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Dual dopamine/serotonin treatment approach for addictive behaviour

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By

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Abbreviations

5 – HT	serotonin
A-O	action-outcome
ANOVA	analysis of variance
CPP	conditioned place preference
DA	dopamine
DAT	dopamine transporter
DP	drug-paired
I.P	intra-peritoneal
I.V	intravenous
MA	methamphetamine hydrochloride
NAcc	nucleus accumbens
NDP	non drug-paired
NE	norepinephrine
NET	norepinephrine transporter
PFC	prefrontal cortex
S-R	stimulus-response
S.C	subcutaneous
SERT	serotonin transporter
TAAR1	trace amine-associated receptor
TRAZ	trazodone hydrochloride
VTA	ventral tegmental area

Abstract

Illicit drug abuse and addiction is a major problem in New Zealand and worldwide with approximately 12% of illicit drug users classified as having drug dependence or drug-use disorders. The chronically relapsing nature of drug addiction is a prominent feature of this disorder and a significant barrier to treating addiction. Amphetamine-type drugs, more than any other class of drugs, have seen an increase in global usage since the early 1990's. The lack of approved medications for the treatment of stimulant addiction together with an increasing treatment demand drives the need for pharmaceutical intervention. Substitute treatment approaches primarily focus on the dopamine (DA) system. However, several lines of research implicate a dual role for serotonin (5-HT). Using a benztropine (BZT) analogue, JHW 007 (JHW), and an atypical antidepressant, trazodone (TRAZ), we sought to test whether the combined modulation of DA and 5-HT during a period of extinction produced greater attenuation to drug-induced reinstatement compared to either DA or 5-HT alone. One hundred and two (102) male Long Evans rats were tested using an extinction-reinstatement model of methamphetamine (MA) addiction in conditioned place preference (CPP) (n=72) and self-administration (n=30) experimental designs. We hypothesised that a combined DA/5-HT treatment would further attenuate MA-induced reinstatement. Findings showed that JHW significantly attenuated MA-induced reinstatement in our self-administration model but not CPP, while TRAZ failed to significantly attenuate reinstatement in both experiments. The combination treatment group showed a mild attenuation to drug seeking with CPP, but this finding was not significant. Due to time restrictions, we did not test the combination group using a self-administration procedure. Unfortunately we were unable to fully address our initially proposed hypothesis, but our results with JHW add further support to this BZT analogue as a promising stimulant abuse medication.

1.0 Introduction

1.1 General overview

Drug addiction is a chronically relapsing disorder of the brain characterised by a compulsion to seek and consume the drug, marked loss of control over limiting intake, and the emergence of a negative emotional state when drug access is restricted (Koob & Le Moal, 2005). Modern views of drug addiction describe three types of use; (1) occasional, controlled or social use, (2) drug abuse or harmful use, and (3) drug addiction. The importance of discriminating between these types of use is highlighted by data showing approximately 15.6% of the US adult population have engaged in nonmedical or illicit drug use at some point in their lifetime, with approximately 3.1% going on to drug abuse and 2.9% on to substance dependence (Grant & Dawson, 1998).

Many factors including trait impulsivity, sensation-seeking and negative affect leading to self-medication, are believed to contribute to addiction vulnerability. Social and environmental factors, together with the inherently reinforcing effects of many drugs, are thought to drive initial drug use. Complex neurobiological processes underlie the shift to more persistent use with the transition from casual use to drug addiction/dependence marking a gradual shift from positive to negative reinforcement (Koob & Le Moal, 2005) and from outcome-driven to habitual drug taking (Belin & Everitt, 2008). With chronic use, drug taking serves to provide relief from an already anxious or stressful state and with time fails to produce the initially desired states of euphoria. Furthermore, as drug taking becomes a means to achieve a state of ‘normality’, repeated attempts at abstinence or control are likely to result in relapse. Similarly, chronic drug abuse can lead to habitual responses and repetitive patterns of behaviour, with drug taking being automatically triggered by conditioned stimuli, such as drug paraphernalia.

The chronically relapsing nature of drug addiction is a prominent feature of this disorder and a significant barrier to treating addiction, as a high susceptibility for relapse typically persists despite extended periods of abstinence. It is immensely difficult to measure relapse prevalence among addicted individuals. However figures are generally reported between 50-90% with factors including, severity of addiction, the class of drug being abused, duration of treatment (if any), and elapsed period of abstinence prior to relapse, proposed to account for this variance. Persistent use of most drugs of abuse results in changes to brain neuroadaptions further facilitating biological addiction processes and increasing the likelihood of relapse (Ernst, Chang, Leonido-Yee, & Speck, 2000). Consequently, drug addiction remains a problematic and costly issue worldwide with increasing treatment demand driving the need for pharmaceutical intervention.

1.2 Drug statistics

Illicit drug abuse and addiction is a major problem in New Zealand and worldwide. In 2010, it was estimated globally that 15.5 - 38.6 million people (approximately 12% of illicit drug users) were classified as problem users including those with drug dependence or drug-use disorders (World Drug Report [WDR], 2012). The Oceania region (predominantly Australia and New Zealand) has a greater annual prevalence for all illicit drugs, except heroin, than the global average. Together with North America, Oceania also has one of the highest rates of drug-related deaths, accounting for approximately 1 in every 20 deaths among the 15-64 year old population. A major cause for concern is the rise in injecting drug users in New Zealand which increased from 40% in 2006 to 50% in 2009 (WDR, 2011). In addition to the risks associated with the drug itself, administration through injection increases the likelihood of contracting infections or diseases such as HIV and AIDS through needle sharing. Since the early 1990's, amphetamines, more than any other class of drugs has seen

an increase in global usage (WDR, 2012). Amphetamine-type stimulants (ATS), including MA, remain the second most widely used class of drugs, behind cannabis, comprising 0.3-1.2% (14.3-52.5 million) of illicit drug use worldwide (WDR, 2012). Northern and Central America and Oceania currently have the highest rates of ATS use.

1.3 Methamphetamine

MA is an amphetamine derivative with high abuse potential and widespread global use as a recreational psycho-stimulant drug. Acutely, MA produces feelings of increased confidence, disinhibition, euphoria, heightened alertness, energy and a sense of wellbeing. However, with repeated chronic exposure, users can experience undesirable effects including paranoia, mood swings, agitation, dizziness and headaches (Sheridan, Bennett, Coggan, Wheeler &, McMillan, 2006). First synthesised in the late 1800's from ephedrine, a basic decongestant, MA was widely used among German, Japanese and American military personnel during World War II as a performance enhancer and to maintain alertness and productivity (Wolkoff, 1997). As a result, widespread abuse of the drug followed, reaching epidemic proportions when military stockpiles of MA became available to the public (Koob & Le Moal, 2005). The passing of the Controlled Substances Act in the United States in 1970 saw a decrease in MA abuse rates (Klatt, Montgomery, Namiki, & Noguchi, 1986). However a dramatic increase in the production of 'designer amphetamines' followed, the most well-known being 3,4-methylenedioxymethamphetamine (MDMA) or ecstasy. MA is currently classified by the Controlled Substances Act (CSA) as a Schedule II narcotic drug with "very high abuse potential" and "acceptance for medical use" in the United States (U.S. Department of Justice [U.S. DOJ], 2002). While rarely prescribed, dextro-methamphetamine, an enantiomer responsible for MA's psychostimulant properties, has been used for the treatment of Attention Deficit Hyperactivity Disorder (ADHD) and obesity (Castle, Aubert, Verbrugge,

Khalid, & Epstein, 2007). While levo-methamphetamine, an enantiomer inactive on the Central Nervous system (CNS), was used in early preparations of the U.S Vicks Vapour inhaler and other early cough suppressants/decongestants.

Throughout the history of ATS manufacture, drug trafficking organisations have shown a remarkable degree of flexibility in adapting their strategies to avoid detection. Ephedrine and pseudoephedrine have been the main precursors used in the illicit manufacture of MA. However control over these substances, in bulk and as ingredients in pharmaceutical medications, has seen a rise in the use of a substitute precursor chemical, 1-phenyl-2-propanone (P-2-P) (WDR, 2012). Constant change in the process of illicit manufacture of these drugs has presented considerable challenges for drug control authorities worldwide (WDR, 2012). The extraction of pseudoephedrine from pharmaceutical preparations still continues for ATS manufacture with large quantities of pseudoephedrine having been seized in Asia and Oceania. Extractions of pseudoephedrine from the ephedra plant were also reported in New Zealand and Kyrgyzstan (WDR, 2012).

The onset and intensity of MA's effects can vary greatly depending on how the drug is consumed. The subjective pleasure of drug use is considered proportional to the rate at which the blood level of the drug increases. Route of administration can therefore affect the potential risk of developing a psychological addiction aside from other factors such as dose and frequency (Winger, Hursh, Casey, & Woods, 2002). Intravenous injection causes blood concentrations to rise fastest with subjective feelings of an intense rush or high being produced almost immediately. Smoking closely follows, with snorting taking approximately 3-5 minutes and ingestion (swallowing), 20-40 minutes (National Institute on Drug Abuse, 1998). Given previous findings, it is not surprising that ingestion is the least common and least addictive route of administration.

Compared to amphetamine, MA passes more readily through the blood-brain barrier, has a much longer half-life, remaining in the CNS for longer and producing psychostimulant effects for up to 12 hours (Tominaga, Garcia, Dzierba, & Wong, 2004). MA's mechanism of action on brain neurotransmitter systems further explains its high abuse liability. It is well established that MA exerts its reinforcing effects through occupation and reversal of monoamine transporters with chronic exposure leading to reduced DA, 5-HT and norepinephrine (NE) levels that can persist for several months (McCann et al., 1998; Volkow et al., 2001). MA binds to the transporter proteins for DA, 5-HT and NE and is also internalised by the pre-synaptic cell. As a consequence, monoamines accumulate in the pre-synaptic cell and are released in abundance into the synapse through reverse transport resulting in significant depletion with repeated use. While the production and reuptake of NE and 5-HT are affected by MA, the psychostimulant and euphoric effects frequently seen as a result of MA use are believed to be primarily mediated by the DA system, particularly at the level of the dopamine transporter (DAT) (Drevets et al., 2001; Lott, Kim, Cook, & de Wit, 2005). Therefore, recent treatment approaches have utilised a series of animal models in evaluating promising pharmaceutical compounds through their ability to stabilise functionality of the DA system.

1.4 Animal models of drug relapse

Relapse is a cardinal feature of drug addiction and one which has brought about a wealth of animal research. Relapse to drug seeking has been successfully demonstrated through the presentation of drug-associated cues, contexts, stress and most notably, re-administration of the drug itself. These addiction models provide important information for relapse in humans. However, in considering the translational value of animal models to human addiction, it is important to distinguish between drug relapse and reinstatement.

Shaham, Shalev, Lu, de Wit, and Stewart (2003) defines reinstatement as occurring after a prolonged period of extinction, while relapse typically follows periods of abstinence/withdrawal in the absence of extinction sessions (Reichel, Moussawi, Do, Kalivas, & See, 2011). Animal models of reinstatement, i.e. those involving a period of extinction, are deemed less relevant for human addiction as therapy procedures do not tend to explicitly extinguish drug cues, whereas abstinence models are thought to more closely resemble human patterns of addiction. Arguably, chronic treatment procedures, most typically occurring within extinction-reinstatement models are believed to possess greater translational relevance to human pharmacotherapies as opposed to single-administration treatment approaches (Reichel & See, 2011). Consequently, various models of drug reinstatement and relapse have been tested within two well-established drug addiction paradigms, CPP and self-administration.

1.4.1 Conditioned place preference. CPP is a frequently used procedure within animal research for assessing the rewarding properties of abused substances such as cocaine, amphetamines, opiates and alcohol. CPP is comparable to the well-known Pavlovian classical conditioning, using the repeated pairing of an unconditioned stimulus (ie, addictive drug) with a particular compartment area to create a conditioned stimulus (CS). When given the opportunity to freely explore both the drug-paired (DP) and non drug-paired (NDP) compartments, animals will typically prefer the DP compartment, thus showing CPP (Itzhak & Martin, 2002). The conditioned response to drugs of abuse seen in animal models of CPP is relevant for human addiction, particularly in regards to relapse after prolonged abstinence.

Early models of CPP are believed to date back as far as the early 1900's with work by Thorndike who, in 1911, conducted an experiment training rats to go to a specific place in a box, unlock a latch, and escape; this marked the initiation of formal learning paradigms for

animals in a laboratory setting. However, procedures most similar to those currently used, began with a study by Garcia, Kimeldorf, and Hunt (1957) who successfully demonstrated conditioned place aversion (CPA) by exposing rats to ionized radiation in a pre-determined environment. When later tested, rats showed a marked aversion to the cues of the radiation-paired environment. Also in 1957, Beach demonstrated, for the first time, the reinforcing properties of morphine with rats using a Y-maze task with conceptual similarities to the CPP method. As with CPP, each goal area in the Y-maze is visually different from the other allowing for the pairing of specific environmental cues with the drug. Following repeated pairing of morphine with its goal box, rats chose the morphine-paired goal box regardless of whether they were actively seeking the drug (drug-free at the time of testing) or were presumably sated, thus emphasising the rewarding properties of the drug.

