

**THE BEHAVIOURAL IMPLICATIONS  
OF POSTNATAL EXPOSURE TO  
BENZYLPIPERAZINE AND  
METHAMPHETAMINE - A  
LONGITUDINAL DOSE-RELATED  
STUDY IN MALE AND FEMALE RATS**

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**by**

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## Abbreviations

<b>ACTH</b>	Andrencorticotropic hormone
<b>ADHD</b>	Attention deficit hyperactivity disorder
<b>ATS</b>	Amphetamine-type stimulants
<b>AMP</b>	Amphetamine
<b>BZP</b>	Benzylpiperazine
<b>C</b>	Celsius
<b>CHDS</b>	Christchurch Health and Development Study
<b>CNS</b>	Central nervous system
<b>CRH</b>	Corticotropin-releasing hormone
<b>DA</b>	Dopamine
<b>DAT</b>	Dopamine transporter
<b>DOA</b>	Drugs of abuse
<b>DSM-V</b>	Diagnostic and Statistical Manual of Mental Disorders, 5th Ed
<b>EPM</b>	Elevated plus maze
<b>fMRI</b>	Functional magnetic resonance imaging
<b>HIV</b>	Human immunodeficiency virus
<b>HPA</b>	Hypothalamic-pituitary-adrenal
<b>i.p.</b>	Intraperitoneal injection
<b>LSD</b>	d-lysergic acid diethylamise
<b>MA</b>	Methamphetamine
<b>MDMA</b>	3, 4-methylenedioxymethamphetamine
<b>mg/kg</b>	Milligram per kilogram
<b>MPH</b>	Methylphenidate
<b>NAc</b>	Nucleus accumbens
<b>NE</b>	Norepinephrine
<b>OF</b>	Open field
<b>PCP</b>	Phencyclidine
<b>PND</b>	Post natal day

<b>PFC</b>	Pre frontal cortex
<b>SI</b>	Social interaction test
<b>SUD</b>	Substance use disorder
<b>TFMPP</b>	trifluoromethylphenylpiperazine
<b>VMAT2</b>	Vesicular monoamine transporter 2
<b>5-HIAA</b>	5-hydroxyndoleacetic acid
<b>5-HT</b>	Serotonin
<b>5-HTT</b>	Serotonin transporter

## **Abstract**

Despite worldwide concern about the consumption of psychoactive substances during earlier development, the long-term behavioural effects of many have remained largely unexplored. By the mid 2000's there was a public epidemic declared on the escalating rate of individuals consuming New Zealand's form of methamphetamine (MA), called 'P' (short for 'pure' methamphetamine). Within this environment the emergence of new unregulated psychoactive compounds appeared. These were known and marketed as "legal or herbal highs". *N*-benzylpiperazine (BZP) became the main ingredient in the legal highs and was marketed as a safe alternative to "P", however there was no research to support this claim. The present study investigated the long-lasting effects on anxiety-like behaviour in rats, after BZP or MA administration during early to late adolescent development, examining variables: drug; age; sex; dose; and whether differences remained over time. Rats were either administered saline, BZP (5.0, 10.0, or 20.0 mg/kg) or MA (0.5, 1.0, 2.0 mg/kg) at three different ages of adolescence (Post Natal Day [PND]: PND31-40, PND41-50, PND51-60) for a period of ten days. After a thirty day wash-out period rats were tested in four different rodent tests of anxiety: Y-maze, Elevated Plus Maze, Light/dark Emergence Box and a Social Interaction Test. Behavioural testing occurred again at PND 120 and PND 200 to assess behavioural change over time. Daily BZP or MA treatment across the three different ages of adolescence increased anxiety-like behaviours in all behavioural measures and this was maintained over time. In summary, rats treated with BZP or MA during PND31-40 and PND41-50 displayed more anxiety-related behaviour comparative to control rats and this effect increased with drug dose, primarily for male-treated rats. The general pattern of results was more complex for PND51-60 treated rats, with MA-treated female rats displaying increased anxiety-like behaviours as the dose of MA increased and rats treated with the highest dose of BZP displaying decreased emotionality. There were more similarities between BZP and MA than differences, yet adolescent BZP treatment appeared to have greater impact on adult behaviour than adolescent MA treatment. Additionally, male rats exposed to both BZP and MA displayed increased anxiety-like behaviours compared to female-treated rats. The implications are far reaching, as there is currently a cohort of the population that consumed BZP legally as adolescents who may have a greater risk for the development of increased anxiety with developing age. In sum, the effect of adolescent exposure to psychostimulant drugs affected anxiety-like behaviours that were observable into middle adulthood, suggesting that the vulnerability to anxiety may be shaped by drug use in adolescence.

## **Chapter 1: Introduction**

### ***General Overview***

Adolescence is the transition period from childhood to adulthood. Typically, adolescence is a time when the individual initiates multiple risk taking behaviours namely, sensation/novelty/reward seeking and impulsivity (Ernst, Pine & Hardin, 2005). These behaviours can be observed in increased delinquency, high risk sexual behaviours and substance use. It is well established that early substance use is associated with a number of functioning difficulties in later life (Rohde, Lewinsohn, Seeley & Klein, 2007). For example, initial consumption of an illicit substance before the age of 15 years is a strong predictor of later drug dependence (Meyer & Neale, 1992; Laviola, Adriani, Terranova & Gerra, 1999; Estelles, Lluch, Rodriguez-Arias, Aguilar & Minarro 2007). Many studies have attempted to explain how drug use or experimentation during adolescence can lead to dependence. Drug experimentation is perceived to be a natural aspect of human behaviour (Smith, 2003) and although substance use occurs across the lifespan, adolescence is proposed to be a time of when long term changes can occur in brain functioning which in turn is manifested in problematic behaviours that further predispose the young person to seek out drugs (Labonte et al., 2012). However, large numbers of individuals experiment with substances for variable periods of time, yet only a few go on to develop an addiction (Piazza & LeMoal, 1998). For example, it is suggested that only 15 to 17 percent of individuals experimenting with drugs become substance- dependent or addicted to the substance (Deroche-Gamonet, Belin, & Piazza, 2004).

### ***Substance Use***

Although substance abuse and dependency is documented throughout history, public concern has markedly increased since the 1960's. Substance use can be defined as a harmful or hazardous use of psychoactive substances. Problematic use of these substances causes significant health and social problems for the individual using them. Likewise the impact on the families of the user and community is significant. In 2008, it was reported that approximately 3.5% to 5.7% of the world's population aged 15 to 64 years used psychoactive substances. Globally cannabis is the most common, followed by amphetamine-type stimulants (ATS), then cocaine and opioids (UNODC, 2012; Labonte et al., 2012). It is reported that 10 to 15 percent of Australian and New Zealand individuals aged between 15 and 64 had smoked

cannabis in the past year, and 2.8 percent had ingested, injected or inhaled ATS (Degenhardt & Hall, 2012).

To assist in the understanding of the increasing worldwide substance abuse problem (Everitt, Dickinson, & Robbins, 2001; Stansfield & Kirstein, 2005), the diagnosis of a substance use disorder is presented. The complex and controversial aetiology of substance use itself is briefly explored. Additionally, before individuals are diagnosed with a substance use disorder (SUD), they must make the transition from experimentation to dependency. A broader construct of the “stage” theory of addiction sheds some light on the possible consequences of earlier drug use.

### ***Substance Use Disorder***

For individuals presenting with SUD, *The Diagnostic and Statistical Manual of Mental Disorders, fifth edition* (DSM-V) is the leading tool used to diagnose substance use disorders (American Psychiatric Association, [APA], 2013). To meet a diagnosis of a SUD, an individual must meet two or more of the eleven criteria during the previous 12 months, with two to three criteria considered to be a mild SUD, four to five criteria moderate and six to seven criteria met a severe SUD (APA, 2013 provides a full description). The defined criteria include: taking the substance in larger amounts or for longer than intended, wanting to cut down but not managing to, spending a long time getting, using or recovering from use, cravings and urges to use, not managing at work, home or school, continuing to use, even when it causes problems in relationships, giving up important social, occupational or recreational activities, using the substance even when it puts the individual in danger, continuing to use even when the individual has a physical or psychological problem that was caused by or made worse by the substance, needing more of the substance to get the desired effect (tolerance) and development of withdrawal symptoms, which can be relieved by taking more of the substance. These criteria are supported by animal drug research. Rats given extended access to self-administered cocaine showed three well-established symptoms of substance dependence, that is: an escalation in drug use, continued drug seeking and an increased motivation to self-administer cocaine (Ferrario, Gorny, Crombag, Li, Kolb & Robinson, 2005). Yet, despite mounting evidence showing detrimental consequences of substance use, individuals still experiment with drugs of abuse (DOA). However, the question of why people use or experiment with substances is complex. As already mentioned, substance use is generally initiated during adolescence, however not all individuals make the transition from recreational or experimental use to dependence or addiction.

### ***Theoretical Frameworks of Addiction***

Two principal theoretical frameworks help to explain the transition from drug experimentation to SUD: The first is the individual-centred concept of addiction, namely, that drug abuse is a pre-existing pathological condition in which certain individuals are biologically predisposed to be vulnerable to the rewarding properties of drugs. For example, some individuals may have increased or sensitised corticosterone levels that induce stress, which in turn increases vulnerability to drugs via the enhancement of the dopamine (DA) reward pathways, resulting in drug dependence (Piazza & LoMoal, 1996). The second theoretical framework is the drug-centred concept of addiction, in which the changes are thought to result from drug use (for example, tolerance, sensitization and conditioning, (see Robinson & Berridge, 2001 or Wolf, 1998) and are primarily responsible for the transition from use to dependency (Ferrario et al., 2005; Robinson & Berridge, 2001). The present study seeks to address this drug-centred concept of addiction, by suggesting that changes in the developing brain during vulnerable windows of time can cause long-term changes in adult behaviour. Regardless of which view is correct, the stage theory of addiction raises important issues that must be considered in relation to young people experimenting with substances.

### ***Stage Theory of Addiction***

In the stage theory of addiction, the development of SUD is considered to follow a lawful progression from legal to illegal drug use. The key predictions of this theory are that individuals using illegal drugs will have previously used legal drugs, and that not all individuals who use legal drugs will move on to illegal drugs (MacCoun, 1998). The common developmental sequence of SUD generally starts with alcohol and/or tobacco, and then moves through inhalants and marijuana to illicit drugs (Smith, 2003; Walker, Venner, Hill, Meyer, & Miller, 2004). These substances have been deemed “gateway” drugs (Fergusson & Horwood, 2000), because the taking of these substances may lead onto use of harder drugs.

