups had a increase in SD (**q**); treatment with KET ($P < 0.001$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) produced an increase in MS and IMP-KET ($P < 0.01$) and FLX-KET ($P < 0.001$) induce higher MS population than IMP and FLX (**r**). DG, dentate gyrus; FLX, fluoxetine; IMP, imipramine; KET, ketamine; MS, mushroom spine; NAc: nucleus accumbens; SD, spinal density; (mean ± s.e.m., $n = 5$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

FLX-KET was able to induce a significant increase in basal dendritic length compared with FLX alone ($P < 0.01$). Neither IMP nor FLX alone increased CA3 basal dendritic length significantly when compared with uCMS animals. Regarding the apical dendritic tree, treatment with KET ($P < 0.01$), IMP-KET ($P < 0.01$), FLX alone ($P < 0.01$) and FLX-KET ($P < 0.01$) induced recovery, whereas treatment with IMP alone failed to increase CA3 apical dendritic length significantly. Both IMP-KET and FLX-KET promoted a higher regrowth than IMP ($P > 0.001$) and FLX ($P > 0.001$; Figures 5m and p). Exposure to uCMS induced an increase in dendritic length in NAc medium spiny neurons ($F_{6,28} = 24.73$ $P < 0.001$), and this feature was reversed within all treatment groups (all $P < 0.01$, Figure 5a).

A significant decrease in spine density (SD) was found in basal ($F_{6,28} = 22.11$, $P < 0.001$) and apical ($F_{6,28} = 6.86$, $P < 0.05$) IL dendrites of uCMS animals. Treatments with KET ($P < 0.001$), IMP ($P < 0.001$), IMP-KET ($P < 0.001$), FLX ($P < 0.001$) and FLX-KET ($P < 0.001$) increased SD in IL basal dendrites; FLX-KET significantly increased SD in basal dendrites when compared with FLX alone ($F_{1,28} = 6.86$, $P < 0.05$). Treatment with FLX ($P < 0.001$) and FLX-KET ($P < 0.001$) increased SD in apical dendrites. No significant increase of SD in apical dendrites was found in animals treated with KET or IMP alone and in animals treated with IMP-KET. Regarding spine morphology, mushroom spines are decreased in uCMS animals both in apical ($F_{6,28} = 5.712$, $P < 0.001$) and basal ($F_{6,28} = 2.668$, $P < 0.001$) dendrites when compared with control animals. Treatment with KET ($P < 0.001$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) reverted this pattern in basal and apical IL dendrites. No significant increase in mushroom spine population was found in animals treated with IMP or FLX alone; although the treatment with IMP-KET and FLX-KET caused a significant rise in mushroom spine population in apical and basal dendrites when compared with IMP ($P < 0.05$) and FLX ($P < 0.01$) alone (Figures 5e, f, h and i).

Hippocampal dentate gyrus neurons in uCMS untreated animals have significant lower dendritic SD ($F_{6,28} = 7.613$, $P < 0.05$) when compared with control animals. Treatment with FLX ($P < 0.01$) and FLX-KET ($P < 0.01$) significantly increased SD. There was no significant increase in SD in animals treated with KET or IMP alone or with IMP-KET. When evaluating the spine morphology, specifically the more developed mushroom spines, uCMS animals present also less number of mushroom dendritic spines in dentate gyrus neurons ($F_{6,28} = 8{,}162$, $P < 0.05$) ($t = 5.267$, $P < 0.001$) but treatment with KET ($t = 5.477$, $P < 0.001$), IMP ($t = 4.339$, $P < 0.01$), IMP-KET ($t = 5.903$, $P < 0.001$), FLX ($t = 4.205$, $P < 0.01$) and FLX-KET ($t = 5.359$, $P < 0.001$) restored mushroom spines numbers (Figures 5k and l).

In the CA3, neurons from uCMS animals displayed a significantly lower dendritic SD in apical ($F_{6,28} = 73.57$, $P < 0.001$) and basal ($F_{1,28} = 22.12$, $P < 0.001$) dendrites, which were recovered upon treatment with all the tested drugs ($P < 0.001$ to all). Addition of KET to FLX treatment statistically increases spine density ($F_{1,28} = 6.86$, $P < 0.05$) in apical dendritic tree. The population of mushroom dendritic spines is decreased in untreated uCMS animals both in apical ($F_{6,28} = 23.86$, $P < 0.001$) and basal ($F_{6,28} = 6.882$, $P < 0.01$) CA3 dendrites. In apical dendrites, treatment with KET ($P < 0.001$), IMP ($P < 0.05$), IMP-KET ($P < 0.001$) and FLX-KET ($P < 0.001$) caused an increase in mushroom spine population. Both IMP-KET ($P < 0.01$) and FLX-KET ($P < 0.001$) induce higher mushroom dendritic spine population. In basal dendrites, treatment with KET ($P < 0.01$), IMP-KET ($P < 0.01$) and FLX-KET ($P < 0.01$) increased the amount of mushroom spines (Figures 5n, o, q and r).

Exposure to uCMS induced a significant increase in SD in medium spiny neurons of the NAc ($F_{6,28} = 7.297$, $P < 0.01$) and this feature was reversed in the treatment with IMP, IMP-KET, FLX-KET ($P < 0.01$) and FLX ($P < 0.05$). Regarding spine morphology, uCMS promoted a decrease in the population of mushroom spines ($F_{6,28} = 9.094$, $P < 0.001$) and all treatments significantly increase mushroom spine compared with the untreated uCMS animals ($P < 0.001$). Importantly, the animals treated with antidepressant and ketamine (IMP-KET and FLX-KET) presented a significant increase of mushroom spine population when compared with those who were only given antidepressant (IMP or KET; Figures 5b and c).

DISCUSSION

The use of KET in subanesthetic dose with antidepressant effect is described both in animal models of stress and in MD patients.[13,24,25] Classic antidepressants are used with success in our animal model, the uCMS.[26] In our study, we aim to test the effect of KET on the response of classic antidepressants. The effect of KET alone is described in the literature, although there is a lack of understanding regarding the synergetic effect of initial antidepressant treatment with KET. Therefore, we propose to investigate the effect of KET on the response of classic antidepressants. In the literature, the methodology described for the administration of ketamine is very heterogeneous; it goes from single to multiple doses, the latest one given either daily or in intermittent days. When designing the protocol of antidepressant treatment we decided to use a multiple continuous method with a short duration (3 days).

The SCT was used as a behavioral hallmark for recovery from depressed phenotype. The first evaluation occurred at the seventh day of treatment and indicated that only animals treated with KET (either alone or in combination with other antidepressant) display behavioral rescue of anhedonia. After one additional week of

treatment, at the 14th day, all treated groups show reversion of anhedonia, except the one only treated with KET, which showed a reversion of the positive behavioral effect. In our results, we can see the specific effect of the interaction of KET added to antidepressant treatment on the results of the treated group KET-FLX. The effect of KET alone in anhedonia does not last until the 14th day, but when given with FLX, it enhances it.

The second hallmark of this model is learned helplessness, a measure of behavior despair. There was an increase in latency to immobility time in all treated animals but the analysis of total immobility time shows that the effect of KET alone is not detected 2 weeks after being administrated. Again, when KET is given with FLX, it is evident that there is an increase in latency time compared with the antidepressant alone.

The analysis of anhedonia and despair in our model showed that combining KET with other treatments seems to anticipate the increase in sucrose preference and to improve the response. KET possibly is acting as an initial booster and keeps the antidepressant response. However, when given alone it failed to show a robust result.

We also assessed anxiety and show that the combination of KET with FLX or IMP was able to significantly reverse the anxiety trait. Again, there seems to be no anxiolytic effect after 2 weeks of KET given alone. Some literature reports long-lasting anxyolitic effect of KET given in a single dose to patients with depressive disorders[27] and in animal models when given in multiple doses.[28] However, we observed a positive effect when given with antidepressants. When given together with the antidepressants, beneficial effect of KET extends beyond the mood domain and also potentiates the anxiolytic effects of SSRIs and tricyclic antidepressant drugs.

The positive effect of initial dual therapy with KET and other antidepressant, concerning behavioral recovery in our animal model, is a faster initial increase in sucrose preference and a more robust response after 2 weeks of treatment not only in anhedonia but also in behavioral despair and anxiety.

MD disorder and stress disorders have been associated with impaired functional connectivity,[29,30] as a result of dysfunction occurring by several pathways. To evaluate the impact of KET in neurotransmission, we have analyzed the levels of both GLU and also we evaluated the dendritic architecture. We assessed the levels of GLU in regions relevant for depressive-like behavior. Our major finding was that NAc, a brain region implicated in anhedonia,[26,31] was the only region where KET, in association with other antidepressants, triggered an increase in the levels of GLU and ASP suggesting that this particular effect is ascribed to glutamate metabolism. The failure to observe significant changes in GLU or ASP levels in other brain regions probably relates to temporal dynamics of the experimental design. However, literature reports that both acute stressors and chronic stressors increase basal release and increased presynaptic reuptake of GLU, but most measurements were performed using mycrodialysis or synaptossomal analysis, thus reflecting extracellular contents, not the overall content of GLU. There is evidence that KET acts as a noncompetitive and nonselective high-affinity NMDA antagonist also on GABAergic neurons and may rapidly increase GLU release, for example, in the PFC.[32,33] Although there is clinical evidence that treatment for depression increases the release of glutamate in the NAc,[34] other studies using ketamine in individuals with MD were not consistent in showing an association between therapy and changes in neurotransmitter content.[35,36] Present data suggest changes in glutamatergic transmission, particularly in the NAc, a mechanism through which KET helps restore mood.

Through the analysis of neuronal architecture, we confirmed the impact of uCMS on shrinkage of dendritic arborizations in the hippocampus and in the IL area of the PFC, and dendritic hypertrophy in NAc medium spiny neurons.[7,8,26,37] Globally, groups treated with antidepressant and KET showed a recovery of the morphology of their dendritic trees, with a more consistent response in FLX than IMP. Yet, the most striking changes were noticed in dendritic spines; we not only found that the addition of KET to the antidepressant treatment boosted a significant recovery in spine density when compared with antidepressants alone, but also with greater spine maturation. KET is known to have effects in synaptic plasticity: the fast-acting antidepressant action of KET is known to be dependent on rapid protein synthesis (namely of BDNF, PSD-95, GLuR1 and synapsin 1) and activation of intracellular pathways such as mTOR, all of them physiologically relevant for synaptic plasticity mechanisms, neuronal growth, differentiation and synaptogenesis.[38–41] Although the specific role of mTOR in NAc is not fully understood in depression, the effect of mTOR modulation is well studied in addiction. Therefore, we can speculate the existence of some similarity between addiction development and in antidepressant action. Indeed, the use of rapamycin not only blocks establishment of addiction, but also the antidepressant effect of KET.[42] The acute and lasting effect of KET in neuronal remodeling might be one of the reasons why it improves behavioral markers of recovery in our experiment. The particular case of combined treatment in increasing more mature dendritic spine population shows that processes related to synaptic functioning and rewiring have an important role in behavioral recovery.

In summary, the present data show that KET has the potential to improve the action of classic antidepressants in brain regions affected by uCMS, a validated animal model of depression. The addition of KET to antidepressants accelerate recovery from anhedonia. The specific addition to FLX improves not only anhedonia but also behavioral despair. Despite lack of robustness in neurochemical data, we show improvement of glutamatergic profile in an area related to anhedonic behavior. The effects in neuronal morphology show a potential mechanism for improvement in synaptic connectivity in brain areas known to be affected and responsible for behavioral hallmarks in MD. These results show that KET has potential to improve the initial response of MD treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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C H A P T E R 3

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Variable delay-to-signal: a fast paradigm for assessment of aspects of impulsivity in rats

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Testing impulsive behavior in rodents is challenging and labor-intensive. We developed a new behavioral paradigm—the Variable Delay-to-Signal (VDS) test—that provides rapid and simultaneous assessment of response and decision impulsivity in rodents. Presentation of a light at variable delays signals the permission for action (nose poke) contingent with a reward. 2 blocks of 25 trials at 3 s delay flank a block of 70 trials in which light is presented with randomly selected 6 or 12 s delays. Exposure to such large delays boosts the rate of premature responses when the delay drops to 3 s in the final block, an effect that is blunted by an acute methamphetamine challenge and that correlates with the delay-discounting (DD) paradigm (choice impulsivity). Finally, as expected, treatment with the NMDA antagonist MK-801 caused a generalized response increase in all VDS blocks. The pharmacological validation, particularly with methamphetamine which has a well established dual effect on response and decision impulsivity, and the correlations between the impulsive behavior in the DD and VDS paradigms, suggests that the later is able to provide, in a single session, a multi-dimensional assessment of impulsive behavior.

Keywords: rodent behavior, decision impulsivity, response impulsivity, delay-discounting, 5-csrtt, methamphetamine, MK-801

INTRODUCTION

Impulsivity is defined as a tendency to act prematurely without foresight (Dalley et al., 2011). It is a non-unitary construct embracing impulsive response and impulsive choice (Evenden, 1999b; Winstanley et al., 2006; Dalley et al., 2011; Dalley and Roiser, 2012). This multifactorial trait depends on a complex morphophysiology involving multiple brain areas and neurotransmitter systems (Dalley et al., 2011; Dalley and Roiser, 2012). Impulsivity is part of the normal behavioral repertoire, but, when out of the normal range, can result in a disruptive behavior, encountered in several psychiatric disorders including obsessive-compulsive disorder (OCD), attention deficit/hyperactivity disorder (ADHD), mania, substance abuse, and schizophrenia (Evenden, 1999a,b; Moeller et al., 2001).

