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COIMBRA

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STUDY OF THE ROLE OF MONOAMINE TRANSPORTERS IN THE VASCULAR EFFECTS OF 3,4-METHYLENEDIOXYMETAMPHETAMINE:

INFLUENCE OF HYPERTHERMIA

Dissertação no âmbito do Mestrado em Farmacologia Aplicada orientada pelo Professor Doutor Diogo André Afonso da Fonseca e pela Professora Doutora Maria Dulce Ferreira Cotrim e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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“Science is a way of thinking, much more than it is a body of knowledge.”

Carl Sagan

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Eternamente grato,

Daniel Filipe Maravilha Ribeiro.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
ANOVA	Analysis of variance
BBB	Blood-Brain Barrier
COMT	Catechol-*O*-methyltransferase
CYP	Cytochrome P450
DAT	Dopamine transporter
E_{max}	Maximum contraction
g	Gram
HHA	3,4-dihydroxyamphetamine
HHMA	3,4-dihydroxymethamphetamine
HMA	4-hydroxy-3-methoxyamphetamine
HMMA	4-hydroxy-3-methoxymethamphetamine
ITA	Human internal thoracic artery
MAO	Monoamine oxidase
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MMPs	Matrix metalloproteinases
mN	milliNewton
NET	Noradrenaline transporter
pEC_{50}	Negative logarithm of the effective concentration of agonist able to induce half of the maximum contraction
SEM	standard error of mean
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
TPH	Tryptophan hydroxylase
VMAT	Monoamine vesicular transporter

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RESUMO

A 3,4-metilenodioximetanfetamina (MDMA) é uma droga de abuso popular entre a população mais jovem devido aos seus efeitos psicotrópicos. Apesar da sua potencial utilização no tratamento de distúrbios de stress pós-traumático ou distúrbios obsessivo-compulsivos, esta droga apresenta diversos efeitos adversos, nomeadamente neurotoxicidade, hepatotoxicidade, nefrotoxicidade, toxicidade cardiovascular e hipertermia. Relativamente aos mecanismos envolvidos, a interação da MDMA com os transportadores de monoaminas (serotonina, noradrenalina e dopamina) e a interação com os recetores serotoninérgicos 5-HT_{2A} têm sido associadas aos efeitos alucinogénicos desta droga. No entanto, os mecanismos envolvidos nos efeitos vasculares permanecem por esclarecer. Neste contexto, demonstrou-se recentemente a potencial interação com recetores 5-HT_1 e 5-HT_2. No entanto, a interação com transportadores de monoaminas ou com recetores adrenérgicos constituem igualmente mecanismos potencialmente envolvidos. O objetivo deste trabalho foi avaliar o papel do transportador da serotonina (SERT) nos efeitos vasculares induzidos pela MDMA, tendo-se baseado na interação da MDMA e da serotonina com o inibidor seletivo da recaptação de serotonina, a fluoxetina, em condições de normotermia e hipertermia (37° e 40° C, respetivamente). Os resultados demonstraram uma diminuição significativa da contração induzida pela MDMA, em ambas as condições térmicas. Estes resultados confirmam assim um papel importante do transportador da serotonina nos efeitos vasculares da MDMA, nomeadamente na indução de vasoconstrição, a qual se pode associar a complicações cardiovasculares decorrentes do abuso desta droga.

Palavras-chave: 3,4-metilenodioximetanfetamina, Vascular, Transportador da serotonina, Fluoxetina, Hipertermia, Artéria torácica interna humana

ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) is a drug of abuse popular among the younger population due to its psychotropic effects. Despite its potential use in the treatment of post-traumatic stress disorders or obsessive-compulsive disorders, this drug has several adverse effects, including neurotoxicity, hepatotoxicity, nephrotoxicity, cardiovascular toxicity, and hyperthermia. Regarding the mechanisms involved, the interaction of MDMA with monoamine transporters (serotonin, norepinephrine and dopamine) and the interaction with 5-HT_{2A} serotonergic receptors have been associated with the hallucinogenic effects of this drug. However, the mechanisms involved in vascular effects remain unclear. In this context, the potential interaction with 5-HT_1 and 5-HT_2 receptors has recently been demonstrated. However, the interaction with monoamine transporters or with adrenergic receptors are also potentially involved mechanisms. The aim of this work was to evaluate the role of the serotonin transporter (SERT) in the vascular effects induced by MDMA, based on the interaction of MDMA and serotonin with the selective serotonin reuptake inhibitor, fluoxetine, in normothermia and hyperthermia conditions (37° and 40° C, respectively). The results demonstrated a significant decrease in the contraction induced by MDMA, in both thermal conditions. These results confirm therefore an important role of SERT in the vascular effects of MDMA, namely in the induction of vasoconstriction, which can be associated with cardiovascular complications resulting from the abuse of this drug.

Keywords: 3,4-methylenedioxymethamphetamine, Vascular, Serotonin transporter, Fluoxetine, Hyperthermia, Human internal thoracic artery

I. INTRODUCTION

1.1. The problematic of drugs of abuse

Drug abuse is a social burden that has a socioeconomic impact in communities. Substances of abuse like alcohol, opiates, amphetamines, and methamphetamine cause numerous health problems and a diminished quality of life. Ecstasy, which active substance is 3,4-methylenedioxymethamphetamine (MDMA), is turning to be a popular drug of abuse worldwide. According to the European Monitoring Centre for Drugs and Drug Addiction [1], 14 million European adults, aged between 15 and 64 years old, have experienced MDMA or ecstasy (data from 2017) and its consumption has been rising in the last decade. More potent MDMA tablets have emerged in the last decade in Europe, and marketing of the product is a factor that contributes for the increase in sales. According to the Statista website [2], the prevalence of ecstasy use in Europe was higher in the Netherlands, where 3.5% of the population may have tried ecstasy at some point of their lives, followed by Ireland with 2.1% and the United Kingdom with 1.7%, compared to only 0.1% of the Portuguese population (data from 2019). This emerging popularity of ecstasy consumption and the concomitant rise of its European market warrants further study of the clinical implications of MDMA abuse, particularly in patients with underlying disease.

1.2. Pharmacology of MDMA

MDMA is a ring-substituted amphetamine that is a popular drug of abuse among young adults. Structurally, it is similar to hallucinogenic compounds such as mescaline, amphetamine, and monoaminergic neurotransmitters [3]. Although primarily used for recreational purposes, MDMA was initially introduced in the market by Merck & Co. for appetite suppression, eventually being considered also for alcoholism and depression treatment [4]. MDMA is a potential drug for abuse amongst the youth population and is commonly found in rave parties. Similarly, to the above-mentioned structural analogues, MDMA can be very addictive, as it may lead to numerous addiction effects and consequently harmful physiological withdrawal effects [5, 6]. More recently, clinical trials have been conducted in the context of the therapy for post-traumatic stress disorders [7–9].

