UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

KETAMINE AND MEDETOMIDINE EFFECTS ON BRAIN:

BEHAVIORAL, HISTOPATHOLOGICAL AND
ELECTROPHYSIOLOGICAL STUDIES

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EM CIÊNCIAS VETERINÁRIAS

PATRÍCIA DO CÉU OLIVEIRA RIBEIRO

***Orientador*:** Professor Doutor Luís Miguel Joaquim Marques Antunes
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O conteúdo desta tese é da responsabilidade do autor.

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GENERAL ABSTRACT

Anesthesia is essential to perform surgical procedures, providing analgesia, hypnosis and muscular relaxation. Moreover, it is a requirement for several procedures in neurobiological research.

The N-metil D-Aspartate (NMDA) receptor antagonist, ketamine, and the α_2-adrenoceptor agonist, medetomidine, are examples of drugs frequently used in veterinary clinics and in research to induce anesthesia, analgesia and sedation. Usually, these drugs are used in combination, improving perioperative hemodynamic stability and reducing the anesthetic requirements. However, it was described that anesthesia with NMDA receptor antagonists may induce deficits of memory in neonates, being uncertain the effects of a single administration of ketamine in adult brain. Moreover, there is a lack of knowledge regarding the effect of a single administration of ketamine combined with the medetomidine on brain. Therefore, the main purpose of this thesis is to explore the impact of a single administration of different doses of ketamine alone or combined with medetomidine on brain of adult mice. To achieve this aim were performed four different studies: two using behavioral, histopathological and immunohistochemistry tests (chapter 3 and 4) and two using electrophysiological tests (chapter 5 and 6).

In the first study, described in chapter 3, were evaluated the effects of different doses of ketamine alone and it combined with medetomidine on memory and neurodegeneration. This study included anesthetic doses of ketamine combined with medetomidine (25 mg/kg of ketamine + 1mg/kg of medetomidine, 75 mg/kg of ketamine + 1mg/kg of medetomidine), subanesthetic doses of ketamine alone (25mg/kg and 75 mg/kg) and a sedative dose of medetomidine alone (1 mg/kg). Some mice were tested in a battery of behavioral tests (T-maze, vertical pole and open field test), and others were used for histopathological (hematoxylin and eosin staining) and immunohistochemical analyses (expression of procaspase-3, activated caspase-3 and brain-derived neurotrophic factor).

The second study, described in chapter 4, complemented the first study, since it included an anesthetic dose of ketamine alone (150 mg/kg) and behavioral tests more complexes (radial-maze test), which allowed to assess different types of memory. Moreover, the neurodegenerative evaluation included some regions of the brain that in the first study were not evaluated.

In the third study, described in chapter 5, were evaluated the effects of different concentrations of ketamine, medetomidine and ketamine/medetomidine combination on basal synaptic transmission and synaptic plasticity (paired-pulse facilitation and long-term potentiation), in hippocampal slices of adult mice. These drugs were applied directly in the bath of slices. For basal synaptic excitatory transmission and paired-pulse facilitation were tested concentrations from 1 µM to 600 µM of ketamine, from 1 µM to 200 µM of medetomidine and from 30 µM +1 µM to 600µM +24 µM of ketamine + medetomidine combination. For long-term potentiation were tested concentrations from 3 µM to 100 µM of ketamine, from 0.1 µM to 0.4 µM of medetomidine and the combination of 3 µM of ketamine with 0.1µM of medetomidine.

In the fourth study, described in chapter 6, different doses of ketamine (25 mg/kg and 75 mg/kg) were administered in adult mice to assess the impact of this drug on hippocampal long-term potentiation 24 hours after anesthesia. This study complemented the previous study (chapter 5) because the ketamine was administered directly in mice by an i.p. injection, similarly to first and second study.

The results of chapter 3 and 4 showed that a single administration of ketamine and medetomidine, alone or in combination, did not affect the memory neither neurodegeneration. These findings were supported by the results obtained in chapter 6, that showed that the ketamine did not affected the long-term potentiation, an essential mechanism for memory formation, 24 hours after anesthesia. Moreover, the combination of ketamine with medetomidine provided a good hemodynamic stability and prevented the hyperlocomotion that arose during the administration of ketamine alone.

The results of chapter 5 showed that ketamine and medetomidine alone or in combination mainly affected the long-term plasticity (long-term potentiation) rather than paired-pulse facilitation (short-term plasticity), at the exact moment of drugs applications. This fact suggested the importance of postsynaptic mechanisms, assessed by long-term potentiation, in detriment of presynaptic mechanisms, assessed by paired-pulse facilitation, in the action of these drugs on hippocampus.

In conclusion, this thesis showed that a single administration of ketamine and medetomidine, alone or in combination, did not affect memory in adult mice. Thus, the ketamine/medetomidine combination showed to be a safe anesthetic combination for clinical and research purposes when adult mice are used.

Palavras-Chave: ketamine, medetomidine, memory, mice, neurodegeneration, synaptic plasticity.

RESUMO GERAL

A anestesia é essencial para a realização de procedimentos cirúrgicos, promovendo analgesia, hipnose e relaxamento muscular; sendo um requisito na realização de vários procedimentos na investigação neurobiológica.

O antagonista dos receptores N-metil D-Aspartato (NMDA), cetamina, e o agonista dos receptores α_2-adrenergicos, medetomidina, são exemplos de fármacos frequentemente utilizados em clínicas veterinárias e em investigação científica para induzir anestesia, analgesia e sedação. Normalmente, estas drogas são utilizadas em combinação, melhorando a estabilidade hemodinâmica e reduzindo os requisitos anestésicos. No entanto, está descrito que a anestesia com antagonistas dos receptores NMDA pode induzir défices de memória em recém-nascidos, sendo ainda incerto o efeito de uma única administração de cetamina na memória dos adultos. Além disso, existe uma lacuna na literatura relativamente ao efeito da administração de cetamina em combinação com medetomidina no cérebro. Assim, o objetivo principal deste trabalho foi explorar o impacto de uma única administração de diferentes doses de cetamina isolada ou em associação com medetomidina no cérebro de murganhos adultos. Para atingir esse objectivo foram realizados quatro estudos diferentes: dois usando testes comportamentais, histopatológicos e imuno-histoquímicos (capítulo 3 e 4) e dois usando testes electrofisiológicos (capítulo 5 e 6).

No primeiro estudo, descrito no capítulo 3, foram avaliados os efeitos de diferentes doses de cetamina sozinha e combinada com medetomidina na memória e na neurodegeneração. Este estudo incluiu doses anestésicas de cetamina combinada com medetomidina (25 mg/kg de cetamina + 1 mg/kg de medetomidina e 75 mg/kg de cetamina + 1 mg/kg de medetomidina), doses subanestésicas de cetamina isolada (25 mg/kg e 75 mg/kg) e uma dose sedativa de medetomidina isolada (1 mg/kg). Alguns murganhos foram testados numa bateria de testes comportamentais (testes “T-maze”, “vertical pole” e “open field”) e outros utilizados para histopatologia (hematoxilina e eosina) e imuno-histoquímica (expressão de procaspase-3, caspase-3 activada e de factor neurotrófico derivado do cérebro).

O segundo estudo, descrito no capítulo 4, complementou o primeiro, visto incluir uma dose anestésica de cetamina isolada (150 mg/kg) e testes comportamentais mais complexos (teste “radial-maze”) que permitiram avaliar diferentes tipos de memória. A avaliação

neurodegenerativa incluiu ainda regiões do cérebro que no primeiro estudo não foram avaliadas.

No terceiro estudo, abordado no capítulo 5, foram estudados os efeitos de diferentes concentrações de cetamina, medetomidina e da combinação cetamina/medetomidina na transmissão sináptica basal e na plasticidade sináptica (facilitação por pulso pareado e potenciação a longo prazo). Os fármacos foram aplicados na solução que contém as fatias de hipocampo. Para a transmissão sináptica basal e para a facilitação de pulso pareado foram testadas concentrações entre 1 e 600 µM de cetamina, entre 1 e 200 µM de medetomidina e desde 30 µM + 1 µM até 600 µM + 24 µM da combinação de cetamina+medetomidina. Para a potenciação de longo prazo foram testadas concentrações entre 3 e 100 µM de cetamina, entre 0,1 e 0,4 µM de medetomidina e a combinação de 3 µM de cetamina com 0,1 µM de medetomidina.

No quarto estudo, descrito no capítulo 6, duas doses de cetamina (25 mg/kg e 75 mg/kg) foram administradas em murganhos adultos para avaliar o impacto deste fármaco na potenciação a longo prazo no hipocampo, 24 horas após a anestesia. Este estudo complementou os estudos anteriores visto a cetamina ter sido administrada diretamente nos murganhos por injeção intraperitoneal.

Os resultados dos capítulos 3 e 4 mostraram que uma única administração de cetamina e medetomidina, isoladamente ou em combinação, não afecta a memória nem a neurodegeneração. Estes resultados foram apoiados pelos resultados obtidos no capítulo 6, que mostraram que a cetamina não afeta a potenciação a longo prazo, um mecanismo essencial para a formação da memória, 24 horas após a anestesia. Além disso, a combinação de cetamina com medetomidina forneceu boa estabilidade hemodinâmica durante a anestesia e preveniu a hiperlocomoção observada aquando da administração isolada de cetamina.

Os resultados do capítulo 5 mostraram que a cetamina e a medetomidina, isoladamente ou em combinação, afectam principalmente a plasticidade de longo prazo em detrimento da plasticidade de curto prazo, aquando da aplicação dos fármacos directamente no hipocampo. Este fato sugere a importância dos mecanismos pós-sinápticos, avaliados por potenciação de longo prazo, em detrimento de mecanismos pré-sinápticos, avaliados por facilitação por pulso pareado, na ação desses fármacos no hipocampo.

Em conclusão, este trabalho mostrou que uma única administração de cetamina e medetomidina, isoladamente ou em combinação, não afecta a memória de murganhos adultos. Assim sendo, a combinação de cetamina/medetomidina mostrou-se uma combinação

anestésica segura para fins clínicos e de pesquisa científica quando são utilizados murganhos adultos.

Palavras-Chave: cetamina, medetomidina, memória, murganhos, neurodegeneração, plasticidade sináptica.

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LIST OF ABREVIATIONS

AC	adenylate cyclase
aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CA	c*ornu ammonis*
Ca^{2+}	calcium
CaMKII	calcium/calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosphate
cm	centimeter
CNS	central nervous system
CREB	cAMP response element binding protein
DG	dentate gyrus
EC_{50}	concentration of a ligand that causes 50% of the maximal response
EC	entorhinal cortex
EMEA	European Medicinal Evaluation Agency
FDA	food and drug administration
fepsp	field excitatory postsynaptic transmission
GABA	γ-amino butyric acid
H&E	hematoxylin–eosin
HFS	high frequency stimulation
Ih	hyperpolarization-activated currents
i.p.	intraperitoneal
K^+	potassium
LC	locus coeruleus
LTP	long-term potentiation
mf	mossy fibers
min.	minute

Mg^{2+}	magnesium
n	number of animals per group
NMDA	N-methyl-D-aspartate
NA	noradrenaline or norepinephrine
Na^{+}	sodium
Nacl	sodium chloride
NAPK	mitogen-activated protein kinase
PBS	phosphate buffered saline
PKA	protein kinase A
PKC	protein kinase C
PPF	paired-pulse facilitation
pp	perforant pathway
PWR	pedal withdrawal reflex
Rc	recurrent collateral
RR	righting reflex
Sc	schaffer collateral/commissural axons
SD	standard deviation
S	second
SUB	subicular complex
SVR	systemic vascular resistance
TBST	mixture of Tris-Buffered Saline and Tween 20

CHAPTER 1 - GENERAL INTRODUCTION

1.1ANESTHESIA

On March 30, 1842, Crawford Long administered the first surgical anesthetic, ether, in Jefferson, Georgia. However, unfortunately, Long did not publish his results until 1849 (Long, 1849) and consequently history remembers October 16, 1846 as the date of the birth of anesthesia and Thomas Morton as the "discoverer of anesthesia". At the Massachusetts general hospital, Morton gave the first successful public demonstration of general anesthesia. He anesthetized Edward Abbot, so that John Warren could remove a vascular tumor from his neck. John Warren then uttered the famous words, "Gentlemen, this is no humbug" (Jeffrey A. Norton, 2008; Robinson and Toledo, 2012). From that moment on, the use of anesthesia spread around the world and human surgery was revolutionized.

Some years later, in 1850s, G.H. Dadd claimed veterinary anesthesia as an important refinement and a scientific principle (Holland, 1973) and finally animals could also benefit from anesthesia routinely. Vertebrate, including humans, and some invertebrate animals share similar pathways and perception of pain (Smith and Lewin, 2009); so also non-human animals should benefit from analgesics and anesthetics when submitted to painful situations.

Several definitions were described for the concept of "anesthesia" but no consensus exists on its definition till date. Anesthesia has been defined as a continuum or progressive depression of the central nervous system (CNS) (TaskForce, 2006) and its effects are commonly described as the combination of three main components: hypnosis, analgesia and muscle relaxation (**figure 1.1**).

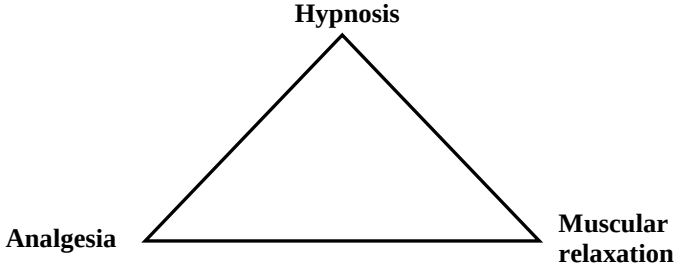


Figure 1.1 Schematic representation of the three main components of general anesthesia.

To achieve all these effects with a reasonable safety margin, a combination of different drugs are generally used. Moreover, when an anesthetic protocol is performed using a combination of drugs, lowering the dose of each drug is necessary to produce the desired effects and to reduce the risk of side-effects such as hemodynamic instability (Arras *et al.*, 2001). This is the concept of balanced anesthesia, the use of a combination of anesthetics that act synergistically in the desired effects and with reduced side-effects (Tonner, 2005).

Each anesthetic drug may induce one or more components of general anesthesia, depending on the molecular targets to which it was specifically developed and presented in a region of the nervous system (Hemmings *et al.*, 2005).

In this work, two different anesthetic drugs were used: ketamine and medetomidine. When these two drugs are administered in combination, the three components of general anesthesia can be achieved, depending of the concentration used.

1.1.1 KETAMINE

Ketamine was first synthesized in 1962 by the Belgian chemist Calvin Stevens at Parke Davis Labs. It was approved by the Food and Drug Administration (FDA) in February of 1970 (Mozayani, 2002). Later the ketamine was also approved by the European Medicinal Evaluation Agency (EMEA). Ketamine has been described as an anesthetic agent that produces a dissociative anesthesia, i.e. produces catalepsy, and analgesia, but not necessarily a common loss of consciousness; the subject seems to be disconnected of the environment (Pender, 1972; Kohrs and Durieux, 1998). These symptoms are the reason why ketamine is also used as a drug of abuse (Morgan *et al.*, 2004).

Nevertheless, recent research continues to clarify different aspects of ketamine pharmacology, and new clinical uses have been suggested for this drug such as its use as an anti-depressant (Berman *et al.*, 2000).

Physicochemical characteristics

Ketamine, 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone, is available as a racemic mixture in a commercial preparation (Imalgene ®, Ketanest®, Ketaject®, Ketalar®) (Mozayani, 2002). There are two optical isomers of the ketamine: S(+) ketamine and R(-) ketamine (**figure 1.2**). Clinically, the anesthetic potency of the S(+)-isomer is approximately three to four times more than of the R(-)-isomer, which is attributable to the higher affinity of

the S(+)-isomer to the phencyclidine binding sites on the N-methyl-D-aspartate (NMDA) receptors (Mozayani, 2002). Ketamine is water- and lipid-soluble, allowing its administration in various routes and providing extensive distribution in the body (Sinner and Graf, 2008). It may be administered by the intravenous, intramuscular, oral, and rectal routes without signs of irritation (Haas and Harper, 1992). In animals it may also be administered by the intraperitoneal (i.p.) route (Cruz *et al.*, 1998).

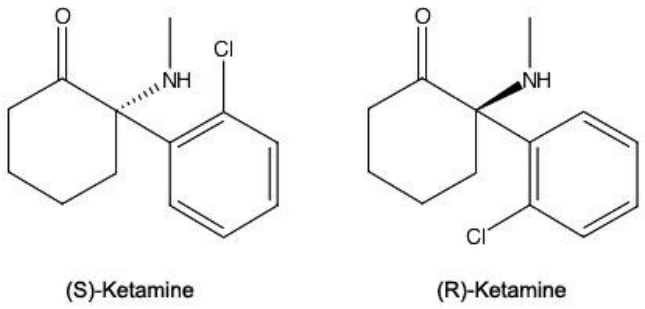


Figure 1.2 Molecular structure of two isomers of ketamine (ChemDoodle® v5.0, iChemLabs, LLC)

Metabolization and Elimination

Biotransformation of ketamine is mediated by hepatic microsomal enzymes resulting in multiple metabolites. The most important pathway involves N-demethylation of ketamine, by cytochrome P450, to norketamine, an active metabolite with one third of the anesthetic potency of ketamine (Mozayani, 2002). Norketamine is then hydroxylated and conjugated to water soluble compounds that are excreted in the urine (90%) (Mozayani, 2002). Five percent is eliminated in the feces and the remaining 5% are excreted unchanged in the urine (Mozayani, 2002).

Dehydronorketamine is an important breakdown product of ketamine, but it is not an important metabolite *in vivo* (Reich and Silvay, 1989) (**figure 1.3**).

Figure 1.3 Ketamine's biotransformation. (ChemDoodle® v5.0, iChemLabs, LLC)

Mechanisms of action

Pharmacologically, ketamine is classified as a noncompetitive NMDA antagonist, but the full range of effects cannot be explained by this inhibition alone. The actions of ketamine are mediated by NMDA, opioid, monoaminergic and muscarinic cholinergic receptors (Hirota and Lambert, 1996). Moreover, ketamine also interacts with voltage-sensitive Ca^{2+} channels and, at higher doses, it can block the sodium channels (Hirota and Lambert, 1996). All these interactions contribute to the anesthetic action of ketamine.

More specifically, ketamine produces dissociative anesthesia by blockage of the NMDA receptor channel pore and by inhibiting the phencyclidine binding site. The effects of ketamine on the opiate receptor may contribute to the analgesic state (Hirota and Lambert, 1996) and the interaction between ketamine and muscarinic receptors, in a dose-dependent manner, may contribute to the neuromuscular rigidity (Haas and Harper, 1992).

Clinical uses

Ketamine is frequently used as an anesthetic/analgesic agent in clinical practice and in research with laboratory animals. In clinical practice, it is used in painful diagnostic procedures, obstetrics, in asthmatic patients, when traumatic and hypovolemic shock occur, and in burn situations (Kuznetsova *et al.*, 1984; Adams and Hempelmann, 1990; Ikechebelu *et al.*, 2003). Moreover, it is very important for emergency procedures and when the equipment for drug delivery and patients´ motorization are limited, since ketamine maintain the hemodynamic stability (Copeland and Dillon, 2005). Ketamine is also recommended for post-operative analgesia (Angst and Clark, 2010; Carstensen and Moller, 2010; Trupkovic *et al.*, 2011) and in the treatment of chronic pain (Sinner and Graf, 2008).

In laboratory animals, ketamine is frequently used as part of the anesthetic protocols. It is combined with other drugs in a high variety of surgeries and short procedures (Cruz *et al.*, 1998) such as collection of blood, biopsies, implementation of monitoring devices, aseptic urine collection, among other situations.

Effects on body system

Ketamine stimulates the sympathetic nervous system (Sinner and Graf, 2008). It has potent vasodilatation properties, which increases intracranial pressure and compromises intracranial complacence (Haas and Harper, 1992).

Regarding cardiovascular system, ketamine appears as an stimulant (Sinner and Graf, 2008). Several investigators have reported increases in heart rate, pulmonary arterial pressure, pulmonary vascular resistance, systemic vascular resistance and in systemic arterial pressure (Greeley *et al.*, 1986; Haas and Harper, 1992).

In respiratory system, ketamine is a mild respiratory depressant with unique properties. It has the ability to maintain functional residual capacity upon induction of anesthesia, thus decreasing the chances of intraoperative hypoxemia (Haas and Harper, 1992). Moreover, it increases lung compliance and decrease airway resistance. Ketamine causes bronchodilation (Sinner and Graf, 2008) and increases salivary and tracheobronchial mucous gland secretions (Haas and Harper, 1992).

Concerning the urinary system, no studies were found about the specific action of anesthetic doses of ketamine in this system. However, in humans, it has been reported that when ketamine is used as recreational drug, it could induce ulcerative cystitis or vesicopathy (Middela and Pearce, 2011; Morgan *et al.*, 2012). Bilateral hydronephrosis and renal papillary necrosis have also been reported in some cases (Morgan *et al.*, 2012).

Regarding gastrointestinal system, ketamine can cause nausea and vomiting. This probably happens because ketamine inhibit neuronal uptake and increase serotonergic activity (Aroni *et al.*, 2009).

1.1.2 MEDETOMIDINE

Medetomidine was developed in the 1970s in Finland and it was approved by the FDA, for veterinary use, in March of 1996. Later, the medetomidine was also approved by the EMEA. It produces sedative and analgesic effects (Sinclair, 2003).

Physicochemical characteristics

Medetomidine, 4-1-[(2,3-dimethylphenyl)ethyl]-1H-imidazole, is found as a commercial preparation (Domitor®, Dormilan®) of a racemic mixture of two steroisomers, dextro-medetomidine and levo-medetomidine (**figure 1.4**) (Sinclair, 2003). The dextro-isomer, dexmedetomidine, is the active isomer of medetomidine formulation and when administered at half the dose induces similar effects to medetomidine. The levo-isomer, levomedetomidine only have pharmacological activity when it is used at very high doses (Savola and Virtanen, 1991).

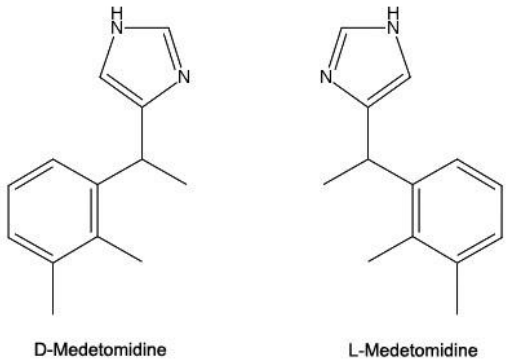


Figure 1.4 Molecular structures of two isomers of medetomidine. (ChemDoodle® v5.0, iChemLabs, LLC)

Metabolization and elimination

Biotransformation of medetomidine occurs mainly in the liver. A metabolic pathway consists in hydroxylation with subsequent glucuronidation, or further oxidation to carboxylic acid (Salonen, 1989).

Most of the metabolites were excreted in urine. Fecal excretion was only significant in the rat (Salonen, 1989).

The major urinary metabolites were the glucuronide of hydroxymedetomidine (about 35% of urinary metabolites) and medetomidine carboxylic acid (about 40%). Unchanged medetomidine represents approximately 1% or 10% of the urinary excretion products, dependent on the dose (Salonen and Eloranta, 1990).

Mechanism of action

Medetomidine is a very potent, efficacious and selective agonist of the α_2-adrenoceptor in the central and peripheral nervous system, that also has affinity for imidazoline receptors (Wikberg *et al.*, 1991). It has an $alpha_2/alpha_1$ selectivity ratio of 1620/1 (Savola, 1989; Virtanen *et al.*, 1989).

Thus, the main medetomidine's mechanism of action is its agonistic activity at the presynaptic alpha-2 adrenergic receptors that results in a decrease in the release of norepinephrine (NA) from adrenergic nerve terminals in the central and peripheral nervous system. (Aggarwal *et al.*, 2001; Sinclair, 2003).

Clinical uses

Medetomidine is frequently used in veterinary medicine to induce analgesia and sedation (Cullen, 1996). It also produces muscle relaxation and decreases the anesthetic requirements of others injectable or inhalant agents to induce anesthesia (Angelini *et al*., 2000; Sinclair, 2003).

In laboratory animals, medetomidine may be used to provide chemical restraint and facilitate clinical examinations (Sinclair, 2003). Moreover, it is also used to induce anesthesia for surgical procedures when combined with other drugs (Hu *et al.*, 1992).

Effects on body systems

Medetomidine decreases sympathetic nervous activity and decreases cerebral blood flow and intracranial pressure (Itamoto *et al.*, 2010). Moreover, medetomidine produces bradycardia, hypotension (Cullen, 1996), increases the systemic vascular resistance and reduces cardiac output (Sinclair, 2003). This drop of cardiac output is not due to a direct negative action of medetomidine on myocardial contractility, but it is a consequence of the heart rate's reduction and increase of systemic vascular resistance (Sinclair, 2003).

Depression of the respiratory rate and an increase of the arterial carbon dioxide tension were observed with medetomidine. Many animals showed marked cyanosis of the skin and mucous membrane after sedation with medetomidine because it causes peripheral vasoconstriction, reducing blood flow to these tissues, consequently decreasing venous oxygen saturation (Sinclair, 2003).

Medetomidine inhibits gastric secretions and reduces gut motility. Additionally, it induces polyuria by decreasing the release of antidiuretic hormone and by the action of medetomidine in renin-angiotensin system (Sinclair, 2003).

1.1.3 KETAMINE/MEDETOMIDINE COMBINATION

Ketamine/medetomidine combination is frequently used in many species, including laboratory animals (Nevalainen *et al.*, 1989; Cruz *et al.*, 1998).

The administration of these two drugs combined allows substantial reduction of the dose required for anesthesia of each drug alone, reducing side-effects (Cullen, 1996; Sun *et al.*, 2003). Moreover, this combination provides a good anesthetic plane, with hemodynamic stability and muscle relaxation (Cruz *et al.*, 1998). The positive chronotropic and inotropic

actions of ketamine help to oppose the depressant actions of medetomidine on the circulatory system (Raiha *et al.*, 1989; Moens and Fargetton, 1990; Verstegen *et al.*, 1990; Cullen, 1996). However, it was also described that this combination may induce hypotension with significant respiratory depression in animals, mainly in rabbits. Therefore, it is advisable to provide supplemental oxygen (Kili, 2004).

In small laboratory animals (mice and rats), this combination has the additional advantage of being administered by an i.p. injection. This technique is a simple procedure, causing minimal distress to the animal (Jang *et al.*, 2009). Moreover, the equipment cost is low and limited number of staff is necessary (Jang *et al.*, 2009). In addition, this combination is particularly useful in veterinary clinics and in laboratory animals, because it has a specific antagonist atipamezole. This can reverse the deep sedative effect induced by medetomidine, shortening the recovery time (Cruz *et al.*, 1998).

1.1.4 ATIPAMEZOLE

Atipamezole (Antisedan®) is a highly potent, selective, and specific antagonist of central and peripheral alpha$_2$-adrenoceptors. It antagonizes all medetomidine´s actions, and so reverses the undesirable side-effects as well as sedation and analgesia (Hu *et al.*, 1992).

Atipamezole, 4-(2-ethly-2,3-dihydro-1Hinden-2yl)-1H-imidazole, has few effects when administered alone, although some people have noted a hyper-alert state in animals (rats, cats, and guinea pigs) following its use (Flecknell, 1997).

1.2 MEMORY

In current language, memory affords the capacity for conscious recollections about facts and events. However, the memory concept is not easy to define and it is important to recognize that there is more than one kind of memory (Zola-Morgan and Squire, 1993). According to Izquierdo et al., memories can be classified according to their content, duration and nature (Izquierdo *et al.*, 1999). Relative to contents, memory can be divided in explicit/declarative and implicit/nondeclarative memory. Declarative memory is the conscious recall of information, involving evaluation, comparison and inference, while nondeclarative memory is the unconscious recall of information for several tasks such as motor skills

(Izquierdo *et al.*, 1999). Declarative and nondeclarative memory can be subdivided in more specific types of memories (**figure 1.5**). The behavioral tests used in this work aimed mainly to evaluate declarative memory.

Declarative memory is frequently replaced by the expression spatial memory, because it is the part of memory responsible for recording information about one's environment and its spatial orientation. Declarative memory not only includes memory for spatial locations, but also memory for word lists, faces, odors, and tactual impressions, among others (Squire and Cave, 1991).

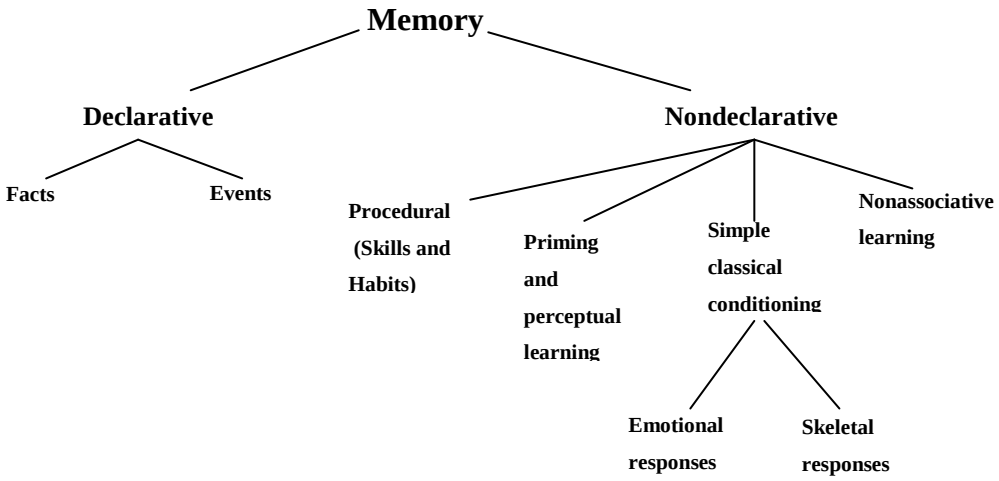


Figure 1.5 Taxonomy of mammalian memory systems. Adapted from (Floel and Cohen, 2007)

Concerning its duration, the memory can be classified as short-term memory or long-term memory. In short-term memory, the information develops in a few seconds or minutes and lasts for several hours, while in the long-term memory, the information could be permanent (Izquierdo *et al.*, 1999).

According to its nature, memory can be divided in archival memories and transient memories. The archival memories (also called reference memory) are preserved offline for later use (long and short term memories) and the transient memories are used moment-to-moment, i.e. involves the retention of information for short periods of time, seconds to a few minutes (working memory) (Hodges 1996; Izquierdo *et al.*, 1999). These two types of memory, reference and working memory, were studied in the present work. The performance of the behavioral task radial maze involves working and reference memory. Since the baited arms are always the same for the entire testing, remembering which arms are baited needs the

use of reference memory, while remembering which arms have already been visited during the trial involves working memory.

1.2.1 NEUROANATOMY AND MEMORY

First of all, it is important to describe the brain anatomy for a later understanding of which regions are involved in memory and learning.

One possible division of the brain is given by Sternberg (Sternberg., 2006). This author divided the brain in three major regions: forebrain, midbrain and hindbrain. These terms are related to the front-to-back arrangement of structures in the developing embryo, however during the course of development the positions change so that the forebrain is almost a cap on top of the midbrain and hindbrain. Nonetheless, the terms are still used to designate areas of the fully developed brain (Sternberg, 2006).

The forebrain comprises the cerebral cortex, the basal ganglia, the thalamus, the hypothalamus and the limbic system (Sternberg, 2006). The limbic system comprises three central interconnected cerebral structures: the amygdala, the septum, and the hippocampal formation(Sternberg, 2006). The midbrain includes the superior colliculi, the inferior colliculi, the grey matter, red nucleus, substantia nigra, ventral region and the reticular activating system(Sternberg 2006). And finally, the hindbrain comprises the medulla oblongata, pons and cerebellum (Sternberg, 2006).

Over the years, several experiments have been made to establish the relationship between the different structures of brain and their specific functions. Tools such as magnetic resonance imaging, positron emission tomography, electrophysiological recordings and behavioral tests contributed to uncover the function of different regions of the brain (Zola-Morgan and Squire, 1993; Sternberg, 2006). However, we have to keep in mind that the established relationships are nonlinear, because the brain acts as a whole, with several connections between different regions, and so the damage of one region can affect the others. **Table 1.1** describes the main functions of the major brain regions.

Table 1.1 Description of the major brain regions' functions. Adapted from the book "Cognitive Psychology" (Robert J. Sternberg *et al.*, 2009)

Region of the brain	Major structures within the regions	Functions of the structures
Forebrain	Cerebral cortex	Involving in receiving and processing sensory information, cognitive processing, planning and sending motor information.
	Basal ganglia	Crucial to the function of the motor system
	Limbic system (hippocampus, amygdala and septum)	Involved in learning, emotions, and motivation (in particular, the hippocampus influences learning and memory, the amygdala influences anger and aggression, and the septum influences anger and fear)
	Thalamus	Primary relay station for sensory information coming into the brain; transmits information to the correct regions of the cerebral cortex thought projection fibers that extend from the thalamus to specific regions of the cortex; comprises several nuclei that receive specific kinds of sensory information and project that information to specific regions of cerebral cortex
	Hypothalamus	Controls the endocrine system; controls the autonomic nervous system, such as internal temperature regulation, appetite and thirst regulation, and other key functions; involved in regulation of behavior related to species survival; plays a role in controlling consciousness, involved in emotions, pleasure, pain, and stress reactions.
Mindbrain	Superior colliculi	Involved in vision (especially, visual reflexes)
	Inferior colliculi	Involved in bearing
	Reticular activating system	Important in controlling consciousness (sleep and arousal), attention, cardiorespiratory function, and movement.
	Grey matter, red nucleus, substantia nigra, ventral region	Important in controlling movement
Hindbrain	Cerebellum	Essential to balance, coordination and muscle tone.
	Pons	Involved in consciousness (sleep and arousal); bridges neural transmission from one part of the brain to another;
	Medulla oblongata	Serves as juncture at which nerves cross from one side of the body to the opposite side of the brain; involved in cardiorespiratory function, digestion and swallowing.

Regarding the memory and learning, hippocampus is one of the most important regions, essentially for declarative memory (Zola-Morgan and Squire, 1993), as showed in **table 1.1**. Moreover, interestingly, it was reported that hippocampus has place cells, i.e. cells that become active only when the animal is located in a specific part of the environment

(O'Keefe and Dostrovsky, 1971). Thus, the cognitive mapping theory postulates that each cell is consigned to a specific location and that the firing pattern of the cells can form a very precise cognitive map about the position of the animal at any given moment (O'Keefe and Dostrovsky, 1971; Jeffery and O'Keefe, 1999).

The hippocampal formation can be divided in four regions: (1) hippocampusproper, which is subdivided in three fields (c*ornu ammonis* (CA) 1, CA2 and CA3), (2) dentate gyrus (DG), (3) subicular complex (SUB), which included 3 fields (subiculum, presubiculum, and parasubiculum) and (4) entorhinal cortex (EC) (**figure 1.6**) (Per Andersen *et al.*, 2007). Although the volume of the hippocampus is about 10 times larger in monkeys than in rats and 100 times larger in humans than in rats, the basic hippocampal architecture is common to all three species (Per Andersen *et al.*, 2007).

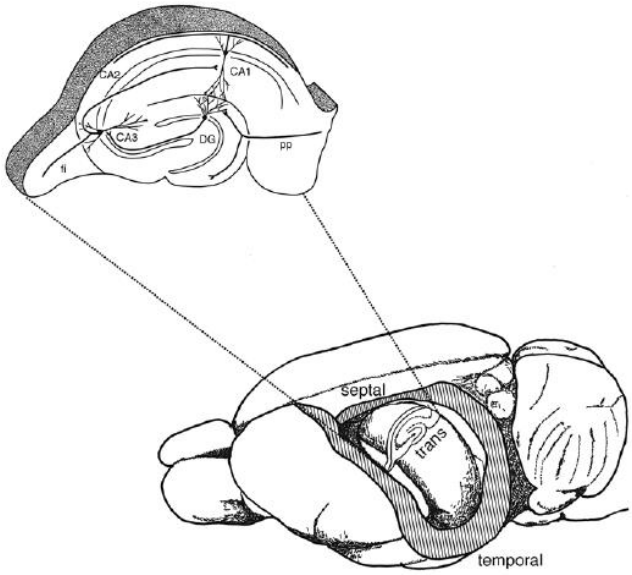


Figure 1.6 Line drawing of the rat brain shows the septotemporal and transverse axes of the hippocampal formation. CA1: *cornu Ammonis* 1, CA2: *cornu Ammonis 2*, CA3: *cornu Ammonis* 3, DG: dentate gyrus, pp: perforant pathway, fi: fimbria. Adapted from the book "Hippocampus book" (Per Andersen *et al.*, 2007).

The dentate gyrus is considered to have three layers: the molecular layer, the granule cell layer, and the hilar region characterized by polymorph neurons. The hippocampus (CA1, CA2 and CA3) consists on seven layers: *stratum moleculare, stratum lacunosum, stratum radiatum, stratum pyramidal, stratum oriens, alveus and epithelial* zone (Cajal, 1909).

1.2.2 MEMORY AND SYNAPTIC PLASTICITY

The brain has the amazing ability to receive inputs and to translate them into memories. It has long been suggested that memory formation in mammalian brain involves modifications in the synaptic connections between neurons (Hebb, 1949). It has been shown that repetitive activation of excitatory synapses in the hippocampus causes an increase in synaptic strength, helping the brain "to learn" (Malenka and Nicoll, 1999). This change in synaptic strength between two neurons is named synaptic plasticity. This term was introduced by Jerzy Konorski to describe the ability of the connection, or synapse, between two neurons that may change in strength in response to either use or disuse of transmission over synaptic pathways (Konorski, 1948).

Nowadays, the activity-dependent synaptic plasticity is considered a cellular mechanism for learning and memory (Bliss and Collingridge, 1993) and most of the excitatory synapses in the hippocampus exhibit various forms of activity dependent of synaptic plasticity. These are generally defined as changes in amplitudes of synaptic potentials that are dependent on the prior activity of the synapse. The different forms are generally distinguished on the basis of their duration or time course. Paired-pulse facilitation (PPF), a form of short-term plasticity, and long-term potentiation (LTP), a form of long-term plasticity, are two different and important forms of synaptic plasticity (Maruki *et al.*, 2001). The first occurs through presynaptic mechanisms and it can be used as a presynaptic index for the probability of neurotransmitter release (Kamiya and Zucker, 1994; Zucker and Regehr, 2002) . It consists in give a pair of pulses (stimuli) to a synaptic pathway and to compare the amplitude or slope of the second excitatory post-synaptic potential to the first one (Schulz *et al.*, 1994). In control conditions the response to a second stimulation is higher than the response provoked by the first pulse/stimulus. Residual presynaptic calcium, left after the first stimulation, is thought to enhance neurotransmitter release in response to the second stimulation.

Long-term potentiation is a long-lasting change in synaptic efficacy and it mainly occurs via postsynaptic mechanisms. It is probably the most studied of all the synaptic plasticity's processes because of its presumed role in learning and memory (Bliss and Collingridge, 1993). It typically starts when one or more trains of high-frequency stimulation are given to a synaptic pathway. It occurs in several regions of the mammalian brain

(Malenka and Nicoll, 1999), but the most studied pathways of LTP are located in the hippocampus, where the main circuit is glutamatergic.

The fundamental information processing pathway in the hippocampal formation is usually considered to be the trisynaptic circuit: the entorhinal cortex sends a perforant path projections to the granule cells of the DG, which sends mossy fibers to the pyramidal cells of the CA3 region, which send Schaffer collaterals to the CA1 pyramidal cells, which then project back to the entorhinal cortex (Tsien *et al.*, 1996; Per Andersen *et al.*, 2007). Moreover, a direct projection from entorhinal cortex to CA1 area (the temporoammonic pathway) and CA3 field of the hippocampus proper (via the perforant pathway), and to the subiculum (via the alvear pathway) are also part of hippocampal processing (Tsien *et al.*, 1996; Per Andersen *et al.*, 2007). Pyramidal cells in CA1 can also project to the subiculum. Both CA1 and the subiculum project back to the deep layers of the entorhinal cortex (**figure 1.7**) (Tsien *et al.*, 1996; Per Andersen *et al.*, 2007).

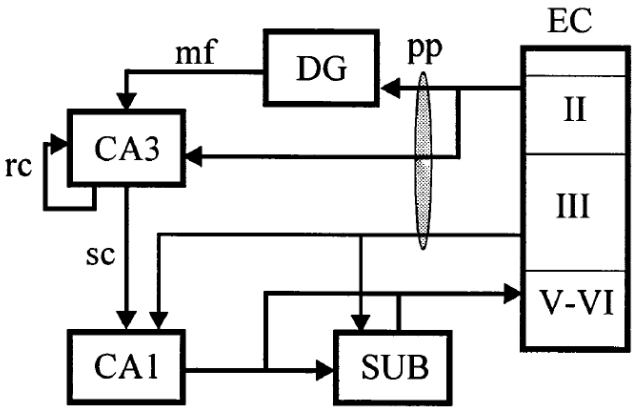


Figure 1.7 Connections in the Hippocampal Memory System. The excitatory pathways in the hippocampal formation. EC, entorhinal cortex; DG, dentate gyrus; mf, mossy fibers; pp, perforant path; rc, recurrent collateral axons of the CA3 pyramidal neurons; sc, Schaffer collateral/commissural axons; SUB, subiculum. Adapted from Tsien et al. (Tsien *et al.*, 1996)

1.2.3 NEUROTRANSMITTER SYSTEMS, SYNAPTIC PLASTICITY AND MEMORY FORMATION

Neurotransmitter is "A substance which is released at the end of a nerve fiber by the arrival of a nerve impulse and by diffusing across the synapse or junction effects the transfer of impulse to another nerve fiber (or muscle fiber or some receptor)" (Webster, 2001). Based on this definition, neurotransmitter systems influence the normal functions of CNS such as

synaptic plasticity (Webster, 2001) that is essential to memory formation (Bliss and Collingridge, 1993).

It was reported that glutamate, followed by γ-amino butyric acid (GABA), dopamine, acetylcholine, serotonin, and noradrenaline are the neurotransmitters with more influence in memory and learning (Myhrer, 2003); and consequently alterations in the normal functioning of the neurotransmitters systems that included neurotransmitters described above can affect the memory.