Support for the stage theory of addiction comes from the Christchurch Health and Development Study (CHDS). This is a 21 year follow up study of 990 New Zealand children born in mid-1977. The authors measured frequency of cannabis use and other illegal drugs in the cohort of individuals when they were aged between 15 and 21 years. They controlled for family, social, behavioural and educational backgrounds prior to the age of 15 and additionally controlled for differences in adolescent lifestyle variables. By the age of 21, nearly 70% of the total cohort of individuals had used cannabis and 26% of the cohort had used other illegal

drugs. This suggests that not all earlier cannabis users went on to using other illicit drugs. Astonishingly though, for the 246 individuals who reported using other illicit drugs, cannabis in all but three of the cases preceded the use of illegal drugs. However 63% of the cannabis users did not progress to use of other illicit drugs. The authors also assessed the frequency of use, using five different levels, from did not use cannabis over the past year to using cannabis over 50 times in the past year. They found a clear dose/response relationship with those who had consumed over 50 times to be 143 times more likely to have progressed to illicit drug use than those that had not consumed any cannabis over the past year. The authors concluded that cannabis in New Zealand may act as a gateway drug, supporting the stage theory of addiction (Fergusson & Horwood, 2000). Proponents of the stage theory of addiction conclude that there is a general progression from legal to illegal drugs.

Research on addiction trajectories has shown that, while initial experimentation with drugs of abuse is largely a voluntary behaviour, continued drug use gradually impairs neural function, eventually impacting the very capacity to exert free will. In persons with genetic vulnerabilities, suffering from chronic stress or comorbid psychiatric conditions, or who have been exposed to drugs, these processes can eventually turn drug use into the automatic and compulsive behaviours that characterize addiction.

### ***Aetiology of Addiction***

The aetiology of addiction is complicated because of interactions among many psychological, environmental and biological risk factors related to drug use (Crombag & Robinson, 2004; Gilvarry, 2000). Examples of internal psychological risk factors include underlying mood disorders (Armstrong & Costello, 2002), sensation seeking (Barnea, Teichman & Rahau, 1992) or impulsivity (Jentsch & Taylor, 1999). Environmental risk factors are one or more of the following: use of the drug in a drug-associated environment, use of drug paraphernalia; individual expectations of the drug being used; and peer pressure or the expectations of the social circle to which the individual belongs (Crombag & Robinson, 2004). Examples of biological risk factors are genetic predispositions (Merikangas et al., 1998) and differences in the structure or biochemistry of the brain (Carlson, 2001).

Addiction can be defined as a chronic, relapsing disorder that has been characterized by a compulsion to seek and take drugs, loss of control over drug intake, and emergence of a negative emotional state (e.g., dysphoria, anxiety, and irritability) that defines a motivational withdrawal syndrome when access to the drug is prevented. Individuals continue to use drugs

despite the negative consequences (Joffe, Grueter & Grueter, 2014; Pierce & Kumaresan, 2006). Addicted individuals typically progress through periods of bingeing (high level consumption of a short time frame), withdrawal (abstinence, in the presence of negative affect and or anxiety), and preoccupation (craving and anticipation of next use). The occasional, limited, recreational use of a drug is clinically distinct from escalated drug use, the loss of control over drug intake, and the emergence of compulsive drug-seeking behaviour that characterise addiction (Koob, 2005).

Several theories have been proposed for the phenomenon of addiction. Bechara (2005) proposes that addiction is an imbalance between two separate but interacting neural systems controlling decision making. On the one hand the impulsive amygdala system for signalling pleasure or pain of the immediate choice compared to the reflective prefrontal cortex for signalling pleasure or pain of future consequences. Blum and colleagues (2012) suggest that addiction occurs due to "reward deficiency syndrome", in that behaviours associated with addiction are due to low DA functioning. However, Berridge (2007) argues that DA's contribution is more to cause "wanting" for hedonic rewards, more than liking or learning for those rewards. Alternatively Koob and colleagues (1989) suggest that addiction occurs through the development of an adaptive process that is initiated to counter the acute effects of the drug (opponent processes). Redish, Jensen and Johnson (2008) propose addicted individuals have vulnerabilities in their decision process. They suggest ten key vulnerabilities that differ for different drugs, different behaviours and different individuals can lead to addiction (see Redish et al., 2008 for a review). Belin, Jonkman, Dickinson, Robins and Everitt (2009) propose that addiction is a maladaptive compulsive habit that has dominance over goal-directed behaviours and highly influenced by Pavlovian incentive mechanisms. Although mentioned, it is beyond the scope of this research to critically analyse different theories of addiction, however it appears sufficient to mention the complexities of addiction will be modulated not only by brain changes but also by genetic, developmental, experiential and environmental factors. The literature also suggests there are differences between individuals and within the same individual at different stages of development.

### ***Neurodevelopment***

Neurodevelopment and neurotransmission play a critical role in the mechanisms of action of addictive drugs. There is evidence indicating differences in development between adolescence and adulthood in brain regions thought to be important in drug abuse (Spear, 2000). After the

synaptic pruning and myelination that typically occurs during adolescence and young adulthood, brain development reaches completion (Andersen, 2005; Rezvani & Levin, 2004; Spear, 2000). Therefore, exposure to drugs during adolescence can have long-lasting implications for brain structure and function (Stansfield & Kirstein, 2005). The adult brain can generally compensate for drug-induced changes within the synaptic clefts, whereas more permanent changes can occur in the immature developing neural systems (Laviola et al., 1999; Stanwood & Levit, 2004). The transition from recreational experimentation to the compulsive patterns of drug use and abuse is understood to arise from changes in the neurotransmitter systems (Robinson & Kolb, 2004).

Most drugs of abuse directly or indirectly target the brain's reward system by flooding the circuit with DA. DA is a neurotransmitter present in regions of the brain that regulate movement, memory formation, retention and extinction, motivation, reward, predispositions for drug abuse and feelings of pleasure. When activated at normal levels, this system rewards the user's natural behaviours. Over stimulating the system with drugs, however, produces euphoric effects, which strongly reinforce the behaviour of drug use (Julien, 2001). The rewarding effects of drugs that are responsible for continuing use are mediated by the mesolimbic DA system (Piazza & LeMoat, 1998), especially DA projections to the nucleus accumbens (NAc) (Robinson & Berridge, 2001). The NAc is implicated not only in levels of self-administration with stimulant drugs (White & Kalivas, 1998) but also in the rewarding properties of all addictive substances (Andersen, 2005). In the human brain, during adolescence DA is overproduced and then subsequently reduced as the brain develops to its mature state (Bolanos, Glatt, & Jackson, 1998). Additionally, DA may play a critical role in shaping neural responses to drugs during this period and these responses might be quite different in adolescence from those of the same receptors in adulthood (Penner, McFadyen, Pinaud, Carrey, Robertson, & Brown, 2002). However, there is evidence that the neurons containing DA and serotonin (5-HT) do not necessarily die after drug use. Instead, it is more likely that their nerve terminals are damaged and further development is limited (Fukami, Hashimoto, Koike, Okamura, Shimizu, & Iyo, 2004). Although there is conclusive evidence of the involvement of DA and the reward pathways in drug abuse, the same does not characterise 5-HT.

The 5-HT system is involved in the regulation of emotion. Emotion consists of stress, sexual behaviour, memory, cognition, mood arousal, pituitary hormone secretion and satiety (Carlson, 2001). Additionally, 5-HT integrates complex brain functions such as motor activity, sensory

processing and cognition (Lesch & Merschedorf, 2000). Like the DA system, during adolescence the 5-HT system undergoes substantial reorganization. For example, it has been reported that in a rat's hippocampus, the 5-HT levels are five times higher at puberty than at either the juvenile or adult ages (Chen, Turiak, Galler, & Volicer, 1997). The decrease of 5-HT uptake after puberty is suggested to be important for the modulation and attenuation of the rewarding properties of stimulant-like drugs (Hashimoto, Harumi, & Guromaru, 1992). It appears safe to suggest that the exposure to psychoactive substances while neurons operated by DA and 5-HT are not fully matured, may have long-lasting implications. As 5-HT is known to be involved in the regulation of mood and the expression of emotions, it is therefore, connected to the development and continuation of many mental illnesses such as anxiety, depression, obsessive-compulsive disorder and panic disorder (Naughton, Mulrooney & Leonard, 2000). As the brain (primarily the pre frontal cortex; PFC), is not fully developed in adolescence and the 5-HT and DA systems are still undergoing changes, stimulant substances administered during this period may affect the regulation of emotion.

The PFC is the last area of the human brain to mature (Smith, 2003; Spear, 2000) and 5-HT is known to be involved in its functioning. The PFC is implicated in impulsive control, attention and executive functioning (Andersen, 2005). There is conclusive evidence that there are difference in executive functioning between adolescents and adults (Spear, 2000); for example, the underdeveloped PFC in adolescence assists to explain impulsivity, risk taking behaviours and use of addictive substance that adolescents engage in (Smith, 2003). Research has also shown that rats with PFC lesions are at a greater risk for the development of SUDs (Chambers, Taylor & Potenza, 2003) because of loss of control over behaviour, or increased impulsivity (Ferrario et al., 2005). Therefore, any alteration to the PFC when it is not fully matured could have long-term negative implications for the normal development of behaviour (Stansfield & Kirstein, 2005).

### ***Neuronal imprinting***

Research is suggesting that exposure to psychoactive substances in early stages of life could affect brain neuroplasticity processes, which may produce long-lasting abnormal behavioural responses (Estelles et al., 2007). Neuroplasticity occurs as a function of experience, through learning and memory and is thought to be due to the re-organization of synaptic connections in brain functioning (Robinson & Kolb, 2004). Most research to date has focused on how learning, stress, environments, recovery of function and pathological states impact on synaptic

or dendrite structure. There is limited research into the impact of how experience at one point in life can change behaviour and an individual's psychological functioning thereafter. The neuronal imprinting hypothesis suggests that long-term effects of drug exposure can be delayed and expressed once the vulnerable brain system reaches maturation. Neuronal imprinting assists to explain the phenomenon of why addicts relapse even years after the discontinuation of drug use and long after withdrawal symptoms have subsided (Andersen & Navalta, 2004). Neuronal imprinting occurs when the effects of the earlier drug exposure outlast the drug itself.

Increasingly, evidence is suggesting that long-term effects of drug exposure in adolescence are delayed and expressed typically during adulthood (Aarons et al., 1999). McFadyen, Brown and Carrey (2002) state that neural development may continue until approximately 20-25 years of age. Therefore, throughout adolescence, the plasticity of the brain continues and also hormonal levels change drastically (Laviola et al., 1999; Smith, 2003). Aarons and colleagues (1999) have researched alcohol and drug use in human adolescents and suggest that even limited or infrequent use may be associated with adulthood depression, lack of a sense of purpose in life and lower self-esteem.

Studies of both adolescent and adult animals suggest that the adolescent brain is more sensitive to the lasting effects of substances than adult brains (Smith, 2003). The most conclusive support for this theory comes from studies with methylphenidate (MP). MP is a stimulant drug used to treat childhood Attention Deficit Hyperactivity Disorder (ADHD). Studies administering MP to adolescent animals have shown behavioural changes long after the drug was withdrawn. For example, after adolescent MPH exposure, adult animals showed depression in a forced swim task (Carlezon, Mague, & Andersen, 2003), reduced responsiveness to response to natural rewards (Bolanos, Barrot, Berton, Wallace-Black, & Nestler, 2003), increased vulnerability to substance abuse (Brandon, Marinelli, Baker, & White, 2001) and increased anxiety-like behaviour (Balanos, et al., 2003).