CPP has only in the last decade or so, been established as a useful paradigm for the extinction/reinstatement model. Variations of this model include the between-session procedure, comprised of drug conditioning followed by a series of reinstatement tests under extinction conditions to examine time-dependent changes (incubation effects) in drug seeking. Using this procedure, Mueller and Stewart (2000) demonstrated, with rats, successful cocaine priming after extinction that was maintained for up to six weeks. This was further supported by Itzhak and Martin (2002) who found comparable results of cocaine-primed reinstatement lasting up to two weeks with mice. Similarly, Cruz, Leao, Marin, and Planeta (2010) showed stress-induced reinstatement to amphetamine seeking in a model of CPP whereby rats reinstated drug seeking following extinction sessions after a 30 minute period of restraint. Other established models include Hoffman and Beninger's (1989) method of giving pre-treatment prior to administration of the drug and subsequent conditioning sessions. This model acts to prevent the development of drug CPP and thus, reinstatement of drug seeking in the absence of both extinction and abstinence. Takamatsu, Yamamoto,

Hagino, Markou, & Ikeda (2011) have successfully demonstrated suppressed MA-induced CPP in mice, with the selective serotonin reuptake inhibitor (SSRI) paroxetine. Conversely, Goeders and Goeders (2004) showed successful attenuation of CPP however by administering a pre-treatment 30 minutes prior to relapse testing, thus as compared to the former study, the development of CPP was not prevented.

Overall, CPP has developed into a highly versatile addiction model contributing substantially to the ever-expanding body of addiction literature. CPP allows us to investigate important aspects of addiction that have translational relevance to human patterns of abuse including drug associated cues, contexts and stress. It is also inherently simplistic so as to allow for additional manipulations to the overall design.

1.4.2 Self administration. Self-administration paradigms are designed to measure drug reinforcement and involve the active administration of drugs of abuse, as opposed to the passive approach taken in CPP models. Animals (most commonly rats or mice), are given the opportunity to respond and maintain intake of a particular drug in operant testing chambers. This paradigm is well-known to produce robust patterns of drug-taking and therefore has been extensively utilised for assessing not only the addictive properties of abused drugs, but also for the ability of drug-associated stimuli to reinstate drug-administration. Operant chambers are generally equipped with a stimulus light, tone and electrified grid which allow for several manipulations to the basic design.

Models of animal self-administration were first developed by Skinner in the early 1930's where he invented his famous operant chamber, also known as the 'Skinner box'. Skinner's initial design of the chamber was equipped with a loudspeaker, two stimulus lights, a response lever, food dispenser and electrified grid. His research exploring operant and classical conditioning in rats set the stage for a multitude of research conducted in the years

that followed. Skinner used various models of reward and punishment to measure operant behaviour including natural reinforcers, such as sucrose or food pellets, as reward for pressing a lever; these models are still frequently used today. He found that behaviour could similarly be shaped by punishment as was demonstrated through the use of mild electric shocks.

However, the first account of drug self-administration was published by Weeks in 1962. Further building on the work of Spragg (1940), Weeks examined the effect of self-administered intravenous injections of morphine on rats and found the drug to be a powerful reinforcer providing almost immediate satiety after administration (Weeks, 1962). Research using the self-administration model became increasingly popular as studies examined the reinforcing efficacy of drugs of abuse in rhesus (Thompson & Schuster, 1964) and macaque monkeys with results indicating the development of drug dependence and toxicity (Deneau, Yanagita, & Seevers, 1969). Drug research during this time focused predominantly on non-human primates and findings were considered somewhat transferrable to human patterns of addiction. However, in 1983, Collins et al. conducted a landmark study exposing rats to a battery of 27 psychoactive drugs through intravenous self-administration. Comparison saline groups were used and findings generally paralleled those previously found in monkeys. Similar observations of the effects of the psychoactive drugs between different animals prompted the use of rats as well as monkeys in drug addiction studies.

As with CPP, the extinction/reinstatement model is well-established in animal self-administration studies. Several studies have used modified protocols to the traditional extinction-reinstatement model by administering a pharmacological treatment prior to training sessions, so as to prevent the development of drug taking, and again prior to a test of reinstatement. This is likely done to measure both the ability of the compound to reduce drug taking and drug-induced reinstatement within the single study. For example, Schmoutz,

Zhang, Runyon, and Goeders (2012) showed attenuation of cocaine intake by administering a neuropeptide antagonist prior to sessions of self administration training. They also showed a reduction in the reinstatement of cocaine-seeking, which was previously found to be reinstated by drug cues, a priming injection of cocaine, and yohimbine. Moffett and Goeders (2007) demonstrated similar findings with MA, as did Kai et al. (2012) using a model of heroin addiction. Given the versatility of the operant chambers, stress- and cue-induced reinstatement to drug seeking has been investigated through this paradigm, further highlighting the importance of targeting these factors within human addiction. Karlsson, Kircher, Shaham, and O'Donnell (2013) observed cue-induced reinstatement to cocaine using an extinction-reinstatement protocol, while Graves and Napier (2011) found mirtazapine to reduce MA-seeking in both models of cue reactivity (without a period of extinction or abstinence) and cue-induced reinstatement. In models of stress-induced reinstatement, two common pharmacological stressors that have been used to study the effects of stress on cocaine-seeking reinstatement are the corticotrophin-releasing factor (CRF) and the alpha-2 receptor antagonist, yohimbine (Brown, Kupferschmidt, & Erb, 2012). In human addiction, stress and drug-associated cues presumably co-occur to produce relapse to drug seeking/taking. Feltenstein and See (2006) found the administration of yohimbine alone to produce reinstatement to cocaine-seeking, but to also potentiate cue-induced reinstatement, suggesting an additive effect of stress and cues on drug reinstatement. Using a model of stress-induced reinstatement, Buffalari and See (2009) studied an intensity-dependent effect of foot-shock on the reinstatement of cocaine in rats. Medium and high levels of foot-shock lead to significant reinstatement of cocaine-seeking. However, consistent with Feltenstein and See (2006), foot-shock at these levels combined with the presentation of previously cocaine-paired cues, further facilitated cocaine-seeking reinstatement.

It is additionally important to note the method of treatment dosing within these drug studies. The aforementioned articles typically utilise single-administration protocols, i.e. one dose of treatment given prior to a test of relapse or reinstatement. This dosing method is common in animal studies. However Reichel and See (2011) proposed that a chronic-dosing regimen may be more relevant to pharmacotherapeutic approaches in human addiction. In studying the therapeutic potential of modafinil to attenuate relapse to MA, Reichel and See (2011) found a single acute dose (30mg/kg) administered prior to tests of relapse seemingly ineffective. However, when administered chronically alongside sessions of extinction, these authors observed successful attenuation to MA-seeking reinstatement. Similarly, chronic treatment with buprenorphine was found to significantly reduce heroin- and cocaine- seeking following extinction, even in the presence of drug-related cues (Sorge, Rajabi, & Stewart, 2005).

Self-administration is perhaps one of the most frequently used paradigms within addiction research. The inherent design of actively seeking a particular substance has high translational value for human addiction. As with CPP models, self-administration allows for the testing of additional factors that commonly contribute to drug relapse including associated stimuli such as drug cues, and thus provides a more comprehensive understanding of other factors that contribute to drug relapse in addition to the pharmacological actions of the abused drug.

1.5 Treatments

Stimulant abuse remains a persistent global problem, particularly in northern and central America and Oceania regions (WDR, 2012). MA and cocaine are the two most commonly abused drugs in this class, exhibiting a high abuse liability and potency. Persistent use of these drugs typically leads to maladaptive patterns of drug taking and a high incidence

of relapse, despite prolonged abstinence. This problem is further exacerbated by the lack of FDA approved medications for the treatment of stimulant addiction. Consequently, research efforts have largely focused on the development of pharmacological compounds to reduce the likelihood of relapse in abstinent individuals that primarily act on the DA system which has long been implicated as a target for drug addiction.

1.5.1 The role of dopamine in addiction. DA plays a fundamental role in reward-driven learning. Reward processing depends upon mesocorticolimbic DA systems whereby DA in the ventral tegmental area (VTA) projects to the nucleus accumbens (NAcc), amygdala, prefrontal cortex (PFC) and other areas of the forebrain (Kelley & Berridge, 2002). Natural rewards, as well as many drugs of abuse have been found to increase extracellular DA in the nucleus accumbens (NAcc) (Spanagel & Weiss, 1999). For example, food deprived rats display increases in DA activity in response to a food reward (Ahn & Phillips, 1999; Mirenowicz & Schultz, 1994; Wilson, Nomikos, Collu, & Fibiger, 1995) and similar results have also been observed with sucrose (Hajnal & Norgren, 2001; Hajnal, Smith, & Norgren, 2004).

Not surprisingly, brain reward pathways play a fundamental role in drug addiction processes. Nearly all abused drugs, when self-administered, acutely stimulate the DA system and increase DA release in the NAcc (Volkow et al., 1997). It is becoming increasingly clear that the ventral striatum plays an important role in mediating initial drug reinforcement, while the dorsal striatum becomes involved in more stimulus-response, cue-controlled habitual patterns that characterise chronic addictive behaviour and occur after prolonged drug taking exposure (Everitt & Robbins, 2005; Pierce & Vandershuren, 2010).

Initial drug taking that produces DA increases in the NAcc, begins as a goal-directed behaviour essentially driven by an action-outcome (A-O) association, whereby drug

administration is contingent upon the action of seeking it, i.e, pressing a lever. However, with prolonged drug taking, behaviour becomes habitual and driven by stimulus-response (S-R) associations. Here, drug taking is likely to be elicited through the presentation of drug cues and other associated stimuli and reflects the dominance of S-R associations on behaviour (Belin & Everitt, 2008).

As addiction develops from initial drug taking, progressively more and more dorsal areas of the striatum are affected. Belin and Everitt (2008) describe a cascading serial connectivity whereby connections with midbrain DA neurons progressively link the NAcc with dorsal regions of the striatum thus promoting a neurobiological shift from initial use to dependence. This pattern of DA brain activity is consistent with the behavioural shift from impulsive to compulsive drug taking (Koob & Le Moal, 2005). Furthermore, Spanagel and Weiss (1999) suggest that neuroadaptions within midbrain DA neurons occurs following chronic drug administration, leading to a disruption or desensitisation of brain mechanisms that mediate reward, further supporting the transition from A-O learning to S-R habit.
As evidence to support this neurobiological change, several studies using primates have shown density changes in the DA D2 receptor and DAT progressively shift from ventral to dorsal regions of the striatum with increased exposure to cocaine self-administration (Moore, Vinsant, Nader, Porrino, & Friedman, 1998; Nader et al., 2002; Porrino, Lyons, Smith, Daunnais, & Nader, 2004). Furthermore, several imaging studies have documented lower than normal levels of the DA D2 receptor in drug-addicted human subjects, including cocaine and MA abusers (Volkow et al., 1990; Volkow et al., 1997; Volkow et al., 2001). Volkow et al. (2001) have also observed an association between DA D2 receptor densities and metabolism rates in the orbitofrontal cortex (a brain region associated with obsessive and compulsive behaviours), and have hypothesized that DA deficits in this region may contribute to the compulsive nature of drug taking behaviour.

The psychostimulant effects of drugs such as cocaine and MA are generally believed to result from an increase in extracellular DA in limbic and striatal regions of the brain (Koob & Bloom, 1988; Self & Nestler, 1995). Stimulant drugs such as cocaine increase DA levels through reuptake inhibition by acting on the DAT (Horn, 1990; Amara & Kuhar, 1993; Giros & Caron, 1993), while amphetamines, including MA, evoke a presynaptic release of DA in addition to inhibiting reuptake (Seiden, Sabol, & Ricaurte, 1993). MA stimulates release of DA through a process called reverse transport. MA readily crosses the plasma membrane and once inside the cell can shift DA from secretory vesicles into the neuronal cytoplasm from which DA can then be released into the extracellular area by outward transport from the DAT (Sulzer & Rayport, 1990; Floor & Meng, 1996). The combined action of enhanced release and reuptake inhibition contributes to MA's high potency and rapid onset.

1.5.2 The substitution treatment approach. Stimuli typically function better as reinforcers when there is a strong response-outcome contingency. This is demonstrated with various drugs of abuse displaying strong reinforcing properties given their direct action on brain neurotransmitters, primarily DA. Weakening the response-outcome contingency by imposing a delay between a response and administration of the drug has been shown with cocaine to reduce drug-maintained behaviour, as well as lower rates of responding when drug delivery was slower (Panlilio et al., 1998; Abreu, Bigelow, Fleisher, & Walsh, 2001). The abuse liability of cocaine is largely dependent upon the speed with which the drug enters the bloodstream, for example, using rhesus monkeys, Balster and Schuster (1973) showed a reduction in self administration rates of up to 80% with slower drug delivery. These findings hold substantial importance for the development of replacement/substitute pharmacotherapies for stimulant addiction. Termed 'replacement' or 'substitution' therapy, this method of treatment involves the substitution of an abused drug for a less potent, less addictive

medication with properties similar to those of the drug. Ultimately, the goal of this treatment approach is to reduce the dose of the substituted drug over time until the individual is no longer dependent and abstinence can be maintained without severe withdrawal symptoms or craving (Panlilio et al., 1998). This approach has been demonstrated by treatment with slow acting forms of nicotine; for example, nicotine patches and gum given to reduce craving and allow abstinence from tobacco (Henningfield & Heishman, 1995). Similar approaches prescribe treatment with a drug from the same class but with slower acting pharmacokinetic properties, such as oral methadone treatment for heroin dependence (Dole & Nyswander, 1965; Simpson, Joe, & Bracy, 1987). Taken together, these findings support the development of similar medications for stimulant addiction.