Impulsive behavior is also present in rodents, both in normal conditions and in models of psychiatric disease (Adriani et al., 2003; Huskinson et al., 2012; Pattij and De Vries, 2013). It has been assessed in a number of paradigms that are well established in terms of their face, construct and predictive validity, with the go/no-go (Harrison et al., 1999), the stop-signal reaction task (SSRT; Eagle et al., 2008), the 5-choice serial reaction time task (5-csrtt; Carli et al., 1983) and the delay-discounting (DD; Evenden and Ryan, 1996) among the most used (for review see Winstanley et al., 2006; Dalley and Roiser, 2012). The construct of each of these paradigms varies substantially, reflecting the non-unitary characteristic of impulsivity (Winstanley et al., 2010; Dalley et al., 2011; Dalley and Roiser, 2012). In the first three paradigms, impulsive responses result from an inability to refrain from an action either when waiting for a go signal or in the presence of an explicit no-go signal, reflecting what is considered "response impulsivity." In contrast, in the DD, impulsive responses are the result of a deliberate choice between a maximal, though delayed, and an immediate but small, reward, reflecting a so-called "decision impulsivity". Although these paradigms have provided valuable tools to study impulsivity in rodents, they present several limitations including the extensive time commitments (spanning over 2 months in some cases), the possibility of confounding by factors like attention and reward valuation, the acquisition of repetitive behaviors (accommodation) due to the sequential performance of the paradigms and the mono-dimensionality of the construct assessed in each test, that limits the behavioral readouts to a single type of impulsivity.

In order to circumvent some of these problems, we developed a new behavioral paradigm, the Variable Delay-to-Signal (VDS) test, consisting of a series of trials, in a single 30 min session, in which the time period (60 s) where an action (nose poke) triggers the delivery of a sugared reward is signaled by a light, presented after a variable delay; a block of 3 s delay trials is followed by a block with large and variable delays (randomized between 6 and 12 s) before a final block again with a 3 s delay. Rats learn the operant sequence (nose poke/reward) rapidly (after a few trials) and the entire protocol lasts for 10 days, a significant reduction comparing to previously described paradigms. In addition, we have validated the VDS by employing two drugs with well-established

actions on impulsive behavior, methamphetamine and MK-801, and by comparing the individual performance against two reference paradigms, 5-csrtt (response impulsivity) and DD (decision impulsivity). Given the observed dual pro- and anti-impulsivity action of methamphetamine (Hayton et al., 2012) in different components of the VDS and their correlations with the reference paradigms, we suggest that the VDS provides an effective assessment of both response and decision impulsivity.

MATERIALS AND METHODS

ORGANIZATION OF THE EXPERIMENTAL PROCEDURES

Three months old, male Wistar Han rats (Charles River Laboratories, Barcelona, Spain) were used in all experiments. Animals were kept in a room with controlled temperature (22 ± 1°C), 12 h light/dark cycle (lights on at 8 a.m.) and housed in pairs in plastic cages with food and water available *ad libitum*. The dietary regimen was restricted to 1 h of food availability (19:00–20:00) 3 days before the initiation of the behavioral experiments. Body weight was thereon controlled to ensure that it did not go below 85% of the initial value. All procedures involving animals were approved by local authorities and the experiments were performed according to the guidelines of European Community Council Directive 2010/63/EU.

We conducted two independent experiments (**Figure 1A**): in the first, 20 animals sequentially performed the VDS under methamphetamine/vehicle (VDS 1), the VDS under MK-801/vehicle (VDS2) and the 5-csrtt; in the second, 12 animals without any treatment performed the VDS (VDS3) followed by the DD. In both the VDS1 and VDS2, animals were assigned to receive either drug or vehicle according to their performance in the preceding training sessions, so that both groups had a similar mean prematurity score; in addition, in VDS2 both vehicle and MK-801 groups had a similar number of animals previously treated with methamphetamine. In VDS 1 session two animals (one from each group) have not finished the task probably due to a failure in the reward delivery system; these were excluded from further analyses.

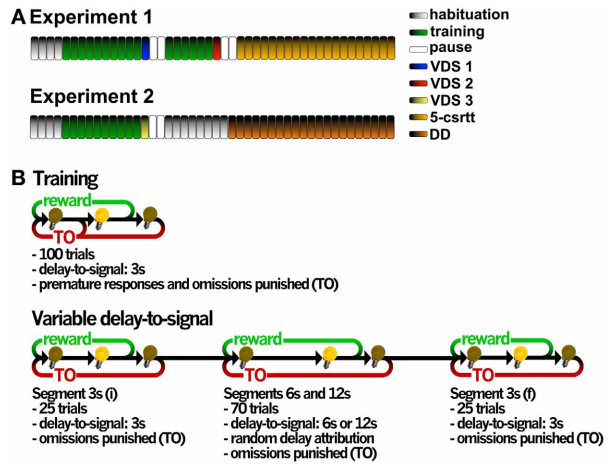

FIGURE 1 | General organization of the validation experiments and operational diagrams of the VDS and preceding training protocol. (A) In experiment 1, two VDS protocols were performed in the same group of animals to test for methamphetamine and MK-801 effects on impulsivity, followed by the 5-csrtt performed in drug-free conditions during the whole protocol. In experiment 2, VDS was followed by DD. Each bar represents a session, except for 5-csrtt and DD whose protocol extension varied between individuals. **(B)** The VDS consisted in two parts: the training protocol (10 sessions) and the VDS proper (1 session); while in the first the delay-to-signal was fixed (3 s) and pre-signal nose pokes were punished (TO), in the VDS proper, 2 blocks of 25 trials at 3 s delay were interposed by a block of 70 trials at 6 and 12 s, pre-signal responses were registered but not punished with a TO. In both training and VDS the signal duration was set to a maximum of 60 s; the absence of a response within this period was registered as an omission. 5-csrtt—5 choice serial reaction time task; DD—delay discounting; TO—timeout; VDS—variable delay-to-signal task.

DRUGS

Methamphetamine was used in VDS 1 session to lessen impulsive behavior. A dose of 1 mg/Kg of the racemic mixture (effective dose of 0.5 mg/Kg) was administered intraperitoneally in a freshly prepared solution at a concentration of 1 mg/mL (in saline) 30 min before the initiation of the session (Hayton et al., 2012). Methamphetamine hydrochloride was synthesized by an adaptation of a previously described method (Milhazes et al., 2007). The NMDA antagonist MK-801 was used in VDS 2 session to boost impulsive behavior. A dose of 0.03 mg/Kg was administered intraperitoneally in a freshly prepared solution at a concentration of 0.03 mg/mL (in saline) 10 min before the initiation of the session (Fletcher et al., 2011). MK-801 was obtained from Calbiochem (CA, USA, Catalog Number: 475878-10MG).

VARIABLE DELAY-TO-SIGNAL

The VDS task was performed in square operant chambers (OC; 25 × 25cm; TSE Systems, Germany) having on a curved wall 5 squared apertures (2.5 cm) elevated 2 cm from the grid floor and, in the opposite wall, a similar aperture (food magazine) connected to a pellet dispenser. Each aperture contained a 3W lamp bulb and an infrared beam system to detect the activity of the animal. Three 5-hole OCs, placed within sound attenuating boxes with individual electrical fans for ventilation and white noise production, were simultaneously used in our studies.

Animals were habituated to the testing conditions in 4, twice daily (am/pm) sessions, 5h apart. In the first 2 sessions, animals were placed in the OC for 15 min with all lights off, access to apertures 1–5 blocked by metallic caps and 10–15 sugared pellets (45 mg, Bioserv Inc., New Jersey, EUA) available in the food magazine. In sessions 3–4, animals were placed in the OCs for 30 min, all lights were on and animals had free pellets available in the center nose poke aperture (response aperture, 2–3) and the food-magazine (10–15). The protocol for VDS was initiated the following day and included two phases, i. training and ii. test (**Figure 1B**).

i. *Training* Sessions were initiated by turning on the house light and delivering one sugared pellet in the food magazine, the collection of which started an intertrial interval (ITI) of 3 s to allow the animal to ingest it. Trials then started, consisting of a 3 s period with only the house light on (delay period), followed by lightning of the response aperture for 60 s (response period). Nose pokes in this aperture were either punished

with a timeout (TO) period in complete darkness (5 S), if performed during the delay period (premature responses), or rewarded with the delivery of a pellet if performed during the response period. Collection of a food reward always triggered a 3 s ITI, before a new trial begun. Except for the TO periods, the house light was always on. Each session comprised 100 trials to be performed in a maximum of 30 min. Training sessions occurred twice daily, with a 5 h interval in between, for 5 consecutive days. The average number of premature responses of the overall group stabilized at ∼30% in the last 4 sessions of training.

ii. *Test* The VDS testing session occurred on a single day and consisted of 120 trials, similar to those previously described, with the exception of the delay, which was 3 s in the first and the last 25 trials and randomly either 6 or 12 s in the middle 70 trials (leading to a 3 s – 6/12 s – 3 s configuration), and, importantly, the fact that multiple nose pokes were allowed during the delay period, i.e., premature responses did not initiate TO punishment periods. (**Figure 1B**).

5-CHOICE SERIAL REACTION TIME TASK

The 5-csrtt was performed in the same apparatus as the VDS, following the general principles originally described by (Carli et al., 1983). Briefly, each session started with the delivery of one pellet. Its collection by the animal initiated the first trial. At this point the house light is on signaling an ongoing trial. After an ITI of 5 s one of the five lights in the rear panel was illuminated for a period of 60 S. Nose pokes in this aperture during the light period or in the subsequent 5 s were rewarded with one pellet in the food aperture whose collection marks the beginning of an ITI that precedes a new light signal. Nose pokes in any of the other 4 apertures initiated a time-out period of 5 s in darkness after which a new trial is started (house light on). Each session consisted in a sequence of 100 trials (or a maximum of 30 min) performed twice a day during the morning/afternoon periods. The performance of the subjects was assessed using the following experimental parameters:

- Accuracy—ratio of correct/total number of responses.
- Prematurity—responses during the ITI in any of the five apertures (triggers a TO).
- Omissions—absence of response in appropriate time.

Other parameters including latency-to-feed and response delay were also registered. Throughout the sessions the level of difficulty was increased by decreasing the stimulus duration from 60 to 30, 10, 2, and 0.5 S. An accuracy ≥80% and omissions ≤20% in two successive sessions were considered as criteria for level change. The last level was performed during 15 sessions. The 5-csrtt was initiated 2 days after the last session of the VDS; no training preceded the sessions.

DELAY-DISCOUNTING

The DD task was performed in (OCs; 30.5 cm L × 24.1 cm W × 21.0 H) equipped with two retractable levers located on either side of a food magazine and a house light placed in the opposite side (MED Associates). Information regarding animals' activity within the OC was registered with MED-PC IV software. Two chambers were used each placed within an individual sound attenuating cubicle. In the first 2 days, animals were placed in the OCs for 5 min with the house light on and both levers retracted. In the food magazine 3–5 pellets were made available. From days 3–5 a continuous reinforcement protocol (CRF) was applied. A single lever was made available and a sugared pellet was delivered for each lever press. Sessions were terminated when 50 pellets were obtained or if 30 min had elapsed and were immediately followed by a similar session differing only in the fact that the levers were switched. The lever presentation order was counterbalanced over days 3–5. The second step of the training protocol consisted in 3 sessions (1 per day; days 6–8) on which the animal was required to nose poke the food magazine in order to trigger the lever presentation and initiate the trial when the house light was on. Only one lever was presented at each trial in a random and balanced fashion (i.e., left and right levers were presented an equal number of times) up to 90 trials with a fixed duration of 70 S. The DD proper sessions consisted in 4 blocks of 10 trials each with an organization similar to that described for training days 6–8 differing in that the nose poke in this case triggers the simultaneous presentation of both levers. One lever is now associated with a small (1 pellet) but immediate reward and the other with a large (4 pellets) but delayed reward (delays: 0, 15, 30, and 45 s respectively in the 1st, 2nd, 3rd, and 4th blocks). The value attributed to each of the two levers is balanced between animals. Each block of 10 trials is preceded by two forced-choice trials in which each lever is individually presented and the pellets are delivered according to the respective block parameters. The DD sessions were repeated uninterruptedly for 20 days and the animals that maintained a robust selection of the favorable lever in the 1st block—5 consecutive days with preferences over 70%—were selected for analysis ($N = 8$). The area under the curve (AUC) was used as the main measure of impulsive DD, but data were also analyzed according to the exponential or the hyperbolic functions (Odum, 2011).

STATISTICAL ANALYSIS

Data is presented as mean ± SEM and analyzed using 1- or 2-way analysis of variance followed by a *post-hoc* (Tukey) for multiple comparisons. Independent-samples *t*-test was used to test for the drug effect within each delay. Intra-individual comparisons in different paradigms were performed by linear regression analyses. Results were considered statistically significant if $p < 0.05$.

RESULTS

PHARMACOLOGICAL VALIDATION

We tested the ability of the VDS to discriminate impulsive behavior in conditions known to decrease or increase impulsivity. As expected, acute challenge with methamphetamine (VDS1) diminished, whereas acute treatment with MK-801 (VDS2) augmented, the absolute number of impulsive responses (IR) [VDS1: $F_{(1,\ 16)} = 5.416$ $p = 0.033$; VDS2: $F_{(1,\ 18)} = 23.258$ $p < 0.001$] (**Figures 2A–D**). Additionally, although the number of premature responses varied in accordance with the delay in both experiments [VDS1: $F_{(3,\ 48)} = 90.248$ $p < 0.001$; VDS2: $F_{(3,\ 54)} = 254.888$ $p < 0.001$], this effect was stronger in the saline group as compared to the methamphetamine group [VDS1: $F_{(3,\ 48)} = 5.478$