The main excitatory transmitter of the mammalian CNS is the glutamate (Fonnum, 1984). It activates the three principal types of ionotropic receptors that mediate excitatory synaptic transmission: α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA), kainate, and NMDA. These receptors are ligand gated ionic channels that, depending on the subtype, are permeable to the divalent cation Ca^{2+} (calcium) and to the monovalent cations Na^{+} (sodium) and K^{+} (potassium) (Danysz and Parsons, 2003). AMPA receptors participate in synaptic transmission conducting mainly Na^{+} and K^{+} ions, and being usually impermeable to Ca^{2+} (Danysz and Parsons, 2003). Kainate receptors are also involved in synaptic transmission and are mostly permeable to Na^{+} and K^{+}, with a low conductivity for Ca^{2+}. It induces bidirectional modulation of both excitatory and inhibitory transmission (Huettner, 2003). In contrast, NMDA receptors do not usually contribute to basal synaptic transmission but are very important to synaptic plasticity and, consequently, to memory and learning (Danysz and Parsons, 2003). NMDA receptors are of particular interest in this work, since ketamine, one of the anesthetics studied, binds to it. Ketamine is a non-competitive NMDAR antagonist (NMDA receptor antagonist) that inhibits NMDARs by binding to allosteric sites (Wang and Slikker, 2008). These receptors are involved in a variety of physiological and pathological processes such as memory and learning and acute neuropathologies related to stroke and traumatic injury (Wang and Slikker, 2008). NMDA receptors present high permeability to Ca^{2+} ions, voltage dependent blockage by Mg^{2+} (magnesium) ions and a slow gating kinetics (Danysz and Parsons, 2003). Moreover, for activation of NMDA receptors it is required simultaneous binding of glutamate and of the co-agonist glycine (Laube *et al.*, 1997).

The best understood model of memory formation based on synaptic plasticity comprises the arrival of a high frequency signal to a glutamatergic synapse, inducing long-term potentiation, and consequently provoking a massive glutamate release which triggers a sequence of events (Danysz and Parsons, 2003). Firstly, AMPA and kainate receptors are activated, but NMDA receptors remained blocked by Mg^{2+}. The continuous activation of

AMPA and kainate receptors leads to a significant influx of Na^{2+} ions into the post-synaptic cell, which in turn decreases the membrane potential removing the Mg^{2+} ion from the blocked NMDA receptor (Voglis and Tavernarakis, 2006). At this stage, Ca^{2+} ions can freely enter in the cell via the NMDA receptor channel and initiate mechanisms that lead to the permanent strengthening of synaptic transmission (**figure 1.8**), including the insertion of more AMPA receptors at the postsynaptic membrane and an increase in protein syntheses (Voglis and Tavernarakis, 2006).

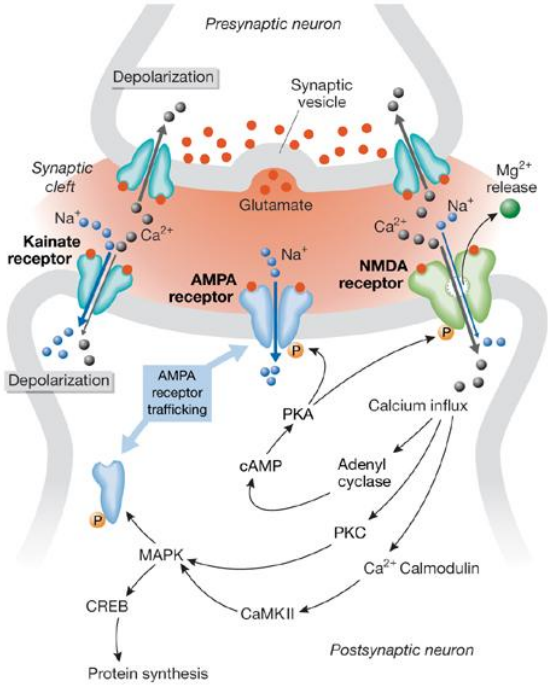


Figure 1.8 Glutamate receptors and synaptic plasticity. The arrival of a series of impulses at the presynaptic terminal triggers the release of glutamate, which binds to glutamate receptors at the postsynaptic membrane. On activation, AMPA and kainate receptors conduct sodium ions, which initiate postsynaptic depolarization. Membrane potential changes initiate the release of magnesium ions that block NMDA receptors. Calcium influx through NMDA channels sets off a chain of events that establish long-term potentiation. Kainate receptors at the presynaptic end also seem to facilitate synaptic transmission at specific synapses by augmenting neurotransmitter release. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CaMKII, calcium/calmodulin-dependent kinase II; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C. Figure and legend from Giannis Voglis and Nektarios Tavernarakis (Voglis and Tavernarakis, 2006).

However, it is important to stress that the model described above is not unique and the situation is actually far more complex with many forms of synaptic plasticity induced by diverse inputs in different neurons. Moreover, synaptic plasticity in the hippocampus does not always depend on NMDA receptors. The synapses between mossy fibers of hippocampal DG and CA3 neurons show a distinctive morphology with many sites for glutamate release, each of which has a corresponding postsynaptic bundle of glutamate receptors (Voglis and

Tavernarakis, 2006). At this case, LTP is NMDA-receptor-independent and requires calcium influx though presynaptic kainate receptors (Lauri *et al.*, 2003). However, the mechanism responsible for this type of synaptic plasticity remains unclear (Voglis and Tavernarakis, 2006).

Other neurotransmitter that is important to the synaptic plasticity in hippocampus is noradrenaline (NA). It acts on the neurons through modifications of currently ongoing events (modulation) and also initiates synaptic plasticity by itself (Marzo *et al.*, 2009). NA has high affinity to bind α_1-adrenoceptors, α_2-adrenoceptors and β-adrenoceptors and consequently modulates synaptic plasticity (Myhrer, 2003; Marzo *et al.*, 2009). The α_2-adrenoceptors are particularly important to this work because medetomidine, one of the anesthetics studied in this work, binds to α_2-adrenoceptors and so it can alter the normal functioning of the noradrenergic system and alter the synaptic plasticity.

Alpha$_2$-adrenoceptors are located on both pre- and postsynaptic sites (Marzo *et al.*, 2009). The presynaptic localization indicates their functions as autoceptors, involved in the control of noradrenaline release by locus coeruleus neuronal axons (Marzo *et al.*, 2009). The postsynaptic localization indicates their involvement in the intrinsic plasticity, i.e. the adaptive alterations of postsynaptic excitability, which are non-synaptic in nature and thus mechanistically internal to a given postsynaptic cell (Marzo *et al.*, 2009). This type of plasticity is crucial for neuronal function given that it directly modulates cellular characteristics such as ion channel opening (Marzo *et al.*, 2009).

As it was reported by Marzo et al., noradrenaline activates a postsynaptic adrenoceptors and consequently a postsynaptic membrane hyperpolarization by intrinsic plasticity can occur. This hyperpolarization can be associated with decrease or increase in input resistance, dependently on the via (Marzo *et al.*, 2009). The decrease in input resistance may derive from the modulation of potassium channels, a decrease in cyclic adenosine monophosphate levels (cAMP) or an opening of adenosine triphosphate (ATP)-dependent potassium channels. The increase in the input resistance may result from the block of the hyperpolarization-activated currents (Ih). In addition, it was reported that α_2-adrenoceptor agonists inhibit voltage-activated calcium currents mediated by the N- or P-type calcium-channels (**figure 1.9**) (Marzo *et al.*, 2009).

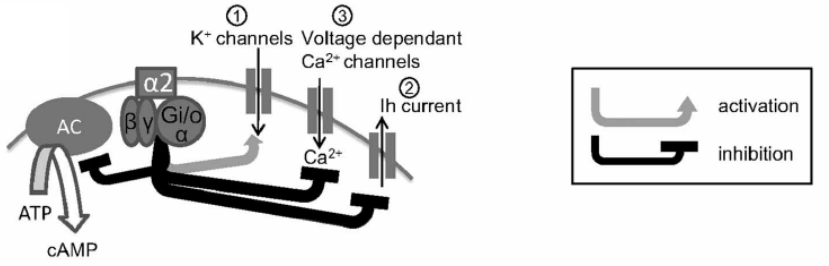


Figure 1.9 Schematic representations of the action of α_2-adrenoceptors that induce intrinsic plasticity at postsynaptic terminal. α_2-adrenoceptors activation generally induce hyperpolarization in postsynaptic terminals coupled to an increase or decrease of input resistance via blockade of Ih current (2) or opening of K+ channels (1) respectively. They also inhibit Ca^{2+} channels (3). This hyperpolarization has as a consequence the increase in postsynaptic excitability that may constitute the mechanism basis for long-lasting potentiation after synaptic stimulation. AC, adenylate cyclase; ATP, adenosine triphosphate; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; Ih, hyperpolarization-activated currents; K+, potassium. From Marzo et al (Marzo *et al.*, 2009).

1.3 ANESTHESIA AND MEMORY

Anesthetics can affect memory. There are several studies about memory deficits detected after anesthesia, mainly in young and old rodents (Culley *et al.*, 2004; Fredriksson *et al.*, 2004) and humans (Monk *et al.*, 2008).

In fact, the nervous system of neonates is highly susceptible to insults. In this developmental state, neurogenesis and synaptogenesis occur at high rates. Neurons have the capacity to trigger cell death quickly in response to a neurotoxic insults (Cottrell, 2008). It was reported that several anesthetics, such as isoflurane, propofol, ketamine, thiopental, and the combinations propofol/thiopental, ketamine/thiopental, ketamine/propofol (in high dose), increased brain cell death and reduced functional performance in neonate rodents (Olney *et al.*, 2000; Fredriksson *et al.*, 2004; Fredriksson *et al.*, 2007; Johnson *et al.*, 2008).

At the other extreme of age, the old brain is also fragile; it has less cognitive reserve, it is less resilient to neuropathologic damage, and needs less anesthetic requirements compared with adult brain. Malfunction of oxidative phosphorylation, genetic mutations and low levels of free radical scavengers such as vitamins C, E and melatonin are possible reasons for the difficult recuperation of old brain after an insult and for the reduction in anesthetic requirements (Cottrell, 2008).

Although neonate and old brains seem to be more susceptible to the anesthetic´s impact than the adult brain, this can also be affected by anesthesia. The formation of new neurons occurs, not only in the developing, but also in the adult and old brain (Gould *et al.*, 1999; Cameron and McKay, 2001) where neurons are also in a constant process of active remodeling and synaptogenesis (Bailey *et al.*, 1996; Engert and Bonhoeffer, 1999) and

consequently also susceptible to insults. Some anesthetics, such as nitrous oxide, kill neurons in adult rat (Jevtovic-Todorovic *et al.*, 2003). Likewise, ketamine may induce neurodegeneration and memory impairment in adult brain. This is a controversial issue, depending on the regime of administration (chronic or acute use) (Chatterjee *et al.*, 2010), type of memory studied (Pitsikas and Boultadakis, 2009), type of cellular death (apoptosis/excitotoxicity), dose administered (sub/anesthetic/analgesic doses) (Morgan and Curran, 2006), and sex of the animals (females are more sensitive than males) (Jevtovic-Todorovic *et al.*, 2001). More consensus exist regarding medetomidine, the other anesthetic used in this work, which had no effect on memory in young adult rats (8.3 months) and improved memory task performance in old rats (Carlson *et al.*, 1992) and old monkeys (Rama *et al.*, 1996). In fact, anesthetics can also be neuroprotective, mainly conferring protection to previously insults. Ketamine has neuroprotective effects in cortical ischemia *in vivo* (Choi *et al.*, 1988; Lees, 1995; Pfenninger and Himmelseher, 1997; Proescholdt *et al.*, 2001) as well as α_2-adrenoceptor agonists as dexmedetomidine (Maier *et al.*, 1993; Kuhmonen *et al.*, 1997; Jolkkonen *et al.*, 1999).

When medetomidine is used, its effects can be reversed by the atipamezole. Depending on the dose, atipamezole can have beneficial effects or impair learning and memory. The dose of 1mg/kg used in this work had no effects in spatial cognitive performance following a single administration in rats (Jolkkonen *et al.*, 2000).

1.4 IMPACT OF ANESTHESIA ON BRAIN

Anesthetics act in CNS changing its functionality. At molecular level, alterations in neurotransmitters may occur and consequently synaptic plasticity and viability of brain cells can be altered. Moreover, these alterations can have impact on memory/learning, causing behavioral modifications. Therefore, alterations in behavior can be a consequence of the brain cellular death or of the alterations in synaptic transmission or plasticity. Tools as histopathological/immunohistochemical studies (to assess cell viability) and electrophysiological studies (to assess alterations in synaptic transmission and plasticity) can be used to assess the impact of anesthesia on brain.

Behavior is the result of the integration of all the processes ongoing in underlying organ systems, in interaction with the external social and physical environment (Bourin *et al.*, 2007). One way of measuring behavior is by using behavioral tests, which have the advantage

of assessing the neurological function in a non-invasive way. The use of animal models can allow the study of mechanisms of specific behaviors and their pathophysiology and can aid in developing and predicting therapeutic responses to pharmacological agents (Bourin *et al.*, 2007).

The choice of the behavioral task to use should be based on the aim of the experiment, type of memory to study, and on the typical and innate behavior of each strain or specie, in order to obtain a normal and natural behavioral pattern.

Histopathological and immunohistochemical studies required the death of the animal but have the advantage of allowing the direct observation of tissues and cells. Thus, histology can detect cell death induced by anesthetics (Majewski-Tiedeken, Rabin et al. 2008). Recent studies used hematoxylin-eosin stain (H&E) to evaluate the alteration in neurons (Shacka *et al.*, 2007). This coloration, despite not being specific to the brain, has provided good information about the neurodegeneration on this organ. However, H&E staining does not distinguish different types of cellular death, such as apoptosis and excitotoxicity (Ikonomidou *et al.*, 2001; Olney, 2002; Shacka *et al.*, 2007). In turn, immunohistochemistry helps to make this distinction. This technique marks protein of interest by applying specific monoclonal or polyclonal antibodies to tissue sections, allowing the visualization of the antigen-antibody reaction sites (Davis, 2002).

Apoptosis implicates the activation of specific death signaling pathways. Caspase-3 is a frequently activated death protease in the final period of apoptosis, and so the detection of this protein is usually used as an indicator of imminent cell death (Porter and Janicke, 1999). This detection can be achieved using the immunohistochemistry technique. Apoptotic neurodegeneration can be induced by anesthetics that cause imbalance in brain-derived neurotrophic factor (BDNF) protein levels resulting in an increase of caspase-3 and caspase-9 activation and, consequently, in apoptosis (Lu *et al.*, 2006). Hence, BDNF determination by immunohistochemistry is also relevant. BDNF level is an important indicator of brain exposure to insults and play a role in activity-dependent plasticity (Ichisaka *et al.*, 2003).

Electrophysiological studies used in this thesis were invasive studies but allowed assess the alterations in synaptic transmission and plasticity. Traditionally, electrophysiological studies in mammalian brain were performed in *in vivo*. However, mechanic disturbances caused by the respiratory and heart rates of the animal are important and confounding variables. One big step ahead in order to solve these limitations was given by McIlwain and co-workers. They developed methods for *ex vivo* CNS preparations

(McIlwain, 1951). In 1957, Li and McIlwain published the first electrophysiological study performed in cortex slices (Li and McIlwain, 1957). Thereafter, the use of electrophysiological recording from slices has vastly increased our understanding of mechanisms underlying synaptic transmission in central nervous system (CNS), offering several technical advantages such as mechanical stability, direct visualization, control of extracellular medium and precise control over concentration of drugs. However, *ex vivo* preparations have also some disadvantages such as damaging neurons and glia, loss of afferent and efferent connectivity, loss of vascular and hormonal regulation and alterations in cellular metabolism due to artificial conditions (Lynch and O'Mara, 1997).

The hippocampus is used extensively in these techniques (Bortolotto *et al.*, 2001), since it has the distinct advantage, over some other areas, of a relatively simple architecture and organization which can be viewed under low power magnification. This simplifies the placement of stimulating and recording electrodes. In addition, the simple circuitry of the hippocampus means that it is relatively easy to study monosynaptic excitatory postsynaptic potentials, without interference from polysynaptic transmission (Lynch and O'Mara, 1997).

1.5 AIMS OF THE THESIS

Several clinical procedures in veterinary medicine have to be performed using anesthesia. Anesthetic agents such as ketamine and medetomidine are routinely used in laboratory animal sciences, and the ketamine/medetomidine combination is probably the most frequent anesthetic mixture used in mice and rats. However, little is known about the impact of these agents, alone or combined, on the brain of adult animals.

In addition, some evidences point to an influence of anesthesia on memory and, consequently, anesthesia may be a variable in research protocols that study memory alterations in animals. Consequently, the main objective of this work is to explore the impact of different doses of ketamine alone or combined with medetomidine in the adult brain of mice.

More specifically, the objectives of this work were to:

- Investigate the effects of different doses of ketamine and ketamine/medetomidine combination on spatial memory, and locomotion;

- Examine if different doses of ketamine and ketamine/medetomidine combination induce apoptosis or/and general cell death in adult brain and which brain areas are more affected;

- Study the effects of different concentrations of ketamine and medetomidine alone or combined on basal excitatory synaptic transmission and on synaptic plasticity (paired-pulse facilitation and long-term potentiation) in adult hippocampus; and

- Clarify the impact of anesthesia with ketamine alone or combined with medetomidine in the results of research projects, so the use of anesthetic drugs during a protocol can interfere with the experimental results.

CHAPTER 2 – GENERAL MATERIAL AND METHODS

Many of the methods of the experiments carried out in this work were common between the studies that are presented in the following chapters. To avoid excessive repetition, these common methods will be explained in detail in this chapter. When variations from these methods exist, they are described as necessary in the particular experimental protocols of each study. A summary of the main characteristics of different experiments performed can be found in **Table 2.1.**

All procedures of all studies were performed under personal and project licenses approved by the national regulatory office for animal experimentation in Portugal (Direcção Geral de Veterinária). The project license refers to the project FCOMP-01-0124-FEDER-009497, PTDC/CVT/099022/2008 named "Effects of ketamine anesthesia in animal models used on research". Moreover, all studies were based in the animal welfare regulations and the application of the Three R's principles– Refinement, Replacement and Reduction" (Russell and Burch, 1959).

Table 2.1 Summary of all the experiments performed. H&E –hematoxylin-eosin, BDNF- brain-derived neurothrophic factor, PPF-paired-pulse facilitation, LTP-long-term potentiation.

Study (chapter)		**Title of the chapter**	**Anesthetic groups (doses or concentrations used)**	**Procedure/technique**
I **(3)**		Apoptotic neurodegeneration and spatial memory are not affected by sedative and anesthetics doses of ketamine/medetomidine combinations in adult mice	• Control • 25 mg/kg of ketamine (ket.) • 75 mg/kg of ket. • 1 mg/kg of medetomidine (med.) • 25 mg/kg of ket. + 1 mg/kg of med. • 75 mg/kg of ket. + 1 mg/kg of med.	**Behavioral studies** • T-maze test • vertical pole test • open field test **Histopathological studies** • H&E • procaspase-3 • activated caspase-3 • BDNF
II **(4)**		A single intraperitoneal injection of ketamine does not affect spatial working and reference memory or neurodegeneration in adult mice.	• Control • 25 mg/kg of ket. • 75 mg/kg of ket. • 150 mg/kg of ket.	**Behavioral studies** • radial-maze test • vertical pole test • open field test **Histopathological studies** • H&E • procaspase-3 • activated caspase-3
III **(5)**	**(5.1)**	Study of the effects of ketamine and medetomidine alone (chapter 5.1 and 5.2, respectively) or in combination (chapter 5.3) on synaptic transmission and on synaptic plasticity (PPF and LTP) in hippocampal slices of adult mice.	• Control • 1 to 600 µM of ket.	**Electrophysiological studies** • synaptic transmission • paired-pulse facilitation • long-term potentiation
	(5.2)		• Control • 0.1 to 200µM of med.	
	(5.3)		• Control • 3 + 0.1 a 600 + 24 µM of ket. + med., respectively.	
IV **(6)**		Study of the effects of an intraperitoneal injection of ketamine on long-term potentiation in hippocampal slices of adult mice – a pilot study.	• Control • 25 mg/kg of ket. • 75 mg/kg of ket.	**Electrophysiological studies** • long-term potentiation

2.1 ANIMALS, HUSBANDRY AND FOOD RESTRICTION

Adult, locally bred, male C57BL/6 mice (for behavior testes, histopathological studies and electrophysiological studies with anesthesia *in vivo*) or female BALB/C mice (for electrophysiological studies with anesthesia *ex vivo*) of conventional microbiological status were used. The animals were housed in groups of three-five mice per cage (Makrolon type II cage, Tecniplast, Dias de Sousa, Alcochete, Portugal) and each cage was provided with standard corncob litter (Probiológica, Lisbon, Portugal), a piece of tissue paper and a cardboard tube. The mice were allowed to acclimate to the facilities at least one week prior to the beginning of the study. The mice were housed in an animal room with controlled temperature (21-23ºC) and relative humidity was maintained at 55%. Lights were on a 12/12h cycle, with lights off at 17.00 h. Water and rodent pellets (4RF25-GLP Mucedola, Settimo Milanese, Italy) were provided *ad libitum.*

A food restriction schedule was established to mice one week before the beginning of the behavioral tasks (chapter 3 and 4). A limited amount of food was fed each day by splitting pellets in small pieces and distributing them over the cage floor once a day. Initially, 2.1 grams per mouse per day were given, and by weighing the mice daily this amount was adjusted to a level that keep the mice on 85-95% of their free-feeding weight. The animals were weighed throughout the experiment to make sure they maintained this weight.

In the first study (Chapter 3) pieces of breakfast cereals with ~ 2 mm (Cheerios, Nestlé Portugal SA, Linda-a-Velha, Portugal) were used as a reward in the T-maze test. However, in the second study (Chapter 4), commercialized 20 mg pellets, chocolate flavor rodent purified diet for laboratory animals (Dustless Precision Pellets, Bio-Serv., New Jersey, USA) were used as a reward in the radial-maze test.

2.2 ANESTHESIA

The anesthetic/analgesic drugs were administered in the animals by intraperitoneal (i.p.) injection in experiments of chapter 3, 4 and 6, or applied directly on hippocampal slices of the animals in experiments of chapter 5.

2.2.1 ANESTHESIA ADMINISTERED BY INTRAPERITONEAL INJECTION (PROTOCOL USED IN CHAPTER 3, 4 AND 6)

Ketamine (Imalgéne® Merial, Portugal; 100 mg/ml) and/or medetomidine (Domitor® Pfizer, Portugal; 1 mg/ml) were administered with the right hand as a single i.p. injection, lateral to the midline next to the umbilicus of the animal. The needle (12 mm / 26 gauges) was inserted in an angle of 45º to the abdominal wall in the lower left quadrant of the abdomen (**figure 2.1**). Regarding the control groups, an isovolumetric i.p. injection of saline solution 0,9% (Soro Fisiologico, Braun Vet, Portugal) with same volume was administered following the same protocol. Injection and restraint were always performed by the same person.

Figure 2.1 Intraperitoneal injection. Figure original of the author.

After i.p. injection, the animal was placed in a type III cage until the lost of the righting reflex (RR). Then, the animals were moved to a homeothermic blanket (N-HB101-S-402, Panlab, Spain) covered with a surgical drape to avoid burns and placed in dorsal recumbence. One hundred per cent oxygen was delivered to the animals with a tube connected to a coaxial circuit. To avoid isolation stress in the non-anesthetized animals, these were returned to their home cage after the i.p. administration. During anesthesia the temperature was monitored and maintained between at 36-37.5ºC, with the homeothermic blanket connected to a rectal thermal probe (UNIT HB 101/2, Panlab, Spain). Pulse rate, respiratory rate, systolic pressure and depth of anesthesia (**table 2.2**) were recorded in intervals of 10 minutes. A cuff and a transducer connected to a pressure meter (LE 5001, Panlab, Barcelona, Spain) were placed on the tail base to measure the pulse rate **(figure 2.2)** and blood pressure**.** Breath was clinically assessed by evaluating movement of the respiratory muscles and the type of movement observed (thoracic or abdominal) during anesthesia. Respiratory depression was defined by inability to use the thoracic muscles; respiratory movements became more abdominal and

shallow leading to potential apnea. The time to onset of sedation, loss of righting reflex (RR), loss of response to tail pinch and loss of pedal withdrawal reflex (PWR) were recorded.

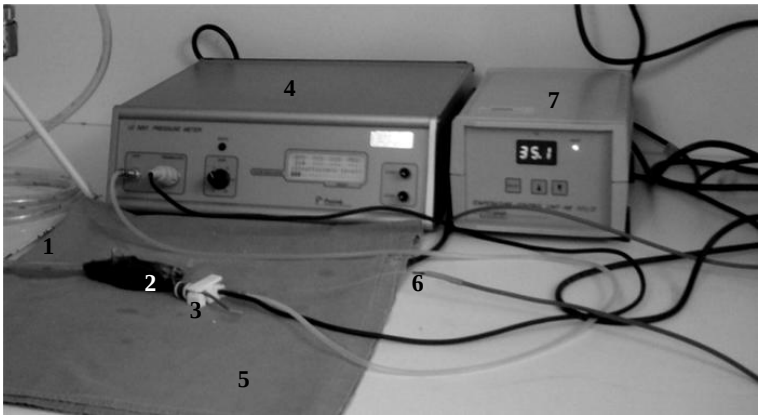


Figure 2.2 Anesthetic setup. 1- oxygen delivery tube; 2- mice in dorsal recumbence, 3- cuff and transducer; 4- pulse rate and blood pressure meter, 5- drape covering the homeothermic blanket; 6- rectal probe to measure temperature; 7- device to measure the temperature. Figure original of the author.

Table 2.2 Scores of clinical signs used to evaluate depth of anesthesia adapted from Antunes et al. (Antunes, 2001). PWR-pedal withdrawal reflex.

Depth	Symptoms
1	Lack of any response to pedal withdrawal reflex (PWR) stimulus, slow and deep respirations
2	Slight rise in muscle tone in response to the PWR stimulus considered to be a superficial reflex response
3	Slight withdrawal response confined to the pinched limb, considered to be a stronger reflex response and related with 'lighter' anesthesia
4	Obvious response in tested leg. Occasional movement elsewhere in response to PWR and increased respiratory rate
5	Onset of slight whisker movement or eye blinking after corneal stimulation, rapid withdrawal of leg and spontaneous movement of other limbs in response to PWR

One hour after loss of RR, the animals that were treated with medetomidine alone or ketamine/medetomidine were injected with 1mg/kg of atipamezole (Antisedan® Pfizer, Portugal; 5 mg/ml) to reverse the effects of medetomidine, leading to animals' RR recovery.

The animals that were only injected with ketamine awaked spontaneously. Animals were considered awake when recovering the righting reflex, when stretching the front paws trying to recover the right posture or when reacting to the approach of the researcher´s finger (stretching the nose to explore and poking). The ketamine-induced muscular rigidity makes it difficult to assess the regain of consciousness, since mice had difficulties in turning to the right position. Animals received 100% oxygen until recovery of RR. The time of return of these responses was recorded. After recovery of the righting reflex, each animal was placed alone in a cage type II with a homeothermic blanket (N-HB101-S-402, Panlab, Spain) where they were observed throughout 30 minutes.

2.2.2 ANESTHESIA APPLIED IN HIPPOCAMPAL SLICES (PROTOCOL USED IN CHAPTER 5)

The animals were euthanized by cervical dislocation followed by decapitation, the brain rapidly removed and hippocampi dissected free in ice-cold artificial cerebrospinal fluid (aCSF) of the following composition: 124 mM of NaCl, 3 mM of KCl, 1.25 mM of NaH_2PO_4, 10 mM of glucose, 26 mM of $NaHCO_3$, 1 mM of $MgSO_4$, 2 mM of $CaCl_2$, gassed with 95% O_2 and 5% CO_2 (pH=7.4). Slices (400 μm thick) were cut perpendicular to the long axis of the hippocampus with a McIlwain tissue chopper (Mickle Laboratory Engineering Co Ltd, Guildford, UK) and maintained for at least 60 minutes in gassed artificial cerebrospinal fluid (aCSF) at room temperature (23-25º) to ensure the energetic and functional recovery of tissue. After this period, each slice was carefully deposited in a submerged recording chamber (1 ml capacity) and superfused at a rate of 3 ml/minutes with aCSF continuously bubbled with 95% O_2 and 5% CO_2, which was maintained at a constant temperature of 32.0±0.1 ºC. The anesthetic agents were diluted in aCSF to obtain the desired concentration and applied in this bath. For paired-pulse facilitation (PPF) and basal synaptic transmission experiments, each slice was cumulatively exposed to increasing concentrations of drugs. For long term potentiation (LTP) experiments, each slice was exposed to only one concentration of drugs. Slices of the control group were superfused only with aCSF.

2.3 BEHAVIOR TASKS

Tasks were chosen based on species-specific or typical behavior of the animals, in order to reduce the stress to a minimum, and to respect animal welfare. Usually, before behavioral testing, animals were submitted to the test habituation period. The animal explored the apparatus during a certain period of time to get used to the environment, avoiding neophobia during the task performance. This is very important since alterations in "emotions" can lead to false-positive or false-negative interpretations. The habituation was performed in the majority of behavioral tests used in this work, except in the open field, where stress levels were assessed.

2.3.1 OPEN FIELD (USED IN PROTOCOL OF CHAPTER 3 AND 4)

The open field is the most widely used test for emotional reactivity and motor function in mice (Gershenfeld *et al.*, 1997; Prut and Belzung, 2003; Wartella *et al.*, 2003). The procedure consists in subjecting an animal to an unknown environment (Walsh and Cummins, 1976). When more anxious, the tendency of rodents is to stay preferably close to the walls (Crawley, 1999) and an increase in defecation is observed. The open field test was used to access locomotor activity, to indicate stress levels (Chapter 3 and 4) and to study the process of habituation to the open field arena (Chapter 4).

The apparatus consisted in a circular arena made of grey polypropylene and surrounded by a wall of 30 centimeter (cm) height. Each animal was released in the center of the arena and allowed to explore it for 20 minutes (**figure 2.3**). At the end of testing the number of fecal boli was counted and the arena was cleaned with alcohol at 70% to avoid the presence of olfactory cues. The test was recorded with a camera placed above the apparatus and recorded into a computer with the multi-camera vigilance system GeoVision (GV-800/8, Taipei, Taiwan). The video analysis was carried out with the program VideoMot 2 (TSE-systems, Bad Homburg, Germany) which measured several parameters: distance walked, time spent, the number of visits on each region, the latency to exit the center, the total distance walked and speed. Three areas were defined in the open field arena: periphery, middle and center.

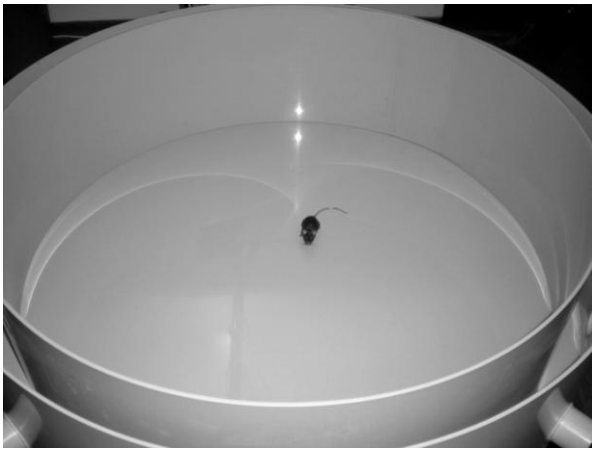


Figure 2.3 Open field arena with a mouse. Figure original of the author.

2.3.2 VERTICAL POLE TEST (USED IN PROTOCOL OF CHAPTER 3 AND 4)

Vertical pole test measures motor coordination and balance and was used to assess post-anesthetic recovery. Deficits in motor coordination and balance were detected when the mouse fell from the pole (Crawley, 2007). This test was performed as previously described by Bellum et al. with minor modifications (Bellum *et al.*, 2007).

Each mouse was placed in the center of a round rough-surfaced pole (50 cm long, 2 cm diameter). The pole was initially positioned horizontally and then slowly inclined to 90º (1º per second (s)); the animal faced the end that was lifted up. Performance was determined by the latency for the mouse to turn downwards and completely descend the pole (**figure 2.4**). Animals had a maximal time of 120 seconds to complete the test. This maximal time was recorded for animals that were not able to turn downwards and instead dropped from the pole.

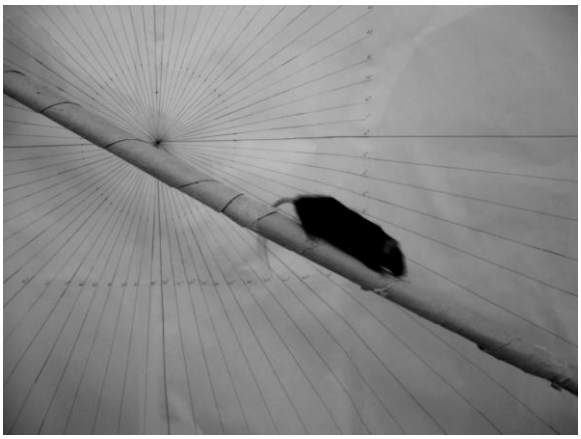


Figure 2.4 Mice descending the pole in the Vertical pole test. Figure original of the author.

2.3.3 T-MAZE TEST (USED IN PROTOCOL OF CHAPTER 3)

T-maze test is a popular task to evaluate learning and memory in animal research (Olton, 1979; Andrade *et al.*, 2001).

The apparatus of T-maze was made of grey acrylic sheets and consisted of a starting arm and two runways positioned in the shape of a T-sign. Each arm measured 10x60x20 cm (width, length, height). A recessed ceramic food pot was placed in the far end of each choice arm (**figure 2.5**). The reward was a 2 mm piece of cereal (Cheerios, Nestlé Portugal SA, Linda-a-Velha).

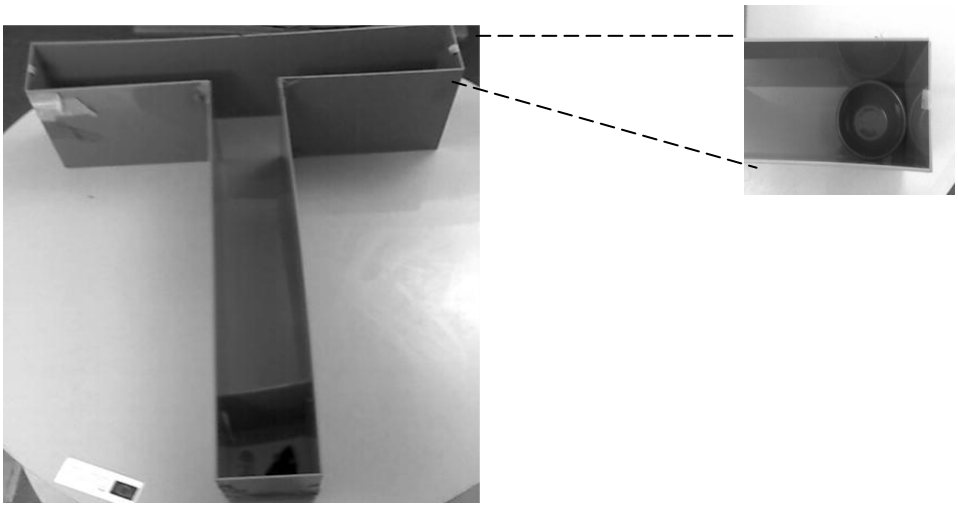


Figure 2.5 T-maze apparatus. Figure at the right represents the magnification of the right arm of the T-maze with the ceramic food pot. Figure original of the author.

The habituation to T-maze was performed on the day before anesthesia. All animals of each cage were left in the starting arm of the T-maze and allowed to explore it for 15 minutes with no food reward present; then they returned to their home cage. Next, the mice were placed individually in the starting arm of the T-maze and allowed to explore it in 15 trials of 2 minutes each, this time with the ceramic pots at the end of each choice arm baited with pieces of the above mentioned reward. The first choice arm entered by each animal in each trial was recorded to analyze whether there was any lateralization bias. If a mouse initially showed a bias for one side, this was corrected in the T-maze test by baiting the opposite arm. A bias was considered if a mouse chose one side 10 times or more in 15 trials. For mice which did not present a bias, the correct arm for the test was selected randomly.

T-maze test was performed 28 hours, 1 week and 2 weeks after anesthesia procedure. The rewarded arm was always the same to the same animal throughout all experiment. All tests

were performed by the same person, blind to the anesthetic procedures that the animals had undergone. During the test, only one of the choice arms was baited, and this arm was considered the correct one. Each animal was placed in the starting arm with a sliding door blocking the access to the choice arms. The trials started when the experimenter lifted the door. The first three trials were learning trials wherein the mouse was allowed to go to both pots and eat the reward from the correct arm. After these learning trials, the next test trials ended when one of the following situations occurred: the mouse entered the unbaited arm (wrong choice), two minutes passed without it making any choice (no choice), or the mouse chose the correct arm and ate the reward (correct choice). Entrance was recorded when the animal entered with all four paws in the selected arm. When a trial ended, the mouse was gently pushed back to the starting position. The test ended when the learning criterion was achieved, i.e. when the mouse entered the correct arm 9 times out of 10 consecutive test trials (excluding the three learning trials); hence every mouse had to perform a minimum of 10 trials, excluding the learning trials. The total time and number of trials to complete the task were measured.

2.3.4 RADIAL-MAZE TEST (USED IN PROTOCOL OF CHAPTER 4)

The radial-maze test has been used to access working and reference memory. The protocol used was based in a previous description (Buccafusco, 2001)**.** Briefly, radial-maze was made of transparent acrylic sheets and consisted of eight equal arms in radial disposition (**figure 2.6**). The central platform measured 180 mm of diameter and the arms measured 80x400x300 mm (width, length, height). All arms have a pit well in the distant end where a piece of commercial pellet of chocolate flavour was placed as food reward.

Habituation to the radial-maze test was performed during two days before anesthesia. At 1st day in the morning, all animals from each cage were left inside the apparatus and allowed to explore it for 15 minutes with no food reward present. At 1st and 2nd days in the afternoon each animal was placed alone inside the full baited apparatus.

The animals started the radial-maze test in the central platform, inside a transparent cylinder. Three arms were baited and the angles between them were always 90º, 135º and 135º. When the cylinder was lifted, the animal was allowed to explore all the apparatus and the trial ended when it visited all arms rewarded or when 5 minutes had elapsed. Then, when the animal returned to the central platform, the cylinder was lowered until the next trial. The

maze was rotated between trials in a clock or anti-clock movement, 45º to 180º in a random way. Each session had 5 trials with 1 minute of inter-trial interval and 1 session per day during 12 consecutive days for each individual animal was performed. Entrance was recorded when the four limbs of the animal passed a landmark position placed outside each arm. Numbers of reference memory errors (number of times that the animal entered in wrong arms) and working memory errors (number of times that the animal entered in an arm previously visited) were measured during 12 consecutive days.

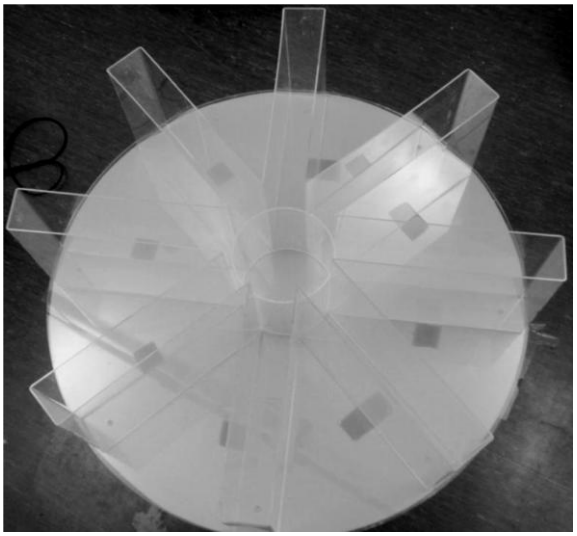


Figure 2.6 Radial arm maze apparatus. Figure original of the author.

2.4 HISTOPATHOLOGICAL (HEMATOXYLIN-EOSIN STAINING) AND IMMUNOHISTOCHEMICAL (PROCASPASE-3, ACTIVATED CASPASE-3 AND BRAIN-DERIVED NEUROTROPHIC FACTOR) STUDIES

Three hours after anesthesia, mice were euthanatized by cervical dislocation followed by decapitation and the brain was quickly removed. This time point was based in previous works (Majewski-Tiedeken *et al.*, 2008; Valentim *et al.*, 2010). Brains were fixed for 48 hours in 4% buffered paraformaldehyde (PBS, pH 7.4, 0.1 M), processed and embedded in *paraplast* (SHANDON, Hypercentre XP and Histotocentre 2, Burlingame, CA, USA). For each block serial sections of 4 µm thick were made cutting coronal sections since Bregma 0.98 mm until Bregma -2.70 mm (Paxinos and Franklin, 2001) with 30 µm of interval (Sanders *et al.*, 2010). The slices were used for different procedures dependently of protocol. In chapter 3, slices were used for H&E staining (observation of general cellular death), procaspase-3, caspase-3 activated expression (observation of apoptotic cells) and for BDNF

detection. In chapter 4, slices were used for H&E staining, procaspase-3 and activated caspase-3 (observation of apoptotic cells) expression. Procaspase-3 antibody (CPP32 Ab-4, Rabbit Polyclonal Antibody, Thermo Scientific), activated caspase-3 antibody (Cleaved Caspase-3 antibody, Asp175, Cell Signalling Technology) and BDNF-antibody [BDNF: (H-117): sc-20981, Santa Cruz Biotechnology, Inc., Heidelberg, Germany] were used.

The technique hematoxylin-eosin staining was performed based in a protocol previously described (Gamble, 2002). After applying this staining, the observation of cellular dead was consistent with morphologies observed in previous study (Nakajima *et al.*, 2000; Shacka *et al.*, 2007). Cells with abnormal morphologies (shrinkage, hypereosinophilic cytoplasm, condensed and hyperchromatic nuclei) were considered positive for cell death.

For immunohistochemical analysis, a streptavidin-biotin immunoperoxidase method was used. In brief, the sections were deparaffinized, hydrated, washed in PBS, quenched in 3% hydrogen peroxide for 20 min, and washed in H_2O. For antigen retrieval, microwave treatment was used (two times 5 min each in citrate buffer, pH 5). After washing in PBS, the sections were incubated in blocking solution for 5 min (Ultra V Block, UltraVision Detection System; Thermo Fisher Scientific, Waltham, MA), followed by overnight incubation in antibody diluted 1:100 in 1% PBS (anti-procaspase-3 and anti-BDNF) or 1:200 in 1% TBST (mixture of Tris-Buffered Saline and Tween 20) (anti-activated caspase-3) at 4°C. For negative control, one extra brain section was incubated in PBS or TBST; as a positive control, a section of newborn and regression stage mouse thymus was processed and analyzed in the same way as the brain. Next day, the sections were washed in PBS or TBST and incubated in biotinylated goat anti-polyvalent antibody (UltraVision Detection System) for 10 min at room temperature. After washing in PBS or TBST, sections were incubated in streptavidin peroxidase complex (UltraVision Detection System) for 10 min at room temperature. To detect the immunoreaction, a diaminobenzidine (Sigma, Madrid, Spain) solution (0.01% 2,3-diaminobenzidine with 0.01% H_2O_2) was used. Finally, the slides were washed in H_2O, counterstained with Gill hematoxylin, and dehydrated. Tissue sections were examined with a microscope (Eclipse E600; Nikon, Tokyo, Japan) equipped with a digital camera (DXM 1200; Nikon). Different areas of the brain were photographed using a 20 objective (numerical aperture, 0.50); to avoid overlapping, the position of each field was recorded using the x-y coordinates of the microscope stage. Digitized images with a resolution of 3840×3072 pixels (3382.34 cm^2) with the final magnification of 960× were exported to Adobe Photoshop CS3 (Adobe Systems Inc., Mountain View, CA).