1.5.3 Benztropine analogues. Consistent with a substitution treatment approach, extensive research over the last two decades has sought to investigate the therapeutic efficacy of agonist-like medications for stimulant addiction. Given that the DA system is a prominent target for psychostimulants, DA uptake inhibitors have provided a fruitful area for investigation. These medications act to substitute the effect of the abused drug by blocking the reuptake of DA through inhibition of the DAT, resulting in increased extracellular DA levels and similar effects to those of the drug. However, DA uptake inhibitors typically have a much slower onset of action, providing steady, long-term elevations of DA in the brain rather than the quick transient 'rush' of the drug. Due to slower and more sustained increases in DA, successful substitute medications would ideally reduce the abuse liability of these treatments allowing for the stabilisation of neurochemical deficits induced by chronic stimulant exposure (Rothman & Baumann, 2003).

One such DA uptake inhibitor is GBR-12909 (GBR) which has high affinity for the DAT and properties consistent with a potential treatment for stimulant abuse. As such, GBR

has been found to attenuate cocaine- and amphetamine-induced increases in extracellular DA (Baumann, Char, de Costa, Rice, & Rothman, 1994) and reduce cocaine self-administration in both rhesus monkeys (Glowa et al., 1995) and rats (Tella, 1995). As with other DA uptake inhibitors, GBR has a slow onset of action, such that peak increases in extracellular DA typically occur 40-45 minutes following administration as compared to cocaine which produces peak DA levels after 10-20 minutes (Baumann et al., 1994). However, GBR also produces effects that suggest potential abuse liability. For example, GBR has been successfully self-administered by rats and non-human primates (Bergman, Madras, Johnson, & Spealman, 1989; Howell & Byrd, 1991). It is thought that perhaps GBR reduces self-administration of cocaine through enhancement of the reinforcing effects of the drug as opposed to attenuating these effects (Holtzman, 2001).

The discovery of a class of highly selective DA uptake inhibitors, BZT analogues, marked an interesting development for substitute treatments. Derived from GBR, the original BZT molecule was initially of interest to researchers as a cocaine abuse medication in part due to its structural similarities with cocaine. Furthermore, BZT has clinical use for Parkinsonian symptoms and is not subject to significant abuse liability (Newman & Katz, 2009). Of the BZT's tested thus far, these compounds readily cross the blood-brain barrier (Raje, Newman, Gao, Eddington, & Natalie, 2003) and produce increases in extracellular DA for even longer periods than cocaine (Raje et al., 2005). Additionally, the effect of BZT analogues remains relatively drug-selective as food-maintained behaviour is not affected by doses that successfully decrease cocaine self-administration (Ferragud et al., 2009; Hiranita, Soto, Newman, & Katz, 2009). This is compared to the DA uptake inhibitor, methylphenidate, which is self-administered at doses that also effectively decrease food-responding (Li, Hiranita, Hayashi, Newman, & Katz, 2013).While BZT analogues are potent DAT inhibitors, they do not display a pharmacological profile consistent with that of cocaine,

possibly due to a different mode of interaction with the DAT (Beuming et al., 2008; Loland et al., 2008). Consistent with an ideal substitute medication, BZT analogues have a slower mechanism of action on the DAT which likely contributes to the reduced reinforcing properties of these compounds (Newman & Katz, 2009). However, traditional DA uptake inhibitors also exhibit slow rates of DAT occupancy but have a tendency to produce cocaine-like effects, in contrast to the more antagonist-like action of the BZT analogues (Li et al., 2013). This property of BZT is highly desirable for the development of substitute or replacement medications for psychostimulant addiction.

Within the class of BZT's, many *N*-substituted analogues have been studied for their therapeutic effects and been found to have a greater than 50-fold selectivity for the DAT over the SERT and NET (Katz, Kopajtic, Agoston, & Newman, 2004). One such analogue, AHN-1055, which also displays affinity for muscarinic receptors, has been examined as a potential substitute medication for the treatment of amphetamine addiction. AHN-1055 has been shown to block the rewarding, stimulant, and sensitizing effects of amphetamine in mice, while failing to produce these effects when administered alone (Velazquez-Sanchez, Ferragud, Renau-Piqueras, & Canales, 2011). AHN-1055 also dose-dependently blocks cocaine-induced CPP (Velazquez-Sanchez et al., 2009) and dose-dependently reduces self-administration of amphetamine (Velazquez-Sanchez et al., 2011). However, at high doses, AHN-1055 produces a weak stimulation of locomotor activity, but does not at low doses (Velazquez-Sanchez et al., 2009).

Another *N*-Substituted BZT analogue, JHW 007 (JHW) has high affinity for the DAT and does not produce cocaine-like behavioural effects. Desai, Kopajtic, French, Newman, and Katz (2005) suggest DAT occupancy as an important contributing factor for the behavioural effects of JHW, given that JHW has a relatively slow action on the DAT. This may help explain the lack of cocaine-like behavioural effects exhibited by this compound. JHW

produces a more antagonistic action compared to traditional DA uptake inhibitors. Past findings have shown JHW to block cocaine- and amphetamine-induced locomotor activity while failing to induce these behaviours when administered alone (Velazquez-Sanchez, Ferragud, Murga, Carda, & Canales, 2010; Velazquez-Sanchez, Garcia-Verdugo, Murga, & Canales, 2013). Remarkably, JHW also prevented amphetamine-induced long term brain neuroadaptions (Velazquez-Sanchez et al., 2013), further promoting its candidacy as a treatment for psychostimulant addiction.

1.5.4 The role of serotonin in addiction. While mesocorticolimbic DA systems play a crucial role in psychostimulant addiction, recent findings have likewise emphasised an involvement of the 5-HT system. Consistent with animal studies, Kish et al. (2009) and Kokoshka et al. (1998) found reductions in the concentration of the SERT in some brain regions of human MA users, although these reductions were less than those of DAT function (Fleckenstein et al., 1999). To accompany findings with DA, low concentrations of 5-HT, 5-H1AA (a metabolite of 5-HT) and SERT were also observed in the orbitofrontal cortex and may be associated with the high rate of suicide attempts in MA users (Zweben et al., 2004). In addition, Parsons, Koob and Weiss (1995) found significantly lower levels of extracellular 5-HT following a period of chronic cocaine self-administration, suggesting that deficits in 5-HT neurotransmission may underlie some of the symptoms of cocaine withdrawal. For example, deficits that deplete 5-HT in healthy subjects induce perseveration, a form of compulsive behaviour, and impaired decision-making, both of which are behaviours associated with chronic cocaine use (Pelloux, Dilleen, Economidou, Theobald, & Everitt, 2012). These findings suggest a relationship between the compulsive behaviour seen in cocaine addiction and reductions in 5-HT transmission (Ersche, Roiser, Robbins, & Sahakian, 2008). To further implicate a role for 5-HT in psychostimulant addiction, several studies have

reported a reduction in adverse addiction behaviours following treatment with various antidepressant medications. For example, Graves and Napier (2011) recently showed approximately 50% reduction in cue-induced MA-seeking following pre-treatment with the atypical antidepressant, mirtazapine, in rats. Similarly, pre-treatment with either citalopram or fluoxetine (both SSRI's) attenuated the rate at which cocaine was self-administered producing an overall downward shift in response function (Spealman, 1993). A novel 5-HT1A receptor agonist, JB0788, has also recently been found to dose-dependently reduce locomotor activity induced by MA (Picard et al., 2010). Transporter knockout (KO) mice studies have revealed compelling findings regarding the role of the SERT in cocaine addiction. DAT knockout (KO) mice still showed preference for, and self-administered cocaine (Sora et al., 1998; Rocha et al., 1998). However, double SERT/DA KO mice did not exhibit cocaine CPP (Sora et al., 2001). In addition to this latter finding, increases in extracellular DA concentrations were observed in the striatum of DAT KO mice but not in SERT/DAT KO mice (Shen et al., 2004), suggesting that SERT inhibition may play an important role in attenuating cocaine CPP. A further study by Takamatsu et al. (2006) revealed that pre-treatment with fluoxetine, an SSRI with inhibitory actions on the SERT blocked MA CPP and reduced locomotor sensitization.

1.5.4.1 Trazodone. TRAZ is a potent 5-HT2A antagonist and serotonin reuptake inhibitor. First introduced in Europe and Asia in the 1970's, TRAZ later became available in the US in 1981 for the treatment of major depression and as a bedtime sedative (Raatjes & Dantz, 2011). Although over time, newer antidepressants exceeded the use of TRAZ with 118%, 142% and 163% increases in prescriptions for sertraline, fluoxetine and citalopram respectively, as compared to a 45% increase for TRAZ in the US between 2001 and 2009 (Raatjes & Dantz, 2011).

TRAZ is characterised as a multifunctional drug, displaying different therapeutic properties and functions at different doses. It exhibits low dose hypnotic and sedative effects through inhibitory actions on 5-HT2A receptors, H1 histamine receptors and alpha-1 adrenergic receptors, and high dose antidepressant actions through blockade of SERT, 5-HT2C receptors and alpha-1 adrenergic receptors (Stahl, 2012). *In vitro* findings show a marked inhibition of the SERT in the hippocampus by 70% following high dose (20 mg/kg/day) administration of TRAZ (Ghanbari, El Mansari, & Blier, 2010), but compared to SSRI's such as citalopram and fluoxetine, TRAZ displays a reduced potency to inhibit SERT (Owens, Morgan, Plott, & Nemeroff, 1997). TRAZ has been shown to have a highly potent action on 5-HT2A receptors, for example, its ability to block the SERT is 100-fold less than its ability to block 5-HT2A (Stahl, 2012). Interestingly, TRAZ also has a moderate affinity for 5-HT1A receptors where it acts as an agonist (Odagaki, Toyoshima, & Yamauchi, 2005); the combined action with SERT inhibition is thought to enhance 5-HT neurotransmission. In addition, TRAZ has also been shown to successfully block the reuptake of 5-HT *in vitro* and *in vivo* (Ghanbari et al, 2010). Taken together, these actions more closely resemble those of traditional SSRI's and as such, TRAZ's dual mechanism of action is frequently categorised as serotonin antagonist-reuptake inhibition (SARI).

TRAZ's active metabolite, meta-chloro-phenyl piperazine (mCPP), has been suggested to contribute to the drug's antidepressant properties. mCPP has affinity for several 5-HT receptors, predominantly with agonistic actions at 5-HT2C receptors and antagonistic actions at 5-HT2A receptors (Fiorella, Rabin, & Winter, 1995). Further supporting the role of mCPP, Pazzagli, Giovannini, and Pepeu (1999) demonstrated that systemic administration of TRAZ lead to an increase of extracellular 5-HT in the frontal cortex of rats. The observed increases in 5-HT levels were suggested to be due to an interaction between the effects of TRAZ and its metabolites, primarily mCPP. Through i.v administration, mCPP was shown to

dose-dependently increase extracellular 5-HT levels in the rat diencephalon (Baumann, Rutter, & Auerbach, 1993); an increase which was prevented by the traditional SSRI, fluoxetine, thus suggesting a pre-synaptic effect involving the SERT. Pazzagli et al. (1999) therefore concluded that TRAZ may influence extracellular levels of 5-HT directly and indirectly through its metabolites.

TRAZ displays a complex pharmacological profile due to its multifunctional and antagonistic properties. While the mechanisms contributing to its therapeutic efficacy as an antidepressant are not fully understood, TRAZ produces actions on 5-HT consistent with current SSRI medications, including blockade of the SERT and moderate agonistic affinity for 5-HT1A receptors. Therefore, provided the correct dose is prescribed, TRAZ acts as a therapeutic antidepressant.

1.5.5 Rothman's hypothesis. While DA has received considerable attention as a therapeutic target for stimulant addiction, increasing evidence has established a key role for 5-HT in addiction processes. Research findings support deficits in both DA and 5-HT function following stimulant abuse, including marked decreases in extracellular DA and 5-HT in the NAcc (Parsons, Koob, & Weiss, 1995; Parsons, Smith, & Justice, 1991; Rossetti, Hmaidan, & Gessa, 1992). The presence of symptoms resembling major depression and suicidal ideation during withdrawal adds further support to the existence of 5-HT deficits within addiction.

Given the abundance of literature implicating a central role for both of these neurotransmitters, Rothman, Blough, and Baumann (2008) proposed a dual/deficit model of stimulant addiction whereby DA and 5-HT dysfunction jointly contribute to withdrawal symptoms, craving and relapse. This model postulates that decreased synaptic DA during stimulant withdrawal underlies anhedonia and craving, whereas decreased synaptic 5-HT

gives rise to depressed mood, obsessive thoughts and lack of impulse control. As a result, treatments targeted to correct or restore abnormalities in both DA and 5-HT function may be more effective in treating stimulant dependence than those that target DA alone.