It is therefore conceivable that both BZP and MA (stimulants) administered during adolescence might affect the developing brain and consequently later behaviour, consistent with MPH research, and the neuronal imprinting theory.

In summary, the sensitivity of adolescence brain development may be related to extensive pruning of synapses and of reorganisation of many neurotransmitter systems (Jacobson-Pick, Audet, Nathoo & Anisman, 2011). The behaviourally activating effects of stimulants have long been thought to be due to its actions on the DA system (Labonte., et al., 2012;

Vanderschuren & Everitt, 2005), although recent evidence suggests 5-HT and norepinephrine (NE) may contribute also (Rothman & Baumann, 2006). As already mentioned drug abuse is a major global health problem and over the last decade there has been a rise in the consumption of new unregulated psychoactive compounds. These substances are often marketed as ‘legal or herbal highs’ and mimic the psychoactive effects of illicit drugs, and evidence suggests that both male and female adolescents in New Zealand have consumed them.

### *Sex Differences*

There is evidence to suggest that there are also differences in brain functioning between sexes (Bridges & Starkey, 2004; Crick & Zahn-Waxler, 2003; Dominguez, Cruz-Morales, Carvalho, Xzvier, & Brandao, 2003; Hallfors, Waller, Bauter, Ford & Halpern, 2005; Koenig, et al., 2005; Opland, Winters, & Stinchfield, 1995) and, as anxiety is predominately diagnosed in females (Baranyi, Bakos, & Haller, 2005; Palanza, 2001) and adolescent stimulant drug users are more likely to be female (Rawson, Gonzales, Obert, McCann & Brethen, 2005), sex differences must be also considered.

The rate that females report MA as their drug of choice is twice the rate for males (Cretzmeyer, Sarrazin, Huber, Block & Hall, 2003). Therefore it appears safe to suggest that there are differences between sexes in their drug of choice. The Iowa Case Management Project sought to identify reasons for and the consequences of MA among rural users and their results suggested that females were more likely than males to use drugs to escape, to deal with emotional and family problems, to be more productive, to lose weight, improve their strength and because of the easy availability. Males reported initiating MA use because their parents use drugs and out of curiosity (Cretzmeyer et al., 2003). Females tend to initiate MA use earlier than males, become more dependent on it but are suggested to respond better to treatment than males (Embry, Hankins, Biglan & Boles, 2009). Rawson et al. (2005) reports that different from other DOA, adolescent MA users are more likely to be female.

Anxiety disorders appear to be predominantly exhibited by women (Baranyi et al., 2005; Palanza, 2001). However, hardly any animal models use female animals: although this discrepancy is lessening, the majority of research still involves only males animals.

Consequently, there are few investigations of sex differences (Bridges & Starkey, 2004). A literature review investigating anxiety and 5-HT by Blanchard and colleagues (1995), revealed that in 750 published studies (ranging from 1991 to 1994) 90% used only male animals, 9% only females and 1% used both sexes (Blanchard, Griebel, & Blanchard, 1995). However, the

generally acknowledged higher frequencies of depression and anxiety are more common in women (Palanza, 2001), and one explanation can be attributed to different socialization practices. Females are more strongly discouraged from engaging in risk taking activities than males (Guilamo-Ramos, Litardo, & Jaccard, 2005). Nevertheless, the similarities between the sexes are more prevalent than differences between them (Armstrong & Costello, 2002).

With the exception of MA, males tend to use a wider variety of drugs and initiate drug use earlier than females; however, in terms of clinical severity females obtain higher scores than males on physical symptoms and the emotional consequences of drug use. Additionally, female drug users scored higher on the emotional scales because they stated that they were using the drug as a way of alleviating emotional unease (Embry et al., 2009; Opland et al., 1995). It is, however, questionable whether this is a true sex (as opposed to gender) difference because males may not readily admit to having any emotional issues or problems as openly as females. Adolescent women are almost equally likely to drink, smoke or take illegal drugs as adolescent men, but are also at a greater health risk (Sarigiani, Ryan, & Petersen, 1999). Therefore the common assumption that drug use is more common in boys than in girls fails to address the current cultural context. Evidence is mounting that there is a growing problem of substance abuse in adolescent females (Litt, 2003). As discussed below, differences between sexes may be due to differences in plasticity during maturation of the brain.

As mentioned, adolescence is an intense period of development of the brain, characterised by extensive pruning of synapses and receptors, and reorganization of many neurotransmitter systems (Spear, 2000). This usually starts earlier for females than for males (Andersen, 2005). There is also a greater over-expression of DA receptors in the Nac and the striatum in males than in females (Andersen, & Teicher, 2000; McCormick, Robarts, Gleason, & Kelsey, 2004). This over-expression may make the male brain more susceptible to stimulation of the DA pathways implicated in addiction (Teicher, Andersen, & Hostetter, 1995). The most common transmitter involved in sex differences in brain development is DA. Male rats have higher levels of D1 receptors in the NAc than female rats and female rats have a greater rate of DA release and reuptake (Andersen, & Teicher, 2000) and higher DAT levels in the striatum than male rats (Spear, 2000). Additionally, the literature on sex differences in psychomotor behaviour suggests that females exhibit more robust sensitization to stimulant substances than males and this is not due to metabolism of the drug, as it is reported to be similar between the sexes (Becker, 1999). Also, it is suggested that drug use by both sexes is initiated increasingly earlier than it has been in previous decades. Notwithstanding, there is conflicting evidence in

regards to sex differences in initiation and prevalence of MA use among adolescents, with some researchers suggesting that prevalence is higher for males (Embry et al., 2009) and others higher for females (Rawson et al. 2005). Simply, as there is evidence of sex differences in the brain during the transition from adolescence to adulthood, exposure to BZP or MA during this period might be expected to affect the two sexes differently.

### *Age*

Drug use is estimated to start from the ages ranging from 11-12 to 17-18 years of age and typically starts with tobacco smoking, followed by cannabis and alcohol, and then stimulants and opiates (Adrianai, Macri, Pacifici & Loviola, 2002). The age of the first use of psychoactive substances appears to be critical, since it impacts on subsequent patterns of drug-related behaviour. The earlier the exposure to psychoactive substances the higher the probability of moving from use to abuse and then to dependence (Labonte et al., 2012). The most commonly used drug prior to the age of 16 years (other than alcohol or tobacco) is cannabis and, although not as prevalent, many users report starting stimulant use prior to 16 years of age also (Cretzmeyer et al, 2003). This is important as Rawson et al. (2005) found that adolescents in treatment for MA use show increased rates of depression and suicide ideation compared to adolescents in treatment for other DOA's.

Preadolescence is the stage of human development following early childhood and prior to adolescence, generally ending at the beginning of puberty. However, there is no exact agreement as to when this period starts or ends but general consensus is from the ages of nine until puberty which is different for each individual based on heredity and environmental factors (e.g. diet and exercise). Adolescence is the transitional period from puberty to legal adulthood and again chronological age only provides a rough marker as each individual is different. Early adulthood is the phase of life span between adolescence and adulthood and Arnett (2000) suggested this to be the period between 18 and 25 years of age. Similarly, in rats these developmental periods are difficult to define. Spear (2000) suggests that that PND28-60 is the periadolescent equivalent to adolescence in humans and Smith (2003) suggests that research needs to incorporate the entire adolescent phase. However, as development is defined by critical periods or timing, it is noted that no critical period ends suddenly rather it gradually tapers off (Herlenius & Lagercrantz, 2004).

This thesis endeavours to capture the periadolescent period in rats and for convenience has defined PND31-40 as early adolescence, PND41-50 as adolescence and PND51-60 as late

adolescence (Vorhees et al., 2005). Additionally, as the research topic involves differences or similarities between two psychoactive substances which are considered to be central nervous system stimulant drugs, prior research conducted during these developmental periods will be briefly examined.

It has not been until the last decade that researchers have reported using animal models to analyse different developmental periods in relation to possible effects of substance use. Importantly, there are limited studies that compare the effects of equivalent treatment regimens under identical conditions at different adolescent developmental stages.

### ***Drug Classifications***

Drugs of abuse are commonly grouped into three large categories defined by their most prominent effects. For example central nervous system stimulants (cocaine, methamphetamine [MA], amphetamine [AMP], caffeine, nicotine, methylphenidate [MP]) produce wakefulness and enhanced alertness, whereas central nervous system depressants (alcohol, benzodiazepines, opioids) induce sedation and drowsiness. The third group is known as hallucinogens (marijuana, mescaline, d-lysergic acid diethylamide [LSD], psilocybin [magic mushrooms], phencyclidine [PCP]) and include a wide variety of plant-derived and synthetic substances. Hallucinogens alter perception and enhance emotional responsiveness so that images are distorted in ways that may be interpreted to the user as interesting or frightening. 3,4-methylenedioxy-methamphetamine (MDMA, commonly known as 'ecstasy') is a combination of a stimulant and a hallucinogen (Julien, 2001) and is mentioned here due to its well-known use in research and its central nervous system (CNS) stimulation ability, similar to AMP and MA (Teixeria-Gomes, Costa, Feio-Azevedo, Bastos, Carvalho & Capels, 2015). However, this thesis will focus on the stimulant category of substances due to the introduction of benzylpiperazine (BZP) as an alternative for individuals experiencing MA dependence in New Zealand.

### ***Benzylpiperazine***

Benzylpiperazine (BZP) is a piperazine derivative which comes as either a hydrochloride salt or a free base and is seen by some as an alternative to amphetamine drugs, as it produces similar behavioural and neurochemical effects (Baumann, Clark, Budzynski, Partilla, Blough & Rothman, 2005; Bishop, McCord, Gratz, Loeliger, & Witowski, 2005; Bye, Munro-Faure, Peck, & Young, 1973; Campbell, Cline, Evans, Lloyd & Peck, 1973; De Boer, Bosman, Hidvegi, Manzoni, Benko, Dos Reys, & Maes, 2001, Fantegrossi, Winger, Woods, Woolverton

& Coop, 2005; Nicholson, 2006; Peters, Schaefer, Staack, Kraemer, & Maurer, 2003; Staack & Maurer, 2005; Wikstrom, Holmgren & Ahlner, 2004). Research suggests that BZP has approximately 10 percent the potency of dexamphetamine (Bye et al., 1973; Campbell et al., 1973).