Number of dead cells and cells showing clear positive immunoreactions, per square millimeter in the retrosplenial and visual cortexes, pyramidal cell layer of the *cornu Ammonis* (CA) 1 and CA3 areas of the hippocampus, granular layer of the DG (chapter 3 and 4), laterodorsal thalamic nucleus, accumbens nucleus and striatum (chapter 4) (**figure 2.7**) were manually counted. The average from three coronal sections selected at 30 µm of interval was calculated (Sanders *et al.*, 2010).

The specific regions of the brain were selected based on the aim of this thesis. So we choose to investigate some regions of brain that are important to memory such as hippocampus (Neves *et al.*, 2008), retrosplenial (Pothuizen *et al.*, 2010) and visual(Super, 2003) cortices, nucleus accumbens (Setlow, 1997), laterodorsal thalamic nucleus (van Groen *et al.*, 2002) and striatum (De Leonibus *et al.*, 2003).

To evaluate the BDNF expression, a relationship between positive cells and negative cells was established.

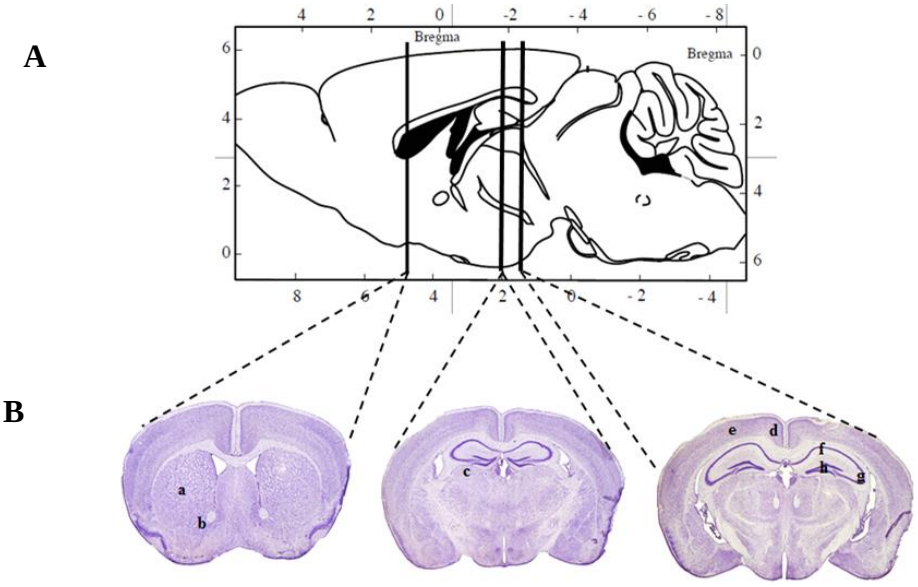


Figure 2.7 A- Mouse brain and its stereotaxic coordinates with bregma, and midpoint of the interaural line as reference points. The vertical lines mark the location of the coronal sections presented in B. a-striatum, b- accumbens nucleus, c- laterodorsal thalamic nucleus, d- retrosplenial cortex, e- visual cortex, f- CA1 region of hippocampus, g- CA3 region of hippocampus, h- dentate gyrus. Figures were adapted from the Paxinos and Franklin (Paxinos and Franklin, 2001).

2.5 EXTRACELLULAR ELECTROPHYSIOLOGY RECORDINGS *(CHAPTER 5 AND 6)*

The electrophysiological recording was based in previous works (Cunha *et al.*, 1994; Coelho *et al.*, 2006) with some modifications. After preparation of slices (explained in previous section: "Anesthesia applied in hippocampal slices"), electrical stimulation was

applied through a bipolar tungsten electrode placed over the Schaffer collateral/commissural fibers. The stimulus duration was 0.1 ms and its intensity was adjusted to evoke field excitatory postsynaptic potentials (fEPSP) with 40-50% of their maximal amplitude. Under basal conditions, stimuli were delivered at a frequency of 0.067 Hz. Evoked fEPSPs were recorded from stratum radiatum layer of the hippocampus CA1 area using a glass micropipette filled with NaCl at concentration of 4 M (2-5 MΩ resistance) (**figure 2.8**).

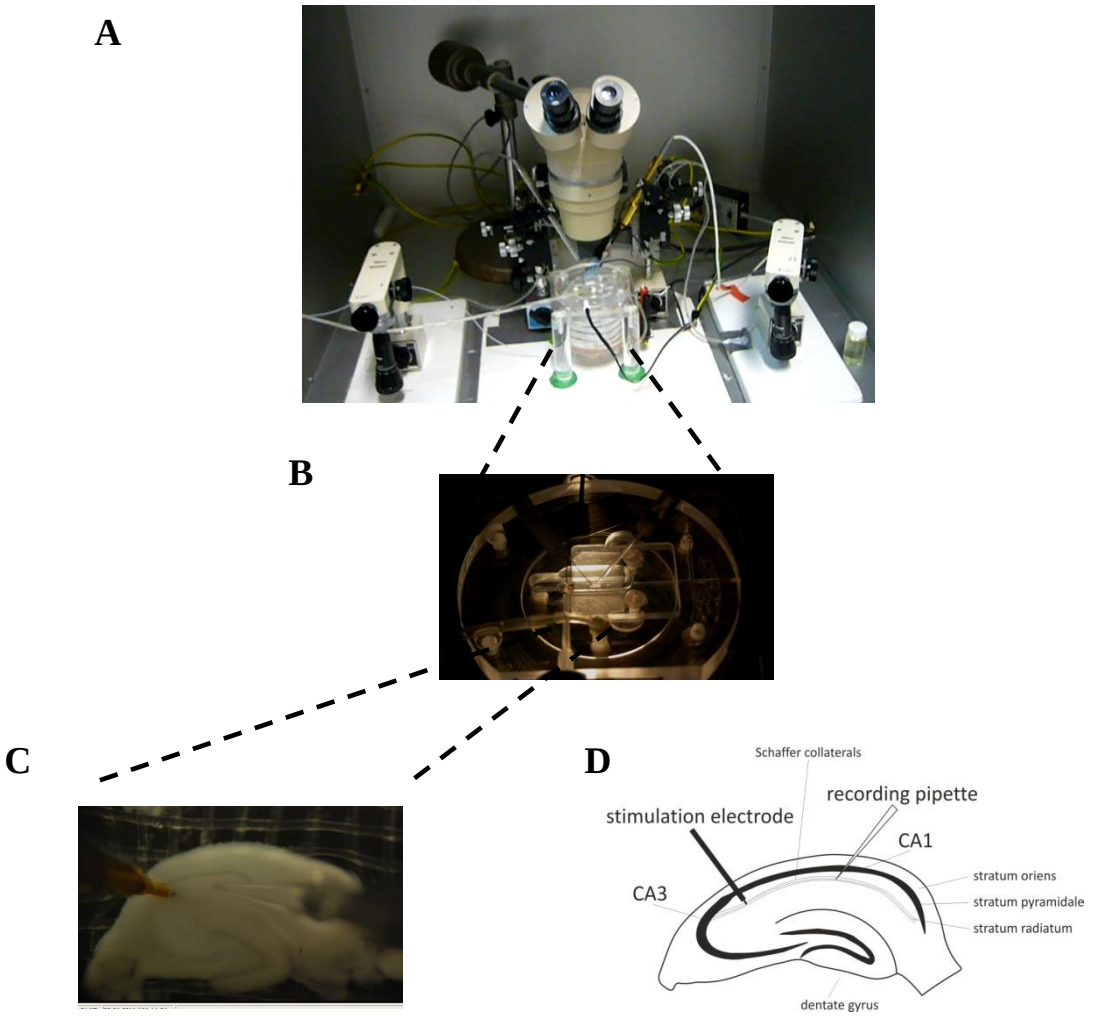
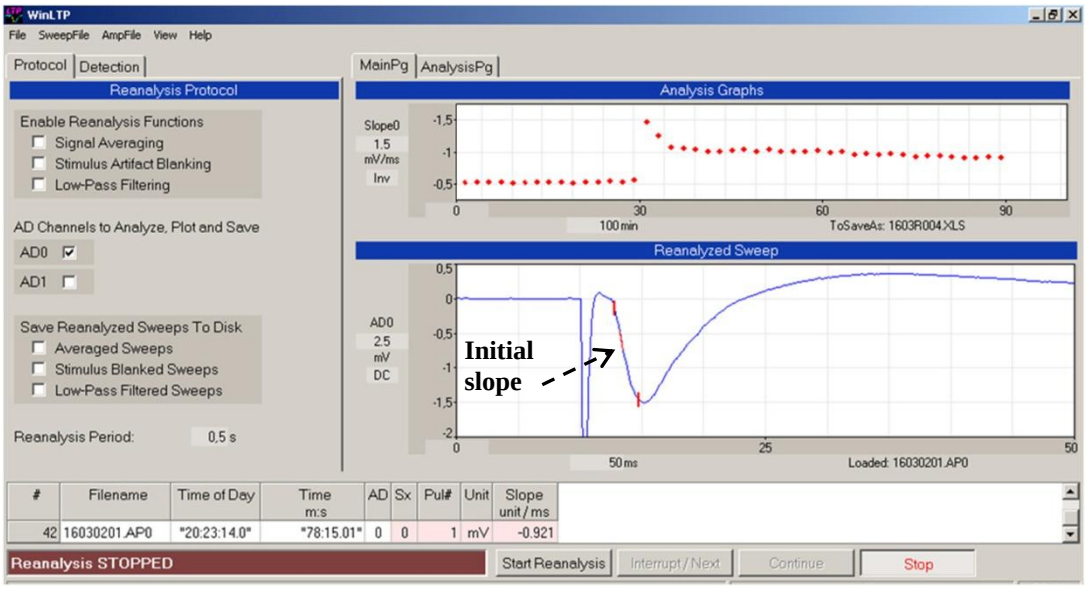


Figure 2.8 A- Electrophysiological setup, B- Acrylic camera with a hippocampal slice, C- Hippocampal slice, stimulating electrode and recording pipette. The recording pipette was placed in the stratum radiatum of the CA1, and the stimulating electrode was placed in the stratum radiatum near the CA3/CA1 border to stimulate the Schaffer collateral pathway. D- Schematic figure of a hippocampal mouse slice with the stimulating electrode and recording pipette. Figure original of the author.

Averages of eight consecutive responses were continuously monitored on a personal computer with LTP 1.3 software (Anderson and Collingridge, 2001) and responses were quantified as the initial slope of the average fEPSPs (**figure 2.9 A e B**). Offline analysis was

performed with WinLTP program version 1.11 (WinLTP Ltd., University of Bristol, Bristol, U.K.) (**figure 2.9 A).**

A



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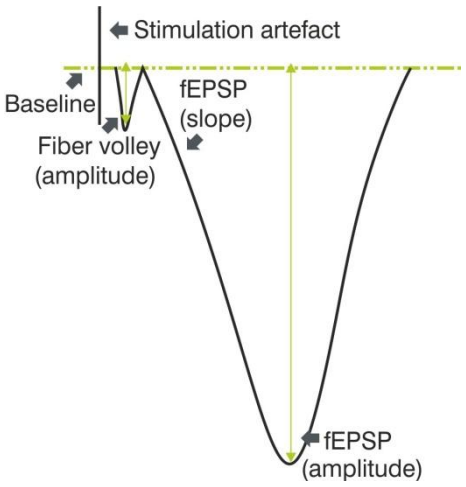


Figure 2.9 A - A "print screen" of the WinLTP 1.11 software (WinLTP Ltd.,University of Bristol, Bristol, U.K.) , during a reanalysis of an experiment of the long-term potentiation. Each red point represent the average of eight consecutive responses (initial slopes of the field excitatory postsynaptic potential) collected during 2 minutes. **B** - Schematic example of a signal of field excitatory postsynaptic potential (fEPSP).

To access **PPF**, two consecutive pulses were applied with a 50 ms inter-pulse interval. Paired pulse facilitation quantification was carried out as the ratio of the fEPSP slopes of the second response over fEPSP slopes of the first response in each pair of stimuli.

Long term potentiation was induced by a high frequency stimulation (HFS) train (100 pulses at 100 Hz). This train was applied 30 minutes after obtain a stable baseline at

frequency of 0.067 Hz, which was resumed immediately after application of the HFS. LTP induction was quantified as the ratio of averaged fEPSP slopes of the first 6 minutes after HFS over the averaged fEPSP slopes during the 10 min before HFS; LTP maintenance was quantified as the ratio of the averaged fEPSP slope from 54 to 60 min after HFS over the averaged fEPSP slope 10 min before HFS.

2.6 STATISTICAL ANALYSIS

Animals were randomly allocated into groups using a web site service available at: http://www.random.org. The number of animals used per group was based on previous works and using a power analysis conducted prior to the research study. Power analysis were performed using an online web site service (available at http://stat.ubc.ca/~rollin/stats/ssize/index.html) with a power of 0.8 and α=0.05.

Data were considered parametric if the following assumptions were fulfilled: independent observations, data from groups with homogeneity of variances (Levene´s test) and each group with data normally distributed (Shapiro-Wilk, an adequate test for small sample sizes).

Repeated measures analysis of variance (ANOVA), Student`s t-test or one-way ANOVA with Bonferroni *post hoc* test were used to analyze parametric data and data expressed as mean ± standard deviation (SD). Non-parametric data were evaluated with Mann-Whitney *U*-test, or Kruskal-Wallis followed by Dunn's test with a Bonferroni correction for multiple comparisons and data expressed as medians and interquartile range (25th percentile; 75th percentile). Details are given in statistical analysis of each chapter.

The concentration of drugs that produces 50% of maximal inhibition (IC_{50}) was calculated from a fitting curve of our data to a Hill equations (nonlinear regression analyses) using GraphPad-Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA).

A value of $P \leq 0.05$ was considered statistically significant. All results were analyzed using Microsoft Office Excel 2003 for data management and SPSS 16.0 for Windows (IBM Corporation, Armonk, NY) for statistical analysis.

CHAPTER 3- APOPTOTIC NEURODEGENERATION AND SPATIAL MEMORY ARE NOT AFFECTED BY SEDATIVE AND ANESTHETICS DOSES OF KETAMINE/MEDETOMIDINE COMBINATIONS IN ADULT MICE

This chapter describes the study derived from practical experiments performed at "Laboratório de Histopatologia e Anatomia Patológica" of UTAD and at Laboratory of Animal Science of IBMC, and led to 2 outputs, which are included in **Appendix B.**

3.1 ABSTRACT

Background: Ketamine is increasingly popular in clinical practice and its combination with α_2-agonists can provide good anesthetic stability. Little is known about the effects of this combination in the brain. Therefore, we investigated the effects of different concentrations of ketamine combined with medetomidine on cognition and its potential apoptotic neurodegenerative effect in adult mice.

Material and methods: Seventy-eight C57BL/6 adult mice were divided into 6 different groups (Saline solution, 1 mg/kg of medetomidine, 25 mg/kg of ketamine, 75 mg/kg of ketamine, 25 mg/kg of ketamine + 1 mg/kg of medetomidine, 75 mg/kg of ketamine + 1 mg/kg of medetomidine). Eight animals per group were tested in the T-maze, vertical pole and open field test. Five animals per group were used for histopathological (H&E staining) and immunohistochemical analyses (procaspase-3, activated caspase-3 and BDNF expressions). Cells showing clear H&E staining and positive immunoreactions for caspase-3 and BDNF expressions in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, and in the granular layer of the DG were counted.

Results: There were no differences between groups regarding number of dead cells and cells showing positive immunoreactions in the different areas of the brain studied. Similarly, no differences were detected in the number of trials to complete the T-maze task. Nevertheless, α_2-agonist decreased hyperlocomotion caused by ketamine in the open field.

Conclusions: Neither apoptotic neurodegeneration nor alterations in spatial memory were observed with different concentrations of ketamine combined with medetomidine in adult mice.

3.2 INTRODUCTION

Ketamine, a non-competitive glutamate NMDA receptor antagonist, is gaining popularity in adult human medicine mainly thanks to its analgesic proprieties (Angst and Clark, 2010; Carstensen and Moller, 2010; Trupkovic *et al.*, 2011) and its importance for emergency procedures (Kuznetsova *et al.*, 1984; Rice *et al.*, 2010). However, ketamine impairs brain activity inducing post-anesthetic delirium in humans (Sussman, 1974; Klausen *et al.*, 1983) and hyperlocomotion in rodents (Irifune *et al.*, 1991).

Ketamine may be combined with α_2-agonists such as dexmedetomidine (Scher and Gitlin, 2003; Brennen *et al.*, 2011) in humans or medetomidine in animals (Verstegen *et al.*, 1990; Hahn *et al.*, 2005). Levanen et al. showed in humans that the combination of ketamine with dexmedetomidine provides stable anesthesia with reduction of adverse effects caused by ketamine, such as post anesthetic delirium (Levanen *et al.*, 1995). However, in Europe, dexmedetomidine has not been approved for human clinical use and was only very recently introduced in veterinary medicine where medetomidine is used routinely.

Dexmedetomidine is the dextro-enantiomer of medetomidine and has the pharmacological activity of medetomidine (Savola and Virtanen, 1991). Ketamine/medetomidine and ketamine/dexmedetomidine have similar effects in induction of anesthesia, heart rate, respiratory rate, temperature, blood gas values and recovery (Bouts *et al.*, 2011).

The use of α_2-agonists reduce anesthetic requirements and improve perioperative hemodynamic stability (Tanaka and Nishikawa, 1994; Hall *et al.*, 2000). In addition, ketamine/(dex)medetomidine anesthesia has the advantage of a rapid recovery with the administration of atipamezole, a specific α_2-antagonist available for animals use, which immediately reverses (dex)medetomidine effects (Vainio and Vaha-Vahe, 1990; Scheinin *et al.*, 1998; Hahn *et al.*, 2005). However, there is a lack of information about potential secondary effects of this combination on the brain, such as effects on memory and neurodegeneration.

The purpose of this study was to assess the influence of two different concentrations of ketamine combined with α_2-agonists on performance a simple spatial cognitive task and the potential neurodegenerative effects in adult mice.

3.3 MATERIAL AND METHODS

This study was performed using seventy-eight 28 weeks-old, male C57BL/6 mice. These animals were randomly divided into 6 different groups: control, 1 mg/kg of medetomidine (1Med.), 25 mg/kg of ketamine (25Ket.), 75 mg/kg of ketamine (75Ket.), 25 mg/kg of ketamine + 1 mg/kg of medetomidine (25Ket./Med.) and 75 mg/kg of ketamine + 1 mg/kg of medetomidine (75Ket./Med.). Ketamine and/or medetomidine were used for sedation/anesthesia. Atipamezole was used (1 mg/kg) to reverse anesthesia promoted by medetomidine.

After i.p. injection, eight animals per group were tested in the behavioral tests (T-maze, vertical pole and open field test). The timing of behavioral tests in relation to i.p. injection is illustrated in figure 3.1. More details on the animals, anesthesia by i.p. injection and behavioral tests used are described in the general methods section ("Chapter 2").

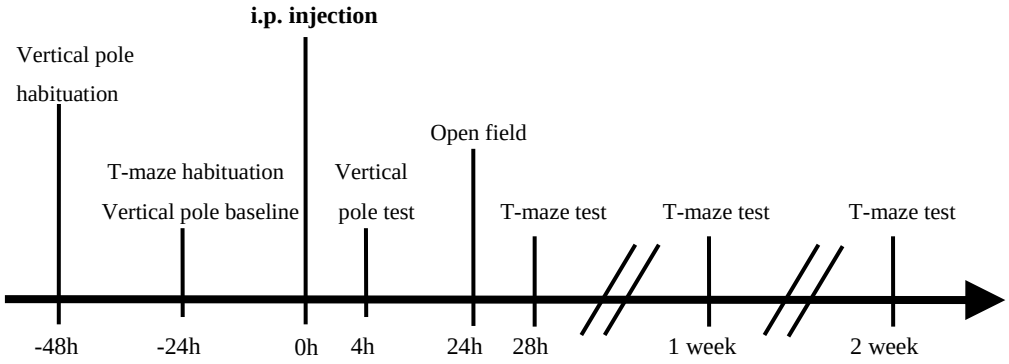


Figure 3.1 - Timeline of the behavior tests.

Five animals per group were euthanized three hours after i.p. injection, by cervical dislocation followed by decapitation, and their brains were used for H&E staining (observation of general cellular death), procaspase-3 and activated caspase-3 expression (observation of apoptosis), and for BDNF detection. Cells showing clear abnormal morphologies in H&E staining and positive immunoreactions for procaspase-3, activated

caspase-3 and BDNF in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, and in the granular layer of the DG were counted. Details about the histopathological and immunohistochemical procedures are found in general material and methods ("Chapter 2").

3.3.1 Statistical Analysis

Data of physiologic parameters and data of open field and histopathological techiques (parametric data) were analyzed using Students t-test and one-way ANOVA with Bonferroni *post hoc* tests, respectively, which is expressed as means ±SD. Data of T-maze and vertical pole tests (non-parametric data) were evaluated with Kruskal-Wallis test followed by Mann-Whitney *U* test and are expressed as median [minimum, maximum]. $P \leq 0.05$ was considered statistically significant.

3.4 RESULTS

All animals from 25Ket./Med. and 75Ket./Med. groups lost the consciousness. Animals with ketamine treatment only did not lose the consciousness. Mice from the 25Ket. group walked quickly with some motor incoordination and mice from 75Ket. group walked slowly with motor incoordination. Animals from the medetomidine group were less active and were generally sited in a corner of the box.

3.4.1 Anesthesia

Results for anesthesia are based on anesthetized groups (25Ket./Med. and 75Ket./Med.). No significant differences were detected between groups for time need to induce anesthesia (2.25±0.71 and 1.87±0.83 minutes, respectively), temperature, pulse rate, systolic pressure and oxygen saturation. Unconscious animals were anesthetized for one hour, afterwards atipamezole was administered for reversal. The group 75Ket./Med. had a higher respiratory rate compared with the group with lower concentration of the ketamine/medetomidine at 10 (p= 0,017), 30 (p= 0,019) and 60 (p= 0,045) minutes following loss of consciousness. The same group also showed higher depth of anesthesia and took longer to recover from anesthesia. **Table 3.1** shows results of hemodynamic and oxygen saturation data.

Table 3.1 Hemodynamic and oxygen saturation data (n=10).

Time (min.)	Group	Pulse rate (beats per min.)	Respiratory rate (respiratory movements per min.)	Systolic Pressure (mm Hg)	SpO_2 (%)	Temperature (ºC)
0	25Ket./Med.	484.1 ± 80.6	128.0 ± 17.8	126.4 ± 26.2	99.0 ± 0.8	37.2 ± 0.3
	75Ket./Med.	498.1 ± 69.1	139.5 ± 28.2	152.5 ± 31.0	98.9 ± 0.8	37.0 ± 0.5
10	25Ket./Med.	474.1 ± 74.6	131.0 ± 14.1	125.8 ± 25.5	98.6 ± 0.9	36.9 ± 0.4
	75Ket./Med.	493.8 ± 70.5	156.0 ± 22.1	124.4 ± 22.9	99.0 ± 0.7	36.5 ± 0.6
20	25Ket./Med	507.0 ± 62.8	147.5 ± 17.3	123.8 ± 24.5	98.8 ± 0.7	36.7 ± 0.5
	75Ket./Med.	518.1 ± 50.2	162.5 ± 20.6	150.1 ± 29.4	98.9 ± 0.8	36.9 ± 0.8
30	25Ket./Med	513.1 ± 68.9	137.8 ± 21.8	129.9 ± 25.4	98.8 ± 0.7	37.0 ±0.2
	75Ket./Med.	488.5 ± 41.5	168.0 ± 23.6	130.8 ± 24.5	98.9 ± 1.0	36.2 ± 0.8
40	25Ket./Med	505.1 ± 54.3	147.0 ± 21.1	134.0 ± 26.7	98.4 ± 1.2	36.7 ± 0.6
	75Ket./Med.	524.6 ± 79.7	162.5 ± 20.8	136.6 ± 24.4	98.6 ± 1.5	36.5 ± 0.3
50	25Ket./Med	518.1 ± 41.4	143.0 ± 18.5	129.4 ± 26.8	99.3 ± 0.7	36.8 ± 0.5
	75Ket./Med.	506.8 ± 58.1	153.5 ± 24.9	135.8 ± 20.6	98.9 ± 1.1	36.5 ± 0.5
60	25Ket./Med	509.6 ± 50.7	144.5 ± 16.3	129.5 ± 17.6	99.3 ± 0.7	36.8 ± 0.7
	75Ket./Med.	530.4 ± 39.7	167.0 ± 23.8	132.9 ± 15.6	99.0 ± 0.8	36.9 ± 0.4

3.4.2 Behavioral tests

T-maze test:

No significant differences were observed between groups regarding the number of trials needed to complete the test at 28 hours, 1 week and 2 weeks after anesthesia (**figure 3.2**).

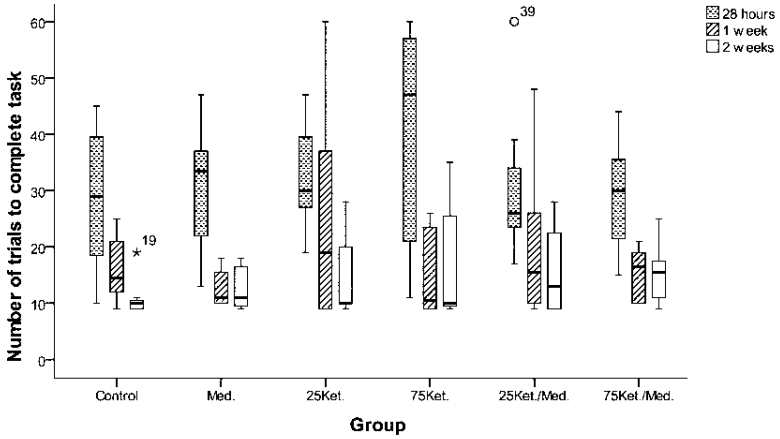


Figure 3.2 Number of trials necessary to complete the T-maze task at 28 hours, 1 week and 2 weeks after intraperitoneal injection in different groups (n=8). Data are presented as a box plot (the median is indicated by

the horizontal bar inside the box; the 25th and 75th percentile are the box borders; and the whiskers are the lowest and highest values for the 5th and 95th percentiles, respectively). ○ = outlier and * = extreme value.

Vertical pole test:

During the vertical pole test, animals from 75Ket/Med. group took longer than the others to completely descend the pole and also showed increased latency to turn downwards (**figure 3.3**). The high values of group 75Ket./Med. were due to the fact that 6 animals fell from the pole.

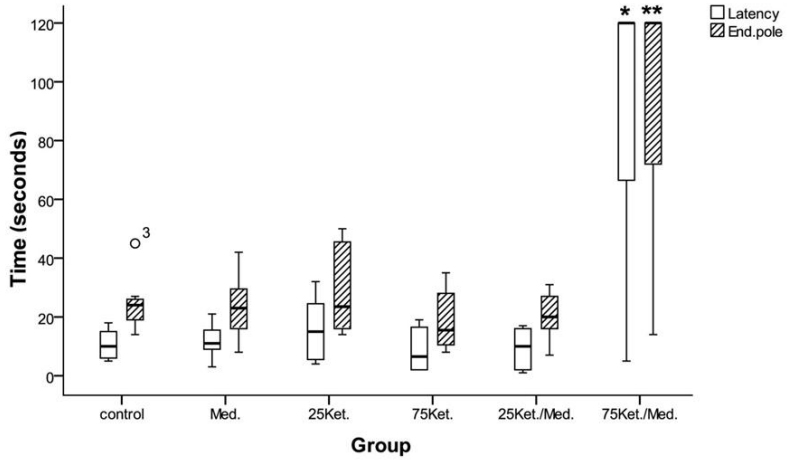


Figure 3.3 Latency time to turn (latency) and completely descend the vertical pole (endpole) in seconds, four hours after anesthesia (n=8). 120 seconds were attributed to the animals that fell. * p ≤0,021 and **p ≤0,028. Data are presented as a box plot (the median is indicated by the horizontal bar inside the box; the 25th and 75th percentile are the box borders; and the whiskers are the lowest and highest values for the 5th and 95th percentiles, respectively). ○ = outlier

Open field test:

The open field test showed significant differences between groups on speed and total distance. The 75Ket. group moved faster and covered a longer distance than the control group. Since the time to explore the arena is the same for all the animals, the speed only depends on the distance walked, hence only speed is represented graphically (**figure 3.4**). Animals anesthetized with 75 mg/kg ketamine (100,61±24,15 m) showed an increase in the distance walked in the peripheral area compared with control group (65,26±13,43 m; p=0,002), Med. group (65,67±13,77 m; p=0,002), and 25Ket./Med. group (66,39±5,74 m; p=0,003). There were no differences detected between groups in the other parameters measured in the open field test.

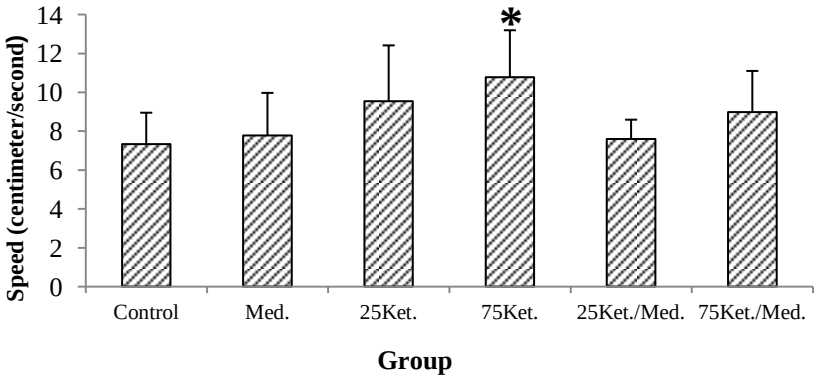


Figure 3.4 Speed in the open field test induced by the different treatment groups (n=8) during 20 minutes, 24 hours post-anesthesia. * p=0.033 compared to the control group. Bars represent means and vertical lines represent SDs.

3.4.3 Brain analyses

Regarding histopathology (H&E stain) there were no significant differences in the number of dead cells between groups in the different brain regions studied. Immunohistochemical studies showed a similar result concerning the number of cells with clear positive immunoreactions (procaspase-3, activated caspase-3 and BDNF expressions) (**figure 3.5 and 3.6**).

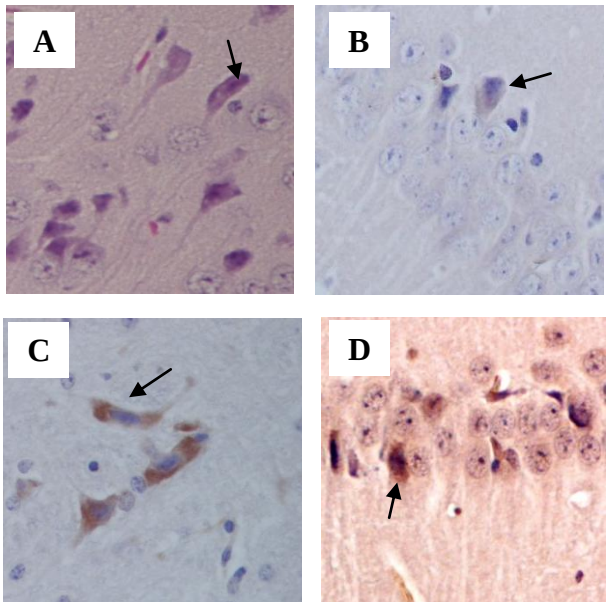


Figure 3.5 Example of cells counted as positive (arrows) in histopathological and immunohistochemical experiment 3 hours after i.p. injection. (**A**) Dead cells for H&E staining. (**B**) Positive cells for procaspase-3. (**C**) Positive cells for activated caspase-3. (**D**) Positive cells for BDNF expression.

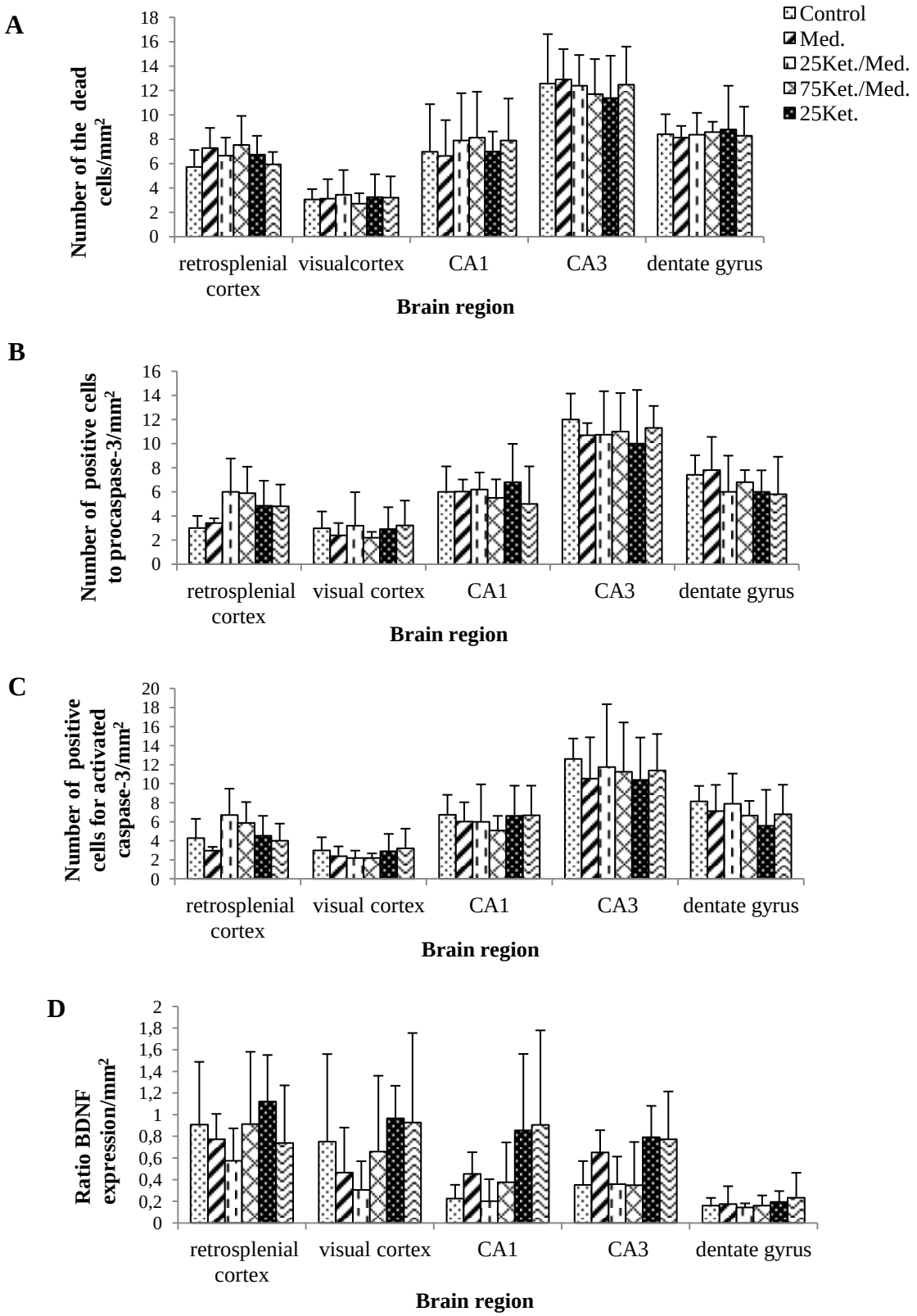


Figure 3.6 Histopathological and immunohistochemical results. (**A**)-Number of dead cells (H&E), (**B**) number of positive cells for procaspase-3, (**C**) activated caspase-3 (apoptosis) and (**D**) ratio of the number of the positive BDNF expression/ negative BDNF expression, per square millimetre (mm^2) in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, and in the granular layer of the DG, 3 hours after anesthesia in different groups (n=5). Bars represent means and vertical lines represent SDs.

3.5 Discussion

The results from this work showed no indication of apoptotic neurodegeneration following different subanesthetic and anesthetic doses of ketamine/medetomidine combination in adult mice. No effect on cell death or on spatial memory and no alterations in BDNF expression were observed. While ketamine induced hyperlocomotion in the open field when administered alone, this effect was reduced by the α_2-agonist medetomidine. The ketamine/medetomidine combination seemed to result in physiological anesthetic stability since there were no significant differences between groups regarding pulse rate and blood pressure values.

The hemodynamic stability in ketamine/medetomidine sedative and anesthetic combinations observed in this work is in accordance with previous studies, which reported that, the action of ketamine help to oppose the depressant actions of α_2-agonists on the circulatory system (Verstegen *et al.*, 1990; Levanen *et al.*, 1995), improving perioperative hemodynamic stability (Hall *et al.*, 2000). Our observation that the higher concentration of ketamine/medetomidine combination increased respiratory rate is supported by reports regarding ketamine respiratory stimulating properties (Morel *et al.*, 1986).

High concentrations of ketamine/medetomidine combination also increased anesthetic depth, with longer latency to recover equilibrium as indicated by the worst performance in the vertical pole test compared with lower concentrations.

In the open field, all animals showed a species-specific response to the aversive condition of the brightly-lit arena, spending more time near the wall than in a central region, with most measures being unaffected by sedative and anesthetic combinations. However, animals from the 75Ket group showed increased distance walked in the periphery and total distance, consequently higher speed was observed. This suggests a hyperlocomotion effect of ketamine, in agreement with previous observations that this drug induces hyperlocomotion by the alteration of pre-synaptic components of dopamine neurons in the nucleus accumbens of mice (Irifune *et al.*, 1991). When the α_2-agonist medetomidine was administered, hyperlocomotion was reduced; perhaps because these drugs reduces dopamine turnover (Koulu *et al.*, 1993). To our knowledge, this is the first study showing that hyperlocomotion induced by ketamine may be inhibited by α_2-agonists in mice.

In the T-maze test, used to assess spatial memory, no differences between groups were detected throughout the experiment. All groups learnt the task at 28 hours after anesthesia

and recalled it 1 and 2 weeks post-anesthesia, as indicated by the decreasing the number of trials to complete the task along time. No data were found concerning the effects of the combined administration of ketamine/medetomidine in spatial memory or neurodegeneration. However, it was reported with consistence that ketamine alone causes memory impairment and neurodegeneration when administered in neonates (Olney *et al.*, 2000; Young *et al.*, 2005). Nevertheless the adult brain is very different from neonates and there is conflicting evidence on the effects of a single administration of ketamine on cognition in the adult brain, with effects depending on the type of memory studied (Morgan and Curran, 2006), acute or chronically use (Morgan and Curran, 2006), dose (Pitsikas and Boultadakis, 2009) and temperature (Pitsikas and Boultadakis, 2009). Chronical administration of ketamine resulted in disrupted spatial working memory after 10 days of treatment (Enomoto and Floresco, 2009). However, this did not happen with less days of treatment (Enomoto and Floresco, 2009) which is in accordance with our results.

The dosages used in our study (25 and 75 mg/kg of ketamine) were subanesthesic when administered alone. It was the addition of the α_2-agonist medetomidine that allowed anesthesia, reducing the amount of ketamine required and consequently reducing the probability of inducing adverse effects caused by high ketamine concentrations.

The alpha$_2$-agonist medetomidine and the drug used to reverse it, atipamezole, did not affect performance in the T-test. This is in agreement with previous works carried out by Carlson and colleagues that reported that medetomidine had no effect on spatial memory in the adult rats (Carlson *et al.*, 1992) and that atipamezole (1 mg/kg) had no effects in spatial cognitive performance following a single administration in rats (Jolkkonen *et al.*, 2000).

Cellular death in this study was evaluated mainly by measuring apoptosis, in which no difference between groups was observed. Other works showed that a single administration of ketamine induced age and sex dependence cell death. Adults were more sensitive than immature rats and females were more sensitive than males; males remain insensitive to ketamine-induced vacuolization of neurons until they reached full adulthood (Jevtovic-Todorovic *et al.*, 2001). In this work, we used adult males and ketamine with medetomidine did not showed any negative impact in brain; however we studied neurodegeneration by apoptosis and not by excitotoxicity (vacuolization of neurons). Furthermore, to study general toxicity it would be necessary to evaluate cell death in more time points. The histopathological analyses were performed only at 3 hours after anesthesia because it was

reported that this time point is a good option (Majewski-Tiedeken *et al.*, 2008; Valentim *et al.*, 2010) to access apoptosis which is the main aim of this work.

Brain-derived neurotrophic factor is an important indicator of brain exposure to insults. This was not altered with ketamine/medetomidine combinations and is in accordance with the results obtained with caspase 3. Usually, BDNF increases after insults (Kokaia *et al.*, 1994). No works were found in the literature, studying the effects of this combination in BDNF expression. There are reports that acute administration of ketamine at low doses increased BDNF expression (Garcia *et al.*, 2008); however, we used higher doses of ketamine. Interestingly, pro-apoptotic anesthetic drugs modulates BDNF protein levels in the developing brain (Lu *et al.*, 2006) resulting in an increase in caspase-3 and caspase-9 activation and, consequently, in apoptotic neurodegeneration (Lu *et al.*, 2006). In our study no differences were detected between groups regarding BDNF level and in caspase-3 expression, this fact reinforced the suggestion that a single administration of ketamine/medetomidine was not an insult to the adult brain.

In conclusion, our work showed that a single administration of sedative and anesthetic concentrations of ketamine / medetomidine combination in adult mice did not affect spatial memory, BDNF expression or neurodegeneration by apoptosis in the hippocampus, retrosplenial cortex and visual cortex. Simultaneously, this study showed that medetomidine prevented ketamine induced hyperlocomotion suggesting that α_2-agonists may be considered to attenuate post-anesthetic delirium and agitation produced by ketamine.

CHAPTER 4- A SINGLE INTRAPERITONEAL INJECTION OF KETAMINE DOES NOT AFFECT SPATIAL WORKING AND REFERENCE MEMORY OR NEURODEGENERATION IN ADULT MICE

This chapter describes the study derived from practical experiments performed at "Laboratório de Histopatologia e Anatomia Patológica" of UTAD and at "Laboratory of Animal Science" of IBMC and led to 2 outputs, which are included in **Appendix C.**

4.1 ABSTRACT

Background: Ketamine is an effective anesthetic and analgesic agent used in clinical practice and in research. Little is known about the effects of this drug on working and reference memory in adult brain and on neuronal cell death. Therefore, we studied the effects of subanesthetics and anesthetics doses of ketamine on working and reference memory and its potential neurodegenerative effect in adult mice.

