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Release of antidiuretic hormone (ADH) in response to the administration of synthetic cathinones: the impact of gender

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RELEASE OF ANTIDIURETIC HORMONE (ADH) IN RESPONSE TO
THE ADMINISTRATION OF SYNTHETIC CATHINONES:
THE IMPACT OF GENDER

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Publications and Communications

In accordance to the nº 2, paragraph a, article 31 from Decree-law n. º115/2013, the following publications and conference communications were prepared under the scope of this dissertation.

Publications in international peer-reviewed journals

- Faria AC, Carmo H, Teixeira C, Rouxinol D, Silva JP, Carvalho F, Bastos ML, Dias da Silva D (2019). Administration of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone increases the release of antidiuretic hormone in female Wistar rats. Accepted for publication in *Toxicology Letters* (abstract number 920).
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- Faria AC, Carmo H, Carvalho F, Bastos ML, Dias da Silva D (2018). Drug-induced hyponatraemia: the case of synthetic cathinones. 17th National Congress of Forensic Medicine and Forensic Sciences. November 22–24. Coimbra, Portugal.
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Abstract

Synthetic cathinones are drugs of abuse obtained by chemical synthesis that have recently emerged in the recreational context, and elicit psychoactive effects similar to those of classic amphetamines. One of the potentially life-threatening consequences of amphetamine abuse, in particular of 3,4-methylenedioxymethamphetamine (MDMA), is serotonin-mediated hyponatraemia, which has been associated with an alteration in the release of the antidiuretic hormone (ADH). The majority of the reported clinical cases indicate a greater susceptibility of the female gender to the development of hyponatraemia after MDMA abuse, which may therefore anticipate gender differences in the MDMA-induced increase of ADH secretion. Recently, a case of hyponatraemia was also documented in a young woman after consumption of synthetic cathinones. Since synthetic cathinones and amphetamines share many of their toxicodynamic mechanisms, the aim of the current study was to investigate i) whether the ingestion of synthetic cathinones increases the release of ADH *in vivo* and, ii) in case of expressed modification of the ADH release pattern, the impact of the gender in that effect.

For this purpose, female Wistar rats were i.p. administered with two doses (20 mg/Kg and 40 mg/Kg) of the synthetic cathinones 3,4-dimethylmethcathinone (3,4-DMMC) or methylone. A group of animals treated with MDMA at 20 mg/Kg was also included for comparison. The quantification of ADH was performed in plasma 1 h after drug administration, through an enzyme competitive immunoassay, using a commercially available kit (Arg⁸-Vasopressin ELISA kit, Enzo). ADH was also evaluated: i) in plasma of male Wistar rats 1 h after administration of 3,4-DMMC or methylone at 20 mg/Kg, and ii) in plasma and urine of female Wistar rats 24 h after administration of 3,4-DMMC or methylone at 20 mg/Kg. The following parameters, which might relate with altered release of ADH, were additionally determined: the rectal temperature of the animals treated for 1 h; the volume of urine excreted, water intake and the brain water content of the animals treated for 24 h; and the ratio of brain to body weight of animals treated for 1 h or 24 h.

A significant increase of the levels of ADH occurred in all animals treated for 1 h with MDMA, methylone and 3,4-DMMC ($p < 0.05$). No significant differences were observed between MDMA and cathinone treatments, as well as between male and female rats. Increased plasma ADH was still observed 24 h after cathinone administration ($p < 0.01$), but less pronounced as compared to animals treated for 1 h. In addition, animals

treated for 24 h with the test cathinones showed higher urinary concentrations of ADH, presenting statistically significant differences to control animals ($p < 0.01$). It was also found that animals administered with 3,4-DMMC for 24 h excreted a smaller urine volume relative to the amount of ingested water, supporting the antidiuretic effect triggered by ADH. This effect was not reflected in cerebral oedema, as no significant differences in brain water content were observed between treated and control animals, and no alterations in the ratio between brain weight and body weight were observed 1 h or 24 h after drug administration. Regarding body temperature, the two cathinones led to significant increases in the body temperature of 1 h-exposed animals ($p < 0.01$), in both genders.

To the best of our knowledge, these results demonstrated for the first time the increased secretion of ADH in rats after the administration of 3,4-DMMC and methylone, and the further antidiuretic response of 3,4-DMMC. Overall, this study is a step forward to elucidate the toxic effects caused by these drugs and will certainly help forensic pathologists to establish the cause of death in fatality cases, as well as health professionals involved in the management of drug-intoxications to establish the most appropriate therapeutic measures in case of non-lethal intoxications.

RESUMO

As catinonas sintéticas são drogas de abuso obtidas por síntese química que emergiram recentemente em contexto recreativo, e que provocam efeitos psicoativos semelhantes aos das anfetaminas clássicas. Uma das consequências potencialmente fatais do abuso de anfetaminas, particularmente da 3,4-metilenodioximetanfetamina (MDMA), é a hiponatremia mediada pela serotonina, que tem sido associada a uma alteração na libertação da hormona antidiurética (ADH). A maioria dos casos clínicos descritos indica uma maior suscetibilidade do sexo feminino para o desenvolvimento de hiponatremia após o abuso de MDMA, o que pode, portanto, antecipar diferenças de género no aumento da secreção de ADH induzido pela MDMA. Recentemente, também foi documentado um caso de hiponatremia numa jovem mulher após o consumo de catinonas sintéticas. Uma vez que as catinonas sintéticas e as anfetaminas partilham muitos dos seus mecanismos toxicodinâmicos, o objetivo do presente estudo foi investigar: i) se a ingestão de catinonas sintéticas aumenta a libertação de ADH *in vivo* e, ii) em caso de modificação evidente do padrão de libertação de ADH, o impacto do género em tal efeito.

Para este fim, ratas Wistar fêmeas foram administradas i.p. com duas doses (20mg/Kg e 40 mg/Kg) das catinonas sintéticas 3,4-dimetilmetcatinona (3,4-DMMC) ou metilona. Um grupo de animais tratados com MDMA a 20 mg/Kg também foi incluído para comparação. A quantificação de ADH foi realizada em plasma 1 h após a administração das drogas, através de um ensaio enzimático competitivo, utilizando um kit comercialmente disponível (kit Arg⁸-Vasopressin ELISA, Enzo). A ADH também foi avaliada: i) em plasma de ratas Wistar machos 1 h após a administração de 3,4-DMMC ou metilona a 20 mg/Kg, e ii) em plasma e urina de ratas Wistar fêmeas 24 h após a administração de 3,4-DMMC ou metilona a 20 mg/Kg. Os seguintes parâmetros, que podem estar relacionados com a libertação alterada de ADH, foram adicionalmente determinados: a temperatura rectal dos animais tratados durante 1 h; o volume de urina excretado, a ingestão de água e o conteúdo de água no cérebro dos animais tratados durante 24 h; e a razão entre o peso do cérebro e o peso corporal dos animais tratados durante 1 h ou 24 h.

Ocorreu um aumento significativo dos níveis de ADH em todos os animais tratados durante 1 h com MDMA, metilona e 3,4-DMMC ($p < 0.01$). Não foram observadas diferenças significativas entre os tratamentos com a MDMA e com as catinonas, nem entre ratas machos e fêmeas. O aumento de ADH no plasma ainda

foi observado 24 h após a administração das catinonas ($p < 0.01$), mas de forma menos pronunciada em comparação com os animais tratados durante 1 h. Para além disso, os animais tratados durante 24 h com catinonas apresentaram maiores concentrações urinárias de ADH, apresentando diferenças estatisticamente significativas em relação aos animais controlo ($p < 0.01$). Verificou-se também que os animais tratados com 3,4-DMMC durante 24 h excretaram um volume urinário menor em relação à quantidade de água ingerida, suportando o efeito antidiurético desencadeado pela ADH. Este efeito não se refletiu em edema cerebral, uma vez que não foram observadas diferenças significativas no conteúdo de água no cérebro entre os animais tratados e os animais controlo e não foram observadas alterações na razão entre o peso do cérebro e o peso corporal 1 h ou 24 h após a administração das drogas. Em relação à temperatura corporal, as duas catinonas provocaram aumentos significativos na temperatura corporal dos animais expostos durante 1 h ($p < 0.01$), em ambos os sexos.

À luz do nosso conhecimento, estes resultados demonstraram, pela primeira vez, o aumento da secreção de ADH em ratazanas, após a administração de 3,4-DMMC e metilona, e a resposta antidiurética da 3,4-DMMC. De um modo geral, este estudo vem elucidar os efeitos tóxicos causados por estas drogas e, certamente, ajudará os patologistas forenses a determinar a causa da morte em casos de intoxicações fatais, bem como profissionais de saúde envolvidos no tratamento de intoxicações associadas a drogas de abuso, a estabelecer as medidas terapêuticas mais apropriadas em casos de intoxicações não letais.

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Figure 2. Pharmacological mechanisms of 3,4-methylenedioxymethamphetamine (MDMA or *ecstasy*) at the serotonergic terminal. The pharmacological action of phenethylamines is quite similar, only differing in the neurotransmitter specificity. For example, due to the methylenedioxy moiety, MDMA has a preferential affinity for the serotonin transporter (SERT) and therefore strongly increases the extracellular concentration of serotonin (5-HT). The presynaptic SERT removes 5-HT from the synapse to be recycled or stored inside vesicles for later use, but MDMA binding to SERT inhibits neuronal reuptake of 5-HT (I). Moreover, after binding to SERT, MDMA translocates into the cytoplasm, stimulating the neurotransmitter exiting from the cell along its concentration gradient, via reversal of the normal reuptake function, in a process designated by diffusion-exchange (II). Also, cytoplasmic concentration of 5-HT is increased due to the drug-induced disruption of vesicular storage (III). At low concentrations, MDMA uses the vesicular monoamine transporter (VMAT) to enter inside the neurotransmitter vesicles, avoiding the storage and promoting the depletion of 5-HT from the vesicles by a transport exchange. At high concentrations, MDMA penetrates into the synaptic vesicles by a passive diffusion process. Once inside these vesicles, MDMA depletes vesicular biogenic amine content by disrupting the gradient of pH, due to a weak base effect that powers VMAT. Furthermore, phenethylamines are known by their monoamine oxidase (MAO) inhibitory properties (IV), thereby increasing the cytosolic content of monoamines through inhibition of their degradation. These coordinated actions explain behaviour of phenethylamines as strong monoamine releasers. In addition to the indirect action of MDMA in stimulating the release of 5-HT, MDMA itself has affinity for 5-HT receptors, activating them directly (V).

Neuroadaptive depletion of 5-HT stores and the reduced ability to synthesize new 5-HT – since MDMA also inhibits tryptophan hydroxylase (TPH), the enzyme for 5-HT synthesis (VI) – underlie the tolerance to the drug (Carvalho et al., 2012; Green et al., 2003; Leonardi and Azmitia, 1994; Rudnick and Wall, 1992; Simantov, 2004). *5-HIAA*, 5-hydroxyindoleacetic acid. *TP*, Tryptophan. 5

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Six female and seven male animals were tested per treatment, except for MDMA (only one male animal was tested). Results are presented as mean \pm standard error of mean. *** p <0.001; **** p <0.0001, vs. female control. & p <0.05, vs. male control. 46

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Figure 14. Effect of 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg and 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 or 40 mg/Kg on ratio brain weight to animal weight in female (A) or male (B) Wistar rats, 1 h after i.p. drug administration; and in female Wistar rats, 24 h after i.p. drug administration (C). At least

six animals were tested per treatment, except for methylone when tested at 40 mg/Kg (one animal). Results are presented as mean \pm standard error of mean. 51

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List of Abbreviations

3,4-DMMC, 3,4-Dimethylmethcathinone

4-MMA, 4-Methylmethamphetamine

4-MMC, 4-Methylmethcathinone or mephedrone

5-HT, Serotonin

5-HT_x, Serotonin receptor, type x

ADH, Antidiuretic hormone

AIDS, Acquired immune deficiency syndrome

AQP2, Aquaporin-2 water channel

AQP3, Aquaporin-3 water channel

AQP4, Aquaporin-4 water channel

ARDS, Acute respiratory distress syndrome

AVP, Arginine vasopressin

BZP, N-Benzylpiperazine

cAMP, Cyclic adenosine monophosphate

CNS, Central nervous system

COMT, Catechol-O-methyltransferase

CK, Creatine phosphokinase

D1, Dopamine receptor, type 1

DA, Dopamine

DAT, Dopamine transporter

Gs, Guanine nucleotide binding protein

HHA, 3,4-Dihydroxyamphetamine or α -methyldopamine

HHMA, 3,4-Dihydroxymethamphetamine or N-methyl- α -methyldopamine

HMA, 4-Hydroxy-3-methoxyamphetamine

HMMA, 4-Hydroxy-3-methoxymethamphetamine

i.c.v., Intracerebroventricular

i.p., Intraperitoneal

i.v., Intravenous

LC-MS/MS, Liquid chromatography-tandem mass spectrometry

MAO, Monoamine oxidase

MDA, 3,4-Methylenedioxyamphetamine

MDMA, 3,4-Methylenedioxymethamphetamine or “ecstasy”

MDMC, 3,4-Methylenedioxy-N-methylcathinone or methylone

MDPV, Methylenedioxypropylone

METH, Methamphetamine

MS, Mass spectrometry

NA, Noradrenaline

NAT, Noradrenaline transporter

NMR, Nuclear magnetic resonance

NPS, New psychoactive substance

NSB, No specific binding

RAAS, Renin-angiotensin-aldosterone system

SA-HRP, Streptavidin conjugated to horseradish peroxidase

s.c., Subcutaneous

SEM, Standard error of the mean

SERT, Serotonin transporter

SIADH, Syndrome of inappropriate secretion of antidiuretic hormone

SSRIs, Selective serotonin reuptake inhibitors

T4, Thyroxine hormone

THP, Tryptophan hydroxylase

UCP, Uncoupling protein

UCP-x, Uncoupling protein, type x

V_xR, Antidiuretic hormone receptor, type x

Chapter 1

General Introduction

1.1 Introduction

Synthetic phenethylamines are among the most important and consumed groups of psychoactive substances (EMCDDA, 2019). This class can be divided into subclasses, according to certain structural characteristics, and may include a large number of new psychoactive substances (NPS) such as synthetic cathinones, but also classic drugs of abuse such as amphetamine, methamphetamine and 3,4-methylenedioxyamphetamine (MDMA or *ecstasy*) (Figure 1).

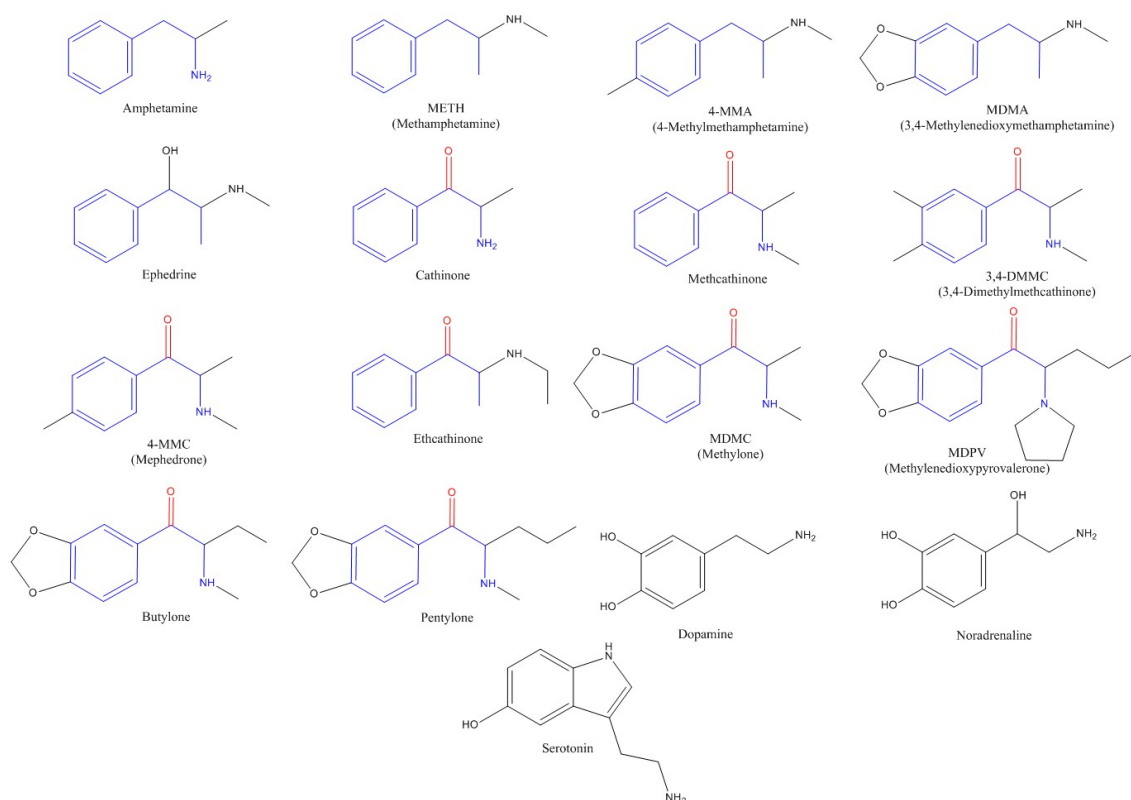


Figure 1. Chemical structures of amphetamine, methamphetamine (METH), 4-methylmethamphetamine (4-MMA), 3,4-methylenedioxyamphetamine (MDMA, *ecstasy*), ephedrine, cathinone, methcathinone, 3,4-dimethylmethcathinone (3,4-DMMC), mephedrone (4-methylmethcathinone, 4-MMC), ethcathinone, methylone (3,4-methylenedioxyethylcathinone, MDMC, β k-MDMA), methylenedioxypropylvalerone (MDPV), butylone, and pentylone. The phenethylamine core is highlighted in blue, and the ketone group of cathinones in red. The neurotransmitters dopamine, noradrenaline and serotonin were included to highlight their structural similarity.

Natural cathinone and amphetamine derivatives have been used for several years through the consumption of plants that produce them, *e.g.* *Catha edulis* (*khat*) and various shrubs of the genus *Ephedra*, including *Ephedra sinica* (*ma huang*). While the effect of *Ephedra sp.* is due to its content in ephedrine (Abourashed et al., 2003), the effect of

Catha edulis derives from its content in cathinone (Valente et al., 2014), an active ketone compound that is also a potent amphetamine found on the leaves of the plant, and from which synthetic cathinones are derived (Carvalho et al., 2012; Sitte and Freissmuth, 2015). Synthetic amphetamine was first synthesised by Lazăr Edeleanu, in Berlin, in 1887, and ever since many derivatives have been produced (Sitte and Freissmuth, 2015; Sulzer et al., 2005). This classical drug is used as a reference for the synthesis of new stimulant substances (Maas et al., 2015), but also the natural occurring cathinone serves as a base for many substitutions (Coppola and Mondola, 2012; Katz et al., 2014). The first synthetic cathinones appeared in the 1920s, initially pursued as a therapeutic application as antidepressants (Valente et al., 2014). Their interest as legal drugs of abuse sprout in the beginning of the current millennium (Coppola and Mondola, 2012) as these derivatives proved to be often more powerful psychoactive drugs than natural cathinone (Adamowicz et al., 2014; Backberg et al., 2015). Currently, unlike classic amphetamines, synthetic cathinones are easily available online or at smartshops, being sold as “research chemicals”, “bath salts” or “plant fertilizers” to facilitate their commercialization. In the last decade, the speed at which new substances have been produced and released on the drug market made it difficult to control their trafficking and abuse (Valente et al., 2014). Regrettably, the risks that arose from their consumption peaked, and synthetic cathinones were mentioned on the death certificate of several fatal intoxications (James et al., 2011; Kovacs et al., 2012; Murray et al., 2012). Consequently, in the USA and in Europe, the authorities have banned several synthetic cathinones, including mephedrone, methyldone and methylenedioxypyrovalerone (MDPV), which became known as the first generation of synthetic cathinones (Sitte and Freissmuth, 2015). Nevertheless, other synthetic cathinones were rapidly designed by exchanging an atom or group of atoms, and novel derivatives emerged. These synthetic molecules are sufficiently different from those of the first generation, enabling circumventing drug testing and legislation, but also sufficiently related to elicit similar biological effects (bioisosterism) (Boulanger-Gobeil et al., 2012; Liechti, 2015). Often, chemical substitutions may also introduce enormous changes in the activity of the drug and on its mode of action (Sitte and Freissmuth, 2015).