Rothman et al. (2008) further justify a dual approach stating that traditional DA uptake inhibitors have the potential to exhibit an inherent abuse liability (Grabowski, Shearer, Merrill, & Negus, 2004) and that in contrast to DA, 5-HT does not tend to produce such behaviour. For example, drugs that produce extracellular increases in DA to a greater extent than 5-HT, such as amphetamine and phentermine (Rothman et al., 2001), tend to exhibit strong locomotor effects and support self-administration. However, drugs that produce extracellular increases of 5-HT greater than DA, including fenfluramine (Baumann et al., 2000), produce minimal locomotor activity and fail to support self-administration behaviour. Given these anti-reward properties of 5-HT, Rothman et al. (2008) propose that, in combination with DA, 5-HT may be useful for decreasing the abuse liability by counteracting the reinforcing and stimulant effects induced by elevations in synaptic DA (Burmeister, Lungren, Kirschner, & Neisewander, 2004; Czoty, Ginsburg, & Howell, 2002; Daw, Kakade, & Dayan, 2002). While BZT analogues do not typically exhibit an abuse liability to the extent of traditional DA uptake inhibitors, it is likely that concurrent modulation of 5-HT would have further therapeutic benefits.

2.0 Rationale, aims and hypotheses

MA is a highly potent drug with potentially devastating consequences following its abuse. Due to the rapid and direct action on brain neurotransmitters, MA exhibits a substantial abuse liability. Initial drug use can quickly progress to more compulsive patterns of drug taking with a high likelihood of relapse despite repeated attempts at abstinence. On the other hand, there is an increasing demand for the development of effective medications for stimulant abuse. Considerable evidence implicates both DA and 5-HT systems in MA addiction. However, to our knowledge no other studies have tested the combined efficacy of DA and 5-HT reuptake inhibition. Hence we conducted our study to further build on the work of Rothman et al. (2008), taking a dual DA/5-HT treatment approach aimed at stabilizing both DA and 5-HT drug-induced deficits during abstinence from MA.

Our highly selective DA uptake inhibitor (BZT analogue), JHW was decided upon for use within our experiments because of its promising therapeutic properties consistent with an effective substitute treatment for stimulant addiction. Yet the majority of studies have assessed JHW's therapeutic treatment efficacy with cocaine. Therefore we sought to test a similar treatment efficacy with MA. Given the proposed additional benefits of 5-HT (Rothman et al., 2008), we developed a combined treatment incorporating the antidepressant properties of TRAZ. Our initial intention was to use a traditional SSRI treatment such as fluoxetine or citalopram as these antidepressant drugs produce a more straightforward enhancement of serotonin transmission through blockade of the SERT (Anderson, 2004). However, due to financial restrictions (i.e., these SSRIs are extremely expensive), we acquired a less expensive medication, TRAZ.

The primary aim of our study was to assess the therapeutic efficacy of a pharmacological treatment combining actions of both DA and 5-HT reuptake inhibition for

MA addiction. Consistent with Rothman et al.'s hypothesis, we expected that compared to the administration of either treatment compound alone, the combination of JHW and TRAZ would attenuate MA-induced reinstatement to a greater extent.

3.0 Methods

3.1 Subjects

Subjects were 102, 2 – 2 ½ month old male Long Evans rats bred at the Animal facility of the Department of Psychology, University of Canterbury. Rats were housed in groups of four in polycarbonate cages (45 x 24 x 20 cm) on a reverse 12 hour light/dark cycle (lights on at 8 PM) under standard conditions of temperature (22 ± 2ºC) and humidity (45 – 55%). Food and water was available *ad libitum* for all 72 CPP rats, while all 30 self-administration rats received approximately 20mg of standard rat chow per day on a maintenance diet. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals, and were approved by the Animal Ethics Committee of the University of Canterbury (protocols 37R and 38R).

3.2 Pharmacological treatments

For the CPP experiments, Methamphetamine hydrochloride (METH, BDG Synthesis, Wellington) was dissolved in 0.9% saline and injected at a dose of 1mg/kg i.p. For both CPP and self-administration experiments, the treatments, JHW 007 and trazodone (Sigma Aldrich), were dissolved through sonication in 0.9% saline and 20% dimethylsulfoxide (DMSO) and injected at doses of 5mg/kg i.p and 10mg/kg i.p respectively. All compounds were prepared fresh daily and administered at a volume of 1mg/ml.

The self-administration experiments required an intravenous route of administration for MA, with rats receiving 0.05 mg/kg/infusion. The MA solution was prepared in a 24 μl bolus and dissolved in 0.9% heparinised saline at a volume of 0.175 mg/ml. The reinstatement dose of MA was 0.75 mg/kg i.p.

3.3 Experiment 1: Conditioned place preference

3.3.1 Conditioned place preference chambers. Each CPP testing chamber consisted of two same-sized compartments (40 x 34 x 45 cm) interconnected by a rectangular corridor (24 x 13 x 45cm). One of the compartments was decorated with white vinyl spots (8 cm diameter) on a black wall background and the other with black vinyl stripes (5 cm wide) on a white wall background. The black to white area ratio was approximately equal for each compartment. Manually removable guillotine doors were used to restrict access to a single compartment during conditioning. Four sets of testing chambers, totalling eight compartment areas, were positioned in a rectangular formation under two video cameras mounted to the ceiling. The location and movements of the rats were recorded using the tracking software, Viewpoint 2.5 (Champagne au Mont D'Or, France) which provided information on the distance travelled and time spent in each compartment.

(a) (b)

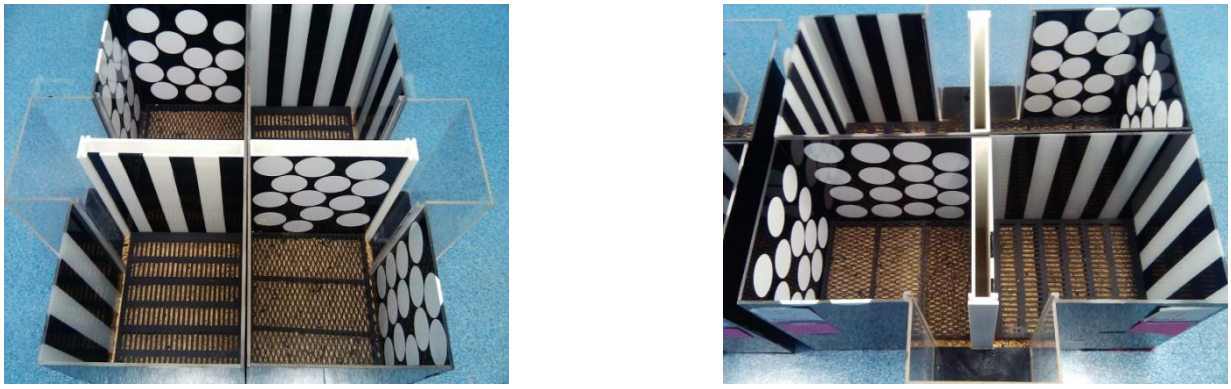

Figure 1. (a) Conditioned place preference chambers. (b) Conditioned place preference chambers: Side alley compartment.

3.3.2 Experimental design.

Table 1

Conditioned Place Preference Experimental Design

	Conditioning	Extinction/ Treatment	Reinstatement	Follow-up reinstatement
Control	Saline	Saline	Saline MA	Saline MA
Saline	MA	Saline	Saline MA	Saline MA
JHW	MA	JHW	Saline MA	Saline MA
TRAZ	MA	TRAZ	Saline MA	Saline MA
J+T	MA	J+T	Saline MA	Saline MA
Pilot study group	MA 0.75mg/kg OR 1.5mg/kg			

Seventy-two (72) rats were randomly assigned to six experimental groups (control n=12; saline n=12; JHW n=12; TRAZ n=12; J + T n=12; pilot n=12). The control group received saline throughout conditioning and extinction, while the saline, JHW, TRAZ and J + T groups were conditioned to MA and received their assigned treatments in a between-groups design during extinction. All animals received counterbalanced saline and MA (within-subjects) in an initial and follow-up reinstatement test, conducted after extinction. Baseline, extinction, conditioning test and reinstatement sessions all lasted 15 minutes while conditioning sessions lasted 30 minutes. MA = Methamphetamine hydrochloride; JHW = JHW 007; TRAZ = trazodone; J+T = JHW 007 + trazodone.

3.3.3 Behavioural assays. A pilot group of 12 rats were initially used to determine an optimum dose of MA that would produce robust conditioning within the main experiment. Two doses of MA were tested; 0.75mg/kg and 1.5mg/kg. Rats were randomly assigned to the low or high dose group with six rats per group in a between-groups design. All rats were first habituated to both CPP compartments in a drug-free state for five consecutive days. Following habituation, conditioning consisted of six days with all rats receiving alternating injections of MA (either 0.75 mg/kg or 1.5 mg/kg i.p) and saline. Rats were restricted to a given compartment during this phase, which was determined by compartment preference during baseline using a biased design. This type of design was used throughout to maximise the effects of the drug, by assigning the drug-paired compartment as that which the rat spent less time in during baseline. Additionally, locomotor activity was recorded during each conditioning session as distance travelled in centimetres. The degree of conditioning produced by each dose of MA was measured during a conditioning test which allowed rats' drug-free access to both compartments. These results were subsequently compared with baseline to determine statistical significance.

Following the pilot study, the main CPP procedure consisted of four phases: baseline, conditioning, extinction/treatment and reinstatement. A total of 60 rats were used for this experiment. Two batches of rats were tested allowing daily exposure and tests. Experimental conditions remained constant throughout the tests. Rats began baseline habituation to the CPP compartments for four consecutive days in a drug-free state. Access was given to both main compartments and time spent in each compartment area was recorded.

During conditioning rats received injections of saline and MA alternating between days with rats confined to one conditioning compartment. However, the control animals in this group (n= 9) received saline across all conditioning days. Locomotor activity was also

recorded each day during this time. A conditioning test measured the degree of conditioning from baseline with rats given access to both compartments in a drug-free state.

Following successful conditioning, rats were withdrawn from MA and exposed to extinction sessions in order to extinguish conditioned preference for the drug-paired compartment. Before each extinction session, rats were treated with either saline, JHW, TRAZ or J + T, and placed in the CPP apparatus for 15 min with free access to both compartments. Essentially, extinction sessions were identical to the test of conditioning except that a pharmacological treatment was administered before the session.

Reinstatement to MA CPP was assessed through MA drug priming following extinction. Rats received saline and MA counterbalanced over two consecutive days in a within-groups design, whereby each animal acted as its own control. Following injections, rats were given access to both compartments with time spent in each compartment recorded. Two weeks later, a follow-up relapse test was conducted involving identical procedures to the original test.

3.4 Experiment 2: Self-administration

3.4.1 Operant self-administration chambers. Drug self-administration procedures were conducted in operant self-administration chambers (Panlab S.L., Barcelona, Spain) fitted with two metal response levers protruding approximately 1 cm from the chamber wall and serving as active (right) and inactive (left) levers. Active lever presses resulted in intravenous infusions of saline or MA, from an infusion pump placed outside of the chamber, while inactive lever presses had no programmed consequences. Levers were positioned either side of an inset 4 x 4 cm water/food receptacle. Chambers were also equipped with a general house light and 4 cm diameter stimulus light located above the active lever which illuminated for all active reinforcements.

(a) (b)

Figure 2. (a) Self-administration chamber with infusion pump. (b) Response levers inside operant chamber.

3.4.2 Surgery. In preparation for surgery, rats were pre-treated daily with the antibiotic, Cephalexin (50 mg/kg s.c) for seven days and habituated to the operant chambers for approximately 15 minutes each day with both active and inactive levers removed so as not to interfere with the training process.

Immediately prior to surgery, rats were anesthetized with Avertin (2,2,2-tribromoethanol, 12.5 mg/ml, in 2.5% tertiary amyl alcohol, 2 ml/100g weight). The right jugular vein was isolated and sterile catheters (O/D 0.63 mm, I/D 0.30 mm, Camcaths, Cambridge, UK) inserted 3.2 cm into the vein. The catheter tubing was secured to the tissue by sutures and the opposite end was pushed through to exit the skin between the scapulae. This end was secured in place with sutures and a mesh collar attached to a threaded tip which was sealed with a protective cap of plastic tubing.

Post-operatively, animals were treated with the analgesic, Carprofen (5 mg/kg s.c) to minimise discomfort and sodium lactate (5ml s.c) to ensure adequate hydration. Cephalexin was administered for a further seven days following surgery to prevent infection and sodium lactate was given during recovery as required. Rats were housed individually following surgery and allowed to recover for seven days before commencing self-administration pre-training.