Material and methods: Forty-eight C57BL/6 adult mice were divided into 4 different groups (Saline solution, 25 mg/kg of ketamine, 75 mg/kg of ketamine and 150 mg/kg of ketamine). Seven animals per group were tested in the radial-maze, vertical pole and open field tests. Five animals per group were used for histopathological (H&E stain) and immunohistochemical analyses (procaspase-3 and activated caspase-3 detections). Death cells and cells showing positive immunoreactions in the retrosplenial and visual cortexes, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, in the granular layer of the DG, in the laterodorsal thalamic nucleus, striatum and accumbens nucleus were counted.

Results: No differences were observed between groups regarding the number of dead cells and cells showing positive immunoreactions in the different areas of the brain. The performance in the vertical pole and the number of reference and working memory errors in the radial-maze were similar in all groups. Nevertheless, the animals treated with 75 mg/kg of ketamine revealed a transient hyperlocomotion in the open field.

Conclusions: Neither neurodegeneration nor alterations in working and reference memory were observed with subanesthetics and anesthetics doses of ketamine in adult mice. The

higher subanesthetic dose of ketamine used produced transitory hyperlocomotion.

4.2 INTRODUCTION

Ketamine is used as an anesthetic and analgesic agent in clinical practice (Domino, 2010). More specifically, this drug is used in painful diagnostic procedures, obstetrics, asthma, traumatic and hypovolemic shock and burn situations (Kuznetsova *et al.*, 1984; Adams and Hempelmann, 1990; Ikechebelu *et al.*, 2003) . Moreover, ketamine is increasingly used in pain therapy, as a preventive pain agent reducing acute postoperative opioid consumption and pain intensity (Unlugenc *et al.*, 2003; Kafali *et al.*, 2004). However, this drug is related with psychomimetic effects (Klausen *et al.*, 1983; Krissel *et al.*, 1994) and there are still uncertainties about the effect of a single administration of anesthetics and subanesthetics doses of ketamine on working and reference memory, and on neurodegeneration in adults.

It was consistently reported that ketamine causes memory impairment and neurodegeneration when administered in neonate rodents (Olney *et al.*, 2000; Fredriksson *et al.*, 2004). However, the adult brain is very different from the neonate's brain and the effects of a single ketamine administration on memory and on neurodegeneration in adults is controversial. Chronic ketamine administration, in rats, disrupted spatial working memory after 10 days of treatment, but these changes were not observed when 5 days of treatment were provided (Enomoto and Floresco, 2009). Furthermore, ketamine disrupted recall of previous information in the rat with anesthetic doses but not with subanesthetic doses (Boultadakis and Pitsikas, 2011).

In vitro, studies showed that ketamine caused neuronal apoptosis via the mitochondrial pathway (Braun *et al.*, 2010). These neurodegenerative effects were also observed in vivo studies showing that a single administration of ketamine induced age and sex dependent cell death by excitotoxicity in rat retrosplenial cortex (Jevtovic-Todorovic *et al.*, 2001). However, apoptosis and excitotoxicity (neuronal vacuolization) are different types of cellular death (Young *et al.*, 2004) and the effect of a single administration of ketamine in apoptosis was not established in adult mice.

Moreover, the effects of subanesthetic and anesthetic doses of ketamine in different regions of the brain were not studied. The purpose of this study was to evaluate the influence of different doses of ketamine on spatial working and reference memory and its potential neurodegenerative effects in different regions of the brain in adult mice.

4.3 MATERIAL AND METHODS

Forty-eight 21 weeks of age, male C57BL/6 mice were used.

Mice were randomly assigned into four treatment groups: control group treated with saline solution, 25Ket. group received ketamine 25 mg/kg (lower subanesthetic dose), 75Ket. group received ketamine 75 mg/kg (higher subanesthetic dose) and 150Ket. group received ketamine 150 mg/kg (anesthetic dose). Ketamine was administered as a single i.p. injection.

After i.p. injection, seven animals per group were tested in the behavioral tests (radial-maze, vertical pole and open field test). More details on the animals, anesthesia by i.p. injection and behavioral tests used are described in the general methods section (chapter 2). The timing of behavioral tests protocol in relation to i.p. injection is illustrated in **figure 4.1**.

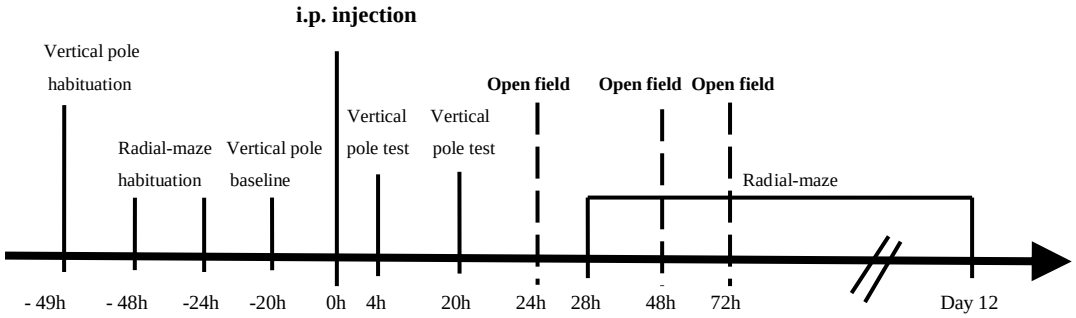


Figure 4.1 Timeline for the behavior tests protocol (in hours to 12 days). Moment of i.p. injection is defined as time zero.

Five animals per group were euthanized three hours after i.p. injection, by cervical dislocation followed by decapitation, and their brains were used for H&E staining (observation of general cellular death), procaspase-3 expression and caspase-3 activation (observation of apoptosis). Cells showing clear abnormal morphologies in H&E staining and positive immunoreactions for procaspase-3 and caspase-3 activated in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, in the granular layer of the DG, in the laterodorsal thalamic nucleus, striatum and accumbens nucleus were counted. More details about the histopathological and immunohistochemical procedures are found in general material and methods (chapter 2).

4.3.1 Statistical Analysis

The data of radial-maze test and vertical pole test are non-parametric data, and so were analysed using a Kruskal Wallis test followed by Mann-Whitney *U*-test, with group as a variable factor.

The parametric data of open field test such as speed, total distance walked and distance walked in center, middle and periphery were analysed using a two-way mixed design ANOVA for repeated measures, with time and group as factors, followed by a Bonferroni post hoc tests for multiple comparisons. In addition, these parameters were analysed on each day using one-way ANOVA with Bonferroni post hoc tests and with group as a factor.

Non-parametric data of the open-field test such as number of fecal boli, latency to exit the center, time spent and number of visits in each region were analysed using a Kruskal Wallis test followed by Mann-Whitney U-test with group as a factor.

The data of H&E, procaspase-3 and activated caspase-3 are parametric data obtained at the same time point, and were analysed using one-way ANOVA with Bonferroni post hoc tests and with group as a factor.

4.4 RESULTS

4.4.1 Physiological parameters

As expected, only animals from the 150Ket group lost consciousness, and so anesthetic parameters were only measured in this group (n=12; i.e. n=7 from behavioral tests +5 animals from histopathological analysis). Mice were unconscious for 30±10.2 minutes. Means± SD for pulse and respiratory rates were respectively 537.18±13.40 beats per minute and 174.76± 29.28 respiratory movements per minute. Oxygen saturation was maintained above 98% throughout anesthesia.

4.4.2 Behavioral tests

Radial-maze test:

No significant differences were observed between groups regarding the numbers of reference and working memory errors during 12 consecutive days after anesthesia, in radial-maze (n=7; **table 4.1**).

Table 4.1 Number of reference memory (Ref.) and working memory (Work.) errors in radial-maze test for 12 consecutive days after intra peritoneal injection of saline or different doses of ketamine. Data are presented as median [minimum-maximum].

Days	Control		25 Ket.		75 Ket.		150 Ket.	
	Ref. errors	Work. errors	Ref. errors	Work. errors	Ref. errors	Work. errors	Ref. errors	Work. errors
1	5.2[3.2-7.4]	2.3[1.2-4.2]	6.2[3.6-8.0]	4.1[1.0-5.8]	5.8[4.0-9.2]	3.1[1.4-6.6]	5.1[3.6-8.6]	3.0[1.0-6.8]
2	4.7[3.2-6.2]	2.1[0.4-2.8]	5.4[3-9.2]	2.8[0.6-6.6]	5.5[4.2-8]	2.8[0.4-6.0]	5.0[3.4-8.4]	3.1[0.4-5.8]
3	3.6[2.4-5.2]	1.4[1-2.6]	4.9[2.2-6.8]	1.8[0.8-3.4]	3.6[3.2-5.2]	1.1[0.2-2.6]	4.2[3.2-4.4]	1.6[1.2-3.8]
4	4.5[2-8]	2.4[0.4-6.4]	4.3[3.2-6.4]	2.1[0.4-3.2]	3.8[2.2-5.6]	1.5[0.8-3]	3[2.4-4.2]	0.9[0.2-1.6]
5	2.9[2.6-3.2]	1[0.2-1.6]	3.5[1.8-6.2]	1.1[0.4-3]	3.3[2.0-4.6]	1.4[0-2.4]	4.1[1.6-6.2]	1.6[0-3.8]
6	2.5[1.2-4.0]	0.2[0-1.2]	3.1[2-3.7]	0.9[0.3-2.3]	2.4[1.6-3.4]	0.3[0-1.6]	3.1[0.8-4]	0.4[0-1.4]
7	2.1[0.4-4.6]	0.7[0-4.2]	2.2[1.8-2.6]	0.9[0.2-1.2]	2.7[1.6-8]	1[0-4.4]	2.8[1.6-3.4]	0.5[0-1.2]
8	1.7[1.2-3]	0.7[0.2-1.8]	2.5[1.4-3]	0.5[0.4-2.2]	2.8[1-3.8]	1[0.4-2]	1.9[1-3.6]	0.3[0-0.6]
9	1.2[0.4-2.8]	0.5[0-2]	2.2[1.2-3.4]	0.5[0.2-3]	1.4[1-3.2]	0.3[0-0.6]	2.1[1.4-4.2]	0.4[0-0.8]
10	1.3[0.8-2.4]	0.6[0.2-1.8]	1.8[1.4-2.2]	0.4[0.2-1]	1.5[0.6-4.6]	0.6[0.2-1.6]	2.3[1.4-3.2]	0.5[0.2-1.4]
11	0.9[0-2]	0.5[0-0.6]	1.4[1-3.4]	0[0-2.8]	0.7[0.4-3.4]	0.3[0-0.6]	1.8[0.8-7]	0.3[0-4.4]
12	0.8[0-1.4]	0.3[0-0.8]	1.4[0.8-1.8]	0.1[0-1.6]	1.1[0.6-3.8]	0.4[0-1]	1.7[0.8-2.2]	0.2[0-1]

Vertical pole test:

During the vertical pole test, no significant differences were detected between groups regarding the latency to turn downwards ($p \geq 0{,}903$) and the time to completely descend the pole ($p \geq 0{,}817$) 4 and 20 hours after anesthesia (**figure 4.2**)

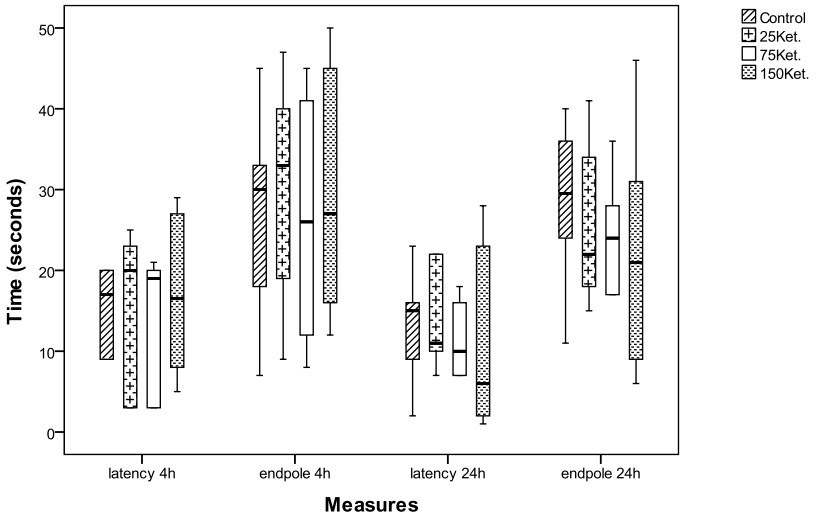


Figure 4.2 Time to turn (latency) and completely descend the vertical pole (endpole) in seconds, 4 and 20 hours after control (saline), 25, 75 or 150 mg/kg ketamine administration (n=7). No differences were detected between groups. Data are showed as a box plot (the median is indicated by the horizontal bar inside the box; the 25th and 75th percentile are the boxes borders; and the whiskers are the lowest and highest values for the 5th and 95th percentiles, respectively).

Open field:

The open field test showed differences between groups on speed and total distance in the first day (n=7). The 75Ket. group moved faster and more (10.22 ±1.61cm/s and 122.68 ± 19.25 m, respectively) than the control group (7.68 ± 0.92 cm/s ; p=0.01 and 92.17 ± 11.04 m; p=0.01 respectively) at 24 hours after anesthesia. Since the time to explore the arena was the same for all the animals, the speed and total distance walked are directly related, hence only speed is represented graphically (**figure 4.3**). There were no differences detected between groups in the other parameters measured in the open field (n=7).

Across time (days) was observed a decrease in total distance walked (p=0.00). This decrease occur at the same rate in all groups (p=0.121), showing a lack of influence of ketamine in the habituation process in the open field arena (n=7).

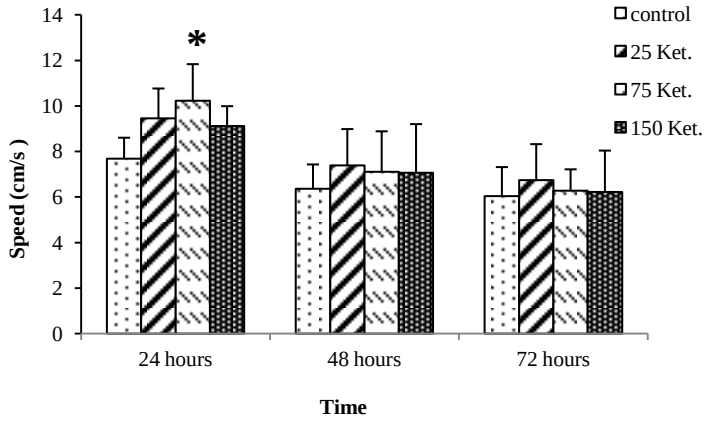


Figure 4.3 Animals' speed in the open field after control (saline), 25, 75 or 150 mg/kg ketamine administration (n=7) measured during 20 minutes at 24, 48 and 72 hours post-anesthesia. * p=0.01 compared to control group. Bars represent means and vertical lines represent SDs.

4.4.3 Brain analyses

There were no significant differences between groups regarding number of dead cells (haematoxylin-eosin) and cells showing positive immunoreactions (procaspase-3 and activated caspase-3) in the different brain regions studied (**figure 4.4 and 4.5**).

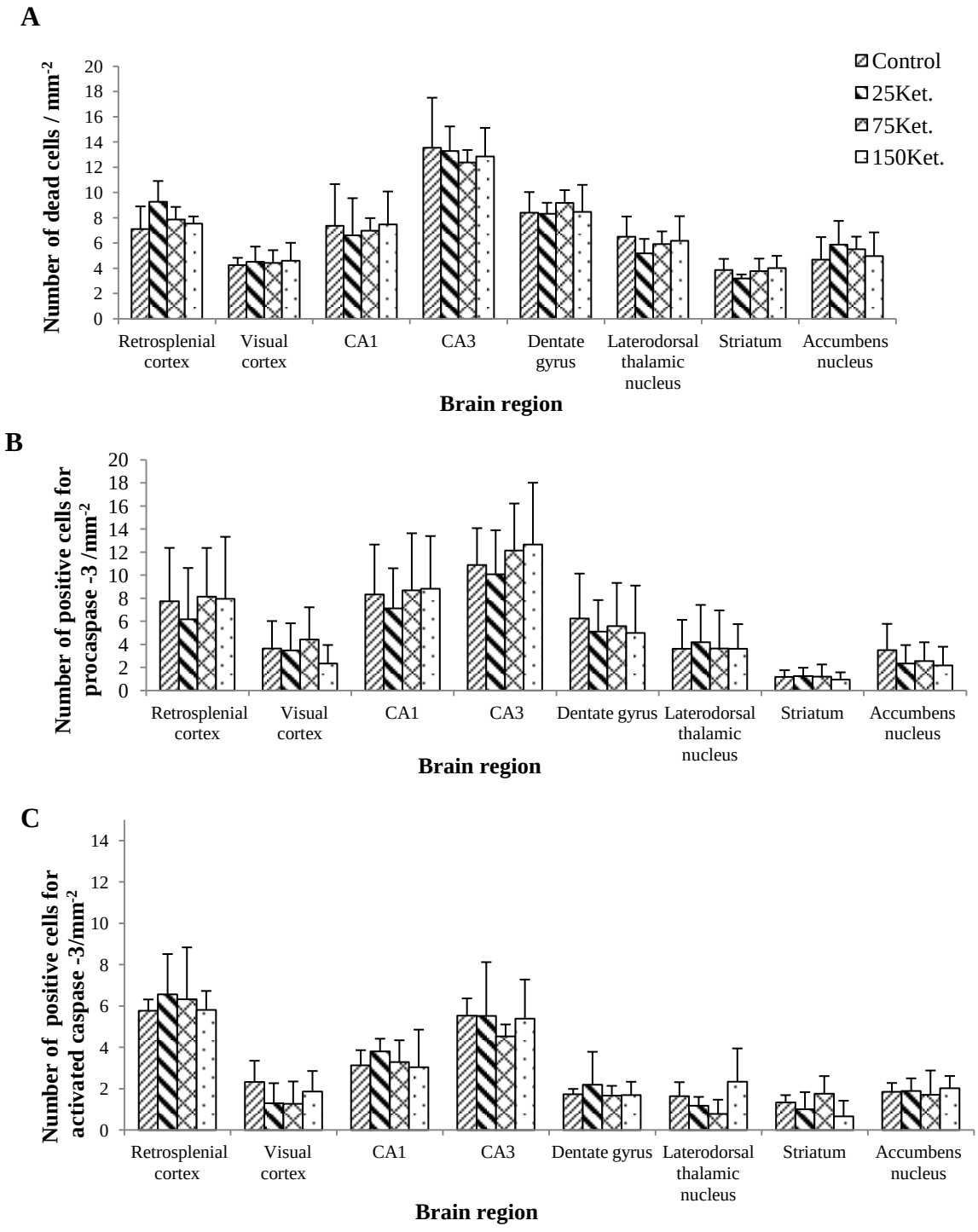


Figure 4.4 Histopathological and immunohistochemical results. (A) The number of dead cells (HE), (B) number of positive cells for procaspase-3 and (C) number of positive cells for active caspase-3 per mm^2 in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, in the granular layer of the dentate gyrus, laterodorsal thalamic nucleus, striatum and accumbens nucleus, 3 h after control (saline), 25, 75 or 150 mg/kg ketamine administration (n=5). Bars represent means and vertical lines represent SDs.

4.5 DISCUSSION

The results from this work showed that a single administration of different doses of ketamine did not affect spatial working and reference memory neither neurodegeneration in adult mice. However, the intermediate dose of ketamine (75 mg/kg) induced transitory hyperlocomotion.

Concerning radial-maze, no significant differences on working and reference memory were detected between groups throughout all the experiment. All groups learned the task and decreased the number of working and reference memory errors along the time. In the literature we found controversial results regarding the effects of ketamine in memory depending on the regime of administration (acute or chronic use) (Chatterjee *et al.*, 2010), type of memory studied (Pitsikas and Boultadakis, 2009), temperature and dose (Morgan and Curran, 2006). When ketamine was chronically used, it disrupted spatial working memory after 10 days of treatment, but the same was not observed when less days of treatment were provided (Enomoto and Floresco, 2009), which is in accordance with our results. It was also reported that low doses of ketamine impaired working and reference memory (1.5-10 mg/kg) (Wesierska *et al.*, 1990) and learning (8 mg/kg) in animal models (Bolton *et al.*, 2012). However, in our study we used higher doses of ketamine.

Regarding equilibrium and motor coordination evaluated in the vertical pole test, no significant differences were detected between groups at 4 and 20 hours after anesthesia. This suggests that all animals recovered from the treatment 4 hours after its administration, and that all animals had equivalent motor capacities when the others behavioral tests were performed. In the literature, there is a lack of information regarding the effects of ketamine on equilibrium and motor coordination using the vertical pole test in mice.

In the open field test, all animals showed a species-typical response to the aversive condition of the brightly-lit arena, spending more time near the wall than in the central region, with most measures being unaffected by ketamine administration. These results suggested similar levels of stress between groups. Moreover, all groups habituated to the open field arena at a similar rate and reduced total activity across sessions. However, 24 hours post-anesthesia, animals from 75Ket.group moved faster and more than the control animals. This effect did not happen at 48 and 72 hours after anesthesia. Interestingly, the hyperlocomotion was not observed with the highest and in the lowest doses of ketamine used in this study. Our result are in agreement with a previous work that showed that an intermediate dose of

ketamine (100 mg/kg) is more effective than 50mg/kg and 200mg/kg for producing hyperactivity (Chatterjee *et al.*, 2010). The exact mechanism that explains these results remain unclear. Hyperlocomotion induced by ketamine was previously observed immediately after administration of drug and justified by the alteration of pre-synaptic components of dopamine neurons in the nucleus accumbens of mice (Irifune *et al.*, 1991). However, we analyzed the effects of ketamine on locomotion at 24, 48 and 72 hours after drug administration and no studies were found in the literature regarding the dopamine levels in nucleus accumbens at these time points.

In this study, neurodegeneration was mainly evaluated by measuring apoptosis, in which no significant differences between groups were observed in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, granular layer of the DG, laterodorsal thalamic nucleus, striatum and in the accumbens nucleus. It was reported that the levels of procaspase-3 (inactive form of caspase-3) in adult rat brain are extremely low when compared with brain in development and so an additional synthesis of procaspase-3 for posterior activation would occurs in order to execute the apoptotic program (Didenko *et al.*, 2002). In this work ketamine did not induced an additional synthesis of procaspase-3 when compared with control group; this suggest that a single administration of ketamine did not cause apoptosis in adult brain. This information is in agreement with our results obtained with activated caspase-3 that was also not affected by ketamine. However, this does not exclude that other types of cell death, such as excitotoxicity may happen. Jevtovic-Todorovic and colleagues previously reported that a single administration of ketamine induced age and sex dependent cell death by excitotoxicity in retrosplenial cortex (Jevtovic-Todorovic *et al.*, 2001). Adults were more sensitive than immature rats, and, females were more sensitive than males. In fact, males remained insensitive to ketamine-induced vacuolization of neurons (excitotoxicity) until they reached full adulthood (Jevtovic-Todorovic *et al.*, 2001). In the present work ketamine did not show any negative impact in the brain. Different results may be potentially explained because we only used adult mice males and we studied neurodegeneration by apoptosis and not by excitotoxicity.

In conclusion, this study showed that a single administration of subanesthetic or anesthesic doses of ketamine, in adult mice, did not affect working and reference memory nor induced apoptotic neurodegeneration in the retrosplenial and visual cortexes, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, the granular layer of the DG, the laterodorsal thalamic nucleus, accumbens nucleus and striatum. Simultaneously, this study

showed that only the intermediate ketamine dose induced hyperlocomotion in the first day, suggesting that psychotomimetic effects caused by ketamine in animals and humans could be avoided by adjusting the dose. These finding can be used to refine anesthesia in laboratory animals. However, the extrapolation of our experimental results to clinical human and veterinary practice requires extreme caution, as differences in drug dosages as well as interspecies variations are important variables which need to be considered.

CHAPTER 5- STUDY OF THE EFFECTS OF KETAMINE AND MEDETOMIDINE ALONE OR IN COMBINATION ON EXCITATORY SYNAPTIC TRANSMISSION AND ON SYNAPTIC PLASTICITY (PPF AND LTP) IN HIPPOCAMPAL SLICES OF ADULT MICE

In this chapter, the studies performed at "Centro de Neurociências e Biologia Celular de Coimbra" (CNC) were described. These experiments resulted in 3 outputs, which are included in **Appendix D.**

This chapter is organized as follows:

- **5.1.** Effects of different concentrations of **ketamine** on excitatory synaptic transmission and on synaptic plasticity

- **5.2.** Effects of different concentrations of **medetomidine** on excitatory synaptic transmission and on synaptic plasticity

- **5.3.** Effects of different concentrations of **ketamine/medetomidine combination** on excitatory synaptic transmission and on synaptic plasticity

5.1. EFFECTS OF DIFFERENT CONCENTRATIONS OF KETAMINE ON EXCITATORY SYNAPTIC TRANSMISSION AND ON SYNAPTIC PLASTICITY (PPF AND LTP)

5.1.1 ABSTRACT

Background: Ketamine, an analgesic/anesthetic drug, is frequently used in clinical practice. However, the effect of ketamine in basal excitatory synaptic transmission and synaptic plasticity are not yet fully understood. Therefore we investigated the effects of different

concentrations of ketamine on basal excitatory synaptic transmission and on two forms of synaptic plasticity: PPF and LTP.

Material and methods: Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in Schaffer fiber - CA1 pyramid synapses of mouse hippocampal slices. Four slices per group and experiment were used. For basal synaptic transmission and PPF increasing concentrations of ketamine (1-600 μM) were applied to each slice and for LTP individual slices were used for each concentration (3-100 μM). High-frequency stimulation (100 pulses at 100 Hz) was used for LTP induction. The initial slope of the fEPSP was measured and LTP induction and maintenance were calculated. PPF was estimated as the ratio between the slopes of the second and first pulses paired with a 50 milliseconds interval.

Results: Clinically relevant concentrations of ketamine decreased LTP in a concentration-dependent manner without changing PPF, whereas basal excitatory synaptic transmission was affected only with higher concentrations of ketamine (300 and 600 μM).

Conclusion: Ketamine impairs LTP in the CA1 region of the mouse hippocampus without affecting PPF, suggesting the predominance of postsynaptic rather than presynaptic effects for ketamine-induced deficits of memory.

5.1.2. INTRODUCTION

Ketamine is used in veterinary and human clinical anesthesia for more than 45 years (Domino, 2010). Ketamine is increasingly popular mainly due to its analgesics properties (Angst and Clark, 2010; Trupkovic *et al.*, 2011) exploited for emergency procedures (Kuznetsova *et al.*, 1984; Rice *et al.*, 2010). However, the use of ketamine has been associated with the disruption of learning and with psychotic effects such post-anesthetic delirium (Sussman, 1974; Irifune *et al.*, 1991).

Alterations in synaptic efficacy in glutamatergic pathways are documented to play a key role in psychopathology (Garcia, 2002). Moreover, activity-dependent synaptic plasticity is considered a cellular mechanism for learning and memory (Bliss and Collingridge, 1993). Paired-pulse facilitation (PPF, a form of short-term plasticity) and long-term potentiation (LTP, a form of long-term plasticity) are two different and important forms of synaptic plasticity (Maruki *et al.*, 2001).

Hippocampal LTP has been closely associated with learning and memory and is well known to be dependent on NMDA receptors (Lynch, 2004), which are considered the main molecular target of ketamine (Davies *et al.*, 1988; Orser *et al.*, 1997). Therefore, it is expected that ketamine may impair synaptic plasticity in the CA1 region of hippocampus. Indeed, an earlier study suggested that dissociative anesthetics including ketamine (30 mg/kg) abolished LTP in rat hippocampus in vivo (Stringer and Guyenet, 1983). However, it is unclear if ketamine selectively affects synaptic plasticity rather than synaptic transmission, which would require comparing the effects of different concentrations of ketamine on synaptic transmission and on synaptic plasticity. Furthermore, LTP is thought to occur mainly via postsynaptic mechanisms (Bliss and Collingridge, 1993), whereas it has been reported that higher concentrations of ketamine (1000 μM) can decrease the amplitude of NMDA population spikes in CA1 neurons induced by paired-pulse stimuli (Wakasugi *et al.*, 1999), a form of short term plasticity dependent on presynaptic mechanisms (Kamiya and Zucker, 1994; Zucker and Regehr, 2002). However, it still remains to be established if clinically relevant concentrations of ketamine indeed affect PPF in the CA1 region of the hippocampus, which would be in agreement with the proposed localization and function of presynaptic NMDA receptors in the glutamatergic terminals in the hippocampus (Musante *et al.*, 2011).

The purpose of this study was to evaluate the effect of different concentrations of ketamine on basal excitatory synaptic transmission and on two forms of synaptic plasticity (LTP and PPF), in order to clarify which type of mechanisms (presynaptic or postsynaptic) are involved in the ketamine action in the CA1 area of mice hippocampus.

5.1.3 MATERIALS AND METHODS

The experiments were performed on hippocampal slices from 5-6 months old females BALB/c mice. Females were chosen since they have been shown more sensitive than males to the psychotomimetic and neurotoxic effects of ketamine (Jevtovic-Todorovic *et al.*, 2001). Details on the animals' husbandry, hippocampal slice preparation and electrophysiological recording are presented in the general methods section (Chapter 2).

5.1.3.1 Drugs used and their administration

Ketamine hydrochloride solution was diluted in aCSF to obtain the desired concentration. For PPF and basal synaptic transmission experiments, each slice was

cumulatively exposed to increasing concentrations of ketamine (1, 3, 10, 30, 100, 200, 300 and 600 μM). For LTP experiments, each slice was exposed to only one concentration of ketamine (3, 10, 30 or 100 μM). For slices of the control group were used aCSF only.

5.1.3.2 Statistical Analysis

The parametric data (effects of different concentrations of ketamine on basal synaptic transmission and PPF) were analyzed using one-way ANOVA followed by Bonferroni post hoc tests and the nonparametric data (effects of different concentrations of ketamine on LTP induction and LTP maintenance) were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn´s multiple comparison test, using SPSS 19 for Windows (IBM Corporation, Armonk, NY). The concentration of ketamine that produces 50% of maximal inhibition (IC_{50}) was calculated from a fitting curve of our data to Hill equations (nonlinear regression analyses) using GraphPad-Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA). All results are expressed as mean±SD. $P \leq 0.05$ was considered statistically significant.

5.1.4 RESULTS

Effects of ketamine on basal excitatory synaptic transmission:

After 20 min of stable baseline recordings with aCSF, applications of consecutive increasing concentrations of ketamine (1, 3, 10, 30, 100 and 200 μM) did not modify significantly ($p>0.05$) excitatory synaptic transmission, as gauged by the lack of alteration of the fEPSP slopes (**figure 5.1A**). Only at the higher concentrations tested (300 and 600 μM) did ketamine decrease synaptic transmission; the concentrations of 300 and 600 μM of ketamine inhibited the fEPSP slope by 22.2±5.3% and 48.1±7.9%, respectively ($n=4$; $p<0.01$) (**figure 5.1A, B, C**). The half-inhibitory concentration (IC_{50}) and Hill coefficient of ketamine to inhibit basal synaptic transmission were 611±34 μM and 2.15±0.26, respectively (n=4). It is worth noting that the inhibition of the basal excitatory synaptic transmission by the higher concentrations of ketamine was not completely eliminated after washout. In fact, following the washout of ketamine, the fEPSPs slopes were 10.6±2.8% lower than before ketamine administration ($n=4$; $p<0.01$) (**figure 5.1A**).

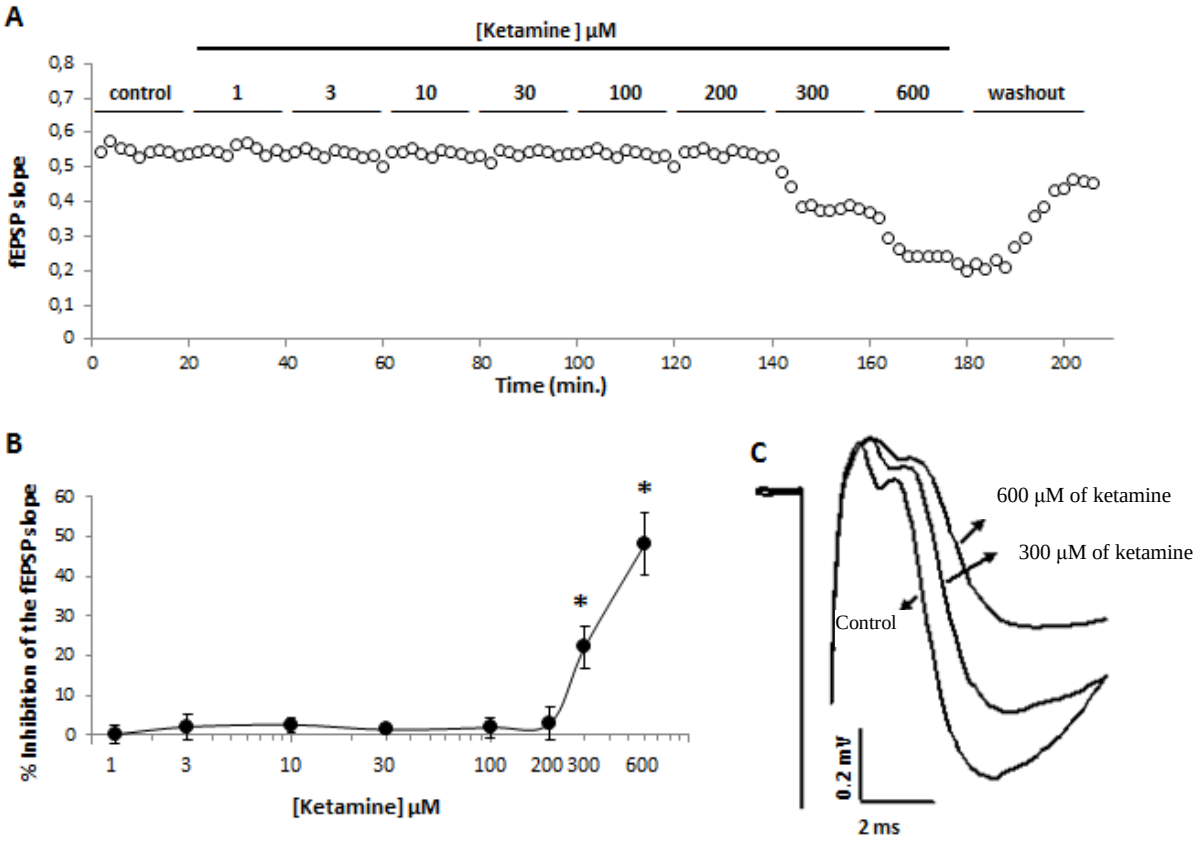


Figure 5.1 Effects of different concentration of ketamine on basal excitatory synaptic transmission **(A)** Time-course of the cumulative effects of increasing concentrations of ketamine on field excitatory postsynaptic potential (fEPSP) slope, regarding an individual experiment. **(B)** Concentration-response curve for cumulative inhibitory effects of ketamine fEPSP slopes using the average results from four experiments; in the ordinates 0% corresponds to the fEPSP slope before ketamine applications and 100% would represent the complete inhibition of fEPSPs (n=4). Significant inhibition of fEPSP slope were observed following 300 and 600µM of ketamine application (*p<0.01). **(C)** Superimposed fEPSP showing the cumulative inhibitions of the higher concentrations of ketamine on fEPSP slope.

Effects of ketamine on paired-pulse facilitation:

As shown in **figure 5.2**, none of tested concentrations of ketamine affected significantly (p>0.05) the paired-pulse facilitation ratio.

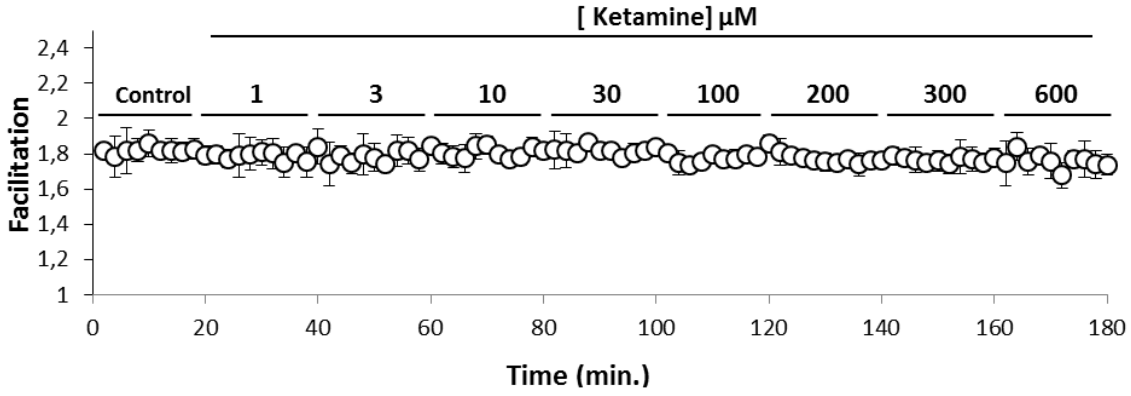


Figure 5.2 Effects of different concentration of ketamine on paired-pulse facilitation (A) Time-course of the effects of increasing cumulative concentrations of ketamine on paired pulse facilitation measured as a ratio of second pulse slope divided by first pulse slope (interpulse interval: 50 ms). No differences were detected between groups (P>0.05).

Effects of ketamine on LTP induction and maintenance:

Under control conditions, HFS effectively triggered LTP since it potentiated fEPSP slope to 225.3±14.8% (n=4) 6 minutes after HFS (LTP induction) (**figure 5.3A**). When present at a concentration of 3 µM, ketamine did not significantly (p>0.05) modify LTP induction. However, at a concentration of 10 µM, ketamine decreased significantly LTP induction to 158.1±9.6% (n=4; p<0.01). Moreover, LTP induction was completely blocked by higher concentrations of ketamine, namely 30 µM (100.6 ±2.4%, n=4; p<0.01) and 100 µM (100.5±0.8%, n=4; p<0.01) (**figure 5.3B and C**). The IC_{50} and Hill coefficient of ketamine to inhibit LTP induction (6 minutes after HFS) were 9.40±1.03 µM and -2.85±0.19 respectively, as calculated from the concentration-response curve shown in **figure 5.3C.**

When we explored the effects of ketamine on LTP maintenance (at 60 minutes after HFS), no significant differences were detected between LTP amplitude in control slices (143.5 ± 8.3%) and in slices treated with 3 µM of ketamine (144.3 ±5.2%; p =1). However, at a concentration of 10 µM, ketamine decreased LTP maintenance to 121.5±4.1% (p<0.01). Moreover, LTP maintenance was abrogated by higher concentrations of ketamine, namely by 30 µM (101.3±0.8%, n=4; p<0.01) and 100 µM (101.5±1.5%, n=4; p<0.01) (**figure 5.3 B and D**).The IC_{50} and Hill coefficient of ketamine to inhibit LTP maintenance (60 minutes after HFS) were 9.97±1.03 µM and -4,103±1,902 respectively, as calculated from the concentration-response curves shown in **figure 5.3D**.

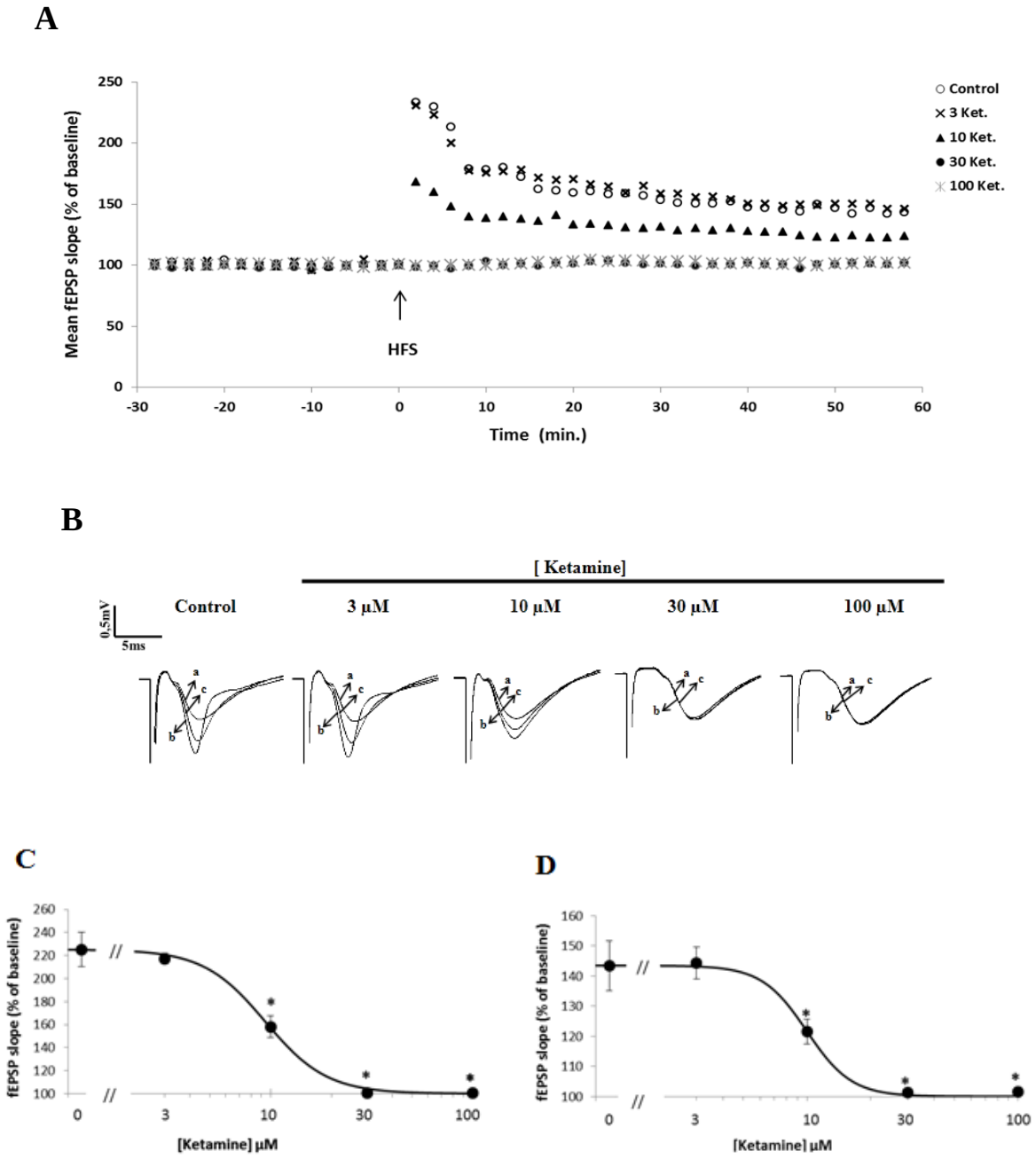


Figure 5.3 Effects of ketamine on long-term potentiation (LTP) induced by high frequency stimulation (HFS) (100 pulses at 100Hz). Mean fEPSP slope (averaged for 10 min.) immediately before HFS is defined as the baseline (100%). **(A)** Time-course of the effects of different concentrations of ketamine on LTP measured by field excitatory postsynaptic potential (fEPSP) slope (n=4). Data is showed as mean. **(B)** Superimposed fEPSP representative of the inhibitory effect of different concentrations of ketamine on LTP. a = fEPSP just before HFS; b= fEPSP immediately after HFS; c= fEPSP 60 min. after HFS. **(C)** Concentration-response curve for LTP induction. Average of fEPSP slope of the first 6 minutes after HFS was taken as a measure of LTP induction. Data is showed as mean ±SDs. *p<0.01. (D) Concentration-response curve for LTP maintenance. Average of fEPSP slope from 54 to 60 minutes after HFS was taken as a measure of maintenance of LTP. Data is showed as mean ±SDs. *p<0.01

5.1.5 DISCUSSION

The outcome of this work shows that clinically relevant concentrations of ketamine affect long-term potentiation (LTP) in a concentration-dependent manner, without affecting paired-pulse facilitation (PPF). Moreover, basal excitatory synaptic transmission was affected by high concentrations of ketamine (300 and 600 µM).