Benzylpiperazine was first developed in 1944, as an antihelminthic agent for livestock. However, because of its lack of efficacy and side effects (namely seizures) it was removed from the market (Gee, Richardson, Woltersdorf & Moore, 2005; Johnstone, Lea, Brennan, Schenk, Kennedy & Fitzmaurice, 2007). In 1970, BZP's potential as an antidepressant was evaluated as it was found to reverse the effects of tetrabenazine in rats and mice (Tekes, Tothfalusi, Malomvolgyi, Herman & Magyer, 1987). Campbell et al., (1973) reported potential antidepressant activity, yet it was never marketed as an antidepressant because studies in rodents showed it to have similar effects to AMP: animals administered BZP displayed hyperactivity and stereotypic behaviour (Campbell et al., 1973), involuntary head movements, and reduced reaction time in shock avoidance studies (Miller, Green & Young., 1971). Any therapeutic effects of BZP were therefore dismissed, as it was concluded that BZP may have the potential to be abused (Bishop et al., 2005). Although aware of the previous findings and warnings, in 1980, BZP was marketed as an anti-depressant named Trebilet® by a pharmaceutical company in Hungary. However, it was withdrawn during phase II of its clinical trials due to its amphetamine-like properties (EMCDDA, 2007a).

Benzylpiperazine has also been trialled in human populations, comparing its effects with that of amphetamine. In a double blind study by Campbell and colleagues (1973), it was reported that former AMP addicts could not distinguish between the effects of 100mg of BZP and 10 mg of AMP, and both were self-reported to be liked by the participants. The subjects reported that the subjective effects of BZP were more enjoyable than AMP (Campbell et al., 1973). The authors concluded that BZP is a compound with a potential for abuse and recommended that it be placed under statutory control similar to that regulating the use of AMP. A similar double-blind study was conducted by Bye and colleagues (1973), in which 12 healthy volunteers were orally administered either AMP (1 mg to 7.5 mg) or BZP (20 to 100 mg). They were scored on performance tests (namely, tapping, addition and hand steadiness tests, and auditory vigilance tests), cardiovascular responses and self-reported subjective effects. The results suggested that both BZP and AMP similarly produced tachycardia and increases in systolic blood pressure. The authors concluded that BZP was an indirectly acting sympathomimetic amine similar to AMP (Bye et al 1973).

The principal mechanisms of action are largely unclear. Brennan et al. (2007) suggested that BZP's abuse potential and self-administration is dependent on DA mechanisms. However, Tekes et al (1987) suggest that BZP has a complex mode of action working directly and indirectly on central monoamine receptors causing stimulation independent release of noradrenaline as well as blocking synaptic uptake. Oberlander, Euvrard, Dumont and Boissier (1979) observed BZP effects similar to AMP and MA, by contralateral circling behaviour in rats that had undergone destruction of the nigrostriatal DA pathway by means of unilateral administration of 6-hydroxydopamine. Recently, Baumann et al (2005) found that in in vivo microdialysis studies that BZP administration caused a parallel rise in extracellular DA and 5-HT dialysate levels in the nucleus accumbens, with the DA effect predominant. However, Simmler, Rickli, Schramm, Hoener and Liechti (2014) investigated BZP's pharmacological mechanism on human transporters and found that BZP inhibited human monoamine transporters and released DA. They concluded that BZP is an indirect DA and NE agonist without 5-HT properties. Overall, the pharmacology and mechanism of action remains inconsistent, however it appears to have similar addiction potential of other stimulant substances (Baumann et al., 2005; Johnstone et al., 2007; Yarosh, Katz, Coop, & Fantegrossi, 2007).

Fantegrossi et al. (2005) found that BZP may be addictive and through their research with rhesus monkeys found that BZP can cause deficits in both the DA and 5-HT systems. Additionally, Fantegrossi et al. (2005) investigated the reinforcing effects of BZP and trifluoromethylphenylpiperazine (TFMPP) in rhesus monkeys previously trained to self-administer cocaine and found that the monkeys would self-administer BZP at rates as high as or higher than they would for cocaine. TFMPP was found in Fantegrossi et al's (2005) study to not be a reliable re-inforcer. TFMPP mimics the effects of MDMA and is often combined with BZP to enhance the subjective effects. Baumann, Clark, Budzynski, Partilla, Blough and Rothman (2004) were concerned with recent accounts of drug users ingesting combinations of BZP and TFMPP. The combinations of these two substances were thought to mimic the subjective effects of MDMA. Therefore, Baumann et al (2004) investigated the effects of BZP and TFMPP on monoamine neurotransmission in the rat brain and reported evidence supporting an increase in extracellular 5-HT and DA similar to MDMA. Additionally, adverse behavioural effects (for example, seizures) were observed in five out of the seven rats that received the higher dose (10 mg/kg) combination of BZP and TFMPP. The drug-induced seizures occurred at a dose that was just 3-times greater than the threshold dose for biological

activity. Baumann et al. (2004), suggest that BZP and TFMPP are potentially dangerous, even life-threatening, and that there appears to be a very narrow window of safety. For example, a single i.p. dose of 200 mg/kg given to a guinea pig caused death through tetanic convulsive seizures and BZP is also suggested to lower the threshold for seizures in human epileptics (National Chemicals Inspectorate, 2004).

In 1999, ‘party pills’ containing 1-benzylpiperazine were introduced into the New Zealand market (Sheridan & Butler, 2010). Party pills were promoted as a safe and legal alternative to illicit drugs and reflected a harm minimisation approach to the escalating MA use being observed in New Zealand (Bowden, 2004). However, there was no research to support these claims and BZP and similar products were unregulated until 2005 and marketed primarily for young people, under the legislation of ‘herbal and dietary supplements’. Collins (2007) reported that 16 percent of the population of individuals aged 15-45 had used BZP in the last year as it was legal and available. BZP was sold at liquor outlets, service stations and corner dairies without advice, sold to any age group without any safety measures or quality control (The Expert Advisory Committee on Drugs [EACD], 2004). The effects of BZP are similar to those of other stimulants, arising from stimulation of the central nervous system, namely, euphoria, a heightened sense of awareness, wakefulness, and increased vigilance. The average duration of these effects is 6 -8 hours (EACD, 2004). Because of the misconception that party pills were “natural” or “herbal”, people appeared to take many times the recommended dose, aiming for stronger effects (Allan, 2005).

In New Zealand, from 2000 a legal market for BZP-based products developed rapidly. An estimated eight million doses were sold in New Zealand (Allan, 2005; Wilkins, Sweetsur & Girling, 2008) and aimed primarily at a youth market. A household survey in 2006 found that out of 2010 individuals, aged between 15 and 45 years of age, the prevalence was higher among those aged 18-29 years and use was greater among males than females (Wilkins, Sweetsur, Huckle & Huakau, 2006). It was not until 2005, when legislation was introduced in a new schedule within the Misuse of Drugs Amendment Act 2005 that BZP was classified as a Class C drug. BZP was the first substance to be regulated this way and made the selling of it illegal (Sheridan & Butler, 2010). In April 2008, BZP and related substances were classified as Class C1 substances under the Misuse of Drugs Act 1975 thereby making the manufacture, exporting, importing or supply of BZP illegal. A six month amnesty period followed, and on 30<sup>th</sup> September 2008 the purchase, possession and use of BZP became illegal (Sheridan & Butler, 2010). A household self-report survey conducted prior to BZP's illegal status, among

people aged 15 to 64 (n = 6784), showed that BZP was the second most used drug over the past 12 months, at 5.6 percent, after cannabis at 14.6% (Ministry of Health, 2010). Since BZP's prohibition, Wilkins and Sweetsur (2013) reported a decline in its use from 15.3% in 2006 to 3.2% in 2009. The self-reported reasons for stopping BZP use were its illegal status (43%), just an experimental phase (26%), non-availability (24%) and 'bad hangover effect' (18%).

At Waikato Hospital, during the mid-2000's Dr Tania Nicholson (2006) surveyed 125 random users of party pills admitted to hospital. She reported that one third of the users were between the ages of 14 and 24 and one third of the individuals had not read the instructions. Likewise, Jamieson (2005) reports that one third of individuals that he had contacted; one third of them had taken more pills than the recommended dose. In 2005, Gee et al. (2005) collected five months of data from 61 patients who had presented at Christchurch Hospital's Emergency Department with BZP toxicity. Sixty one patients attended on a total of 80 occasions with adverse effects following ingestion of party pills. The ratio of male to female was 1:1.3, age range was 15-36 years and the average number of pills consumed was 4.5. The authors concluded that seizures (grand mal type) occurred in 14 patients and these patients had not taken more pills than the non-seizure patients. Mild symptoms included insomnia, anxiety, nausea, and heart palpitations. Serious cases in addition to toxic seizures included epileptic attacks and severe respiratory and metabolic acidosis. Two cases were admitted to the hospital's intensive care unit. Females presented with more adverse effects more frequently than males. Gee et al. (2005) concluded that BZP products had a narrow safety margin possibly due to intrinsic pharmacodynamic properties, dosage variability, and/or genetic factors. A literature search found 82 scientific articles related to BZP (Cohen & Butler, 2011), with only nine of these examining the psychoactive effect in humans. Cohen and Butler reported limited social and health harm associated with BZP and were undecided about whether or not BZP serves as a gateway to illegal drugs. They concluded that the lack of long-term effects of BZP use was a significant gap in the literature.

In Ireland a 48 year old male diagnosed with schizophrenia presented with acute onset of unexplained confusion. He was speaking incoherently and behaving in a 'bizarre' manner. He had been stable for a number of years and was treated with clozapine 300mg twice daily. It was discovered that he had developed an acute delirium secondary to BZP use (Tully, Hallahan, & McDonald, 2011). Additionally, Gee et al. (2005) stated that there have been numerous reports of BZP causing either a toxic paranoid psychosis or exacerbating an existing mental illness. With the exception of the clinical trials mentioned (Bye et al., 1973; Cambell et

al., 1973), there has been limited research into the subjective effects of BZP in human populations. Thompson and colleagues (2010) conducted a randomised double-blind placebo-controlled trial looking at the clinical effects of BZP/TFMPP alone and in combination with alcohol in 35 healthy adults. The outcome measures were driving performance, cardiovascular effects, psychological function and sleep. However, the study was terminated early on because of severe adverse events in four of the 10 BZP/TFMPP-only subjects and three of the combined BZP/TFMPP and alcohol subjects. None of the control participants experienced the severe agitation, anxiety, hallucinations, vomiting, insomnia and migraines experienced by the experimental groups. The authors concluded that BZP/TFMPP either alone or in combination with alcohol carried a significant risk of severe adverse events even when taken in doses recommended by manufactures (Thompson et al., 2010). Curley, Kydd, Kirk and Russell (2013) looked at the effects of a single acute dose of BZP or BZP and TFMPP on reward circuitry in healthy adults. From an fMRI result recorded during an event-related gambling task, they concluded that BZP alone decreased the activation of the inferior frontal gyrus, insula and occipital regions in comparison to placebo. This suggested that BZP increased positive arousal and subsequently reduced the response to uncertainty.