3.4.3 Experimental design.

Table 2

Self-administration Experimental Design

	Pre-training	Training	Extinction/ Treatment	Reinstatement test
Control		Saline	Saline	Saline MA
Saline	FR1, FR2, FR3	MA	Saline	Saline MA
JHW	FR1, FR2, FR3	MA	JHW	Saline MA
TRAZ	FR1, FR2, FR3	MA	TRAZ	Saline MA

Thirty (30) rats were randomly assigned to one of four experimental groups (control n=9; saline n=8; JHW n=8; TRAZ n=5). All rats were initially trained on FR1, FR2 and FR3 overnight sessions to press an active lever for MA. Animals in the control group were assigned on the basis of failing to learn to respond for MA. Following pre-training, the remaining three MA groups worked to respond for MA (and control for saline) for 10 consecutive days on 90 minute FR3 sessions. Rats then underwent an extinction phase for a further 10 days while concurrently receiving one of the following treatments: saline, JHW or TRAZ. Reinstatement involved the i.p administration of saline and MA across two consecutive days to assess reinstatement to MA drug seeking. Training and extinction sessions lasted 90 min while reinstatement sessions lasted 3 h. MA = Methamphetamine hydrochloride; control = animals receiving saline during training and extinction; saline = animals receiving MA during training and saline during extinction; JHW = animals receiving MA during training and JHW during extinction; TRAZ = animals receiving MA during training and TRAZ during extinction.

3.4.4 Behavioural assays. The self-administration procedure consisted of four phases: pre-training, training, extinction/treatment and reinstatement. Rats were initially pre-trained to press the right (active) lever for MA on extended sessions lasting approximately 15 h on a Fixed Ratio 1 (FR1) schedule of reinforcement (with one active lever press resulting in one infusion). A time-out period of one second was used throughout so as to encourage as much lever pressing as possible. Once rats reached the criterion of 30 reinforcements in an FR1 session, they progressed to FR2, now requiring two responses per one reinforcement. A criterion of 30 active reinforcements was needed to move from FR2 to FR3 where three active responses were needed per reinforcement. The computer program, Packwin (Panlab, S.L., Barcelona, Spain) provided information on the total number of active and inactive lever presses and active reinforcements per session. Before and after each self-administration session, rats had their catheters flushed with heparinised saline (0.1 ml, 70 U.I./ml) to help maintain catheter patency. Food and water was available in the chambers during extended sessions.

Once rats met the criterion of 30 active reinforcements during an extended FR3 session, they began self-administration training on 90 minute FR3 sessions for 10 consecutive days to stabilise responding. A minimum of 15 active reinforcements was needed with less than 20% variance during the last three days of training in order to begin extinction.

Following stable responding at FR3, rats were withdrawn from MA for 10 consecutive days during an extinction phase. Concurrently, rats received three treatments in a randomised between-groups design: saline, JHW and TRAZ, and were then placed in the chambers receiving infusions of saline on the active lever and no consequences for the inactive lever. By the last extinction session, a criterion of 10 or less active lever presses was required prior to tests of reinstatement. Our initial intention was to replicate the number of groups used in our CPP experiment, including a J + T group. While a sufficient number of

animals were cannulated in order to fill all five groups, we encountered problems training rats to respond for MA and therefore had to reduce our experiment to four groups, excluding the combination treatment group.

MA-induced reinstatement was assessed across two consecutive days. On day 1, rats received saline (i.p) prior to going in the chamber. This session acted as a control comparison to MA reinstatement which was conducted the following day. Saline sessions were conducted before MA sessions for all animals so as to minimise potential carry-over effects of MA. Both reinstatement sessions lasted 3 h with the infusion pump disconnected, therefore both levers produced no consequences when pressed, however, active and inactive lever presses were still recorded.

3.5 Perfusions

Immediately following MA reinstatement, rats were deeply anaesthetised with pentobarbital (125 mg/kg) and perfused transcardially to fix (using 4% paraformaldehyde in 0.1 M phosphate buffer) and remove the brain. Once the rat was under deep anaesthesia (totally unconscious and insensitive to pain) we opened up the thoracic cavity to expose the heart. A needle was inserted into the left ventricle and secured therein, and a heparinised saline solution was perfused. This method is preferred to killing the animal before perfusion as it helps the process of complete blood removal from the brain. This is important because blood interferes with histological techniques. Following saline perfusion, fixative was perfused and the brain removed.

4.0 Results

4.1 Data analysis

For the CPP experiments, all raw data (time in seconds spent in each compartment area) was converted into ratio values, expressed as the relative time rats spent in the drug-paired (DP) and non drug-paired (NDP) compartment (DP/NDP). However, due to substantial variability within the treatment group ratios, floor and ceiling values were introduced to limit variability, i.e., a ratio of nine, equating to 90% of time spent in the DP compartment was used as a ceiling cut-off, and a ratio of 0.11, equating to 10% of time spent in the DP compartment, as a floor cut-off.

For self-administration experiments, data was recorded as the number of active and inactive lever presses with the number of active reinforcements received dependent on the fixed-ratio score, i.e., FR1, FR2 or FR3.

4.2 Statistical analysis

Data was analysed through analyses of variance (ANOVA's) using the Statview 5.0 statistics programme. Repeated measures ANOVA's were conducted to determine significance for conditioning tests (as compared with baseline), extinction with treatment as a between-groups factor, and reinstatement tests using drug treatment (saline or MA) as a within-subjects factor. Fisher's post-hoc comparisons were used to further examine individual differences between each of the MA groups and the control for all significant results.

4.3 Experiment 1: Conditioned place preference

4.3.1 Introduction. MA addiction is a persistent problem in New Zealand and worldwide, the consequences of abuse often negatively impacting upon social, emotional and financial domains of life. The highly potent nature of MA, in combination with the relative ease of accessibility to its ingredients has largely contributed to MA's widespread abuse throughout New Zealand. Expressed in monetary terms, approximately US$ 200-250 billion (NZD$ 236-295 billion) would be required to cover global drug treatment-related costs. However, in actuality, the current amount spent on treatment is substantially lower as less than 20% of those needing treatment actually receive it (WDR, 2012); hence the increasing demand for sufficient medical intervention for MA addiction.

Research over the last 30 years, has implicated a fundamental role for DA, as both a target and treatment focus, in MA addiction. Studies investigating MA neurotoxicity have repeatedly found enduring deficits/depletion to DA levels and in particular change at the level of the DAT following prolonged MA exposure (Ricaurte, Schuster, & Seiden, 1980; Schmidt & Gibb, 1985). Consequently, medications serving to normalise DA deficits induced by MA are being thoroughly explored as treatment options to reduce relapse. Furthermore, serotonin has received increasing attention as a therapeutic target for MA addiction, (Rothman et al., 2008) with reports of similar deficits in 5-HT and transporter levels (Ricaurte et al., 1980). Research generally indicates DA and 5-HT as the main targets for MA-induced neuroadaptions leading us to question whether a combined treatment aimed at restoring levels of both DA and 5-HT would provide additional therapeutic benefits in reducing relapse.

In answering this question, we referred to models of CPP as a well-established experimental design used to assess the rewarding and motivating properties of various drugs of abuse. Derived from Pavlov's early classical conditioning, CPP has also frequently been

used to evaluate the efficacy of potential addiction treatments (Voigt, Mickiewicz, & Napier, 2011; Goeders & Goeders, 2004; Takamatsu et al., 2011) through the extinction-reinstatement model. Proposed by Shaham et al. (2003), this model consists of a series of extinction sessions, designed to extinguish a previously conditioned response, together with daily administration of a chosen treatment and a series of cue- , stress- or drug-primed reinstatement tests. We therefore decided upon use of this model within our experiment as it was consistent with our aims to evaluate drug reinstatement and provided an established approach to answering our research question.

The high prevalence and recurring nature of drug relapse highlights the need for successful treatments to aid in the prevention of relapse. This study intended to further build on the work of Rothman et al. (2008), taking a dual dopamine/serotonin treatment approach to MA addiction. Our current study tested the pharmacological compounds JHW 007, a BZT analogue, TRAZ, an antagonist of some serotonin receptor subtypes, and a combination of both treatments in their ability to attenuate relapse to MA CPP. Additionally, the stimulant properties of MA were assessed through measures of locomotor activity which were obtained during conditioning.

4.3.2 Methods. Long Evans rats were used as subjects in the CPP experiments. As we were uncertain of the optimum dose of MA needed to produce sufficient conditioning in these animals, we conducted a pilot study to test this prior to the main experiment. All rats were between 2-2 ½ months old at the time of testing. The pilot study consisted of 12 rats, six receiving a low dose (0.75mg/kg i.p) and another six a high dose (1.5mg/kg i.p) of MA alternately with saline across six consecutive days. The degree of conditioning produced by each dose was compared with baseline. Similarly, during the main experiment, using 45 rats, we tested the degree of conditioning produced by MA across all drug-treated animals. Rats were conditioned with MA alternately with saline for six consecutive days. Rats then underwent extinction sessions, receiving treatments of either saline, JHW 007, TRAZ, or a JHW 007 + TRAZ combination, and placed in the CPP chamber with access to both compartments. Following 10 days of extinction training, counterbalanced reinstatement sessions were conducted whereby rats received saline and MA i.p prior to going in the chambers (See Table 1, page 27). Additionally, locomotor activity was measured using a within-subjects design and recorded for each day during conditioning for both the pilot and main experiments.

4.3.3 Pilot study.

4.3.3.1 Conditioning locomotor activity. MA-induced hyperlocomotion was greater for animals receiving the high dose (1.5mg/kg); however, substantial standard errors in this group raised concerns over the reliability of these results ($F[1, 10] = .403$, $p = .6737$). Overall, animals receiving saline produced a relatively consistent baseline comparison with higher values for the third saline session possibly due to MA carry over effects or to contextual sensitization. It is unclear as to the true degree of hyperlocomotion induced by both low and high doses of MA; however, it is clear that both doses produced a stimulatory increase compared to saline.

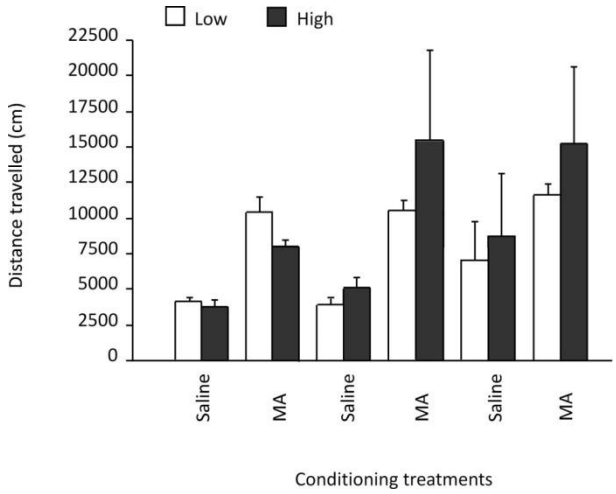

Figure 3. Locomotor activity for rats receiving saline (n=12) and MA (n=12) during conditioning. Error bars show standard errors of the means. *Significantly different from saline ($p < .05$). MA = Methamphetamine hydrochloride.

4.3.3.2 Conditioning test. Neither dose of MA produced significant conditioning from baseline in a test of conditioning ($F[1, 10] = .049$, $p = .8297$). Large within-group variability, as shown in Figure 4 demonstrates the inconsistency of both MA doses. Consequently, conditioning results were deemed unreliable and ultimately inconclusive.

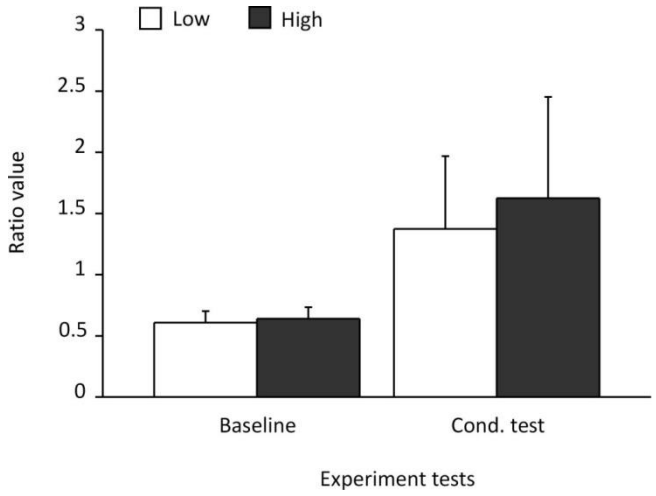

Figure 4. Ratio values of time (seconds) spent in DP and NDP compartments during the conditioning test as compared with baseline, for low (n=6) and high (n=6) dose MA groups. Error bars show standard errors of the means. *Significantly different from control group ($p < .05$).

4.3.3.3 Pilot study conclusions. Locomotor and conditioning test results for our pilot study did not clearly indicate a preferential dose of MA for conditioning, with large standard errors across both experiments posing problems for interpreting data. Overall, the low dose of MA appeared to produce smaller but relatively consistent increases in locomotor activity, whereas the high dose seemed to produce larger albeit variable increases in both locomotor activity and conditioning. However, it is important to note that we did obtain a trend of conditioning in the right direction for both low and high dose groups compared to baseline, even though this difference was not significant. We therefore decided upon a MA dose of 1mg/kg and proceeded with our main experiment in the hope that using more animals per group, we would achieve robust levels of conditioning. This dose is also supported by several recent CPP studies showing significant levels of MA-conditioning in rats (Voigt & Napier, 2012; Baracz et al., 2012; Herrold, Shen et al., 2009; Zakharova, Leoni, Kichko, & Izenwasser, 2009).

4.3.4 Main experiment.

4.3.4.1 Conditioning locomotor activity. In the main experiment, rats that received MA during conditioning showed significant hyperlocomotion compared to saline (F[1,58] = 4.964, p = .0086), as clearly illustrated in Figure 5.