The concentrations of ketamine tested in the current study included subanesthetics and anesthetics concentrations in mice. Furthermore, the current study included some plasma concentrations used in clinical human settings. Human plasma concentrations of ketamine for analgesia are around 0.55 µM and for anesthesia about 10 µM (White *et al.*, 1980; Grant *et al.*, 1983). In mice, anesthetic doses are around 100-200 mg/kg and the corresponding serum concentration are between 30-60 µg/ml (Maxwell *et al.*, 2006) or 100-200 µM of ketamine. This drug in plasma exists in two states: free unbound form and bound to plasma proteins. The levels of free unbound determine the rate of diffusion to the site of action. In humans, as much as 47% of ketamine is bound to plasma proteins (Dayton *et al.*, 1983). However, ketamine is highly lipid soluble with brain-to-serum/plasma ratios estimated to be 3.33:1 in mice (Maxwell *et al.*, 2006) and 6.5:1 in rat, respectively, indicating that ketamine preferentially accumulates in the brain. Thus it is possible that, during anesthesia the concentration of ketamine present in brain is considerably higher than the plasma concentration. With that in mind, we studied the effects of a wide range of relevant concentrations of ketamine (1, 3, 10, 30, 100, 200, 300 and 600µM) in hippocampal slices. However, the extrapolation of our experimental results to clinical human practice requires extreme caution, as differences in drug concentrations and exposure times as well as interspecies variations are all important variables which need to be considered.

Regarding the effects of ketamine on basal excitatory synaptic transmission, our results showed no effects induced by 1, 3, 10, 30, 100, 200 µM of ketamine at 0.067 Hz. Only higher concentrations (300 and 600 µM) decreased basal excitatory synaptic transmission. These results are in agreement with our expectations because NMDA receptors usually do not contribute to basal excitatory synaptic transmission and are only activated under certain conditions (Danysz and Parsons, 2003). The decrease observed with higher concentrations could be explained by nonspecific toxic depression of the neuronal events (Lewis *et al.*, 2001), since 300 and 600 µM are very high concentrations.

Few studies were found regarding the effects of ketamine on basal excitatory synaptic transmission. However, it was reported that 10 and 100μM of ketamine increased the fEPSP amplitude at 0,05 and 0,2Hz in male rats with 7-8 weeks (Narimatsu *et al.*, 2002). These results are in disagreement with our results; differences between species, age, sex and stimulation frequency could be responsible for the discrepancy.

Results from the current study also showed that ketamine (1-600 μM) did not affect CA1 hippocampal paired-pulse facilitation. This form of plasticity occurs through presynaptic mechanisms and it can be used as a presynaptic index for probability of neurotransmitter release (Kamiya and Zucker, 1994; Zucker and Regehr, 2002) . In control conditions (aCSF slices) the response to the second of a pair of stimulation pulses is higher than that of the first pulse. Residual presynaptic calcium, left after the first stimulation, is thought to enhance neurotransmitter release in response to the second stimulation. Therefore, our findings suggest that ketamine does not affect dynamic changes in transmitter release that are required for paired-pulse facilitation at Schaffer collateral terminals. As found in literature, higher concentrations of ketamine (1000 μM) decreased the NMDA population spikes in the area CA1 of rat hippocampus induced by paired-pulse stimuli (Wakasugi *et al.*, 1999). However, no studies were found regarding the effect of clinically relevant concentrations of ketamine in PPF in CA1 region of hippocampus. Usually, PPF has been shown to increase after manipulations that reduce calcium-mediated glutamate release from the Schaffer-collateral-commissural pathway (Manabe *et al.*, 1993). In contrast, manipulations that depress CA1 neuron fEPSPs *via* postsynaptic actions do not alter PPF (Zucker, 1989; Manabe *et al.*, 1993). This last topic was confirmed with our results regarding the effect of ketamine on LTP at Schaffer collateral-CA1 synapses. LTP, a long-lasting change in synaptic efficacy, occurs mainly via postsynaptic mechanisms (Bliss and Collingridge, 1993) and we observed that ketamine decreased/inhibited the induction and maintenance of LTP in the CA1 region of the hippocampus. Therefore, it seems that ketamine affects postsynaptic events required for LTP in the CA1 region of hippocampus. This effect was concentration dependent; concentrations above 30 μM of ketamine blocked completely the LTP induction. These results could be explained because the induction of LTP is dependent of NMDA receptors and ketamine induced blocking of NMDA receptor (Davies *et al.*, 1988; Orser *et al.*, 1997). Moreover, the effect of ketamine on LTP was pronounced in the early phase of LTP (LTP induction: up to approximately 6 min after HFS). A major mechanism of LTP induction is an increased calcium influx through both N-methyl-D-aspartate (NMDA) receptors and voltage-gated

calcium channels (Grover and Teyler, 1990). When ketamine (NMDA antagonist) was present this mechanism was decreased/blocked in a concentration-dependent manner and so LTP induction was decreased/blocked; consequently LTP maintenance (60 min after HFS) was also affected. Few studies were found that tested directly the effects of ketamine in hippocampal LTP. An earlier study showed that dissociative anesthetics, including ketamine, may abolish in vivo hippocampal LTP in rats (Stringer and Guyenet, 1983). Our work was performed in *ex vivo* conditions and showed results in agreement with that study.

In summary, we have shown that ketamine decreased long-term potentiation in CA1 region of mouse hippocampus without affecting paired-pulse facilitation, suggesting the importance of postsynaptic mechanisms, in detriment of presynaptic mechanisms, for ketamine induce deficits in memory after anesthesia or analgesic procedures.

5.2. EFFECTS OF DIFFERENT CONCENTRATIONS OF MEDETOMIDINE ON EXCITATORY SYNAPTIC TRANSMISSION AND ON SYNAPTIC PLASTICITY (PPF AND LTP)

5.2.1 ABSTRACT

Background: Alfa$_2$-adrenoceptors agonists are frequently used in veterinary and human anesthesia. Medetomidine, a α_2-adrenoceptors agonist, is a sedative/analgesic drug. However, it has been reported that the pharmacological manipulation of the noradrenergic system can affect the memory. Little is known about the effect of this drug on basal excitatory synaptic transmission and synaptic plasticity. Therefore we studied the effects of different concentrations of medetomidine on basal excitatory synaptic transmission and on two forms of synaptic plasticity: PPF and LTP.

Material and methods: Evoked field excitatory postsynaptic potentials were recorded in Schaffer fiber collaterals - CA1 pyramidal cell synapses of mouse hippocampal slices. Four slices per group and experiment were used. For basal synaptic transmission, and PPF, increasing concentrations of medetomidine (from 1 to 200 μM) were applied to each slice. For LTP experiments, individual slices were used for each medetomidine concentration (from 0.1 to 0.4 μM). LTP was induced by high-frequency stimulation (100 pulses at 100Hz). The synaptic transmission strength was assessed by measuring the initial slope of the fEPSP. LTP

induction and maintenance were calculated. PPF was estimated as the ratio between the slopes of the second and first paired pulses, applied 50 milliseconds apart.

Results: Medetomidine decreased basal excitatory synaptic transmission and LTP in a concentration-dependent manner. PPF only was affected by the highest concentration (200 μM) of medetomidine used.

Conclusion: Medetomidine decreased mainly LTP and basal excitatory synaptic transmission rather than PPF in the CA1 region synapses of the mouse hippocampus, suggesting the importance of postsynaptic mechanisms in detriment of presynaptic mechanisms to the sedative action of medetomidine on brain.

5.2.2 INTRODUCTION

Alfa$_2$-adrenoceptors agonists are frequently used in veterinary and human clinical anesthesia (Maze and Tranquilli, 1991). These drugs produce sedation, analgesia, reduce anesthetic requirements and improve perioperative hemodynamic stability (Tanaka and Nishikawa, 1994; Hall *et al.*, 2000).

Medetomidine is a potent and selective α_2-adrenoceptor agonist (α_2/α_1 selectivity ratio of 1620/1, as measured by the displacement of [^{3}H]clonidine (Virtanen *et al.*, 1988)), that also has affinity for imidazoline receptors (Wikberg *et al.*, 1991). This drug provides good anesthetic stability and reduces the post-anesthetic delirium and agitation produced by other drugs, such as ketamine (Ribeiro *et al.*, 2012). However, it was reported that pharmacological manipulation of the noradrenergic system can affect the memory performance (Chamberlain *et al.*, 2006). PPF and LTP are two important forms of short- and long-term plasticity, respectively (Maruki *et al.*, 2001). These mechanisms are considered cellular instruments for learning and memory formation (Bliss and Collingridge, 1993). The effect of medetomidine on hippocampal basal excitatory synaptic transmission and/or on synaptic plasticity on adult mice remains to be studied. Previous works reported that α_2-adrenoceptor activation can reduce noradrenergic synaptic transmission and block synaptic plasticity at glutamatergic synapses of the basolateral amygdala (DeBock *et al.*, 2003). Beyond that the α_2-adrenoceptor agonist clonidine reduced LTP in the occipital cortex of rats (Mondaca *et al.*, 2004) and the α_2-adrenoceptor agonist dexmedetomidine decreased LTP in the hippocampus young mice, without affecting PPF (Takamatsu *et al.*, 2008).

The purpose of this study was to evaluate the effect of different concentrations of medetomidine on basal excitatory synaptic transmission and on synaptic plasticity in order to clarify which type of mechanisms (presynaptic or postsynaptic) are affected by medetomidine in the CA1 area of adult mice hippocampus; that may underlie the memory deficits caused by this compound.

5.2.3 MATERIALS AND METHODS

The experiments were performed on hippocampal slices from 5-6 months old females BALB/c mice. Details on the animals' husbandry, hippocampal slice preparation and electrophysiological recording are presented in the general methods section (Chapter 2).

5.2.3.1 Drugs used and their administration

Medetomidine, ((±)-4-[1-(2,3-dimethylphenyl) ethyl]-1H-imidazole monohydrochloride) solution. The drug was diluted in aCSF to obtain the desired concentration. For PPF and basal synaptic transmission experiments, each slice was cumulatively exposed to increasing concentrations of medetomidine (1, 2, 4, 8, 12, 24, 48, 100 and 200 μM) for 20 min each. The effect of a given medetomidine concentration on PPF was determined using the last 6 min of application. For LTP experiments, each slice was exposed to only one concentration of medetomidine (0.1, 0.2 or 0.4 μM). The drug was introduced 30 min before LTP induction and was maintained throughout the experiment. In control slices were applied only aCSF.

5.2.3.2 Statistical Analysis

All data (effects of different concentrations of medetomidine on basal synaptic transmission, on PPF and on LTP induction and LTP maintenance) were parametric data and were analyzed using one-way ANOVA followed by Bonferroni post hoc tests, using SPSS 19 for Windows (IBM Corporation, Armonk, NY). The concentration of medetomidine that produces 50% of maximal inhibition (IC50) was calculated from a fitting curve of our data to a sigmoidal log dose-response normalized inhibition curve with variable slope (nonlinear regression analysis) using GraphPad-Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA). All results are expressed as mean ±SD. $P \leq 0.05$ was considered statistically significant.

5.2.4 RESULTS

Effects of different concentrations of medetomidine on basal synaptic transmission:

After 20 min of stable baseline recordings with aCSF, application of consecutively increasing concentrations of medetomidine (1, 2, 4, 8 and 12 μM) did not modify significantly ($p>0.05$) synaptic transmission, as gauged by the lack of alteration of the fEPSP slopes (**figure 5.4A**). When higher concentrations of medetomidine were applied (24, 48 and 100 μM), synaptic transmission decreased. More specifically, at medetomidine concentrations of 24, 48, 100 and 200 μM the fEPSP slope was inhibited by 9.65±1.77%, 18.40±0.94%, 44.31±5.01% and 92.16±2.77%, respectively ($n=4$; $p<0.01$) (**figure 5.4A, B and C**). This inhibition was completely reverted after washout ($p=1$; $n=4$) (**figure 5.4A**). The half-inhibitory concentration (IC_{50}) and Hill coefficient of medetomidine to inhibit basal synaptic transmission were 98.9±6.4 μM and 2.46±0.35, respectively ($n=4$).

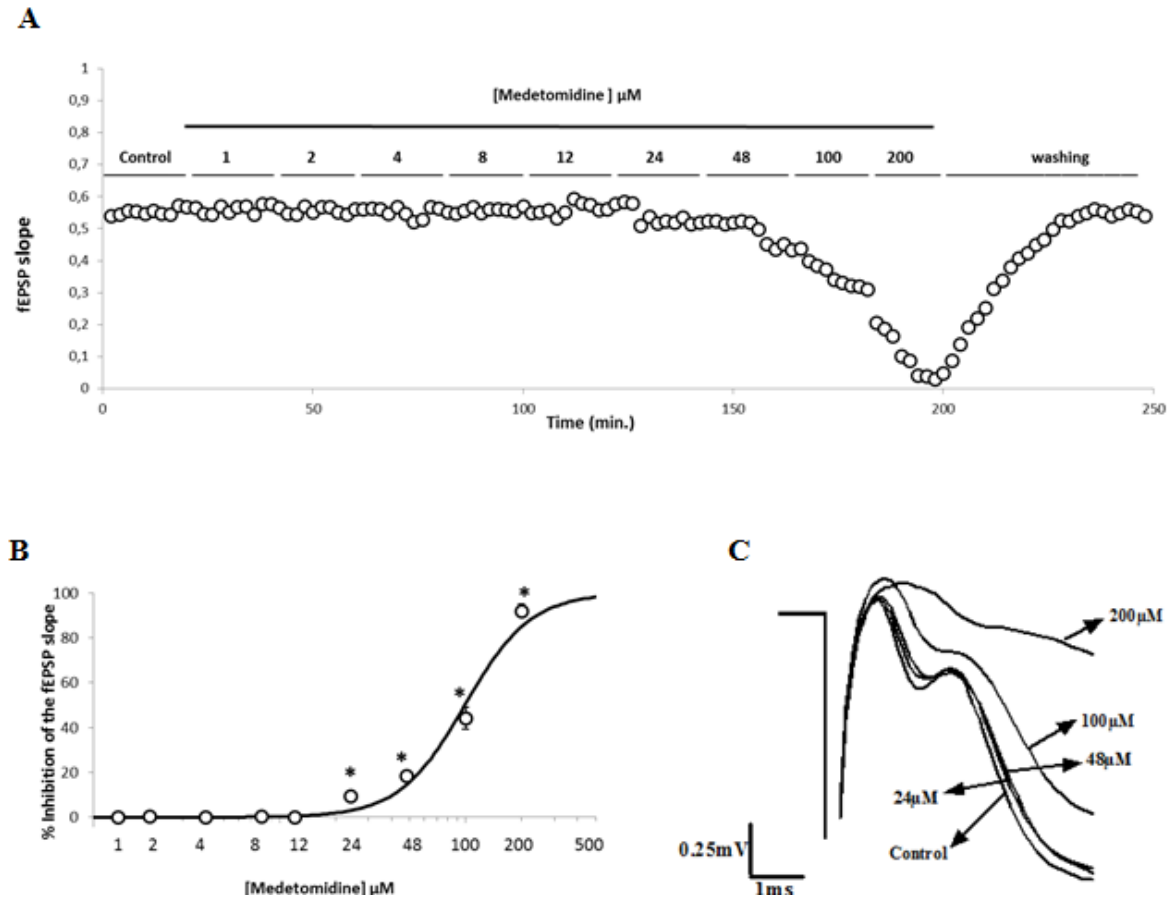


Figure 5.4 Effects of different concentration of medetomidine on basal synaptic transmission **(A)** Time-course of the cumulative effects of increasing concentrations of medetomidine on field excitatory postsynaptic potential (fEPSP) slope, regarding an individual experiment. **(B)** Concentration-response curve for cumulative inhibitory effects of medetomidine fEPSP slopes using the averaged results from four experiments; in the ordinates 0% corresponds to the fEPSP slope before medetomidine applications and 100% would represent the complete inhibition of fEPSPs (n=4). Significant inhibition of fEPSP slope were observed following 24, 48, 100 and 200 µM of medetomidine application (*$p<0.01$). **(C)** Superimposed fEPSP showing the cumulative inhibitions of the higher concentrations of ketamine on fEPSP slope.

Effects of medetomidine on paired-pulse facilitation:

Only the highest concentration of medetomidine tested (200 µM) significantly affected the paired-pulse facilitation ratio ($p<0.01$) (**figure 5.5**).

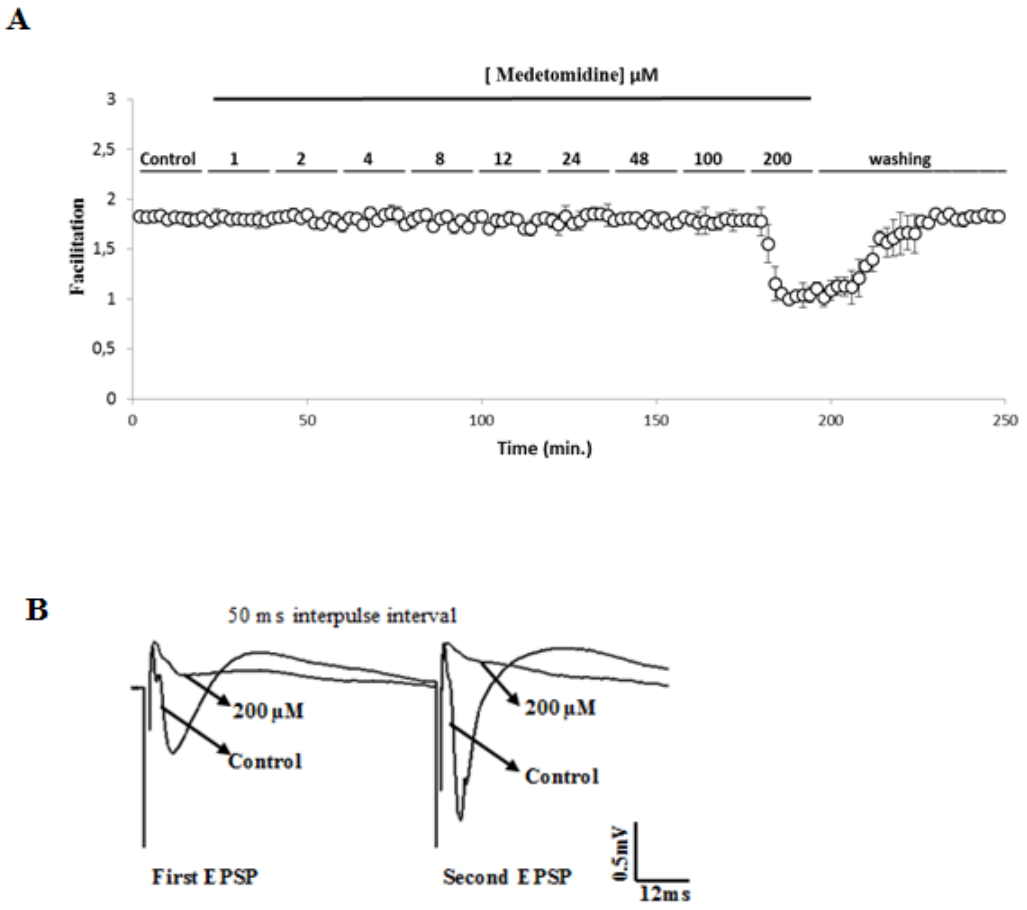


Figure 5.5 Effects of different concentration of medetomidine on paired-pulse facilitation (A) Time-course of the effects of increasing cumulative concentrations of medetomidine on paired pulse facilitation measured as a ratio of second pulse slope divided by first pulse slope (interpulse interval: 50 ms). No differences were detected between groups, except with the highest concentration of medetomidine that decreased significantly PPF ($p=0$). (B) Superimposed fEPSP showing the cumulative inhibitions of higher concentrations of medetomidine on first and second fEPSPs.

Effects of medetomidine on LTP induction and maintenance:

Under control conditions, HFS increased fEPSP slope to 230.35±11.20% (n=4) in first 6 minutes after its application (LTP induction), relative to the baseline value (**figure 5.6A**). When present at a concentration of 0.1 μM, medetomidine did not change significantly ($p>0.05$) LTP induction. However, at concentrations of 0.2 and 0.4 μM, medetomidine decreased significantly LTP induction to 192.19±14.33% and 159.37±5.35%, respectively (n=4; $p<0.01$) (**figure 5.6B and C**). The IC_{50} and Hill coefficient values for the inhibition of LTP induction by medetomidine were 0.215 μM and -2.66 respectively, as calculated from the concentration-response curve shown in **figure 5.6C**.

When we explored the effects of medetomidine on LTP maintenance (between 54 and 60 minutes after HFS), no significant differences were detected between LTP amplitude in control slices (148.82±2.27%) and in slices treated with 0.1 μM of medetomidine (146.86±2.24%; $p =1$). However, medetomidine at 0.2 μM decreased LTP maintenance to 125.09±7.44% ($p<0.01$). Moreover, LTP maintenance was abrogated by the highest concentration of medetomidine, namely 0.4 μM (103.03±5.72%, n=4; $p<0.01$) (**figure 5.6B and D**). The IC_{50} and Hill coefficient values for the inhibition of LTP maintenance by medetomidine were 0.2033±0.0021 μM and -4.20±0.23 respectively, as calculated from the concentration-response curves shown in **figure 5.6D**.

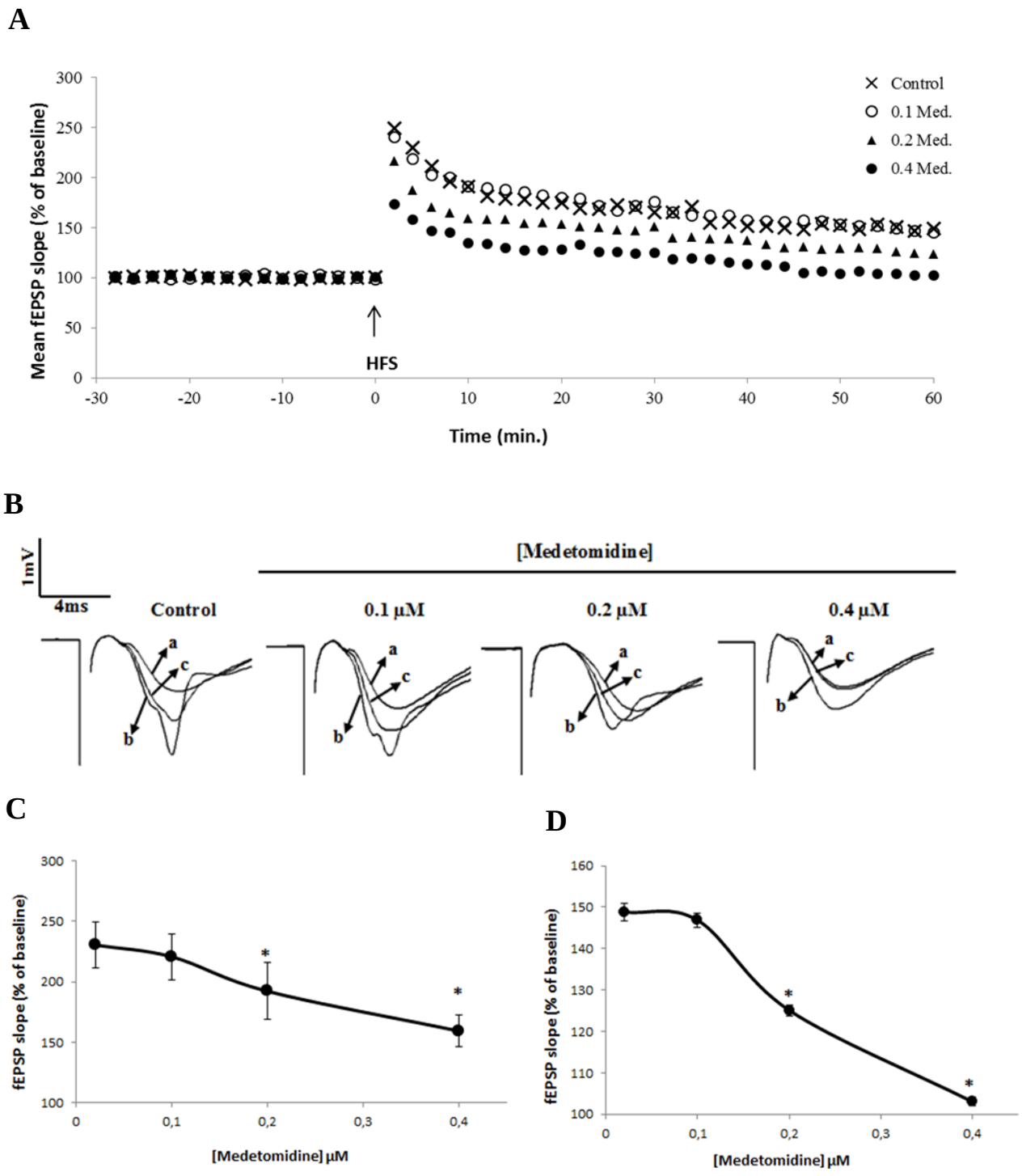


Figure 5.6 Effects of medetomidine on long-term potentiation (LTP) induced by high frequency stimulation (HFS) (100 pulses at 100Hz). Mean fEPSP slope (averaged for 10 min.) immediately before HFS is defined as the baseline (100%). **(A)** Time-course of the effects of different concentrations of medetomidine on LTP measured by field excitatory postsynaptic potential (fEPSP) slope (n=4). Data is showed as mean.
(B) Superimposed fEPSP representative of the inhibitory effect of different concentrations of medetomidine on LTP. a = fEPSP just before HFS; b= fEPSP immediately after HFS; c= fEPSP 60 min. after HFS. **(C)** Concentration-response curve for LTP induction. Average of fEPSP slope of the first 6 minutes after HFS was taken as a measure of LTP induction. Data is showed as mean ±SDs. *p<0.01
(D) Concentration-response curve for LTP maintenance. Average of fEPSP slope from 54 to 60 minutes after HFS was taken as a measure of maintenance of LTP. Data is showed as mean ±SDs. *p<0.01

5.2.5 DISCUSSION

In this work, we showed that medetomidine affected significantly basal excitatory synaptic transmission and LTP in a concentration-dependent manner. PPF was only affected by the highest concentration of medetomidine tested.

Regarding the effects of medetomidine on basal excitatory synaptic transmission, our results showed that, at lower concentrations, medetomidine (1, 2, 4, 8 and 12 μM) did not affect synaptic transmission in hippocampal slices of mice stimulated at 0.067 Hz. However, when higher concentrations of medetomidine were applied (24, 48, 100 and 200 μM), synaptic transmission decreased in a concentration-dependent manner. These results are in agreement with the observation that activation of presynaptic noradrenaline α_2-adrenergic receptors inhibits transmission at glutamatergic hippocampal synapses (Boehm, 1999).

The current study also showed that medetomidine (1-100 μM) did not affect CA1 hippocampal PPF. This decreased only when the highest concentration of medetomidine was applied (200 μM). This form of plasticity occurs through presynaptic mechanisms and it can be used as a presynaptic index for probability of neurotransmitter release (Kamiya and Zucker, 1994; Zucker and Regehr, 2002) . Under control conditions in aCSF slices, the response to the second of a pair of stimulation pulses is higher than that of the first response. Residual presynaptic calcium, after the first stimulation, is thought to enhance neurotransmitter release in response to the second stimulation. Therefore, our findings suggest that medetomidine did not affect dynamic changes in transmitter release that are required for paired-pulse facilitation at Schaffer collateral terminals, except when the highest concentration was applied. However, this concentration is so high that nonspecific events cannot be excluded. No studies were found regarding the direct effects of the medetomidine in paired-pulse facilitation in the CA1 region from the hippocampus. When dexmedetomidine (dextro enantiomer of medetomidine) were applied in concentration of 50 nM did not affect paired-pulse facilitation in CA1 region of hippocampus of young mice (Takamatsu *et al.*, 2008). As similar pharmacological profiles were observed between dexmedetomidine and the racemate medetomidine (Savola and Virtanen, 1991), this result is in agreement with our results. However, we study the effects of different concentrations of medetomidine (1, 2, 4, 8, 12, 24, 48, 100 and 200 μM) in fully into adulthood mice.

Usually, PPF has been shown to increase after manipulations that reduce calcium-mediated glutamate release from the Schaffer-collateral-commissural pathway (Manabe *et al.*, 1993). In

contrast, manipulations that depress CA1 neuron fEPSPs *via* postsynaptic actions do not alter PPF (Zucker, 1989; Manabe *et al.*, 1993). This last topic was confirmed by our results regarding the effect of medetomidine on LTP at Schaffer collateral-CA1 synapses. LTP, a long-lasting change in synaptic efficacy, occurs mainly via postsynaptic mechanisms (Bliss and Collingridge, 1993) and we observed that very low concentrations of medetomidine decreased the induction and maintenance of LTP in the CA1 region of the hippocampus. Therefore, it seems that medetomidine affects postsynaptic events required for LTP in the CA1 region of hippocampus. This observation showed a concentration-dependent response; concentrations of 0.2 μM and 0.4 μM of medetomidine decreased or blocked completely the LTP maintenance, respectively. No studies were found regarding the effects of medetomidine on LTP. The alpha$_2$-adrenoceptor agonist clonidine, reduced dose-dependently the long-term potentiation elicited in vivo in the occipital cortex of anesthetized rats (Mondaca *et al.*, 2004) and dexmedetomidine decreased long-term potentiation in the hippocampal slices of young mouse. Our results are in agreement with these previous studies. The mechanisms by which medetomidine decreased/blocked may be explained by the effects of medetomidine in synaptic transmission; i.e. the alterations of noradrenergic system. Moreover, it was reported that others α_2-adrenoceptors agonist decreased LTP via imidazoline system (Takamatsu *et al.*, 2008) and so the affinity of medetomidine for activation of imidazoline receptors may have implications in LTP reduction observed in our results.

In summary, we have shown that medetomidine affect mainly long-term potentiation in CA1 region of mouse hippocampus and basal excitatory synaptic transmission. Paired-pulse facilitation was not affected, except when a very high concentration was applied, suggesting the importance of the postsynaptic mechanisms in detriment of the presynaptic mechanisms for medetomidine induce deficits in memory after sedation or analgesic procedures.

5.3 EFFECT OF KETAMINE/MEDETOMIDINE COMBINATION ON BASAL EXCITATORY SYNAPTIC TRANSMISSION AND ON SYNAPTIC PLASTICITY (PPF AND LTP) IN HIPPOCAMPAL SLICES OF ADULT MICE

5.3.1ABSTRACT

Background: The combination of ketamine with α_2-adrenergic agonists is frequently used in clinical and research to induce anesthesia. However, little is known about the action of these drugs in combination on hippocampal synapses. Therefore we investigated the effects of ketamine/medetomidine combination on basal excitatory synaptic transmission and on synaptic plasticity: PPF and LTP, in the CA1 region of mouse hippocampal slices.

Methods: Evoked field excitatory postsynaptic potentials were recorded in Schaffer fiber collaterals - CA1 pyramidal cell synapses of mouse hippocampal slices. Three slices per group and experiment were used. For basal synaptic transmission, and PPF, increasing concentrations of ketamine combined with medetomidine: 30+1, 100+4, 200+8, 300+12 and 600+24 (μM of ketamine + medetomidine, respectively) were applied in hippocampal slices. For LTP experiments, 3 μM of ketamine combined with 0.1 μM of medetomidine were applied to slices. LTP was induced by high-frequency stimulation (100 pulses at 100 Hz). The synaptic transmission strength was assessed by measuring the initial slope of the fEPSP. LTP induction and maintenance were calculated. PPF was estimated as the ratio between the slopes of the second and first paired pulses.

Results: Ketamine/medetomidine combination did not affected paired-pulse facilitation, whereas the basal excitatory synaptic transmission only was affected when higher concentrations were applied. Moreover, LTP decreased when ketamine concentrations were combined with medetomidine.

Conclusion: The combination of ketamine with medetomidine impairs LTP in the CA1 region of the mouse hippocampus without affecting PPF, suggesting the importance of postsynaptic mechanisms in the action of ketamine/medetomidine combination on hippocampus.

5.3.2 INTRODUCTION

The combination of ketamine with medetomidine is frequently used in human and veterinary clinical anesthesia (Verstegen *et al.*, 1990; Scher and Gitlin, 2003; Hahn *et al.*, 2005; Brennen *et al.*, 2011). This combination reduce anesthetic requirements and improve perioperative hemodynamic stability (Levanen *et al.*, 1995; Hall *et al.*, 2000) and an interesting reduction of adverse effects on the CNS caused by ketamine (Levanen *et al.*, 1995).

Previously we reported that ketamine alone decreased basal synaptic transmission and synaptic plasticity in hippocampal slices of mice in a concentration-dependent effect (Chapter 5.1). Similarly, medetomidine alone also decreased synaptic transmission and synaptic plasticity in hippocampal slices of mice (Chapter 5.2). However, the action of these two drugs combined on basal synaptic transmission and synaptic plasticity in the hippocampus has yet to be established. Therefore, the purpose of this study was to evaluate the effect of different concentrations of ketamine combined with α_2-adrenoceptor agonist medetomidine on basal excitatory synaptic transmission and synaptic plasticity (LTP and PPF) in Schaffer collateral CA1 hippocampus slices in mice, in order to clarify which type of mechanisms (presynaptic or postsynaptic) are involved in the ketamine/medetomidine combination action in the CA1 area of mice hippocampus.

5.3.3 MATERIALS AND METHODS

The experiments were performed on hippocampal slices from 5-6 months old females BALB/c mice. Details on the animals' husbandry, hippocampal slice preparation and electrophysiological recording are presented in the general methods section (chapter 2).

5.3.3.1 Drugs used and their administration

Ketamine and medetomidine were diluted in aCSF to obtain the desired concentration.

For basal excitatory synaptic transmission and PPF experiments, increasing concentrations of ketamine combined with medetomidine were applied in hippocampal slices (30+1, 100+4, 200+8, 300+12 and 600+24 μM of ketamine+ medetomidine concentrations, respectively). These concentrations were based in previous works (chapter 5.1 and 5.2) and in

one approximate proportion used in laboratory animals to induce anesthesia by i.p. injection in mice, 25:1 (25 mg/kg ketamine to 1 mg/kg medetomidine). Slices of the control group were superfused only with aCSF. Each concentration was applied during 20 minutes. Besides that, a new concentration only was initiated after a stable value of slope of fEPSP was obtained for the previous concentration.

For LTP experiments, each slice was exposed to only one concentration of ketamine combined with medetomidine (3 μM of ketamine with 0.1 μM of medetomidine). This combination of concentrations was selected after analyze the effect of the drugs when were applied alone in LTP (chapter 5.1 and 5.2). Concentrations of ketamine and medetomidine that had not affected LTP were selected to make this combination and consequently to assess one possible synergic or additive effect between drugs on LTP.

5.3.3.2 Statistical Analysis

Data regarding the effects of different concentrations of ketamine combined with medetomidine on basal synaptic transmission, PPF and LTP, showed a normal distribution and were analyzed using one-way ANOVA followed by Bonferroni post hoc tests. All results were analyzed using SPSS 19 for Windows (IBM Corporation, Armonk, NY) and expressed as mean ±SD. $P \leq 0.05$ was considered statistically significant.

All results obtained previously with ketamine and medetomidine alone (Chapter 5.1 and 5.2) were compared with results obtained with ketamine/medetomidine combination to assess the type of interaction between these two drugs on excitatory synaptic transmission and on synaptic plasticity.

5.3.4 RESULTS

Effects of ketamine/medetomidine combination *versus* ketamine and medetomidine alone on basal excitatory synaptic transmission:

Applications of consecutive increasing concentrations of ketamine combined with medetomidine (30+1, 100+4 and 200+8μM respectively) did not modify ($p>0.05$) synaptic transmission, as gauged by the lack of alteration of the fEPSP slopes (**figure 5.7 A**). Higher concentrations of ketamine combined with medetomidine (300+12 and 600+24 μM) decreased the excitatory synaptic transmission; thus the concentrations of the 300+12 and

600+24 µM of ketamine+medetomidine inhibited the fEPSP slope by 23.3±1.7% and 51.3±1.6%, respectively (n=4; p<0.01) (**figure 5.7 B**)

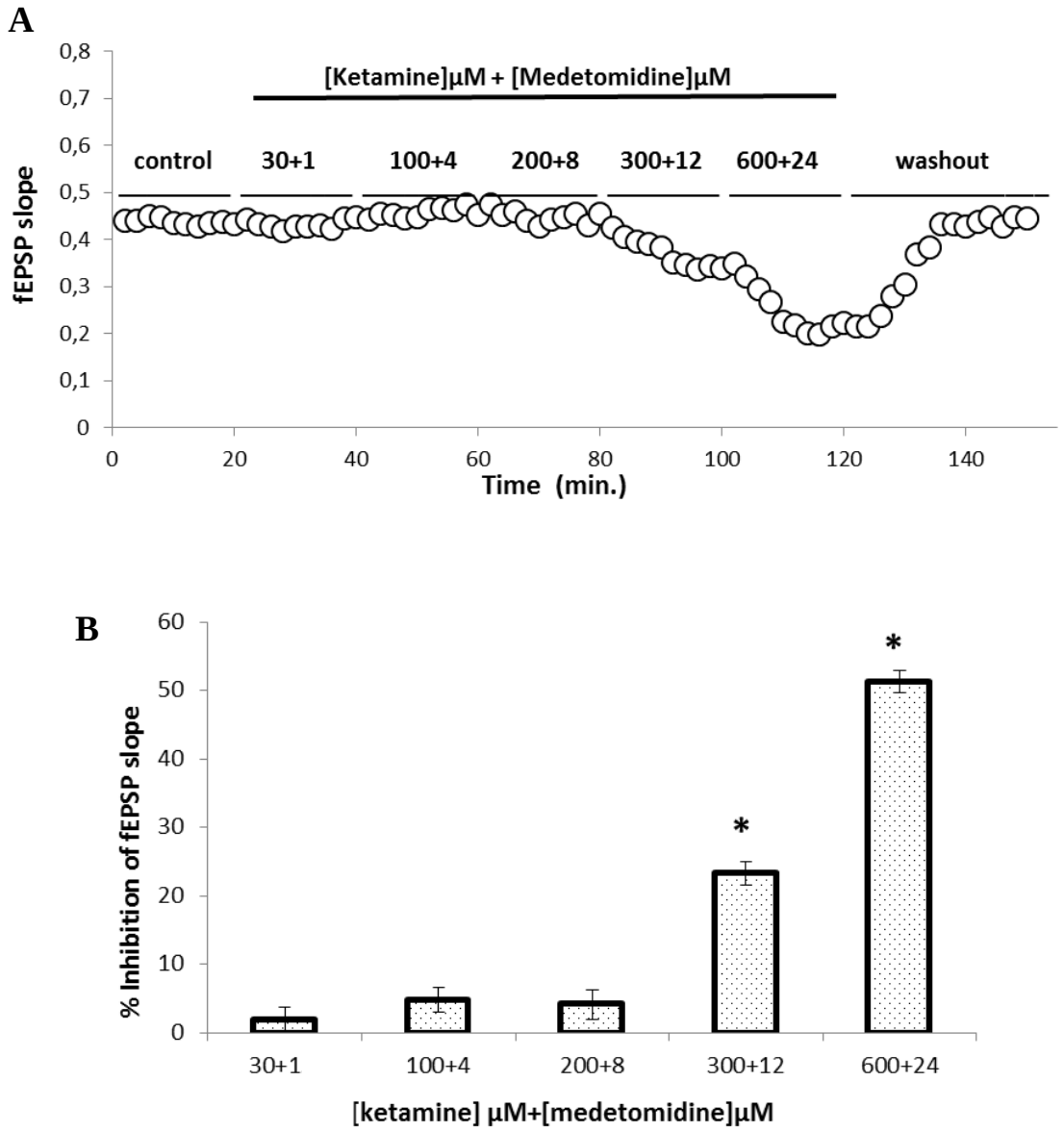


Figure 5.7 Effects of different concentration of ketamine combined with medetomidine on basal synaptic transmission **(A)** Time-course of the cumulative effects of increasing concentrations of ketamine combined with medetomidine on field excitatory postsynaptic potential (fEPSP) slope, regarding an individual experiment. **(B)** Concentration-response bars for cumulative inhibitory effects of ketamine/ medetomidine combination fEPSP slopes using the averaged results from three experiments; in the ordinates 0% corresponds to the fEPSP slope before drugs applications and 100% would represent the complete inhibition of fEPSPs. Significant inhibition of fEPSP slope were observed following application of the 300+12 and 600+24µM of ketamine + medetomidine concentration, respectively (*p<0.01).