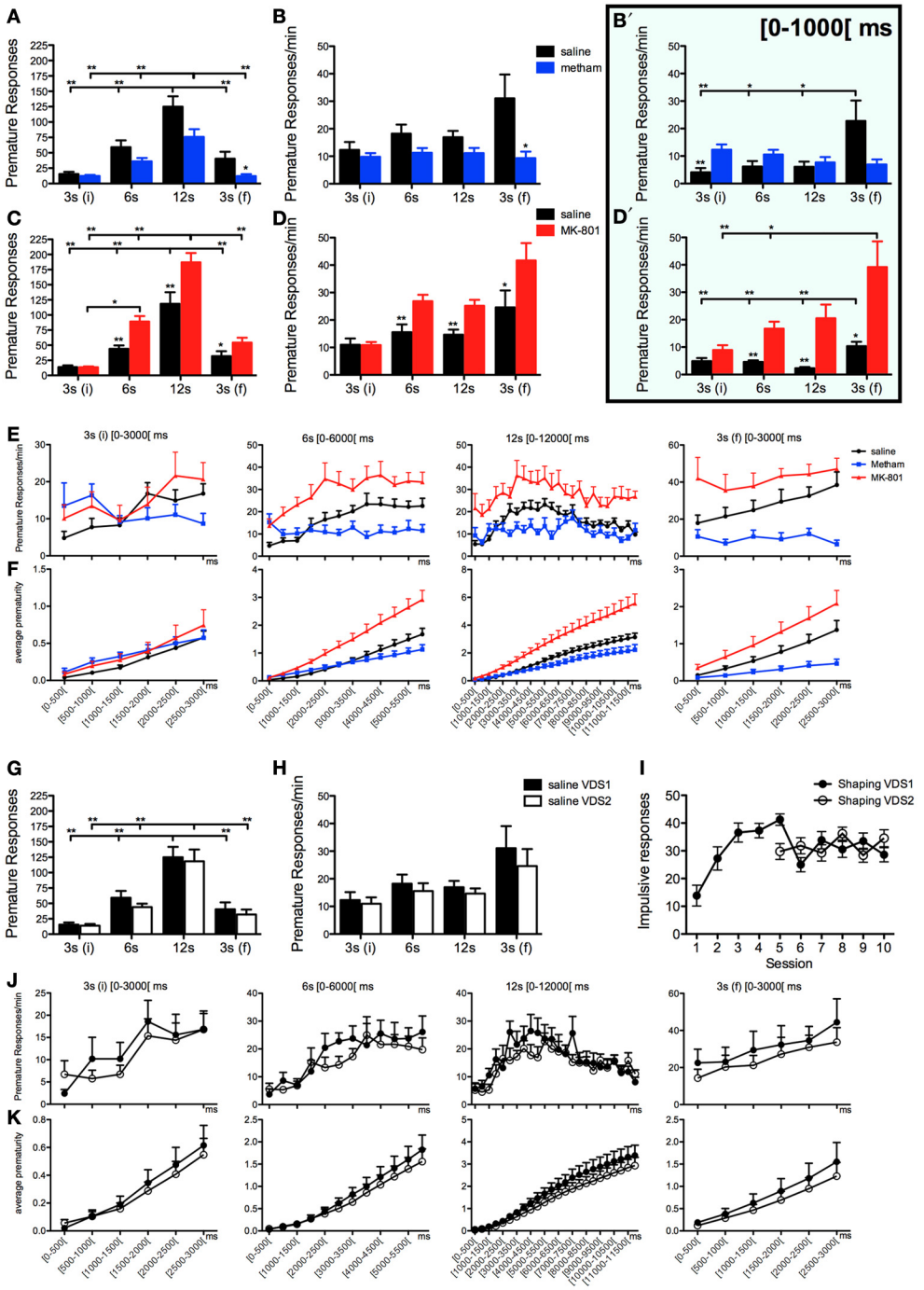

FIGURE 2 | Pharmacological validation of the VDS with methamphetamine and MK-801. (A–F) Methamphetamine (VDS1) and MK-801 (VDS2) decreased and increased, respectively, the absolute number of premature responses **(A, C)** and the number of premature responses per available minute of delay **(B, D)**. In the case of methamphetamine, but not MK-801, this pattern was inverted in the initial second of the 3 s (i) delay segment **(B′, D′)**. The absolute **(E)** and the accumulated **(F)** average prematurity/trial response profile is presented in a segmented (500 ms periods) fashion for each delay segment 3 s (i), 6, 12, and 3 s (f). The same analyses are presented to compare the saline controls in VDS1 and 2 experiments **(G–K)**. No differences were observed between VDS 1 and 2 nor between the impulsive behavior in the preceding training sessions **(I)** indicating that the VDS permits multiple tests without significant alterations of the basal behavior. Statistically significant comparisons between delay segments are marked with a horizontal line over the relevant graph bars; statistically significant comparisons between groups are marked over the graph bar of lowest value. $^{*}P < 0.05$; $^{**}P < 0.01$; data presented as mean + S.E.M. 5-csrtt—5 choice serial reaction time task; DD—delay discounting; VDS—variable delay-to-signal task.

$p = 0.016$] and in the MK-801 compared with the respective saline group [VDS2: $F_{(3, 54)} = 26.066$ $p < 0.001$] (**Figures 2A,C**). Importantly, the ability of the protocol to detect changes in impulsivity was further confirmed by an analysis of response rates (responses per minute of delay–IR/m), which were decreased by methamphetamine treatment and increased by acute MK-801 injections [VDS1: $F_{(1, 16)} = 4.815$ $p = 0.043$; VDS2: $F_{(1, 18)} = 17.449$ $p = 0.001$]. Interestingly, this analysis also revealed that although animals from all groups kept their premature response rate approximately constant across the 3 delay blocks, they increased it in the last 3 s delay [VDS1: $F_{(3, 48)} = 6.931$ $p = 0.011$; VDS2: $F_{(3, 54)} = 28.166$ $p < 0.001$], an effect that was present in the saline group but not in the methamphetamine group [VDS1: $F_{(3, 48)} = 8.767$ $p = 0.005$] and was stronger in the MK-801 compared with the respective saline group [VDS2: $F_{(3, 54)} = 12.973$ $p < 0.001$] (**Figures 2B,D**).

To further explore the possibilities offered by VDS in the characterization of impulsivity, we decided to partition data from each delay block into 500 ms intervals, the results of which (pooling data from VDS1 and VDS2 together) are depicted in **Figures 2E,F**. Overall, this analysis was in line with our previous data in that methamphetamine treated animals showed decreased, whereas animals treated with MK-801 had increased, premature responses and response rates compared with saline treated animals, particularly near the end of each delay block and more obvious across the entire final delay block [3 s(f)]. This way of looking at our data also revealed an alteration to the overall response pattern, specifically of methamphetamine treated animals, in the initial 1000 ms of delay, which prompted us to analyze premature response rates on this initial period across delays and treatment groups (**Figures 2B′,D′**). In this initial period of each delay block, MK-801 maintained its pro-impulsivity effect [$F_{(1, 18)} = 13.780$ $P = 0.002$] whereas methamphetamine failed to reduce impulsivity [$F_{(1, 16)} = 0$ $P = ns$], with pairwise comparisons even revealing a significant increase in response rates of methamphetamine treated animals in the first (3 s) delay block ($t_{16} = -3.180$ $p = 0.006$). However, in line with results from the entire delay period, data from the first 1000 ms also showed that methamphetamine treatment, but not MK-801, prevents the increase in premature response rates observed in the last delay block [3 s(f)] of the other experimental groups [VDS1: delay $F_{(3, 16)} = 5.163$ $P = 0.012$ drug′delay $F_{(3, 48)} = 7.533$ $P = 0.002$; VDS2: delay $F_{(3, 18)} = 11.918$ $P < 0.001$ drug*delay $F_{(3, 54)} = 3.209$ $P = 0.063$] (**Figures 2B′,D′**).

To test the presence of a putative effect of VDS repetition on impulsive behavior (**Figures 2G–K**), we compared the performance of the saline groups in VDS1 and VDS2. Apart from confirming a significant effect of the delay block both in the number of responses, that varied according to the amount of delay, [$F_{(3, 48)} = 193.262$ $P < 0.001$] and in the response rate, which was constant in the first 3 delay blocks but increased dramatically in the last (3 s) period [$F_{(3, 48)} = 12.460$ $P = 0.001$], we could not find any significant effect of experiment on either parameter [number of responses VDS1 vs. VDS2: $F_{(1, 16)} = 2.607$ $P = ns$; response rate VDS1 vs. VDS2: $F_{(1, 16)} = 2.468$ $P = ns$] nor any interaction between experiment and the effects of delay block described above [number of responses experiment*delay: $F_{(3, 48)} = 2.660$ $P = ns$; response rate experiment*delay: $F_{(3, 48)} = 3.422$ $P = ns$] (**Figures 2G,H**). Similarly, the response pattern along the delay blocks was similar in both saline groups (**Figures 2J,K**). Importantly, methamphetamine and MK-801 at the selected doses had no sedative or motivational effects, as no differences were observed in the latency to feed (**Figure 3A**). On the contrary, the two drugs had contrasting effects in response latency. While methamphetamine treatment had no effect in this parameter [$F_{(1, 16)} = 0.002$ $P = ns$] and failed to abrogate a decrease in response latency in the last delay block [3 s(f)] [delay: $F_{(3, 48)} = 14.508$ $P < 0.001$; drug*delay: $F_{(3, 48)} = 2.494$ $P = ns$], MK-801 had a profound effect, not only shortening response latencies [$F_{(1, 18)} = 7.011$ $P = 0.016$] but also reducing the influence of delay block on them [delay: $F_{(3, 54)} = 24.352$ $P < 0.001$; drug*delay: $F_{(3, 54)} = 10.403$ $P < 0.001$]. (**Figures 3B,C**).

COMPARISONS WITH REFERENCE PARADIGMS

In order to further characterize the profile of impulsivity assessed by the VDS, we compared it against reference paradigms, namely the 5-csrtt (in experiment 1) and DD (in experiment 2). The parameters used to correlate assessments of impulsivity of the same animal made in different paradigms were: i) the average number of impulsive responses in the last 5 days of training before VDS, a period where impulsive responses were stable (**Figure 2I**), ii) the average rate of impulsive responses per minute of delay, partitioned in segments of 3 S, during the VDS, iii) the average number of impulsive responses in the different stages of the

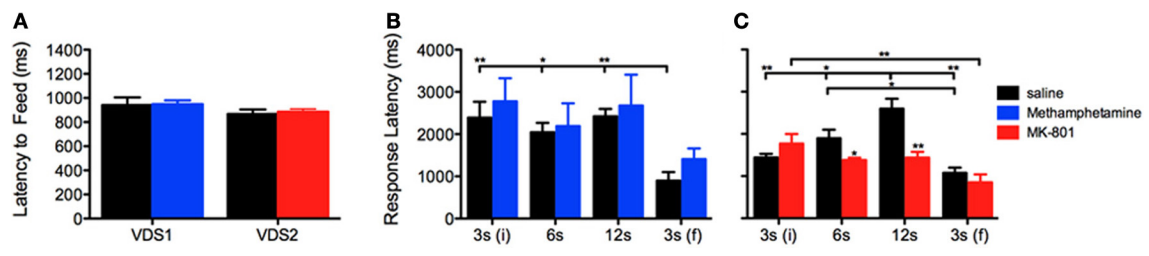

FIGURE 3 | Methamphetamine and MK-801 influence on (A) latency to feed and (B,C) response latency measures. Statistically significant comparisons between delay segments are marked with a horizontal line over the relevant graph bars; statistically significant comparisons between groups are marked over the graph bar of lowest value. *$P < 0.05$; **$P < 0.01$; data presented as mean + S.E.M. VDS—variable delay-to-signal task.

5-csrtt - 60, 30, 10, 2, and 0.5 s of signal duration—and iv) the AUC in the DD. While comparisons 5-csrtt and training included all animals of experiment 1, only animals injected with saline were used for comparisons with VDS 1 and 2 (**Figure 4**). The number of impulsive responses in the training protocol of both VDS 1 and 2 was strongly correlated with the first stage of the 5-csrtt, but not with later stages of increasing attentional demand (**Figure 4**; **Table 1**). On the contrary, the best correlate of the rate of impulsive responses in the VDS was performance in the 10 s stage of the 5-csrtt, but not in periods with shorter or longer stimulus presentations (**Figure 4**; **Table 1**). Finally, the rate of premature responses per min in the last delay block of the VDS (as well as in part of the 12 s delay) was negatively correlated with the AUC of the DD (of note, in this test a smaller area corresponds to a higher impulsivity) (**Figure 4**; **Table 1**). Importantly, this significant correlation also holds true when using the coefficients derived by fitting an exponential or hyperbolic function to the response curve.

DISCUSSION

The VDS paradigm was designed according to some simple principles: (i) The task (and its training protocol) is performed in a standard 5-csrtt apparatus in which only the center nose poke aperture is available. Such approach has been tried before by Dalley and colleagues (2002) and is intended to increase the efficiency of the task by reducing the attentional load. (ii) The training protocol consists in twice daily sessions on a "differential-reinforcement-of-low-rate" (DRL)-like schedule that quickly (4 days) achieve stability and a high degree of learning. Of notice, premature responses under DRL are often considered a measure of impulsivity, particularly under stable schemes such as ours (Pizzo et al., 2009) and can even be conceptualized as a delay discounting (Monterosso and Ainslie, 1999) which, although not the focus of the present paper, can also contribute to enrich the assessment of impulsivity obtained with the VDS. (iii) The initial block of 25 trials with 3 s delays until light presentation establishes a baseline against which results of the other blocks can be compared and assesses the acquisition of the training protocol. In our pharmacological assays, baseline behavior did not differ between saline and methamphetamine (VDS1) or MK-801

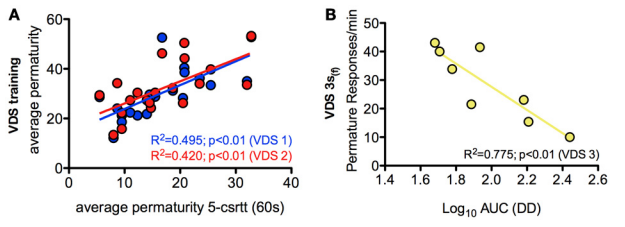

FIGURE 4 | Relevant correlations between 5-csrtt and DD performances and the VDS. (A) The average number of premature responses in the 60 s stage of the 5-csrtt correlated with the number of premature responses of both VDS 1 and VDS 2 training periods. **(B)** Preference for delayed choices (as measured by the AUC) in DD was inversely correlated with premature responses in the 3 s (f) period of the VDS. See **Table 1** for complete analyses. 5-csrtt—5 choice serial reaction time task; AUC—area under the curve; DD—delay discounting; VDS—variable delay-to-signal task.

(VSD2) groups. (iv) The following block of 70 trials exposes animals to larger delays of 6 and 12 s, randomly presented. Importantly, this probably induces two sources of behavioral control, similarly to what happens in mixed-fixed interval (FI) experiments (Whitaker et al., 2003), that might contribute to the increased responding observed in the final 3 s-delay block (Baron and Leinenweber, 1995). (v) The last block consists of 25 trials with a delay of 3 s before light presentation, in which control animals present an increased response rate; this is in accordance with current concepts in behavioral timing, in which the rate of responding has an inverse relationship with the duration of the interval (Kirkpatrick, 2002; Guilhardi et al., 2005).