Phenylethylamines present distinct affinity and selectivity towards monoamine transporters and receptors, and therefore distinctly disrupt dopamine (DA), noradrenaline (NA) and serotonin (5-HT) neurotransmission (Figure 2). Due to the structural similarity with monoamine neurotransmitters (Figure 1), phenylethylamines act as competitive substrates of monoamine membrane uptakers, such as dopamine (DAT), serotonin

(SERT) and noradrenaline transporters (NAT), resulting in an over-stimulation after drug administration (Carvalho et al., 2012; de la Torre et al., 2004; Morton, 2005; Sitte and Freissmuth, 2015; Valente et al., 2014). This effect is responsible for the attractive outcomes of amphetamines, which include their stimulating, euphoric, hallucinogenic, anorexigenic and empathogenic or entactogenic effects – the latter two describing the effects of MDMA on socialization, *i.e.* improved social skills and feelings of empathy (Carvalho et al., 2012; de la Torre et al., 2004). Chronic abuse may, however, drive to the decline of relationships (Ferreira et al., 2019).

Over decades, phenethylamine-related fatalities, as well as non-fatal severe intoxications, greatly attracted the attention of both public and scientific literature (Berney-Meyer et al., 2012; Claffey, 2011; Rosenson et al., 2007; Thakkar et al., 2017; Vakde et al., 2014). There are cases of deaths related with MDMA that occurred in first-time users or after consumption of a single tablet of *ecstasy*. On the other hand, there are cases of mild intoxication symptoms and recovery happening after the ingestion of a large number of *ecstasy* tablets consumed on a single occasion, indicating a wide interindividual variability in the susceptibility to the toxic effects of these drugs (Coore, 1996; De Letter et al., 2004; Henry et al., 1992; Milroy et al., 1996; Ramcharan et al., 1998; Regenthal et al., 1999; Schifano, 2004). In fact, the consumption of phenethylamines often results in unexpected and random severe toxicity, for which there is no clear explanation (Barrios et al., 2016; Boulanger-Gobeil et al., 2012; Ikeji et al., 2018; Lusthof et al., 2011; Murray et al., 2012; Potocka-Banas et al., 2017; Warrick et al., 2012; Wood et al., 2010). Often, the quantity of drug found in intoxicated individuals varies immensely, consequently threshold toxic concentrations and potentially lethal doses are mostly unknown for these substances (De Letter et al., 2004; Libiseller et al., 2005). In addition, the toxicity of synthetic phenethylamines might not be merely related with the dose of drug ingested and/or drug blood concentration, but also a consequence of the toxicological potentiation exerted by the environmental conditions at which these drugs are consumed (e.g. the rave scene).

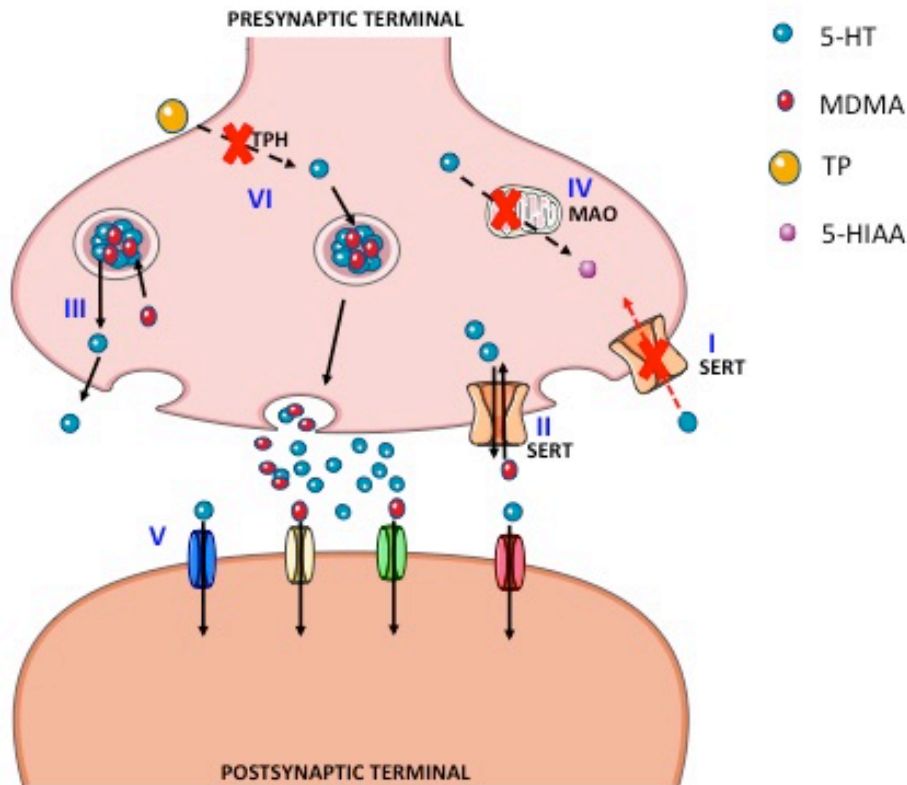


Figure 2. Pharmacological mechanisms of 3,4-methylenedioxymethamphetamine (MDMA or *ecstasy*) at the serotonergic terminal. The pharmacological action of phenethylamines is quite similar, only differing in the neurotransmitter specificity. For example, due to the methylenedioxy moiety, MDMA has a preferential affinity for the serotonin transporter (SERT) and therefore strongly increases the extracellular concentration of serotonin (5-HT). The presynaptic SERT removes 5-HT from the synapse to be recycled or stored inside vesicles for later use, but MDMA binding to SERT inhibits neuronal reuptake of 5-HT (I). Moreover, after binding to SERT, MDMA translocates into the cytoplasm, stimulating the neurotransmitter exiting from the cell along its concentration gradient, via reversal of the normal reuptake function, in a process designated by diffusion-exchange (II). Also, cytoplasmic concentration of 5-HT is increased due to the drug-induced disruption of vesicular storage (III). At low concentrations, MDMA uses the vesicular monoamine transporter (VMAT) to enter inside the neurotransmitter vesicles, avoiding the storage and promoting the depletion of 5-HT from the vesicles by a transport exchange. At high concentrations, MDMA penetrates into the synaptic vesicles by a passive diffusion process. Once inside these vesicles, MDMA depletes vesicular biogenic amine content by disrupting the gradient of pH, due to a weak base effect that powers VMAT. Furthermore, phenethylamines are known by their monoamine oxidase (MAO) inhibitory properties (IV), thereby increasing the cytosolic content of monoamines through inhibition of their degradation. These coordinated actions explain behaviour of phenethylamines as strong monoamine releasers. In addition to the indirect action of MDMA in stimulating the release of 5-HT, MDMA itself has affinity for 5-HT receptors, activating them directly (V). Neuroadaptive depletion of 5-HT stores and the reduced ability to synthesize new 5-HT – since MDMA also inhibits tryptophan hydroxylase (TPH), the enzyme for 5-HT synthesis (VI) – underlie the tolerance to the drug (Carvalho et al., 2012; Green et al., 2003; Leonardi and Azmitia, 1994; Rudnick and Wall, 1992; Simantov, 2004). 5-HIAA, 5-hydroxyindoleacetic acid. TP, Tryptophan.

The most noticeable clinical manifestation of phenethylamines is the sympathomimetic syndrome, which is characterized by tachycardia, tachypnoea, hypertension, hyperreflexia, tremor, hyperthermia, diaphoresis, and a variety of central nervous system (CNS) manifestations such as agitation, hyperexcitation, hallucinations, paranoia, disinhibition, convulsions and coma (Barrios et al., 2016; Gillman, 1999; Greene et al., 2008; Schifano, 2004; Schifano et al., 2003; Warrick et al., 2012; Wood et al., 2010). In addition, nausea, dry mouth, bruxism, muscle pain or tension, hot flashes, nystagmus, insomnia, coagulopathy, thrombocytopenia, acidosis, hypoglycaemia, pulmonary congestion, and oedema may also occur (Green et al., 2003; Greene et al., 2008).

Hyperthermia, in particular, can potentiate all other detrimental drug effects (Carvalho et al., 2012; Dias da Silva et al., 2013) and is frequently responsible for complications that contribute to a fatal outcome, including rhabdomyolysis and metabolic acidosis that can lead to impairment of renal function and acute kidney failure, disseminated intravascular coagulation, cardiovascular collapse, intracranial haemorrhage and multiple organ failure (Berney-Meyer et al., 2012; Henry et al., 1992; Kalant, 2001; Kendrick et al., 1977).

An important consequence and also a predictable pharmacological effect of phenethylamine administration is hyponatraemia (Backberg et al., 2015; Berney-Meyer et al., 2012), which is a consequence of fluid and electrolyte disorders, resulting from i) the excessive intake of water by users with the intent of preventing drug-induced hyperthermia, and/or a consequence of ii) the serotonin-mediated inappropriate secretion of antidiuretic hormone (ADH) (Berney-Meyer et al., 2012; Traub et al., 2002). In the particular case of MDMA, the drug induces the secretion of the hormone via activation of the serotonin system, resulting in an inappropriate retention of water (Traub et al., 2002) and consequently leading to the significant decrease of sodium in plasma. Ultimately, severe hyponatraemia may culminate in seizures, cerebral oedema and death (Berney-Meyer et al., 2012; Matthai et al., 1996; O'Connor et al., 1999; Traub et al., 2002). Recently, a case of ethcathinone and methylone ingestion followed by development of hyponatraemia was reported, possibly attributed to the MDMA-like characteristics of methylone (Boulanger-Gobeil et al., 2012).

1.2 Hyponatraemia

Hyponatraemia is an alteration of the hydroelectrolytic equilibrium, which occurs due to a deregulation in the water balance that results in decreased plasma osmolality (Lee and Noronha, 2016; Rocha, 2011; Zilberberg et al., 2008). Hyponatraemia consists of a sodium plasma concentration below the level that is expected to be found in a healthy individual, i.e. 136 mmol/L (Nathan, 2007; Rai et al., 2006; Rocha, 2011), and its severity depends upon the osmolality or sodium amount per Kg of total body water (Rai et al., 2006). In most clinical reports, the hyponatraemic effect is an asymptomatic condition, only detected by biochemical tests. In more severe acute situations, signs and symptoms of hyponatraemia develop (Gankam Kengne and Decaux, 2018); these are nonspecific and include nausea and vomiting, headache, loss of energy, drowsiness and fatigue, restlessness, irritability, confusion, muscle weakness, and spasms or cramps (Bora et al., 2016). Extreme cases may end up in serious morbidity, being the brain the main organ affected. In these cases, the absence of rapid interventions can result in seizures, coma, cerebellar tonsillar herniation, severe neurological damage and even death (Lee and Noronha, 2016; Verbalis et al., 2007).

1.2.1 Role of antidiuretic hormone (ADH) in hyponatraemia

Antidiuretic hormone, also referred to as arginine vasopressin (AVP), vasopressin, or argipressin, is a hormone composed of 9 amino acids that acts in the kidney to regulate the volume and osmolality of the urine through renal retention of water. The hormone is produced in the hypothalamus, and stored in the posterior pituitary or hypophysis (Figure 3).

The mechanism most likely to be related with the induction of ADH release is the serotonergic regulation of neurohormonal secretion (Jorgensen et al., 2003a), according to which the activation of 5-HT receptors leads to increased levels of systemic ADH. In a previous study using 5-HT receptor selective agonists and antagonists, it was observed that 5-HT_{2C}, 5-HT₄ and 5-HT₇ receptors are important in the regulation of the pituitary secretion of ADH (Jorgensen et al., 2003a).

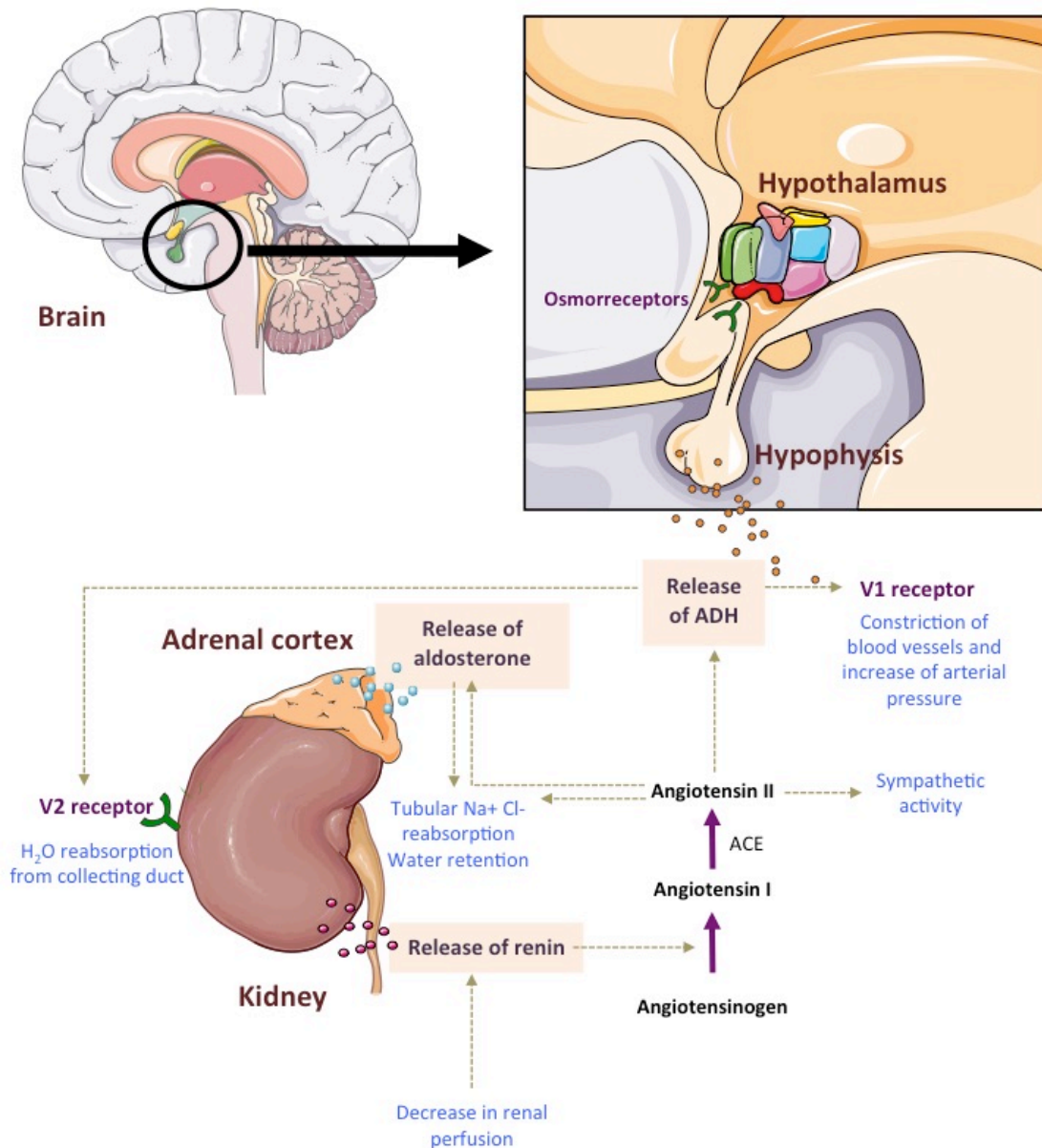


Figure 3. Synthesis, storage, secretion and action of antidiuretic hormone (ADH). The synthesis of ADH occurs in the supraoptic and paraventricular nuclei in the hypothalamus. The small peptide is then transported to the posterior pituitary gland via the neurohypophysial capillaries, where its synthesis is completed. The hormone is stored there until alterations to plasmatic osmolality (hyperosmolar stimulus) or decrease of blood pressure trigger a negative feedback mechanism through activation of osmoreceptors located in the hypothalamus, with subsequent stimulation of the gland to release ADH into the blood stream. Once secreted, the hormone regulates the electrolyte equilibrium through the increase of free water reabsorption by kidneys. In addition, the decrease of water in the blood, associated with a decrease in blood pressure, triggers the release of renin from juxtaglomerular cells. Renin induces conversion of angiotensinogen into angiotensin I, which is then converted to angiotensin II. Angiotensin II acts on pituitary to stimulate ADH release and on the adrenal cortex to stimulate the synthesis and release of aldosterone. Finally, both aldosterone and angiotensin II increase sodium reabsorption by increasing the number of active sodium channels and the action of Na⁺-K⁺ ATPases in the distal tubules. *ACE*, angiotensin-converting enzyme.

Serotonin is not only involved in the pituitary secretion of ADH but also in the synthesis of the hormone that occurs in the magnocellular neurons of the supraoptic hypothalamic nucleus and paraventricular hypothalamic nucleus. Using male Wistar rats, the 5-HT receptors involved in the mRNA expression of ADH were investigated. It was possible to observe that activation of 5-HT_{2A} and 5-HT_{2C} receptors drove to an increase of the ADH mRNA at the level of the paraventricular hypothalamic nucleus, although the synthesis of ADH at the hypothalamic supraoptic nucleus did not seem to be affected by serotonergic stimulation (Jorgensen et al., 2003a).

The synthesis of ADH is completed in the posterior pituitary gland, where the hormone is stored until further afferent signalling is sent from the hypothalamus, triggering its release. Secretion of ADH is usually regulated by the increase of plasma osmolality through the activation of osmoreceptors located in the organum vasculosum of the lamina terminalis and the subfornical organ of hypothalamus (Johnson and Thunhorst, 1997), or through the decrease of blood pressure detected at the carotid sinuses, aortic arch, cardiac atrium and pulmonary vessels (Brennan et al., 1971; Clark and Silva, 1967; Johnson and Thunhorst, 1997; Shimamoto et al., 1979; Thames and Schmid, 1981).

Osmoreceptors are cells sensitive to alterations in plasma osmotic pressure, which depends on the plasma volume and osmolality. Osmolality of plasma increases with dehydration and decreases with overhydration. So, if the total plasma volume falls, then the osmolality of the plasma will rise, as there will be sodium plasma concentration. This will lead to an osmotic shift of water from the intracellular compartment to the plasma, stimulating the osmoreceptor cells to shrink and signalling the posterior pituitary gland to increase the hormone release. Once released into the plasma, ADH mainly exerts its action on the kidney, which leads to an antidiuretic response through water retention.

On the other hand, a rise in plasma volume will cause plasma osmolality and sodium concentration to fall down the osmotic threshold, which in turn leads to the movement of water down its concentration gradient from the plasma into osmoreceptors, causing them to expand. Osmoreceptors then send afferent signals to the pituitary gland to decrease the release of ADH. The decrease of circulating ADH levels leads to the excretion of water (Brunton et al., 2005). This excretion of the excess of water occurs without electrolyte depletion (Verbalis et al., 2007). Through this mechanism, the organism avoids the reduction of plasma sodium and the electrolyte equilibrium is restored (Rai et al., 2006; Rocha, 2011; Verbalis et al., 2007). If the secretion of ADH is

not inhibited in the presence of a hypoosmolar stimulus, the increased levels of ADH then lead to excessive renal water retention and extracellular fluid expansion, which is compensated by the increase of urinary sodium excretion through the renin–angiotensin–aldosterone system (RAAS) (Figure 3). The retention of water in conjunction with increased sodium excretion increases the risk of hyponatraemia (Rai et al., 2006; Traub et al., 2002; Verbalis et al., 2007).