Research with rats has provided similar findings. Herbert and Hughes (2009) compared the acute effects of either BZP or MA on several measures of activity and unconditioned choice in rats. They found that both BZP and MA increased open field rearing and ambulation, and possible anxiety-related novelty avoidance in a Y-maze. They concluded that there were more similarities between the two drugs than differences. Brennan et al. (2007) investigated the effects of acute and repeated BZP and MA exposure, to determine whether there was sensitization and cross-sensitization between the two drugs. Both MA and BZP produced dose-dependent hyperactivity and stereotypy and repeated use of both drugs resulted in a potentiated locomotor but not stereotypy response. The researchers concluded that repeated exposure to BZP results in sensitization and cross-sensitization to MA. Meririnne, Kajos, Kankaanpaa and Seppala (2006) administered BZP (1.25, 5 & 20 mg/kg) to rats for three days and tested them in a place preference paradigm the following day. They concluded that BZP induced dose-dependent place preference and suggested that BZP possesses rewarding properties in the rat, possibly due to adaptations to DA and 5-HT. They concluded that BZP may be susceptible to human abuse.

In summary, even though prior to regulation many people in New Zealand were taking herbal highs, little is known about their possible long-term effects. BZP was initially marketed as an

alternative to illicit and poor quality MA available in New Zealand (Bowden, 2004; EMCDDA, 2007a). Consequently, many individuals were under the impression that BZP was a safe and legal alternative to illegal drugs. Evidence to date suggests that, although BZP is seen by some as an alternative to other stimulant drugs, it produces very similar effects (Baumann et al., 2005; Bishop et al., 2005; Bye et al., 1973; Campbell et al., 1973; De Boer et al., 2001; Fantegrossi et al., 2005; Peters et al., 2003; Wistrom et al., 2004). General consensus has emerged that BZP's potency is one-tenth that of amphetamines (Robinson & Kolb, 2004), yet no research has addressed whether there are differences in long-term outcomes between BZP and other stimulant drugs, especially methamphetamine.

### *Methamphetamine*

Methamphetamine is a potent psychomotor stimulant drug with strong physiological effects on peripheral and central systems. Its use has increased significantly over the last few decades, fast becoming a world-wide problem that is now recognised by the public, the criminal system and researchers (Sheridan, Bulter & Wheeler, 2009; Wu, Pilowsky, Schlenger & Galvin, 2007). Initially, developed as an amphetamine derivative, methamphetamine quickly became a popular medication during the 1940s and 1950s, prescribed for a variety of concerns, including obesity, narcolepsy, depression and sinus inflammation. MA's chemical structure is similar to AMP, but it has a more potent effect on the central nervous system. That is, MA is the N-methylated analogue of AMP and it metabolizes into amphetamine in the body (Julien, 2001). Moreover, it is commonly accepted that MA is more addictive and preferred to AMP by drug users. By the 1960's, the easy availability of MA resulted in its use becoming popular by students and truck drivers to stay awake and athletes as a diet aid. In the 1970's the U.S government made MA illegal resulting in 'black market' manufacturing and distribution primarily by motor cycle gangs. In the 1990's large clandestine laboratories appeared, and over the past 30 years the illicit manufacture and use of MA has increased substantially.

MA is popular as a "street drug" because of its ease and cost-efficiency of manufacturing and low price compared to heroin or cocaine (Slamberova, Pometlova and Charousova, 2006). MA is produced, or "cooked" quickly, reasonably simply, and cheaply by using legal and readily available ingredients. In New Zealand, these include ephedrine or pseudoephedrine (available in cold and flu tablets), red phosphorous (used in matches), iodine (health food retailers), ammonia (supermarket), paint thinner and lye (hardware stores), methylated spirits and drain cleaner (McFadden & Matuszewich, 2007, New Zealand Police, 2009; Volkow et al., 2001;

Wilkins, Griffiths & Sweetsur, 2009). Recipes' for "cooking" MA can be found on the Internet. It is known in New Zealand by the street names of "P", meth, ice, pure and crack, and is generally smoked or injected. Over the past two decades the purity of available MA has increased with new technology and manufacturing techniques. A higher risk of dependency and compulsive patterns of use are associated with users that smoke or inject purer forms of MA (McKetin, Kelly & McLaren, 2006).

Immediately after use of MA, the individual experiences a number of sensations arising from the interaction of the drug with various neurotransmitter systems, primarily the dopaminergic but also the epinephrine and norepinephrine systems. The greatest subjective effect is the sense of euphoria, increasing productivity, experiencing heightened attentiveness and curiosity, hypersexuality, decreased anxiety, and increased energy (Cretzmeyer et al., 2003). These pleasurable feelings may vary in intensity and duration, depending on the mode of administration, with intravenous injection or smoking the preferred options. The MA subjective effects are estimated to last between four and 24 hours (Panenka, Procyshyn, Lecomte, MacEwan, Flynn, Honer, & Barr, 2013) and even after just one dose of MA, it persists in the body for nearly one day (Cook et al., 1993). The effects on the body are long-lasting due to the relatively slow rate at which MA is metabolized. MA has a half-life of 8 to 24 hours with 50 percent still remaining in the body after 24 hours, whereas cocaine is metabolized rapidly and has a half-life of approximately 30 minutes (National Institute on Drug Abuse: NIDA). Despite the short-term desirable effects the long term use of MA has significant negative implications for the psychological, physiological and social wellbeing of the individual (McKetin et al., 2006).

Psychological effects include decreased sleep, depression, paranoia, hallucinations and irritability (Sommers, Baskin & Baskin-Sommers, 2006), decreased appetite, increased libido increased confidence and elevated mood (Panenka et al., 2013). Higher doses can cause dysphoria which can be evident as restlessness and anxiety and associated with tremors and dyskinesia (Panenka et al., 2013). With prolonged methamphetamine use, consumers may experience nutritional and sleep deprivation, which can result in a number of mood and/or cognitive problems including fatigue, anxiety and depression (Cretzmeyer et al., 2003). Disorganized lifestyle, poor coping abilities and unprovoked incidents of violence are common. MA users tend to indulge in a 'binge-crash' cycle of 1 to 3 weeks duration, which intensifies the negative effects as the binge period progresses. The crash symptoms include irritability, depression, fatigue, anxiety, impaired social functioning, aggression and suicidal

and homicidal ideation. Binge users can exhibit highly focused and repetitive behaviours, known as “punding” which is the most severe form of Repetitive Reward-Seeking Behaviours (Fasano, Barra, Nicosia, Rinaldi, Bria, Bentivoglio & Tonioni, 2008). This is where an individual may have an intense fascination with complex, excessive, non-goal orientated, repetitive activities like manipulation of technical equipment, examining or sorting through common objects, writing lists, grooming or hoarding objects. These behaviours are reported to be calming or soothing. However the individual can become irritable when distracted from their chosen task (Fasano et al., 2008).

Physiological effects include increased heart rate, elevated blood pressure, vasoconstriction, strokes, heart attacks, irreversible damage to blood vessels, tooth decay (meth mouth), loss of appetite, malnutrition, anorexia, self-inflicted wounds, bone and hair loss, dry mouth, respiratory failure, chest pain, liver damage, kidney and lung disorders and permanent nerve damage (Panenka et al., 2013). These effects are attributed to continuous use rather than occasional or recreational use of MA. The rates of infectious disease, including human immunodeficiency virus (HIV) and hepatitis C are increased by intravenous MA use and the engagement in more risky sexual behaviours (Paneka et al., 2013). Also, certain individuals who use MA experience compulsive scratching which results in skin lesions or delusional parasitosis, in that the individual believes that bugs are crawling under their skin (Hinkle, 2011). The common street term for this is ‘P worms’ in New Zealand.

Social implications are far reaching. The relationship between violence and aggressive behaviour related to MA is not well understood. Sleep deprivation and paranoia may be a factor related to violence while under the influence, yet social and biological factors, namely gang and other culture affiliation may also be a contributing factor (Cretzmeyer et al., 2003). The Methamphetamine Treatment Project examined the history of abuse and violence in a sample of 1016 MA users in treatment for MA dependence. Cohen and colleagues (2003) report that history of abuse and violence experienced by the individuals was extensive, with 80% of women reporting violence or abuse from a partner. Men were more likely to experience violence from friends and others. The researchers concluded that current and past interpersonal violence is a characteristic of the lifestyle of MA users. Children of MA users can suffer neglect and abuse and the use of MA by pregnant women can cause growth retardation, premature birth, and long-term cognitive deficits in children (Anglin, Burke, Perrochet, Stamper & Dawnd-Noursi, 2000). Risky sexual behaviour and practices increases the likelihood of HIV and other sexually transmitted diseases. Lifetime costs of MA exposure

to adults and their children are estimated to exceed \$1.5 million per user when considering child-protection services, special education, criminal justice, healthcare, mental health and other domains (McDaniel & Embry, 2001).

MA interacts with nerve terminals that use DA and NE (catecholamines) or 5-HT (indoleamine), as neurotransmitters. Regardless of route of administration (swallowed, snorted, smoked or taken intravenously), MA crosses the blood-brain barrier and enters these terminals, inducing a non-exocytotoxic transmitter 'release' (Marshall and O'Dell, 2012). Acutely this accounts for the initial euphoric and other stimulatory actions of MA. In addition to acute effects, MA is capable of inducing long-lasting brain changes. Volkow et al. (2001) and McCann et al. (1998) found DA transporter binding reductions persisting for at least eleven months after last MA use in human subjects.

MA affects neuronal functioning through the continuous overstimulation of the neural transmitters DA, epinephrine, and norepinephrine (NE). NE is released first, followed by DA then 5-HT. Panenka and colleagues (2013) reviewed the reported mechanism of action of MA and concluded that the acute effects of MA modulate DA release by acting at two main molecular substrates on DA neuronal terminals: the vesicular monoamine transporter-2 (VMAT-2) and the plasmalemmal DAT. According to the 'exchange-diffusion model' (Fleckenstein, Volz, Riddle, Gibbs & Hanson, 2007), MA competes with synaptic DA at the extracellular site on the DAT. Because the DAT can transport DA in a bi-directional manner, and concentrations of DA are greater inside the cell, the binding of MA on the extracellular side causes cytosolic DA to be reverse-transported outside of the cell. Additionally, MA is taken up into the presynaptic terminal where it binds to and inhibits vesicular monoamine transporter 2 (VMAT2). This mechanism of action prevents cytosolic DA from being re-vascularised, leaving more DA (and 5-HT in 5-HT terminals) in the cytosol where it is either pumped back out into the synaptic cleft by MA-induced reversal of DAT and SERT or escapes by passive diffusion. Through these interactions individuals can experience psychotic states, such as paranoia and hallucinations, which in turn can lead to extremely irrational behaviours as well as suicidal and homicidal ideation (Zweben, Cohen, Christian, Galloway, Salinardi, Parent & Iguchi, 2004). Chronic long-term use is associated with alterations to the DA system, including depletion of DA, a decrease in the number of DAT neurons, and a degeneration of nerve endings (De Vito & Wagner, 1989). Behaviourally a reduction in DA transported is associated with poor motor activity, decreased memory performance, and reduced verbal learning.