An approach similar to ours, a go/no-go task using delays of variable duration (9 to 24 s) has been already described by Mitchell and co-workers (Gubner et al., 2010; Moschak and Mitchell, 2012). However, while the variable delays constitute the core of their task, from which measures of impulsive behavior are taken, the variable intervals in the VDS act as a trigger of increased impulsivity between the basal 3 s block and the final 3 s block, contributing to unmask manifestations of impulsive behavior. In addition, while the VDS can be conducted in a single session after a training protocol of fixed duration (10 sessions, 5 days), the total number of training sessions in the previously described task can amount to 11 days, depending on each animal's performance.

In a first attempt to characterize VDS, we assessed animals acutely treated with MK-801 or methamphetamine, two drugs with well described and opposite effects on impulsive behavior. Supporting its validity as a test of impulsivity, acute MK-801 treatment (VDS2), which induces enhanced impulsivity (Fletcher et al., 2011), caused a generalized increase in the number and rate of premature responses across all delay blocks, whereas acute challenge with methamphetamine (VDS1), which acts as a stabilizer of impulsive decisions in animals (Richards et al., 1999; Winstanley et al., 2003) and humans (De Wit et al., 2002), prevented the increase in premature response rates displayed by control animals in the last (3 s) block. Interestingly, the latter results seem to be critically dependent on the existence of a previous block of randomly presented 6/12 s delays in our protocol, since it was shown that only variable delays, as opposed to fixed delays, trigger the inhibitory action of acute methamphetamine upon premature responses on a FI protocol (Hayton et al., 2012). Besides this effect on the last block, metamphetamine treatment also resulted in a completely stable response profile across blocks, independently of the delay. Of notice, this enhanced delay tolerance is in accordance with data from DD tasks in which acute amphetamine induces a delay insensitive behavior (Winstanley et al., 2003).

To further characterize VDS, we correlated performance in our test with results of the same animals in one of two standard paradigms of impulsivity (Winstanley et al., 2010; Dalley et al., 2011; Dalley and Roiser, 2012): the 5-csrtt (VDS1) and the DD (VDS2). For these comparisons, VDS delays were divided in bins of 3 s for several reasons: (i) all delays were multiple of 3 and therefore this was a convenient option for analyses; (ii) the training protocol was set at a periodicity of 3 S/trial and therefore this could be considered the basal value of delay tolerance; (iii) we

Table 1 | Linear regression of the comparisons between VDS and standard impulsivity paradigms: 5-csrtt and DD.

5-csrtt		Training		3 s(i) [0–3] s		6 s [0–3] s		6 s [3–6] s		12 s [0–3] s		12 s [3–6] s		12 s [6–9] s		12 s [9–12] s		3 s(f) [0–3] s	
		VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2	VDS 1	VDS 2
60 s	**R2**	0.495	0.420	0.164	0.274	0.159	0.043	0.057	0.002	0.234	0.097	0.055	0.061	0.110	0.004	0.052	0.007	0.057	0.018
	P	**0.001**	**0.002**	0.319	0.121	0.327	0.563	0.570	0.897	0.225	0.382	0.576	0.490	0.423	0.871	0.586	0.819	0.567	0.715
30 s	**R2**	0.044	0.042	0.043	0.239	0.216	0.036	0.164	0.005	0.213	0.087	0.073	0.276	0.121	0.198	0.119	0.159	0.162	0.011
	P	0.375	0.388	0.622	0.152	0.246	0.601	0.320	0.849	0.250	0.409	0.518	0.119	0.398	0.198	0.402	0.253	0.323	0.774
10 s	**R2**	0.088	0.102	0.185	**0.701**	0.290	**0.614**	**0.703**	0.179	**0.700**	**0.733**	0.417	0.214	**0.549**	**0.561**	0.461	0.277	0.486	0.194
	P	0.203	0.170	0.288	**0.003**	0.168	**0.007**	**0.009**	0.223	**0.010**	**0.002**	0.084	0.178	**0.035**	**0.013**	0.064	0.118	0.055	0.203
2 s	**R2**	0.003	0.014	0.057	0.052	0.414	0.275	0.311	0.161	0.376	0.241	0.363	0.159	0.402	0.248	0.325	0.220	0.402	0.106
	P	0.833	0.621	0.569	0.526	0.085	0.120	0.151	0.251	0.106	0.150	0.114	0.254	0.091	0.143	0.140	0.172	0.092	0.358
0,5 s	**R2**	0.000	0.001	0.001	0.330	0.086	0.149	0.183	0.096	0.197	0.208	0.100	0.179	0.141	0.239	0.123	0.289	0.189	0.111
	P	0.994	0.925	0.931	0.082	0.480	0.271	0.291	0.383	0.270	0.186	0.445	0.224	0.360	0.152	0.394	0.109	0.282	0.347

DD		3 s(i) [0–3] s	6 s [0–3] s	6 s [3–6] s	12 s [0–3] s	12 s [3–6] s	12 s [6–9] s	12 s [9–12] s	3 s(f) [0–3] s
		VDS3	VDS3	VDS3	VDS3	VDS3	VDS3	VDS3	VDS3
AUC	**R2**	0.432	0.237	0.389	0.198	0.409	**0.763**	0.172	**0.755**
	P	0.077	0.221	0.098	0.270	0.088	**0.010**	0.307	**0.005**
K^1	**R2**	0.436	0.097	0.350	0.029	0.418	0.609	0.407	**0.810**
	P	0.328	0.836	0.441	0.95	0.351	0.146	0.365	**0.027**
K^2	**R2**	0.532	-0.053	0.339	−0.147	0.547	0.643	0.501	**0.807**
	P	0.219	0.91	0.458	0.753	0.204	0.119	0.252	**0.028**

Concerning the later, comparisons were made with the AUC and with the hyperbolic/exponential decaying coefficients, k^1 and k^2, respectively. Significant comparisons ($P < 0.05$) are highlighted in bold.

observed that the impulsivity response profile along the delay was not stable and varied in periods of ∼3 s (see **Figure 2E**).

The strong positive correlation between impulsive behavior in the initial stage of the 5-csrtt and the training period preceding the VDS fits with the fact that both tests use a DRL-like schedule, in which premature responses lead to a time-out period. In contrast, there were almost no significant correlations between impulsivity behavior in the 5-csrtt and the VDS, which likely relates to the fact that, in contrast to the training period, premature responses in the VDS are not "punished." Indeed, this protocol difference implies that parameters used as measures of impulsivity in both tests are of a fundamentally different nature (percentage of prematurely interrupted trials in the 5-csrtt vs. number/rate of premature responses in the VDS) and probably correspond to different types of impulsivity. This idea is supported by the fact that methamphetamine administration decreased impulsivity in the VDS (present study) and decision impulsivity paradigms, including the DD (Richards et al., 1999; Winstanley et al., 2003), but increased response impulsivity in the 5-csrtt (Cole and Robbins, 1987; Fletcher et al., 2011). In line with this, and despite its overall inhibitory action, is the observation that metamphetamine increased the rate of premature responses in the first second of the delay period might reflect increased response impulsivity. More importantly, the rate of premature responses in the final 3 s block of the VDS was strongly and significantly correlated with preference for delayed choices in the DD, either quantified by the AUC or by an equivalent parameter in terms of hyperbolic or exponential discounting functions (Odum, 2011). As the latter is the gold-standard in decision impulsivity assessment, this, together with data on metamphetamine and MK-801 discussed above, strongly supports the validity of VDS as a test of impulsive behavior.

The VDS presents a number of advantageous characteristics over the available impulsivity paradigms: (i) It has a significantly shorter training period (10 twice daily sessions) when compared with the ≈35–55 days of 5-csrtt and DD (Winstanley, 2011), (ii) it requires only one test session, (iii) it is resistant to multiple testing (has almost no test-retest effect) making it particularly suitable for longitudinal assays. Our validation assays, namely in the comparisons with the 5-csrtt and DD, relied in the intrinsic behavioral variability of an outbred population (Wistar Han), and not in artificially induced variability (e.g., by drug treatments or genetic manipulations), reinforcing the sensitivity of our paradigm in terms of impulsivity assessment. It should, however, be stated that the VDS does not replace paradigms like the 5-csrtt, where impulsivity is measured in conditions of high attention demand or like the DD, where an actual choice is presented. Recently, we have used an earlier version of the VDS to successfully demonstrate alterations in impulsive behavior in animals with neuropathic pain (Leite-Almeida et al., 2012).

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CHAPTER 4

António Melo, Hugo Leite-Almeida, Clara Ferreira, Nuno Sousa, José M. Pêgo

Exposure to Ketamine Anesthesia Affects Rat Impulsive Behavior

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Exposure to Ketamine Anesthesia Affects Rat Impulsive Behavior

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Introduction: Ketamine is a general anesthetic (GA) that activates several neurotransmitter pathways in various part of the brain. The acute effects as GA are the most well-known and sought-after: to induce loss of responsiveness and to produce immobility during invasive procedures. However, there is a concern that repeated exposure might induce behavioral changes that could outlast their acute effect. Most research in this field describes how GA affects cognition and memory. Our work is to access if general anesthesia with ketamine can disrupt the motivational behavior trait, more specifically measuring impulsive behavior.

Methods: Aiming to evaluate the effects of exposure to repeat anesthetic procedures with ketamine in motivational behavior, we tested animals in a paradigm of impulsive behavior, the variable delay-to-signal (VDS). In addition, accumbal and striatal medium spiny neurons morphology was assessed.

Results: Our results demonstrated that previous exposure to ketamine deep-anesthesia affects inhibitory control (impulsive behavior). Specifically, ketamine exposed animals maintain a subnormal impulsive rate in the initial periods of the delays. However, in longer delays while control animals progressively refrain their premature unrewarded actions, ketamine-exposed animals show a different profile of response with higher premature unrewarded actions in the last seconds. Animals exposed to multiple ketamine anesthesia also failed to show an increase in premature unrewarded actions between the initial and final periods of 3 s delays. These behavioral alterations are paralleled by an increase in dendritic length of medium spiny neurons of the nucleus accumbens (NAc).

Conclusions: This demonstrates that ketamine anesthesia acutely affects impulsive behavior. Interestingly, it also opens up the prospect of using ketamine as an agent with the ability to modulate impulsivity trait.

Keywords: ketamine, ketamine anesthesia, rat model, impulsive behavior, nucleus accumbens, striatum

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INTRODUCTION

General anesthetics (GA) are widely used in invasive surgeries and diagnostic procedures. Their application is considered to be safe, despite evidences suggesting post-surgery behavioral impairments. Following the pioneer work by Bedford (1955), several studies have investigated the relationship between surgical anesthetics and several clinical disturbances involving deteriorated memory and executive function (for review see Steinmetz et al., 2009). These

functional alterations are now collectively called *postoperative cognitive dysfunction* (POCD). POCD is normally a transient phenomenon in the majority of patients, although it might gain a persistent character in aged individuals (Abildstrom et al., 2000; Selnes et al., 2008). POCD is not entirely understood and seems to be linked to a multitude of factors, such as the duration of surgery (Canet et al., 2003), specific procedures such as cardiac (Goto and Maekawa, 2014) and orthopedic surgery (Shi et al., 2014), immune response to surgery (Riedel et al., 2014) and to individual characteristics of each subject such as age or previous cognitive impairments (Moller et al., 1998; for review see also Krenk et al., 2010).

Ketamine is an intravenous GA, classified as a dissociative agent that acts as an antagonist in N-methyl-D-aspartate (NMDA) glutamate receptor (Maeng et al., 2008). There is evidence that ketamine interferes with dopamine release as well (Hancock and Stamford, 1999; Masuzawa et al., 2003) explaining its effect on executive function and motivation behavior. Others have argued that its effects mainly stem from its psychotomimetic properties and its ability to induce psychosis-like behavior in several animal models (Frohlich and Van Horn, 2014). Despite the potential behavioral secondary effects, ketamine remains a very versatile drug used in pain treatment, in general anesthesia and, more recently, in antidepressant therapy (DeWilde et al., 2015; Melo et al., 2015). Additionally, it is also used as a recreational drug with known addictive properties (Sun et al., 2014).

Despite the growing number of studies on this drug and associated effects, its impact on impulsive behavior remains largely unexplored. Impulsivity is a complex construct encompassing the domains of inhibitory control and decision-making (Dalley and Roiser, 2012). It is in most cases considered adaptive (trait impulsivity) but it can assume a disruptive character in several neuropsychiatric diseases as ADHD, substance dependence or obsessive-compulsive disorders. Impulsive behavior is often classified in impulsive action—compromised ability to inhibit a pre-potent behavior—and impulsive choices—preference for immediate though suboptimal over delayed but more compensating options (Evenden and Ryan, 1996).

Our objective is to study if repeated exposure to ketamine in the context of general anesthesia might induce changes in impulsivity using a paradigm that allows evaluating both impulsive action and delay intolerance.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles-River Laboratories, Barcelona), weighing 300–400 g and aged 6 months, were housed (two per cage) under standard laboratory conditions (12 h light: 12 h dark cycle, at 22°C, relative humidity of 55%; free access to food and water). Sixteen animals were randomly assigned to two experimental groups—a control group (CONT) without anesthetic exposure and a group with ketamine exposure (KET). The dietary regimen was restricted to 1 h of food availability (19:00–20:00), 3 days preceding the initiation of the behavioral experiments. Body weight was controlled to ensure that it did not decrease below 85% of the initial value.

A different set of animals was assigned to perform behavioral evaluation after anesthetic exposure with the same protocol applied to the variable delay-to-signal (VDS) group. Animals performed the following behavioral tests: Elevated Plus Maze, Open Field, Forced Swim Test and Novel Object Recognition test.

Experiments involving the use of animals followed the guidelines of European Community Council Directive 2010/63/EU and were approved by the university ethical committee.

Drugs

For the experimental design the following drug was used: Ketamine (50 mg/ml, Ketalar®, Pfizer, New York City, NY, USA) and as vehicle, saline (NaCl 0.9%).

Anesthetic Procedure

The anesthetic procedure was performed through intra-peritoneal injections of ketamine according to the following scheme: an initial anesthetic dose of 100 mg/Kg of ketamine to induce anesthesia and then two subsequent injections of 50 mg/Kg. Anesthetized animals were placed over a warming pad with feedback control at 37°C in order to maintain body temperature. Anesthesia was considered adequate when animals lost the righting reflex and were irresponsive to tail pinch. The following dosage (50 mg/ml) was given when animals regain response to pressure stimuli in the tail. The anesthesia protocol was applied between the end of the training sessions and before the VDS test session (see below) in three GA procedures. Average time of anesthesia was 2.5 h. A washout period of at least 12 h was applied between the last anesthetic procedure and the initiation of the VDS protocol (see **Figure 1**).