Most states of hyponatraemia result from excessive high levels of circulating ADH and are related with pre-existing pathologies (Verbalis et al., 2007). The syndrome of inappropriate secretion of antidiuretic hormone (SIADH) is a common cause of hyponatraemia that results from aberrant or continued secretion of ADH even in the presence of hypoosmolar stimulus, or from continuous action of this hormone on its receptors (Rai et al., 2006; Yasir and Mechanic, 2019). In this syndrome, the excessive secretion of ADH leads to retention of water, which prevents the re-establishment of the hydroelectrolytic equilibrium (Rai et al., 2006). The SIADH is characterized by the presence of hypotonic hyponatraemia, increased natriuresis and inappropriate rise of urine osmolality, absence of oedema or volume depletion, and regular renal and adrenal functions (Cuesta and Thompson, 2016; Rai et al., 2006). There are several reasons for SIADH, including the administration of drugs (e.g. phenethylamines) that modify the mechanisms responsible for regulating ADH secretion (Rocha, 2011; Verbalis et al., 2007).

1.2.1.1 Antidiuretic hormone (ADH) receptors

After being released, the action of ADH is mediated by three subtypes of G protein-coupled receptors that are structurally distinct, the V_{1A} , V_2 , and V_3/V_{1B} receptors (Ferguson et al., 2003; Thibonnier et al., 2001).

The V_{1A} receptor is expressed in vascular smooth muscle cells, myocardium, platelets and liver. This receptor is responsible for activating phosphoinositide pathways that induce vasoconstriction, myocardial contraction, platelet aggregation and glycogenolysis (Rai et al., 2006). Of note, activation of V_{1A} receptor promotes vasoconstriction through increase of cyclic adenosine monophosphate (cAMP) (Gheorghiade et al., 2003) and is responsible for the negative impact of ADH on hemodynamic and cardiac remodelling, such as chronic vasoconstriction and vascular hypertrophy (Goldsmith, 1999; Lee et al., 2003).

Nonetheless, the major effects of ADH are mediated by V_2 receptors, which trigger the increase of the permeability of the collecting duct to water (Gheorghide et al., 2003). The V_2 receptor is located in vascular endothelium and in cells of the renal distal tubule and collecting duct (Gheorghide et al., 2003). Upon ADH binding, the receptor activates a guanine nucleotide binding protein (Gs) and, subsequently, adenylate cyclase that increases cAMP synthesis. The cAMP activates a protein kinase A, which in turn stimulates the synthesis of aquaporin-2 water channels (AQP2) (Gheorghide et al., 2003; Lee et al., 2003) and its further insertion into the apical membrane of the collecting duct cells (Figure 4), resulting in increased water reabsorption (Ferguson et al., 2003; Nielsen, 2002). Once ADH is removed from the V_2

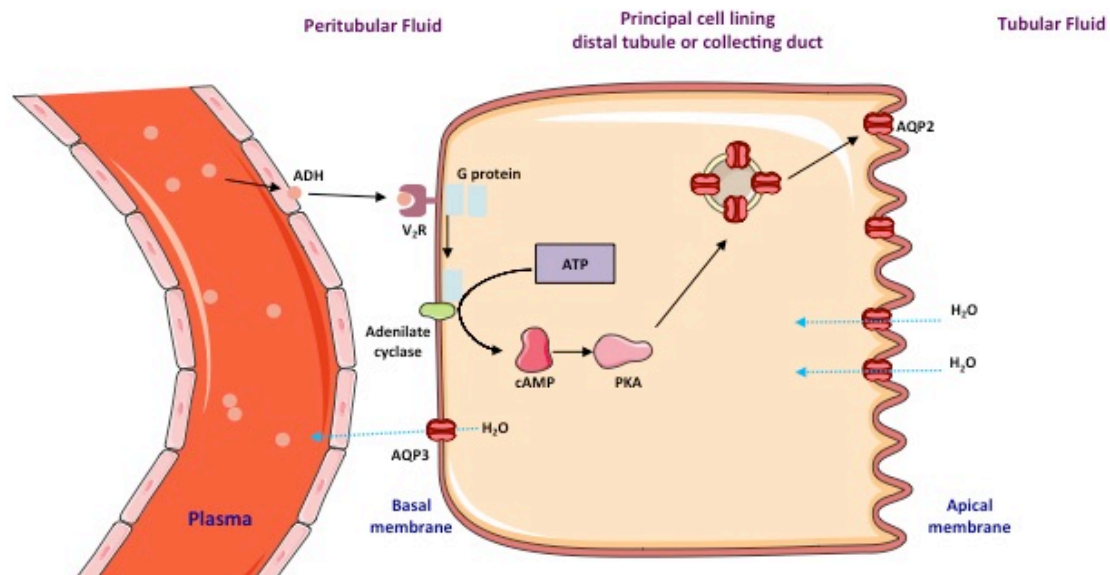


Figure 4. Mechanism of action of antidiuretic hormone (ADH) in the distal convoluted tubule and collecting duct of the kidney. The hormone modulates the permeability of the renal tubule and collecting duct to water, and consequently diuresis. Upon ADH binding to V_2 receptors (V_2R) located in the principal cells of the collecting duct and distal tubule, activation of G protein-mediated signalling pathways stimulates the recruitment and insertion of aquaporin-2 water channels (AQP2) to the apical membrane of the renal cell, increasing its permeability and allowing the diffusion of water that moves out of the nephron and back into the blood stream. Consequently, water reabsorption from urine is increased through passive transport (osmolality of urine increases). Alternatively, when ADH release is suppressed, the number of AQP2 transcript and inserted into the apical membrane decreases and the volume of water reabsorbed is reduced. *AQP3*, aquaporin-3 water channel. *cAMP*, cyclic adenosine monophosphate. *PKA*, protein kinase A.

receptor, AQP2 are internalized, which reflects in a decrease of membrane permeability to water. This is an adaptive process that participates in the regulation of the fluid and electrolyte balance (Rai et al., 2006).

Finally, the V_3 receptor is expressed by cells of the anterior pituitary and is involved in the regulation of the adrenocorticotrophic hormone secretion through phosphoinositide pathways (Ferguson et al., 2003). Recent works have also demonstrated its presence in other organs, such as pancreas, brain, kidneys and adrenal medulla (de Keyzer et al., 1996; Lolait et al., 1995). This receptor presents a unique pharmacological profile that is distinct from the other subtypes of receptors, i.e. V_2 and V_{1A} (Thibonnier et al., 2001), but also involves activation of various signaling pathways through different G proteins (Thibonnier et al., 2001).

1.2.2 Types of hyponatraemia

Regardless of individual variations in the general population, under conditions of normal hydration, osmolality ranges from 280 to 295 mOsm/Kg water (Verbalis et al., 2007). The osmolality of body fluids is maintained within physiological limits through the secretion of ADH and the ingestion of water (Rai et al., 2006; Verbalis et al., 2007).

Only the solutes for which the membranes are impermeable contribute to the effective osmolality, being responsible for creating gradients through the membranes. These gradients influence the osmotic movement of water between the extra and intracellular compartments (Verbalis et al., 2007). The concentration of solutes that in fact contribute to the osmotic movement of water allows to evaluate if a state of hypoosmolality or hyperosmolality is set. Generally, plasma hypoosmolality corresponds to hyponatraemia, since the solutes that most contributes to the osmotic equilibrium is sodium and its respective anions, of these, the most common is chloride (Verbalis et al., 2007).

It is assumed that plasma hypoosmolality translates into hypotonic hyponatraemia resulting from an excessive amount of water relative to the solute or, on the other hand, results from solute depletion (Diringer, 1992). In most situations of hyponatraemia, both co-occur: excess of water and solute depletion. In the context of hyponatraemia induced by synthetic phenethylamines, it is important to mention the three types of hypotonic hyponatraemia, i.e. hypervolaemic, euvolaemic and hypovolaemic hyponatraemia (Verbalis et al., 2007).

1.2.2.1 Hypervolaemic hyponatraemia

Generally, the manifestation of clinically detectable increase in extracellular volume is synonymous with hypervolaemia. In this situation, the secretion of ADH secondary to

associated pathologies (Table 1) leads to reduction of excretion and consequent body accumulation of water, resulting in hypoosmolality (Nathan, 2007). Sodium urinary concentration is usually below 30 mmol/L due to the activation of RAAS, which prevents renal excretion of sodium despite the excess of fluid (Verbalis et al., 2007). In these situations, the correction of hyponatraemia can be clinically difficult and may depend on the treatment of the underlying pathology (Rocha, 2011).

Table 1. Etiology of hyponatraemia whose origin is sodium dilution.

DECREASED RENAL EXCRETION OF WATER	
Euvolaemic Hyponatraemia	Hypervolaemic Hyponatraemia
SIADH	Congestive heart failure
Tumours	Cirrhosis
Central nervous system disorders	Nephrotic syndrome
Drug induced	Renal failure
Pulmonary diseases	
AIDS	
Prolonged exercise	
Glucocorticoid deficiency	
Hypothyroidism	
EXCESSIVE WATER INTAKE	
Primary polydipsia	

AIDS, acquired immune deficiency syndrome. *SIADH*, syndrome of inappropriate secretion of antidiuretic hormone.

1.2.2.2 Euvolaemic hyponatraemia

There are several situations of hypoosmolality with normal extracellular volume or presenting such small modifications that are not clinically detectable (Table 1). Most cases of euvolaemic hyponatraemia are related to SIADH (Verbalis et al., 2007). Clinically, there are no signs of changes in the extracellular volume and the concentration of urinary sodium is increased. While the water that is ingested is retained by the action of ADH, there is a progressive decrease of the sodium plasma concentration (Verbalis et al., 2007). Since ADH secretion is not suppressed when plasma osmolality falls lower than the osmotic threshold, irregular or continuous secretion of ADH in the absence of an adequate osmotic stimulus results in hyponatraemia (Rai et al., 2006). In this situation, the urinary concentration of sodium is usually greater than 30 mmol/L. Lower concentrations secondary to sodium depletion may also be observed (Verbalis et al., 2007).

1.2.2.3 Hypovolaemic hyponatraemia

Hypovolaemic hyponatraemia is related to a decrease of extracellular fluid volume and hypovolaemia. There are several causes for volume depletion secondary to sodium loss, as depicted in Table 2. Usually, as the extracellular volume cannot be measured, the clinical diagnosis of volume depletion is done by medical history, physical examination and laboratory results (Adroque and Madias, 2000; Traub et al., 2002). After correction of hypovolaemia, the baroreceptor stimulus for ADH secretion is inhibited and, consequently, there is a decrease in the release of ADH (Rocha, 2011).

Table 2. Etiology of hyponatraemia caused by sodium loss.

Renal sodium loss	Extra renal sodium loss
Antidiuretic treatments	Gastrointestinal loss
Cerebral salt-wasting syndrome	Diarrhoea
Mineralocorticoid deficiency	Vomit
Salt-wasting nephropathy	Sweat loss
Bicarbonaturia, glycosuria, ketonuria	Physical exercise resistance (prolonged)
	Other losses
	Pancreatitis
	Muscle injuries
	Burns
	Intestinal obstructions

1.3 Hyponatraemia triggered by phenethylamines

There are many cases reported in the scientific literature of hyponatraemia and cerebral oedema after consumption of MDMA (Farah and Farah, 2008; Ghatol and Kazory, 2012; Libiseller et al., 2005; Rosenson et al., 2007; Wilkins, 1996). More recently, one case associated with the abuse of “*legal ecstasy*” was described in a 22-year-old woman that was taken to the emergency room following several episodes of tonicoclonic seizures (Boulanger-Gobeil et al., 2012). As the toxicological exam of the seized material revealed the presence of a mixture of methylone and ethcathinone, hyponatraemia was explained by the MDMA-like characteristics of methylone, which may have led to SIADH. In addition, the joint action of methylone and ethcathinone (both 5-HT reuptake inhibitors) might have triggered the neurologic manifestations compatible with serotonin toxicity,

and the prolonged rhabdomyolysis (CK 34.537 U/L) that compelled the 6-day hospitalisation of the patient, even after resolution of the severe hyponatraemia and patient's mental status (Boulanger-Gobeil et al., 2012). Table 3 summarizes all clinical cases described in literature involving hyponatraemia associated with the abuse of phenethylamines. Most of these concerned intoxications that occurred in young women, which indicates a probable predisposition of the female gender (Ajaelo et al., 1998; Aramendi and Manzanares, 2010; Ayus et al., 2008; Balmelli et al., 2001; Ben-Abraham et al., 2003; Boulanger-Gobeil et al., 2012; Box et al., 1997; Braback and Humble, 2001; Brvar et al., 2004; Budisavljevic et al., 2003; Chen and Viola, 1991; Cherney et al., 2002; Claffey, 2011; Farah and Farah, 2008; Gankam Kengne and Decaux, 2018; Ghatol and Kazory, 2012; Gomez-Balaguer et al., 2000; Holden and Jackson, 1996; Holmes et al., 1999; Kalantar-Zadeh et al., 2006; Libiseller et al., 2005; Magee et al., 1998; Matthai et al., 1996; Maxwell et al., 1993; Niemeijer et al., 2009; Nuvials et al., 1997; O'Connor et al., 1999; Parr et al., 1997; Rosenson et al., 2007; Salathe et al., 2018; Satchell and Connaughton, 1994; Thakkar et al., 2017; Traub et al., 2002; Watson et al., 1997; Wilkins, 1996). In most of the situations described, only phenethylamines and their metabolites have been detected (Ajaelo et al., 1998; Aramendi and Manzanares, 2010; Ben-Abraham et al., 2003; Box et al., 1997; Braback and Humble, 2001; Brvar et al., 2004; Budisavljevic et al., 2003; Chen and Viola, 1991; Cherney et al., 2002; Claffey, 2011; Farah and Farah, 2008; Ghatol and Kazory, 2012; Gomez-Balaguer et al., 2000; Holden and Jackson, 1996; Holmes et al., 1999; Kalantar-Zadeh et al., 2006; Karlovsek et al., 2005; Magee et al., 1998; Matthai et al., 1996; Maxwell et al., 1993; Nuvials et al., 1997; Parr et al., 1997; Rosenson et al., 2007; Satchell and Connaughton, 1994; Thakkar et al., 2017; Traub et al., 2002; Watson et al., 1997; Wilkins, 1996). The absence of other drugs that may induce hyponatraemia supports the contribution of phenethylamines to the appearance of this condition.

Table 3. Clinical cases involving hyponatraemia associated with the abuse of phenethylamines.

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
Methamphetamine	Unknown	Female	19	Unknown	SIADH; Hyponatraemia	Unknown	Unknown	(Salathe et al., 2018)
MDMA	Unknown	Female	20	A quarter tablet	Seizures; SIADH; Hyponatraemia; Cerebral oedema; Pulmonary oedema; Respiratory failure; ARDS	112 mmol/L	Recovery	(Thakkar et al., 2017)
Molly (pure form of MDMA); Cannabinoids; Benzodiazepines; “Purple drank” (promethazine and codeine)	Unknown	Male	24	Unknown	Seizure; Acute kidney injury; Respiratory failure; Tachycardia; Multiorgan failure	137 mmol/L (day one); 129 mmol/L (day three)	Recovery	(Vakde et al., 2014)
MDMA	Unknown	Female	20	Two tablets (273 mg each)	Hyperthermia; Mydriasis; Lactic acidosis; Parenchymal oedema; Hyponatraemia; Cerebral oedema	123 mmol/L	Recovery	(Ghatol and Kazory, 2012)
MDMA BZP	Unknown	Male	23	Two tablets	Acute kidney injury; Hyponatraemia; Cerebral oedema	Unknown	Death	(Berney-Meyer et al., 2012)

11-Hydroxy-THC, 11-Hydroxy- Δ 9-tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ 9-tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
Ethcathinone; Methylone	Unknown	Female (60 Kg)	22	Unknown	Tonicoclonic seizures (20s each); Hyponatraemia; Rhabdomyolysis	120 mmol/L	Recovery	(Boulanger-Gobeil et al., 2012)
MDMA	Unknown	Female	26	Unknown	Tachycardia; Pulmonary oedema; Cerebral oedema; Renal failure; Brain dead	126 mmol/L	Death	(Claffey, 2011)
MDMA	Unknown	Female	Unknown	50 mg	Hyponatraemia; Pulmonary oedema; Cerebral oedema; Brain dead	109 mmol/L	Death	(Aramendi and Manzanares, 2010)
MDMA Benzodiazepines	Unknown	Female	19	Unknown	Mydriasis; SIADH; Hypothermia; Hyponatraemia	118 mmol/L	Recovery	(Niemeijer et al., 2009)
MDMA	Unknown	Female	13	Unknown	Stupor; Mydriasis; Tachycardia; SIADH; Hyponatraemia	123 mmol/L	Recovery	(Farah and Farah, 2008)
MDMA	Unknown	Female	19	Unknown	Hyponatraemia; Cerebral oedema	122 mmol/L	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	20	Unknown	Hyponatraemia Cerebral oedema	124 mmol/L	Death	(Rosenson et al., 2007)

11-Hydroxy-THC, 11-Hydroxy- Δ 9-tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ 9-tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA	Unknown	Female	20	Unknown	Hyponatraemia; Respiratory depression; Hypothermia	119 mmol/L	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	16	Unknown	Seizure; Hyponatraemia; Cerebral oedema; Pulmonary oedema	123 mmol/L	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	29	Unknown	Cardiac arrest; Seizure; Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	16	Unknown	Cardiac arrest; Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	14	Unknown	Cardiac arrest; Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)
MDMA	Unknown	Male	18	Unknown	Hyperthermia; Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	18	Unknown	Cardiac arrest; Hyperthermia; Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)
MDMA	Unknown	Female	16	Unknown	Hyponatraemia	Unknown	Death	(Rosenson et al., 2007)

11-Hydroxy-THC, 11-Hydroxy- Δ 9-tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ 9-tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA	Unknown	Female (55 Kg)	20	Several tablets	Tachycardia; Hypothermia; Hyponatraemia	117 mmol/L	Death	(Kalantar-Zadeh et al., 2006)
MDMA	Blood: 3.2 mg/L Urine: 280 mg/L	Male	21	Two tablets	Hypertension; Tachycardia; Metabolic acidosis; Rhabdomyolysis; Renal failure; Cerebral oedema; Pulmonary oedema	Unknown	Death	(Karlovsek et al., 2005)
MDMA Insulin (diabetic individual)	Blood: 9.1 mg/L MDMA; 510.4 mE/L insulin Urine: 13.5 mg/L MDMA	Male	49	Unknown	Hypertension; Cerebral oedema; Pulmonary oedema	Unknown	Death	(Karlovsek et al., 2005)
MDMA MDA Cannabinoids (11-hydroxy-THC and 11-nor-9-carboxy-THC)	Blood: 3.8 mg/L MDMA; 1.6 µg/L 11-hydroxy-THC; 65 µg/L 11-nor-9-carboxy-THC	Female (52 Kg)	19	Four tablets	Pulmonary oedema; General hypoxia; Cardiopulmonary failure; Cerebral oedema	Unknown	Death	(Libiseller et al., 2005)
MDMA	Blood: 0.87 mg/L	Female (65 Kg)	18	5 tablets (60 mg each)	Headache; Mydriasis; Tachycardia; Hyponatraemia	130 mmol/L	Recovery	(Brvar et al., 2004)
MDMA	Unknown	Female	24	Unknown	Coma; Seizures; Tachycardia; Rhabdomyolysis; Lactic acidosis; Hyponatraemia	Unknown	Recovery	(Ben-Abraham et al., 2003)