Despite the therapeutic potential of this compound, extensive literature has emerged on the deleterious effects of MDMA, which include diverse toxic effects, such as:

neurotoxicity, hepatotoxicity, nephrotoxicity, cardiotoxicity and hyperthermia [10]. In this context, the knowledge of the physiological processes and mechanisms that are triggered or affected by MDMA is vital in understanding the numerous effects of this compound, namely at the neuronal, hepatic, kidney and cardiovascular level [11–16]. In fact, several factors may contribute to this array of toxic effects, e.g. human metabolism of MDMA, monoaminergic effects such as enzymatic and nonenzymatic oxidation of monoamines (i.e. 5-hydroxytryptamine or 5-HT, noradrenaline and dopamine) and inhibition of their synthesis, hyperthermia, inflammation or even oxidative stress.

1.2.1. Human metabolism of MDMA

As several reports have emerged suggesting that MDMA metabolites may produce marked effects on human health, it is important to consider the human metabolism of this compound. As shown in Figure 1, cytochrome P450 (CYP), namely CYP2D6, is the main responsible for MDMA metabolism in the liver [17, 18]. The half-life is about 8 to 9 hours, independently of the dose, and follows non-linear pharmacokinetics, i.e. the plasmatic concentration does not increase proportionately with the dosage intake [17, 18]. In addition, MDMA has autoinhibitory properties, as it inhibits the isoenzyme CYP2D6 decreasing its own metabolism. Consequently, increased MDMA and/or its metabolites plasma concentrations may cause potential harm to the human body [17, 18]. Moreover, genetic differences in CYP2D6 isoenzyme also influence MDMA plasma concentrations, as slow metabolizers present higher MDMA concentrations than rapid metabolizers [18, 21]. The main MDMA metabolites are 3,4-methylenedioxyamphetamine (MDA), obtained through *N*-demethylation by CYP1A2 and CYP2D6, and 3,4-dihydroxymethamphetamine (HHMA), obtained through *O*-demethylenation by CYP2D6, CYP1A2 and CYP3A4. Other metabolites include 3,4-dihydroxyamphetamine (HHA, also known as α-methyldopamine), obtained through *O*-demethylenation of MDA by CYP2D6 and CYP3A4. HHMA and HHA can be *O*-methylated by catechol-O-methyltransferase (COMT), giving place to their ortho-quinones equivalents, 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) [22, 23].

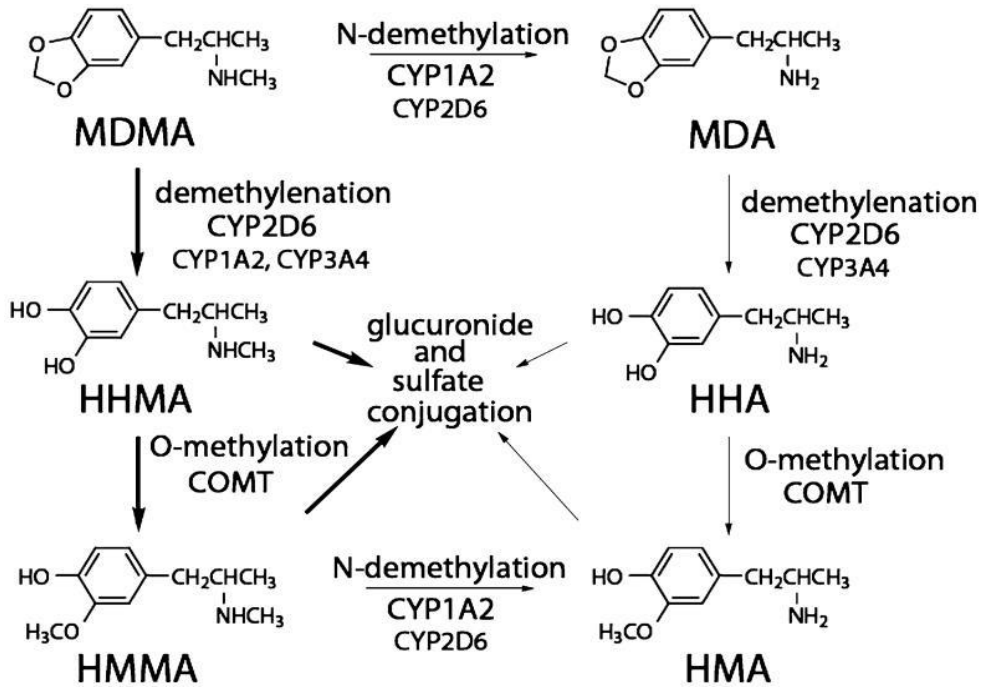

Figure 1 Human metabolism of MDMA (adapted from [23]).

1.2.2. Monoaminergic effects

The majority of the experimental studies have focused on the interference of MDMA in the peripheral and the central nervous system functions, in which this compound acts mainly on the monoaminergic system and particularly on the serotonergic neurotransmission [3].

In fact, MDMA has been proposed as an inhibitor of monoamine reuptake, particularly 5-HT (serotonin). In part, this assumption is based on the interaction of MDMA with the serotonin transporter (SERT), which leads both to (a) the release of 5-HT into the synaptic cleft and (b) the uptake of MDMA into the presynaptic terminal [3, 24]. Inside the presynaptic terminal, MDMA promotes the release of 5-HT from storage vesicles to the cytoplasm by interacting with the monoamine vesicular transporter (VMAT) 2, thus increasing intracellular levels of the neurotransmitter [3, 24, 25]. Furthermore, MDMA elicits an inhibition of tryptophan hydroxylase (TPH) which limits the synthesis of 5-HT, and also a partial inhibition of monoamine oxidase (MAO) B in the mitochondrial membrane, thus influencing the degradation of 5-HT.

An interaction with 5-HT receptors has also been proposed, as an agonistic action on 5-HT2A receptors may be responsible for the hallucinogenic effects of this drug [3, 24]. Furthermore, an interaction with central 5-HT1 receptors has also been reported [26–29].

a) Serotonergic neurotoxicity

Chronic consumption of MDMA may later lead to central nervous system complications related to serotonin toxicity. However, the underlying mechanisms of neurotoxicity remain unclear. Furthermore, as most studies use animal models to elucidate this mechanistic basis, interspecies differences may constitute an obstacle for the understanding of the toxic effects of MDMA intake, in humans. However, previous literature has been consistent in identifying 5-HT as a key factor in the psychophysiological effects of MDMA [30].

MDMA-induced serotoninergic damage is associated with many neuropsychological complications that are consistent with serotonergic damage in the prefrontal cortex, hippocampus, and striatum. The combination of these cerebral changes with other neurological system disruptions might lead to neuropsychiatric symptoms and increase the vulnerability to develop psychoses [31].

In this context, Daumann et al. [32] assessed the neural mechanisms of working memory in MDMA users using functional magnetic resonance imaging and showed that these individuals exhibited altered patterns of cerebral activation during cognitive processing, which did not reverse until at least 18 months or several years of abstinence. The memory performance has been assessed by Kuypers et al. [33] who showed an influence of MDMA on the middle frontal gyrus processes that resulted in a loss in memory encoding performance.

In regard to altered mood and impulsivity, 5-HT_2 receptors seem to be the mainly responsible, as the 5-HT_1 receptors do not appear to be involved [34]. Moreover, changes in impulsivity may be correlated with the synaptic effects of MDMA, as 5-HT depletion could play a role in the increased impulsivity during abstinent periods [35].