Variable Delay to Signal (VDS)

The VDS task (Leite-Almeida et al., 2013) was performed in a 25 cm × 25 cm × 40 cm (W × L × H) operant chamber (OC; TSE Systems, Bad Homburg, Germany). The selection wall was slightly curved and presented five squared apertures (2.5 cm^2), equally distributed and elevated 2 cm from the grid floor (during the VDS procedures only the middle aperture is available; see below). Similarly, on the opposite wall, an aperture (food access) led to a pellet dispenser. Each aperture contained a 3W lamp bulb and an infrared beam system to detect the activity of the animal. Four OCs were used simultaneously, each placed in soundproof boxes with individual electrical fans for ventilation and white noise production. Animals were habituated to the testing conditions once a day for 4 days (pm session). In the first 2 sessions, animals were placed in the OC for 15 min with all lights off, access to apertures 1–5 blocked by metallic caps and 10–15 sugared pellets (45 mg, Bioserv Inc., New Jersey, EUA) available in the reward-deliver aperture. In sessions 3–4, animals were placed in the OCs for 30 min, all

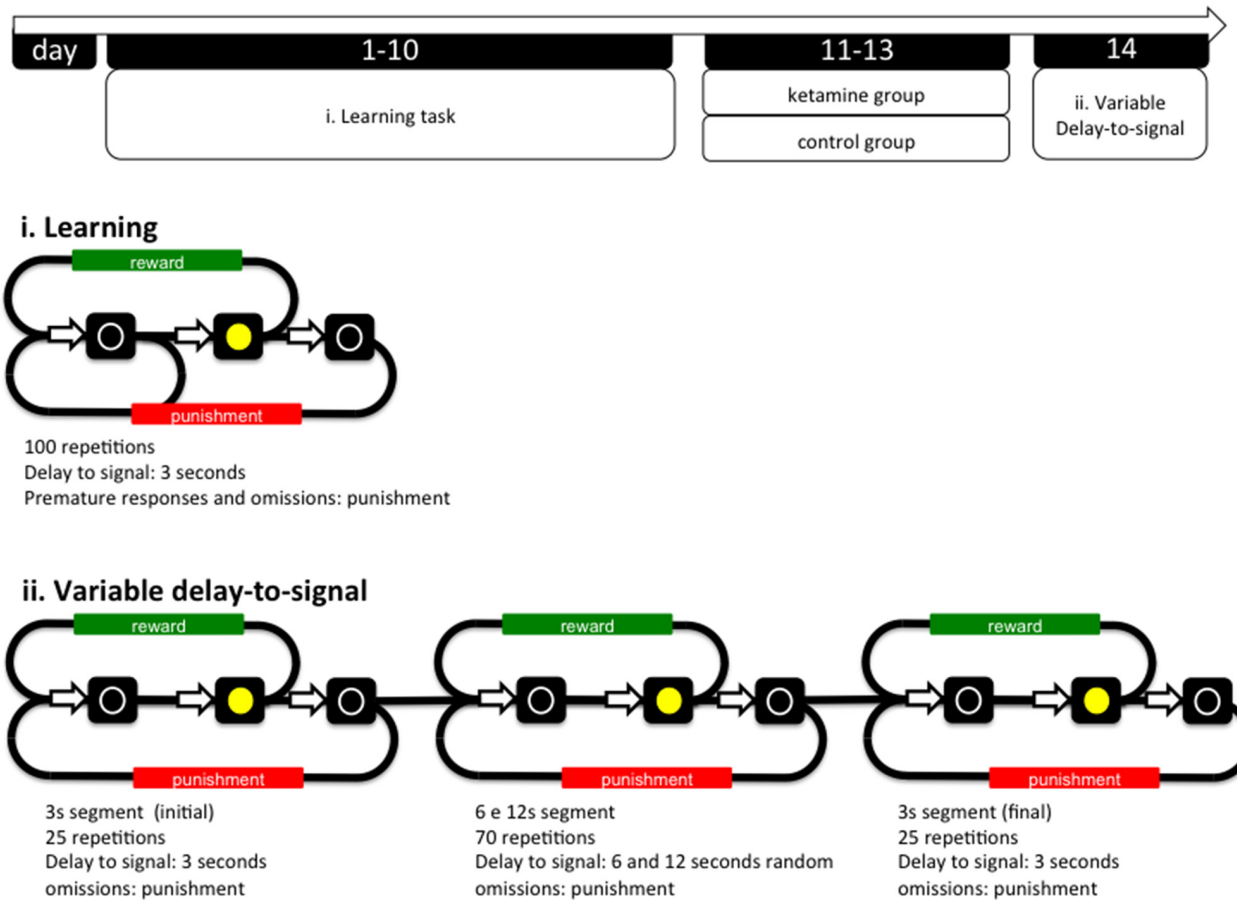

FIGURE 1 | General organization of the experiment and operational diagrams of the VDS and preceding training protocol. The VDS consisted in two parts: the training protocol (10 sessions) and the VDS proper (1 session); while in the first the delay-to-signal was fixed (3 s) and pre-signal nose pokes were punished (TO), in the VDS proper, 2 blocks of 25 trials at 3 s delay were interposed by a block of 70 trials at 6 s and 12 s, pre-signal responses were registered but not punished with a TO. In both training and VDS the signal duration was set to a maximum of 60 s; the absence of a response within this period was registered as an omission. TO, timeout; VDS, variable delay-to-signal task.

lights were on and animals had free 2–3 pellets available in the central (#3) nose poke aperture 10–15 in the reward-deliver aperture. The protocol for VDS was initiated the following day and included two phases: (i) training sessions; and (ii) VDS test (see **Figure 1**).

(i) Training. Sessions were initiated by turning the house light on and delivering one sugared pellet in the reward-deliver aperture, the collection of which started an inter trial interval (ITI) of 3 s to allow the animal to ingest it. Trials then started, consisting of a 3 s period with only the house light on (delay period), followed by lightning of the response aperture #3 for 60 s (response period). Nose pokes in the delay period were punished with a timeout (TO) period in complete darkness (5 S) and in the response period (aperture #3 light on) were rewarded with the delivery of one pellet. These responses were respectively labeled as premature (PR) or correct responses. Collection of a food reward always triggered a 3 s ITI, before a new trial begun. Except for the TO periods, the house light was always on. Each session consisted of 100 trials or a maximum time of 30 min (whichever was reached first). Training sessions occurred twice per day, with a 5 h interval in between, for five consecutive days. The average number of PR of the overall group stabilized at ∼30% in the last four sessions of training (see **Figure 2**).

(ii) Test. The VDS testing session occurred on a single day and consisted of 120 trials, similar to those previously described, with the exception of the delay, which was 3 s in the first and the last 25 trials, and, either 6 or 12 s in the middle of the 70 trials randomly attributed by the software leading to a 3 s (i)—6/12 s—3 s (f) configuration. Importantly, nose pokes were allowed during the delay period; these premature responses (PR) were registered and did not trigger TO or reward deliver.

VDS parameters analyzed are: PR/minute—number of PR per amount of delay; cumulative prematurity—is the average of PR accumulated along consecutive bins of 500 ms; latency to feed—average latency to collect the reward: response latency—amount of time between the light signal at #3 and

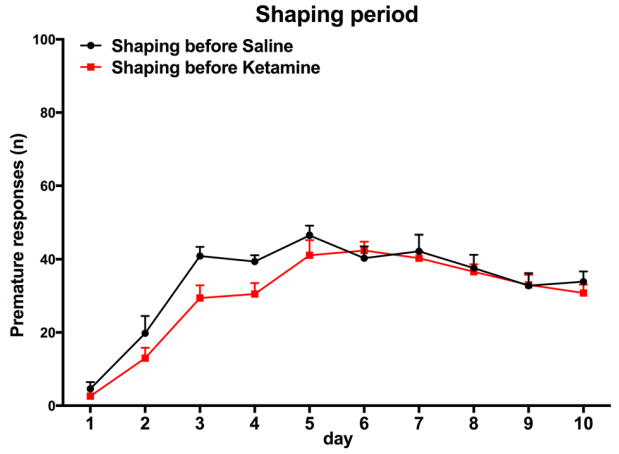

FIGURE 2 | Training session. Animal performance in both groups does not differ significantly. Group assignment is performed after the end of the learning period, with animal randomly assigned to the groups and matched for performance in premature responses (PR).

response. Latencies are presented as averaged times, i.e., sum of response times divided by the number of trials at each block—3 s (i), 6 s, 12 s or 12 s.

Elevated Plus Maze

To assess anxiety-like behavior, animals were tested on the elevated-plus maze (MED-NIRPMNR; Med Associates, St Albans, VT, USA) as previously described (Pêgo et al., 2008). Animals were placed at the central hub facing the open-closed arm intersection and were allowed to explore the maze for 5 min. An arm entry was considered if the four paws were positioned within the arm. The test was filmed and the ratio between time in open arms and time in closed arms was subsequently quantified. Activity in the open arms was calculated as open arm entries percentage (entries into the open arms/total entries into all arms) and time spent in open arm percentage (time spent in the open arms/total time spent in all arms). The degree of anxiety was indirectly related to the time spent in the open arms and the number of open arm entries.

Open Field

To assess locomotor activity, rats were placed in an open-field apparatus (43.2 (length) cm × 43.2 (width) cm × 30.5 (height) cm, transparent acrylic walls and white floor, Med Associates) in a room illuminated by white light. Instant position was monitored over a period of 5 min by an array of two 16 beam infrared arrays. Total distance and average speed were used as a measure of locomotor activity.

Forced Swim Test

Learned helplessness was evaluated in the forced-swim test on the last day of exposure to uCMS. Twenty-four hours after a pre-test session (10 min), rats were placed in cylinders filled with water (25°C; depth 30 cm) for a period of 5 min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint, Lyon, France); the system automatically calculated immobility time and latency to immobility. Learned helplessness behavior was defined as an increase in time of immobility and a decrease in latency to immobility.

Novel Object Recognition

Recognition memory of the animals was assessed using an adapted version of the non-matching-to-sample learning task as previously described (Bevins and Besheer, 2006).

Histological Procedures

After behavioral evaluation, five rats from each group were perfused transcardially with saline (NaCl 0.9%) under deep pentobarbital anesthesia. Brains were removed and kept in Golgi-Cox solution for 15 days and then transferred to a 30% sucrose solution for 5 days (Glaser and Van der Loos, 1981). Coronal sections (200 μm) were obtained using a vibratome and collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene-cleared before coverslipping. Dendritic arborization was then analyzed in the nucleus accumbens (NAc) and Striatum (Str). Selected neurons had every branch of the dendritic tree reconstructed using a motorized microscope (Olympus BX51; Olympus, Tokyo, Japan) and Neurolucida v10 software (Microbrightfield, Williston, VT, USA) and three-dimensional analysis of the reconstructed neurons was performed using Neurolucida Explorer v10 software (Microbrightfield). For each animal, 20 neurons were reconstructed and measurements from individual neurons from each animal were averaged. The following dendritic morphology parameters were examined: dendritic length and the number of primary dendrites and dendritic branching points were compared across experimental groups. The observer who made the neuronal reconstruction was blind on the constitution of the groups.

Statistical analysis

Data is presented as mean ± SEM and analyzed using two-way analysis of variance followed by a *post hoc* (Bonferroni) for multiple comparisons for analysis of the VDS results. Independent-samples *t*-test was used to test for the drug effect within each delay; behavioral results in the elevated plus maze, open field, forced swimming and novel object recognition test; and for analysis of neuronal dendritic length. The spearman rank correlation test was used to test correlation between different variables. Results were considered statistically significant if $p < 0.05$.

RESULTS

Impulsivity

We tested the effect of repeated anesthetic exposure to ketamine in impulsivity behavior. General anesthesia with ketamine had