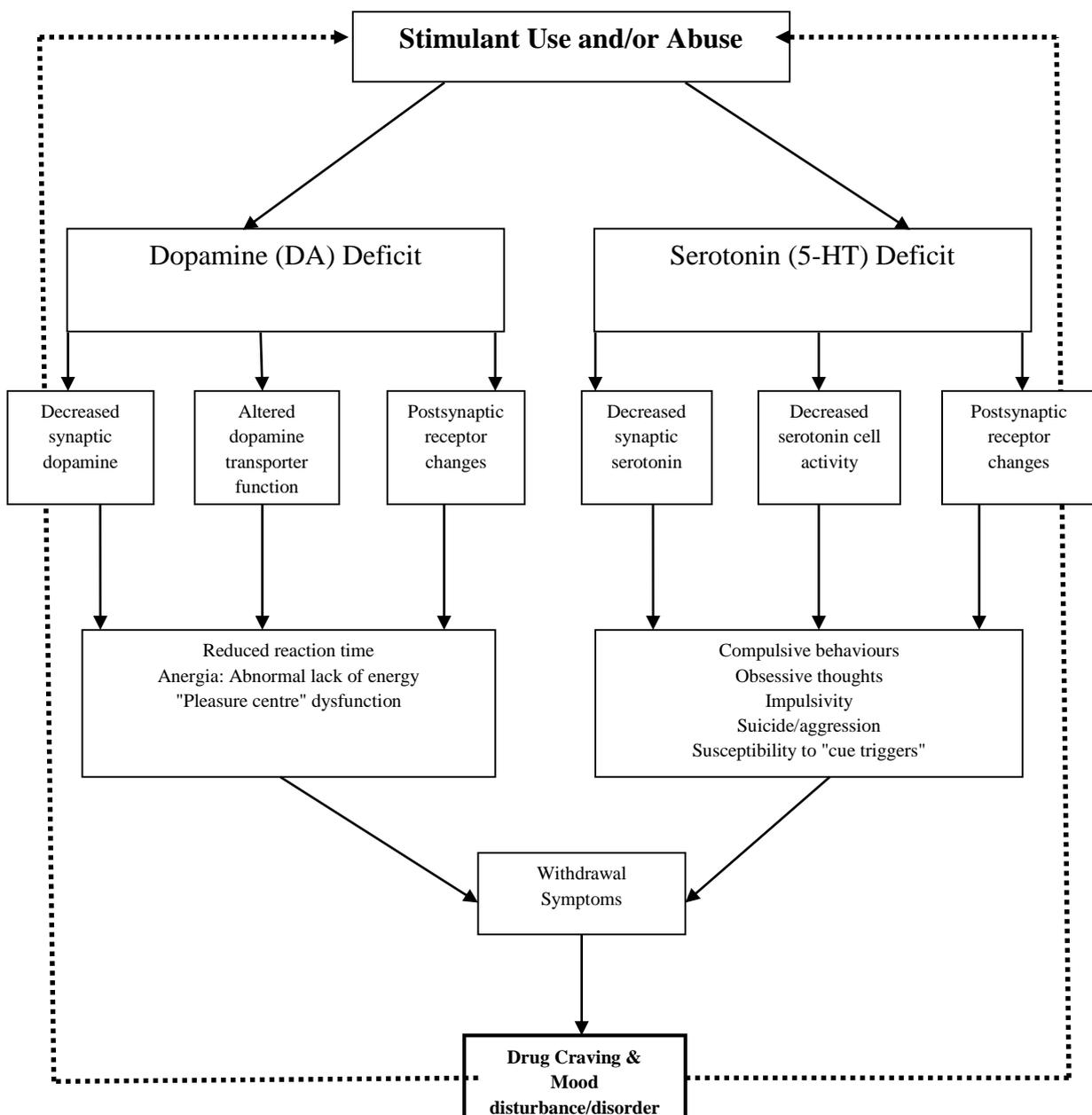
Kalechstein and colleagues (2003) found that former MA users continued to demonstrate impairments across a variety of neurocognitive domains (attention, psychomotor speed and executive functioning) even after a short period of abstinence. The impact of MA on executive functioning has been well documented (Paulus, Hozack, Zauscher, Frank, Brown, Braff & Schuckit, 2002). Block, Erwin, and Ghoneim (2002) compared the cognitive functioning of adult drug users in treatment, and non-using controls on achievement tests. Two to three weeks after last use, drug users scored lower than non-drug users on all four achievement tests and could be classified according to their primary drug dependence, namely stimulant, alcohol or polydrug users. The same tests were administered 11 to 15 weeks after last use and non relapsers were compared to relapsers. Only one test showed relative improvement for the abstinent drug users and there was no difference in improvements among the stimulant, alcohol and polydrug users. They concluded that the greater cognitive impairment was greater for stimulant users than other classes of drugs. Overall, it appears that humans who use MA, similar to rats exposed to this drug can suffer long-lasting brain injury (Marshall & O'Dell, 2012).

Among adolescents, MA is now the second most used illicit substance after marijuana in over 36 countries, and this age group is now identified as the key group at risk of MA use (New Zealand Police, 2009; Wu et al., 2007). A United Nations Survey found that New Zealand and Australia were second only to Thailand for MA use in 2001, with 3.4% of the population using MA (Degenhardt & Hall, 2012). New Zealand experienced what was termed a 'P' epidemic (McCrone, 2008) and under the harm reduction umbrella BZP was proposed to be a safe and legal alternative for MA use. It appears there is sufficient evidence to suggest that BZP and MA share similar mechanisms of action and have the same potential for abuse. However, with the exception of one study the long term effects of BZP are largely unknown. Aitchison and Hughes (2006) found that BZP treatment during adolescence led to heightened anxiety in later development possibly because of interference with the maturation of anxiety-associated forebrain mechanisms operated by 5-HT.

### ***Long-term effects of Stimulant exposure***

Psychostimulant drugs such as MA, AMP or cocaine have profound and long-lasting neurobiological effects, which may affect anxiety or social behaviours. There is evidence that use of any stimulant drug leads to similar long-term consequences. Although, many other neurotransmitters are involved in and contribute to substance abuse, Figure 1 simplifies the

roles of stimulant drug use on both DA and 5-HT neurotransmitters. Deficits in these neurotransmitters can lead to a range of negative outcomes that can constitute withdrawal symptoms and mood disturbances. To alleviate these negative outcomes, the individual may continue to use the drug, leading to escalating drug use and a circular pattern that may eventually constitute a SUD. Additionally, a number of behavioural, cognitive and psychological consequences can occur. The DA deficits produce predominantly psychomotor deficits, whereas, 5-HT deficits may produce cognitive behavioural dysregulation and emotional changes. In other words, fundamental aspects of pleasurable human behaviour and survival are compromised by continued use of the stimulant drugs (Rothman & Bauman, 2003).



**Figure 1: Stimulant use and/or abuse and the corresponding deficits in dopamine and serotonin transmission. Dopamine deficits underlie psychomotor disturbances, whereas, serotonin deficits causes mood disturbances and lack of impulse control, characteristic of withdrawal symptoms, in turn, contributing to increased drug craving and mood disorders. (Adapted from Rothman & Baumann, 2003).**

Evidence from long-term studies of human MA abuse suggests that detrimental effects occur in tests of memory, mental flexibility and abstract thinking (Simon, Dacey, Glynn, Rawson, & Ling, 2004) and these deficits continue for periods lasting for five days to several years. Additionally, there is evidence that abusers of stimulant drugs may develop abnormalities in brain regions implicated in mood disorders. London et al. (2004), found that human abusers of MA provided higher self-ratings of anxiety and depression than controls. In addition, social withdrawal, paranoia and anxiety are well documented occurrences in human MA users (Rawson, Gonzales, & Brethen, 2002). King, Alicata, Cloak and Chnag (2010) found in adolescent abstinent MA users that even after 11 months of not using MA, that MA had induced psychological and behavioural alterations displayed as increased depression and anxiety scores and increased cortisol secretion following a social stressor compared to non-MA users, with female MA-users having the most symptoms.

The ability to quickly respond to objects, events and threats in the environment is essential for the survival of humans and animals alike (Gawronski & Cesario, 2013). Fear and anxiety are behaviourally very similar, however anxiety is defined as a long-lasting state of apprehension that can become pathological if it becomes extreme, whereas, fear is prompted by imminent and potential real danger and serves to activate the individual to respond. Anxiety is a future-oriented mood state that is associated with arousal and vigilance (Davis, Wlaker, Miles & Grillon, 2010) and behavioural avoidance is a central feature of anxiety disorders. In rats, anxiety is an avoidance behaviour that is considered to reflect fear of novelty, whereas approach behaviour (e.g. exploration) reflects motivation to explore novelty. In the current research, the conflict between approach and avoidance was used, however, it is proposed that they do not represent ends of a continuum, but instead are independent biological functions (Green, Barnes & McCormick, 2012).

Commencing early in life, anxiety disorders are one of the most common mental disorders with an estimated one in fourteen people meeting diagnostic criteria at any one point in time (Baxter, Scott, Vos and Whiteford, 2012). No one single brain structure or neurotransmitter controls the anxiety response system. It is reported that several interrelated systems operate together in complex ways to induce anxiety. The hypothalamic-pituitary-adrenal (HPA) axis and the limbic system (particularly the amygdala) act as a mediator between the brain stem and the cortex, the PFC and other cortical and subcortical structures to evoke a behavioural responses to potential threats and fear (Chaby, Cavigelli, Hirrlinger, Caruso & Braithwaite, 2015). The HPA axis is the central factor of the brain's neuroendocrine response to stress. The

hypothalamus, when stimulated, secretes the corticotropin-releasing hormone (CRH), which stimulates the pituitary gland to secrete the adrenocorticotrophic hormone (ACTH) into the bloodstream. In order to prepare the body to meet a challenging situation, ACTH then causes the adrenal gland to release cortisol. This system works on a feedback loop and it is reported that damage or disruption to this feedback loop can effect normal responses to stress and abilities to regulate emotions, such as anxiety or mood disorders (Chaby et al., 2015).

There are three possible views concerning the association between substance use and anxiety disorders. One of these is based on a causal link between anxiety disorders and substance use in order to alleviate the anxiety symptoms. This is described as the "self-medication hypothesis" (Harris & Edlund, 2005). For example, an individual will use stimulants to decrease inhibition and increase confidence, or conversely will use depressants to decrease tension or anger. A second opposing view is that the causality runs from substance use to anxiety disorders (Chilcoat & Breslau, 1998); specifically that substance use may advance the onset of anxiety symptoms. The final view is that there is a shared etiology or a third variable, such as a genetic predisposition. Merikangas et al. (1998), suggest that genetic factors, environmental risk factors and/or prenatal environment may predispose an individual to both substance use disorders and anxiety disorders. The present study was influenced by the view that causality progresses from substance use to anxiety disorders. However, neither of the other two approaches should necessarily be discounted because animals used in the present study were normal rats, without genetically increased anxiety or any differences in environmental or prenatal manipulations. Therefore, it would be likely that if BZP or MA induces behavioural changes in adulthood, these would have arisen purely from earlier exposure to the stimulant substances, relatively independent of other influences.