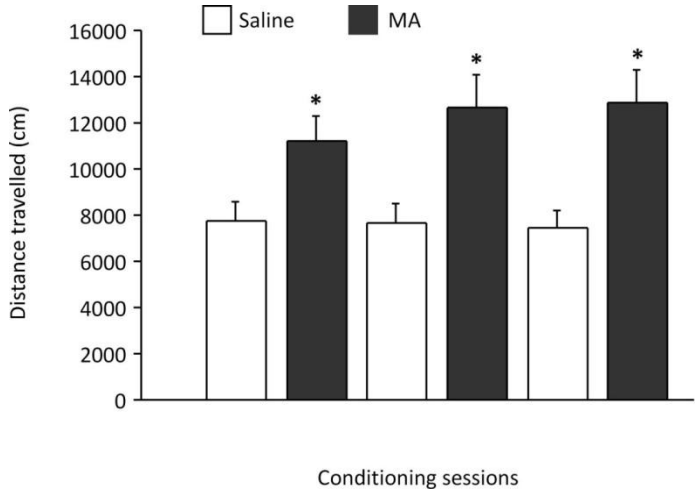

Figure 5. Locomotor activity for rats receiving saline (n=60) and MA (n=60) during conditioning. Error bars show standard errors of the means. *Significantly different from saline ($p < .05$). MA = Methamphetamine hydrochloride.

4.3.4.2 Conditioning test. We initially encountered difficulties getting some rats to show successful conditioning for the DP compartment. As this was essential prior to extinction training and the administration of treatment, we excluded a total of 15 rats, reducing the total number of rats to 45. The test of conditioning revealed a significant interaction between compartment preference ratio and treatment group ($F[4,40] = 2.822$, $p = .0375$) with all MA groups in the conditioning test displaying significant DP compartment preference compared to baseline. A Fisher's post-hoc test did not reveal any significant differences between MA-treated groups.

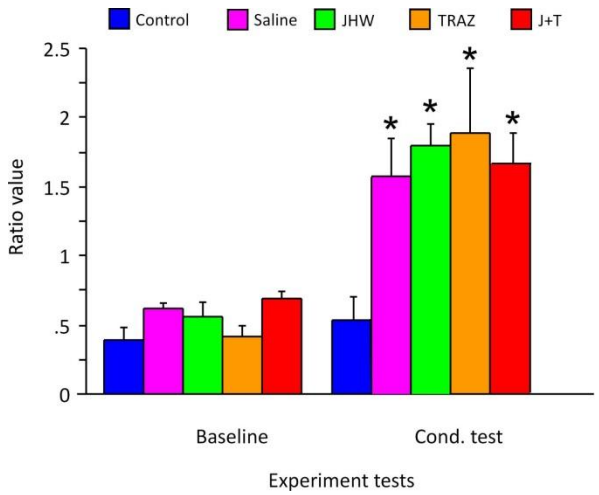

Figure 6. Ratio values of time (seconds) spent in DP and NDP compartments during the conditioning test as compared with baseline, for control (n=9) and MA groups (n=36). Error bars show standard errors of the means. *Significantly different from control group ($p < .05$). JHW = JHW007; TRAZ = trazodone hydrochloride; J+T = JHW007 + trazodone hydrochloride.

4.3.4.3 Extinction/treatment. Extinction results showed varied data trends for each of the MA-treated groups. All groups except TRAZ displayed a somewhat stable pattern of compartment preference across extinction with no significant change in ratio values throughout. The TRAZ group, however, showed a distinct peak in DP compartment preference around days five and six followed by a steep decline whereby its group values ultimately match those of the control group. While there was no significant effect of extinction day or group, there was a significant extinction*treatment interaction (F[4,40] = 1.999, p = .0160), but this was likely due to the pattern of extinction in the TRAZ group (p = .0245, by post-hoc Fisher's test).

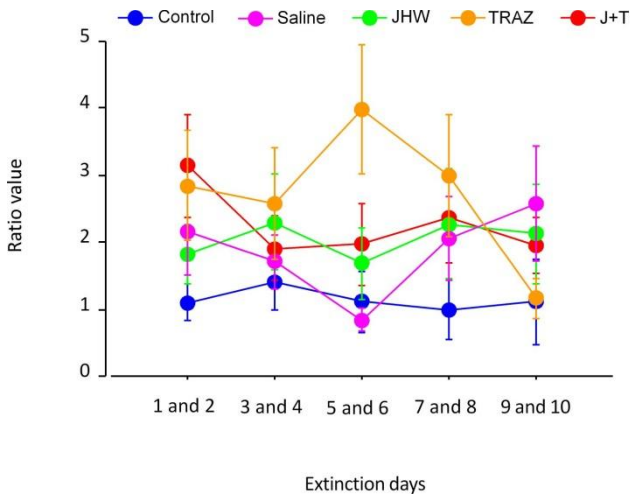

Figure 7. Ratio values of time (seconds) spent in the DP compartment during extinction for control (n=9) and MA groups (n=36). *Significantly different from control group ($p < .05$). JHW = JHW007; TRAZ = trazodone hydrochloride; J+T = JHW007 + trazodone hydrochloride.

Overall, the data (Figure 7) showed no clearly discernible pattern of extinction (i.e., a gradual decrease in ratio values for all MA groups across extinction days), and therefore it cannot be confidently stated that all animals successfully extinguished preference for the DP compartment prior to undergoing tests of reinstatement. However, we proceeded with the tests because saline animals in the first batch of tests showed sufficient levels of extinction after 10 days, and therefore this fixed time period was applied to all groups to ensure consistency. Furthermore, an extinction period of between 8-12 days has been shown to be adequate in similar CPP experiments (Itzhak & Martin, 2002; Mueller & Stewart, 2000).

4.3.4.4 Reinstatement tests. As illustrated in Figure 8a, the first reinstatement test following extinction did not produce a significant interaction. Animals treated with saline showed residual DP compartment preference with substantial standard errors similar to that of MA-treated animals. While not significant (p = .5652), visually, the TRAZ group showed attenuation of DP compartment preference following treatment with MA compared to other groups, with results more closely resembling those of the control group. Interestingly, the TRAZ group was also the only group to match control levels at the end of extinction. However, large standard errors for all groups made interpretation difficult. In addition, the saline-treated animals did not provide a reliable comparison for MA reinstatement.

A follow-up reinstatement test conducted two weeks later (Figure 8b), similarly did not produce a significant interaction with ratio values (equating to compartment preference) looking remarkably similar for both saline and MA reinstatement. However, both JHW and J+T groups showed evidence of drug seeking attenuation suggesting a possible effect of treatment. Interestingly, ratio values for all groups (excluding the control) receiving saline and MA during reinstatement were noticeably lower than those obtained during the first reinstatement test two weeks prior (Figure 8a).

(a) (b)

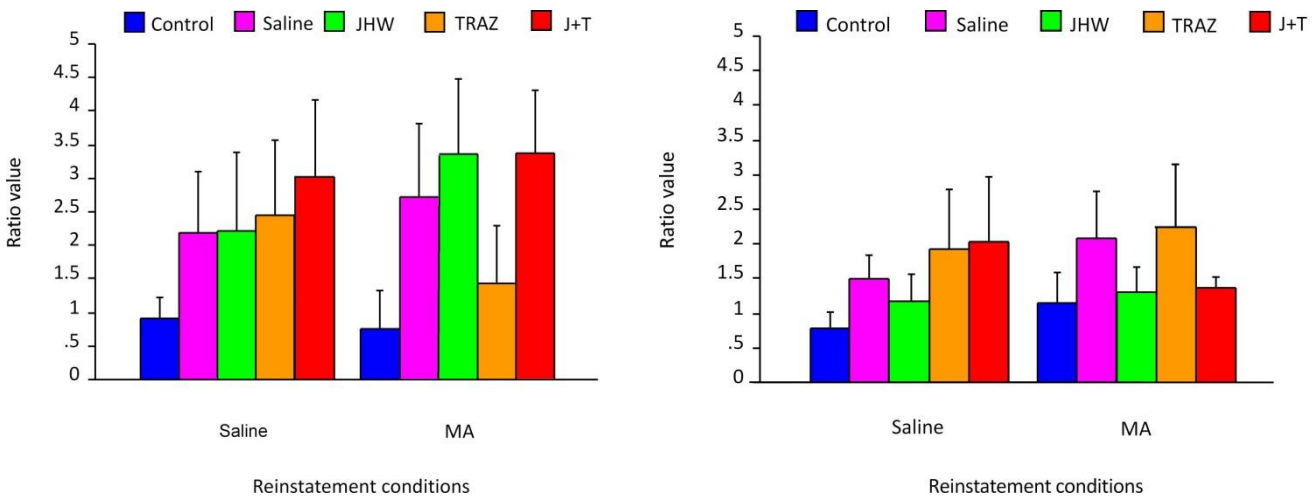

Figure 8. (a) Reinstatement test 1: ratio values for animals receiving counterbalanced i.p injections of saline and MA during reinstatement, for control (n=9) and MA (n=36) groups; (b) Reinstatement test 2: ratio values for animals receiving counterbalanced i.p injections of saline and MA during reinstatement. *Significantly different from control group ($p < .05$). JHW = JHW007; TRAZ = Trazodone hydrochloride; J+T = JHW007 + Trazodone hydrochloride.

4.3.5 Discussion. In this CPP experiment, we aimed to evaluate the potential efficacy of JHW, TRAZ, and a combination thereof, as treatments to attenuate relapse to MA-seeking in Long Evans rats. All rats were first habituated to CPP compartments through baseline testing sessions which established existing compartment preference prior to drug intervention. Animals were then conditioned to MA alternately with saline in DP and NDP compartments before a test of conditioning, with subsequent extinction sessions conducted to extinguish the DP compartment preference. Concurrently, animals were treated daily with the aforementioned compounds and following a pre-determined period of extinction, priming injections of saline and MA were administered to test the efficacy of treatments to reduce reinstatement to MA-seeking.

We established a significant effect of conditioning, with all MA-conditioned groups displaying greater preference for the DP compartment compared to baseline. However throughout extinction, contrary to expectation, animals previously conditioned to MA did not appear to gradually extinguish preference for the DP compartment, rather patterns of extinction reflected minimal change in preference throughout. The trend of behaviour displayed by the TRAZ group raised doubts as to whether this group underwent successful extinction, despite ultimately reaching control levels. TRAZ animals showed evidence of possible residual responding to MA during the first test of saline reinstatement following extinction. Conversely, these animals also showed attenuated DP compartment preference during the test of MA reinstatement suggesting an effect of treatment whereby extinction did occur. While this latter outcome seems likely given the comparatively high DP compartment preferences produced by other groups in this test, an unreliable saline reinstatement comparison coupled with large standard errors made the TRAZ data trend overall difficult to interpret. However it is possible that counterbalancing of MA and saline throughout reinstatement tests may have contributed to an unstable saline comparison, as animals first

receiving MA may have exhibited residual drug responding when administered saline the following day.

The follow-up reinstatement test similarly produced unexpected results. During a two week abstinence period between tests, we predicted an incubation of drug craving effect (Pickens et al., 2011) whereby drug craving intensifies with prolonged abstinence. However, our results failed to support this hypothesis, instead resulting in noticeably lower levels of reinstatement overall compared to the first test. Many studies that observe successful incubation effects use self-administration paradigms (Shepard, Bossert, Liu, & Shaham, 2004; Bienkowski et al., 2004; Abdolahi, Acosta, Breslin, Hemby, & Lynch, 2010), therefore we must consider that CPP may not be the best model to generate states of drug craving, at least not to the same extent as self-administration. This may be partially due to the passive nature of drug administration in CPP models compared to active drug-seeking and -taking in self administration. However, it is noteworthy that in the follow-up reinstatement test, both JHW and J+T groups showed no MA-induced reinstatement as compared with control values, suggesting an attenuation of MA-seeking. However, we cannot draw definitive conclusions given the lack of sufficient evidence for reinstatement in the saline group. These reinstatement findings, together with those displayed by the TRAZ group during extinction, warrant further replication of this study with some technical changes, as detailed below.

Throughout our experiment we encountered problems with large standard errors, indicating varied within-group compartment preferences and complicating data interpretation. Our study relied on measures of duration in each compartment area to determine MA conditioning, extinction and most importantly, reinstatement. However, the inherent design of CPP has the potential to be largely influenced by external factors.

A potentially significant limitation to our study may have been the use of spots and stripes as stimuli to discriminate the testing compartments. Using a white adhesive vinyl

material, these shapes were mounted to the chamber walls. When testing, rats were frequently observed picking and gnawing at any imperfections on the walls, usually edges of the vinyl material, thus prolonging their duration in one area and reducing locomotor activity. In retrospect, it is likely that this behaviour influenced our results and contributed to the large within-group variation seen throughout. In replicating this study, we propose the use of plain black and white compartments and a permanently implemented biased design so as to limit distractibility factors and simplify testing areas. Furthermore, this design is supported by several other CPP drug studies using MA (Berry, Neugebauer, & Bardo, 2012; Takamatsu et al., 2006; Gehrke, Harrod, Cass, & Bardo, 2003; Goeders & Goeders, 2004) and cocaine (Itzhak & Martin, 2002).