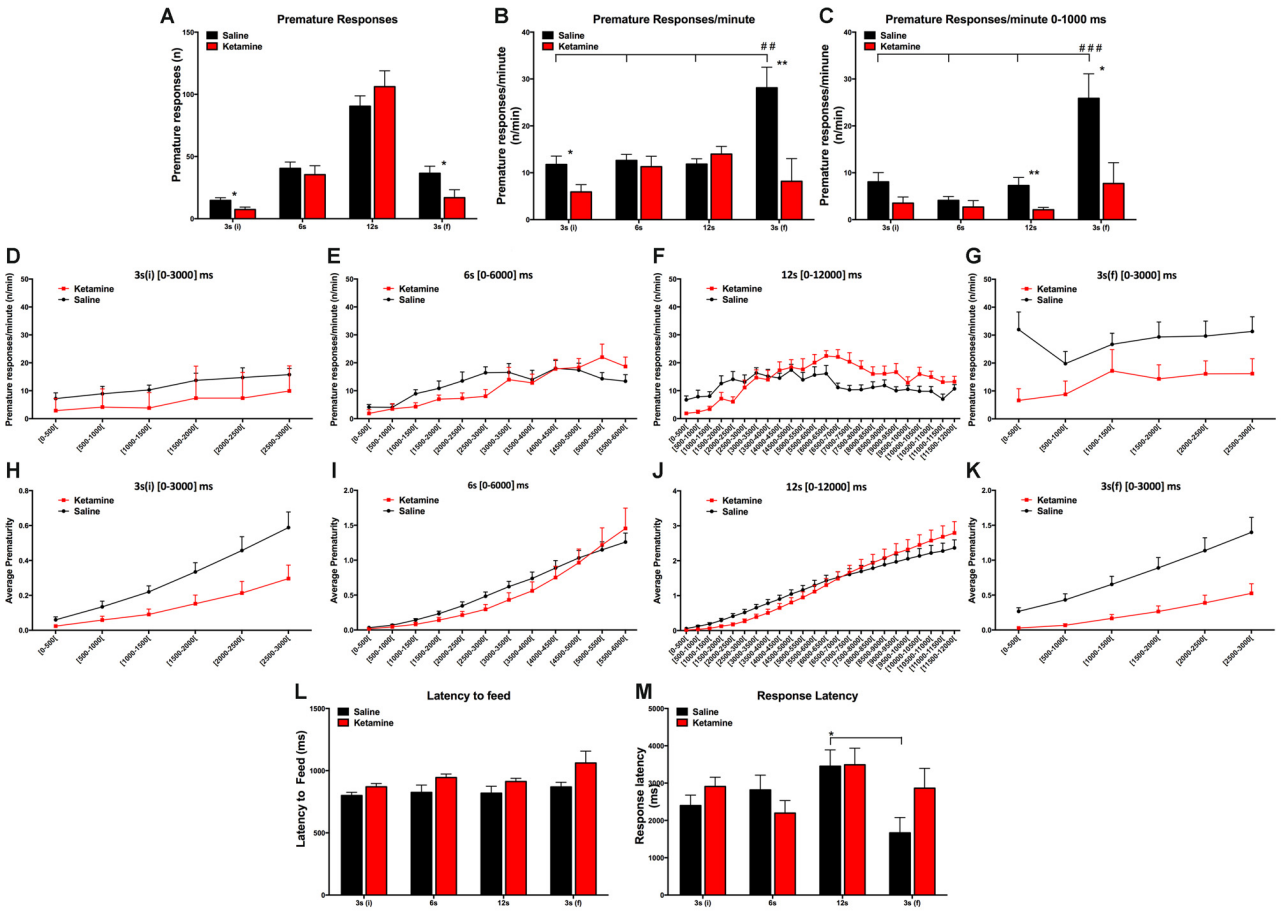

FIGURE 3 | VDS test results general anesthesia with ketamine had no effect in the absolute number of PR; however, the number of PR varied according to the delay (A). Normalized PR per minute of available delay (PR/m) varied according to the delay 3 s (i), 6 s, 12 s and 3 s (f). Decrease of PR/m in animals treated with ketamine compared to the control animals in both 3 s (i) and 3 s (f) delay periods **(B)**. Decrease of PR for the KET animals when compared with CONT animals in the 0–1000 ms period **(C)**. Absolute (**D–G**) and the accumulated (**H–K**) average prematurity/premature responses profile is presented in a segmented (500 ms periods) fashion for each delay segment 3 s (i), 6 s, 12 s and 3 s (f). No differences were observed in the latency to feed and no overall effect was detected across different delays **(L)**. No effects were observed in response latencies between experimental groups **(M)**. ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; *significances refer to differences between groups, $^{\#}$significances refer to differences between different delays).

no effect in the absolute number of PR ($F_{(1,112)} = 0.6795$ $P = ns$); however, the number of PR varied according to the delay ($F_{(3,112)} = 59.74$, $P < 0.0001$; **Figure 3A**). We also observed that the normalized PR per minute of available delay (PR/m) varied according to the delay 3 s (i), 6 s, 12 s and 3 s (f) ($F_{(3,112)} = 4.087$, $P = 0.0085$) and that it was significantly decreased for animals treated with ketamine compared to the control animals ($F_{(1,112)} = 10.66$, $P = 0.0015$) in both 3 s (i) and 3 s (f) delay periods (**Figure 3B**). Our results revealed that animals from both groups, CONT and KET, kept their PR/m rate relatively constant across the initial blocks, while for the last 3 s delay, we observed a difference in the IR: CONT animals showed a normal increase in PR/m, while KET were insensitive to the exposure to large delays ($F_{(1,112)} = 6.931$, $P = 0.0015$; **Figure 3B**).

To further explore the possibilities offered by VDS in the characterization of impulsivity and according to our previous studies (Leite-Almeida et al., 2013), we partitioned each delay block into 500 ms intervals (**Figures 3D–K**). This analysis confirmed our previous data showing a decrease of PR for the KET animals when compared with CONT animals (delay: $F_{(3,112)} = 10.44$, $P < 0.0001$; exposure to ketamine: $F_{(1,112)} = 14.73$, $P = 0.0002$; **Figure 3C**). In the 3 s (i) and 6 s delays, we observed no differences between groups. But in the 12 s delay, KET exposure decreased PR rate in the first part (up to 6000 ms) but not in the second part (6000–12,000 ms) of the delay, suggesting a heightened impulsive behavior ($p_{(t = 0.880,\ dF = 28)} = 0.0075$; **Figure 3C**). On the contrary, in the last delay period [3 s (f)] ($p_{(t = 2.654,\ dF = 28)} = 0.0130$), ketamine prevented an increase in

PR that in normal conditions is observed after exposure to long delays (**Figure 3C**). These observations are not due to sedative or motivational effect at the selected doses as no differences were observed in the latency to feed and no overall effect was detected across different delays ($F_{(3,112)}$ = 2.504, P = 0.0629; **Figure 3L**). No effects were observed in response latencies

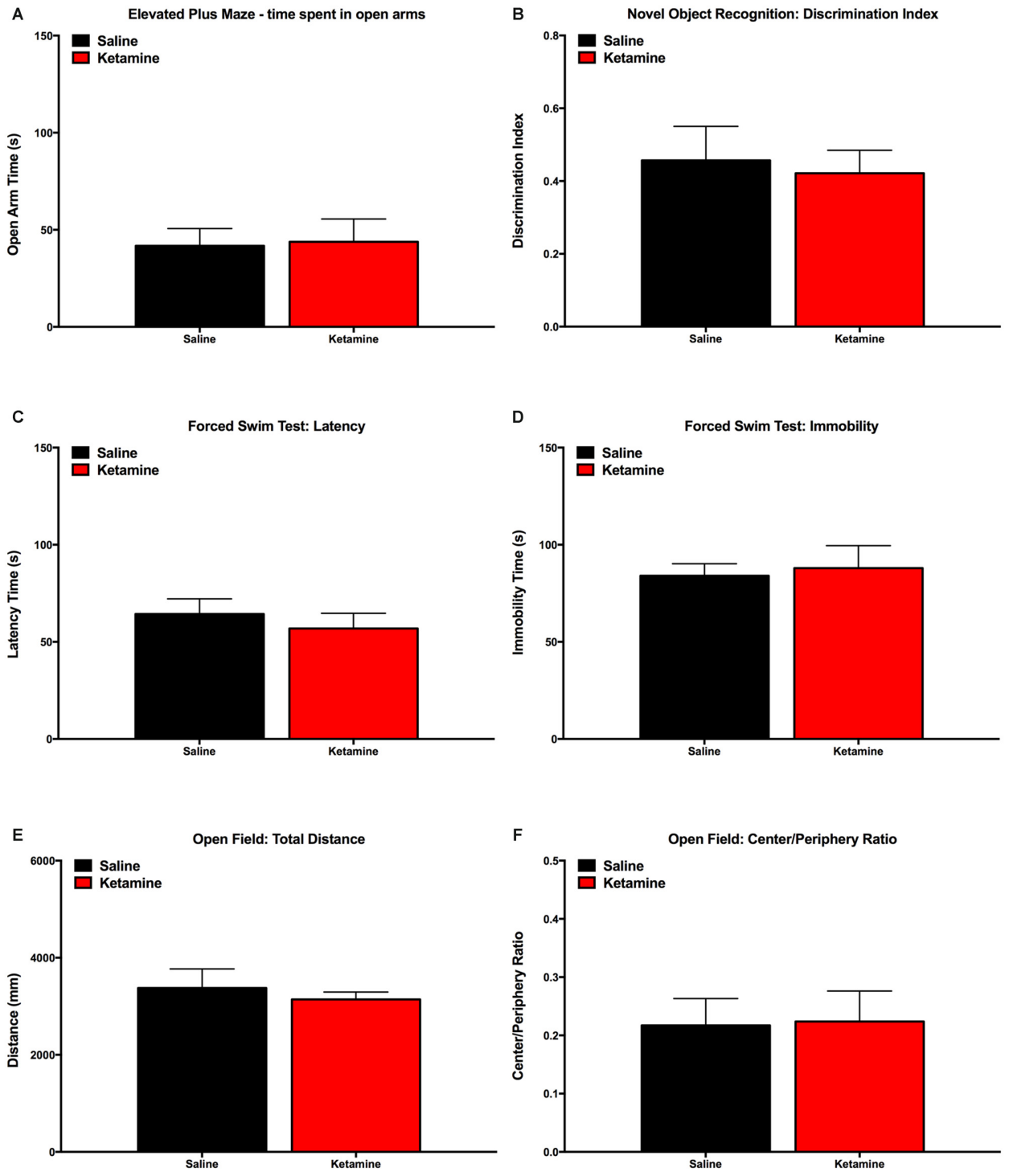

FIGURE 4 | Behavior evaluation performed to validate the model of anesthesia exposure: animals exposed to ketamine anesthesia performed similarly to control animals in the Elevated Plus Maze **(A)**, Novel Object Recognition Test **(B)**, Open Field **(C,D)**, Forced Swimming Test **(E,F)**.

between experimental groups (drug*delay: $F_{(3,112)} = 1.886$, $P = ns$; **Figure 3M**).

Other Behavioral Evaluations

In order to rule out the effect of ketamine anesthesia behavioral dimensions that could interact with VDS results, we performed evaluations in a different group of animals not assigned to perform the VDS paradigm. Results show that animals exposed to ketamine anesthesia have similar performance to control group concerning Elevated Plus Maze (**Figure 4A**), Novel Object Recognition Test (**Figure 4B**), Forced Swimming test (**Figures 4C,D**) and Open field (**Figures 4E,F**).

Neuronal Morphology

We performed neuronal dendritic reconstruction. The analysis of dendritic branching in the NAc showed a significant increase in dendritic length in animals exposed to ketamine anesthesia $p_{(t = 3.880,\ dF = 28)} = 0.0047$ (**Figure 5A**) and no difference in the striatum analysis (**Figure 5B**). No differences were found concerning dendritic spine analysis.

Correlation of VDS Performance With Neuronal Morphology

We found a significant negative correlation between NAc neuronal dendritic length and the number of absolute PR in the initial 3 s delay ($R = -0.67$, $P = 0.039$) and final 3 s delay PR 3 s (i) ($R = -0.66$, $P = 0.041$). Concerning the ratio of PR/minute we also found a negative correlation between the values in the initial 3 s period and dendritic length of NAc neurons ($R = -0.740$, $p = 0.001$) (**Figures 6A,B**; **Table 1**).

DISCUSSION

In this work, we investigated the existence of possible alterations in executive function induced by consecutive ketamine anesthetic administration using impulsivity as surrogate readout as assessed by the VDS. We observed that KET-multiple exposure abolished the increase in the rate of PR (PR/minute) that is generally observed in the last block [3 s (f)] after the long-delay trials (6 and 12 s; Leite-Almeida et al., 2013). This effect was preserved in control animals. Curiously, over the course of long 6 s and 12 s delays, KET animals increased their PR above normal levels. It is important to notice that we found no differences in the latencies to feed or changes in animal weight, indicating that both KET-exposed animals and controls were equally motivated during the performance of the test. Additionally, we have analyzed the neuronal dendritic architecture in the NAc and striatum; our analysis revealed an increased average dendritic length in the first. Also, we observed increased impulsivity at the 3 s (i) and 3 s (f) blocks were associated with longer dendritic length in the NAc medium spiny neurons.

The VDS, which was developed in our lab, was designed and validated to evaluate impulsive behavior in a rat model (Leite-Almeida et al., 2013). In this paradigm, delay intolerance manifests as a robust increase of PR in the 3 s (f) segment when compared to the 3 s (i), i.e., after exposure to large 6 s and 12 s delays. This increment in the rate of impulsive responses as well as the rate of impulsive responses in the later periods of the 12 s segment has been shown to correlate with discounting steepness in the delay discounting (DD) paradigm (Evenden and Ryan, 1996). Additionally, impulsive responses in the training phase reflect action impulsivity; the construct at this stage is similar to that of the 5-choice serial reaction time task (5-CSRTT; Carli et al., 1983).

The KET group displayed a smaller PR/min in the 3 s (i) delay, even when the analysis was confined to the first 1000 ms, a parameter that is closely associated with impulsive action (Leite-Almeida et al., 2013). In previous works, using Sprague Dawley (Nemeth et al., 2010) and Lister Hooded (Smith et al., 2011) rats under the influence of acute sub-anesthetic doses of KET showed no difference in impulsive responses in the 5-CSRTT. Similarly, Nikiforuk and Popik (2014) observed that exposure to both single or repeated sub-anesthetic doses of ketamine produced no differences in the baseline performance of any of the 5-CSRTT performance measures, such as correct/incorrect responses, omissions, accuracy and perseverant responding). However, in both experimental conditions an increase in omissions was detected without alterations in motivational aspects of the response. On the contrary, Oliver and coworkers revealed that acute administration of sub-anesthetic doses of ketamine increased the CD1 mice impulsive behavior, when performing the 5-CSRTT (Oliver et al., 2009). Indeed, several studies reported that NMDA antagonists (other than ketamine) increase impulsivity in the 5-CSRTT. That is the case of acute treatment with MK-801 or phencyclidine (Paine and Carlezon, 2009; Thomson et al., 2011; Barnes et al., 2012). Similarly, our group has shown that an acute challenge with MK-801 is able to increase all measures of impulsive behavior in the VDS (Leite-Almeida et al., 2013). On the other hand, chronic treatment may display a different behavioral pattern, either reduced PR during a 24-h drug withdrawal (Paine and Carlezon, 2009) or had no effect (Thomson et al., 2011; Barnes et al., 2012). Until now, most research seems to show a tendency of NMDA antagonists to increase impulsive behavior after an acute exposure, while on sub-chronic and chronic regimens the response seems to be towards a decrease in impulsive response particularly if the test is made during the withdrawn period. Our study shows a more complex picture. In line with the above studies, we observed a decrease in basal impulsive response. However, when analyzing the patterns of response along the delay period in intervals of

TABLE 1 | Correlation table (Pearson r^2 values; P values; $*p < 0.05$).