These results do not differ significantly from those obtained with the administration of ketamine alone in chapter 5.1. The concentrations of the 300 µM and 600µM of ketamine

alone inhibited the fEPSP slope by 22.2±5.3% and 48.1±7.9%, respectively ($p<0.01$) (**figure 5.8**) and lower concentrations did not affect the basal synaptic transmission ($p\geq0.05$)

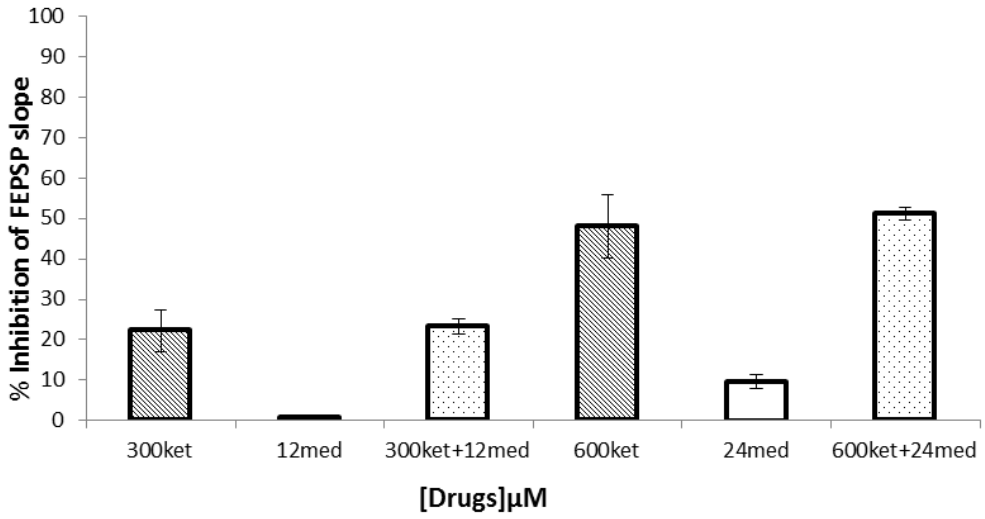


Figure 5.8 Percentage of inhibition of the fEPSP slope caused by different concentrations of ketamine (ket) and medetomidine (med) combined or alone; in the ordinates 0% corresponds to the fEPSP slope before drugs applications and 100% would represent the complete inhibition of fEPSPs. No significant differences were observed between the % of inhibitions of fEPSP slope caused ketamine alone or combined with medetomidine.

Effects of ketamine and metetomidine combined versus ketamine and metetomidine alone on paired-pulse facilitation:

As shown in **figure 5.9**, none of tested concentrations of ketamine combined with medetomidine affected significantly ($p>0.05$) the paired-pulse facilitation ratio. Similarly, the same doses of ketamine and medetomidine alone also did not affect paired-pulse facilitation (chapter 5.1 and 5.2).

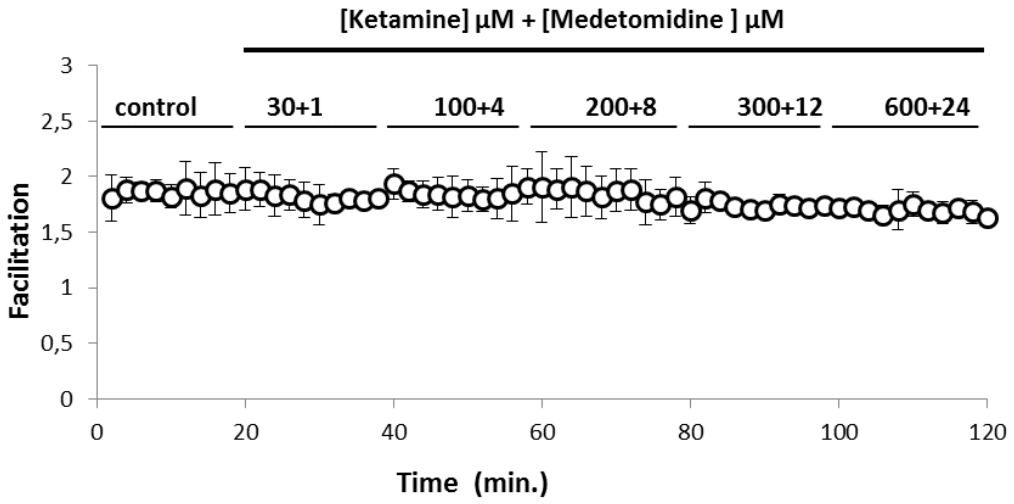
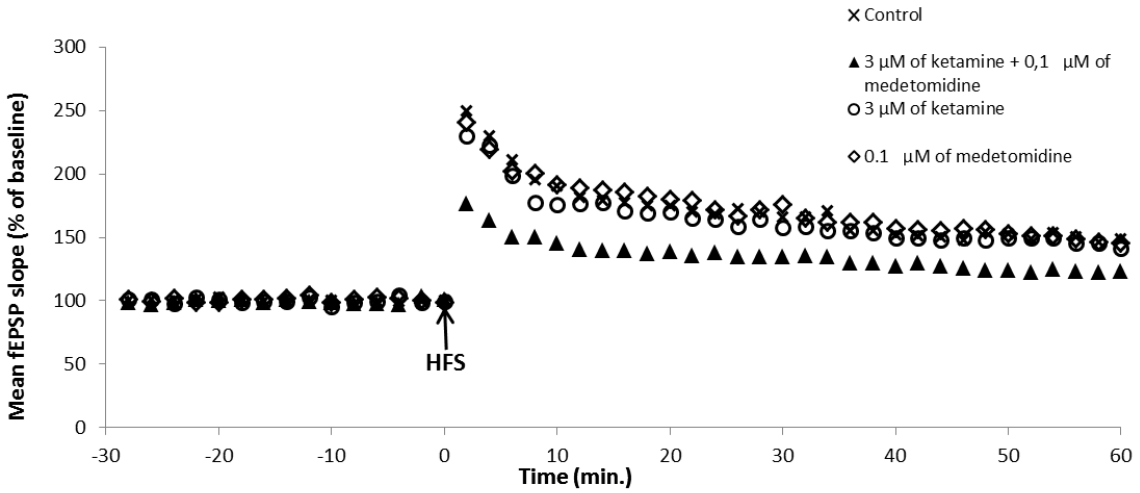


Figure 5.9 Time-course of the effects of increasing cumulative concentrations of ketamine combined with medetomidine on paired pulse facilitation measured as a ratio of second pulse slope divided by first pulse slope (interpulse interval: 50 ms).

Effects of ketamine and metetomidine combined versus ketamine and metetomidine alone on LTP induction and maintenance:

The LTP induction and LTP maintenance, in hippocampal slices, decreased significantly, when 3 µM of ketamine was combined with 0.1 µM of medetomidine, in comparison with control slices (**figure 5.10 A and B**). Moreover, the effect of this combination on LTP differs significantly of the drugs administered alone. As we reported in chapter 5.1 and 5.2, when only one of these two drugs was administered, no significant differences were detected on LTP induction and maintenance in comparison with the control slices (**figure 5.10 A and B**).

A



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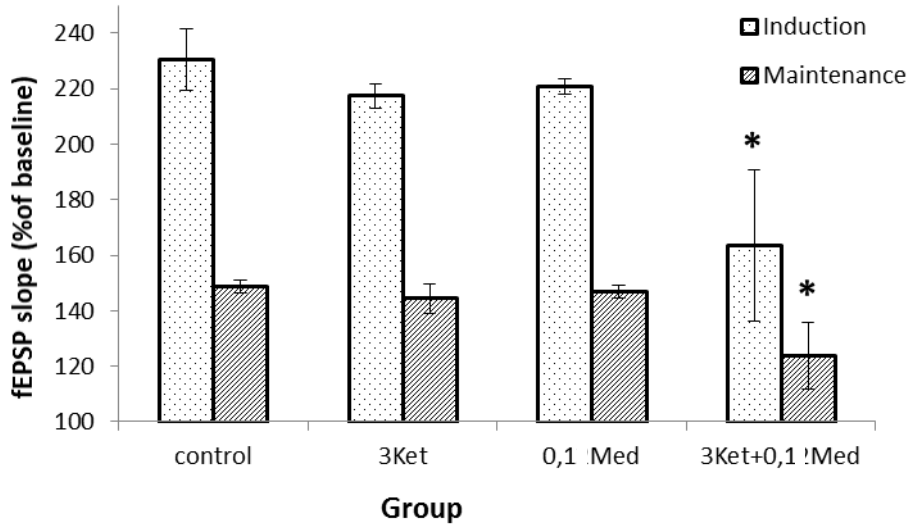


Figure 5.10 Effects of ketamine/medetomidine combination on long-term potentiation (LTP) induced by high frequency stimulation (HFS) (100 pulses at 100Hz). Mean fEPSP slope (averaged for 10 min.) immediately before HFS is defined as the baseline (100%). **(A)** Time-course of the effects of different concentrations of ketamine/medetomidine combination on LTP measured by field excitatory postsynaptic potential (fEPSP) slope (n=4). Data is showed as mean. (B) Concentration-response bars for LTP induction (average of fEPSP slope of the first 6 minutes after HFS was taken as a measure of LTP induction) and LTP maintenance (average of fEPSP slope from 54 to 60 minutes after HFS was taken as a measure of maintenance of LTP). Data is showed as mean ±SDs. *p<0.01 in comparison with control, 3µM of ketamine and 0.1µM of medetomidine**.**

5.3.5 DISCUSSION

The results of this work show that the ketamine/medetomidine combination did not affect paired-pulse facilitation, whereas basal excitatory synaptic transmission was affected only when high concentrations of ketamine/medetomidine combination were tested. Moreover, the combination of ketamine with medetomidine decreased long-term potentiation (LTP).

Regarding the effects of ketamine/medetomidine combination on hippocampal basal excitatory synaptic transmission, our study showed that only when higher concentration of ketamine combined with medetomidine were applied (300+12 and 600+24 µM, [ketamine]+[medetomidine] respectively) the synaptic transmission decreased.

In literature no studies were found regarding the effect of ketamine/medetomidine combination on hippocampal synaptic transmission. However, the results obtained with this combination on basal synaptic transmission are similar to the results obtained only with ketamine (chapter 5.1). So, this suggests that the addition of medetomidine did not interfere and potentiate ketamine` effects on basal synaptic transmission.

Results from the current study also showed that ketamine/medetomidine combination did not affect CA1 hippocampal paired-pulse facilitation. No studies were found regarding the effect of this combination on paired-pulse facilitation in literature. However, the results obtained in this study are in agreement with results obtained when these two drugs were tested alone in a previous study (chapter 5.1 and chapter 5.2). This study points that ketamine and medetomidine did not interacted to alter the presynaptic events required for paired-pulse facilitation on hippocampal synapses, so the paired-pulse facilitation occurs through presynaptic mechanisms (Kamiya and Zucker, 1994; Zucker and Regehr, 2002).

The effects of ketamine/medetomidine combination on postsynaptic events were tested using LTP studies. LTP is considered a cellular instrument for learning and memory formation (Bliss and Collingridge, 1993) and so the decrease of LTP can be related with the possible effects on memory of animals and humans after anesthesia. In this study, when 3 µM of ketamine were combined with 0.1 µM of medetomidine, LTP induction and LTP maintenance measured in hippocampal slices decreased. These results are very interesting when we have in mind the results of chapter 5.1 and 5.2, i.e. that 3 µM of ketamine and 0.1 µM of medetomidine alone did not affected LTP. Probably these two drugs have a synergic interaction regarding some postsynaptic events required for LTP in the CA1 region of adult

hippocampus and consequently the LTP measures was altered. However, the specific action of this combination of drugs on postsynaptic terminals remain unclear. Moreover, no studies were found regarding the effects of ketamine combined with medetomidine on LTP.

In summary, this study shows that ketamine/medetomidine combination decreased long-term potentiation in CA1 region of mouse hippocampus without affecting paired-pulse facilitation, suggesting the importance of postsynaptic mechanisms for ketamine/medetomidine combination induce deficits in memory. Moreover, the outcome of this work suggests that ketamine and medetomidine has a synergic interaction on LTP.

CHAPTER 6- STUDY OF THE EFFECTS OF AN INTRAPERITONEAL INJECTION OF KETAMINE ON LONG-TERM POTENTIATION IN HIPPOCAMPAL SLICES OF ADULT MICE – A PILOT STUDY

This chapter included experiments performed at "Centro de Neurociências de Coimbra" (CNC) and resulted in 1 output which is described in **Appendix E**.

6.1 ABSTRACT

Background: Ketamine is frequently used to induced analgesia or anesthesia in research and in veterinary medicine. However, its effects on memory and learning after anesthesia are unknown. Long-term potentiation (LTP) is considered a cellular mechanism for learning and memory. Therefore we investigated the effects of different concentrations of ketamine on LTP 24 hours after administration, in mice.

Material and methods: Nine C57BL/6 male adult mice were divided into 3 different groups (Saline solution, 25 mg/kg and 75 mg/kg ketamine). Twenty-four hours after anesthesia, animals were euthanized and their hippocampal slices were used to record the evoked fEPSP. High-frequency stimulation (100 pulses at 100 Hz) was used for LTP induction. The initial slope of the fEPSP was measured and LTP induction and maintenance were calculated.

Results: No differences were detected between groups regarding induction and maintenance of the hippocampal long-term potentiation, recorded 24 hours after ketamine administration.

Conclusion: An intraperitoneal single administration of ketamine did not affect hippocampal long-term potentiation 24 after injection. These results suggest that ketamine may not induced deficits of memory in male adult mice after anesthesia.

6.2. INTRODUCTION

Ketamine, an NMDA antagonist, is frequently used to induced analgesia and anesthesia in veterinary and human medicine. However, it was reported that anesthesia can induce a decline in memory function (Jevtovic-Todorovic *et al.*, 2003; Culley *et al.*, 2004).

Little is known about the effect of a single administration of ketamine in memory of adults. The activity dependent of synaptic plasticity is considered a cellular mechanism for learning and memory (Bliss and Collingridge, 1993). Long-term potentiation (LTP) is an important form of synaptic plasticity (Maruki *et al.*, 2001), being well known to be dependent on NMDA receptors (Lynch, 2004), which are considered the main molecular target of ketamine (Davies *et al.*, 1988; Orser *et al.*, 1997).

In a previous study, described in chapter 5.1, it was observed that different concentrations of ketamine (0.2 and 0.4 μM) decreased the long-term potentiation (LTP), during drug application directly on hippocampal slices. Similarly, previous studies from Stringer and Guyenet suggested that dissociative anesthetics, including ketamine (30 mg/kg), abolished hippocampal LTP *in vivo*, immediately after drug administration in rat (Stringer and Guyenet, 1983). However, no studies were found regarding the effect of different doses of ketamine on LTP after anesthesia. Therefore, the purpose of this study was to evaluate the effect of different doses of ketamine on hippocampal long-term potentiation 24 hours after anesthesia.

6.3 MATERIALS AND METHODS

This study was performed using nine 28 weeks-old, male C57BL/6 mice. These animals were randomly divided into 3 different groups: control (saline solution), 25 mg/kg ketamine, 75 mg/kg ketamine. The drug was administered by i.p. injection. Twenty-four hours after injection, animals were euthanized and their brains removed. Hippocampal slices were used to record the evoked fEPSP in Schaffer fiber - CA1 pyramid synapses. High-frequency stimulation (100 pulses at 100 Hz) was used for LTP induction. All slices were superfused only with aCSF continuously bubbled with 95% O_2 and 5% CO_2. The initial slope of the fEPSP was measured and LTP induction and maintenance were calculated.

More details on the animals' husbandry, anesthesia by i.p. injection, hippocampal slice preparation and electrophysiological recording are showed in the general methods section (chapter 2).

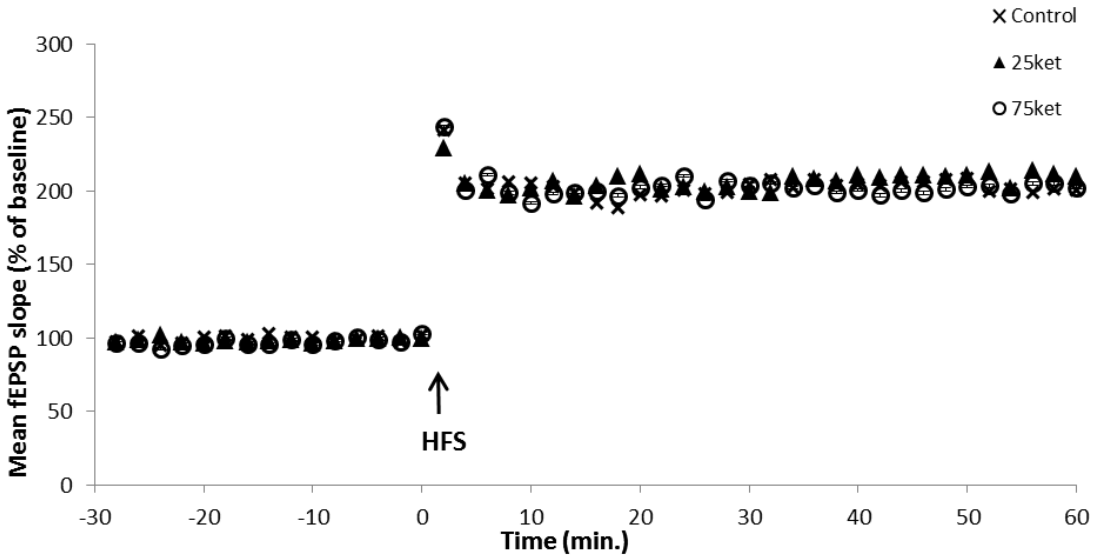
6.3.1 Statistical Analysis

The data were analyzed using one-way ANOVA followed by Bonferroni post hoc tests and, using SPSS 19 for Windows (IBM Corporation, Armonk, NY). All results are expressed as mean ±SD and $p \leq 0.05$ was considered statistically significant.

6.4 RESULTS

High frequency stimulation effectively triggered LTP induction, since it potentiated fEPSP slope to 216.3±16.9% in control group (**figure 6.1**). No significant differences were detected between the induction of LTP in the control group and the groups that received 25 mg/kg (211.8±12.8%) or 75 mg/kg of ketamine (218.6±13.8%). Similarly, no significant differences were found between all groups regarding the LTP maintenance (control: 200.6±40.4%; 25 mg/kg of ketamine: 212.2±14.5% and 75 mg/kg of ketamine: 204.3±35.1%) (**figure 6.1**).

A



B

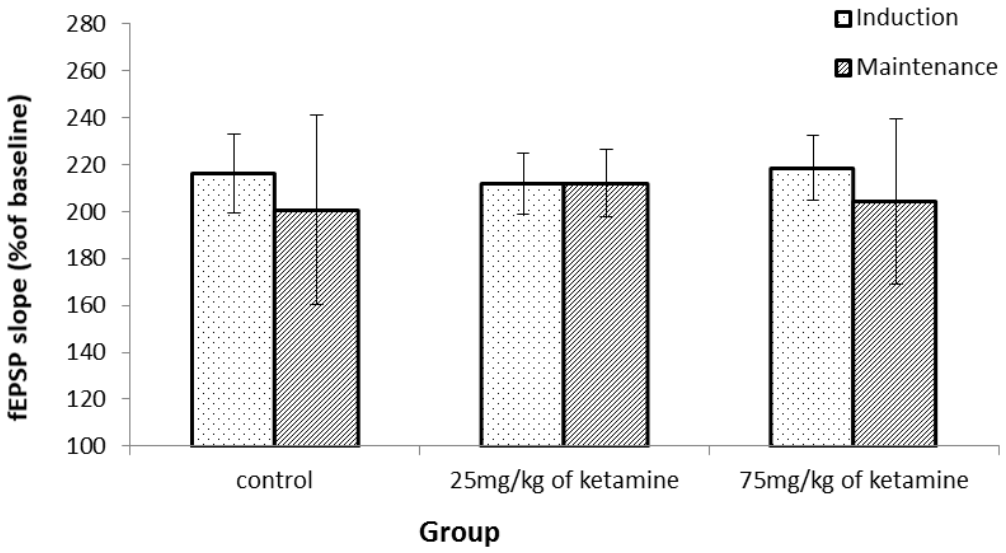


Figure 6.1 Effects of ketamine on long-term potentiation (LTP) induced by high frequency stimulation (HFS) (100 pulses at 100Hz), 24 hours after intraperitoneal administration of saline solution (control) or ketamine. Mean fEPSP slope (averaged for 10 min.) immediately before HFS is defined as the baseline (100%). **(A)** Time-course of the effects of different doses of ketamine on LTP measured by field excitatory postsynaptic potential (fEPSP) slope (n=3). Data is showed as mean. **(B)** Concentration-response bars for LTP induction and LTP maintenance. Average of fEPSP slope of the first 6 minutes after HFS was taken as a measure of LTP induction and average of fEPSP slope from 54 to 60 minutes after HFS was taken as a measure of maintenance of LTP. Data is showed as mean ±SDs.

6.5 DISCUSSION

The outcome of this work shows that different doses of ketamine did not affect long-term potentiation (LTP) 24 hours after anesthesia in adult mice.

The doses of ketamine tested in the current study included subanesthetics doses of ketamine, when it is administered alone, and anesthetic doses, when it is administered in combination with medetomidine, in mice (see chapter 3).

Regarding the effect of ketamine on LTP, we observed that ketamine did not affect induction and maintenance of LTP in hippocampal slices. Few studies were found in literature that tested the effects of ketamine on hippocampal LTP. An earlier study showed that dissociative anesthetics, including ketamine, may abolish hippocampal LTP in *in vivo* rats, immediately after drug administration (Stringer and Guyenet, 1983). Similarly, in chapter 5.1, ketamine decreased LTP induction and LTP maintenance in *ex vivo* hippocampal slices, at the moment of drug application. By the contrary, in this study, ketamine did not affected hippocampal LTP. This disagreement between this study and the previous studies can be explained by the fact that this study was performed in a later time point i.e. 24 hours after drug administration. In fact, the factor time may be an important variable to consider when exploring consequences from anesthesia. When LTP was measured during application of ketamine (chapter 5) or immediately after anesthesia (Stringer and Guyenet, 1983), LTP is reduced/blocked, because ketamine blocked NMDA receptor (Davies *et al.*, 1988; Orser *et al.*, 1997) that is a crucial receptor to induction of LTP (Grover and Teyler, 1990) and to induction of anesthesia (Irifune *et al.*, 1992). However, when LTP was measured 24 hours after anesthesia, the action of ketamine in NMDA receptor is reverted and ketamine is eliminated of the body of the animal; consequently no effects on LTP were observed. Apparently, the blocking of NMDA receptors during anesthesia did not induced significant secondary effects on LTP 24 hours after anesthesia.

In conclusion, this work shows that an i.p. single administration of ketamine did not affect hippocampal long-term potentiation 24 after injection. Moreover, as LTP is considered a cellular mechanism for learning and memory, our results also suggest that a single administration of ketamine by i.p. injection not affect memory events-dependent of hippocampal LTP

.

CHAPTER 7 – GENERAL DISCUSSION

Anesthesia is used in surgery and other interventions to control pain, anxiety and awareness. In veterinary practice, anesthesia is also essential to immobilize animals for clinical examination. Likewise, anesthesia is a requirement for several procedures in research, namely in neurobiological research. However, recently, concerns have been raised about potential persistent effects of anesthesia on the CNS, such as the post-operative cognitive dysfunction described in humans. The concerns regarding post-operative cognitive effects are also present in research that uses laboratory animals to study cognitive abilities after a procedure requiring anesthesia, as the use of anesthetic drugs can interfere with the experimental results.

Ketamine, an NMDA receptor antagonist, and medetomidine, an alpha$_2$-adrenoceptor agonist, are examples of drugs that are routinely used in clinics and in research procedures with laboratory animals. These drugs can be used alone or in combination. The combination of these two drugs reduces the anesthetic requirements, and improves perioperative hemodynamic stability (Levanen *et al.*, 1995; Hall *et al.*, 2000). However, little is known about the effect of these drugs on the adult brain.

The main purpose of this thesis is to explore the impact of different doses and concentrations of ketamine alone or combined with medetomidine in the adult brain of mice. Thus, this work was divided in four different main studies (**table 7.1**).

The first study (chapter 3) included two different anesthetic doses of ketamine combined with medetomidine (25 mg/kg of ketamine + 1 mg/kg of medetomidine, 75 mg/kg of ketamine + 1 mg/kg of medetomidine), two subanesthetic doses of ketamine alone (25 mg/kg or 75 mg/kg) and one sedative dose of medetomidine alone (1 mg/kg). These different doses of drugs combined or administered isolated did not influence spatial memory of animals in the T-maze task neither neurodegeneration in different regions of the brain such as the hippocampus, retrosplenial and visual cortices. Interestingly, the group treated only with 75 mg/kg of ketamine showed hyperlocomotion, and the group with the highest dose in combination (75 mg/kg of ketamine+ 1 mg/kg of medetomidine) performed worse than the other groups in the vertical pole test.

The second study (chapter 4) complemented the first study by evaluating also an anesthetic dose of ketamine (150 mg/kg). Besides, this study used more complex behavioral

tests to assess different types of memory (working and reference memory) and the habituation process was based on the open field performed during 3 consecutive days. Neurodegeneration was evaluated in more brain regions also important to memory, such as nucleus accumbens, laterodorsal thalamic nucleus and striatum. Results showed that two subanesthetic doses (25 mg/kg and 75 mg/kg) and one anesthetic dose of ketamine (150 mg/kg) did not induced significant effects on working and reference memory in the radial-maze task. Similarly, no differences were detected between groups regarding the vertical pole task or the neurodegeneration evaluation. In the open field test, all animals showed a species-specific response to the aversive condition of the brightly-lit arena with most measures being unaffected by anesthesia. However, the group with intermediate dose of ketamine (75 mg/kg) showed hyperlocomotion, similar to the result observed during the first study.

In the third study (chapter 5), the effects of different concentrations of ketamine (chapter 5.1), medetomidine (chapter 5.2) or ketamine/medetomidine combination (chapter 5.3) on basal excitatory synaptic transmission and synaptic plasticity (PPF and LTP) were evaluated in hippocampal slices of adult mice. These drugs at different concentrations were applied directly in the solution with brain slices. The study of these two forms of synaptic plasticity, PPF and LTP, complemented the prior studies (chapter 3 and chapter 4) involving memory evaluation, as memory formation and learning are dependent of synaptic plasticity. Therefore, alterations on synaptic plasticity in hippocampus can have impact on memory/learning, causing behavioral modifications.

In the first part of the third study (chapter 5.1), to assess the effect of ketamine on basal excitatory synaptic transmission and on paired-pulse facilitation concentrations from 1 to 600 μM of ketamine were tested; while to study the effect, of ketamine on long-term potentiation concentrations from 3 to 100 μM of ketamine were used. This study showed that only high concentrations of ketamine (300 and 600 μM) decreased synaptic transmission, and that none of the concentrations of ketamine tested affected paired-pulse facilitation ratio. However, ketamine decreased long-term potentiation in a concentration-dependent manner.

In the second part of the third study (chapter 5.2), to assess the effect of medetomidine on basal excitatory synaptic transmission and on PPF, concentrations from 1 to 200 μM of medetomidine were tested while to assess the effect of medetomidine on long-term potentiation, concentrations from 0.1 to 0.4 μM of medetomidine were tested. Concentrations above 24 μM of medetomidine decreased excitatory synaptic transmission. Furthermore,

paired-pulse facilitation ratio was only affected by the highest concentration of medetomidine tested (200 μM) and long-term potentiation decreased in a concentration-dependent manner.

In the third part of the third study (chapter 5.3), the effects of different concentrations of ketamine and medetomidine were evaluated in combination on basal synaptic transmission, paired-pulse facilitation, and LTP. The synaptic transmission only decreased with high concentrations of ketamine combined with medetomidine (300+12 and 600+24 μM). Moreover, none of the tested concentrations of ketamine combined with medetomidine significantly affected the paired-pulse facilitation ratio. The LTP decreased significantly when 3 μM of ketamine were combined with 0.1 μM of medetomidine.

In the fourth study (chapter 6), different doses of ketamine (25 mg/kg and 75 mg/kg) were administered by i.p. injection in adult mice to assess the impact of this drug on hippocampal long-term potentiation 24 hours later. This study complemented the previous study (chapter 5.1) by evaluating LTP in mice injected with ketamine. The doses used are similar to the ones from the first and second study and try to replicate what happens in clinic or research contexts. Moreover, LTP was evaluated at approximately the same time point that behavioral tests were performed in the first and second study. Results showed that the concentrations of ketamine studied did not affect the long-term potentiation when compared with control group.

Table 7.1 Summary of all results. ketamine (Ket.), medetomidine (Med.)..

Study (chapter)	**Anesthesia**	**Procedure/ Technique**	**What was evaluated**	**Results**
Study I (chapter 3)	Control, 25 mg/kg of Ket. (25Ket.) 75 mg/kg of Ket. (75Ket.) 1 mg/kg of Med. (1Med.) 25Ket.+ 1Med. 75Ket.+ 1 Med.	Open-field (1 day)	Exploratory activity and stress levels	75Ket. group showed Hyperlocomotion
		T-maze	Spatial memory	No effects
		Vertical-pole test	Anesthetic recovery and equilibrium	75Ket.+1Med. group had worse performance
		H&E, procaspase-3, activated caspase-3 and BDNF	Number of dead cells, and apoptotic cells	No effects
Study II (chapter 4)	Control, 25Ket. 75Ket. 150Ket.	Open field tests (3 consecutive days)	Stress levels exploratory activity and habituation	75Ket. group showed hyperlocomotion in first day
		Radial-maze	Working and reference memory	No effect
		Vertical pole	Anesthetic recovery and equilibrium	No effect
		H&E, procaspase-3 and caspase-3 activity	Number of dead cells and apoptotic cells	No effect
Study III - first part (chapter 5.1)	Control, 1, 3, 10, 30, 100, 200, 300 and 600µM of Ket.	Electrophysiology study on hippocampal slices	Basal excitatory synaptic transmission and paired-pulse facilitation	No effects on paired-pulse facilitation, No effects on excitatory synaptic transmission except with 300 and 600µM of Ket.
	Control, 3, 10, 30, and 100µM of Ket.		Long-term potentiation	Long-term potentiation decreased in a concentration-dependent manner
Study III – second part (chapter 5.2)	Control, 1, 2, 4, 8, 12, 24, 48, 100 and 200µM of Med.	Electrophysiology study on hippocampal slices	Basal excitatory synaptic transmission and paired-pulse facilitation	No effects except with higher concentrations
	Control, 0.1, 0.2 and 0.4µM of Med.		Long-term potentiation	LTP decreased in a concentration dependent manner

Study III - third part (chapter 5.3)	Control, 30µM of Ket.+1µM of Med. 100µM of Ket.+ 4µM of Med. 200µM of Ket.+8µM of Med. 300µM of Ket.+12µM of Med. 600µM of Ket.+24µM of Med.	Electrophysiology study on hippocampal slices	Basal excitatory synaptic transmission and paired-pulse facilitation	No effects on paired-pulse facilitation, No effects on excitatory synaptic transmission, except with higher concentrations, similar to results of 5.1
	Control 3µM of Ket. + 0.1µM of Med.		Long-term potentiation	3µM of Ket. + 0.1µM of Med decreased long-term potentiation
Study IV (chapter 6)	Control 25Ket. 75Ket.	Electrophysiology study on hippocampal slices 24 h after i.p. injection	Long-term potentiation	No effects

Regarding the general results of the first and second study (chapter 3 and 4), ketamine alone or in combination with medetomidine did not affect memory independently of the dose used, when it was administered in a single i.p. injection in adult mice. Several articles reported that the NMDA antagonists such as ketamine may cause learning deficits (Mickley *et al.*, 2000; Peng *et al.*, 2011; Turner *et al.*, 2012). However, the majority of those studies were conducted in neonatal animals, the brain of which is more fragile to insults than the adult brain. Moreover, the studies that were found in literature using adult animals showed a conflicting evidence about the effects of a single administration of ketamine on cognition, depending on the type of memory studied, acute or chronic administration (Morgan and Curran, 2006) and dose used (Pitsikas and Boultadakis, 2009). The first and second studies of this thesis contribute to clarify the effects of a single administration of ketamine alone or combined with medetomidine on memory of adult mice.

The results obtained in behavioral tests were supported by the results obtained in a neurodegenerative evaluation that included the study of the general cell death (H&E staining) and the evaluation of the apoptosis, using immunohistochemistry techniques to assess BDNF, procaspase-3 and activated caspase-3 expressions. All these indicators of apoptosis are related with neurodegeneration and important to evaluate it incidence. Levels of procaspase-3 (inactive form of caspase-3) in adult rat brain are extremely low when compared with brain in development and so an additional synthesis of procaspase-3 for posterior activation would

occurs in order to execute the apoptotic program(Didenko *et al.*, 2002). In the first and second studies, ketamine alone or combined with medetomidine did not induce an additional synthesis of procaspase-3 when compared with control group, suggesting that it did not cause apoptosis in adult brain. This information is in agreement with our results obtained with activated caspase-3 and BDNF expressions. However, these results did not exclude that other types of cell death, such as excitotoxicity may happen. The hematoxilin-eosin staining were performed 3 hours after anesthesia to evaluate the general cell death, however this time of observation may not be sufficient to detect lesions in brain cells induced by excitotoxicity.

Interestingly it was observed, in study I and II, during the open field test that an intermediated dose of ketamine (75 mg/kg) induced hyperlocomotion one day after anesthesia. But this response did not appear in mice treated with the lowest or with the highest dose administered. Moreover, this effect was reduced by medetomidine. Our result are in agreement with a previous work that showed that an intermediate dose of ketamine (100 mg/kg is more effective than 50 mg/kg and 200 mg/kg to induce hyperactivity (Chatterjee *et al.*, 2010). However, the mechanism that explains these results remains unclear. A possible explanation can be based in the alteration of pre-synaptic components of dopamine neurons in the nucleus accumbens of mice induced by ketamine(Irifune *et al.*, 1991), increasing dopamine turnover. Moreover, medetomidine administration reduced dopamine turnover (Koulu *et al.*, 1993) which may explain the reduction of hyperlocomotion caused by medetomidine.

Furthermore, the 75 mg/kg of ketamine + 1 mg/kg of medetomidine combination produced the highest anesthetic depth. According with this observation only the group with this combination did not recover the equilibrium 4 hours after anesthesia as observed in the vertical pole test. This is explained because this group needed more time than the others to recover after anesthesia.

Regarding the results of the hemodynamic data evaluation from the first and second studies, it was observed that the group with 150 mg/kg of ketamine had higher pulse and respiratory compared with all others anesthetic regimes. These results are in agreement with our expectation because it was reported that ketamine stimulates the circulatory (Verstegen *et al.*, 1990; Levanen *et al.*, 1995) and respiratory systems (Morel *et al.*, 1986).

Concerning, the results of the study III (chapter 5), ketamine and medetomidine, isolated or combined, decreased basal excitatory synaptic transmission only when very high concentrations were applied in hippocampal slices. In addition, these drugs did not affect

paired-pulse facilitation, except when the highest concentration of medetomidine alone (200 μM) was applied. Interestingly, the results obtained with the ketamine isolated and with ketamine/medetomidine combination on basal synaptic transmission and on PPF did not differ significantly, suggesting that medetomidine did not interact with ketamine regarding basal synaptic transmission or paired-pulse facilitation with the concentrations used. However, we need to have in mind that the results of the basal synaptic transmission and paired-pulse facilitation are based in cumulative concentrations, i.e. the different concentrations of drugs were administered in consecutive increasing concentrations; so the detectable effects can correspond to a concentration slightly higher.

In third study (chapter 5), also it was observed that ketamine and medetomidine, isolated or combined, decreased long-term potentiation in a concentration dependent manner, when drugs were applied directly in bath with the slices. However, these results are in disagreement with the results obtained during study IV (chapter 6). In that study, ketamine did not affect hippocampal LTP. This disagreement may be explained based on the moment when LTP measurements were taken. The results on LTP in study III were recorded in the exact moment of drug application while in study IV LTP was measured 24 hours after anesthesia. Moreover, in study III the drugs were applied in brain slices and in fourth study were administered in the animal. The administration of the drug in the animal has the advantage that the metabolization of the drug can occur normally in the animal's body and so the results resemble more the real clinical practice. However, applications of the drugs directly in brain slices allow a precise control over concentration of drugs and reduce the variability. Moreover, the study IV supported the results obtained in study I and II. In those studies, a single i.p. injection of ketamine did not affect the memory of adult male mice. Similarly, LTP measured 24 hours after ketamine administration showed no alterations in the same mice strain and sex.

Interestingly, in study III (chapter 5.3), when concentrations of ketamine and medetomidine that did not alter LTP were combined (3 μM of ketamine + 0.1 μM of medetomidine), the LTP decreased significantly. This suggests a synergic interaction between these two drugs.

In summary, a single i.p. injection of ketamine or ketamine / medetomine combination did not affect the memory of male adult mice in the tested conditions, and may be used in research without interfering with the results of the experiments. Furthermore, this combination seems to be safe to be used in veterinary practice without causing memory deficits to the animals. The use of the ketamine/medetomidine combination is more advisable

than the use of ketamine alone to induce anesthesia/sedation, because it has higher physiological parameters stability and avoids the hyperlocomotion in animals and possibly post-anesthetic delirium in humans induced by ketamine administered alone.

CHAPTER 8- GENERAL CONCLUSION

This project aimed to contribute to a better understanding of the effects of the anesthetics ketamine and medetomidine, isolated or combined, on adult mice.

The main target organ from this thesis is the brain, and the main objective is to study the influence of these anesthetic drugs in research projects in which memory is assessed. Moreover, this thesis brings important new information for veterinary anesthesia, with potential interest for translational research into humans. This was the first time that the implications of ketamine/medetomidine combination on memory were studied.

The following conclusions can be drawn based on the results achieved with the studies in this thesis:

- Ketamine/medetomidine combination may provide a good anesthetic plane, with stability of the physiologic parameters;

- A single intermediate dose of ketamine alone induced hyperlocomotion, and medetomidine can inhibit hyperlocomotion produced by ketamine suggesting that medetomidine may be considered to attenuate postanesthetic delirium and agitation produced by ketamine in animals and humans;

- Ketamine/medetomidine may be regarded as a safe anesthetic combination concerning learning and memory, with no clinical and research implications in adults mice. In particular, a single administration of ketamine and medetomidine alone or in combination did not affect apoptosis in adult mice brain and no particular benhavioral changes were induced;

- Different concentrations of ketamine and medetomidine alone or in combination decreased hippocampal long-term potentiation rather than paired-pulse facilitation, showing the implication of postsynaptic mechanisms in the action of these drugs on brain;

- A single intraperitoneal injection of ketamine did not affect the long-term potentiation measured 24 hours after injection, suggesting that ketamine did not affect memory after anesthesia.

These findings suggest that the ketamine/medetomidine combination is a good option to induce anesthesia in veterinary clinical settings and in research using adult mice.

FUTURE STUDIES

Interestingly, only the intermediated dose of ketamine induced hyperlocomotion. Moreover, this hyperlocomotion disappeared at the second day after anesthesia and when medetomidine was also administered. As hyperlocomotion can be related with alterations in neurotransmitters such as dopamine, norepinephrine and serotonin; it would be relevant to evaluate the level of neurotransmitters on brain.

As the study reported in chapter 6 is a pilot study, it would be relevant to perform other study similar to this investigation, using higher number of animals per group and more doses of ketamine such as an anesthetic dose of ketamine alone. In addition, it is pertinent to study the effects of medetomidine alone and ketamine/medetomidine combination in LTP measured at different time points after anesthesia. In chapter 5, the effects of these drugs were tested in LTP; however it was evaluated in slices at the moment of drug application.

As we collected other organs of mice during this thesis, such as liver and kidney; it will be interesting to study the effects of ketamine and medetomidine in these organs; because the liver and the kidney are very important to metabolization and elimination of ketamine and medetomidine.

All these studies should also benefit for more broad investigations to establish if there are any strain and/or sex differences. In particular it will be interesting to perform all electrophysiological studies in both sexes, since there is a potential sex implication on synaptic transmission and synaptic plasticity.

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APPENDIX A

LIST OF OUTPUTS

LIST OF OUTPUTS

Articles published from this thesis:

Patrícia Ribeiro, Ana Valentim, Paula Rodrigues, Anna Olsson, Luís Antunes (2012). Apoptotic neurodegeneration and spatial memory are not affected by sedative and anaesthetics doses of ketamine/medetomidine combinations in adult mice; British Journal of Anaesthesia **108**(5):807-14.

Patrícia Ribeiro, Paula Rodrigues, Ana Valentim, Luís Antunes (2013). A single intraperitoneal injection of ketamine does not affect spatial working, reference memory or neurodegeneration in adult mice; European Journal of Anaesthesiology **30**(10): 618-26.

Articles submitted or in preparation based in material from this thesis:

Patrícia Ribeiro, Ângelo Tomé, Henrique Silva, Rodrigo Cunha, Luís Antunes; Clinically relevant concentrations of ketamine affect basal excitatory synaptic transmission and long-term potentiation without changing paired-pulse facilitation in mice hippocampal slices (submitted for Brain Research, manuscript nº BRES-D-13-01017).

Patrícia Ribeiro, Luís Antunes, Henrique Silva, Rodrigo Cunha, Ângelo Tomé; Effects of medetomidine on basal excitatory synaptic transmission and on synaptic plasticity in mice hippocampal slices (submitted for European Journal of Pharmacology, manuscript nº EJP-38661).

Patrícia Ribeiro, Ângelo Tomé, Henrique Silva, Rodrigo Cunha, Luís Antunes; Synergic effects of ketamine and α_2-adrenoceptor agonist medetomidine on long-term potentiation in hippocampus (In preparation)

Abstracts

Patrícia Ribeiro, Ana Valentim, Paula Rodrigues, Luis Antunes; Cognitive and Neurodegenerative Effects of the Medetomidine/Ketamine Anesthesia Combination in Mice; American Society of Anesthesiologists, Annual Meeting 2010; 16-20 October, San Diego, Califórnia.

Patrícia Ribeiro, Ana Valentim, Paula Rodrigues, Anna Olsson, Luis Antunes; Cognitive and neurodegenerative effects of the ketamine/medetomidine anesthesia combination in adult mice; SCAND-FELASA Joint Meeting 2010, 14-17 June, Helsinki, Finland.

Patrícia Ribeiro, Ana Valentim, Paula Rodrigues, Luis Antunes; Different Concentrations of Ketamine did not Affect Cognition or Neurodegeneration in Adult Mice; American Society of Anesthesiologists, Annual Meeting 2011; 15 - 19 October, Chicago, Illinois.

Patrícia Ribeiro, Henrique Silva, Rodrigo Cunha, Ângelo Tomé, Luís Antunes; Ketamine affects basal excitatory synaptic transmission and long-term potentiation without affect paired-pulse facilitation in hippocampal slices of adult mice; The European Anaesthesiology Congress 2012; 9-12 June, Paris, France; European Journal of Anesthesiology, Vol 29, Supp.50, p.108-109

Patrícia Ribeiro, Luís Antunes, Henrique Silva, Rodrigo Cunha, Ângelo Tomé; Effects of medetomidine on basal excitatory synaptic transmission and on synaptic plasticity in mice hippocampal slices. Abstract accepted for presentation al the European Anaesthesiology Congress 2013; 1-4 June, Barcelona, Spain.

Patrícia Ribeiro, Henrique Silva, Rodrigo Cunha, Ângelo Tomé, Luís Antunes; Effects of ketamine/medetomidine combination on basal synaptic transmission and synaptic plasticity in hippocampal slices of adult mice. Accepted for presentation at the 12th FELASA-SECAL Congress 2013, Barcelona, Spain.

Patrícia Ribeiro, Henrique Silva, Rodrigo Cunha, Ângelo Tomé, Luís Antunes; A single intraperitoneal injection of ketamine did not affect hippocampal long-term potentiation of adult mice. Accepted for presentation at the 12th FELASA-SECAL Congress 2013,Barcelona, Spain.