However, the results from experimental and clinical studies on the neurotoxicity of MDMA and its metabolites (e.g. MDA, which also promotes 5-HT release) are still inconclusive, as several other pathways have been identified, namely: oxidative stress and production of reactive oxygen species, hyperthermia, inflammation-related signaling pathways that lead to the production of proinflammation mediators, serotonergic effects and also glutamate-related neuronal excitotoxicity [31, 36–43].

b) Serotonin syndrome

Serotonin syndrome is defined by the occurrence of varied clinical features such as altered mental status, autonomic nervous system overactivity, and neuromuscular hyperactivity, and its caused by an excess of serotonin in the central nervous system [44, 45]. The MDMA-induced increase in both peripheral and central serotonin has different consequences. While peripheral serotonin can stimulate vasoconstriction and platelet aggregation, while central serotonin plays a role in thermoregulation, for example. These effects correlate with the manifestations of the serotonin syndrome, namely cardiovascular complications, such as hypertension and tachycardia and other systemic complications as hyperthermia, which may lead to rhabdomyolysis in extreme cases. [46, 47].

The synergetic actions of selective serotonin reuptake inhibitors (SSRIs) and MDMA seem to play a major role in serotonin syndrome. This drug-drug interaction may be responsible for an increased serotonin concentration in the central nervous system, which in turn leads to an excessive activation of postsynaptic serotonin receptors. Furthermore, the metabolism of both MDMA and SSRIs may also be a factor, as SSRIs also inhibit the isoenzyme CYP2D6, similarly to MDMA, thus decreasing MDMA metabolism and therefore increasing its plasma concentration This pharmacokinetic interaction may also compromise liver function, decreasing the clearance of both SSRIs and MDMA, leading to an increased risk of developing serotonin syndrome [21, 47].

1.3. Cardiovascular effects of MDMA

Cardiovascular complications after MDMA use have been studied both in pre-clinical and clinical setups. In humans, dose-dependent increases in blood pressure and heart rate are the most recognized cardiovascular effects of MDMA. In fact, this has been shown in several settings, namely controlled experimental and clinical settings [48–57], recreational use settings [58, 59] and also in children intoxications [60, 61].

Gender differences were assessed by Liechti et al [62] and Papaseit et al [63] indicating that female individuals have greater subjective effects after MDMA ingestion. Metabolic differences, as well as SERT and receptor genetic differences have been hypothesized to play a role in MDMA acute effects, with enhanced physiological and subjective effects. In regard to genetic differences, SERT and COMT high-functionality genotypes have been associated with

increased systolic blood pressure and heart rate [64]. In contrast, Vizeli et al. [65] recently suggested that interindividual genetic differences in the 5-HT system (i.e. TPH, SERT and 5-$HT_{1A/1B/2A}$ receptors) would only play a minor role in the cardiostimulant effects of MDMA. These findings are in accordance with other studies which suggested that genetic modifications in SERT and receptors did not significantly influence these effects [63–65]. Moreover, ambient temperature-related enhancement of core body temperature might exacerbate neurotoxicity and physiological effects of MDMA [16, 67, 68]. Finally, MDMA in conjugation with other drugs and alcohol binge ingestion assessed both in human and animal models [34, 35, 69–72] may contribute to an enhancement of these adverse cardiovascular effects, subjective and behavioral responses to MDMA .

In addition to changes in blood pressure and heart rate, MDMA abuse may also lead to a myriad of cardiac effects, namely: (a) cardiac arrhythmias [15, 73–80]; (b) cardiomyocyte necrosis, tissue inflammation and myocardial infarction [77, 78, 81, 82]; and (c) valvular heart disease [83, 84].

1.3.1. Vascular-specific effects

a) Vasoconstriction

Considering that BP derives from cardiac and vascular factors, an increased BP could result both from an increased cardiac output and from increased peripheral vascular resistance. Although there is limited evidence on the effect of MDMA on cardiac output [84], several reports have emerged on the direct effect of MDMA on the vascular tone. Furthermore, MDMA consumption has been associated with reversible cerebral vasoconstriction syndrome [85]. In the rat small mesenteric artery, but not in the rat aorta, MDMA significantly increased the potency of noradrenaline, as this effect was abolished by cocaine therefore suggesting the involvement of NET in these effects [86]. Interestingly, MDMA and MDA produced concentration-dependent increases in the contraction to the α_1-agonist phenylephrine in rat aortic rings, which could suggest the involvement of α_1-adrenoceptors [52]. Also in rat aortic rings, MDMA did not alter significantly the efficacy and potency of 5-HT induced vasoconstriction [87].

In the porcine coronary artery, MDMA mainly produced vasoconstriction, even though the concentration of 1000 μM produced a relaxation [88]. The presence of cocaine or prazosin

and endothelium removal did not affect the observed vasoconstriction, thus suggesting the involvement of mechanisms other than indirect sympathomimetic activity or α_1-adrenoceptors [88].

In aortic rings from rats chronically exposed to MDMA, Cannon et al. [89] showed that chronic MDMA exposure decreased maximal contraction to 5-HT at 1 and 7 days post-treatment, but not at 14 or 21 days. Interestingly, the authors suggested that the effect of MDMA on the vasculature does not result from a direct effect of MDMA on peripheral 5-HT transport.

Using isolated human arterial specimens, our group has explored the vascular effects exerted by an acute exposure to MDMA and its metabolites. In fact, we have shown that MDMA, HHMA and HHA elicit a concentration-dependent vasoconstrictor effect, which is potentiated in hyperthermic conditions (40°C), whereas 5-(GSH)-HHMA and 5-(GSH)-HHA elicit no change on vascular basal tone [91, 92]. Furthermore, we have also shown that MDMA and its metabolites (HHMA, HHA, 5-(GSH)-HHMA, 5-(GSH)-HHA) alter serotonergic activity in the vasculature both in normothermic and hyperthermic conditions [90]–[92]. Based on these findings, we have hypothesized that an increased blood pressure may result from a direct vasoconstrictor effect involving 5-HT receptors, specifically 5-HT_1 and 5-HT_{2A} (Figure 2). However, additional mechanisms may be involved, namely SERT-mediated release of 5-HT [90].