	Dendritic length nucleus accumbens	
	R^2	p
Premature responses/minute 3 s (i)	−0.89*	0.001093474
Premature responses/minute 6 s	−0.27	0.448276565
Premature responses/minute 12 s	0.09	0.811282518
Premature responses/minute 3 s (f)	−0.57	0.093003748

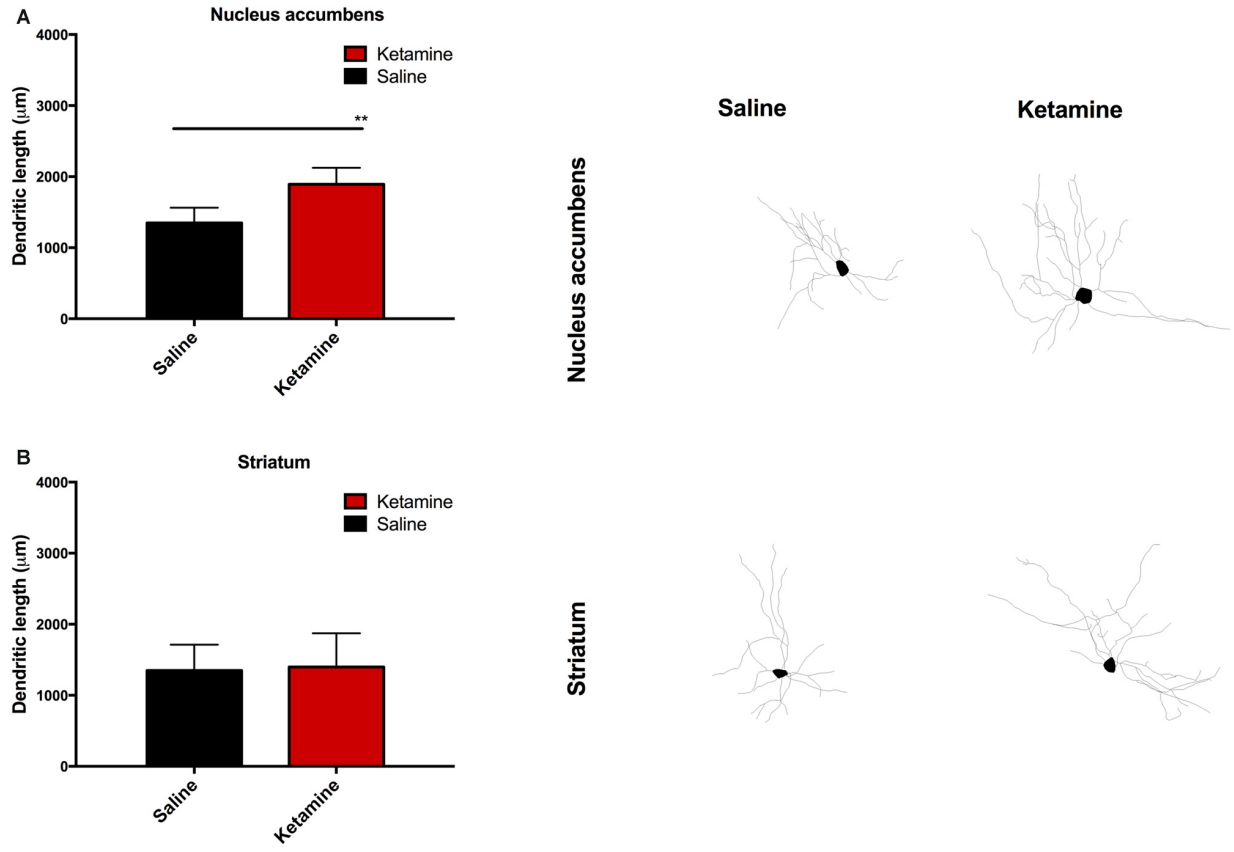

FIGURE 5 | Neuronal dendritic reconstruction in the nucleus accumbens (NAc). Increase in dendritic length in animals exposed to ketamine anesthesia **(A)** and no difference in the striatum analysis **(B)**. (**$p < 0.01$).

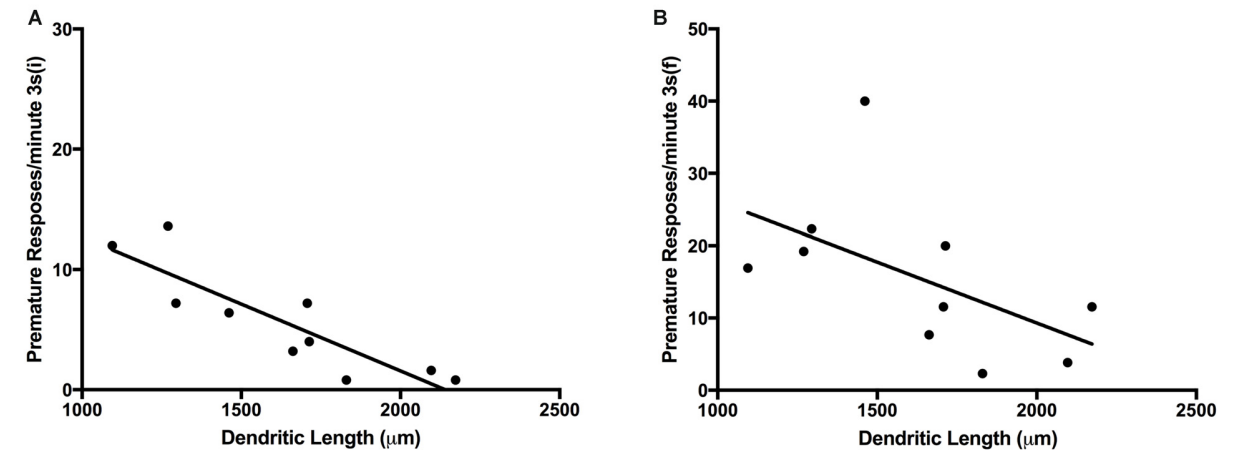

FIGURE 6 | Plot graph; values of ratio of PR/minute in the 3 s initial period **(A)**; values of ratio of PR/minute in the 3 s final period **(B)**; (PR, premature responses).

500 ms, it was evident that KET animals increased their PR/min towards the end of the delay overcoming the CONT group. In other words, while CONT animals, after some premature unrewarded responses, were able to correct their behavior, KET animals failed to adjust and steadily increase their prematurity rate, suggesting failures in their inhibitory control. In addition,

KET animals failed to show a robust increase in the PR/min between [3 s (i)] and [3 s (f)], which is normally observed after the exposure to large delays (Leite-Almeida et al., 2013). The PR/min in the [3 s (f)] is strongly correlated with the DD behavior, i.e., higher PR/min is associated to a preference for immediate (but smaller) choices in the DD (Leite-Almeida et al., 2013). Our findings suggest that ketamine disrupted a response correlated with performance in DD. These findings are supported by data from studies that show changes in DD performance after administration of ketamine and other NMDA antagonists such as memantine and MK-801 (Floresco et al., 2008; Cottone et al., 2013; Yates et al., 2015). Specifically, Yates et al. (2015) observed that ketamine decreased the sensitivity to reinforcer amount in DD, while Cottone showed that ketamine increased the choice for small immediate reward (Cottone et al., 2013) and Floresco reported that ketamine induced a decrease in tolerance for delayed rewards (Floresco et al., 2008).

No significant differences were observed in response and feed latencies which is concordant with the work of Nelson et al. (2002) where repeated ketamine administration was shown to have no impact on animals' capacity to perform the sustained attention task.

Food availability is also a concern and a limitation in operant behavior protocols. Research animals are food restricted in order to get the motivation for performing the task. The specific feeding regimen was applied according to the previously published protocol of the VDS paradigm (Leite-Almeida et al., 2013) and following the recommendations for daily food intake previously reported in other operant protocols (Bari et al., 2008). However, binge access to food might also promote the development of a higher impulsive trait (Vanderschuren and Ahmed, 2013).

VDS results were also not affected by emotional, locomotory or cognitive altered behavior as there were no differences between experimental and control group in these behavioral tests.

Chambers and Potenza (2003) proposed two main circuits to control impulsive response. A primary circuit consisting of parallel loops of neuronal projections from the prefrontal cortex, to the ventral Str (including the NAc), to the thalamus and then back to the cortex and a second circuit would supply the primary with autonomic, affective, motor and memory information necessary for a proper shaping of the output. The role of NAc in impulsive behavior was first shown by Cardinal et al. (2001), who described that bilateral lesion of the NAc but not of the anterior cingulate cortex or medial prefrontal cortex, resulted in more impulsive choices in delayed reinforcement choice task. In the DD, NAc lesioned rats were less impulsive, but only when the delay was changed between sessions, suggesting that NAc lesions impaired learning or adaptation to changes in delay reinforcement but did not affect tolerance to delays (Acheson et al., 2006). On the contrary, da Costa Araújo et al. (2009) showed that NAc lesioned rodents had changes in inter-temporal choice behavior preferring immediate over delayed reinforcement in an adjusting-delay approach. In our work, KET exposure increased dendritic arborization in the NAc medium spiny neurons but not in the striatum. Drugs of abuse with known effect in impulsive behavior, such as amphetamine and cocaine, are known to induce changes in neuronal architecture in the NAc (Robinson and Kolb, 2004). Ketamine is a GA that is also used as a drug of abuse. Despite its relatively short half-life, some of the effects of ketamine are known to be long-lived, namely through the activation of signaling pathways following the acute blockage of NMDA receptors and that are responsible for the neuronal changes namely synaptic and dendritic modeling. Exposure to a single dose of ketamine is known to increase the levels of activity regulated cytoskeletal protein (Arc), glutamate AMPA receptor-1 (GluR1), postsynaptic density protein-95 (PSD95) and synapsin I, all of them of importance in dendritic and synaptic remodeling (Li et al., 2010). Ketamine also has been shown to regulate the levels of neurotrophic factors such as brain derived neurotrophic factor (BDNF), which has been related with processes such as neurogenesis and synaptic protein expression (Kovalchuk et al., 2002). Ketamine also affects the regulation of signaling pathways such as mammalian target of rapamicin (mTOR), is a critical mediator of protein synthesis (Ma and Blenis, 2009) including dendritic/synaptic proteins (Liu-Yesucevitz et al., 2011). Indeed, the inactivation of mTOR pathway blocked the antidepressant effect of ketamine (Li et al., 2010). Interestingly, Sabino et al. (2013) showed that mTOR activation is needed to achieve a reduction of alcohol consumption in alcohol-preferring rats, showing that this pathway is also implicated in the build-up of other behaviors such as addiction. Our findings of changes in neuronal architecture after exposure to ketamine supports the known effect in synaptic protein expression factors and regulation of protein synthesis signaling pathways such as the aforementioned mTOR pathway. The synthesis of synaptic proteins important for neuroplasticity is one of the potentially critical steps for ketamine action (Duman et al., 2012). Our findings on dendritic organization largely reflect these observations.

In summary, this study demonstrated that a multiple exposure to ketamine anesthesia affects impulsive behavior. We demonstrated that this abnormal inhibitory control is associated with an increase in neuronal dendritic length of the NAc. In human patients exposed to anesthetic procedures, the postoperative cognitive deficits, manifested by their inability to engage successfully tasks at home or work, have also been reported, though impulsive behavior has not been specifically measured. We cannot therefore exclude the possibility that alterations in impulsive behavior might also influence postoperative period impairments.

AUTHOR CONTRIBUTIONS

AM, HL-A, CF, NS and JMP are all substantial contributors to the conception, design, acquisition, analysis and interpretation of the experimental work; all took part in revising it and approval of the version to be published.

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Ketamine is a versatile drug used in clinical practice for sedation, analgesia or anesthesia. The work described in this thesis provides new insights for ketamine use in the modulation of several behavioral dimensions, namely mood and impulsive behavior.

Several neurological networks within the brain are of paramount importance in the establishment of normal behavior. Neurons are the unitary base that support this network. Brain connectivity is a concept based in physical and functional wiring of the neuronal activity through the whole brain. Changes in brain connectivity are a hallmark of both pathological changes in the central nervous system, and of behavioral traits. Through this thesis, we show that ketamine has the ability to induce changes in neuronal network architecture, and cause behavioral changes in animal models of depressed like behavior and increased impulsivity. These characteristics make ketamine a potential game changer in different settings of behavioral modulation.

Our work focuses in the action of Ketamine as both antidepressant and impulsive behavior modulator. The fast acting antidepressant effect of ketamine and the acute effect in impulsivity are both known. Our main objective was to study the effect of repeated use of ketamine, while avoiding the establishment of models of chronic administration. The objective was to establish animal models, in which ketamine was studied in an interventional and therapeutic manner avoiding the establishment of models of addiction or schizophrenic-like behavior.

We used two distinct animal models and approaches to answer questions regarding the effect of ketamine in two different behavior domains: depression and impulsivity.

Animal model of depression

The main aim of our work was to characterize the impact of ketamine on current antidepressant therapy strategies. To answer this question, we selected an animal model that allowed us to reproduce a depressive-like phenotype in rodents that could be reverted by antidepressants. We chose the unpredictable CMS animal model of depressive-like behavior, which allowed us to mimic the changes induced in the central nervous system and behavior observed in humans with major depression. This animal model has been extensively used and reviewed as one of the gold standards in the study of the etiology of major depression and in preclinical development of antidepressant drugs (Willner *et al*, 2005). This model replicates not only behavioral changes but also neuronal structural changes that are associated with depression in humans, specifically in brain areas such as the hippocampus, PFC and NAc. This animal model is also known for being responsive to antidepressant treatment with reversal of the behavioral correlates of the clinical core symptoms of depression, namely, anhedonia and helplessness. It is a very valuable tool to assess the cellular and systemic mechanisms underlying depression and antidepressant drugs mechanisms of action.

The first study in rodents exposed to chronic stressors as a model for depressive-like behavior was developed by Katz (1982) and the main behavioral endpoint was the evaluation of sucrose preference, believed to be akin to the impairments in reward processing, which are the foundation for one of the core symptoms of major depression, anhedonia. The concept of exposure to stressors evolved with the elimination of noxious stimuli, and in the late 80's Paul Willner's group developed the chronic mild stress (CMS) paradigm (Willner *et al*,1987). The construct for this model is that depressive disorders, in humans, typically, develop after long lasting exposure to uncontrollable and unpredictable mild daily stressors (Kessler *et al*,1997). The validity of the model was confirmed by a reversion of the deficit in hedonic behavior as a response to chronic treatment with conventional antidepressants (Willner *et al*,1987). The adoption of the CMS model in rodents as a gold standard to study major depression produced extensive knowledge about the behavioral endpoints. The exposure of rodents to CMS

produced for example: increased immobility in the forced swim test, reduced self-care, changes in sexual behavior, increase in REM sleep latency (Willner *et al*, 1995). Nowadays, with more than two decades of use, the CMS model is known to be of paramount relevance to the study of the etiology of major depression. The exposure of rodents to CMS protocol produces behavioral, neuro-biological and neuronal structure changes that can be reversed with antidepressant treatment (Hill *et al,* 2012, Bessa *et al*, 2009; Bessa *et al*, 2013). Additionally, the CMS model is extensively used in the evaluation of antidepressant potential of drugs in rodents, as well as, allowing to investigate potential means of shortening the onset latency of antidepressant action using co-administration of drugs (Papp *et al*, 1996).