11-Hydroxy-THC, 11-Hydroxy- Δ^9 -tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ^9 -tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA	Unknown	Female	23	Unknown	Coma; Seizures; Rhabdomyolysis; Metabolic acidosis; Hyponatraemia; Coma	Unknown	Recovery	(Ben-Abraham et al., 2003)
MDMA	Unknown	Male	21	Unknown	Seizures; Hyperthermia; Rhabdomyolysis; Lactic acidosis; Hyponatraemia	Unknown	Recovery	(Ben-Abraham et al., 2003)
MDMA	Unknown	Female (65 Kg)	18	One tablet	Mydriasis; Hyponatraemia	124 mmol/L	Recovery	(Budisavljevic et al., 2003)
MDMA	Unknown	Female (50 Kg)	20	One tablet	Seizure; Hyponatraemia; Cerebral oedema	112 mmol/L	Unknown	(Cherney et al., 2002)
MDMA	Unknown	Female	19	One tablet	Seizure; Hyponatraemia; Cerebral oedema	121 mmol/L	Recovery	(Traub et al., 2002)
MDMA	Unknown	Female	20	One tablet	SIADH; Hyponatraemia	Unknown	Death	(Braback and Humble, 2001)
MDMA	Unknown	Female	21	One tablet	Rhabdomyolysis; Hyponatraemia; Cerebral oedema	126 mmol/L	Death	(2001)

11-Hydroxy-THC, 11-Hydroxy- Δ 9-tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ 9-tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA A2 (BZP)	Unknown	Female	23	Unknown	Bradycardia; Hypertension; Seizures; Hyponatraemia; Cerebral oedema	115 mmol/L	Death	(Balmelli et al., 2001)
MDMA	Unknown	Female	18	Three tablets	Mydriasis; Tachycardia Hyponatraemia; SIADH	120 mmol/L	Recovery	(Gomez-Balaguer et al., 2000)
MDMA	Unknown	Female	27		Hyponatraemia; Cerebral oedema			(O'Connor et al., 1999)
MDMA	Unknown	Female	26	One tablet	Seizures; Pulmonary oedema; Hyponatraemia	101 mmol/L	Recovery	(Holmes et al., 1999)
MDMA	Unknown	Female	17	One tablet	Seizure; Stupor; Mydriasis; Hyponatraemia; Cerebral oedema	124 mmol/L	Recovery	(Magee et al., 1998)
MDMA	Unknown	Female	19	Unknown	Mydriasis; Stupor; SIADH; Hyponatraemia; Cerebral oedema	115 mmol/L	Recovery	(Ajaelo et al., 1998)

11-Hydroxy-THC, 11-Hydroxy- Δ^9 -tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ^9 -tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA	Unknown	Female	20	One and a half tablets	Hyponatraemia	119 mmol/L	Recovery	(Nuvials et al., 1997)
MDMA	Unknown	Female	15	Unknown	Sinus; Tachycardia; Respiratory arrest; Coma; Hyponatraemia; Pulmonary oedema; Cerebral oedema; Brain death	125 mmol/L	Death	(Parr et al., 1997)
MDMA	Unknown	Female	30	One tablet	Seizures; Hypothermia; Hyponatraemia; Pyrexia; Rhabdomyolysis; Oliguria	117 mmol/L	Unknown	(Box et al., 1997)
MDMA	Unknown	Female	24	Unknown	Stupor; SIADH; Hyponatraemia	113 mmol/L	Recovery	(Watson et al., 1997)
MDMA	Unknown	Female	15	Unknown	Hyponatraemia; Cerebral oedema	119 mmol/L	Recovery	(Matthai et al., 1996)
MDMA	Unknown	Female	16	Unknown	Hyponatraemia; Cerebral oedema	112 mmol/L	Recovery	(Matthai et al., 1996)
MDA	Unknown	Female	16	Unknown	Hyponatraemia; Cerebral oedema	112 mmol/L	Recovery	(Wilkins, 1996)
MDMA	Unknown	Female	15	Unknown	Hyponatraemia; Cerebral oedema	119 mmol/L	Recovery	(Wilkins, 1996)

11-Hydroxy-THC, 11-Hydroxy- Δ^9 -tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ^9 -tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Table 3 Clinical cases involving hyponatraemia associated with the abuse of phenethylamines (cont.)

Substances	Concentration	Gender (weight)	Age	Amount	Effects	Plasma sodium concentration	Outcome	Reference
MDMA	Unknown	Female	20	One tablet	Mydriasis; Hyponatraemia; Cerebral oedema	112 mmol/L	Recovery	(Holden and Jackson, 1996)
MDMA	Unknown	Female	24	Unknown	Hyponatraemia	120 mmol/L	Recovery	(Satchell and Connaughton, 1994)
MDMA; MDA	Unknown	Female	17	One capsule	Mydriasis; Hyponatraemia	118 mmol/L	Recovery	(Maxwell et al., 1993)
MDMA; MDA	Unknown	Female	17	One and a half tablets	Seizures; Hyponatraemia	130 mmol/L	Recovery	(Maxwell et al., 1993)
MDMA	Unknown	Female	23	Half a tablet	Mydriasis; Hyponatraemia	123 mmol/L	Recovery	(Chen and Viola, 1991)

11-Hydroxy-THC, 11-Hydroxy- Δ^9 -tetrahydrocannabinol. *11-Nor-9-carboxy-THC*, Nor-9-carboxy- Δ^9 -tetrahydrocannabinol. *ARDS*, Acute respiratory distress syndrome. *BZP*, N-Benzylpiperazine. *MDA*, 3,4-Methylenedioxyamphetamine. *MDMA*, 3,4-Methylenedioxymethamphetamine. *SIADH*, Syndrome of inappropriate secretion of antidiuretic hormone.

Typically, the characteristics of drug-related hyponatraemia are similar to those described for SIADH, however, in the later case, cerebral oedema does not occur (Ajalo et al., 1998; Brvar et al., 2004; Henry et al., 1998; Holden and Jackson, 1996; Parr et al., 1997). When hyponatraemia is triggered by phenethylamines, oedema may occur not only due to water retention mediated by ADH, but also due to over-hydration. The excessive ingestion of water by abusers to prevent hyperthermia induced by phenethylamines, and the increased loss of sodium in sweat as a consequence of the rise in body temperature, often lead to an increased risk of developing hyponatraemia and consequent oedema (Traub et al., 2002).

1.3.1 Mechanisms involved in phenethylamine-induced hyponatraemia

Among the mechanisms contributing to phenethylamine-induced hyponatraemia, metabolic activation, genetic variability, serotonergic regulation of neurohormonal secretion, hyperthermia, gender susceptibility and hormonal influence might be considered.

1.3.1.1 Metabolic activation

The rise in the number of reported cases of hyponatraemia related to phenethylamine abuse, mostly related to consumption of MDMA, has led some authors to try to establish a possible relationship between the concentration of plasma MDMA and the concentration of ADH. When low doses of MDMA (40 mg) were administered to healthy, hydrated male individuals, a strong and acute increase of plasma ADH occurred within 1 to 2 h after the oral administration, which was accompanied by a slight decrease of sodium plasma levels and an increase of urine osmolality (Henry et al., 1998). This study demonstrated an exaggerated release of ADH in treated individuals, in comparison with controls, and also evidenced that there was no direct correlation between the variation of plasma levels of MDMA and ADH (Henry et al., 1998). This was later related to the possibility that the release of ADH is induced by MDMA metabolites (Fallon et al., 2002; Forsling et al., 2001; Forsling et al., 2002). Hence, when Forsling et al (2001) revised the results published by Henry et al. (1998), providing a full description of the relations between concentrations of ADH and MDMA and between ADH and the metabolite 3,4-methylenedioxyamphetamine (MDA), a significant negative correlation between the concentrations of MDMA and ADH was further established (Forsling et al., 2001). In

contrast, no statistically significant correlation was observed between the concentrations of MDA and ADH, 1 h after the administration of MDMA, supporting the possibility that MDA is not the responsible for the stimulation of ADH release (Forsling et al., 2001). Briefly, MDMA is mainly metabolized in the liver by CYP2D6 and its biotransformation mainly occurs through two pathways: O-dealkylation (demethylenation) and N-demethylation (main pathway) into MDA, that also retains biological activity. However, there are other pathways of metabolism, namely deamination and conjugation with methyl, glucuronide or sulphate, which are also shared by MDA. O-Demethylenation of MDMA and MDA originate two catechol metabolites, N-methyl- α -methyldopamine (HHMA) and α -methyldopamine (HHA). Both catechols are afterwards methylated by catechol-O-methyltransferase (COMT) (Carvalho et al., 2012).

Following these findings on the correlation of ADH and MDMA plasma concentrations, a study was carried out to verify the role of MDMA metabolic activation in the release of ADH and consequent SIADH (Forsling et al., 2002). For such purpose, an *in vitro* assay was performed in isolated rat hypothalamus with MDMA and its major metabolites MDA, HHMA, HHA, and the methylated catechols 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA). All tested substances affected the secretion of ADH but HMMA and HHA had greater ability to induce secretion of ADH than MDMA. As the study was performed in isolated rat hypothalamus, the results show that MDMA and its metabolites act directly on the hypothalamus, excluding any contribution of the systemic effects induced by the drug for the release of ADH (Forsling et al., 2002).

In this regard, it is possible to anticipate the occurrence of effects similar to those of MDMA for cathinones exhibiting similar metabolic bioactivation, specifically those displaying the methylenedioxy moiety such as methyldone and MDPV. Based on the structural and metabolic similarity, and knowing that clinical intoxications involving the abuse of cathinones were also associated with the development of hyponatraemia, it is expected that cathinone metabolites may also act directly to induce release of ADH.

1.3.1.2 Genetic variability

The importance of metabolism in the induction of ADH secretion by phenethylamines was established, thus reflecting the importance of differences in individual metabolic capacity. Since the metabolism of MDMA is mainly mediated by the CYP2D6 isoenzyme

of cytochrome P450, genetic polymorphisms of this enzyme may heavily influence the individual susceptibility to the release of ADH (Bertilsson et al., 2002; Lin et al., 1997). Moreover, the formation of HMMA is dependent on COMT, so the individual susceptibility can also be affected by polymorphisms of this transferase (Aitchison et al., 2012; Forsling et al., 2002; Pardo-Lozano et al., 2012). In the human population, the genetic polymorphism of COMT presents three variants and, therefore, there are individuals with low, intermediate and high enzymatic activity (Mannisto and Kaakkola, 1999). In individuals who metabolize the drug more slowly, it remains for a longer period in the organism, consequently increasing the risk of acute toxicity (Bora et al., 2016). In addition, as MDMA metabolism is not linear, an insignificant increase in the drug dose may lead to a great increase in MDMA blood and brain concentrations (Thakkar et al., 2017). On the other hand, since MDMA is bioactivated into metabolites that more profoundly disturb ADH secretion, rapid and ultra-rapid drug metabolisers may also be at increased risk. These important variations in the process of metabolism/excretion might explain why users taking the same dose of MDMA do not experience the same adverse effects (de la Torre et al., 2004; Forsling et al., 2002).

1.3.1.3 Serotonergic regulation of neurohormonal secretion

The mechanisms responsible for increased ADH secretion subsequent to phenethylamines administration are not yet completely understood. However, since the action of phenethylamines, in particularly of MDMA, results in over-stimulation of the serotonergic system, and the release of ADH is mediated by 5-HT, it is predictable that phenethylamine-induced hyponatraemia is due to changes in serotonergic regulation of neurohormonal secretion of ADH (Berney-Meyer et al., 2012; Bora et al., 2016; Traub et al., 2002). In fact, in rats, MDMA has a greater affinity to SERT than DAT and NAT (Rothman and Baumann, 2003). Although in humans MDMA has a higher affinity to NAT than for SERT or DAT (Han and Gu, 2006), in the human brain, the drug has a greater capacity for releasing 5-HT than DA and NA (Verrico et al., 2007).

In a previous study whose purpose was to investigate whether 5-HT is involved in the regulation of ADH release, the hormone was measured in the plasma of male Wistar rats administered with drugs capable of increasing 5-HT neurotransmission and with 5-HT depleting drugs; 5-HT in the forebrain was also measured (Iovino and Steardo, 1985). It was observed that drugs inducing increase of 5-HT were able to induce increases in

plasma ADH levels in normohydrated animals. However, this effect was prevented by a 5-HT depleting agent. In addition, treatment with 5-HT depleting drugs led to a decrease in the content of 5-HT in the forebrain, indicating that 5-HT neurotransmission is required for the hormonal response to osmotic stimuli (Iovino and Steardo, 1985). Serotonin was further implicated in the physiological release of ADH from neurohypophysis, in an *in vivo* study conducted in male Sprague-Dawley rats (Pergola et al., 1993). In this study, the i.c.v. administration of 2.5 µg 5-HT led to a rapid increase of plasma ADH, with a maximal response observed 5 min after the administration (Pergola et al., 1993). This effect was brief since 15 min after administration ADH levels returned to baseline. Noteworthy, the administration of 5-HT_{2/1C} antagonists blocked ADH release, further supporting the role of 5-HT in the secretion of ADH (Pergola et al., 1993). Moreover, in a retrospective study of the cases admitted in 1996 in the Department of Health Care of the Elderly, Nottingham, UK, Bouman and collaborators (1998) observed that from the thirty-two individuals that received treatment with selective serotonin reuptake inhibitors (SSRIs), four developed symptomatic hyponatraemia due to SIADH, while another four developed asymptomatic hyponatraemia with laboratorial confirmation (Bouman et al., 1998). Jorgensen and colleagues (2003b) also investigated the involvement of 5-HT receptors in serotonergic regulation of ADH secretion, concluding that 5-HT-induced ADH secretion is mainly mediated by 5-HT_{2C}, 5-HT₄ and 5-HT₇ receptors (Jorgensen et al., 2003b). In an earlier work, Bagdy and colleagues (1992) investigated the effects of the i.v. administration of 5-HT agonists with different structures and receptor binding profiles to conscious male Sprague-Dawley rats on the ADH plasma levels, blood pressure and plasma renin activity (through the amount of angiotensin I generated). The authors observed that neither a selective, high affinity 5-HT₂ receptor agonist, nor a 5-HT_{1A} agonist led to changes in plasma ADH. On the other hand, 5-HT_{1C} agonism triggered significant increases in plasma ADH concentrations, suggesting that it mediates ADH secretion (Bagdy et al., 1992).

1.3.1.4 Hyperthermia

One of the most well-known effects of serotonergic drugs is the alteration of body temperature (Carvalho et al., 2012; Morton, 2005). The increase in body temperature occurs due to an increase in heat production, through the stimulation of metabolic activity of the CNS (Carvalho et al., 2012). The incidence of the hyperthermic effect varies

depending on the phenethylamine administered, being especially frequent after the use of MDMA and methamphetamine (Green et al., 2004; Henry et al., 1992; Imanishi et al., 1997; Jaehne et al., 2007; Kamijo et al., 2002; Kojima et al., 1984; Uemura et al., 2003). After i.p. administration of 12.5 mg/Kg MDMA to male Dark Agouti rats placed at a room temperature (20–22 °C), a significant increase of approximately 1.5 °C in rectal temperature of the animals was observed. This increase was maintained for at least 3 h, but the peak of the hyperthermic response occurred between 40 and 60 min after drug administration (Mechan et al., 2002). Also, several synthetic cathinones have shown ability to induce a thermogenic response (Fantegrossi et al., 2013; Shortall et al., 2013; Tariq et al., 1989). Accordingly, 10 mg/Kg MDPV induced an increase of approximately 2 and 1.5 °C in body temperature of adult male NIH Swiss mice kept at a room temperature of 28 and 20 °C, respectively (Fantegrossi et al., 2013). In addition, Tariq et al. (1989) demonstrated significant dose-dependent increases in rectal temperature of male Wistar rats, after oral administration of cathinone or amphetamine at 5, 10 or 15 mg/Kg.

The origin of hyperthermia is diffuse and may be related to the action of these drugs in the CNS and peripheral nervous system (Parrott, 2012). Classically, the thermogenic response of MDMA is attributed to the stimulation of the serotonergic system by the drug (Shankaran and Gudelsky, 1999) and, although the serotonergic system is the main target of MDMA in the brain, it has been argued that hyperthermia mainly results from the ability of 5-HT to induce DA release, rather than a serotonergic direct action (Mills et al., 2004). Indeed, DA involvement in hyperthermic response was confirmed after using a selective D1 receptor antagonist, in Male Dark Agouti rats (Mechan et al., 2002). This antagonist elicited a dose-dependent inhibitory response of MDMA-induced hyperthermia, suggesting that temperature elevation induced by the drug more likely results from the increased release of DA, rather than 5-HT (Mechan et al., 2002). In addition, further evidence suggests that an interaction between the hypothalamic-pituitary-thyroid axis, the sympathetic system and the activity of uncoupling proteins (UCP) may underlie the thermogenic response (Sprague et al., 2003). Accordingly, the contribution of the hypothalamic-pituitary-thyroid axis to the drug-induced hyperthermia was demonstrated by the absence of hyperthermic response after administration of 40 mg/Kg MDMA to rats without hypothalamus or without thyroid. In addition, hyperthermia was strongly associated with thyroxine (T4 hormone) when MDMA-induced hyperthermia was re-established in thyroidectomized animals that were

administered with thyroxine (Sprague et al., 2003). Furthermore, the increased activity of thermogenic tissues, such as brown fat (Rusyniak and Sprague, 2005; Zhao et al., 1997) and muscle (Rusyniak and Sprague, 2005; Rusyniak et al., 2005), and the vasoconstriction induced by the drug (Pedersen and Blessing, 2001) may also contribute to the hyperthermia triggered by MDMA.

In addition, environmental temperature has been shown to influence the hyperthermic effect (Banks et al., 2007; Capela et al., 2009; Carvalho et al., 2002; Green et al., 2005; Malberg and Seiden, 1998; Von Huben et al., 2007). Malberg and Seiden (1998) demonstrated that small changes in room temperature led to significant alterations in core temperature of Holtzman rats that were s.c. administered with 20 or 40 mg/Kg MDMA, as animals kept at 20–22 °C showed hypothermic responses, when compared with control animals; while animals kept at 28–30 °C exhibited hyperthermia. Also, after injecting 5 or 7.5 mg/Kg MDMA to male Wistar rats, high susceptibility to small changes in ambient temperature was observed when rats placed at 22 °C showed hyperthermic response, while those at 17 °C presented hypothermia (Dafters, 1994). Furthermore, in an earlier study aiming to determine metabolic activity, water loss by evaporation and body temperature in male Long-Evans rats, kept at 10, 20 or 30 °C, 1 h after s.c. injection of 30 mg/Kg MDMA, it was observed an increase in metabolic rate for MDMA-treated rats, at 20 and 30 °C. Similarly, animals kept at 20 and 30 °C presented hyperthermia, with increases up to 3.2 °C in core body temperature (Gordon et al., 1991). In addition, water loss was higher in animals administered with the drug, compared to control animals.

In most cases, phenethylamines consumption occurs in hot and crowded environments, which are often associated with prolonged and intense dancing. These facts may further contribute to the acute hyperthermia observed in substance abusers. Consequently, the diaphoresis associated with the physical effort and aggravated by hyperthermia, leads to significant sodium losses (Campbell and Rosner, 2008). Moreover, to prevent the hyperthermic effect, ecstasy users are often advised to ingest copious amounts of liquids (Bora et al., 2016), and polydipsia might be further intensified by the ready availability of fluids at the dance scene and by the xerostomia and thirst, which are common effects of phenethylamines (Campbell and Rosner, 2008). The elevated intake of fluids combined with loss of solutes in sweat, may therefore contribute to the development or aggravation of hyponatraemic effect triggered by these drugs (Bora et al., 2016).

On the other hand, temperature itself might also alter the release of ADH (El-Nouty et al., 1980; Groza et al., 1974). Accordingly, exposure of Holstein cows to heat (35 °C) triggered a rapid increase in plasma ADH levels, while in animals submitted to dehydration (30 h without water) under the same heat conditions a sharp increase in ADH levels and a decrease in urine output were observed (El-Nouty et al., 1980). In rats, hyperthermic stress also elicited a rise in plasma ADH concentration, which was proportional to its intensity (Groza et al., 1974). The parallel histochemical studies using methods for demonstrating RNA, protein and the neurosecretory material in the supraoptic nucleus, showed the synthesis of the hormone in correlation with the plasma concentration of ADH (Groza et al., 1974). Moreover, in dogs, an increase in plasma ADH from $2.4 \pm 0.6 \mu\text{U/mL}$ to $24.0 \pm 4.7 \mu\text{U/mL}$ was observed 10 min after heating of the brain tissue induced by a thermode implanted near the preoptic area, indicating that the increase of temperature in the basal forebrain may be responsible for the activation of the hypothalamo-hypophysial antidiurectic system (Szczepanska-Sadowska, 1974).