Our particular CPP design used a side alley corridor (24 x 13 x 45cm) as a means for rats to move between compartments. This specific design has been used successfully in mice to produce cocaine and amphetamine conditioning (Velazquez-Sanchez et al., 2009; Velazquez-Sanchez et al., 2010) and we expected similar results in rats. However, levels of activity and exploration in rats and mice differ considerably. We suggest that this compartment set-up may have made it difficult for rats to navigate their way between compartments, with rats sometimes spending long periods of time in the neutral corridor space rather than either of the compartments. Other CPP studies frequently utilise a dual compartment apparatus equipped with a cut-out hole in the shared compartment wall so as to allow direct and easy access between areas, without the need for an additional neutral area (Takamatsu et al., 2006; Itzhak & Martin, 2002; Goeders & Goeders, 2004). Overall, we propose that the use of black and white compartments and an archway door between compartments would reduce distractibility issues and hesitancy moving around the apparatus. Additionally, it should be considered whether mice would be more preferable in CPP studies

compared to rats. As CPP relies on the animals' movement between compartments as a measure of preference, the highly active nature of mice may be better suited to this paradigm.

In conclusion, our CPP experiment did not yield significant reinstatement results to MA in tests performed immediately following extinction or two weeks later. In addition to possible CPP design flaws, problems during extinction likely contributed to these findings, as groups did not appear to extinguish DP preferences. However, the apparent attenuation of MA-seeking displayed by both the JHW and J+T groups warrants further investigation.

4.4 Experiment 2: Self-administration

4.4.1 Introduction. Self-administration is an established experimental paradigm, effective in measuring the reinforcing or aversive properties of various substances. Beginning with Skinner's initial design of the operant chamber (Skinner box) in the 1930's, self-administration has become a widely popular method for assessing drugs of abuse. This model of testing is deemed highly relevant for human addiction models as animal subjects are motivated to self-administer the drug rather than passively receive it. Furthermore, in animal addiction studies, this model has been consistently shown to produce robust states of addiction (Pickens et al., 2011) and thus provides a useful tool for evaluating potential treatments to aid in the prevention of drug relapse.

In line with our previous CPP study, we sought to test the efficacy of JHW and TRAZ to attenuate reinstatement to MA using the extinction-reinstatement model within a self-administration design. Interestingly, food deprivation studies have revealed marked increases in the self-administration of abused drugs (Carroll & Meisch, 1984) with Carr, Kim, and Cabeza de Vaca (2000) showing increases in reward magnitude by several drugs of abuse and some DA agonists following food restriction. Complementing these findings, impairments in the acquisition of cocaine self-administration (Wellman, Nation, & Davis, 2007) and amphetamine CPP (Davis et al, 2008) have been found in animals with free access to a high fat diet. Therefore, in an attempt to heighten reward sensitivity to MA in our study, we began all rats on a food maintenance diet prior to the initiation of MA training.

As with CPP, our design additionally incorporated the chronic administration of a pharmacological treatment alongside extinction; an approach believed to further facilitate the extinction process through opportunities for learning new contingencies under the effects of a neuroprotective treatment (Quirk & Mueller, 2008). While there is limited research using this particular design in CPP models, Reichel and See (2012) suggest evidence for the use of a

chronic treatment regimen as more beneficial compared to a single dose regimen in a model of self-administration. In their study, modafinil, a cognitive enhancing drug was investigated for its therapeutic potential in attenuating relapse to MA. A single acute dose of modafinil administered prior to tests of relapse was found seemingly ineffective, however, when administered chronically alongside sessions of extinction, produced successful attenuation of reinstatement to MA-seeking. Further supporting the chronic treatment approach are findings by Sorge et al (2005) who found chronic maintenance of buprenorphine significantly reduced heroin and cocaine-seeking during extinction. Given the suggested benefits of a chronic dosing regimen over a single dose treatment approach, we hoped to essentially potentiate the effects of both JHW and TRAZ through concurrent administration of these treatments with sessions of extinction.

4.4.2 Methods. Following recovery from cannulation surgeries, rats began pre-training for MA. Rats were placed in operant testing chambers and presented with two levers, active and inactive. For all chambers, responses on the right lever (active) resulted in infusions of MA (0.05mg/kg/infusion i.v.), while the left lever (inactive) had no programmed consequences. In order to facilitate learning for MA self-administration, rats were exposed to both levers within the chamber during extended pre-training sessions lasting several hours. During this time, rats were pre-trained on FR1, FR2 and FR3 schedules of drug reinforcement, i.e., during FR1 sessions, one press on the active lever corresponded to one MA reinforcement. Once animals reached a criterion of 30 reinforcements in an extended session, they progressed to FR2, now requiring two active lever presses per reinforcement. Similarly, once 30 reinforcements were met during this phase, animals moved to FR3. Once rats had stably achieved the 30 reinforcement criteria for all extended fixed-ratio schedules, they began training on 90 minute FR3 sessions whereby 10 consecutive days of MA responding with at least 15 reinforcements per session was required to begin extinction.

Throughout extinction, rats received their assigned treatment i.p. (saline, JHW, TRAZ) and were placed in the operant chambers, receiving saline on the active lever and no consequence on the inactive lever. Across this 10-day extinction period, rats were expected to extinguish preference for the active lever with a criterion of 15 or less active lever presses required on the final day of extinction.

Tests of MA reinstatement were conducted in a non-counterbalanced fashion across two days with all animals receiving saline on the first day and MA (i.p) on the second day, before being placed in the operant chambers for three hours. Non-counterbalancing was done in order to collect sufficient brain samples from animals showing MA reinstatement. During reinstatement, as with extinction sessions, rats received saline on the active lever and no consequences on the inactive lever.

4.4.3 Self-administration training. Figures 9(a) and (b) below show the number of (a) active and (b) inactive lever presses for each group across the 10 day training period. All MA-treated animals showed significantly higher rates of responding for MA on the active lever compared to controls (F[3, 27] = 2.126, p = .0281) while there were no significant differences between all groups for inactive lever presses (F[3, 27] = .485, p = .8843) showing rats were able to successfully discriminate MA from no infusion.

(a) (b)

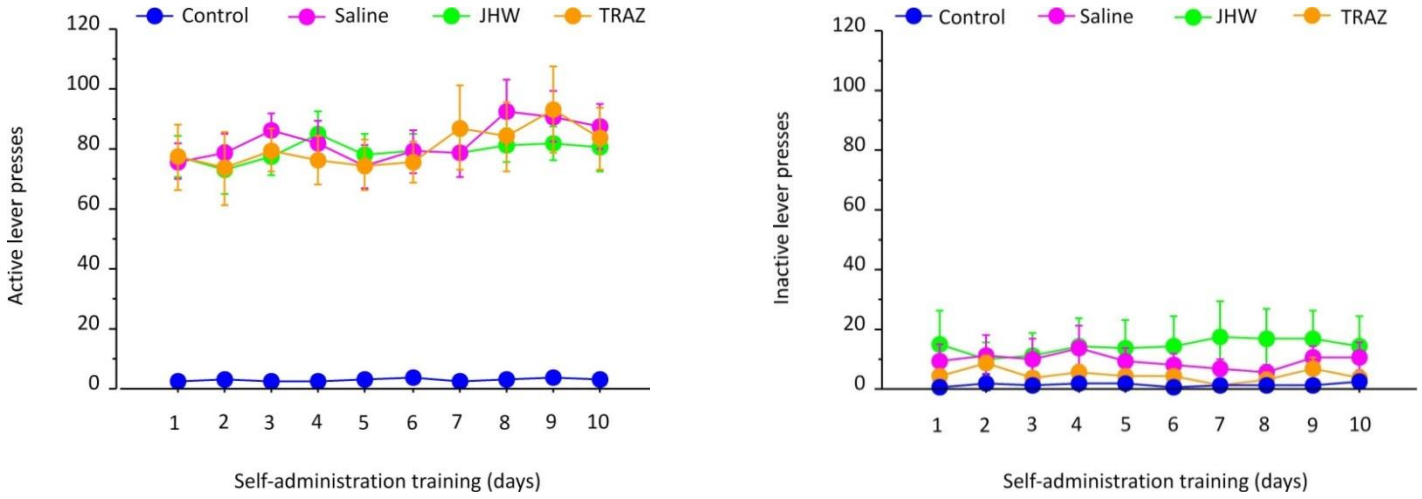

Figure 9. (a) Active lever presses during self-administration training for control (n=9) and MA groups (n=21). (b) Inactive lever presses during self-administration training for control (n=9) and MA groups (n=21). Error bars show standard errors of the means. Significance level is p = .05. JHW = JHW 007; TRAZ = trazodone hydrochloride.

4.4.4 Self-administration extinction. Following a large initial decrease from MA training, both saline and JHW groups demonstrated a gradual decline in active lever presses throughout extinction with the control group maintaining a steady baseline (Figure 10a). Interestingly, on the first day of extinction for TRAZ animals, treatment drastically attenuated responding on the active lever and thereafter, responses remained approximately at the level of control animals. We observed a significant effect of extinction day (F[3, 27] = 14.236, p = <.0001) showing the successful extinction of responding on the active lever as established during MA training, and a significant interaction effect between active lever presses and treatment group (F[3, 27] = 1.567, p = .0418). Fisher's post-hoc test did not reveal a significant difference between the MA training groups. However, responses on the inactive lever produced intriguing results for the TRAZ group, as these animals showed significantly greater lever presses compared to all other groups (F[3, 27] = 5.971, p = .0031) despite large standard errors.

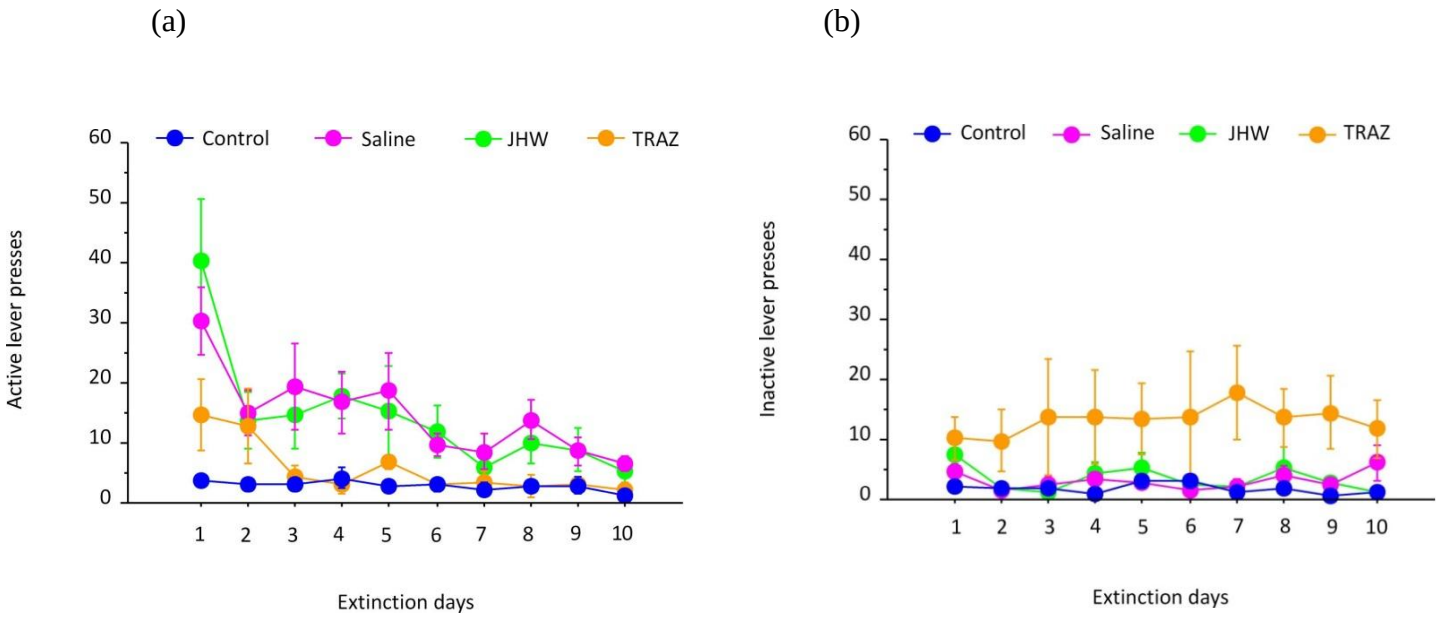

Figure 10. (a) Active lever presses during extinction training for control (n=9) and MA (n=21) groups. (b) Inactive lever presses during extinction training for control (n=9) and MA (n=21) groups. Error bars show standard errors of the means. Significance level is p = .05. JHW = JHW 007; TRAZ = trazodone hydrochloride.

4.4.5 Self-administration reinstatement. Tests of saline- and MA-induced reinstatement produced a significant interaction effect in the main ANOVA (F[3, 26] = 6.838, p = .0015). As hypothesised, the JHW group showed a significant attenuation of MA-seeking compared to the saline group (p = .0420, by post-hoc Fisher's test), whereas, TRAZ animals clearly showed increased MA-seeking relative to saline.