For these characteristics, we used the CMS animal model of depressive-like behavior to test the effect of ketamine when given in co-administration with other antidepressants.

The effect of Ketamine in the reversion of depressive like behavior

Several molecular players take part in the etiology of depressive disorders. The most commonly defended theory, the so called “monoaminergic hypothesis”, postulates that depression is the result of an imbalance in brain monoamines, and is the basis of the development of current antidepressant strategies (Delgado *et al*, 2000). The first developed antidepressants had a less selective action, such as tricyclic antidepressants, but they evolved to more selective mechanisms of action, such as SSRIs (Alamo *et al*, 2009). Despite a wide range of antidepressant drugs, the major issues concerning current treatments are the lack of efficacy and the delayed onset of clinical efficacy (Rush *et* al, 2006). In recent years, it has become clear that neurotransmitters other than monoamines are involved in major depression, namely the glutamatergic system (Sanacora *et al*, 2012). Glutamate is the most common excitatory neurotransmitter in human brain, in particular in cortical areas, and plays a major role in processes such as cell plasticity and neuronal development. Ketamine is a NMDA competitive antagonist with known antidepressant effects reported both in animal models and in clinical studies (Garcia *et al*, 2008; Berman *et al*, 2000; Zarate *et al*, 2006,

Phelps *et al*, 2008). The most striking feature concerning ketamine antidepressant activity is the fast acting and long lasting behavioral effect. We hypothesized that these characteristics are important to achieve a therapeutic strategy that shortens the time lag between the beginning of treatment and the clinical response that can endanger the vulnerable patient inside this time frame.

In order to test our hypothesis, we used antidepressants belonging to two different families with established efficacy in CMS: imipramine, a tricyclic antidepressant, and fluoxetine, a selective 5-HT reuptake inhibitor. The efficacy of both antidepressants in this model has already been documented (Bessa *et al*, 2009). The efficacy of ketamine in sub-anesthetic doses as a fast-acting antidepressant in rodents has also been previously reported (Li et al, 2011). Additionally, ketamine also as anxiolytic, sedative and psychomimetic effects. In fact, one of the applications of ketamine in rodents is the development of an animal model of schizophrenia. In order to focus our behavioral evaluation on the antidepressant endpoints, we ruled out that the used dosage (10 mg/Kg) could induce psychomimetic changes in rodents.

The behavioral endpoints of the CMS are to develop anhedonia and learned helplessness, both characteristic traits of major depression in humans. The use of the sucrose preference test allowed us to observe the temporal response to the antidepressant treatment in anhedonia. We were able to confirm that the addition of ketamine during the beginning of the antidepressant treatment was able to anticipate recovery from anhedonia and that this response was sustained in time. In the remaining behavioral evaluation, ketamine increased the efficiency in recovery when given with fluoxetine, but not with imipramine. Given that fluoxetine usually is associated with a slower onset of recovery, probably the effect of addition of ketamine is a stronger one. The addition of ketamine to both antidepressants also produced anxiolytic effects. (See Chapter 2)

Morphological correlates of the antidepressant effect of ketamine

In the last several years it has become increasingly recognized that maladaptive changes in the structure and function of excitatory/inhibitory circuits have a primary role in the pathophysiology of mood disorders. The use of neuroimaging technics revealed morphological changes in brains of depressed patients. Study of the brain structure in the CMS rodent model also revealed that stress induces changes in structure and morphology in cortical and limbic areas, such as the hippocampus and NAc and treatment with both monoaminergic and non-monoaminergic antidepressants was able to reverse the changes in dendritic arborization and spine density induced by CMS (Bessa *et al* 2009; Bessa *et al*, 2013). In the present work, we found similar changes in neuronal morphology, namely in dendritic arborization and in spine density, induced by chronic exposure to stressors. The effect of treatment was a generalized reversal of the effects induced by chronic stress, with special focus on the shift of spine morphology to a more mature phenotype (mushroom spines). The acute effect of ketamine in dendritic spines has already been shown and is present as soon as 2 hours after administration, with a peak of maturation to mushroom spines at 24h (Lie *et al*, 2010). In our work addition of ketamine to the antidepressants resulted in a higher degree of differentiation of spine morphology in some areas (Chapter 2). Despite not having performed serial time point analysis we postulate that changes were the summation of the effect of the antidepressant with the immediate acute effects of ketamine. Time point analysis is a tool that we can further develop in order to evaluate the individual effect of each drug and the temporal profile of dendritic spine development.

The molecular targets of ketamine are extremely important to establish the effects of this drug. Beside the fast antidepressant effect, ketamine has well known described acute effects in behavior. It is an established recreational drug with widespread use as abuse substance. However, ketamine is also a very useful drug in the practice of contemporary medicine. In our

work, we used the behavioral dimension impulsivity to measure the lasting effect of exposure to ketamine anesthesia.

Animal model of impulsivity

Impulsive response can be measured with a wide range of behavioral paradigms: stop-signal task (SST)(Logan *et al,* 1984), the go/no-go task (de Wit *et al*, 2002), the 5-CSRT (Robbins *et al*, 2002) the Differential Reinforcement of Lower Rates of Behavior (DRL) task (Seiden *et al*, 1979), and the simple reaction time task (SRTT) (Amalric and Koob, 1987). Despite the existence of all these behavioral paradigms, the test that is most commonly used and is considered the gold standard is the 5-CSRT. The common feature of all these 5 tests is the ability to evaluate premature responses (PR). The specific design of the different tasks allows further evaluation of certain parameters: the SST and go/no-go tasks can measure the ability to inhibit a motor response (Eagle and Baunez, 2010); the 5-CSRT, DRL and SRTT involve "waiting" before making a response to obtain a reinforcement.

The measurement of impulsive choice, which reflects the preference for immediately available small rewards over larger but more delayed ones, is commonly evaluated with temporal discounting procedures such as the delay discounting task (DDT)(Ainslie *et al*, 1975), effort discounting paradigms (Floresco *et al*, 2008), probabilistic discounting tasks (St Onge and Floresco, 2009) and gambling-like tasks, such as the Iowa Gambling Task (Bechara *et al*, 1994). Most of these tasks might be applied both to rodents and humans, obviously with the necessary technical adaptations.

To clarify if chronic ketamine anesthesia affected impulsive behavior, we used a new in-house developed paradigm of assessment of impulsivity (Leite-Almeida *et al,* 2013), the Variable Delay to Signal Task (VDS; View Chapter 3). This new paradigm provides rapid and simultaneous assessment of response and decision impulsivity in rodents. Being aware that DA plays an important role in the process of impulsive response modeling, and that DA agonist medication is related to the onset of various impulse-control behavior problems such

as pathological gambling or compulsive drug-seeking (Gallagher *et al*, 2007; Dagher and Robbins, 2009; Moore *et al,* 2014), it became particularly relevant to pharmacologically validate the VDS protocol which was achieved with the use of two drugs known to modulate DA: MK-801 and methamphetamine.

The VDS presented several advantageous characteristics over the other available impulsivity paradigms, such as: a significantly shorter training period when compared with the 5-CSRT and DD; only one test session required; and the suitability to retest the animals without losing the test effect.

The characteristics of the VDS test, and the fact that it has earlier been used to successfully demonstrate alterations in impulsive behavior in a rodent model of neuropathic pain (Leite-Almeida *et al*, 2012), made this test a good tool to access the long-lasting effect of ketamine after recovery of an anesthetic procedure. Procedures that take a longer period of time to access impulsivity could induce bias in the results when trying to evaluate the acute effect of the exposure. The short duration of the learning period (shaping) as well as the assessment period be accomplished in one day were the reasons that led us to choose the paradigm. Other feature was the feasibility to repeat the test, with the possibility of new experimental designs.

So far, most of the work concerning ketamine effect in impulsive behavior focus either on the acute effect of sub anesthetic acute dosages or on the potential of development of addiction response with chronic administration of the drug. One particular study by Agnieska focuses on the use of ketamine either in a chronic scheme or an acute scheme. In the chronic scheme ketamine was administered for 10 days, and the evaluation was performed 22 hours after and for 4 consecutive days (Nikiforuk and Popik, 2014). Despite some similarities, there are important differences: ketamine in this study has always been administered in sub-anesthetic dosages, and the timeline is similar to protocol used to induce schizophrenia-like behavior. For these reasons, our work is unique in the aim to assess the short-term/non-acute effects of repeated general anesthesia using ketamine, on motivational behavior, because the experimental protocol avoids development of both schizophrenia and addiction phenotypes, while focusing in the effect in impulsive behavior after ketamine anesthesia.

As previously discussed, in VDS, delay intolerance manifests itself as a robust increase of PR in the 3 s (f) segment when compared to the 3 s (i), i.e., after exposure to large 6 s and 12 s delays. This increment in the rate of impulsive responses as well as the rate of impulsive responses in the later periods of the 12 s segment has been shown to correlate with discounting steepness in the delay discounting paradigm (Evenden and Ryan, 1996). Additionally, impulsive responses in the training phase reflect action impulsivity; the construct at this stage is similar to that of the 5-choice serial reaction time task (5-CSRTT; Carli e*t al*, 1983) (See Chapter 3).

The exposure of animals to variable delays in order to perform the task was specifically designed to boost the impulsivity response during the last period of fixed delay of 3 seconds. That is the expected response in controls (See Chapter 3 and Chapter 4).

In our study there was a decrease in basal impulsive response, in accordance with expected response (Leite-Almeida *et al*, 2013). However, when analyzing the patterns of response along the delay period in intervals of 500 ms, it was evident that animals exposed to ketamine increased their PR/min towards the end of the delay. In other words, while control animals were able to correct their behavior, after some premature unrewarded responses, ketamine exposed animals failed to adjust and steadily increase their prematurity rate, suggesting failures in their inhibitory control. In addition, the exposed animals failed to show a robust increase in the PR/min between [3 s (i)] and [3 s (f)], which is normally observed after the exposure to large delays (Leite-Almeida *et al*, 2013).

The morphological changes associated to the antidepressant effect of ketamine shows that this drug has a potent neurotrophic effect, causing acute changes in neuronal morphology. In accordance, we investigated whether similar changes occurred in neuronal dendrites in key areas known to be involved in decision-making processes, such as the Str and the NAc. In animals exposed to ketamine we found an increase in the length of neuronal dendrites in NAc. We previously have witnessed an increase in neuronal dendritic length in NAc in animals exposed to CMS (Bessa *et al*, 2013). Our work (see Chapter 2) shows that when depressed rodents are treated with sub-anesthetic doses of ketamine, there is a recovery toward the neuronal morphology of control animals.

In addition to the neurotrophic effect, ketamine also induces an increase in extracellular DA acutely. Most studies show that within minutes of cessation of exposure to ketamine, extracellular DA starts to decrease back to normal levels. We did not evaluate the concentration of DA in the brain, but we performed western blot analysis to access dopamine 2 receptor (D2R) isoforms in the same areas. The result of our analysis was a decrease in relative ratio in both glycosylated and non-glycosylated isoforms of D2R in the Str. Subjects with chronic consumption of metamphetamine have an increased impulsive trait, which correlates with a decrease in the availability of D2R in the Str and NAc (Lee *et al*, 2009).

The molecular actions of ketamine are known to set effect in a short period of time, and to be dependent on rapid protein synthesis, namely of BDNF, PSD-95, GLuR1 and synapsin and activation of intracellular pathways such as mTOR, all of them physiologically relevant for synaptic plasticity mechanisms, neuronal growth, differentiation and synaptogenesis (Hoeffer *et al,* 2010; Kavalali *et al*, 2015).

This is the main mechanism that is thought to underlie the fast antidepressant effect of ketamine. When we evaluated the effect of ketamine as an adjuvant to antidepressants, we found morphological changes as well as molecular adaptations in the NAc. The NAc is a determining region in the formation of motivational behavior as well in anhedonia which suggests that, putatively, ketamine induced improvement in anhedonia involves these alterations.

Ketamine has different effects that are dose-dependent. For instance, the activity of ketamine as an antidepressant is only described for sub-anesthetic doses. However, we hypothesize that the molecular targets and the activation of the mechanisms that underlie the behavioral responses of ketamine may be similar.

Behavior is thought of as the sum of bottom-up influences of factors ranging from those existing at the level of a single cell, those involving cell to cell communication, and those derived from the circuits and networks that are present in the brain. However, the process of communication in the brain is not a strict product of information going through the anatomical pathways. The degree of activation of different brain regions and the synchrony between neural activity patterns is one of the determinants that influences brain processes and behavior. In our studies, we explored ketamine as a tool to modulate two behavioral constructs: mood and impulsivity. Ketamine, which is commonly used as a GA, has activity over specific receptors: it is mainly an NMDA antagonist, with known dopaminergic modulator activity and activator on fast acting molecular pathways responsible for its behavioral responses.

The main aim of our work was to establish the potential role of the GA ketamine as a therapeutic agent in other areas of intervention not related with anesthesia, namely, mood and impulsive behavior.

Concerning mood, we were able to show that the addition of ketamine to antidepressants treatment is effective in achieving a faster recovery on the antidepressant response; we also evaluated structural changes, and we found that addition of ketamine is especially important concerning dendritic spine maturation.

When our work focused in the effect of ketamine in impulsive response we found that exposure to ketamine anesthesia in consecutive GAs induces a change in impulsive response in the VDS paradigm, with a decrease in impulsive response rate after challenged with variable time delays to get a reward and a parallel change in dendritic structure.

The versatility in behavioral responses after exposure to ketamine in different settings and the known fast acting effect behavioral responses are striking features of this drug. Our work further contributes to clarify the potential use ketamine as a therapeutic agent as well as increase knowledge on mood and impulsive disorders. Ketamine is by itself an example that currently used drugs can be used to increase standard of care in domains different from those they were initially developed for.

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