1.3.1.5 Gender susceptibility and hormonal influence

Previous reports showed that women are at higher risk of developing drug-induced hyponatraemia, and most cases of death or permanent neurological injury from hyponatraemic encephalopathy were reported in women between 15 and 30 years old who had consumed a single dose of MDMA (Aramendi and Manzanares, 2010; Box et al., 1997; Braback and Humble, 2001; Cherney et al., 2002; Claffey, 2011; Kalantar-Zadeh et al., 2006; Parr et al., 1997; Rosenson et al., 2007; Wilkins, 1996). In a study using a randomized placebo-controlled crossover design, plasma and urine osmolalities, as well as the levels of ADH and of copeptin were measured in eight healthy men and eight healthy women, at baseline and after administration of 125 mg of MDMA. Copeptin is an ADH secretion marker derived from the C-terminus of the ADH precursor, which is produced at an equal proportion relative to ADH. It was observed that MDMA increased plasma copeptin in women but not in men. A similar trend was observed for ADH, but this difference was not statistically significant and no significant differences were also observed in sodium plasma levels (Simmler et al., 2011). In a retrospective review of the cases related to the abuse of this drug in California that aimed to describe the clinical characteristics of patients with ecstasy-associated hyponatraemia, the authors concluded that females were at increased risk of developing both hyponatraemia and coma

(Rosenson et al., 2007). Furthermore, the fact that most of the reported manifestations of SIADH secondary to drug ingestion occurred in women lead to the interrogation of which factors regulate gender susceptibility at the onset of SIADH.

Several physiological features may be involved in these gender differences, including metabolic interactions and hormonal mechanisms involved in the homeostatic regulation (Rosenson et al., 2007). Accordingly, it has been observed in studies with female rats that the susceptibility to the action of ADH varies during the reproductive cycle (Forsling et al., 1996), and this variation was also observed in women (Claybaugh et al., 2000). One of the mechanisms involved is related with the oestrogen stimulation of ADH secretion. In women, ADH secretion by the pituitary gland seems to be controlled in part by the action of oestrogen (Rosenson et al., 2007), which leads to an increase in ADH secretion by decreasing the osmotic threshold in humans. Accordingly, in a study whose purpose was to determine the effects of oestrogen on the osmotic regulation of ADH in women, the reproductive function (endogenous oestrogen and progesterone) was suppressed (Stachenfeld and Keefe, 2002). Following suppression, the volunteers received either oestrogen (transdermal patch) or a combination of oestrogen and progesterone (vaginal gel), for four days. During the treatment, the ADH and body fluid regulatory responses to an hypertonic stimulus were compared. It was observed that oestrogen led to a decrease of the osmotic threshold for the stimulation and release of ADH and led to an increase of sodium and fluid retention in response to 3% NaCl infusion. When combined, the hormones reduced the ADH increase observed in response to 3% NaCl infusion and led to the activation of RAAS with subsequent water and sodium retention (Stachenfeld and Keefe, 2002). Overall, the authors concluded that fluid retention increased with both hormone treatment, and renal sensitivity to ADH may be lower in the presence of oestrogen due to renal effects on water and sodium excretion. Also, oestrogen has been shown to decrease the antidiuretic response to ADH in rats (Wang et al., 1995) as it inhibits the $\text{Na}^+\text{-K}^+\text{-ATPase}$ ion pump, which is the primary defence against the osmotic shifts caused by severe hyponatraemia. So, this inhibition by oestrogen may impair resolution of cerebral oedema by reducing the capacity to eliminate intracellular sodium (Arieff et al., 1995; Campbell and Rosner, 2008), which may be reflected in the increased frequency of seizures and/or coma in drug-induced hyponatraemic women. Oestrogen may also prompt increased cerebral vasoconstriction in response to ADH, causing cerebral ischemia (Ayus et al., 2008; Campbell and Rosner, 2008). As oestrogen levels are lowest during the menstrual period and highest at ovulation, the differential secretion

of hormones during the menstrual cycle may be linked with a differential susceptibility of drug-induced complications during the different phases of the cycle (Rosenson et al., 2007). A previous study, aimed to determine the factors related with the development of encephalopathy and with its clinical course in individuals with postoperative hyponatraemia, showed that when hyponatraemic encephalopathy develops, menstruant women are about 25 times more likely to die or have permanent brain injury, compared with either men or postmenopausal women (Ayus et al., 1992). Thus, it is possible to conclude that the variation of ADH response related to hormonal gender differences such as fluctuations of ovarian steroids, and variations in glomerular, antidiuretic and natriuretic actions of ADH induced by these fluctuations, may explain the differences in susceptibility to the hyponatraemic effect induced by phenethylamines (Forsling et al., 1996). During the ovulation phase women present higher levels of ADH, which decrease in the menstrual period (Claybaugh et al., 2000). In addition, the ovarian hormones can trigger variations in renal function during the menstrual cycle, influencing the sodium balance through alterations in the renal control of salt and water (de Vries et al., 1972; Rafestin-Oblin et al., 1991). Thus, the cyclic variations in circulating ovarian steroids may be a possible cause for the altered renal responsiveness to ADH (Forsling et al., 1996). Also, the ovarian steroids can affect the water and electrolyte balance through direct action on ingestion of water (Tarttelin and Gorski, 1973) and the increase of body temperature in women during the ovulation may additionally lead to sodium loss in sweat and to the intensification of the water intake (Claybaugh et al., 2000).

Finally, the higher incidence of hyponatraemia in women may be related with decreased muscle mass and lower body weight, as compared to men (Campbell and Rosner, 2008). As half of the total body water is present in skeletal muscle cells, an individual presents higher risk for severe hyponatraemia after a given water load, if he has a very small muscle mass (Cherney et al., 2002). Since the movements of water between the extracellular and intracellular compartments are dictated by the osmotic pressure, if the osmolar concentration within the cells of skeletal muscle is higher than the osmolar concentration of the extracellular compartment, the displacement of water from extracellular to intracellular compartment will occur (Hoorn et al., 2005; Welt et al., 1950) attenuating the impact of hyponatraemia in vital organs such as the brain. In general, since women have a greater amount of adipose tissue and a lower amount of muscle mass, the female gender is more predisposed to the rapid onset of hyponatraemia.

1.3.2 Treatment of hyponatraemia after intoxication with synthetic phenethylamines

The diagnostics of hyponatraemia is based on the clinical evaluation of the volume of the extracellular fluid through laboratory parameters such as plasma osmolality, urine osmolality and/or sodium urine concentration (Hoorn et al., 2005). However, the diagnosis of hyponatraemia induced by phenethylamines requires the history of the administration of these substances and a level of plasma sodium lower than 135 mmol/L. Individuals presenting phenethylamine-induced hyponatraemia usually have levels of serum sodium of 115-125mmol/L (Traub et al., 2002).

The treatment of severe symptomatic hyponatraemia is complex, varying according to the initial degree of hyponatraemia, and generally will require intensive unit care with nephrologic and/or endocrinologic consultation (Campbell and Rosner, 2008; Traub et al., 2002). Normally, interventions are based on the symptoms presented and aim the correction of the sodium and water balance (Claffey, 2011).

Treatment of hyponatraemia in individuals who ingested phenethylamines begins with an evaluation of the airway, breathing and circulation (Traub et al., 2002). In general, fluid restriction is the treatment of choice since evidence suggests that hyponatraemia induced by phenethylamines is caused by SIADH. Therefore, imprudent use of high-volume intravenous fluids in individuals without hypovolaemia should be avoided. If the individual displays profound hypovolaemia, negative free water clearance and high urine osmolality at the time of presentation, 0.9% sodium chloride should be administered (Campbell and Rosner, 2008; Traub et al., 2002). Of note, hypotonic fluids may worsen the outcome. On the other hand, hypertonic saline, i.e. 3% NaCl, should be administered to individuals with acute symptomatic hyponatraemia that present seizures or alteration in mental status due to hyponatraemia (Campbell and Rosner, 2008). Hypertonic saline is used to increase the serum sodium rapidly and its administration should be as fast as possible, since irreversible changes in the brain function may arise in a short period of time (Hoorn et al., 2005; Traub et al., 2002). As soon as the levels of plasma sodium are restored, the acute treatment should be discontinued. At this point, fluid restriction is the treatment of choice for hyponatraemia induced by phenethylamines. Normally, sodium levels return to normal in approximately 24 h, as the effects of the ingested substances decrease (Traub et al., 2002; Verbalis et al., 2007).

Chapter 11 Objectives

Substituted phenethylamines, such as synthetic amphetamines and cathinones, are drugs of abuse with the capacity to interact with the serotonergic system and, therefore cause toxicity. One of the main consequences of amphetamines, in particular of MDMA, is the altered release of ADH, which may lead to SIADH, a condition potentiated by hyperthermia, excessive intake of water, and loss of sodium in sweat, all of them potentially exacerbated in the corresponding consumption settings. In turn, SIADH may end up in hyponatraemia. Generally, the significant decrease of sodium concentration in plasma may trigger important changes in the CNS, including cerebral oedema, which may ultimately culminate in death. Of note, the majority of the reported cases concerning hyponatraemia induced by MDMA indicates a greater susceptibility of the female gender. Recently, a case of hyponatraemia was also documented in a young woman after consumption of synthetic cathinones.

Since synthetic cathinones and amphetamines display similar mechanisms of action, the overall objectives of this work were to demonstrate the ability of the synthetic cathinones 3,4-DMMC and methylone to induce ADH secretion *in vivo*, and elucidate the consequences of the altered secretion of this hormone to the antidiuretic response that could lead to hyponatraemia. In addition, it was intended to investigate the female gender susceptibility to the expression of this effect.

In accordance, six specific aims were outlined:

1. To evaluate alterations in plasma ADH 1 h after the i.p. administration of 3,4-DMMC and methylone to female (20 mg/Kg and 40 mg/Kg) and male (20 mg/Kg) Wistar rats. Based on previous knowledge for MDMA, increased ADH release of synthetic cathinones is expected to be acute, emerging rapidly;
2. To compare differences between ADH release subsequent to the administration of 3,4-DMMC or methylone in male and female Wistar rats, when tested at the same dose (20 mg/Kg) for 1 h. This will help disclose hypothetical female gender susceptibility;
3. To compare differences between ADH release subsequent to the administration of MDMA and 3,4-DMMC or methylone, when tested at the same dose (20 mg/Kg) for 1 h in female Wistar rats, as MDMA is a well-known releaser of ADH;

4. To quantify the hormone in plasma and urine 24 h after 3,4-DMMC and methylene i.p. administration to female Wistar rats. This will enable to evaluate if the drug effect persists over this time period;
5. To measure the acute increase of body temperature 1 h after 3,4-DMMC and methylene i.p. administration to male and female Wistar rats, when tested at the same dose (20 mg/Kg). Hyperthermia might be an additional factor contributing to the serotonin-induced altered release of ADH;
6. Finally, to evaluate the volume of water intake and urine excreted, as well as the existence of cerebral oedema after 3,4-DMMC and methylene i.p. administration to male and female Wistar rats, when tested at the same dose (20 mg/Kg) for 24 h. These parameters are indicators of the antidiuretic response of ADH.

Chapter III Material and Methods

3.1 Chemicals

Methylone and 3,4-DMMC were acquired online at sensearomatics.net on March 2013. MDMA (HCl salt) was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department, at REQUIMTE/Toxicology Laboratory, Biological Sciences Department of Faculty of Pharmacy, University of Porto. Chemical purity and identity of all drugs were verified by nuclear magnetic resonance (NMR) and mass spectrometry (MS) methodologies. Analytical data were consistent with the assigned structures with about 99% purity. All reagents used were of analytical grade and unless stated otherwise were obtained from Sigma Aldrich (Saint Louis, Missouri).

3.2 Animals

The *in vivo* study was performed at the highest standards of ethics after approval by the local Ethical Committee for the Welfare of Experimental Animals (University of Porto-ORBEA; project 251/2018) and by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV). Housing and all experimental procedures were performed by investigators accredited for laboratory animal use and complied with the Portuguese and European legislation (law DL 113/2013, Guide for Animal Care; Directives 86/609/EEC and 2010/63/UE). Adult male and female Wistar rats weighing 200–300g were used. The animals were kept in sterile facilities under controlled temperature ($20 \pm 2^\circ\text{C}$), humidity (40–60%), and lighting (12 h-light/dark cycle) conditions, and fed with sterile standard rat chow and tap water *ad libitum*. On the day of the experiments, the vaginal smears of the female rats were collected and examined unstained under a microscope to identify the estrous cycle (proestrus, estrus, metestrus or diestrus) through the analysis of the proportion among nucleated epithelial cells, non-nucleated cornified cells and leukocytes. All animals used under the same experiment were synchronised.

3.3 Drug challenge

On the day of the experiments, the animals (at least six animals per group; the exceptions are clearly indicated in the Results section) were weighed and placed separately in polyethylene cages with metallic mesh at the top, having access to water and food *ad libitum*. The animals were injected intraperitoneally, at a maximum volume of 0.5 mL/300 g body weight, with saline (0.9% NaCl) or 20 mg/Kg MDMA, 20 or 40 mg/Kg 3,4-DMMC, or 20 or 40 mg/Kg methylone solutions (prepared in saline).

The effects of MDMA, 3,4-DMMC and methylone on the body temperature and release of ADH were studied after 1 h of administration. During the experimental period, animal behaviour was carefully monitored. After the treatment period, anaesthesia was induced by an i.p. injection of a combination of 20 mg/kg xylazine (Rompun® 2 %, Bayer HealthCare, Germany) and 100 mg/kg ketamine (Clorketam® 1000, Vétoquinol, France) and intrarectal temperature was immediately assessed using a digital thermometer. Anaesthesia was maintained through inhalation of isoflurane vapour (IsoVet® 1000 mg/g, B. Braun VetCare, Germany) and, through the use of a catheter, the inferior vena cava was punctured. The first millilitre of venous blood was quickly collected and transferred into an EDTA tube, using an appropriate technique to avoid ADH oscillations attributed to hypovolaemia triggered during collection. The remaining blood was collected into a new EDTA tube and used only for biochemical analysis (these results will not be presented and discussed in the current dissertation).

In the case of animals treated for 24 h, the animals were kept in metabolic cages. Food and water intake were monitored and the 24 h urine collected into tubes kept on ice and added with 200 µL of the non-specific proteases inhibitor phenylmethanesulfonyl fluoride (PMSF; 100 mM). During the experimental period, animal behaviour was carefully monitored. After treatment, the animals were anaesthetised and blood collected as described. The brain was excised, rinsed and weighted and its water content was assessed by weighing the organ before and after incubation at 104 °C, until no weight variations were observed.

The blood and urine samples were centrifuged at 1,600 g and 3,000 g, respectively, for 15 min at 4 °C, and plasma and urine supernatants transferred into microcentrifuge tubes that were stored at -80 °C until further analysis.

3.4 Quantification of antidiuretic hormone (ADH)

Quantification of ADH was performed on plasma (animals treated for 1 h and 24 h) and urine supernatants (animals treated for 24 h) through an enzyme competitive immunoassay, using a commercially available kit (Arg⁸-Vasopressin ELISA kit, Enzo) according to the manufacturer's instructions (Figure 5). Briefly, plasma and urine were subjected to an extraction procedure, in which proteins and lipophilic interferents were removed. The extraction of ADH also enabled to concentrate the samples and obtain more accurate determinations of ADH, as the hormone is present at low endogenous levels. For

this purpose, 200 μL of sample were added with 400 μL of ice cold acetone (Valente & Ribeiro, Sintra, Portugal), vortex mixed and centrifuged at 3,000 g for 20 min at 4 $^{\circ}\text{C}$. The supernatant was transferred into a new tube and added with 1,400 μL of ice cold petroleum ether (Pronalab, City of Mexico, Mexico), with subsequent centrifugation at 3,000 g for 10 min at 4 $^{\circ}\text{C}$. The top organic layer was discarded, and the remaining aqueous layer carefully transferred into a glass tube. The samples were dried under nitrogen gas and the residue reconstituted with 120 μL of assay buffer (consisting of phosphate buffered saline, bovine serum albumin and detergent).

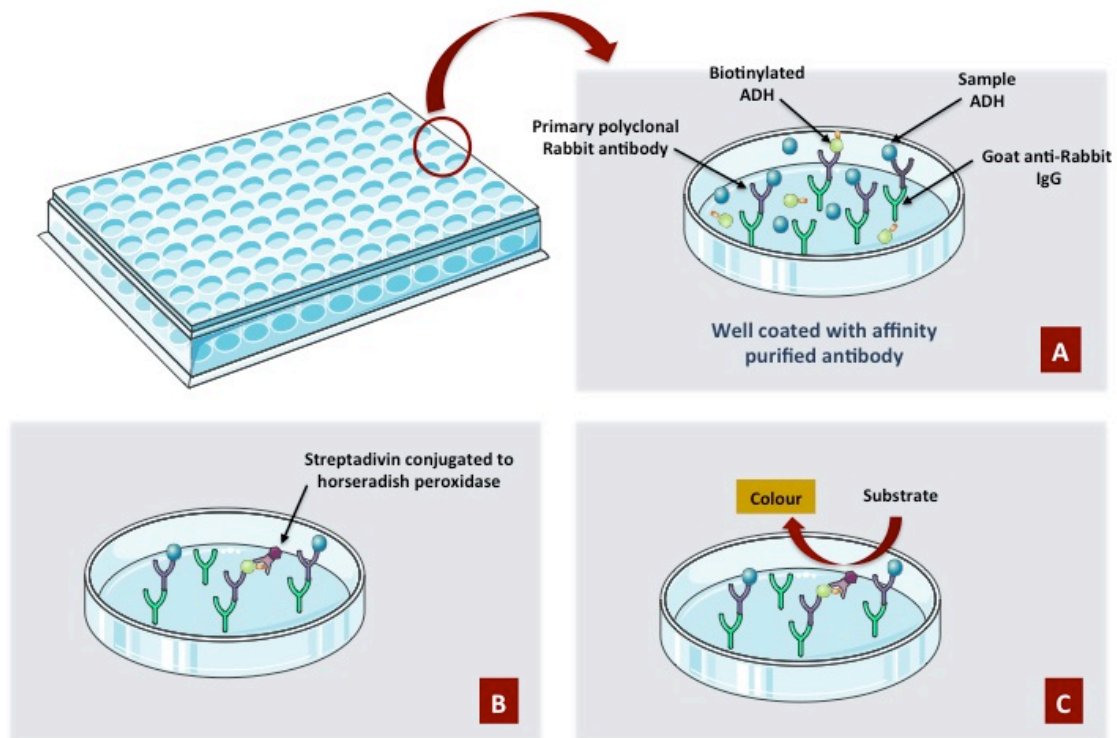


Figure 5. Antidiuretic hormone (ADH) quantification method. A polyclonal antibody specific for ADH binds competitively to the ADH present in the standards and samples or to the ADH associated with a biotin molecules. The complexes formed are fixed in the wells coated with anti-IgG antibodies (A). The wells are then washed for removal of unbound ADH and biotinylated ADH conjugates. Streptavidin conjugated to horseradish peroxidase (HRP) is added and binds the biotinylated ADH (B). The unbound HRP conjugate is removed and, after addition of substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine, an HRP-catalysed colorimetric reaction generates a yellow colour in the solution (C). Thus, the intensity of the colour formed is inversely proportional to the amount of ADH present in the samples or in the standards.