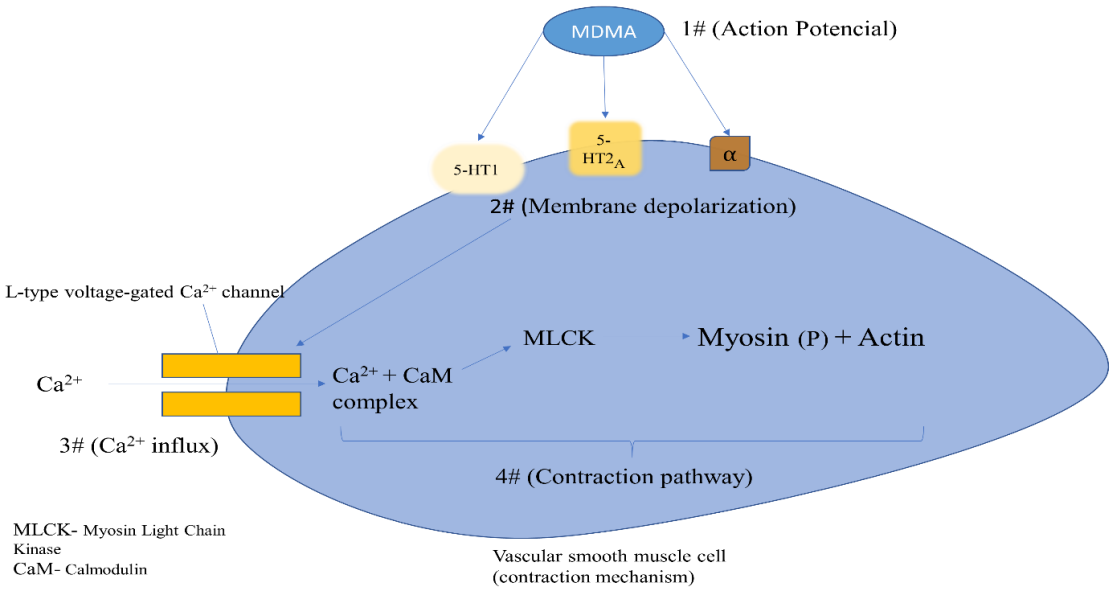

Figure 2 Proposed mechanism of MDMA-induced vascular smooth muscle contraction involving 5-HT receptors and adrenoceptors

b) Blood-Brain Barrier disruption

Several observations have been reported which suggest that MDMA induces BBB disruption, as previously reviewed [94, 95]. These include increased BBB permeability, increased expression of pro-inflammatory cytokines, production of reactive oxygen species and also upregulation of heat-shock protein 72, although a direct effect on the BBB may also contribute to the MDMA-induced BBB dysfunction [94, 96], in which MDMA-induced activation of the mitogen-activated protein kinase (MAPK) JNK1/2 has been suggested to play a role [94].

Interestingly, Rubio-Araiz et al. [96] suggested that MDMA-induced increase in BBB permeability is mediated by a mechanism involving $P2X_7$ receptors, which in turn promotes the activity of matrix metalloproteinases (MMPs), specifically MMP-9 and MMP-3, and subsequently extracellular matrix degradation. These findings have been recently confirmed by Pérez-Hernández et al. [97] who showed that a single dose of MDMA (12.5 mg/kg) increases BBB permeability to Evans Blue dye and produces an oedema of short duration associated with changes in the expression of aquaporin 4 and decreased expression of claudin-5, that are prevented by SB-3CT (inhibitor of MMP-9). Furthermore, the MDMA-induced oedema was confirmed in vivo by magnetic resonance imaging. Also, MDMA promotes the activity of tissue plasminogen activator (tPA) and induces an increase in the expression of tPA and lipoprotein receptor-related protein 1 (LRP-1) and a decrease in the expression of plasminogen.

Overall, these findings suggest that MDMA disrupts BBB integrity and promotes vasogenic diffuse oedema through MMP-9 activation and tight-junction degradation.

c) Disruption of vascular integrity

Even though several cases of methamphetamine-related aortic dissection have been reported [98], only two have reported a connection between MDMA consumption and aortic dissection: one case in a young adult without previous medical conditions [99] and one case in a 37-year-old female with previous history of hypertension [100], both without history of connective tissue disorders or underlying valvular abnormalities. So far, no controlled study has been carried out on this subject, thus the precise mechanisms are still not known.

d) Altered haemostasis

Amphetamine and methamphetamine intake has been associated with acute vascular syndromes, including myocardial infarction and ischaemic stroke, which mainly involve arterial thrombosis [102, 103]. Similarly, MDMA consumption has been associated with acute myocardial infarction [81, 104–108], renal venous thrombosis [108] and cerebral venous sinus thrombosis [109] in several case reports. Interestingly, MDMA has also been associated with retinal [110], intracerebral [111] and subarachnoid [113–115] haemorrhage.

Although primarily focused on amphetamine consumption, Gebhard et al. [101] showed that MDMA (at nontoxic concentrations) significantly enhanced the expression and activity of tissue factor, the main trigger of haemostasis. These findings provide a preliminary mechanistic basis for the connection between MDMA consumption and thrombotic events.

1.4. Serotonergic activity in the vasculature

At the vascular level, 5-HT plays a fundamental role in the regulation of vascular tone and also in platelet aggregation [44, 116]. Furthermore, this neurotransmitter is mainly stored in platelets, which contain SERT on their surface [116]. There are many 5-HT receptors involved in cardiovascular responses, namely 5-HT_{1B}, 5-HT_{2A}, 5-HT_3, 5-HT_4, 5-HT_7. As some of these receptors are mainly involved in the vascular contraction mechanism (e.g. 5-HT_{2A}), others promote endothelium-dependent vascular relaxation through nitric oxide production (e.g. 5-HT_{1B}) [118,119]. Concerning the vascular smooth muscle contraction mechanism induced by receptor agonism, it happens by phosphorylation and dephosphorylation of myosin light chain, by myosin light chain kinase and myosin light chain phosphatase (relaxation pathway). In particular, 5-HT_{1B} receptors coupled with G proteins, initiating a signal transduction pathway by inhibition of adenylyl cyclase. 5-HT_{2A} receptors also coupled with G proteins by activation of phospholipase C, leading to the accumulation of inositol triphosphate and diacylglycerol, thus increasing intracellular Ca^{2+} concentration and activating protein kinase C [118–120].

SERT is a sodium-dependent transporter from the solute-carrier 6 neurotransmitter transporter family [121]. This monoamine transporter has been described in many studies, using animal models, in cardiovascular tissues such as: pulmonary arteries, heart, endothelial cells in systemic rat arteries and peripheral arterial smooth muscle. Also, mRNA and SERT

proteins are found in platelets [117, 122]. SERT is involved in the uptake and release of intracellular 5-HT. As 5-HT binds to SERT, this 5-HT-SERT complex induces a conformational change in SERT, turning this transporter protein to an inward faced position (intracellularly), thus promoting the release of 5-HT and other ions. The binding of intracellular potassium ions to SERT turns it to the extracellular position once again. Together, these elements (i.e. 5-HT, sodium, chloride, and potassium) act on this multifunctional binding site, making SERT a 5-HT-gated ion channel [116]. As previously reviewed by Ni and Watts [116], SERT has a leading role in the cardiovascular system particularly within platelets, where an impairment of the 5-HT system has been associated with hypertension.

II. AIMS

Driven by the fact that MDMA is a drug that can lead to toxic effects both at central (i.e. central nervous system) and peripheral levels (e.g. cardiovascular), it is of particular interest to further assess these complications and the underlying mechanisms.

In this MSc thesis, we aimed to further explore the acute vascular effects of MDMA, using an ex vivo human arterial model, the human internal thoracic artery (ITA). The general aim was to understand the influence of monoamine transporters, specifically SERT, in the vascular effects elicited by MDMA. Accordingly, the following specific aims were outlined:

i. Evaluate if SERT is involved in the acute vascular effects of MDMA;
ii. Evaluate the influence of hyperthermic conditions on the SERT-mediated vascular effects of MDMA.