APPENDIX B

OUTPUTS FROM CHAPTER 3

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BJA

Apoptotic neurodegeneration and spatial memory are not affected by sedative and anaesthetics doses of ketamine/medetomidine combinations in adult mice

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Editor's key points

- The combined effect of ketamine and medetomidine on cognition and apoptosis was studied in mice.
- No differences were seen between groups in terms of spatial memory or apoptosis.
- Hyperlocomotive activity in mice receiving ketamine was attenuated by medetomidine.
- Co-administration of ketamine and medetomidine may be clinically useful.

Background. Ketamine is increasingly popular in clinical practice and its combination with α_2-agonists can provide good anaesthetic stability. Little is known about the effects of this combination in the brain. Therefore, we investigated the effects of different concentrations of ketamine combined with medetomidine on cognition and its potential apoptotic neurodegenerative effect in adult mice.

Methods. Seventy-eight C57BL/6 adult mice were divided into six different groups (saline solution, 1 mg kg^{-1} medetomidine, 25 mg kg^{-1} ketamine+1 mg kg^{-1} medetomidine, 75 mg kg^{-1} ketamine+1 mg kg^{-1} medetomidine, 25 mg kg^{-1} ketamine, and 75 mg kg^{-1} ketamine). Eight animals per group were tested in the T-maze, vertical pole, and open-field test. Five animals per group were used for histopathological [haematoxylin and eosin (HE) staining] and immunohistochemical analyses [caspase-3 activation and expression of neurotrophin brain-derived neurotrophic factor (BDNF)]. Cells showing clear HE staining and positive immunoreactions for caspase-3 and BDNF in the retrosplenial cortex, visual cortex, pyramidal cell layer of the *cornu Ammonis* 1 and *cornu Ammonis* 3 areas of the hippocampus, and in the granular layer of the dentate gyrus were counted.

Results. There were no differences between groups regarding the number of dead cells and cells showing positive immunoreactions in the different areas of the brain studied. Similarly, no differences were detected in the number of trials to complete the T-maze task. Nevertheless, α_2-agonist decreased hyperlocomotion caused by ketamine in the open field.

Conclusions. Neither apoptotic neurodegeneration nor alterations in spatial memory were observed with different concentrations of ketamine combined with medetomidine in adult mice.

Keywords: anaesthetics; apoptosis; cognition; ketamine; medetomidine

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Ketamine, a non-competitive glutamate *N*-methyl-D-aspartate acid receptor antagonist, is gaining popularity in adult human medicine mainly thanks to its analgesic properties[1–3] and its importance for emergency procedures.[4 5] However, ketamine impairs brain activity inducing post-anaesthetic delirium in humans[6 7] and hyperlocomotion in rodents.[8]

Ketamine may be combined with α_2-agonists such as dexmedetomidine[9 10] in humans or medetomidine in animals.[11 12] Levanen and colleagues[13] showed in humans that the combination of ketamine with dexmedetomidine provides stable anaesthesia with a reduction in adverse effects caused by ketamine, such as post-anaesthetic delirium. However, in Europe, dexmedetomidine has not been approved for human clinical use and was only very recently introduced in veterinary medicine where medetomidine is used routinely.

Dexmedetomidine is the dextro enantiomer of medetomidine and has the pharmacological activity of medetomidine.[14] Ketamine/medetomidine and ketamine/dexmedetomidine have similar effects in induction of anaesthesia, heart rate, respiratory rate, temperature, blood gas values, and recovery.[15]

The use of α_2-agonists reduce anaesthetic requirements and improve perioperative haemodynamic stability.[16 17] In addition, ketamine/(dex)medetomidine anaesthesia has the advantage of a rapid recovery with the administration of atipamezole, a specific α_2-antagonist available for the use of animals, which immediately reverses (dex)medetomidine effects.[11 18 19] However, there is a lack of information

about potential secondary effects of this combination on the brain, such as effects on memory and neurodegeneration.

The purpose of this study was to assess the influence of two different concentrations of ketamine combined with α_2-agonists on performance of a simple spatial cognitive task and the potential neurodegenerative effects in adult mice.

Methods

This study was reviewed and ethically approved by the Portuguese competent authority for animal protection, Direcção Geral de Veterinária (Lisbon, Portugal).

Animals

Seventy-eight 28-week-old, male C57BL/6 mice bred in the animal facility of the institute (F1-F2 offspring of animals bought from Charles River, Barcelona, Spain) were used. The mice were housed with controlled temperature (21°C) and a relative humidity of 55%.

Animals received a commercial pellet diet (4RF25-GLP Mucedola, SRL, Settimo Milanese, Italy) and water *ad libitum*. A food restriction schedule 1 week before the T-maze habituation and during the task was applied to animals used in behavioural tests. A limited amount of food administered once daily was adjusted to a level that kept the mice at 85–95% of free-feeding weight.

Anaesthesia

Ketamine (Imalgéne® Merial, Portugal; 100 mg ml^{-1}), medetomidine (Domitor® Phizer, Portugal; 1 mg ml^{-1}), or both were used for anaesthesia. Standard physiological saline 0.9% (Soro Fisiologico, Braun Vet, Portugal) was used in the control group and for diluting the drugs to ease handling of small volumes.

Mice were randomly assigned into six treatment groups, described in Table 1; all groups consisted of 13 animals (eight mice for behavioural tests and five for histology).

The mice were weighed using an electronic scale and volumes to be injected were calculated for each animal. Ketamine and medetomidine were mixed and administered as a single intraperitoneal (i.p.) injection. After administration, each animal was placed alone in a cage until it lost its righting reflex (RR), thereafter the animal moved to a homeothermic blanket connected to a rectal thermal probe (50-7061-F, Harvard Apparatus Ltd, Kent, UK) maintaining the temperature at 36–37.5°C throughout anaesthesia. One hundred per cent of oxygen was delivered to the animals with a tube connected to a coaxial circuit (0.8 litre min^{-1}) and ophthalmic gel was placed in the eyes of the animals (LACRYVISC, Alcon, Paço D'Arcos, Portugal).

To avoid isolation stress, the animals that did not lose consciousness were returned to their home cage after the i.p. injection and heating was provided. Animals in which consciousness was achieved after the i.p. injection, the time to loss of RR, loss of response to tail pinch, and loss of pedal withdrawal reflex were recorded. In addition, the respiratory rate, depth of anesthesia,[20] pulse, and systolic pressure were recorded at 10 min intervals. Systolic pressure and heart rate values were obtained with pressure meter (LE 5001, Panlab, Spain). A pulse oximeter (S&W 9040, Athena, Germany), placed on the upper right hind leg of each mouse, was used for monitoring oxygen saturation (Sa_{O_2}).

Animals were anaesthetized for 1 h and were observed throughout the period of anaesthesia. After 1 h, the α_2-antagonist, atipamezole (Antisedan® Phizer, Portugal; 5 mg ml^{-1}) was administered (1 mg kg^{-1} i.p.) to reverse anaesthesia induced by medetomidine. This drug was administered to the animals from the 25Ket./Med.; 75Ket./Med., and Med. groups.

Behavioural tests

The timing of behavioural tests in relation to anaesthesia is illustrated in Figure 1.

T-maze

The T-maze test was used to access spatial memory. The procedure used in this study was based on a protocol used in our laboratory[21] with the exception that more time points were studied. The test ended when the learning criterion was achieved, that is, when the mouse entered the correct arm nine times out of 10 consecutive tests trials (excluding the three learning trials); hence, every mouse had to perform a minimum of 10 trials. The number of trials to complete the task was measured.

Vertical pole test

The vertical pole test was used to assess post-anaesthetic recovery. Deficits in motor coordination and balance were detected by the mouse falling off the pole.[22] This test was performed as previously described by Bellum and colleagues[23] with minor modifications.

Each mouse was placed in the centre of a round rough-surfaced pole (50 cm long, 2 cm diameter). The pole was initially positioned horizontally and then slowly inclined to 90° (1° s^{-1}); the animal faced the end that was lifted up. Performance was determined by the latency (s) for the mouse to turn downwards and completely descend the pole (s).

Table 1 Treatment groups

Group	Control	Med.	25Ket.	75Ket.	25Ket./Med.	75Ket./Med.
Dose of drugs administered	Saline solution	1 mg kg^{-1} medetomidine	25 mg kg^{-1} ketamine	75 mg kg^{-1} ketamine	25 mg kg^{-1} ketamine+1 mg kg^{-1} medetomidine	75 mg kg^{-1} ketamine+1 mg kg^{-1} medetomidine

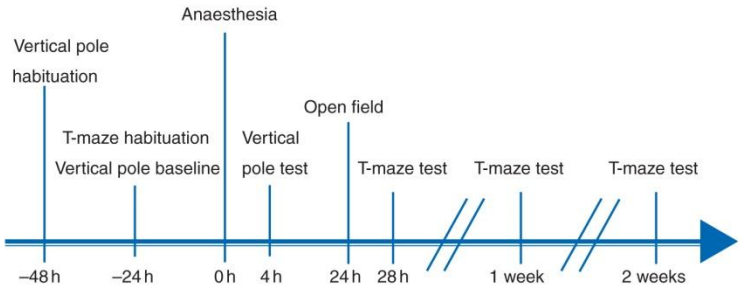

Fig 1 Timeline of the behaviour tests.

Animals had a maximal time of 120 s to complete the test. This maximal time was recorded for animals that were not able to turn downwards and instead dropped from the pole.

Open field

The open-field test was used to measure the locomotor and exploratory activity as an indicator of any difference in anxiety.

The apparatus consisted of a circular arena (1 m of diameter) made of grey polypropylene and surrounded by a wall of 30 cm height. Each animal was released in the centre of the arena and allowed to explore it for 20 min.

The number of fecal boli was counted and the arena was cleaned with alcohol at 70% to avoid the presence of olfactory cues. The test was recorded with a camera placed above the apparatus and collected into a computer with a multi-camera vigilance system GeoVision (GV-800/8, Taipei, Taiwan).

The video analysis was carried out using the software VideoMot 2 (TSE-systems, Bad Homburg, Germany) which measured several parameters: distance walked, time spent, and the number of visits on each region. The latency to exit the centre, the total distance walked, and the total speed were also calculated. Three areas were defined in the open-field arena: periphery, middle, and centre.

Histopathological (haematoxylin–eosin staining) and immunohistochemical (caspase-3 activation and neurotrophin brain-derived neurotrophic factor) studies

Three hours after anaesthesia, the mice were killed by decapitation and the brain was removed. Brains were fixed for 48 h in 4% buffered paraformaldehyde (PBS, pH 7.4, 0.1 M), processed, and embedded in paraplast (SHANDON, Hypercentre XP and Histocentre 2, Burlingame, CA, USA). For each block, serial sections of 4 μm thick were made making coronal cuts from Bregma −2.06 mm until Bregma −2.70 mm[24] at 30 μm intervals. Slices were used for haematoxylin and eosin (HE) staining (observation of cellular death) and for caspase-3 activation (apoptosis) and neurotrophin brain-derived neurotrophic factor (BDNF) detection. Anticaspase-3-antibody (CPP32 Ab-4, Rabbit Polyclonal Antibody, Thermo Scientific, Walthan, USA) and anti-BDNF-antibody [BDNF: (H-117): sc-20981, Santa Cruz Biotechnology, Inc., Heidelberg, Germany] were used. Details regarding immunohistochemical protocols and image capture are found in our previous work.[25]

Cellular death was based on morphological definitions from previous studies.[26 27] Cells with abnormal morphologies (shrinkage, hypereosinophilic cytoplasm, condensed and hyperchromatic nuclei, marginated chromatin, and apoptotic bodies) were considered positive for cell death. Dead cells and cells showing clear positive immunoreactions were manually counted per square millimetre in the pyramidal cell layer of the *cornu Ammonis* 1 (CA1) and *cornu Ammonis* 3 (CA3) areas of the hippocampus, the granular layer of the dentate gyrus, retrosplenial cortex, and visual cortex. For BDNF expression, a relationship between positive cells and negative cells was established. The average of the neuronal counts from three coronal sections selected at 30 μm intervals from each brain was calculated.

Statistical analysis

Student's *t*-test or one-way ANOVA with Bonferroni *post hoc* tests were used to analyse parametric data which are expressed as mean (SD), while non-parametric data were evaluated with the Kruskal–Wallis test followed by the Mann–Whitney *U*-test and are expressed as median (range). A value of $P \leq 0.05$ was considered statistically significant. All results were analysed by using Microsoft Office Excel 2003 for data management and SPSS 16.0 for Windows (Apache Software Foundation, Forest Hill, MD, USA) for statistical analysis.

Results

All animals from 25Ket./Med. and 75Ket./Med. groups lost consciousness. Animals treated with ketamine only did not lose consciousness. Mice from the 25Ket. group walked quickly with some motor inco-ordination and mice from the 75Ket. group walked slowly with motor inco-ordination.

Table 2 Haemodynamic and oxygen saturation data ($n=10$). Data are expressed as mean (SD)

Time (min)	Group	Heart rate (beats min^{-1})	Respiratory rate (respiratory movements per minute)	Systolic pressure (mm Hg)	Sp_{O_2} (%)	Temperature (°C)
0	25Ket./Med.	484.1 (80.6)	128.0 (17.8)	126.4 (26.2)	99.0 (0.8)	37.2 (0.3)
	75Ket./Med.	498.1 (69.1)	139.5 (28.2)	152.5 (31.0)	98.9 (0.8)	37.0 (0.5)
10	25Ket./Med.	474.1 (74.6)	131.0 (14.1)	125.8 (25.5)	98.6 (0.9)	36.9 (0.4)
	75Ket./Med.	493.8 (70.5)	156.0 (22.1)	124.4 (22.9)	99.0 (0.7)	36.5 (0.6)
20	25Ket./Med	507.0 (62.8)	147.5 (17.3)	123.8 (24.5)	98.8 (0.7)	36.7 (0.5)
	75Ket./Med.	518.1 (50.2)	162.5 (20.6)	150.1 (29.4)	98.9 (0.8)	36.9 (0.8)
30	25Ket./Med	513.1 (68.9)	137.8 (21.8)	129.9 (25.4)	98.8 (0.7)	37.0 (0.2)
	75Ket./Med.	488.5 (41.5)	168.0 (23.6)	130.8 (24.5)	98.9 (1.0)	36.2 (0.8)
40	25Ket./Med	505.1 (54.3)	147.0 (21.1)	134.0 (26.7)	98.4 (1.2)	36.7 (0.6)
	75Ket./Med.	524.6 (79.7)	162.5 (20.8)	136.6 (24.4)	98.6 (1.5)	36.5 (0.3)
50	25Ket./Med	518.1 (41.4)	143.0 (18.5)	129.4 (26.8)	99.3 (0.7)	36.8 (0.5)
	75Ket./Med.	506.8 (58.1)	153.5 (24.9)	135.8 (20.6)	98.9 (1.1)	36.5 (0.5)
60	25Ket./Med	509.6 (50.7)	144.5 (16.3)	129.5 (17.6)	99.3 (0.7)	36.8 (0.7)
	75Ket./Med.	530.4 (39.7)	167.0 (23.8)	132.9 (15.6)	99.0 (0.8)	36.9 (0.4)

Animals from the medetomidine group were less active and generally remained in a corner of the box.

Anaesthesia

Results for anaesthesia are based on anaesthetized groups (25Ket./Med. and 75Ket./Med.). No significant differences were detected between groups for time needed to induce anaesthesia [2.25 (0.71) and 1.87 (0.83) min, respectively], temperature, heart rate, systolic pressure, and oxygen saturation. Unconscious animals were anaesthetized for 1 h; afterwards, atipamezole was administered for reversal. The 75Ket./Med. group had a higher respiratory rate compared with the mice receiving lower concentrations of ketamine/medetomidine, at 10 ($P=0.017$), 30 ($P=0.019$), and 60 ($P<0.045$) min after loss of consciousness. The same group also had a greater depth of anaesthesia and took longer to recover from anaesthesia. Table 2 shows the haemodynamic and oxygen saturation data.

Behavioural tests

No differences were observed between groups regarding the number of trials needed to complete the T-maze test at 28 h, 1 week, and 2 weeks after anaesthesia (Fig. 2). During the vertical pole test, animals from the 75Ket/Med. group took longer than the others to completely descend the pole and also showed increased latency to turn downwards (Fig. 3). The high values of the 75Ket./Med. group were due to the fact that six animals fell from the pole.

The open-field test showed differences between groups in terms of speed and total distance. The 75Ket. group moved faster and covered a greater distance than the control group. Since the time to explore the arena is the same for all the animals, the speed only depends on the distance walked; hence, only speed is represented graphically (Fig. 4). Animals anaesthetized with 75 mg kg^{-1} ketamine walked a larger distance in the peripheral area compared with the control group ($P=0.002$), the Med. group ($P=0.002$), and the 25Ket./Med. group ($P=0.003$). There were no differences detected between groups in the other parameters measured in the open field.

Brain analyses

HE staining revealed no differences in the number of dead cells between groups in the different brain regions studied. Immunohistochemical analysis showed a similar number of cells with clear positive staining for caspase-3 and BDNF expression (Fig. 5).

Discussion

The results from this work showed no apoptotic neurodegeneration after different sedative and anaesthetic doses of ketamine/medetomidine combinations in adult mice. No effect on cell death or on spatial memory and no alterations in BDNF expression were observed. While ketamine induced hyperlocomotion in the open field when administered alone, this effect was reduced by the α_2-agonist medetomidine. The ketamine/medetomidine combination seemed to result in physiologically anaesthetic stabile anaesthesis since there were no significant differences between groups regarding heart rate and arterial pressure.

The haemodynamic stability observed in ketamine/medetomidine combinations is in accordance with previous studies, which reported that the action of ketamine helps to oppose the depressant actions of α_2-agonists on the circulatory system,[12 13] improving perioperative haemodynamic stability.[16] Our observation that the higher concentration of ketamine/medetomidine increased respiratory rate is supported by reports of the respiratory-stimulating properties of ketamine.[28]

High concentrations of ketamine/medetomidine also increased anaesthetic depth, with longer latency to recover equilibrium as indicated by the worst performance in the vertical pole test compared with lower concentrations.

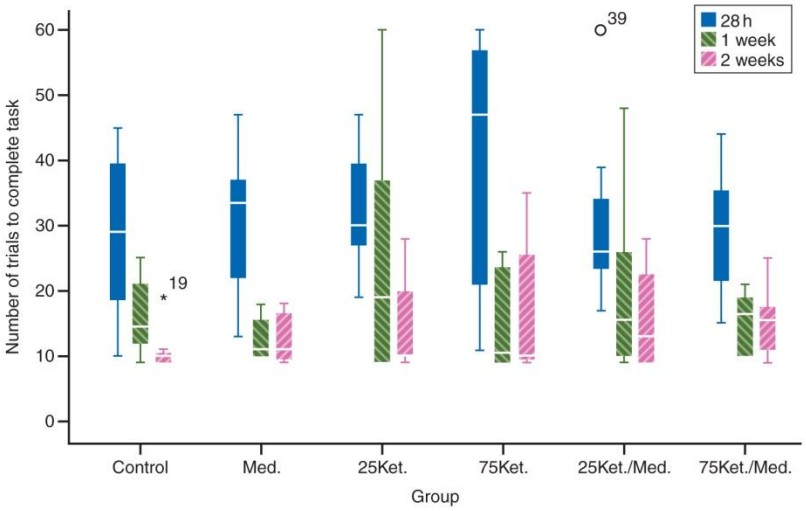

Fig 2 Number of trials necessary to complete the T-maze task at 28 h, 1 week, and 2 weeks after i.p. injection in different groups (n=8). Data are presented as box and whisker plots showing the median, inter-quartile range, and 5th and 95th percentiles. ○=outlier and *=extreme value.

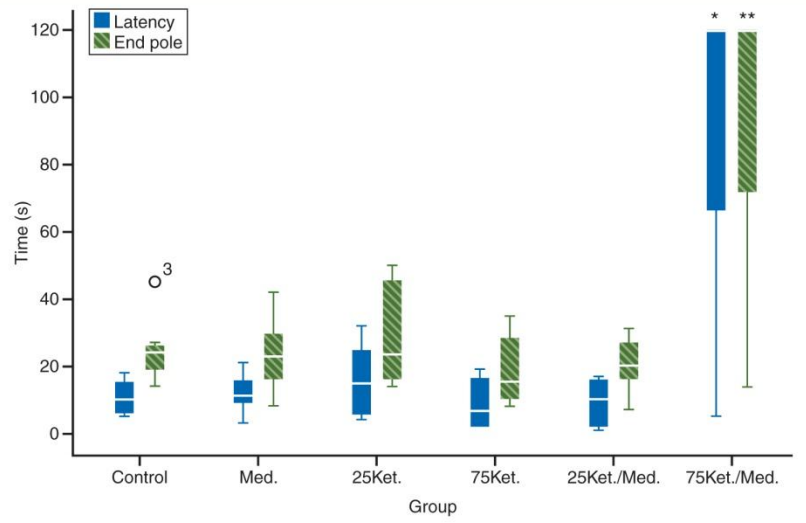

Fig 3 Latency time to turn (latency) and completely descend the vertical pole (end pole) in seconds, 4 h after anesthesia (n=8); 120 s was attributed to the animals that fell. *$P \leq 0.021$ and **$P \leq 0.028$. Data are presented as box and whisker plots showing the median, inter-quartile range, and 5th and 95th percentiles. ○=outlier.

In the open field, all animals showed a species-specific response to the aversive condition of the brightly lit arena, spending more time near the wall than in the central region, with most measures being unaffected by the different sedative and anaesthetic combinations. However, animals from the 75Ket group showed increased distance walked in

the periphery and total distance; consequently a higher speed was observed. This suggests a hyperlocomotion effect of ketamine, in agreement with previous observations that this drug induces hyperlocomotion by the alteration of pre-synaptic components of dopamine neurones in the nucleus accumbens of mice.[8] When the α_2-agonist medetomidine was administered, hyperlocomotion was reduced; perhaps because this drug reduces dopamine turnover.[29] To our knowledge, this is the first study showing that the hyperlocomotion induced by ketamine may be inhibited by α_2-agonists in mice.

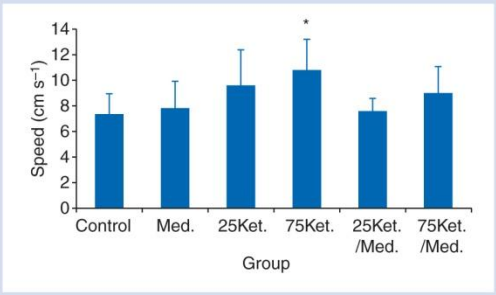

Fig 4 Speed in the open-field test induced by the different treatment groups (n=8) during 20 min, 24 h post-anaesthesia. *P=0.033 compared with the control group. Mean (SD) shown.

In the T-maze test, used to assess spatial memory, no differences between groups were seen. All groups learned the task at 28 h after anaesthesia and recalled it 1 and 2 weeks post-anaesthesia, as indicated by the decreasing number of trials to complete the task with time. No data were found concerning the effects of the combined administration of ketamine/medetomidine in spatial memory or neurodegeneration. However, it has been consistently reported that ketamine alone causes memory impairment and neurodegeneration when administered in neonates.[30 31] Nevertheless, the adult brain is very different from neonates and there is conflicting evidence on the effects of a single dose of ketamine on cognition in the adult brain, with effects depending on the type of memory studied,[32] acute or chronic use,[32] dose,[33] and temperature.[33] Chronic administration of ketamine resulted in disrupted spatial working memory after 10 days of treatment.[34] However, this did not happen with less days of treatment[34] which is in accordance with our results.

The dosages used in our study (25 and 75 mg kg^{-1} ketamine) were subanaesthetic when administered alone. The addition of the α_2-agonist medetomidine allowed anaesthesia, reducing the amount of ketamine required and consequently reducing the probability of inducing adverse effects caused by high ketamine concentrations.

The α_2-agonist medetomidine and the drug used to reverse it, atipamezole, did not affect performance in the t-test. This is in agreement with the previous work carried out by Carlson and colleagues[35] that reported that

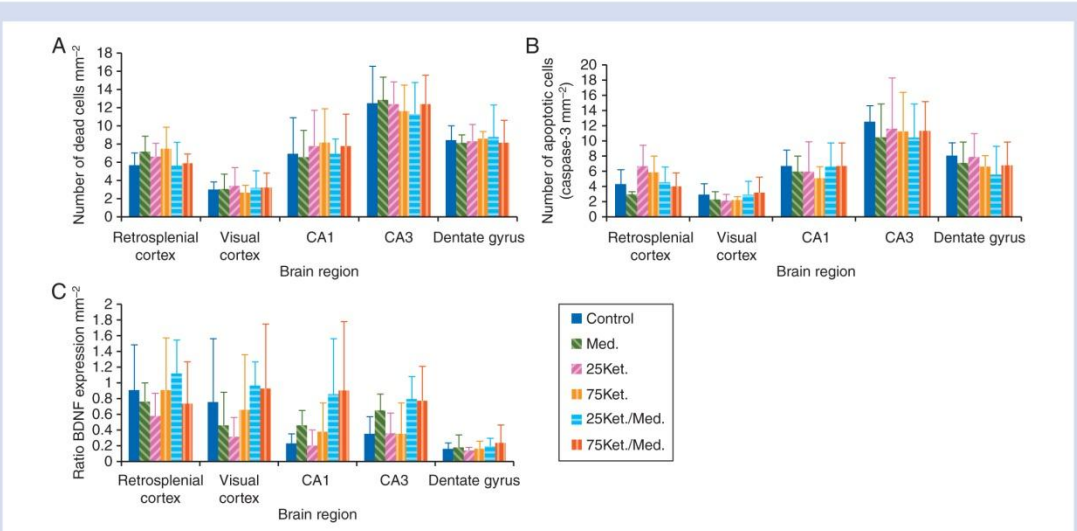

Fig 5 (A) The number of dead cells (HE), (B) number of caspase-3 activation positive profiles (apoptosis), and (C) ratio of the number of positive BDNF expression/negative BDNF expression (mm^2) in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, and in the granular layer of the dentate gyrus, 3 h after anaesthesia in different groups (n=5). Mean (SD) shown. No significant difference between groups was observed.

medetomidine had no effect on spatial memory in the adult rats and that atipamezole (1 mg kg^{-1}) had no effect on spatial cognitive performance after a single dose in rats.[36]

Cellular death in this study was evaluated mainly by measuring apoptosis, in which no difference between groups was observed. Other works showed that a single dose of ketamine induced age- and sex-dependent cell death. Adults were more sensitive than immature rats and females were more sensitive than males; males remained insensitive to ketamine-induced vacuolization of neurones until they reached full adulthood.[37] In this work, we used adult males and we found no negative impact of ketamine with medetomidine in the brain. However, we studied neurodegeneration as apoptosis and not by excitotoxicity (vacuolization of neurones). Furthermore, to study general toxicity, it would be necessary to evaluate cell death in more time points. The histopathological analyses were performed only at 3 h after anaesthesia because it was reported that this is a good time point [25 38] to study apoptosis.

BDNF is an important indicator of brain exposure to insults. This was not altered with ketamine/medetomidine combinations and is in accordance with the results obtained with caspase-3. Usually, BDNF increases after insults,[39] but we found no evidence in the literature of the effects of this combination on BDNF expression. Acute administration of low doses of ketamine has been reported to increase BDNF expression.[40] However, we used higher doses of ketamine. Interestingly, pro-apoptotic anaesthetic drugs modulate BDNF protein levels in the developing brain,[41] resulting in an increase in caspase-3 and caspase-9 activation and, consequently, in apoptotic neurodegeneration.[41] In our study, no differences were detected between groups regarding the BDNF level and in caspase-3 activation, reinforcing the suggestion that a single dose of ketamine/medetomidine was not an insult to the adult brain.

In conclusion, our work showed that a single administration of sedative and anaesthetic concentrations of ketamine/medetomidine combinations in adult mice did not affect spatial memory, BDNF expression, or neurodegeneration by apoptosis in the hippocampus, retrosplenial cortex, and visual cortex. Simultaneously, this study showed that medetomidine prevented ketamine-induced hyperlocomotion, suggesting that α_2-agonists may possibly attenuate post-anaesthetic delirium and agitation produced by ketamine.

Acknowledgement

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Declaration of interest

None declared.

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Room Hall B1-Area C

Cognitive and Neurodegenerative Effects of the Medetomidine/Ketamine Anesthesia Combination in Mice

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Background: Medetomidine (MED)/Ketamine (KET) is a commonly used combination in laboratory animals. Several studies have shown that KET can enhance neuronal death in neonates (1). Nerveless, little is known about its effect in combination with Med and in adult. The aim of this work was to study the effect of different concentrations of MED/KET in cognition and its potential neurodegenerative effect in adult mice.

Methods: Fifty-two mice males, inbred C57BL/6, with 28 weeks old, were divided into 4 different groups (I-saline, II-1mg/kg MED, III-1mg/kg MED + 25mg/kg KET, IV-1mg/kg MED + 75mg/kg KET). Drugs were administered intraperitoneally (i.p.). After one hour, anesthesia was reversed with atipamazole 1mg/kg (i.p.). Thirty-two animals (n=8 per group) were behavioral tested with the T-maze task (28 h, 1 and 2 weeks after anesthesia) and open field (24 h after anesthesia). The remaining 20 animals (n=5 per group) were sacrificed 3 hours after anesthesia by cervical dislocation followed by decapitation and their brains analyzed by hemotoxylin-eosin staining and caspase-3 activation to access neurodegeneration in the retrosplenial cortex, visual cortex, pyramidal cell layer from CA1 and CA3 areas of the hippocampus, and in the granular layer of the dentate gyrus. Death cells (H&E) and cells showing clear positive immunoreactions (caspase-3) were counted. Statistical analysis was performed using univariate ANOVA.

Results: No significant differences were detected between groups on the behavior performance of the T-maze and open field. These observations were supported by similar results with histopatological studies (H&E and caspase 3 activation).

Conclusion: This study showed no cognitive impairment or neurodegenerative differences induced by different concentrations of MED/KET anesthesia in the brain of adult mice.

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APPENDIX C

OUTPUTS FROM CHAPTER 4

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Eur J Anaesthesiol 2013; **30:**1–9

ORIGINAL ARTICLE

A single intraperitoneal injection of ketamine does not affect spatial working, reference memory or neurodegeneration in adult mice

Patrícia O. Ribeiro, Paula C. Rodrigues, Ana M. Valentim and Luís M. Antunes

CONTEXT Ketamine is an anaesthetic and analgesic drug used in research and clinical practice. Little is known about the effects of different doses of this drug on memory and brain cellular death.

OBJECTIVE To study the effects of different doses of ketamine on working and reference memory, and neurodegeneration in adult mice.

DESIGN A randomised study.

SETTINGS The study was carried out in a basic science laboratory, between March 2011 and August 2012.

ANIMALS Forty-eight 7-month-old, male C57BL/6 mice were used.

INTERVENTION Animals received a single intraperitoneal injection of saline solution or one of three doses of ketamine (25, 75 or 150 mg kg^{-1}). Each group consisted of 12 animals (seven animals for behavioural tests and five animals for histopathological and immunohistochemical studies). The animals used for histopathology studies were sacrificed 3 h after anaesthesia.

MAIN OUTCOME MEASURES Working and reference memories were assessed using the radial-maze test over 12 consecutive days. The equilibrium was tested using the vertical pole (4 and 24 h after injection), whereas locomotion was assessed using the open field (24, 48 and 72 h after injection). Histopathological (haematoxylin-eosin staining) and immunohistochemical analyses (procaspase-3 and activated caspase-3 detections) were performed 3 h after injection to assess neurodegeneration in the retrosplenial and visual cortices, pyramidal cell layer of the *cornu Ammonis* 1 and *cornu Ammonis 3* areas of the hippocampus, in the granular layer of the dentate gyrus, in the laterodorsal thalamic nucleus, striatum and accumbens nucleus.

RESULTS No significant differences were observed between different groups regarding the number of dead cells and cells showing positive immune-reactivity in the different regions of the brain studied. The performance in the vertical pole test and the number of reference and working memory errors in the radial-maze were similar in all groups. Nevertheless, the animals treated with ketamine 75 mg kg^{-1} were transiently more active, walking a greater total distance at a greater speed in the open field than other groups (power of 0.96).

CONCLUSION These data indicate that a single intraperitoneal injection of ketamine at subanaesthetic and anaesthetic doses does not impair working memory, reference memory or neurodegeneration in adult mice, but an intermediate dose of ketamine produces transitory hyperlocomotion.

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Introduction

Ketamine is a noncompetitive antagonist of glutamate at the level of its N-methyl-D-aspartate receptor subtype. It is used as an anaesthetic and analgesic in clinical practice,[1] particularly in painful diagnostic procedures, in obstetrics, in asthmatic patients, in traumatic and hypovolaemic shock, and in burns treatment.[2–4] It is increasingly used in pain therapy, reducing acute postoperative opioid consumption and pain intensity.[5,6] However, it is associated with psychomimetic effects[7,8] and there are still uncertainties about the effect of a single

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administration of ketamine on working and reference memory, and on neurodegeneration in adults.

There are consistent reports that ketamine causes memory impairment and neurodegeneration when administered to neonatal rodents.[9,10] However, the adult brain is very different from that of the neonate, and the effect of a single ketamine administration on memory, and on neurodegeneration in adults, is controversial. Chronic ketamine administration in rats disrupted spatial working memory after 10 days of treatment, but these changes were not observed at 5 days.[11] Also, ketamine disrupted recall in the rat at anaesthetic, but not at subanaesthetic doses.[12]

In-vitro studies have shown that ketamine induces neuronal apoptosis via the mitochondrial pathway.[13] These neurodegenerative effects were also observed in in-vivo studies, which showed that a single administration of ketamine induced age and sex-dependent cell death by excitotoxicity (neuronal vacuolisation) in rat retrosplenial cortex.[14] However, apoptosis and excitotoxicity are different types of cellular death[15] and the effect of a single administration of ketamine on apoptosis has not been established in adult mice. Moreover, the effects of subanaesthetic and anaesthetic doses of ketamine in different regions of the brain were not studied.

The purpose of this study was to evaluate the influence of different doses of ketamine on spatial working and reference memory and its potential neurodegenerative effects in different regions of the brain in adult mice.

Materials and methods

This project was reviewed and ethically approved by the Portuguese competent authority for animal protection, Direcção Geral de Veterinária, Lisbon, Portugal, protocol/project no. 003053, from 19 March 2012.

Experimental animals, housing and husbandry

Forty-eight 7-month-old, male C57BL/6 mice bred in the animal facility of the institute (F1–F2 offspring of animals bought from Charles River, Barcelona, Spain) were used. The mice were housed with controlled temperature (21°C) and relative humidity at 55%. Lights were on a 12/12 h cycle, with lights off at 1700 h. The animals were housed in groups of three to five mice per cage (Makrolon type II cage, Tecniplast, Dias de Sousa, Alcochete, Portugal). Each cage was provided with standard corncob litter (Probiológica, Lisbon, Portugal), a piece of tissue paper and a cardboard tube. The mice were allowed to adjust to the facilities at least 1 week prior to the study. Animals received a commercial pellet diet (4RF25-GLP Mucedola, SRL, Settimo Milanese, Italy) and water *ad libitum*. A food restriction schedule 1 week before radial-maze habituation and during the test was applied to animals used in behavioural tests. A limited amount of food administered once daily was adjusted to a level that kept the mice on 85 to 95% of free-feeding weight.

General study design

Mice were randomly assigned into four treatment groups: the control group treated with saline solution, the 25Ket. group received ketamine 25 mg kg^{-1} (lower subanaesthetic dose), the 75Ket. group received ketamine 75 mg kg^{-1} (higher subanaesthetic dose) and the 150Ket. group received ketamine 150 mg kg^{-1} (anaesthetic dose). Animals were randomly allocated into the groups using a web site service available at http://www.random.org. All groups consisted of 12 animals (seven for behavioural tests and five animals for histology).

Experimental procedure

Anaesthesia

Ketamine (Imalgéne Merial, Portugal; 100 mg ml^{-1}) was used for anaesthesia. Standard physiological saline 0.9% (Soro Fisiologico, Braun Vet, Portugal) was used in the control group and for dilution of the ketamine. All animals received an isovolumetric volume of 0.3 ml, including the control group.

Ketamine or saline solution was administered as a single intraperitoneal (i.p.) injection. Injection and restraint were always performed by the same person. After i.p. injection, each animal was placed alone in a cage until it lost its righting reflex; thereafter, the animals were moved to a homeothermic blanket connected to a rectal thermal probe (50-7061-F, Harvard Apparatus Ltd, Kent, UK) that allows the maintenance of the temperature at 36°C to 37.5°C throughout anaesthesia. One hundred per cent of oxygen was delivered to the animals with a tube connected to a coaxial circuit (0.8 l min^{-1}) and ophthalmic gel was applied to the eyes (Lacryvisc, Alcon, Paço D'Arcos, Portugal).

To avoid isolation stress, animals that did not lose consciousness (control, 25Ket. and 75Ket.) were returned to their home cage; heating was provided.

Time to loss of the righting reflex, tail pinch and pedal withdrawal reflex were recorded in unconscious animals (150 Ket. group). In addition, the respiratory rate and pulse were recorded at 10-min intervals. Systolic pressure and pulse rate values were obtained with a pressure meter (LE 5001, Panlab, Spain). A pulse oximeter (S&W 9040, Athena, Germany) placed on the right hindleg of each mouse was used for monitoring oxygen saturation (SpO_2).

Behavioural tests

The timing of behavioural test protocol in relation to anaesthesia is shown in Fig. 1.

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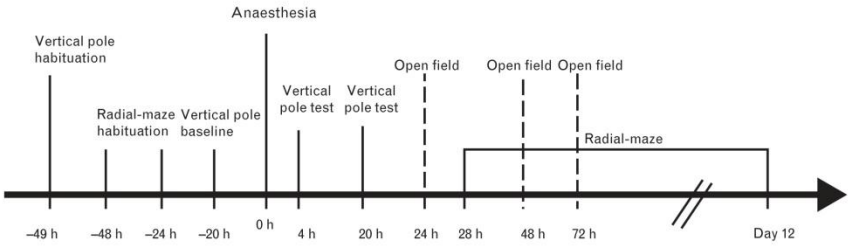
Fig. 1



Timeline for the behaviour tests protocol (in hours to 12 days). Moment of anaesthesia administration is defined as time zero.

Radial-maze

The radial-maze test was used to assess learning, working memory and reference memory. In this study, our setting was based on one previously described.[16] The radial-maze was made of transparent acrylic sheets and consisted of eight equal arms in a radial pattern. The central platform measured 180 mm across and the arms measured 80 x 400 x 300 mm (width, length, height). All arms have a depression in the distal end wherein a piece of commercial chocolate flavoured pellet (Dustless Precision Pellets, 20 mg, chocolate flavour rodent purified diet, Bio-Serv, Frenchtown, New Jersey, USA) was placed as food reward.

Habituation to the radial-maze test was performed over 2 days before anaesthesia. In the morning of the first day, all animals from each cage were left inside the apparatus and allowed to explore it for 15 min with no food reward present. In the afternoons of the first and second days, each animal was placed alone inside the full baited apparatus. The animals started the test in the central platform, inside a transparent cylinder. Three arms were baited and the angles between them were always 90°, 135° and 135°. When the cylinder was lifted, the animal was allowed to explore all the apparatus and the trial ended when it had visited all the arms with rewards or when 5 min had elapsed. Then, when the animal returned to the central platform, the cylinder was lowered until the next trial. The maze was rotated between trials in a clock or anticlock movement, from 45° to 180° in a random way. Each session had five trials with 1 min of inter-trial interval. Each animal had one session per day over 12 consecutive days. Entrance was recorded when the four limbs of the animal passed a landmark position placed outside each arm. Numbers of reference memory errors (entries to nonbaited arms) and working memory errors (re-entries to arms already visited) were measured over the 12 consecutive days.

Vertical pole test

The vertical pole test measures motor coordination and balance and was used to assess postanaesthetic recovery. Deficits in motor coordination and balance were detected when the mouse fell from the pole.[17] This test was performed as previously described by Bellum *et al.*[18] with minor modifications.

Each mouse was placed in the centre of a round rough-surfaced pole (50 cm long, 2 cm diameter). The pole was initially positioned horizontally and then slowly inclined to 90° [1° per second (s)]; the animal faced the upright end. Performance was determined by the latency (s) for the mouse to turn downwards and completely descend the pole. The vertical pole test was performed four times in four consecutive periods: on the same day of the habituation to the radial-maze test (habituation trial); on the day before anaesthesia (to determine the baseline coordination of the animals), and 4 and 20 h postrighting reflex recovery (testing).

Open field

The open field test was used to assess locomotor activity, stress levels[17] and the habituation to the open field arena.[19,20] The apparatus consisted of a circular arena 1 m in diameter, made of grey polypropylene and surrounded by a wall 30 cm high. Three areas were defined in the open field arena: periphery, middle and centre. The test was performed 24, 48 and 72 h post-anaesthesia. Each animal was released in the centre of the arena and allowed to explore it for 20 min.

At the end of testing, the number of faecal boli was counted and the arena was cleaned with 70% alcohol to remove olfactory cues. The test was recorded with a camera placed above the apparatus attached to a computer with the multicamera vigilance system GeoVision (GV-800/8, Taipei, Taiwan). The video analysis was carried out with the program VideoMot 2 (TSE-systems, Bad Homburg, Germany), which measured several variables: distance walked, time spent and the number of visits to each region. The latency to exit the centre, the total distance walked and the total speed were also calculated.

Histopathological (haematoxylin-eosin staining) and immunohistochemical (procaspase-3 and activated caspase-3) studies

Three hours after anaesthesia, a time point used in previous studies,[21,22] mice were euthanised by cervical dislocation followed by decapitation and rapid removal of the brain. All brains were fixed for 48 h in 4% buffered paraformaldehyde (PBS, pH 7.4, 0.1 mol/l), processed and embedded in *paraplast* (SHANDON, Hypercentre XP and Histotocentre 2, Burlingame, California, USA). For each block, serial sections of 4 μm thickness were made making coronal cuts from Bregma 3.08 mm to Bregma 2.70 mm[23] with a 30 μm interval.[24] Three slices per animal per region of brain were made for each stain used. These slices were used for haematoxylin-eosin staining (observation of cellular death), procaspase-3 and activated caspase-3 detections (observation of apoptotic cells). Procaspase-3 antibody (CPP32 Ab-4, Rabbit Polyclonal Antibody; Thermo Scientific) and active caspase-3 antibody (Cleaved Caspase-3 antibody, AQ2 Asp175; Cell Signaling Technology) were used. The immunohistochemical protocols and image capture have been reported elsewhere.[21]

Cellular death (HE) was consistent with morphology from previous studies.[25,26] Cells with abnormal morphology such as cell shrinkage, hypereosinophilic cytoplasm or/and condensed and hyperchromatic nuclei were considered positive for cell death.