Then, for the quantification of ADH, 100 μL of assay buffer (blank and No Specific Binding, NSB, wells), standard (4.1–1000 pg/mL ADH) or sample were added

to a 96-well microtiter plate coated with goat antibody specific to rabbit IgG, followed by addition of 50 μ L of the blue ADH-biotin conjugate solution and of 50 μ L of the yellow ADH rabbit polyclonal antibody (except in the NSB well, which was added with 50 μ L of assay buffer instead). At this point, all wells were green in colour, except the NSB well, which was blue. The plate was sealed, gently mixed, and incubated at 4 $^{\circ}$ C for 18–24 h. After incubation, each well was washed four times with 200 μ L of wash solution (tris buffered saline containing detergents) and the plate carefully dried. Then, 200 μ L of the streptavidin conjugated to horseradish peroxidase (SA-HRP) solution were added into each well. The plate was sealed and incubated at room temperature on a plate shaker, for 30 min. The wells were again emptied and washed four times with 200 μ L of wash solution, and the plate carefully dried before addition of 200 μ L of the 3,3',5,5'-tetramethylbenzidine substrate solution (containing hydrogen peroxide). The plate was sealed and incubated at room temperature on a plate shaker, for 30 min. Then, 100 μ L of 1 M HCl were added into each well to stop the reaction, and the plate immediately read at 450 nm. This experimental procedure for the quantification of ADH is summarized in Table 4.

Table 4. Summary of the experimental procedure for antidiuretic hormone (ADH) quantification.

REAGENT	VOLUME ADDED PER WELL (μ L)			
	Blank	NSB	Standards	Samples
Assay buffer	100	150	–	–
Standards (4.1–1000 pg/mL ADH)	–	–	100	–
Samples (plasma or urine)	–	–	–	100
ADH-biotin conjugate	50	50	50	50
ADH rabbit polyclonal antibody	50	–	50	50
18–24 h-incubation at 4 $^{\circ}$ C				
Wash Buffer	200 (x4)	200 (x4)	200 (x4)	200 (x4)
SA-HRP	200	200	200	200
30 min-incubation at RT, shaking				
Wash Buffer	200 (x4)	200 (x4)	200 (x4)	200 (x4)
Substrate	200	200	200	200
30 min-incubation at RT, shaking				
Stop solution	100	100	100	100
Read at 450 nm				

ADH, Antidiuretic hormone. *RT*, Room temperature. *SA-HRP*, Streptavidin conjugated to horseradish peroxidase solution.

The percentage of binding of biotin-ADH to the anti-ADH antibody was calculated as follows: % bound = (Net sample or standard OD / Net blank OD) x 100.

The ADH binding data of standards were fitted to a logistic calibration curve of 4 parameters (Figure 6), which was performed for every determination, and the ADH concentration of samples (pg ADH/ μ L plasma or urine) calculated by interpolation.

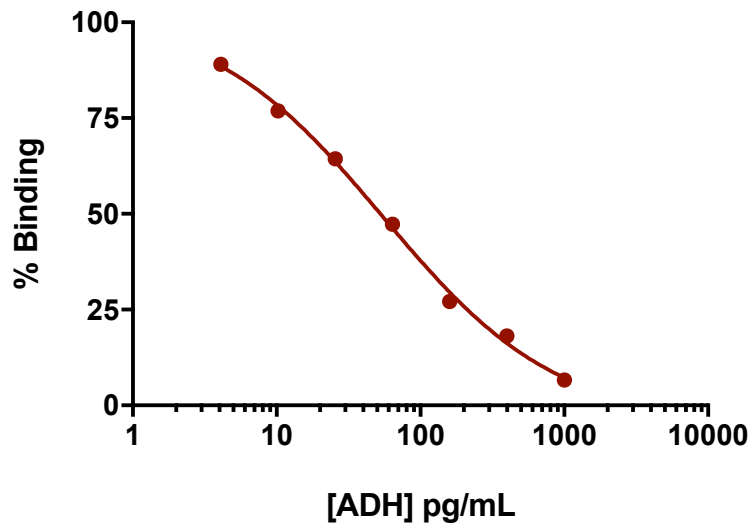


Figure 6. Representative calibration curve for the quantification of antidiuretic hormone (ADH).

3.5 Statistics

All data obtained in the present study were presented as mean \pm standard error of the mean (SEM). Normality was assessed using the Kolmogorov-Smirnov test. Statistical comparisons between groups were performed by Kruskal-Wallis test followed by uncorrected Dunn's test, and differences considered significant at $p < 0.05$. All statistical calculations were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

Chapter IV

Results

4.1 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-dimethylmethcathinone (3,4-DMMC), and methylone trigger an acute increase of antidiuretic hormone (ADH) plasma levels in female Wistar rats, an effect still observed after 24 h

Both cathinones tested, i.e. 3,4-DMMC and methylone, led to a significant increase in plasma levels of ADH, 1 h after their administration to female rats ($p < 0.01$, vs. control), as shown in Figure 7. The mean plasma ADH concentration was 28.01 ± 5.09 pg/ μ L for the control animals, injected with 0.9% NaCl, while in animals injected with 20 mg/Kg of 3,4-DMMC or methylone the mean plasma ADH concentrations were 367.6 ± 90.69 pg/ μ L and 285.0 ± 84.41 pg/ μ L, respectively. These values were close to that observed for the reference drug, i.e. MDMA, at the same dose (349.0 ± 59.48 pg/ μ L), thus, no statistical differences were observed between MDMA and methylone or 3,4-DMMC. The observed increases in plasma ADH seem to be dose-independent, since at 40 mg/Kg the values obtained for 3,4-DMMC and methylone were 296.1 ± 45.60 pg/ μ L and 135.5 ± 26.92 pg/ μ L, respectively. However, at this higher dose only two animals were tested with methylone as during the preliminary assays the animal discomfort was too severe; consequently, this dose was discarded from the subsequent experiments.

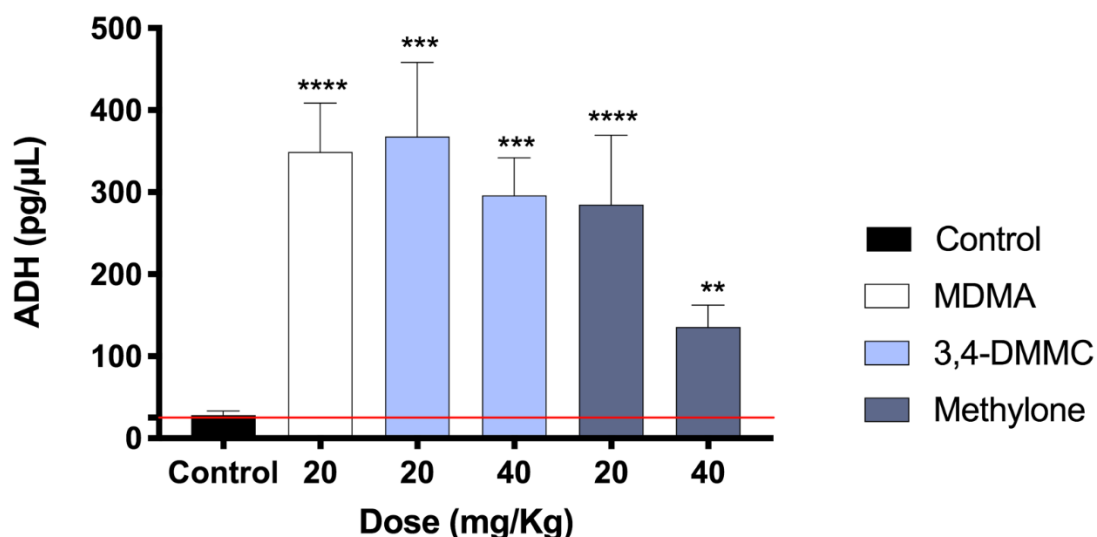


Figure 7. Effect of 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 or 40 mg/Kg on the plasma concentration of ADH in female Wistar rats, 1 h after i.p. drug administration. Six animals were tested per treatment, except for methylone at 40 mg/Kg (two animals). Results are presented as mean \pm standard error of mean. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, vs. control.

Increased ADH plasma levels were still observed 24 h after administration of 20 mg/Kg 3,4-DMMC and methylone, although to a lesser extent as compared with the 1 h-administration period (Figure 8). Accordingly, animals injected with 3,4-DMMC and methylone at 20 mg/Kg presented values of 77.59 ± 13.4 pg/ μ L and 107.9 ± 19.73 pg/ μ L, respectively, which were significantly higher ($p < 0.01$) than that of controls (17.82 ± 2.1 pg/ μ L). Nevertheless, ADH concentrations achieved for 3,4-DMMC ($p < 0.01$, vs. 1 h) and methylone ($p < 0.05$, vs. 1 h) at 20 mg/Kg after 24 h, were significantly lower than those observed in animals treated for 1 h, at the same dose. Due to the animal discomfort noted at the higher 40 mg/Kg dose of the cathinones and also at the 20 mg/Kg dose of MDMA, these drug treatments were excluded from the 24 h experiments.

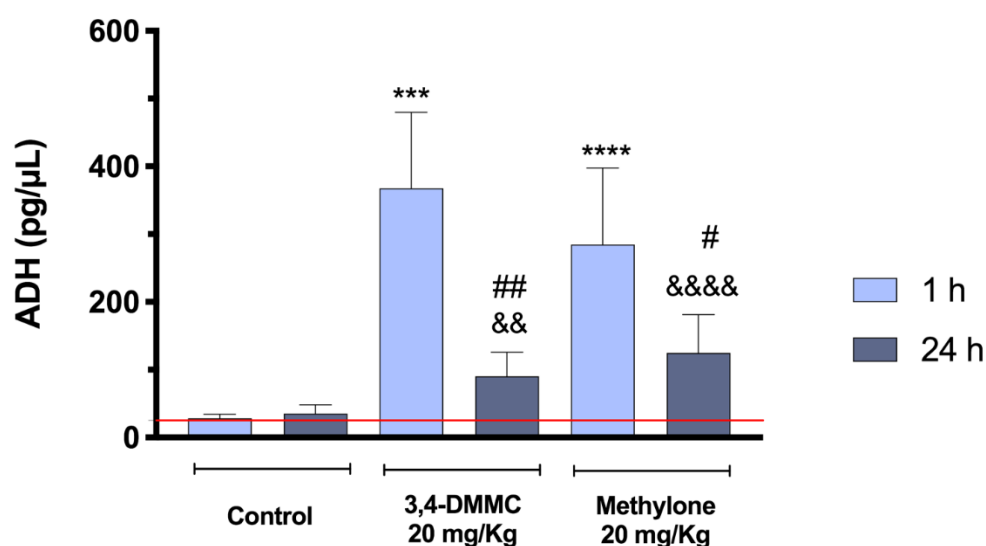


Figure 8. Effect of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 mg/Kg on the plasma concentration of ADH in female Wistar rats, 1 h or 24 h after i.p. drug administration. Six animals were tested per treatment. Results are presented as mean \pm standard error of mean. *** $p < 0.001$; **** $p < 0.0001$, vs. control at 1 h. && $p < 0.01$; &&&& $p < 0.0001$, vs. control at 24 h. # $p < 0.05$; ## $p < 0.01$, vs. the same drug at 1 h.

4.2 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone trigger an increase of antidiuretic hormone (ADH) plasma levels in male Wistar rats less exuberant than that observed for female rats

The effect of all test substances on ADH plasma levels seems to be more evident in females (Figure 9). Nevertheless, no statistical differences were observed between males

and females, when tested with the same substance at the same dose – but a high variability was noted in these data, as shown by the relatively high SEM values.

In males, the cathinones tested, i.e. 3,4-DMMC and methylone, led to a significant increase in plasma levels of ADH, 1 h after their administration ($p < 0.05$, vs. control). For control males the mean plasma ADH concentration was 26.35 ± 5.41 pg/ μ L, while in males treated with 20 mg/Kg of 3,4-DMMC or methylone the mean plasma ADH concentrations were 290.2 ± 142.9 pg/ μ L and 149.2 ± 49.45 pg/ μ L, respectively. Furthermore, the value obtained for methylone is close to that observed for the reference drug, i.e. MDMA, at the same dose (167.5 pg/ μ L), although only one male animal was dosed with MDMA. The same does not apply to the ADH value obtained after the administration of 20 mg/Kg 3,4-DMMC, which was slightly higher than that obtained for the same MDMA dose (the limited sample size of MDMA precluded statistical comparisons).

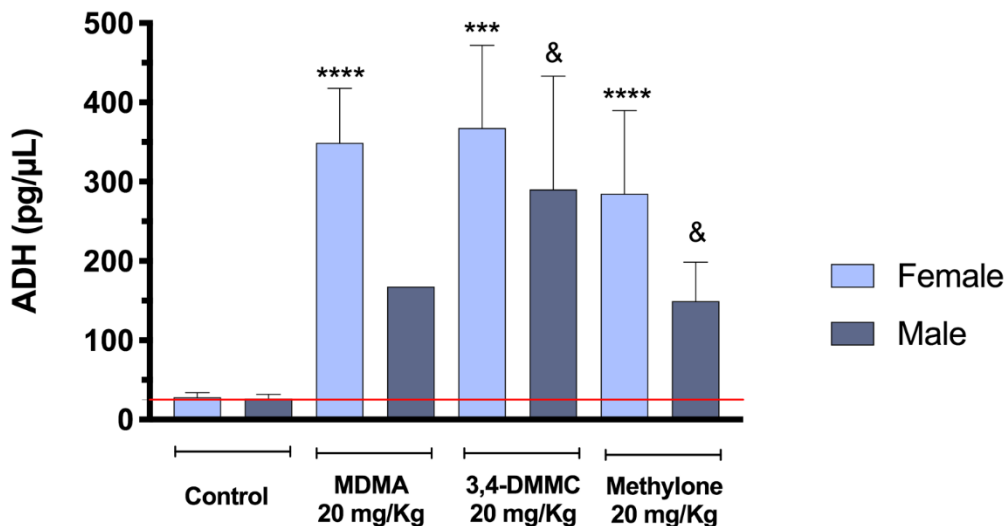


Figure 9. Effect of 3,4-methylenedioxymethamphetamine (MDMA), 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 mg/Kg on the plasma concentration of ADH in female and male Wistar rats, 1 h after i.p. drug administration. Six female and seven male animals were tested per treatment, except for MDMA (only one male animal was tested). Results are presented as mean \pm standard error of mean. *** $p < 0.001$; **** $p < 0.0001$, vs. female control. & $p < 0.05$, vs. male control.

4.3 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone trigger an increase in levels of antidiuretic hormone (ADH) in urine collected for 24 h

Increased levels of ADH in 24 h-urines of female Wistar rats were observed after the drug treatments ($p < 0.01$, vs. control), as shown in Figure 10. The mean urinary concentration of ADH was 146.8 ± 35.65 pg/ μ L for control animals. In animals injected with 20 mg/Kg of 3,4-DMMC or methylone, the mean concentrations of ADH in urine were 692.5 ± 111.0 pg/ μ L and 408.0 ± 53.08 pg/ μ L, respectively. Compared to methylone, 3,4-DMMC seems to display a greater potency for deregulating ADH secretion ($p < 0.05$), since urinary excretion of ADH during 24 h was more pronounced in animals treated with the latter drug.

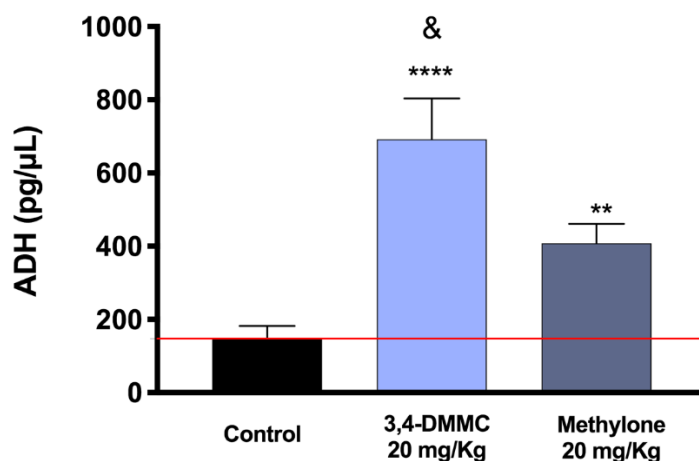


Figure 10. Effect of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 mg/Kg on the ADH concentration of 24-h urine after i.p. drug administration, in female Wistar rats. Six animals were tested per treatment. Values are presented as mean \pm standard error of mean. ** $p < 0.01$; **** $p < 0.0001$, vs. control. & $p < 0.05$, vs. methylone.

4.4 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone increase body temperature in Wistar rats

The effect of the tested phenethylamines on body temperature, in female and male Wistar rats, are represented in Figure 11.

One hour after administration of both cathinones, i.e. 3,4-DMMC and methylone, there was a significant increase of body temperature in female Wistar rats ($p < 0.0001$, vs. control). The mean body temperature was 36.14 ± 0.19 °C for the control animals, while the mean body temperatures of animals treated with 20 mg/Kg of 3,4-DMMC or

methylone were 37.6 ± 0.08 °C and 38.41 ± 0.32 °C, respectively. Since the mean body temperature for animals injected with 3,4-DMMC at 40 mg/Kg was 37.5 ± 0.15 °C, this effect seems to be dose-independent. The value observed for the reference drug, i.e. MDMA, at 20 mg/Kg (40.3 °C) is higher than those obtained for 3,4-DMMC and methylone at the same dose. Nevertheless, as only one animal was tested with MDMA at 20 mg/Kg, comparisons to this drug were not performed.

In addition, a significant increase of body temperature was also observed in male Wistar rats, 1 h after administration of the two cathinones ($p < 0.01$, vs. control). Accordingly, animals treated with 3,4-DMMC and methylone at 20 mg/Kg presented mean body temperatures of 37.77 ± 0.24 °C and 38.70 ± 0.28 °C, respectively, which were significantly higher than that of controls (36.56 ± 0.17 °C).

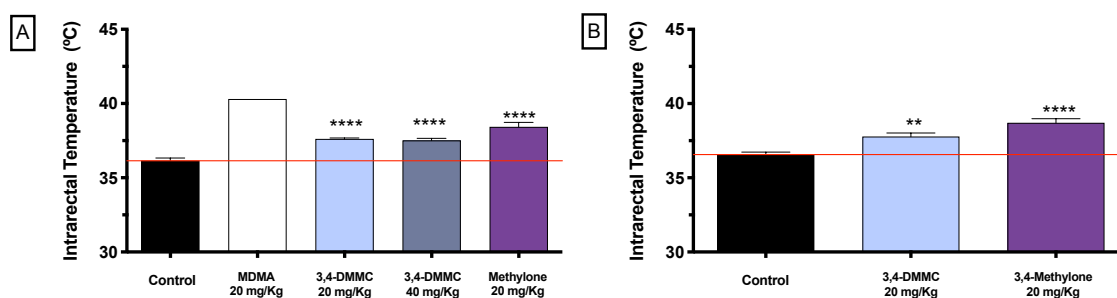


Figure 11. Effect of 3,4-methylenedioxymethamphetamine (MDMA) and methylone at 20 mg/Kg, and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg on body temperature in female (A) or male (B) Wistar rats, 1 h after i.p. drug administration. In females, six animals were tested per treatment, except for MDMA (only one animal was tested). In males, seven animals were tested per treatment. Values are presented as mean \pm standard error of mean. ** $p < 0.01$; **** $p < 0.0001$, vs. control.

4.5 Administration of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone does not trigger a significant increase in the brain water content 24 h after administration

Twenty four hours after cathinones administration, a small increase in brain water content was observed in female Wistar rats, but this tendency is not statistically significant (Figure 12). For animals injected with 3,4-DMMC and methylone at 20 mg/Kg, the mean percentages of water in brain were 9.67 ± 0.56 % and 9.96 ± 0.45 %, respectively, which were only slightly higher than that obtained for the control animals (9.01 ± 0.65 %).