The experimental methodology involved tissue organ bath experiments in which the isometric tension of arterial rings was recorded in normothermic and hyperthermic conditions. This approach allows us to visualize and understand directly the vascular acute effects of MDMA. Our results may be important in real-life conditions, particularly when considering the recreational consumption of MDMA by individuals with underlying cardiovascular disease.

III. MATERIALS & METHODS

3.1. Human arterial specimens

The ITA was used as an *ex vivo* human arterial model in a tissue organ bath setup. Arterial samples were harvested with the approval by the Ethics Committees of Coimbra University Hospitals (reference PC-388/08) and Faculty of Medicine of University of Coimbra (reference CE-084/2020), from patients undergoing coronary artery bypass grafting and after informed consent.

3.1.1. Vessel harvesting

Arterial segments were harvested from 22 male patients, aged between 47 and 80 years. The arteries were dissected as a pedicle from the anterior internal surface of the chest after sternal incision, externally irrigated with papaverine, a vasodilator used to prevent perioperative vasospasm, and cut distally. The distal segments remaining from surgery were placed in cold (4°C) Krebs-Henseleit bicarbonate buffer – with the following composition in mM: NaCl 118.7, KCl 5.4, $CaCl_2.H_2O$ 1.9, KH_2PO_4 0.9, $MgSO_4.7H_2O$ 0.6, $NaHCO_3$ 25 and glucose 11.1 – and transferred to the Laboratory of Pharmacology and Pharmaceutical Care, Faculty of Pharmacy of University of Coimbra.

3.1.2. Vessel preparation and ring mounting

To fully isolate the vessel and avoid the potential influence on tissue reactivity, the perivascular tissue - fat, muscle, and vasa vasorum - was carefully removed with surgical tweezers and scissors to maintain tissue viability. The vessel segments were then cut into small rings (about 3 mm in length), mounted on platinum wires, and suspended in organ bath chambers filled with 10 mL of freshly prepared Krebs-Henseleit solution, previously adjusted to pH 7.4 and aerated with a gas mixture of 95% O_2/5% CO_2. Arterial rings were washed multiple times during a stabilization period of 2 hours, under an optimal resting tension, which corresponded to the equilibrium state achieved after the application of a passive force of 19.6 mN (2 g). The organ bath was maintained in normothermic (37°C) or hyperthermic (40°C) conditions using a thermostat (PanLab®). The platinum wires were attached to force

transducers (AD Instruments®) that allowed the recording of the isometric tension of the rings during the experiment. Isometric tension variation data was collected using the PowerLab® data acquisition package, in gram (g) and afterwards converted to milliNewton (mN).

3.2. Experimental protocols

The viability of arterial rings throughout the experiments was assessed with a single dose of 60mM of potassium chloride (KCl) at the beginning and at the end of each experiment. Then, arterial rings were washed up with Krebs-Henseleit buffer and the vasoreactivity assessed after complete relaxation and tension stabilization.

To evaluate the role of SERT on the vascular response to MDMA, cumulative concentration-response curves to MDMA (10-1360 μM) were first performed in the absence and then in the presence of fluoxetine (0.4, 2 or 10 μM), after pre-incubation for 30 minutes. These curves were obtained by cumulative addition of increasing doses of MDMA, each dose being added when the tissue reached a sustained contracted state ("plateau"), after the previous dose. Control curves were obtained in the absence of fluoxetine. The same experimental setup was also used for 5-HT (0.1-70 μM).

The protocols detailed in this section were primarily carried out at normothermic conditions (37°C).

The influence of hyperthermia on the role of SERT was evaluated through concentration-response curves to MDMA using an equivalent protocol, in the presence or absence of fluoxetine (10 μM). Only this concentration was tested, based on the results obtained in normothermic conditions.

3.3. Statistical analysis

Data was analyzed and reported as previously [91]. In particular, the experimental protocol involved two cumulative concentration-response curves, in which the second was performed after a 30-minute incubation with fluoxetine, which data was initially converted from g to mN. The results of the second curve were then expressed as a percentage of the

maximum contraction (E_{max}) achieved in the respective control (% E_{max}), which was considered as parameter of intrinsic activity.

The analysis of the results also included the determination of potency, expressed as the negative logarithm of the effective concentration (in mol/L or M) of agonist able to induce half of the maximum contraction (pEC_{50}, -log[M]), by interpolation in a half-logarithmic scale (% Emax vs. -log [M]) using GraphPad Prism 7® (GraphPad Software, Inc., La Jolla, CA, USA).

The control curves both to MDMA and 5-HT at 37°C (i.e. first and second curves of control rings) were compared in terms of absolute (in mN) and relative (% E_{max}) contraction, to monitor the reactivity of the tissue with repeated exposure. This analysis was also applied to the comparison of the response to MDMA between thermal conditions, i.e. normothermic vs hyperthermic conditions, in order to discard any potential influence of the temperature on the reactivity of the arterial rings.

In terms of statistics, concentration-response curves obtained at 37°C were first evaluated with analysis of variance (ANOVA) with Tukey's multiple comparisons test, to understand mean differences among curves. Afterwards, Multiple t tests were used to determine differences among the curves between concentrations. Pharmacological parameters of efficacy (E_{max}) and potency (pEC_{50}) were compared by ANOVA with Tukey's multiple comparisons test.

For curves obtained at 40°C, paired t test was used for comparing curves and unpaired t test for comparing pharmacological parameters. This analysis was also employed to the following comparisons: (a) first vs second control curves and (b) normothermia vs hyperthermia.

Data were presented as mean ± standard error of mean (SEM), and *n* corresponds to the number of patients (2-8 rings were used from each patient). Values of $p<0.05$ were considered to indicate statistically significant differences.

3.4. Reagents

MDMA (hydrochloride salt) was extracted and purified from high-purity MDMA tablets that were kindly provided by the Portuguese Criminal Police Department. The obtained hydrochloride salt was fully characterized by nuclear magnetic resonance and mass spectrometry, as previously detailed and reported [123]. All other chemicals were purchased

from Sigma-Aldrich® (St. Louis, Missouri) and correspond to the highest grade commercially available.

IV. RESULTS & DISCUSSION

4.1. Vascular effects of MDMA

4.1.1. Contractile effects at normothermic conditions

As expected, both 5-HT and MDMA induced a concentration-dependent contraction in the ITA at normothermic conditions (Figure 3). This contractile effect is in accordance with previous studies from our group [91, 92] and also with previous findings in other vascular beds, e.g. porcine coronary artery [88]. As can be seen, MDMA displayed a lower intrinsic activity (E_{max} = 2.79±1.03 mN) compared to 5-HT (E_{max} = 5.16±1.31 mN), even though the difference was not statistically significant (P = 0.20). Moreover, the potency for 5-HT (pEC_{50} = 6.22±0.44) was significantly (P = 0.045) higher compared to MDMA (pEC_{50} = 2.62±1.63). In the case of ITA, the main receptors involved in vasoconstriction are 5-HT_{1B} and 5-HT_{2A} and are found in smooth muscle cells [91, 93, 118, 119, 124, 125]. Furthermore, this contractile response may also involve α1-adrenoceptors as previously suggested [52].