The number of dead cells and cells showing clear positive immunoreactions, per square millimetre in the retrosplenial and visual cortices, pyramidal cell layer of the *cornu Ammonis* 1 and *cornu Ammonis 3* areas of the hippocampus, the granular layer of the dentate gyrus, the laterodorsal thalamic nucleus, accumbens nucleus and striatum, were manually counted and the average from three coronal sections selected at 30 μm intervals was calculated.[24] These specific regions of the brain such as hippocampus,[27] retrosplenial[28] and visual[29] cortices, nucleus accumbens,[30] laterodorsal thalamic nucleus[31] and striatum[32] were selected because they are important to memory.

Statistical analysis

Two sample size calculations were performed: one to achieve the sample size for the behavioural studies and another to achieve the sample size for histopathologic studies. For the first, we used the information from the study by Fredriksson *et al.*[9] in which, in controls, the number of working memory errors in the first day of radial maze using adult mice was 15 ± 4 (mean ± SD). Considering an estimated treatment effect of 40% from this reference value, a two-tailed hypothesis and a power of 0.8 with type error I at 0.05, we concluded that the sample size was seven animals per group.

The second sample size calculation was based on the information from a study performed in our laboratory using the same histopathological methodology and mice strain,[21] in which controls had 8.2 ± 1.7 caspase-3 activated cells in retrosplenial cortex per square millimetre. Using an estimated treatment effect of a 40%, and two-tailed hypothesis testing to achieve a power of 0.8 with type error I at 0.05, we would need five animals per group.

The data were considered parametric if the following assumptions were fulfilled: independent observations, data from groups with homogeneity of variances (Levenés test) and normal distribution (Shapiro–Wilk).

Parametric data were expressed as mean ± SD, whereas nonparametric data were expressed by median [minimum, maximum]. A value of P of 0.05 or less was considered statistically significant. All results were analysed using Microsoft Office Excel 2003 for data management and SPSS 16.0 for Windows (Apache Software Foundation, Forest Hill, Maryland, USA) for statistical analysis.

Results

The results from all 48 mice were analysed. Twenty-eight animals were used for behavioural tests analysis (seven animals per group), and 20 animals were used for histopathologic analysis (five animals per group). As expected, only animals from the 150Ket group lost consciousness, and so, anaesthetic variables were only measured in this group. Mice were unconscious for 30 ± 10.2 min. Pulse and respiratory rates were, respectively, 537.18 ± 13.40 beats per minute and 174.76 ± 29.28 respiratory movements per minute. Oxygen saturation was maintained above 98% throughout anaesthesia.

Behavioural tests

In the radial-maze test, the number of reference and working memory errors during 12 consecutive days after anaesthesia were similar between groups ($n = 7$; Table 1).

In the vertical pole test, the latency to turn downwards at 4 h ($\chi^2 = 0.597$, df = 3, $P = 0.897$) and 24 h ($\chi^2 = 0.877$, df = 3, $P = 0.831$) after anaesthesia were similar between groups. Similarly, little difference was detected between groups regarding the time to completely descend the pole at 4 h ($\chi^2 = 0.553$, df = 3, $P = 0.907$) and 20 h ($\chi^2 = 1.292$, df = 3, $P = 0.731$) after anaesthesia (Fig. 2).

The open field test showed differences between groups in speed and in total distance walked in the first day ($n = 7$). The 75Ket. group moved faster over a greater distance ($10.22 \pm 1.61\ \mathrm{cm\,s^{-1}}$ and 122.68 ± 19.25 m, respectively) than the control group ($7.68 \pm 0.92\ \mathrm{cm\,s^{-1}}$ and 92.17 ± 11.04 m, respectively; $P = 0.013$, df = 3 and $F = 4.612$ for speed and $P = 0.013$, df = 3, $F = 4.607$ for total distance travelled), at 24 h after anaesthesia. As the

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Table 1 Number of reference memory (Ref.) and working memory (Work.) errors in the radial-maze test over 12 consecutive days after intraperitoneal injection of saline (control) or different doses of ketamine

Days	Control		25 Ket.		75 Ket.		150 Ket.		Results of Kruskal–Wallis test for Ref. errors between groups	Results of Kruskal Wallis test for Work. errors between groups
	Ref. errors	Work. errors	Ref. errors	Work. errors	Ref. errors	Work. errors	Ref. errors	Work. errors		
1	5.2 [3.2–7.4]	2.3 [1.2–4.2]	6.2 [3.6–8.0]	4.1 [1.0–5.8]	5.8 [4.0–9.2]	3.1 [1.4–6.6]	5.1 [3.6–8.6]	3.0 [1.0–6.8]	$\chi^2 = 0.835$, df = 3, $P = 0.841$	$\chi^2 = 0.960$, df = 3, $P = 0.811$
2	4.7 [3.2–6.2]	2.1 [0.4–2.8]	5.4 [3–9.2]	2.8 [0.6–6.6]	5.5 [4.2–8]	2.8 [0.4–6.0]	5.0 [3.4–8.4]	3.1 [0.4–5.8]	$\chi^2 = 1.226$, df = 3, $P = 0.747$	$\chi^2 = 1.843$, df = 3, $P = 0.606$
3	3.6 [2.4–5.2]	1.4 [1–2.6]	4.9 [2.2–6.8]	1.8 [0.8–3.4]	3.6 [3.2–5.2]	1.1 [0.2–2.6]	4.2 [3.2–4.4]	1.6 [1.2–3.8]	$\chi^2 = 2.458$, df = 3, $P = 0.483$	$\chi^2 = 4.432$, df = 3, $P = 0.218$
4	4.5 [2–8]	2.4 [0.4–6.4]	4.3 [3.2–6.4]	2.1 [0.4–3.2]	3.8 [2.2–5.6]	1.5 [0.8–3]	3 [2.4–4.2]	0.9 [0.2–1.6]	$\chi^2 = 4.298$, df = 3, $P = 0.231$	$\chi^2 = 6.619$, df = 3, $P = 0.085$
5	2.9 [2.6–3.2]	1 [0.2–1.6]	3.5 [1.8–6.2]	1.1 [0.4–3]	3.3 [2.0–4.6]	1.4 [0–2.4]	4.1 [1.6–6.2]	1.6 [0–3.8]	$\chi^2 = 3.733$, df = 3, $P = 0.292$	$\chi^2 = 1.599$, df = 3, $P = 0.660$
6	2.5 [1.2–4.0]	0.2 [0–1.2]	3.1 [2–3.7]	0.9 [0.3–2.3]	2.4 [1.6–3.4]	0.3 [0–1.6]	3.1 [0.8–4]	0.4 [0–1.4]	$\chi^2 = 1.945$, df = 3, $P = 0.584$	$\chi^2 = 4.157$, df = 3, $P = 0.245$
7	2.1 [0.4–4.6]	0.7 [0–4.2]	2.2 [1.8–2.6]	0.9 [0.2–1.2]	2.7 [1.6–8]	1 [0–4.4]	2.8 [1.6–3.4]	0.5 [0–1.2]	$\chi^2 = 2.179$, df = 3, $P = 0.536$	$\chi^2 = 1.244$, df = 3, $P = 0.743$
8	1.7 [1.2–3]	0.7 [0.2–1.8]	2.5 [1.4–3]	0.5 [0.4–2.2]	2.8 [1–3.8]	1 [0.4–2]	1.9 [1–3.6]	0.3 [0–0.6]	$\chi^2 = 3.006$, df = 3, $P = 0.391$	$\chi^2 = 6.773$, df = 3, $P = 0.080$
9	1.2 [0.4–2.8]	0.5 [0–2]	2.2 [1.2–3.4]	0.5 [0.2–3]	1.4 [1–3.2]	0.3 [0–0.6]	2.1 [1.4–4.2]	0.4 [0–0.8]	$\chi^2 = 4.894$, df = 3, $P = 0.180$	$\chi^2 = 2.711$, df = 3, $P = 0.438$
10	1.3 [0.8–2.4]	0.6 [0.2–1.8]	1.8 [1.4–2.2]	0.4 [0.2–1]	1.5 [0.6–4.6]	0.6 [0.2–1.6]	2.3 [1.4–3.2]	0.5 [0.2–1.4]	$\chi^2 = 2.792$, df = 3, $P = 0.425$	$\chi^2 = 0.737$, df = 3, $P = 0.864$
11	0.9 [0–2]	0.5 [0–0.6]	1.4 [1–3.4]	0 [0–2.8]	0.7 [0.4–3.4]	0.3 [0–0.6]	1.8 [0.8–7]	0.3 [0–4.4]	$\chi^2 = 4.196$, df = 3, $P = 0.241$	$\chi^2 = 2.440$, df = 3, $P = 0.486$
12	0.8 [0–1.4]	0.3 [0–0.8]	1.4 [0.8–1.8]	0.1 [0–1.6]	1.1 [0.6–3.8]	0.4 [0–1]	1.7 [0.8–2.2]	0.2 [0–1]	$\chi^2 = 7.082$, df = 3, $P = 0.069$	$\chi^2 = 0.103$, df = 3, $P = 0.992$

The respective results of Kruskal Wallis tests using group as a variable are also shown. Data are presented as median [minimum-maximum]. χ^2: chi-square, df: degrees of freedom, *P*: significance.

time to explore the arena was the same for all the animals, speed and total distance walked are directly related; hence, only speed is graphically represented (Fig. 3). With time (days), there was a decrease in total distance walked (df = 2, $F = 63.63$, $P = 0.00$). This decrease was at the same rate in all groups (df = 6, $F = 1.815$, $P = 0.121$), showing a lack of influence of ketamine in the habituation process in the open field arena ($n = 7$).

There were no important differences detected between groups in the other variables measured in the open field ($n = 7$).

Brain analyses

Differences were similar between groups regarding the number of dead cells (haematoxylin-eosin) and cells showing positive immunoreactions (procaspase-3 and activated caspase-3) in the different brain regions studied ($n = 5$; Fig. 4).

Discussion

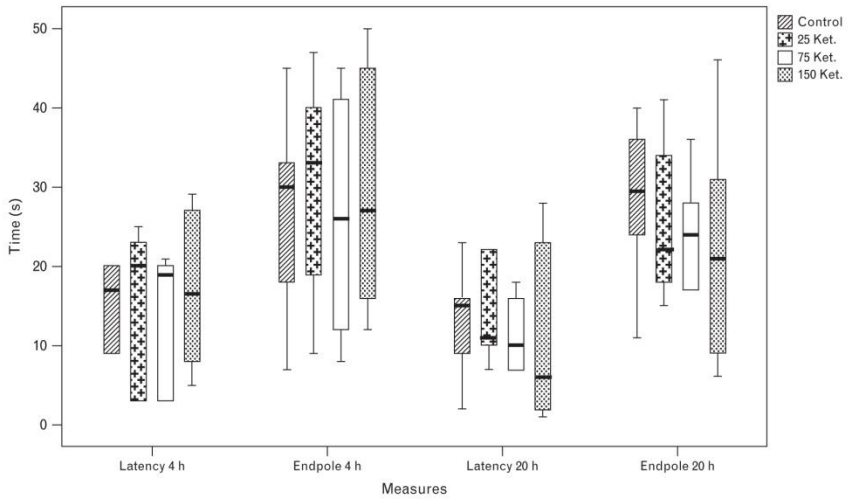
Our results show that a single administration of different doses of ketamine does not affect spatial working, reference memory or neurodegeneration in adult mice. The intermediate dose of ketamine (75 mg kg^{-1}) induced transitory hyperlocomotion.

The effects of the drug on working and reference memory were assessed by the radial-maze tests. The results were similar between groups throughout the entire experiment. All groups learned the task and reduced the number of working and reference memory errors with time. Earlier reports give controversial information on the effects of ketamine on memory depending on the regimen, acute or chronic,[33] type of memory studied,[34] temperature and dose.[35] With chronic administration, after 10 days of treatment, the spatial working memory was disrupted, but not with a shorter duration of treatment,[11] and this is in accordance with our results. It has also been reported that low doses of ketamine impaired working and reference memory (1.5 to 10 mg kg^{-1})[36] and learning (8 mg kg^{-1})[37] in animal models.

The vertical pole test was used to evaluate equilibrium and motor coordination. Again, groups were similar at 4 and 20 h after anaesthesia. This suggests that all animals recovered from the drug 4 h after its administration, and that all animals had equivalent motor capacities when the other behavioural tests were performed. There is insufficient published information on the effects of ketamine on the vertical pole test in mice to comment further.

In the open field test, all animals showed a species-typical response to the aversive conditions of the brightly lit arena, spending more time near the wall than in the central region, with most measures being unaffected by ketamine administration. These results suggest similar levels of stress between groups. Moreover, all groups

Fig. 2



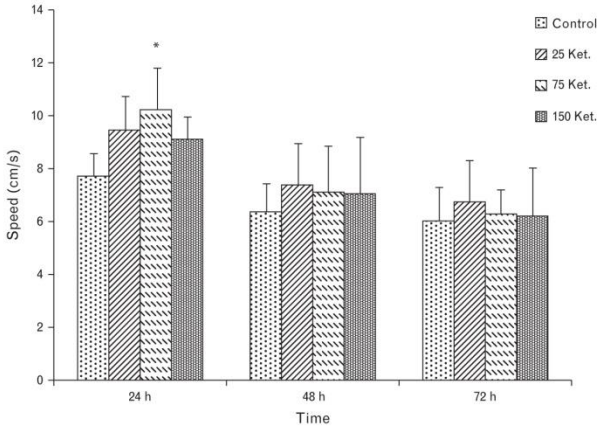
Time to turn (latency) and completely descend the vertical pole (endpole) in seconds, 4 and 20 h after control (saline), or ketamine 25, 75 or 150 mg kg^{-1} ($n = 7$). All groups were similar. Data are shown as a box plot (the median is indicated by the horizontal bar inside the box; the 25th and 75th percentile are the boxes borders; and the whiskers are the lowest and highest values for the 5th and 95th percentiles, respectively).

habituated to the open field arena at a similar rate and reduced total activity across sessions. However, 24 h postanaesthesia, animals from 75Ket.group moved more and faster than the control animals. This effect was not seen at 48 and 72 h after anaesthesia. Interestingly, this hyperlocomotion was not observed with the highest and the lowest doses of ketamine used in this study. Our results agree with a previous study that showed that an intermediate dose of ketamine (100 mg kg^{-1}) is more effective than 50 and 200 mg kg^{-1} for producing hyperactivity.[33] The explanation for this remains unclear. Hyperlocomotion induced by ketamine occurs immediately after administration of the drug and is explained by the alteration of presynaptic components of dopamine neurons in the nucleus accumbens of mice.[38] We were unable to find other reports of the effects of ketamine on locomotion at 24, 48 and 72 h after drug administration, nor were there studies of dopamine levels in the nucleus accumbens at these time points.

In this study, neurodegeneration was mainly evaluated by measuring apoptosis, for which no significant differences between groups were observed in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, granular layer of the dentate gyrus, laterodorsal thalamic nucleus, striatum and in the accumbens nucleus. It has been reported that the levels of procaspase-3 (inactive form of caspase-3) in adult rat brain are extremely low compared with the developing brain, and the apoptotic programme requires additional synthesis for posterior activation.[39] We did not see an additional synthesis of procaspase-3 compared with the control group, suggesting that a single administration of ketamine does not cause apoptosis in the adult brain. This is in agreement with our results with activated caspase-3. However, this does not exclude the occurrence of other types of cell death, such as excitotoxicity. Jevtovic-Todorovic *et al.*[14] have previously reported that a single administration of ketamine induced age and sex-dependent cell death by excitotoxicity in the rat retrosplenial cortex. Adults were more sensitive than immature rats, and, females were more sensitive than males. In fact, males remained insensitive to ketamine-induced vacuolisation of neurons (excitotoxicity) until they reached full adulthood.[14] In the present study, ketamine did not show any negative impact on the brain. Other studies may differ from ours because we only used adult male mice and we studied neurodegeneration by apoptosis, not by excitotoxicity.

Unexpectedly, we did observe some cerebral cell death after the injection of saline solution. This may be explained by programmed cell death that still occurs during neurogenesis in adults, mainly in the dentate

Fig. 3

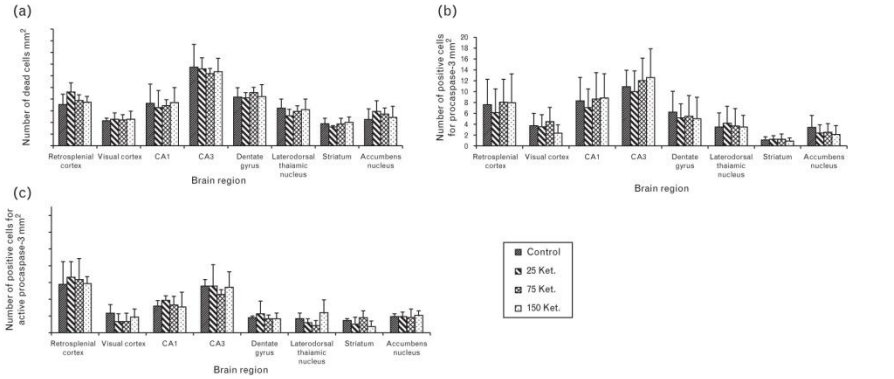


Animals' speed in the open field after control (saline), or ketamine 25, 75 or 150 mg kg^{-1} ($n = 7$) measured over 20 min at 24, 48 and 72 h postanaesthesia. *$P = 0.01$ compared with control group. Bars represent mean and vertical lines represent SD.

gyrus.[40–42] In addition, the fixation method used could have induced artefact in the assessment of cellular death, but this would have been common to all groups. The study animals were not subject to transcardiac perfusion because this would have required additional anaesthetic drugs.

There are no experimental data from humans that clearly demonstrate ketamine neurotoxicity. In in-vitro studies, ketamine induced apoptosis in human neurons[43] just like the neurons of rats,[44] indicating that extrapolation of data from animals to humans might be possible. However, further work is needed both in the laboratory and in the

Fig. 4



(a) The number of dead cells (HE), (b) number of positive cells for procaspase-3 and (c) number of positive cells for active caspase-3 per square millimetre in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, in the granular layer of the dentate gyrus, laterodorsal thalamic nucleus, striatum and accumbens nucleus, 3 h after saline (control), or ketamine 25, 75 or 150 mg kg^{-1} ($l = 5$). Bars represent mean and vertical lines represent SD.

operating room to clarify that neurotoxicity occurs in rodents and humans in the same way.

The doses of ketamine that we have selected for this study are based on a comparison between mice and children,[45] and then extrapolating this to adult humans. Ketamine doses administered for induction of anaesthesia in adult mice (around 100 mg kg^{-1} i.p.)[46] are approximately 8 to 15 times higher than intramuscular (i.m.) doses used for anaesthesia induction in adult humans (in the range of 6.5 to 13 mg kg^{-1} i.m.).[47] Thus, the doses used in our experiment, 25, 75 and 150 mg kg^{-1} i.p, would be equivalent to 1.7 to 3.1, 5.0 to 9.4 and 10.0 to 18.8 mg kg^{-1} i.m. used for an adult human. These values are 25, 75 or 150 mg kg^{-1} doses divided by 15 or 8, respectively. Our study administered ketamine at doses that were subanaesthetic and anaesthetic for an adult mouse, and equivalent to subanaesthetic/anaesthetic doses for an adult human. However, the extrapolation of our results for human clinical practice requires extreme caution; differences in administration route, exposure times and interspecies variations are important variables that need to be considered.

In conclusion, this study showed that a single administration of a subanaesthetic or anaesthetic dose of ketamine, in adult mice, did not affect working and reference memory nor induced apoptotic neurodegeneration in the retrosplenial and visual cortexes, pyramidal cell layer of the *cornu Ammonis* 1 and *cornu Ammonis 3* areas of the hippocampus, the granular layer of the dentate gyrus, the laterodorsal thalamic nucleus, accumbens nucleus and striatum. Only the intermediate ketamine dose induced hyperlocomotion on the first day, suggesting that psychotomimetic effects caused by ketamine in laboratory animals and maybe in humans could be avoided by adjusting the dose. These findings can also be used to refine anaesthesia in laboratory animals.

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Conflicts of interest: None declared.

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Hall A1 South Area E

Different Concentrations of Ketamine did not Affect Cognition or Neurodegeneration in Adult Mice

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Background: Ketamine is used in clinic how anesthetic and analgesic agent (1). However, little is known about the effects of the acute administration of this drug in brain of adult mice. The aim of this work was to study the effect of different concentrations of ketamine on cognition and its neurodegenerative effects.

Methods: Forty-eight mice males, inbred C57BL/6, with 28 weeks old, were divided into 4 different groups (I-saline, II-25 mg/kg ketamine (KET), III-75mg/kg KET, IV-150mg/kg KET). Drugs were administered intraperitoneally (i.p.). Twenty-eight animals (n=7 per group) were behavioral tested with the Radial-maze task (during 12 consecutive days after anesthesia). Number of reference memory and working memory errors and time to finish the test were checked. The remaining 20 animals (n=5 per group) were sacrificed 3 hours after anesthesia by cervical dislocation followed by decapitation and their brains analyzed by hemotoxylin-eosin staining and caspase-3 activation to access neurodegeneration in the retrosplenial cortex, visual cortex, pyramidal cell layer from CA1 and CA3 areas of the hippocampus, and in the granular layer of the dentate gyrus. Death cells (H&E) and cells showing clear positive immunoreactions (caspase-3) were counted. Statistical analysis was performed using univariate ANOVA.

Results: No significant differences were detected between groups on the behavior performance of the Radial-maze (fig.1). These observations were supported by similar results with histopatological studies (H&E and caspase 3 activation).

Conclusion: This study showed no cognitive impairment or neurodegenerative differences induced by different concentrations of ketamine in the brain of adult mice.

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Acknowledgements: FCT-Portugal (PTDC/CVT/099022/2008 and SFRH/BD/48883/2008)

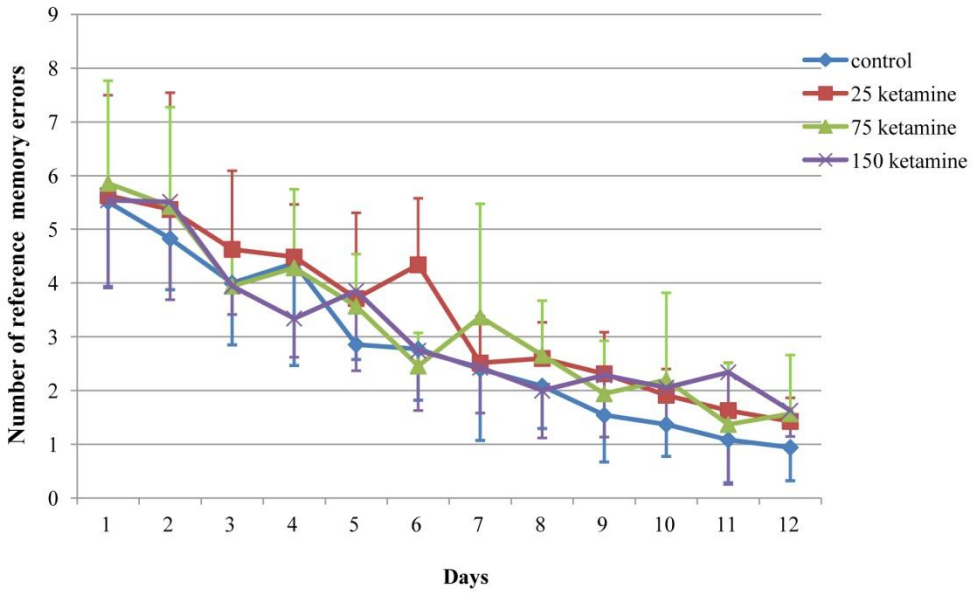

Figure 1. Number of errors in radial-maze task during twelve consecutive days. Data showed as average + or - standard deviation; n= 7. Group I- saline; Group II - 25mg/kg KET; group III- 75mg/kg KET; group IV- 150 mg/kg KET.

APPENDIX D

OUTPUTS FROM CHAPTER 5

Consequently AP-frequency was significantly increased from 62.9±4.0Hz to 69.0±4.0Hz (n=5; p< 0.05).
Conclusion(s): In this study we could show that Xe enhances excitability of RTN neurons which are the major source of inhibitory input to TC neurons. TC neurons in the VB gather somatosensory information from the periphery and project to the cortex (3). An inhibition of TC neurons could disrupt the passage to the cortex and this might be a mechanism how Xe induces the anaesthetic state.
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7AP2-6

Xenon reduces excitability in thalamocortical relay neurons in a cyclic adenosine monophosphate (cAMP)-dependent manner

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Background and Goal of Study: The mechanisms, how the anaesthetic xenon (Xe) mediates its hypnotic properties are not fully understood. The thalamus is the major gateway for the passage of somatosensory information to the cortex. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels regulate neuronal excitability and are highly expressed in thalamocortical (TC) neurons (1). HCN channels are gated voltage-dependent and binding of cAMP facilitates channel activation (2). We investigated the effects of Xe on HCN channel currents (I_h) in acute brain slices.
Materials and Methods: Horizontal slices were prepared from male C57BL/6 mice. I_h currents were recorded from TC neurons using the patch-clamp technique. I_h was activated by hyperpolarizing steps of increasing amplitude (ΔV=-10mV) to -133mV. Rebound bursts were elicited by a hyperpolarizing current step and rebound delay was measured (time course from start of repolarization to the first action potential). In a subset of experiments, intracellular cAMP level was increased by adding 30 μM cAMP to the pipette solution. Slices were kept in carbogenated artificial cerebro-spinal fluid (ACSF). For Xe application, ACSF was additionally saturated with 65% Xe.
Results: The Xe-mediated reduction of I_h current amplitudes ranged from 11.0±3.3% to 31.9±12.9% (n=7; p< 0.05) depending on the voltage step. Half-maximum activation ($V_{1/2}$) of HCN channels was shifted to -108.1±3.6mV under Xe (n=8;control: -99.4±1.5 mV; p< 0.05). 30μM cAMP in the pipette solution shifted $V_{1/2}$ to more depolarized levels (-87.3±1.6mV). With cAMP, Xe did neither affect I_h current amplitude nor $V_{1/2}$ (n=5; p>0.05). Xe prolonged rebound burst delay to 139.5±8.8% of control (n=5; p< 0.05) whereas no effect could be seen when cAMP was added intracellulary (99.9±2.5% of control; n= 5; p>0.05).
Conclusion(s): In TC neurons Xe reduces I_h current amplitudes dependent on intracellular cAMP.This led to a decreased neuronal excitability resulting in a prolonged rebound delay. When cAMP was added intracellularly, Xe did not affect neuronal excitability. Thus, the effect of Xe on HCN channels in TC neurons might be mediated by a Xe-induced reduction of intracellular cAMP levels. A reduction of TC neuron excitability by HCN channel modulation might be a mechanism how Xe induces loss of consciousness.
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7AP2-7

The toxic effect of ketamine on the central nervous system - potential hazard or safe to use?

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Background and Goal of Study: Increasing evidence points to a potential neurotoxic effect of the NMDA receptor antagonist ketamine when administered systemically and/or neuraxially. We present an overview on recent preclinical and clinical literature investigating the neurotoxic potential of the sole use of ketamine on nerve or brain tissue.
Materials and Methods: We searched Pubmed(1970 to 2011) and Embase(1988 to 2011). For data extraction, we followed the Prisma Statement in its current version.
Results and Discussion: From 1013 primary hits, 63 records were included into this systematic review. Animal studies: Out of 35 studies, 28 studies showed a dose-dependend neurotoxicity in doses ranging from 5-75 mg/kg systemically, and 0.25-10 mg/kg intrathecally (i.th.). Additionally, the younger the animal, the more vulnerable were cells in even lower doses. Eight studies failed to show neurotoxicity.
Cell lines: Out of 17 studies, 13 studies observed neurotoxicity in neuronal cells of young animals in doses ranging from 0,002-3 mM and incubation time varying from1-96 h. In most studies, either a dose or time dependent relation was reported, in some even both. Three articles revealed neurotoxicity in human cell lines. A dose dependent increase of apoptosis was reported after 24 h of incubation with 0,5-12 mM S(+)-ketamine, or of 48 h with 100 -2500 μg/ml ketamine respectively. Five studies failed to present neurotoxocity after incubation for up to 24 h in a dosesfrom 10-100 μM, and 1-20 μg/ml, respectively.
Human data: Four case reports described neuropathological findings after i.th. administration of ketamine in dose ranges of
(1) 5 mg/d for 3 weeks,
(2) 7 days with 67,2 mg (mean daily dose),
(3) 28 days of S(+)-ketamine (peak dose: 50mg/kg),
(4) 2 mg/kg as a bolus, up to 7,5 mg/kg/h titrating for the first 48-72 hours.
One randomized controlled clinical trial presented no neurotoxicity of 2 mg/kg ketamine in children.
Ketamine can exert neurotoxicity in animals and humans when administered systemically or neuraxially. Neurotoxic events depended on ketamine dose, exposure time and the developmental age of the central nervous system, indicating that young mammals are more susceptible to ketamine toxicity than older. Interestingly, 53 articles reported neuroprotective effects of ketamine when added to chemically or ischaemically injured neuronal tissue underlying it's potential in particular clinical conditions. Further research needs to answer this question.

7AP2-8

Ketamine affects basal synaptic transmission and long-term potentiation without affect pair-pulse facilitation in hippocampal slices of adult mice

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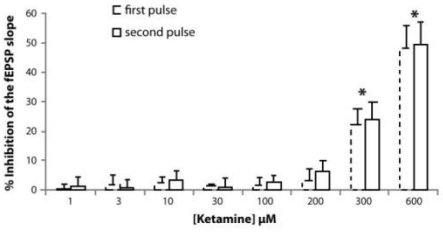
Background and Goal of Study: Ketamine, an anesthesic and analgesic drug, has been associated with disruption on learning and psychotic effects. Alterations in synaptic efficacy in glutamatergic pathways are documented to play a key role in psychopathology. Therefore we investigated the effects of different concentrations of ketamine on basal synaptic transmission (BST) and on synaptic plasticity (paired-pulse facilitation (PPF) and long-term potentiation (LTP)).
Materials and Methods: Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in Schaffer collateral in CA1 stratum radiatum, from mouse hippocampal slices. Four slices per group and type of experiment were used. For BST and PPF consecutive applications of increasing concentrations of ketamine (1, 3, 10, 30, 100, 200, 300 and 600 μM) were used and for LTP individual slices were used for each concentration. High-frequency stimulation (HFS) conditioning pulses were applied for LTP induction (100 pulses at 100Hz). The slope of the fEPSP was measured and its % of inhibition was calculated. To access PPF, a ratio of second pulse slope was divided by the first pulse slope. LTP potentiation was analyzed 60 minutes after HFS. Statistical analysis was performed using univariate ANOVA.
Results and Discussion: Ketamine attenuated LTP induction and LTP potentiation in a concentration dependent manner. BST was not affected by lower concentrations of ketamine (1, 3, 10, 30, 100 and 200μM) but higher concentrations of ketamine decreased (300 and 600 μM) the BST (figure 1). Ketamine did not affect PPF.



[Figure 1]

Conclusions: Ketamine impairs BST and LTP in CA1 region of the mouse hippocampus without affect PPF, suggesting the importance of the postsynaptic mechanisms in detriment of the presynaptic mechanisms for ketamine induce deficits in memory.
Acknowledgements: FCT (Lisbon, Portugal) and COMPETE:-01-0124-FEDER-009497 through the project grant PTDC/CVT/099022/2008 and personal grant SFRH/BD/48883/2008.

7AP3-1

Permutation does not react to the paradoxical EEG activation during propofol induction

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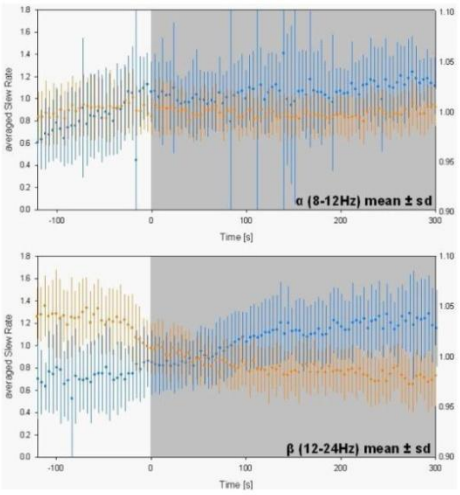
Background and Goal of Study: Propofol is supposed to have a biphasic effect on EEG during induction.[1,2] This paradoxical behaviour is most prominent in the α- and β-range.[1] EEG activation might have a negative effect on EEG anaesthesia monitors based on spectral analysis methods, i.e., high index values at unconscious patients[3] during transition. Nonlinear measures, e.g., permutation entropy PeEn proved to be better in reflecting the anaesthetic level compared to spectral approaches.This abstract raises the question if PeEn is also activated during transition period in the α- and β-band like spectral parameters do.
Materials and Methods: 39 EEG segments recorded during propofol induction were analysed. Loss of consciousness LOC was defined when the subject failed to respond to a repeated command to squeeze the hand. The segments were band pass filtered to the α- (8-12Hz) and the β- (12-24Hz) band using zero phase shift filtering routine and down sampled to 100Hz. PeEn and slew rate were calculated from non-overlapping, 4s EEG segments. The slew rate characterizes frequency and amplitude of the waveform. Each parameter series was normalized by division through its mean.
Results and Discussion: The course of slew rate and PeEn is displayed in figure1.



[Figure1: slew rate: blue; PeEn: orange]

Slew rate reflects activation in the α- and β-band and increases, whereas PeEn follows deepening of anaesthesia and decreases with time. The results suggest that PeEn is able to reflect the anesthetic level during LOC, whereas spectral parameters show a biphasic course around LOC. The decreasing trend of PeEn also bears valuable information regarding possible cortical mechanisms during LOC.
Conclusion: Although α- and β-frequencies are activated, signal complexity and hence their information content decreases.
References:
[1]BJA 2001; 86:354-360.
[2]Anesthesiology 1996; 84:52-63.
[3]BJA 1999; 82:666-671.

7AP3-2

Analysis of analgesic effect of propofol by in-vivo patch clamp recordings from the somatosensory cortex of rats

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Background and Goal of Study: Although propofol is in general considered to have a poor analgesic potency, several electrophysiological studies have reported that clinical dosage of propofol depressed nociceptive transmission in the spinal cord.
To address this conflict, we examined whether propofol affects the response of tactile stimuli to the skin in the primary somatosensory cortex (SI) which is thought to play important roles in the pain perception.
Materials and Methods: Sprague-Dawley rats (3-4 weeks old) were anesthetized with urethane (1.5 g/kg). In vivo whole-cell patch-clamp recordings were performed from SI neurons which responded to the mechanical stimuli to the receptive field of the hind paw area. Propofol (5mg / kg) was administered intravenously to the rat after starting recordings. We analyzed the responses evoked by pinch stimuli to the hind paw on the contralateral side before and after administration of propofol.
Results and Discussion: In the current clamp mode, propofol didn't change the baseline membrane potential of SI neurons, but significantly inhibited the firing of action potentials evoked by the pinch stimuli. In the voltage clamp mode, the barrage of excitatory postsynaptic currents (EPSCs) evoked by the stimuli was depessed, but the baseline holding current was not significantly changed.
These results reveal that propofol directly and/or indirectly inhibits the pain perception in the SI.
Conclusion(s): This study strongly suggests that propofol inhibits the nociceptive transmission in the central nervous system including the spinal cord and has antinociceptive effects.

7AP3-3

Inhibitory effect of intrathecal EGCG on mechanical allodynia and nitric oxide synthase expression in spinal cord in neuropathic pain of rat

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Background and Goal of Study: Epigallocatechin-3-gallate (EGCG), the major catechin in green tea, is known to have antioxidant activity against nitric oxide (NO) by scavenging free radicals, chelating metal ions, and inducing endogenous antioxidant enzymes, which contributes to the neuroprotective effects of EGCG. NO generated by nitric oxide synthase (NOS) is also one of the key players in nociceptive processing. We examined the effect of intrathecal EGCG on mechanical allodynia and NOS expression in spinal nerve ligation of rat.
Materials and Methods: Mechanical allodynia was induced by L5,6 spinal nerve ligation of male Sprague-Dawley rat. Effect of intrathecally administered EGCG(1,3,10,30μg) or L-arinine (100 μg; NO precursor) on mechanical allodynia was measured using von Frey test, in which rats were randomly assigned into 4 groups; saline/saline, saline/EGCG, L-arginine/EGCG, L-arginine/saline.
To examine dose-responsiveness, maximal possible effect (MPE, %) was calculated as follows: (PWT[paw withdrawal threshold] after experiment drug - PWT of baseline) / cut-off threshold [15 g] - PWT of baseline) × 100(%). Change in the expression of nitric oxide synthase of spinal cord was compared using Western blotting among rats with sham operation, SNL, or SNL+EGCG.
Results and Discussion: Intrathecal EGCG attenuated mechanical allodynia in rats with SNL, compared to sham-operated rats, with MPE of 69.2%. This antinociceptive effect was reversed by intrathecal pretreatment with L-arginine (L-arginine/EGCG). Intrathecal EGCG also blocked the increase in neuronal NOS (nNOS) expression in the spinal cord of SNL rats, but inducible NOS (iNOS) expression was not significantly suppressed.
Conclusion(s): Intrathecal EGCG produced an antiallodynic effect against spinal nerve ligation-induced neuropathic pain, mediated by blockade of nNOS protein expression and possibly by inhibition of the pronociceptive effects of NO.
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Abstract 1330

Effects of medetomidine on basal excitatory synaptic transmission and on synaptic plasticity in mice hippocampal slices

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Background and Goal of Study: Alfa$_2$-adrenoceptors agonists are frequently used in veterinary and human anesthesia. Medetomidine, a α_2-adrenoceptors agonist, is a sedative/analgesic drug. However, it has been reported that the pharmacological manipulation of the noradrenergic system can affect the memory. Little is known about the effect of this drug on basal excitatory synaptic transmission and synaptic plasticity. Therefore we investigated the effects of different concentrations of medetomidine on basal excitatory synaptic transmission and on two forms of synaptic plasticity: paired-pulse facilitation (PPF) and long-term potentiation (LTP).
Materials and Methods: Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in Schaffer fiber collaterals - CA1 pyramidal cell synapses of mouse hippocampal slices. Four slices per group and experiment were used. For basal synaptic transmission, and PPF, increasing concentrations of medetomidine (from 1 to 200 µM) were applied to each slice. For LTP experiments, individual slices were used for each medetomidine concentration (from 0.1 to 0.4 µM). LTP was induced by high-frequency stimulation (100 pulses at 100Hz). The synaptic transmission strength was assessed by measuring the initial slope of the fEPSP. LTP induction and maintenance were calculated. PPF was estimated as the ratio between the slopes of the second and first paired pulses, applied 50 milliseconds apart.
Results and Discussion: Medetomidine decreased basal excitatory synaptic transmission and LTP in a concentration-dependent manner. PPF only was affected by the highest concentration (200 µM) of medetomidine used.
Conclusion(s): Medetomidine decreased mainly LTP and basal excitatory synaptic transmission rather than PPF in the CA1 region synapses of the mouse hippocampus, suggesting the importance of postsynaptic mechanisms in detriment of presynaptic mechanisms for medetomidine-induced deficits in memory after sedation or analgesic procedures.
Acknowledgements: This research was funded in part by FCT (Lisbon, Portugal) and co-funded by COMPETE: 01-0124- FEDER-009497 (Lisbon, Portugal), through the project grants PTDC /CVT /099022/2008 and PTDC/SAU-NSC/122254/2010 and through a personal PhD grant (SFRH /BD/48883/2008).

Assigned speakers:
Patricia Ribeiro , Institute for Molecular and Cell Biology / Center for Neuroscience in Cell Biology , Laboratory Animal Science , Porto/Coimbra , Portugal

Assigned in sessions:
02.06.2013, 16:00-17:30, Abstract Presentation Session, 07AP3, Mechanisms of anaesthesia and cognition, Poster Area - ROW 13A

12th FELASA-SECAL Congress 2013, June, Barcelona, Spain,
Abstract number: P-313 (accepted for presentation)
Authors: P Ribeiro, A Tomé, H Silva, R Cunha, L Antunes

Effects of ketamine/medetomidine combination on basal synaptic transmission and synaptic plasticity in hippocampal slices of adult mice

The ketamine/medetomidine anaesthetic combination is frequently used in research procedures with animals and routine veterinary medicine. However, there is a lack of information about its effect on hippocampal synaptic activity. This activity is essential for learning and memory formation. Therefore we investigated the effects of ketamine/medetomidine combination on basal synaptic transmission and on two forms of synaptic plasticity (PPF) and LTP) in the CA1 region of mouse hippocampal slices. Evoked field excitatory postsynaptic potentials (fEPSP) were recorded CA1 pyramidal cell synapses of mouse hippocampal slices. Three slices per group and experiment were used. For basal synaptic transmission and PPF, increasing concentrations of ketamine combined with medetomidine: 30+1, 100+4 and 200+8 (μM of ketamine + medetomidine, respectively) were applied in hippocampal slices. For LTP experiments, 3 μM of ketamine combined with 0.1μM of medetomidine were applied to slices. LTP was induced by high-frequency stimulation. The synaptic transmission strength was assessed by measuring the initial slope of the fEPSP. LTP induction and maintenance were calculated. PPF was estimated as the ratio between the slopes of the second and first paired pulses. Ketamine/medetomidine combination did not affected PPF neither basal excitatory synaptic transmission but decreased LTP in the CA1 region of the mouse hippocampus. These findings suggest that ketamine/medetomidine combination may induce deficits in memory after anaesthesia mainly by alteration of postsynaptic mechanisms involved in LTP.

APPENDIX E

OUTPUTS FROM CHAPTER 6

12th FELASA-SECAL Congress 2013, June, Barcelona, Spain,
Abstract number: P-312 (accepted for presentation)
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A single intraperitoneal injection of ketamine did not affect hippocampal long-term potentiation of adult mice

Ketamine is frequently used to induce analgesia or anaesthesia during research procedures with animals. However, little is known about its effect on memory and learning. Long-term potentiation (LTP) is considered a cellular mechanism for learning and memory. Therefore we investigated the effects of different concentrations of ketamine on long-term potentiation. Eight C57BL/6 male adult mice were divided into 3 different groups (Saline solution, 25mg kg^{-1} ketamine, 75mg kg^{-1} ketamine). Twenty-four hours after injection, animals were euthanized and hippocampal slices were used to record the evoked field excitatory postsynaptic potentials (fEPSP). High-frequency stimulation (100 pulses at 100 Hz) was used for LTP induction. The initial slope of the fEPSP was measured and LTP induction and maintenance were calculated. The data were analyzed using one-way ANOVA followed by Bonferroni post hoc tests. No significant differences were detected between groups regarding the induction or maintenance of the long-term potentiation. This finding suggests that a single intraperitoneal injection of ketamine did not affect hippocampal long-term potentiation 24 after injection, suggesting that ketamine did not induced deficits of memory in male adult mice.