Another long-term outcome of earlier stimulant abuse is behavioural sensitization. Behavioural sensitization occurs after the repeated administration of a substance and cross-sensitization occurs when an increased behavioural response occurs to a different drug after previous repeated administration of another drug (Landa, Machalova and Sulcova, 2014). The expression of sensitization is associated with the reinstatement of self-administration of the substance after a long period of abstinence (Everitt & Wolf, 2002) and is thought to underlie certain aspects of addiction and drug-induced psychosis (Vanderschuren, Schmidt, Vries, Van Moorsel, Tilders & Schoffelmeer, 1999). Psychostimulant sensitization is characterised by progressive increases in locomotor response after repeated drug administration and is thought to result from experience-induced plasticity in brain regions involved in mediating motivation

for drugs (Clark & Bernstein, 2004). Behavioural sensitization is well documented in humans (see Leyton, 2007 for a review) and animals models suggest this occurs through sensitized activations of the DA system. In rodents, sensitization from BZP to BZP (Brennan et al., 2007; Meririnne et al., 2006), AMP to AMP (McPherson & Lawrence, 2006; Vanderschuren et al., 1999) and cocaine to cocaine (Snyder, Katovic & Spear, 1998) has been documented, and cross-sensitization from BZP to MA (Brennan et al., 2007), AMP to nicotine (Santos, Marin, Cruz, DeLucia & Planeta, 2009) and cocaine (Vanderschuren et al., 1999), MP to AMP (Valvassori et al., 2007) and cocaine (Brandon et al., 2001) and MDMA to cocaine (Fone, Beckett, Topham, Sweetenham, Ball & Maddocks, 2002) to name a few. In the few studies that look at long-term behavioural change, these effects appear to more prominent with increasing abstinence (Vanderschuren et al., 1999). Behavioural sensitization is important, as there may be a cohort of young people who consumed BZP (as a legal substance) that are now more sensitive to the effects of other stimulants and Wilkins et al. (2006) reported that 13.5% of individuals they surveyed reported using BZP-based pills prior to any other illicit substance.

### ***Long-term Outcomes of Adolescent Exposure to Amphetamine (AMP) in Animals***

Amphetamine effects result from elevation of the extracellular DA and prolonging DA signaling in the striatum. Three mechanisms have been proposed, first AMP is a substrate for DAT that inhibits DA uptake; secondly, AMP facilitates the movement of DA out of the vesicles into the cytoplasm; and third, AMP promotes DAT-mediated reverse-transport of DA into the synaptic cleft independently of action-potential-induced vesicular releases (Fleckenstein et al., 2007). Low doses of AMP (1 mg/kg) are suggested to be therapeutically enhancing, yet high doses (10 mg/kg) can produce a behavioural profile similar to the positive symptoms associated with schizophrenia (Daberkow et al., 2013). McPherson and Lawrence (2006) administered either a low (2 mg/kg) or high (10 mg/kg) dose of AMP to male rats, during PND33-41. After a 4-week period of abstinence, rats received either AMP or saline and their locomotor activity was assessed for 60 minutes, then anaesthetized. The researchers concluded that exposure to AMP during the periadolescence period in rats, established behavioural sensitization to an AMP re-challenge in adulthood. Rats previously treated with 10 mg/kg of AMP had a higher Fos expression compared with rats treated with 2 mg/kg, however the locomotor activity displayed was similar between the two groups. The researchers concluded that adolescent exposure to AMP led to a neuronal sensitisation in adulthood and this was accompanied by widespread neuronal activation.

As most studies of the effects of adolescent stimulant exposure in adulthood, follow a re-challenge with the drug, Labonte et al. (2012) were interested in the subsequent altered emotionality of the rodents in a drug-free condition. They treated male rats during PND30 and PND50 with either saline, 0.5, 1.5 or 5.0 mg/kg of AMP. After a 20 day wash-out period the rats were either randomly assessed in an open-field test and EPM or used for electrophysiological analysis. 5-HT and DA firing was increased for rats treated with 1.5 mg/kg AMP but not NE firing, in contrast with the rats treated with 5 mg/kg, in which NE firing was increased but not 5-HT or DA firing. Similarly, the rats treated with the middle dose of AMP significantly increased the total distance travelled in the EPM, while this effect did not occur for the other two doses. All three doses of AMP increased the time spent in the open arms and central platform, as well as the number of stretch-attend postures made. Labonte et al. (2012) concluded that AMP exposure during the adolescence developmental stage, in rats, may promote long-lasting neurophysiological alterations that influence their future behaviour.

Sherrill, Stanis and Gulley (2013) administered 1 or 3 mg/kg of AMP, every other day to adolescent rats (PND37 to PND55) and adult rats (PND98 to 116). The authors were interested in the long-lasting effects of repeated AMP exposure on locomotor sensitisation and cognition in groups of rats treated during adolescence or adulthood. The rats were tested at adulthood on cognitive performance (delayed matching-to-position and delayed nonmatching-to-position). Adolescent treated rats were less sensitive to the psychomotor effects of AMP and more vulnerable to exposure-induced cognitive impairment, compared to adult-treated rats. The authors suggested that adolescent rats are more susceptible to AMP-induced neurobehavioral deficits than adult AMP treated rats. They proposed that the cognitive impairment is possibly due to the adolescent brain still undergoing neural development and tentatively suggested that the PFC may be an important mediating factor in their results. A study assessing sustained attention after 1.0, 2.0 or 3.0 mg/kg of AMP administered to male PND54 rats, for five alternative days, found that after sensitisation had occurred, the effects of AMP treatment led to attention consequences. Deller & Sarter (1998) reported that treated rats had increased false alarms, like "claims" for hits in non-signal trials, suggestive of hyperattentional dysfunctions that may contribute to the development of psychostimulant-induced psychotic symptoms.

Overall, it appears that exposure to AMP during adolescence leads to long-lasting neurophysiological alterations, which contributes to changes in the rats' behaviour in adulthood. The cognitive impairments displayed after AMP treatment seem to be dependent on the age of exposure and possibly due to complex neural mechanisms.

### *Long-term Outcomes of Adolescent Exposure to Cocaine in Animals*

Cocaine is an indirect agonist at the DA receptors by inhibiting DAT in neuron terminals, causing an increase of concentration and intensity of DA action on postsynaptic receptors (Pereira, Andrade and Valentao, 2015). Estelles et al. (2007) treated both PND26 (defined as adolescent) or PND46 (defined as early adult) male mice to either daily injections of 25 mg/kg of cocaine for nine days, or the same total amount of cocaine over a three day period (defined as binge administration). Three variables were examined: age; pattern of drug administration and the housing conditions (either isolated or in groups). 15 days later they were evaluated in measures of cocaine-induced motor activity; anxiety, using the Elevated plus maze (EPM) and their social profiles using the social interaction test (SI). The mice treated with cocaine from PND26 and housed individually displayed increased avoidance and flee in the social interaction measure, and the same age treated mice that had been housed in groups displayed decreased social contacts. The binge-treated PND26 mice increased the time spent on the open arms of the EPM. Both PND26 and PND46 mice daily treated with cocaine displayed an increase in cocaine-induced motor activity when challenged with 6 mg/kg of cocaine two weeks after drug treatment. Similarly, Snyder et al. (1998) administered 30 mg/kg cocaine to PND14 male and female rats for a period of seven days. The drug treatment was paired with placement in a novel test context. The rats were then challenged with a 15 mg/kg of cocaine in the same environment either 1, 3, 7, 14 or 21 days after the administration phase. They concluded that behavioural sensitization was evident in terms of squares crossed in the test environment and stereotypy behaviours and suggested that behavioural sensitisation can be expressed for weeks following chronic treatment during late pre-weaning period. Santucci & Rosario (2010), administered daily 10 mg/kg or 20 mg/kg cocaine to PND30 male rats for eight days and measured their anxiety-like behaviour twice following a ten to 11 day withdrawal period. Group differences of both cocaine-treated rats were displayed only on the second test day, whereas the control animals increased the amount of time spent in the open arms of a zero maze, relative to the cocaine-treated rats. The authors concluded that an anxiety-like response produced by adolescent treatment with cocaine emerges after a relatively short abstinence period. The results of Santucci and Rosario's (2010) research are supported by Santucci and Maderia (2008) study, which found the same anxiogenic response after an abstinence period of eight and thereafter twelve weeks. Overall, it appears that adolescent exposure to cocaine largely affects anxiety-related behaviours in the treated mice and rats tested in adulthood.

### ***Long-term Outcomes of Adolescent Exposure to 3,4-methylenedioxymethamphetamine (MDMA) in Animals***

Methylenedioxymethamphetamine (MDMA) is a ring-substituted amphetamine derivative that acts by increasing the release of 5-HT, noradrenaline and DA from their respective axon terminals. MDMA does not act by directly releasing 5-HT but rather by binding to SERT and preventing the re-uptake (Kalant, 2001). Piper and Meyer (2004) administered (PND35-60) with MDMA (10 mg/kg) twice daily for six days, to male rats, and observed a significant weight loss over this period. Five days after the last doses of MDMA the rats were assessed in an open field (OF), an object recognition task and the EPM. The results suggested that MDMA did not affect ambulatory behaviour in the OF. However the MDMA rats showed impairment on the object recognition test and reduced anxiety in the EPM. These results suggest that MDMA exposure during adolescence can alter subsequent cognitive and affective function, and Piper and Meyer (2004) reported that this occurred in the absence of severe damage to the 5-HT. Similarly, Gurtman, Morley, Li, Hunt and McGregor (2002) and Morley, Gallate, Hunt, Mallet and McGregor (2001) found deficits in working memory but also reported increased anxiety-like behaviour in adult rats treated with MDMA in adolescence. However, Kolyaduke and Hughes (2013) administered MDMA (10 mg/kg) daily to PND35 (defined as early adolescence) and PND45 (defined as late adolescence) male and female rats for ten days. Rats were subsequently tested in an OF, Y-maze, light/dark box and a novel object test at PND90 in a drug-free state. Female rats that had been treated with MDMA during early adolescence were heavier than saline controls at PND90, whereas male rats treated with MDMA during late adolescence were heavier than saline treated rats at PND90. MDMA treated rats displayed decreased OF ambulation (for male treated rats only), increased defecation, plus fewer entries into the light compartment of the light/dark box and entries into both arms of the Y-maze. Of note, there was no evidence for any effects on spatial or working memory. Overall, Kolyaduke and Hughes (2013) suggested that rats exposed to MDMA during adolescence, especially late adolescence had increased anxiety in adulthood.

Piper, Fraiman and Meyer (2005) administered MDMA (5 mg/kg) four times daily for six days to male rats during PND35-60 and tested them on a variety of anxiety and memory tests at PND64-70. They reported that MDMA altered habituation to an OF, increased motor activity in the EPM, decreased attention in a novel-object recognition test and reduced transporter binding in the neocortex. They concluded that repeated exposure to MDMA during adolescence produced later changes in behaviour and neurochemistry. Similarity, Fone et al.