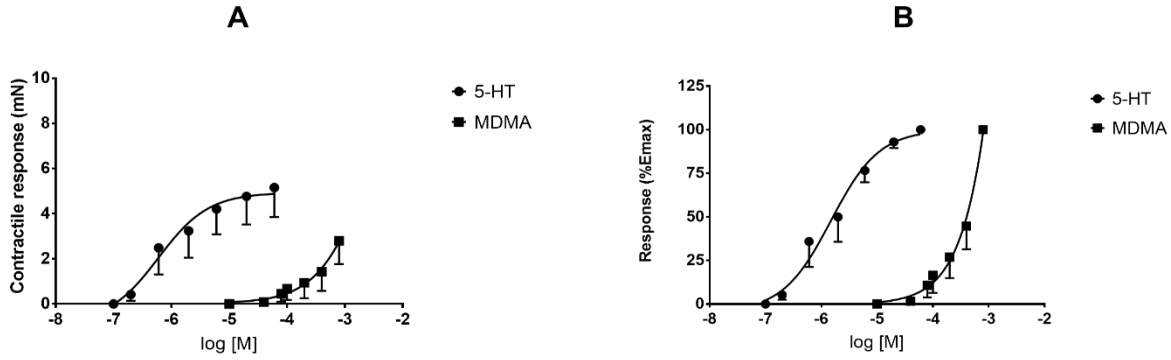

Figure 3 Contractile responses of ITA rings to MDMA and 5-HT

In order to monitor the reactivity of the tissue with repeated exposure to the tested compounds, we compared the first and second concentration-response curves in control rings in terms of absolute (in mN) and relative (as % of E_{max} from the first curve) contraction. At normothermic conditions (Table 1), MDMA elicited a similar contractile response in both curves (Figure 4), as the differences were not statistically significant in terms of absolute (P = 0.34) or relative (P = 0.52) contraction.

Table I Pharmacological parameters from concentration-response control curves to MDMA and 5-HT at normothermic conditions (*n* corresponds to the number of experiments).

Compound	Curve	E_{max} (mN)	pEC_{50} (-log[M])	E_{max} (% 1st curve)	*n*
MDMA	1st	2.79 ± 1.03	2.62 ± 1.63	100.00 ± 0.00	5
	2nd	3.01 ± 1.03	3.12 ± 0.67	128.83 ± 30.11	
5-HT	1st	5.16 ± 1.31	6.22 ± 0.44	100.00 ± 0.00	6
	2nd	3.52 ± 0.90	6.35 ± 0.44	58.82 ± 12.00	

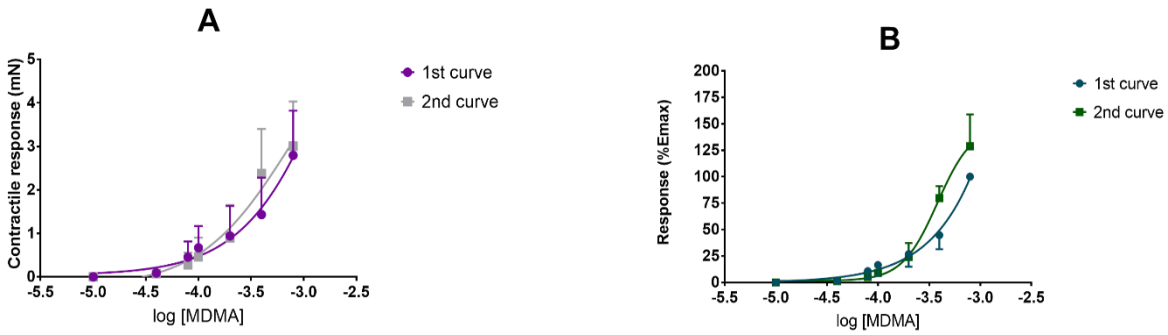

Figure 4 Comparison between first and second concentration-response curves to MDMA at 37°C in control rings. A, Comparison of absolute contraction (in mN). B, Comparison of relative contraction (as % of E_{max} from the first curve).

Regarding 5-HT (Figure 5), an apparent decrease in response was observed in the second concentration-response curve compared to the first curve. A statistically significant difference was found when comparing curves expressed in absolute contraction (P = 0.024). However, this difference was not found in specific concentrations. In a general comparison of the curves expressed as relative contraction, no statistically significant difference was initially found (P = 0.054), but the E_{max} was significantly different (P = 0.006). In general, these results suggest a loss of reactivity of ITA to 5-HT of about 41%, possibly by desensitization of 5-HT receptors [126]. In the case of ITA, 5-HT_{1B} and 5-HT_{2A} receptors are the mainly involved in the contractile response to this neurotransmitter [125, 127, 128].

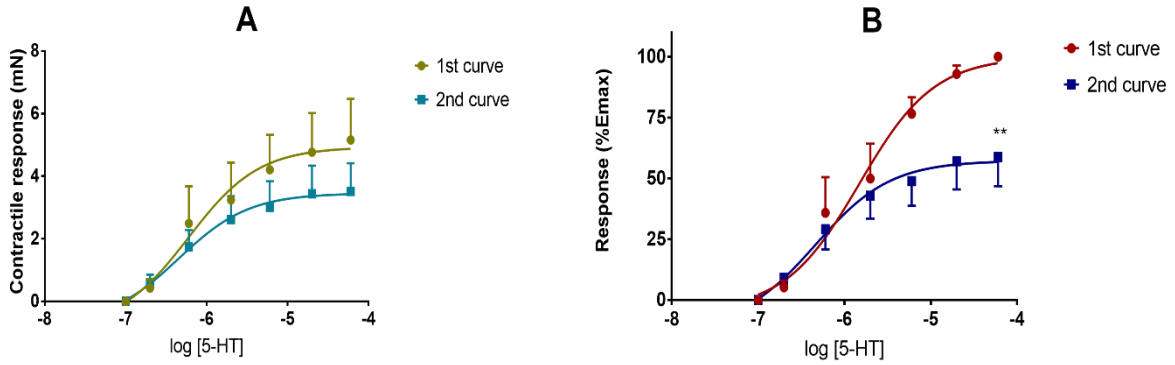

Figure 5 Comparison between first and second concentration-response curves to 5-HT at 37°C in control rings. A, Comparison of absolute contraction (in mN). B, Comparison of relative contraction (as % of E_{max} from the first curve).

4.1.2. Contractile effects at hyperthermic conditions

At hyperthermic conditions, a similar contractile response was found to MDMA (Table 2). In comparison with normothermic conditions (Figure 6), MDMA displayed a higher intrinsic activity (E_{max} = 7.23±4.54 mN at 40°C vs E_{max} = 2.79±1.03 mN at 37°C), although this difference was not statistically significant (P = 0.37). Moreover, the potency was also nonsignificantly different between conditions (pEC_{50} = 2.62±1.63 at 37°C vs pEC_{50} = 3.40±0.78 at 40°C, P = 0.68).