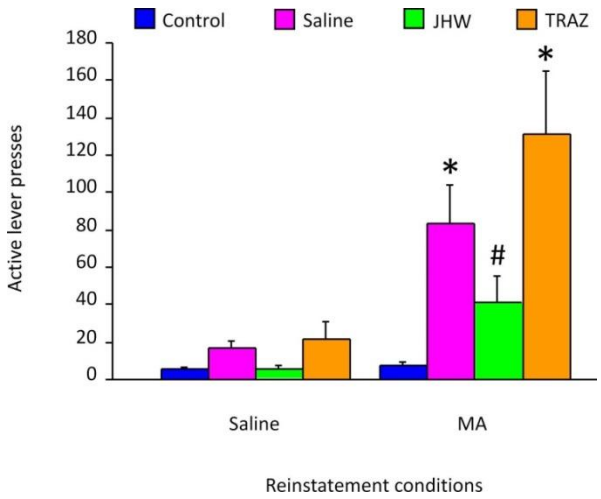

Figure 11. Active lever presses for animals receiving non-counterbalanced i.p injections of saline and MA during reinstatement, for control (n=9) and MA (n=21) groups. *Significantly different from control group ($p < .05$). #Significantly different from saline group ($p < .05$). JHW = JHW007; TRAZ = trazodone hydrochloride.

4.4.6 Discussion. Our current experiment aimed to assess JHW and TRAZ as potential pharmacological treatments to attenuate MA-induced reinstatement in a model of self-administration. Rats were pre-trained to respond for MA on extended sessions until criterion values were met. MA training took place across 10 consecutive days whereby stable responding at FR3 was required in order to begin sessions of extinction and the concurrent administration of each group's assigned treatment (See Table 2, page 32). Finally, priming injections of saline and MA across two consecutive days aimed to measure the relative degree of MA-seeking attenuation produced by both JHW and TRAZ groups. As compared to our CPP experiment, we only conducted one reinstatement test with self-administration as we observed significant effects after extinction and attenuation with JHW, which is what we had hypothesised and were pursuing to demonstrate. Indeed, we could have conducted further tests but having obtained a result already and considering time limitations, we thought it best to finish the experiment there and then and collect the brains.

Following extended pre-training sessions, animals successfully met criteria for MA-training, showing significantly greater rates of responding on the active lever compared to controls. All MA-treated groups then appeared to successfully extinguish responding for the MA-paired lever, with all active lever presses ultimately falling to the level of controls. Interestingly, TRAZ appeared to accelerate the extinction process, as relative to saline and JHW groups, TRAZ animals showed a rapid attenuation of MA-seeking on the active lever. However, it is unclear as to whether TRAZ acted to solely attenuate drug seeking behaviour or whether it actually inhibited motor and/or motivational processes. We did not additionally test for this possibility (for example by testing the effects of TRAZ on responding for natural reinforcers such as food or sucrose) in our current experiment, and previous research on TRAZ has likewise not investigated the inhibitory action of TRAZ on drug vs. natural reinforcement. Therefore, further research exploring TRAZ's effects on food or sucrose-

maintained responding would provide additional insight and aid in the interpretation of our results. Unexpectedly, inactive lever presses during extinction were significantly greater for the TRAZ group compared to all other MA-treated groups, further illustrating TRAZ's complex mechanism of action. We can assume an initial increase in inactive lever presses during extinction as rats fail to receive an infusion of MA through the active lever and therefore try the inactive lever. However, if this was indeed the case for the TRAZ group, we should have seen this trend in behaviour for all MA-treated animals and not just those receiving TRAZ, thus perhaps suggesting a degree of confusion in TRAZ-treated animals. During MA reinstatement (Figure 11), TRAZ produced an increase in response rates compared to saline. This finding is unexpected given the rapid trend of extinction exhibited by this group. Clearly TRAZ has a complex mechanism of action. In our experiment, it is not clear whether this drug exclusively inhibited drug reinforcement or if natural reinforcement pathways were additionally affected.

In contrast to the paradoxical findings obtained with TRAZ, the result produced by JHW treatment was straightforward and highly relevant. As expected, animals showed a gradual decline in responses on the active lever throughout extinction (Figure 10a) and consistently low responses on the inactive lever (Figure 10b). Reinstatement findings with JHW showed a clear attenuation of MA-seeking compared to the saline group (Figure 11), further supporting previous research implicating JHW as a promising treatment target for stimulant addiction (Velazquez-Sanchez et al., 2010; Velazquez-Sanchez et al., 2013).

To further build on Rothman's dual/deficit hypothesis, it would have been beneficial to substitute TRAZ for a more traditional SSRI treatment, such as fluoxetine, and also include a JHW + antidepressant combination group. A self-administration design was effective in producing reliable results for our control, saline and JHW groups; therefore it is likely the

action of TRAZ itself that contributed to this group's contradictory results evident throughout our study.

5.0 General Discussion

In line with Rothman et al. (2008), the aim of the present experiments was to test whether dual modulation of DA and 5-HT produces greater attenuation to MA-seeking reinstatement as compared to either DA or 5-HT alone. JHW was found to significantly attenuate MA-induced reinstatement in a model of self-administration, while TRAZ produced an unexpected behavioural profile in both experiments. Notably, our combination treatment group failed to reliably attenuate MA reinstatement using CPP as we did not establish a strong reinstatement comparison with saline. However, problems with design and treatment dose were believed to have impacted upon results.

The dual deficit hypothesis of Rothman et al. assumes the successful attenuation of drug-seeking with DA or 5-HT treatment alone, and a further reduction in drug-seeking when both treatments are administered. In our CPP experiment, treatment with JHW or TRAZ alone did not produce a reliable and significant attenuation of MA drug-seeking, nor did our combination group. Although, a mild attenuation of MA reinstatement was observed with JHW alone and the combination group in a follow-up reinstatement test two weeks after treatment (see Figure 8b). However, we did not observe this attenuation in an initial test immediately following treatment (see Figure 8a). It is possible that, while TRAZ exhibited an unexpected trend of behaviour when administered alone in both CPP and self-administration experiments, the combination of JHW and TRAZ produced a synergistic effect supporting the attenuation of MA-induced reinstatement in our CPP experiment. However, it is unclear as to why we see such attenuation only after a period of abstinence between reinstatement tests. The interpretation of these results were complicated by the fact that animals treated with saline during extinction (i.e., those expected to show high MA reinstatement), did not show sufficient levels of reinstatement to act as a reliable comparison for the attenuation seen in both the JHW and combination groups. Together with various CPP design flaws, it is likely

that additional factors aside from drug- or non-drug pairing influenced time spent in each compartment. Therefore we are not confident that our findings with JHW and J+T using CPP are a true reflection of the effect that these treatments had on MA-induced reinstatement. In addition, we were unable to test this treatment combination using a self-administration paradigm due to time constraints, thereby making it difficult to conclude findings for this group based solely on results from our CPP experiment. Thus, in order to more reliably test Rothman's hypothesis, we need to replicate our CPP and self-administration experiments implementing our previously proposed changes to the CPP design, and substituting TRAZ for a traditional SSRI treatment such as fluoxetine or citalopram.

When administered alone, TRAZ did not attenuate reinstatement to MA-seeking in either CPP or self-administration experiments. We must consider that given the multifunctional properties of TRAZ, our given dose was not sufficient to produce antidepressant effects but rather induced sedative, hypnotic effects common of lower doses of the drug (Stahl, 2012). There is limited research regarding optimum doses of TRAZ for use in rats, as the majority of studies investigate the effects of TRAZ within the human population (Khouzam, Mayo-Smith, Bernard, & Mahdasian, 1994; Papakostas & Fava, 2007; Raatjes & Dantz, 2011). However, Balsara, Jadhav, Gaonkar, Gaikwad, and Jadhav (2005) observed evidence of possible sedation and ptosis in rats receiving a dose range of 10-50 mg/kg, suggesting that even higher doses may be required to produce antidepressant effects. It is possible that the drastic reduction in active lever responding during extinction for our self-administration experiment was not in fact due to an inhibition of drug reinforcement but rather sedation, i.e, rats were too tired to press the lever. This could additionally explain why rats still reinstated responding after a period of extinction. If rats were indeed tranquilised by the effects of TRAZ and rendered less mobile, this may also help to explain the large ratio

values, indicating greater time spent in one compartment over the other, evident during extinction in our CPP experiment.

However, the trend of inactive lever presses during extinction in the self-administration experiment does not support our sedative explanation. TRAZ animals showed significantly greater responses on the inactive lever relative to all other groups. A significant limitation to this finding was the TRAZ groups' small sample size of only five animals. Through closer examination of each animal's results, we observed that only two out of five animals showed high inactive lever responses while the other three did not, and consequently we are not confident that this significant result is a true representation of the effect that TRAZ had on animals in this group.

Although we were unable to fully address Rothman's hypothesis, our self-administration findings with JHW showed promising results for this compound as a substitute medication. Previous research has found JHW to block amphetamine- and cocaine-induced locomotor activity (Velazquez-Sanchez et al., 2010; Velazquez-Sanchez et al., 2013) and also to prevent long-term brain neuroadaptions induced by amphetamine (Velazquez-Sanchez et al., 2013). As such, several lines of research have attributed JHW's therapeutic efficacy as a potential stimulant abuse treatment, to its antagonist-like properties (Katz et al., 2004; Li et al., 2013). However, new and emerging research with this compound suggests that JHW may act to reduce drug-induced behaviours through mechanisms that agonise the DA system. For example, pre-treatment with JHW (10mg/kg) marginally reduced self-administration for a low dose of MA (0.04mg/kg/inf), but significantly reduced responding when rats received a higher dose (0.12mg/kg/inf) (Ferragud et al. [submitted manuscript]). In the same experiments, JHW 007 enhanced MA reinforcing efficacy in a progressive ratio schedule of reinforcement. Given these findings, it is possible that JHW acts to potentiate the effects of MA by increasing its rewarding efficacy. Therefore, animals do not feel the need to respond

for MA as often. If JHW does indeed increase the rewarding effects of MA, treatment-induced reductions in drug-associated behaviours attributed to antagonistic pharmacological actions may actually be the result of an enhanced rewarding effect. While these properties may not seem desirable for a therapeutic drug treatment, provided that MA is not taken in combination with JHW, its induced elevation of the DA system would likely alleviate craving and thus ultimately reduce drug intake. Nevertheless, if MA were taken under the effects of JHW, due to reward enhancement, MA consumption would most likely decrease. This research sheds new light on JHW's possible mechanism of action and has implications for other BZT analogues yet to be evaluated as well as those currently under evaluation.

5.4 Future directions in pharmacotherapy

Given our result with JHW, BZT analogues provide a promising avenue of research for the development of stimulant abuse medications. This treatment approach is in line with the development of similar substitute medications for other drugs of abuse including heroin and nicotine. In addition to current research into pharmacotherapies targeting the classical neurotransmitter systems (i.e., monoamine and indoleamine systems), new research is beginning to focus on molecules resulting from monoamine metabolism and their receptors as targets for medication development in neuropsychiatry. These molecules are referred to as traces amines. Trace amines exist in the mammalian brain but at concentrations approximately 1000-fold lower than the classical neurotransmitters, DA, 5-HT, and noradrenaline (Berry, 2004). Trace amines are believed to act as neuromodulators or co-transmitters within classical neurotransmitter systems (Saavedra & Axelrod, 1976) and have been found to induce amphetamine-like effects at high concentrations through primary interaction with the DAT (Berry, 2004). As such, a dysregulation of trace amines has been

associated with various disorders involving DA dysfunction, including schizophrenia, depression, Parkinson's disease, and ADHD (Grandy, 2007; Sotnikova et al., 2009).

A recently discovered class of G protein-coupled receptors, trace amine-associated receptors (TAAR1's) that can be activated by trace amines, have been found in brain areas associated with reward including the VTA, dorsal raphe nucleus and amygdala (Lindemann et al., 2007). Recent research has therefore investigated the role of these receptors in models of stimulant addiction. TAAR1 KO mice have been found to exhibit greater locomotor stimulation and greater levels of DA release in the striatum following a single injection of amphetamine compared to mice with intact TAAR1 (Wolinsky et al., 2007; Lindemann et al., 2008). TAAR1 is also believed to play a role in the modulation of MA reward, as TAAR1 KO mice show CPP after two MA conditioning sessions while control mice do not (Achat-Mendes et al., 2012). Lindemann et al. (2008) also suggest that TAAR1 may modulate DA firing in the VTA through interaction with the DA D2 receptor. As TAAR1 appears to play a key role within reward systems that mediate stimulant addiction, current findings with these receptors merit further investigation.

Research with TAAR1-based medications complements well the investigations with compounds that interact more directly with the brain monoamine systems, such as the BZT analogues used in the present research work.

5.5 Conclusions

MA is a highly potent and dangerous psychostimulant drug with very high abuse liability. Despite periods of prolonged abstinence, relapse is relatively common. Given the financial, mental, and emotional impact of MA abuse, the development of pharmacotherapies for stimulant addiction is of high importance. The current study observed a significant attenuation of reinstatement to MA-seeking following chronic treatment with the BZT

analogue, JHW. This finding further supports the development of substitute medications for stimulant addiction and adds support to BZT analogues as a promising avenue for future research in this area. New and emerging research with JHW contradicts our previous views of this compound's antagonist-like actions, suggesting that JHW may in fact serve to potentiate drug-induced reward thus appearing to reduce or block drug-induced effects. Indeed, further research is needed to fully understand the actions of this compound and its therapeutic potential as a stimulant replacement medication.

6.0 References

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