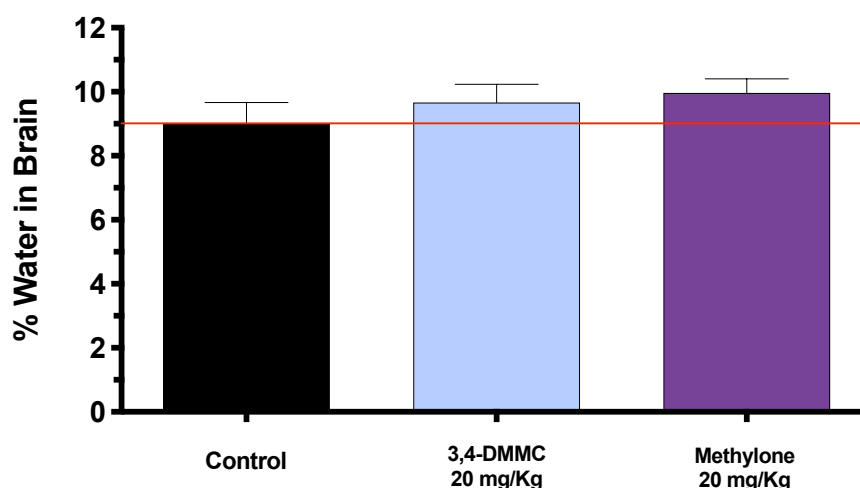


Figure 12. Effect of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 mg/Kg on brain water content in female Wistar rats, 24 h after i.p. drug administration. Six animals were tested per treatment. Values are presented as mean \pm standard error of mean. No statistical differences were observed between the treatment groups, i.e. 3,4-DMMC and methylone, and the control group.

4.6 Administration of 3,4-dimethylmethcathinone (3,4-DMMC) induces an antidiuretic effect in female Wistar rats, but no significant alteration in the water intake pattern was observed

Following 24 h after the administration of both cathinones at 20 mg/Kg, no significant differences in water intake were noted, in female Wistar rats. 3,4-DMMC led to a slight increase (24.50 ± 2.22 mL) but methylone led to a small decrease of water intake (17.40 ± 3.52 mL), when compared to the control animals (21.93 ± 1.99 mL). Furthermore, 3,4-DMMC reduced 24 h-urine excretion (mean volume 4.67 ± 0.21 mL; $p < 0.001$), compared to control animals (8.33 ± 0.76 mL), while no alteration was observed for methylone (8.67 ± 0.21 mL). Finally, with regard to the ratio between the mean water intake and the mean volume of urine excreted, no significant differences were observed between methylone and control groups. In contrast, there was a significant difference between animals treated with 3,4-DMMC and controls ($p < 0.001$). In the case of control animals, the mean ratio was 2.80 ± 0.40 , whereas in animals treated with 20 mg/Kg of 3,4-DMMC or methylone the values obtained were 5.21 ± 0.31 and 2.00 ± 0.38 , respectively. These data are presented in Figure 13.

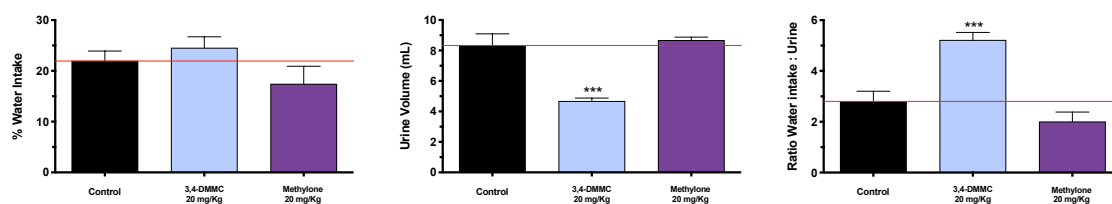


Figure 13. Effect of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 mg/Kg on water intake, urine excretion and ratio water intake to urine excretion within 24 h after i.p. drug administration, in female Wistar rats. Six animals were tested per treatment. Results are presented as mean \pm standard error of mean. *** $p < 0.001$, vs. control.

4.7 Administration of 3,4-dimethylmethcathinone (3,4-DMMC) and methylone does not disturb the ratio between brain and animal weights

After administration of the drugs, no statistically significant differences were observed in the ratio between brain weight and animal weight, as can be seen in Figure 14. In females treated for 1 h, the mean of the ratios for control group was 0.75 ± 0.02 , a value close to those obtained for females injected with the 3,4-DMMC, MDMA and methylone at 20 mg/Kg (0.82 ± 0.05 , 0.72 ± 0.01 and 0.76 ± 0.03 , respectively) and 3,4-DMMC and methylone at 40 mg/Kg (0.75 ± 0.02 and 0.72 , respectively). As previously stated, the discomfort of the animals was severe following administration of methylone at 40 mg/Kg and, for this reason, only one animal was tested with this dose.

In male rats treated for 1 h, also no significant differences were found between control animals (0.48 ± 0.02) and treated animals. In this case, the values obtained for animals treated with 3,4-DMMC and methylone at 20 mg/Kg were 0.48 ± 0.01 and 0.49 ± 0.01 , respectively.

Finally, the same was observed for females treated for 24 h. The mean ratios obtained in animals treated with 3,4-DMMC (0.84 ± 0.04) or methylone (0.89 ± 0.04) were close to that obtained for the control group (0.84 ± 0.03).

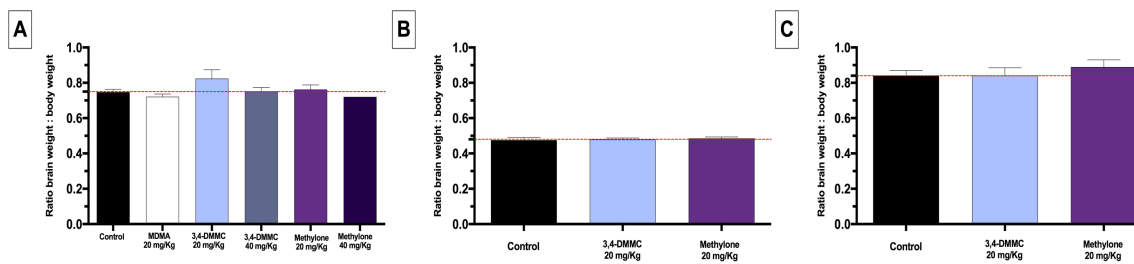


Figure 14. Effect of 3,4-methylenedioxyamphetamine (MDMA) at 20 mg/Kg and 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 or 40 mg/Kg on ratio brain weight to animal weight in female (A) or male (B) Wistar rats, 1 h after i.p. drug administration; and in female Wistar rats, 24 h after i.p. drug administration (C). At least six animals were tested per treatment, except for methylone when tested at 40 mg/Kg (one animal). Results are presented as mean \pm standard error of mean.

Chapter V

DÍSCUSSION

5.1 3,4-Dimethylmethcathinone (3,4-DMMC) and methyldone trigger an acute increase of antidiuretic hormone (ADH) levels in female Wistar rats, an effect still observed for 3,4-DMMC and methyldone after 24 h

Herein we observed that the administration of both cathinones under study, i.e. 3,4-DMMC and methyldone, triggered the increase of ADH secretion in female Wistar rats. This increase occurred both at 1 h (Figure 7) and 24 h (Figure 8) after the administration of the substances, and at all tested doses. As expected, an increase in ADH secretion was also observed after the administration of 20 mg/Kg MDMA, under the same experimental conditions (Figure 7). MDMA increases 5-HT levels in CNS, which in turn leads to an increase of ADH release (Traub et al., 2002). This may result in the development of SIADH, triggering water retention and consequent reduction of plasma sodium levels (Traub et al., 2002). In fact, MDMA has been previously reported to induce ADH secretion both *in vivo* (Baggott et al., 2016; Henry et al., 1998; Silva and Carmo, 2008) and *in vitro* (Forsling et al., 2002). *In vitro* studies showed that 500 nM MDMA and its metabolites MDA (500 nM), HMMA (≥ 10 nM), HMA (≥ 10 nM), 3,4- HHMA (≥ 10 nM), and HHA (≥ 10 nM) triggered ADH secretion on isolated hypothalamus, obtained from male Wistar rats, after 20 min-incubations (Forsling et al., 2002). Although all tested compounds affected ADH release, HMMA and HHA were shown to be more active than MDMA, while HHMA was the less active compound. Thus, that work allowed to establish that the main oxidative metabolites of MDMA might contribute to the secretion of ADH (Forsling et al., 2002). Similar to what is herein reported, a previous study had already demonstrated that the *in vivo* i.p. administration of 20 mg/Kg MDMA to female Wistar rats induced an acute release of ADH to levels up to 236.38 ± 60.94 pM (vs. 53.01 ± 19.60 pM in controls) (Silva and Carmo, 2008). Studies in humans have also shown increased ADH plasma levels after oral administration of 40 mg of MDMA that peaked at 1 to 2 h after administration ($2.46\text{--}9.16$ μM vs. $1.14\text{--}1.88$ μM in controls) (Henry et al., 1998).

The mechanisms associated with MDMA-induced hyponatraemia have been previously investigated in two different experiments (Baggott et al., 2016). In the first, volunteers received 1.5 mg/Kg MDMA or placebo, either alone or in combination with the $\alpha 1$ adrenergic inverse agonist prazosin (a positive control of ADH secretion). In order to minimize the effects of water consumption, individuals were only allowed to ingest up to 473 mL of water per hour. MDMA alone and in combination with prazosin triggered a

significant decrease in serum sodium supporting the postulate that MDMA can increase the risk of development of hyponatraemia. In addition, prazosin led to increased levels of ADH but the same was not observed for MDMA. In the second experiment, volunteers received 1.5 mg/Kg MDMA or placebo, followed by an oral water challenge that took place 1 h later. The water challenge consisted in the ingestion of 20 mL/Kg water within 30 min. No intake of other liquids was allowed up to 6 h of the administration of MDMA or placebo. When compared with placebo, MDMA was associated with a more pronounced decrease in serum sodium, but no significant increase in ADH was again detected. Thus, no relationship was established in that study between the hyponatraemia induced by MDMA and the altered release of ADH. However, it was observed that MDMA lowered serum sodium and overstressed the hyponatraemic response to fluid intake, supporting that MDMA associated to fluids intake displays greater ability to lower serum sodium than the drug or water administration alone (Baggott et al., 2016), which is also related with the increased risk of developing hyponatraemia and cerebral oedema. Accordingly, there are several reports of severe and even fatal intoxications involving MDMA abuse and the development of hyponatraemia, which have been argued to be associated with increased ADH secretion (Ghatol and Kazory, 2012; Rosenson et al., 2007; Thakkar et al., 2017; van Dijken et al., 2013). In a retrospective analysis of MDMA-induced intoxications in patients admitted to the Tel Aviv Sourasky Medical Center from October to December 2001, it was observed that one of the main clinical manifestations was hyponatraemia (Ben-Abraham et al., 2003). One man and two women (out of thirty-two patients) presented coma associated with seizures, after ingestion of one to three MDMA tablets of 150 mg, outcomes that were related to hyponatraemia, with the three individuals presenting plasma sodium levels between 115 and 127 mEq/L (note that the expected plasma sodium concentration is 136 mEq/L) (Ben-Abraham et al., 2003). Other relevant clinical manifestations included high fever, rhabdomyolysis, dehydration and metabolic acidosis accompanied by impaired liver and renal functions.

It is well known that amphetamines and cathinones share several toxicodynamic mechanisms, producing a strong monoaminergic stimulation. It has been described that methylone exhibits potency and selectivity similar to those of MDMA, with a preferential effect on SERT (Baumann et al., 2012). Such serotonergic drugs are often associated with the serotonergic syndrome, that is accompanied by hyperthermia, seizures and hyponatraemia (Batisse et al., 2016; Garrett and Sweeney, 2010; Liechti, 2015; Mugele et al., 2012; Warrick et al., 2012; Zaami et al., 2018). Given the structural similarity

between 3,4-DMMC and methamphetamine, and between methylone and MDMA, it is reasonable to assume that these cathinones induce ADH secretion also via the stimulation of the serotonergic system. In fact, a case was reported in which a woman developed hyponatraemia after consumption of a mixture of methylone and ethcathinone. In this case, the 22-year-old woman presented euphoria, agitation, sweating and intense thirst after administration of the so-called “legal ecstasy”. She ingested 3.5 L of water and, posteriorly, developed several episodes of tonicoclonic seizures. Laboratory tests revealed severe hyponatraemia (plasma sodium was 120 mmol/L), metabolic acidosis, an elevation of creatine phosphokinase (CK), and SIADH. The woman was diagnosed with acute “ecstasy-like intoxication”. She was perfused with 3% NaCl solution until correction of hyponatraemia. Twenty four hours later, the sodium level returned to normal, and the patient was extubated without complications on the next day (Boulanger-Gobeil et al., 2012).

In addition to hyponatraemia, adverse renal effects elicited by these substances are also commonly described (Pearson et al., 2012; Romanek et al., 2017; Warrick et al., 2012). A retrospective study aimed at evaluating substance ingestion patterns (including co-ingestion with other drugs) and complications arisen from synthetic cathinones use in abusers treated at a specialized hospital Toxicology Unit in Southern Germany, between January 2010 and January 2016, concluded that methylone was among the most consumed cathinones. Thirty-five per cent of the eighty one cases analysed were laboratory-confirmed for the use of methylone, and in seventy-two per cent of these, elevated levels of CK were observed, which could be related with rhabdomyolysis and renal failure (Romanek et al., 2017). Similarly, Murphy et al. (2013) reviewed all cases of human exposure to “bath salts” and “plant food” products, which mostly contain β -keto-phenylalkylamines such as 4-methylmethcathinone (mephedrone), MDPV and methylone, reported to the Carolinas Poison Centre from 2010 to 2011. The authors paid special attention to the clinical effects, observing that most reported outcomes were tachycardia, agitation, hallucinations/delusions and hypertension. Other serious complications included rhabdomyolysis, renal failure, excited delirium syndrome and death (Murphy et al., 2013). Rhabdomyolysis, seizures and renal failure were also reported in a 23-year-old male, following ingestion of methylone (Pearson et al., 2012), and in a 24-year-old female, after ingestion of two capsules containing methylone and butylone (Warrick et al., 2012). Our *in vivo* data with 3,4-DMMC and methylone suggest

that the hyponatraemic effect reported in many of these cases might be linked to the ability of these cathinones to increase ADH secretion.

Our investigation shows that the magnitude of the ADH-releasing acute effect was quite similar among the tested cathinones and MDMA, with a slight but not statistically significant increase noted for 3,4-DMMC and MDMA, when compared to methylone (Figure 7). This effect seems to be more persistent in the case of the cathinones, since after 24 h of the drug administration, increased plasma levels of ADH were still detected for both drugs. On the contrary, for MDMA, it was previously noted that, under similar experimental conditions, the ADH plasma levels of the drug-treated rats had returned to control levels 24 h after MDMA administration (Silva and Carmo, 2008). Nevertheless, after 24 h of the drug treatment with both 3,4-DMMC or methylone, the observed ADH plasma levels were significantly lower than those measured after 1 h (Figures 7 and 8). This less pronounced plasma concentration of ADH at this later time-point, may have occurred due to the depletion of the hormone reserves, to the suppression of its synthesis (Schmidt, 1987), or to the decline of ADH release as a consequence of the reduction of the drug plasma concentration. For 3,4-DMMC, this last hypothesis is supported by the rapid biodistribution of the drug (Rouxinol et al., 2019). In fact, 1 h after 3,4-DMMC administration to Wistar rats, this cathinone was detected in practically all the body fluids and organs (plasma, brain, heart, kidneys, liver, lungs, intestine, testicles, spleen, adipose tissue and muscle), but after 24 h the drug was only present in urine and absent from all other tested biological samples (Rouxinol et al., 2019). This seems to be also the case of methylone. In a previous study, 15 or 30 mg/Kg methylone were orally administered to male Sprague-Dawley rats; blood samples were collected from 5 min to 24 h after the drug administration, and methylone was quantified in plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Lopez-Arnau et al., 2013). It was observed that maximum plasma concentrations of methylone were achieved rapidly, 0.5 to 1 h after treatment, decreasing to undetectable values within 24 h (Lopez-Arnau et al., 2013). Likewise, MDMA also appears to have a rapid elimination in humans (i.e. maximum plasma concentrations achieved during the first 4 h after drug administration and the majority of the drug excreted in 24 h) (Fallon et al., 1999). Altogether, this justifies the similarity between the results observed for 3,4-DMMC, methylone and MDMA with respect to the decline of ADH secretion at 24 h.

Finally, the quantification of ADH in the 24 h urine samples showed that animals injected with 3,4-DMMC or methylone had higher urinary ADH concentrations than the

control animals. This effect was much more exuberant in the case of 3,4-DMMC (Figure 10). On the other hand, although it is possible to detect high plasma levels of ADH 24 h after cathinones administration, a greater increase in the excretion of this hormone was observed after this period. These observations support the acute effect of cathinones on the induction of ADH secretion, an effect that is attenuated as cathinones are excreted from the organism.

5.2 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone trigger an increase of antidiuretic hormone (ADH) plasma levels in male Wistar rats, albeit less exuberant than that observed for female rats

The effect on ADH secretion 1 h after administration of the two cathinones, i.e. 3,4-DMMC and methylone, was also evaluated in male animals, for which statistically significant increases were also noted, compared to the control animals (Figure 9).

Increased ADH plasma levels were also previously described after the administration of MDMA in human males (Forsling et al., 2001). In that study, each of eight normally hydrated men received a capsule containing 40 mg of MDMA, and blood samples were collected at 0.5, 1, 2, 4, 6, 8 and 24 h after administration. The analysis of plasma revealed that there was a significant increase in plasma ADH ($4.0 \text{ pM} \pm 0.72$) compared with basal values ($1.5 \text{ pM} \pm 0.09$) (measured 1 and 4 h before administration of MDMA). This was accompanied by a small decrease in serum sodium levels (a decrease of 1–2 mM from basal values) in six of the eight volunteers, which returned to normal 8 h after drug administration (Forsling et al., 2001). Nevertheless, there are several literature reports that indicate a greater female gender susceptibility towards the development of hyponatraemia after MDMA abuse (Ajalo et al., 1998; Aramendi and Manzanares, 2010; Balmelli et al., 2001; Box et al., 1997; Braback and Humble, 2001; Brvar et al., 2004; Budisavljevic et al., 2003; Chen and Viola, 1991; Cherney et al., 2002; Claffey, 2011; Farah and Farah, 2008; Ghatol and Kazory, 2012; Gomez-Balaguer et al., 2000; Holden and Jackson, 1996; Holmes et al., 1999; Kalantar-Zadeh et al., 2006; Libiseller et al., 2005; Magee et al., 1998; Matthai et al., 1996; Maxwell et al., 1993; Niemeijer et al., 2009; Nuvials et al., 1997; O'Connor et al., 1999; Parr et al., 1997; Rosenson et al., 2007; Satchell and Connaughton, 1994; Thakkar et al., 2017; Traub et al., 2002; Watson et al., 1997; Wilkins, 1996), which could anticipate gender differences in the MDMA-related ADH-secretion. This might also be the case of cathinone-induced

hyponatraemia, as the only clinical report described in the literature occurred in a 22-year-old woman (Boulangier-Gobeil et al., 2012). Based upon our *in vivo* data with this Wistar rat animal model, although slightly lower ADH plasma levels were detected in male rats, no significant gender differences were noted after 1 h of the administration of both cathinones (Figure 9). Our data agrees well with the previous mentioned study in which 20 mg/Kg MDMA were administered i.p. to male and female Wistar rats, where no significant gender differences were noted in the measured plasma ADH levels (Silva and Carmo, 2008). Contrarily, investigations conducted in humans signpost marked gender differences, with respect to ADH release (Baggott et al., 2016; Rosenson et al., 2007; Simmler et al., 2011; van Dijken et al., 2013). In a previous work, the incidence of hyponatraemia in individuals who consumed MDMA at a rave party was investigated. From the one hundred and seven participants, sixty-six used MDMA, one man and eight women developed hyponatraemia (van Dijken et al., 2013). Also, Rosenson et al. (2007) studied the characteristics of patients with MDMA-related hyponatraemia, and observed that females are more prone to development of this effect, since from the seventy-three cases of hyponatraemia reported, fifty-five occurred in women; and that the female gender in combination with hyponatraemia was related to increased odds of developing seizures and coma. Accordingly, the modulation of ADH secretion for the maintenance of the hydroelectrolyte balance is influenced by the female sex hormones, in particularly by the action of oestrogen, and this fact may contribute to a higher incidence of hyponatraemia in the female users of this type of substances (Rosenson et al., 2007; Sladek and Somponpun, 2008; Somponpun and Sladek, 2003). In addition, the inhibitory activity of oestrogen on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ system, reducing its catalytic activity, may trigger a decrease in the capacity to eliminate intracellular sodium, affecting the resolution of cerebral oedema (Ayus et al., 2008; Chen et al., 2006). Furthermore, oestrogen also appears to interfere with the movement of water and neurotransmission, by affecting AQP4 expression (Ayus et al., 2008, Sun, 2007 #458), increasing susceptibility of menstruant women to phenethylamine-induced hyponatraemia. Since there is a cyclic variation in circulating female hormones, this susceptibility may additionally vary during the ovarian cycle (Forsling et al., 1996). Therefore, the occurrence of hyponatraemia in women may be associated with hormonal gender differences in the susceptibility to the action of ADH.