Table 2 Pharmacological parameters from concentration-response control curves to MDMA at hyperthermic conditions (*n* corresponds to the number of experiments).

Compound	Curve	E_{max} (mN)	pEC_{50} (-log[M])	E_{max} (% 1st curve)	*n*
MDMA	1st	7.23 ± 4.54	3.40 ± 0.78	100.00 ± 0.00	5
	2nd	6.04 ± 3.21	2.69 ± 1.32	95.10±28.45	5

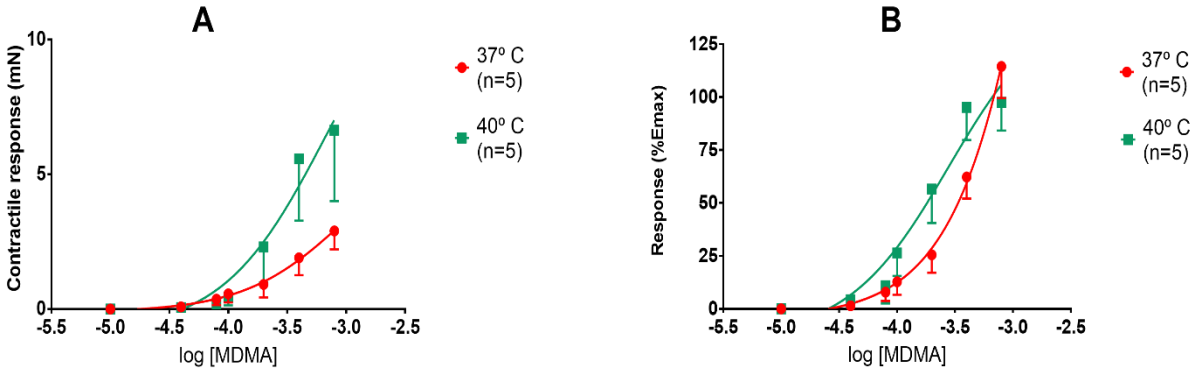

Figure 6 Comparison between concentration-response curves to MDMA at 37°C vs 40°C in control rings. A, Comparison of absolute contraction (in mN). B, Comparison of relative contraction (as % of E_{max} from the first curve).

Based on these results, we may assume that the arterial tissue displays a similar response in both thermal conditions to MDMA. Interestingly, this assumption is not in accordance with our previous report [91], in which we showed a significantly higher response to MDMA at hyperthermic conditions (E_{max} = 8.03±0.49 mN at 40°C vs E_{max} = 1.98±0.77 mN at 37°C, P < 0.001).

Regarding the general comparison between first and second curves (Figure 7), a borderline statistically significant difference was retrieved when results were expressed as absolute contraction (P = 0.0497). However, this difference was not found neither in specific concentrations nor when comparing as relative contraction (P = 0.35).

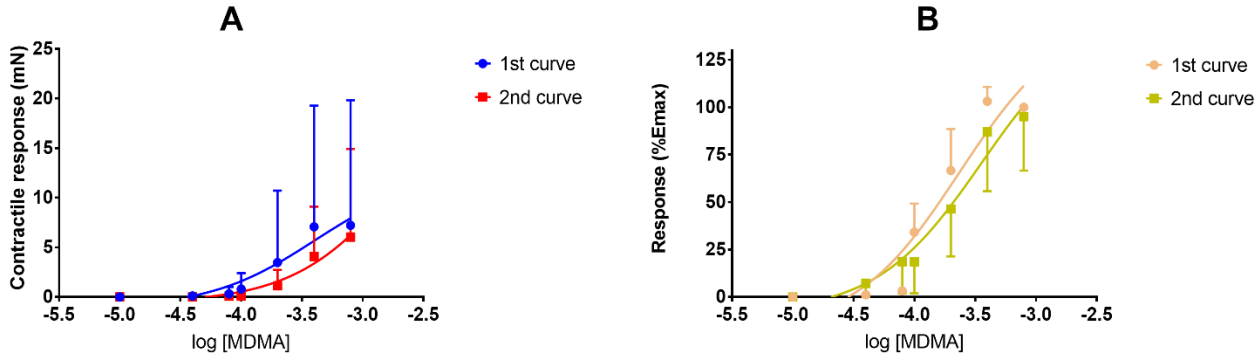

Figure 7 Comparison between first and second concentration-response curves to MDMA at 40°C in control rings. A, Comparison of absolute contraction (in mN). B, Comparison of relative contraction (as % of E_{max} from the first curve).

4.2. Role of SERT on the vascular effects of MDMA

As mentioned above, SERT has been the most recognized target of MDMA. However, findings from experimental and clinical studies which evaluate the specific role of this transporter on the cardiovascular effects of this drug are not conclusive. In particular, Cannon et al. [89] have previously suggested that the effect of MDMA on the vasculature does not result from a direct effect of MDMA on peripheral 5-HT transport in rat aortic rings. Baker et al. [88] further suggested the involvement of additional mechanisms other than indirect sympathomimetic activity or α_1-adrenoceptor-mediated effects, which were initially proposed by Bexis and Docherty [52]. However, a role for SERT may not be discarded [90] and requires further clarification. In this context, we aimed to evaluate the influence of monoamine transporters, specifically SERT, in the vascular effects elicited by MDMA. Pharmacological parameters from concentration-response curves to MDMA in the presence and absence of fluoxetine are detailed in Table 3.

At normothermic conditions (Figure 8), a statistically significant ($P < 0.05$) reduction was observed on the maximal contraction (% E_{max}) to MDMA after 30-minute preincubation with 0.4, 2 and 10μM of fluoxetine (E_{max} decrease of 34.13%, 27.53% and 65.95% vs control, respectively). Potency changes were not statistically significant.

As shown above, the first and second concentration-response curves to MDMA were equivalent (Figure 4). Therefore, we may hypothesize that the decrease in arterial tissue reactivity to MDMA in the presence of fluoxetine is a result from the inhibitory effect on 5-HT reuptake of this compound, thus suggesting the involvement of SERT on the MDMA-associated vascular effects. The presence of SERT in peripheral arterial smooth cells comes in agreement with previous studies in animal models [122, 129].

Comparing our results with the findings from a previous study from our group, Silva et al. [90] showed that fluoxetine induced a decrease of about 23% of the maximal contraction of ITA to MDMA. However, the range of concentrations of fluoxetine used in our study were higher (0.4, 2 and 10 μM) in comparison with 0.1 μM in Silva et al. [90] which could explain the different findings.

Regarding 5-HT, a significant decrease in maximal contraction was observed for 2 μM (E_{max} reduction of 40.07% vs control) and 10 μM (E_{max} reduction of 44.4% vs control) of

fluoxetine (Table 3 and Figure 8). Changes in potency were not statistically significant. Considering the decrease in tissue reactivity to 5-HT discussed above (Figure 5), the decrease in maximal contraction in the presence of fluoxetine may be a result of this factor and not of a real SERT-mediated effect.

Table 3 Pharmacological parameters from concentration-response curves to MDMA and 5-HT in the presence and absence of the SSRI fluoxetine at different thermal conditions (*n* corresponds to the number of experiments).

Compound	Temperature	Fluoxetine	E_{max} (%)	pEC_{50} (-log[M])	*n*
MDMA	37 °C	Control	100.00 ± 0.00	2.68 ± 3.43	21
		0.4 µM	65.87 ± 19.95	2.50 ± 1.26	5
		2 µM	72.47 ± 20.65	–	5
		10 µM	34.05 ± 9.72	2.55 ± 1.36	6
	40 °C	Control	100.00 ± 0.00	3.33 ± 0.22	10
		10 µM	15.45 ± 8.07	2.03 ± 6.65	5
5-HT	37 °C	Control	100.00 ± 0.00	5.76 ± 0.08	15
		0.4 µM	92.38 ± 22.84	5.77 ± 0.37	5
		2 µM	59.93 ± 17.66	5.77 ± 0.41	5
		10 µM	55.60 ± 9.71	5.42 ± 0.33	5

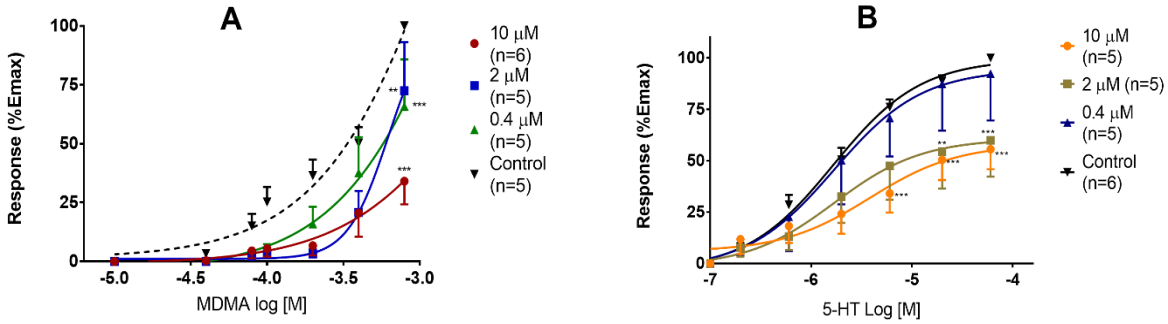

Figure 8 Cumulative concentration-response curves to MDMA (A) and 5-HT (B) in the presence and absence of the SSRI fluoxetine under normothermic conditions. Statistical information: A, *** $P < 0.001$ for 0.4 and 10 µM vs control and ** $P < 0.01$ for 2 µM vs control; B, *** $P < 0.001$ for 10 and 2 µM vs control and ** $P < 0.01$ for 2 µM.

4.2.1. Influence of hyperthermia

At hyperthermic conditions, we only tested the 10 μM concentration of fluoxetine, based on the findings at normothermic conditions. As can be seen in Figure 9, a significant decrease in maximal contraction was observed (E_{max} reduction of 84.55% vs control, P = 0.0491). Changes in potency were not statistically significant.

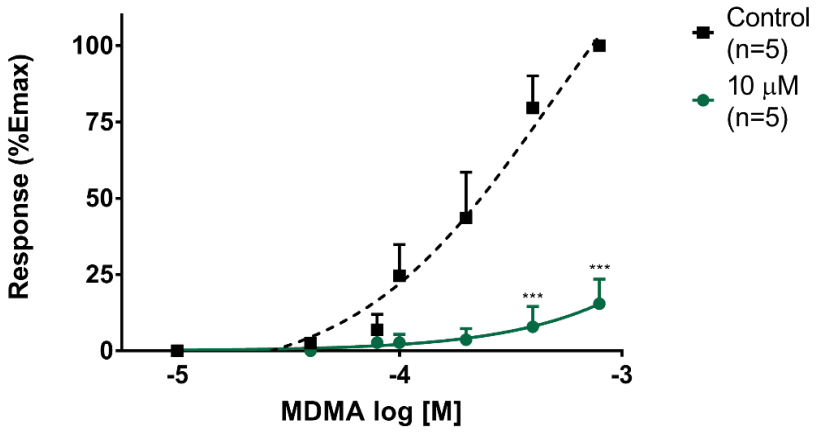

Figure 9 Cumulative concentration-response curves to MDMA in the presence and absence of the SSRI fluoxetine under hyperthermic conditions. Statistical information: *** P < 0.001 vs control.

Together our results both at normothermic and hyperthermic conditions confirm the potential involvement of SERT in the vascular effects of MDMA. The presence of SERT in the cardiovascular system has been documented in several tissues, namely the heart, blood vessels and platelets [116]. In the arteries, this transporter has been implicated in the modulation of smooth muscle contractility [116]. Although its specific physiological role remains largely unexplored, previous studies have shown that SERT inhibition, e.g. with SSRIs (fenfluramine, paroxetine or citalopram) may be involved in primary pulmonary hypertension [130, 131] and even in valvular disease [132, 133]. Furthermore, SERT activity has been shown to be increased in arterial hypertension [116, 122].

The comparison between normothermic and hyperthermic conditions, revealed no statistically significant differences in terms of pharmacological parameters. Although a similar inhibitory effect was observed, the MDMA-induced hyperthermia may represent an important

factor when considering the potential cardiovascular risk of MDMA abuse, as previously suggested [91].

V. CONCLUSION

According to the World Health Organization, cardiovascular diseases are the leading cause of death worldwide. The mortality is influenced mostly by socioeconomic disparity between developed and non-developed countries, where access to healthcare is difficult and expensive, and therefore only accessible for a niche of the population. Moreover, behavioural risk factors such as tobacco use, alcohol consumption, dietary negligence that can lead to obesity problems, sedentary habits and drug abuse are the main causes of cardiovascular diseases.

To this purpose, the 2nd year of the Master's degree in Applied Pharmacology at the Faculty of Pharmacy of University of Coimbra was devoted to the study of the vascular effects of MDMA and its potential to impair cardiovascular regulatory mechanisms, with a particular focus on the vascular system. Regarding the vascular studies performed in the human ITA, we sought to improve the scientific knowledge on this subject and showed that:

- MDMA consistently produces a contractile response of human arterial tissue, further confirming the vasoconstrictor potential of this drug;
- The SSRI fluoxetine modulates the contractile response elicited by MDMA in a concentration-dependent manner;
- The modulatory effect of fluoxetine on the MDMA-induced contractile response is independent of thermal condition;
- In general, a role for SERT in the acute vascular effects.

In summary, MDMA abuse and underlying physiological outcomes, such as hyperthermia, the influence of the ambient temperature in which its use takes place, underlying cardiovascular diseases, binge ingestion of multiple MDMA tablets, alcohol and other drugs, gender differences and genetic metabolic variations, must be taken into account for the consequent harmful cardiovascular outcomes.

For future studies, it will be important to fully characterize the human vascular tissue impairment and which agents, at a molecular level, are involved in this matter with the objective to raise awareness against MDMA abuse and in order to develop secure structural analogues that can be useful for the treatment of psychological disturbances.

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