Independent of gender, there are also behaviours and/or other factors typical of users of these substances that may facilitate hyponatraemia, such as excessive water

intake, intense exercise/dancing, hyperthermia, and sodium loss in sweat (Box et al., 1997; Budisavljevic et al., 2003; Chandra et al., 2016). Therefore, for females, the hormonal factor may also potentiate some of these effects since during the ovulation phase the body temperature of women increases, which in the presence of substances such as MDMA or cathinones, can lead to the worsening of sodium loss in sweat and to a higher intake of water (Claybaugh et al., 2000; Forsling et al., 1996; Rosenson et al., 2007).

5.3 3,4-Dimethylmethcathinone (3,4-DMMC) and methyldone trigger the increase of body temperature in Wistar rats

Statistically significant increases on body temperature were noted in animals treated with 3,4-DMMC or methyldone, for both female and male rats, when compared to control animals (Figure 11). This ability of phenethylamines, in particularly MDMA, to induce changes of body temperature in animal models (Baumann et al., 2012; Coman et al., 2015; Costa et al., 1972; Fantegrossi et al., 2013; Grecco and Sprague, 2016; Jaehne et al., 2007; Mehan et al., 2002; Shortall et al., 2013; Tariq et al., 1989) as well as in humans (Freedman et al., 2005; Ginsberg et al., 1970; Jordan and Hampson, 1960; Kojima et al., 1984) has been vastly reported in the literature. In a previous study, ten healthy volunteers received 2 mg/Kg MDMA or placebo; core body temperature was measured with a radiotelemetry pill that was ingested before each session, in addition to skin temperature, heart rate, blood pressure, metabolic rate (indirectly by calorimetry), electrical muscle activity (to detect shivering) and sweat rate. It was observed that MDMA produced elevations in metabolic rate and in core body temperature of approximately 0.6 and 0.3 °C, when individuals were kept in hot (30 °C) or cold environments (18 °C), respectively (Freedman et al., 2005). Furthermore, MDMA elevated the average temperature from 36.9 ± 0.21 to 37.8 ± 0.07 °C and maximum temperature from 37.2 ± 0.17 to 38 ± 0.28 °C, 4 h after an intramuscular administration of 1.7 mg/Kg MDMA to rhesus monkeys (Taffe et al., 2006).

It is known that the deregulation of body temperature results from an imbalance between the mechanisms of heat production and dissipation, and the increase of heat production stems from overstimulation of the metabolic activity at the CNS. In the case of MDMA, the effects of the drug on the regulation of body temperature are complex, since it interacts with the three major monoamine neurotransmitter systems by stimulating

neurotransmitter release or directly acting on their receptors, ultimately deregulating central thermoregulation and promoting peripheral alterations in blood flow and in thermogenesis of brown adipose tissue (Carvalho et al., 2012; Docherty and Green, 2010; Simmler and Liechti, 2018). The initial release of 5-HT induced by MDMA leads to an increase of DA biosynthesis, and consequent overactivation of D1 receptors that triggers hyperthermia (Mills et al., 2004). A complex interaction between the hypothalamic-pituitary-thyroid axis, the sympathetic nervous system, and the activity of UCP seems to be involved in this process (Sprague et al., 2003). Sprague et al. (2003) showed that hypophysectomia and thyroparathyroidectomy of rats subcutaneously administered with 40 mg/Kg MDMA, abolished the hyperthermic response observed in normal rats treated with the same dose of the drug. Accordingly, 1 h after the treatment, the mean rectal temperature was approximately 35 °C in hypophysectomised rats and 33 °C in thyroparathyroidectomised rats, while in MDMA control animals, the mean rectal temperature was approximately 40 °C. Drug activation of the hypothalamic-pituitary-thyroid axis and, consequently, thermogenesis are dependent on the circulating levels of adrenal and thyroid hormones (Burns et al., 1996; Mills et al., 2004; Parrott, 2016; Sprague et al., 2018). In accordance, the authors also proved that hyperthermia was re-established if thyroid hormone was replaced (Sprague et al., 2003). In addition, MDMA also triggers cutaneous vasoconstriction (Pedersen and Blessing, 2001) and increases activity of thermogenic tissues, such as brown fat and muscle, through the uncoupling of oxidative phosphorylation prompted by the activity of UCP. These mitochondrial UCP proteins act by providing a conduit for the passive flow of electrons across the inner membrane of mitochondria and, consequently, under physiological normal conditions constitute a regulated source of heat production (Rusyniak and Sprague, 2005), contributing to the increase of body temperature. Three different UCP proteins are known: UCP-1 in brown fat of rodents, UCP-2 in the liver and UCP-3 in human skeletal muscle (Carvalho et al., 2012). The skeletal muscle thermogenic protein UCP-3 appears to play an important role in MDMA induced hyperthermia, since this drug is capable of directly stimulating its activity but is also capable of inducing the activation of β_3 -adrenoreceptors, which leads to the conversion of triglycerides to free fatty acids and consequently to the activation of UCP-3 for the thermogenesis (Dao et al., 2014). In this mechanism, thyroid hormones play a permissive role through the regulation of the UCP-3 gene and protein expression (Mills et al., 2004).

The fact that MDMA consumption is associated with hot and crowded environments and prolonged physical activity may also contribute to the hyperthermic effect (Dafters, 1994; 1995; Freedman et al., 2005; Gordon et al., 1991; Malberg and Seiden, 1998; Patel et al., 2005). In a study aimed to determine whether MDMA-induced hyperthermia depended on environmental temperature, MDMA was intramuscularly administered to rhesus monkeys (doses ranging 0.56–2.4 mg/Kg). However, this study failed to establish such a dependence since there was only a mean temperature alteration of approximately 1 °C across an ambient temperature range of 18–30 °C (Von Huben et al., 2007). As already mentioned, similar conclusions could be drawn in humans when Freedman and colleagues (2005) observed that MDMA increased body temperature regardless of the ambient temperature.

Synthetic cathinones also exert their effects through the interaction with monoamine neurotransmitters, culminating in an increased concentration of monoamines in synapses (Baumann et al., 2012; Cameron et al., 2013; Simmler et al., 2013; Sogawa et al., 2011). Therefore, their administration also triggers hyperthermia, among other effects (Baumann et al., 2012; Fantegrossi et al., 2013; Shortall et al., 2013; Tariq et al., 1989; Valente et al., 2014), which is hypothesised to be induced through inhibition of reuptake by SERT and/or DAT. Piao et al. (2015) studied the toxicity of methylone in SERT or DAT knockout mice and observed that, compared with wild-type animals, the drug-induced lethality was significantly lower in DAT knockout mice, but not in SERT knockout mice. Conversely, only a small reversion of the hyperthermic effect elicited by methylone was observed in DAT knockout mice, whereas in SERT knockout mice a small increase of body temperature was observed. Thus, it was concluded that DAT, but not SERT, is associated with lethal toxicity induced by methylone, which did not seem to be dependent on the hyperthermic effects of the drug – although the study supports, at least in part, the role of DAT in the hyperthermia induced by the drug. Also, cathinone and methcathinone significantly increased the rectal temperature of male Lister hooded rats after i.p. injection of 4 or 10 mg/Kg of each of these substances, but the same did not occur after the administration of mephedrone or MDMA (Shortall et al., 2013). Also, MDPV induced an increase in body temperature of adult male NIH Swiss mice kept at a room temperature of 28 °C (Fantegrossi et al., 2013).

It is well acknowledged that MDMA is bioactivated through O-demethylation of the methylenedioxy ring into catechol metabolites (de la Torre et al., 2004; Kreth et al., 2000; Segura et al., 2005) that exhibit higher toxicity than the parent drug (Antolino-

Lobo et al., 2011; Carmo et al., 2006), and these metabolites have been implicated in the hyperthermic effect elicited by MDMA (Bexis and Docherty, 2006; Colado et al., 1995; Miller and O'Callaghan, 1994). Methylone, which shares the methylenedioxy ring with MDMA, has a metabolic pattern similar to that of the classic drug and, therefore, also originates active metabolites which are likely implicated in the hyperthermic effect of the cathinone (Baumann et al., 2012; de la Torre et al., 2004; Pedersen et al., 2013). Accordingly, the metabolites 3,4-dihydroxy-N-methylcathinone, 4-hydroxy-3-methoxy-N-methylcathinone and 3,4-methylenedioxycathinone of this 3,4-methylenedioxy ring-substituted stimulant have also been shown to interact with monoamine transporters (Elmore et al., 2017; Luethi et al., 2019), and therefore, might be able to induce changes in thermoregulation. These results relating metabolism at the methylenedioxy ring with the drug-induced hyperthermic effect may justify the fact that in our study methylone elicited a higher temperature increase, when compared with 3,4-DMMC. The hyperthermia induced by methylone in our study is also supported by a previous study in which a total of three s.c. injections of 3.0 and 10 mg/Kg methylone were administered every 2 h to male Sprague-Dawley rats, inducing temperature increase (+1.4 °C at 3.0 mg/Kg and +2.1 °C at 10 mg/Kg) in the animals. Nevertheless, this effect was not accompanied by a long-term alteration in cortical and striatal amines. On the other hand, the administration of three s.c. injections of 2.5 and 7.5 mg/Kg MDMA induced a robust hyperthermia in the animals (+1.1 °C at 2.5 mg/Kg and +2.2 °C at 7.5 mg/Kg), in this case accompanied by a persistent depletion of cortical and striatal 5-HT (Baumann et al., 2012). Although it was observed that repeated high-dose administration of MDMA and methylone cause acute hyperthermia, methylone seems to be less likely to cause persistent depletion of brain 5-HT, when compared to other substrates of SERT such as MDMA, supporting a less detrimental outcome for this cathinone (Baumann et al., 2012). Furthermore, after s.c. injection of a single dose of seven phenethylamines [4-methylmethamphetamine (4-MMA), methylone, mephedrone, butylone, pentylone, MDPV and MDMA] at 30 mg/Kg to Sprague-Dawley rats, all drugs produced a significant increase of the core body temperature. The exception was MDPV, which only increased core body in 0.5 °C. Methylone was the cathinone that induced the most effective thermogenic response (with changes of approximately 3.5 °C to baseline temperature) (Grecco and Sprague, 2016), while MDMA was the substance that triggered the greatest change in body temperature (+4.86 °C), surpassing the response to methylone. A structure-activity comparison of the drugs indicates that the oxidation at the benzylic

position, the extending of the α -alkyl chain to ethyl and propyl, and/or the addition of a pyrrolidine on the N-terminus, significantly reduces the thermogenic response (Grecco and Sprague, 2016). Our results are in line with this postulate, since methylone elicited the highest temperature increase when compared with 3,4-DMMC, but did not overcome the hyperthermia induced by administration of MDMA (although these results await confirmation, as only one animal was treated with MDMA). In fact, methylone, a β -keto amphetamine only differs from MDMA by the presence of a ketone at the benzylic position. The small differences between the effects of methylone and MDMA are consistent with the fact that this cathinone is a potent substrate for monoamine transporters, displaying a profile of DA- and 5-HT-releasing activity which resembles that of MDMA (Baumann et al., 2012). On the other hand, the thermogenic response induced by methylone was more pronounced than that of 3,4-DMMC, and in this case, the difference in molecular structures resides on the aromatic ring (3,4-methylenedioxy substitution for methylone and 3,4-dimethyl substitution for 3,4-DMMC). Actually, 3,4-DMMC exhibits a structure similar to amphetamine and methamphetamine, which have also been shown to trigger hyperthermia (Bowyer et al., 1994; Brown et al., 2003; Cadet et al., 2007). However, methylone is more similar to the MDMA, which is the amphetamine derivative most often implicated in drug-induced hyperthermia (Carvalho et al., 2012).

In regard to the possible impact of gender, there are many reports of increased body temperature following MDMA consumption in males (Ben-Abraham et al., 2003; Karlovsek et al., 2005; Logan et al., 1993; Nimmo et al., 1993; Rosenson et al., 2007; Tehan et al., 1993), as well as in females (Ghatol and Kazory, 2012; Nimmo et al., 1993; Rosenson et al., 2007). More recently, cases of hyperthermia in both genders have also been reported after consumption of methylone (Pearson et al., 2012; Warrick et al., 2012). Apparently, there are no significant gender differences in the hyperthermic effect of these drugs. In accordance, our data also showed that in Wistar rats the thermogenic action of the tested drugs was gender independent (Figure 11).

5.4 Administration of 3,4-dimethylmethcathinone (3,4-DMMC) and methyldone does not change the brain water content 24 h after drug administration

As previously discussed, due to their effects on the serotonergic nervous pathways, phenethylamines may induce disturbances in the balance of water, which can culminate in hyponatraemia (Campbell and Rosner, 2008). The decrease of the plasma levels of sodium leads to an osmotic shift of water into the intracellular compartment and, consequently, to the increased risk of developing cerebral oedema and associated complications, such as seizures (Campbell and Rosner, 2008). Accordingly, a previously published *in vivo* study showed that the administration of a single neurotoxic dose of 12.5 mg/Kg MDMA leads to water accumulation from 2.5 h to 6 h after drug administration, expanding the extracellular space and producing cerebral oedema of short duration in rats (values returned to normal levels at 24 h). (Perez-Hernandez et al., 2017). It is therefore reasonable to expect that brain oedema could also occur to some extent in cathinone-treated animals. Herein, although a slight tendency for the increase in the brain water content was observed for both cathinones, no statistically significant difference was noted (Figure 12). As an additional indicator of brain oedema, the ratio between brain weight and body weight of each animal was calculated, but no differences were observed when compared to control animals (Figure 14), which also argues against the occurrence of cerebral oedema under our experimental conditions.

Although there is strong forensic evidence that MDMA-induced hyponatraemia may lead to the development of cerebral oedema (Ajaelo et al., 1998; Aramendi and Manzanares, 2010; Balmelli et al., 2001; Berney-Meyer et al., 2012; Cherney et al., 2002; Claffey, 2011; Ghatol and Kazory, 2012; Holden and Jackson, 1996; Karlovsek et al., 2005; Libiseller et al., 2005; Magee et al., 1998; Matthai et al., 1996; Parr et al., 1997; Rosenson et al., 2007; Thakkar et al., 2017; Traub et al., 2002; Wilkins, 1996), these evidence have not always been supported by studies with animal models (Kwack et al., 2014). In accordance, Kwack et al. (2014) did not observe differences in organ weights of male and female C57BL/6 mice after a single daily oral administration of 1.25, 5 or 20 mg/Kg MDMA for two to four weeks. On the other hand, in a previous study, it was observed that 4 h after i.p. administration of 40 mg/Kg MDMA to Wistar rats and C57 Balb mice, the animals presented cerebral swelling indicative of oedema, evidence that was later confirmed by measuring the brain water content (Sharma and Ali, 2008).

Accordingly, in the already mentioned study of Henry et al. (1998) it was demonstrated that after administration of 40 mg/Kg MDMA to male individuals, the increase of ADH levels was accompanied by a decrease of plasma levels of sodium at 0.5 to 2 h after administration, which may lead to the onset of cerebral oedema. In this same study, concentrations of ADH returned to normal levels 8 h after administration. According to our data, 3,4-DMMC and methylone do not appear to induce the development of cerebral oedema, but these results would be benefited by the testing of other doses and time-points, as the water content in brain was measured only 24 h after administration, which may have contributed to oedema attenuation.

5.5 Administration of 3,4-dimethylmethcathinone (3,4-DMMC) significantly induces an antidiuretic effect in female Wistar rats, but no alteration in the water intake pattern was observed

The results of the present study showed that animals treated with 3,4-DMMC excreted less urine than control animals, whereas in the case of methylone-treated animals a slight, no statistically significant increase in urine excretion occurred. With regard to water consumption, both 3,4-DMMC-treated and methylone-treated animals ingested an amount of water similar to that ingested by controls (Figure 13). Although there were no alterations in the ingestion of water pattern, after calculating the ratios between water intake and excretion of urine, the obtained results clearly demonstrate the antidiuretic action of 3,4-DMMC, that is likely related to the increased ADH secretion previously detected. Similar results were obtained in Wistar rats after i.p. administration of 20 mg/Kg MDMA (Silva and Carmo, 2008), which linked the antidiuretic action of MDMA to the increase of ADH secretion, and to water retention. The overexpression of AQP2 channels that mediate reabsorption of water in the apical membrane of the collecting tubules and prompt rapid development of hyponatraemia in Wistar rats after i.p administration of 10 mg/Kg MDMA (de Braganca, 2017), might be responsible for the 3,4-DMMC-induced antidiuretic effect observed herein.

Chapter VI Conclusions

In the study presented herein, both 3,4-DMMC and methylone at 20 or 40 mg/Kg triggered the increase of ADH secretion in female Wistar rats, 1 h after administration. This increase was similar to that observed for MDMA at 20 mg/Kg. Albeit less pronounced, increased plasma ADH concentration was still detected 24 h after administration of the cathinones at 20 mg/Kg. In addition, a great increase in ADH was found in the urine collected during the 24 h after the administration of 3,4-DMMC and methylone. Although this effect seems to be more evident in females, the increase of ADH release also occurred in male rats under the same experimental conditions. No significant gender differences were found. A body temperature increase induced by 20 mg/Kg 3,4-DMMC or methylone was also evidenced for both genders.

Contrary to the expected, the administration of 20 mg/Kg 3,4-DMMC or methylone did not trigger significant changes in the brain water content 24 h after administration of the cathinones. Also, no changes in the ratio brain weight to body weight were observed, 1 h and 24 h after the administration. In fact, cerebral oedema is an extreme consequence of increased ADH secretion, therefore, although an increase in the release of ADH has been observed, this may not have been reflected in the development of oedema. The antidiuretic effect observed after administration of 3,4-DMMC is in agreement with the increase in release of ADH induced by this cathinone.

In conclusion, this work is a starting point in the research concerning cathinones-induced hyponatraemia. Toxicological studies of NPS are important and necessary, since these substances are legally marketed in many countries to circumvent the restrictions on the production, trafficking, and consumption of classic amphetamines. The rapid emergence of synthetic cathinones and the increase in their use, leading to an increased number of intoxications, demonstrate the importance of the recognition and treatment of such intoxications. These substances have the ability to cause serious damage in several organs systems, which can ultimately lead to death. So, accurate knowledge on the toxic effects caused by them is crucial, as it helps to establish the cause of death in fatality cases, as well as to establish the most appropriate therapeutic measures in case of non-lethal intoxications. Overall, this study is a step forward to elucidate the health professionals involved in the management of the drug-intoxications and the forensic pathologists who investigate the cause of death, that hyponatraemia is a potentially fatal consequence associated with abuse of 3,4-DMMC and methylone.

Chapter VII References

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