(2002) found that rats tested in a SI paradigm 12 days after the last dose of a 7 day regime of MDMA, showed decreased rearing in the OF and a SI reduction of 41%. Additionally on a cocaine challenge, the MDMA-treated rats showed significant conditioned place preference compared to control rats. Some immediate effects of MDMA given to PND39 male and female rats included the females displaying significantly greater locomotor activation than males at a later MDMA challenge. However males showed greater hyperlocomotion at the second challenge and all of the ten male rats died at the third challenge (Koenig et al., 2005).

Morley and colleagues (2001) administered moderate (1x5 mg/kg) or high dose (4 x 5 mg/kg) of MDMA or AMP (4 x1 over four hours) for a two day period and, 12 weeks later, tested the animals on three anxiety-provoking measures (emergence box, SI test and the EPM) and a test of object recognition memory. They reported that high MDMA treated rats displayed greater anxiety on all three anxiety measures compared to saline- or AMP-treated rats. The moderate dosed rats showed increased anxiety, but not to the extent of the high MDMA treated rats and the AMP animals did not differ from control animals. However, Clemens, van

Nieuwenhuyzen, Li, Cornish, Hunt & McGregor (2004) found that after an acute dose of either MDMA (2.5 or 5 mg/kg x 4), or MA (2.5 or 5 mg/kg x 4) or MDMA and MA (1.25 + 1.25 or 2+2 mg/kg) combined, four to five weeks later the MDMA/MA- treated rats, MA and high dose MDMA rats displayed less social interaction in a SI test. A lower dose of MDMA also produced increased anxiety on the emergence test. Seven weeks after the last drug administration, these researchers conducted a neurochemical analysis and concluded that MDMA caused 5-HT and 5-HIAA depletion in several brain regions (PFC, striatum, hippocampus and amygdala) whereas MA pre-treatment reduced DA in the PFC. McGregor, Gurtman, Morley, Clemens, Blokland, Li, Cornish and Hunt (2003) administered 5 mg/kg MDMA every four hours for two days to late adolescent rats and tested them in a variety of anxiety measures (SI, emergence test and forced swim) eight to ten weeks after last drug use. Two to 4 months later MDMA pre-treatment resulted in increased anxiety in the SI test and emergence test and reduced escape attempts and increased immobility in the forced swim test.

Cox, Shah, Cichon, Tancer, Galloway, Thomas and Perrine (2014) were interested in how the effects of MDMA (5.0 or 10 mg/kg) administered once daily for four days to rats during PND38-41, would affect place conditioning to MDMA, anxiety-like behaviour and their subsequent monoamine levels once abstinent from the drug. Rats treated with 5 mg/kg of MDMA during adolescence were similar to saline-treated rats on all measures. However, rats treated with 10 mg/kg displayed place aversion one day after the last exposure to MDMA and

similar to Kolyaduke and Hughes (2013) study, avoidance behaviours in the light/dark box and increased anxiety-like behaviours in the OF after a five day absence period. Cox et al. (2014) found an opposing result to Clemens et al. (2004) on 5-HT alteration due to adolescent MDMA treatment. Whereas, Clemens et al. (2004) reported a depletion of 5-HT and 5-HIAA in the brain regions mentioned above, Cox et al. (2014) found decreased 5-HT in the dorsal raphe, and increased 5-HT and 5-HIAA in the amygdala and no change in the hippocampus. It is noted though that Clemens et al. (2004) administered MDMA to adult male rats, whereas Cox et al. (2014) treated adolescent male rats, suggestive of different 5-HT receptor functioning possibly due to age of treatment. Similarly, Bull et al. (2004) treated PND28 male rats with MDMA (5 mg/kg) hourly for four hours on two consecutive days. Social interaction occurred on PND84 in a drug free state, and Bull et al. (2004), found that social interaction was reduced by 27% in the pre-treated rats compared to control rats. On PND86, rats received MDMA (5 mg/kg) followed by either saline or 5-HT<sub>2A/2C</sub> receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 1 mg/kg) and locomotor activity, wet-dog shakes and back muscle contractions were monitored, followed by an EPM test. On the EPM, pre-treatment with MDMA prevented the anxiogenic effect of DOI. On PND92, neurochemical analysis showed significant reductions in hippocampal 5-HT and 5-HIAA concentrations, and 5-HT levels were also depleted in the frontal cortex and striatum. The researchers concluded that the inability of DOI to decrease anxiety-like responses suggests that the 5-HT<sub>2A</sub> receptor may have mediated the observed behaviour in the MDMA pre-treated rats.

Kelly, Ritchie, Quate, McBean & Olverman (2002) treated pregnant female rats on gestational day 15 (E15) with MDMA (20 mg/kg), twice daily for four consecutive days, neonatal male rats (from untreated dams) with the same MDMA treatment on PND10, 15, 20, 25 or 30 and another group of rats with the same MDMA treatment on PND90. This study investigated the toxicity induced by MDMA in the perinatal rat brain and its relation to 5-HTT sites and whether early exposure to MDMA alters subsequent brain functions in adulthood. Kelly et al. (2002) found no difference in the density of [<sup>3</sup>H]-paroxetine binding sites, measured at PND40, in the brains of MDMA treated rats from E15 to PND20, compared to controls. However, MDMA treatment from PND25 onwards resulted in significant reductions in [<sup>3</sup>H]-paroxetine binding, with decreases of 46% at PND25, 63% at PND30, and 90% at PND90, compared to control rats. They concluded that MDMA-induced neurotoxicity is absent in the perinatal period and increases markedly from PND25 onwards, although not to the same extent as what is found in the adult rat brain. Klomp, den Hollander, de Bruin, Booij and Reneman (2012)

investigated the effects of MDMA on the 5-HT system and whether MDMA effects in 5-HT densities were dependent on the age of first exposure. They treated male rats from PND27 or PND63 with 10 mg/kg MDMA twice daily, for four consecutive days, and following a seven day wash-out period, were injected with [ $^{123}\text{I}$ ] $\beta$ -CIT and then anaesthetised. MDMA treatment produced significant reductions in 5-HTT binding in several brain regions and this was more pronounced in the PND63 rats (ranging from 35% to 75%) compared to the PND27 treated rats (ranging from 20% to 69%). The effect of age and treatment was observed in the frontal cortex of rats and not in the midbrain. They concluded that the difference on the developing and mature brain of MDMA effects may be due to the different maturational stages of the 5-HT projections at the age of first exposure.

In summary, it appears that MDMA-induced neurotoxicity to the 5-HT system occurs from PND25 and is largely absent during gestation or the perinatal period. Adult rats seem to be more susceptible to neurotoxic effects than adolescent rats. Notwithstanding, the literature suggests long-term decreases in 5-HT, 5-HIAA and 5-HTT binding sites within the adolescent rat brain due to MDMA administration, however further research is required into the effects of MDMA exposure on other neurotransmitter functions.

### ***Long-term Outcomes of Adolescent Exposure to Methylphenidate (MPH) in Animals***

Methylphenidate (MPH, commonly known as Ritalin®) is the prominent pharmacological treatment in the management of attention deficit hyperactivity disorder (ADHD) and the pharmacological pathways and psychotropic effects are similar, although not identical to those produced by other stimulant substances. MPH acts mainly through the modification of the DA system, by blocking DAT, and consequently by increasing meso-corticolimbic DA levels (Volkow et al., 2001). The majority of published research into the effects of MPH on later behaviour largely exposes juvenile animals (<PND40), due to MPH's abilities to treat childhood ADHD, and the prominent area of investigation focuses on the potential increased risk of drug addiction in adulthood following MPH earlier use.

Brandon et al. (2001) found that MPH (2 mg/kg) administered to PND14 male rats, enhanced the rewarding effects of cocaine administration at PND56, without inducing locomotor sensitisation. However, Carlezon et al. (2003) treated male rats aged PND20 until PND35 with MPH (2 mg/kg) or cocaine (15 mg/kg twice daily) for 15 days and then tested them as adults on PND60. These authors were interested in how early exposure to either MPH or cocaine would affect sensitivity to the rewarding and aversive properties of cocaine using a place

preference paradigm. They reported that early exposure to MPH or cocaine made moderate doses of cocaine aversive and high doses less rewarding in adulthood. Early MPH use also caused depressive-like effects in a forced swim test. They concluded that earlier exposure to MPH caused long-term behavioural changes enduring into adulthood, some of these changes were considered to be beneficial (reduced sensitivity to reward) whereas others (increases in depression) may be detrimental. Andersen et al. (2002) found similar results with earlier MPH treatment reducing self-administration of cocaine and rewarding properties. Additionally, Bolanos et al. (2003) found the MPH-treated animals were significantly less responsive to rewards such as sucrose, novelty and sex compared with control animals. They also found that the earlier MPH-exposed animals were significantly more sensitive to stressful situations and showed increased anxiety-like behaviours and these effects lasted well into adulthood, compared to the saline-exposed group (Balanos, et al., 2003). They concluded that this effect was because of deficits in the mesolimbic DA system induced by early exposure to stimulants. Likewise, Crawford, Der-Ghazarian, Britt, Varela & Kozanian (2013) investigated juvenile male and female (PND11-PND20) treatment with MPH (2 or 5 mg/kg) and the effects in adulthood (PND60) on novelty-induced conditioned place preference, sucrose preference and the EPM. They found that early MPH exposure altered the rats' preference for the white compartment of the of the placement chamber. Crawford et al. (2013) suggested that the MPH pre-treated rats displayed an anxiolytic response to novelty, behaviourally expressed as decreased fear or anxiety. However, the MPH-treated rats displayed a long-term increase in anxiety, as observed by reduced time in the open arms of the EPM, with no significant sex effects, yet female-treated rats spent more time in the open arms, compared to male treated rats. Overall, they reported that the results of changes in anxiety-like behaviour may be dependent on the type of anxiety-provoking stimulus.

As mentioned above, the published research on MPH's ability to increase or decrease an animal's vulnerability to later substance abuse is conflicting. However, there appears to be increasing evidence of the use of MPH to treat young children with ADHD does not effect the risk of substance abuse in adulthood. For example, Biederman et al. (2008) conducted a 10-year follow-up study on 140 male children with ADHD (ages 6 to 17 years) and found no association between earlier stimulant treatment and alcohol, drug or nicotine use disorders (Biederman, Monuteaux, Spencer, Wilens, MacPherson & Faraone, 2008). Similarly, Mannuzza et al. (2008) were interested in the age of initiation of MPH treatment of ADHD children (ages 6 to 12 years) on the later development of substance use and concluded that

treatment prior to the age of eight years of age did not increase the risk of later substance use and possibly had beneficial long-term effects (Mannuzza, Klein, Truong, Moulton, Roizen, Howell & Castellanos, 2008). Additionally, Mannuzza et al. did not find any association between age of MPH treatment and mood or anxiety treatments in adulthood.

Another study of MPH treatment in rats during adolescence, on subsequent anxiety-like behaviours, investigated sex differences (Vendruscolo, Izidio, Takahashi, & Ramos, 2008). From PND23 to PND38 male and female rats were treated with MPH (2.0 mg/kg x 2 daily) for 16 days and tested in the OF, an EPM and a protocol of ethanol consumption, four weeks after last drug exposure. Earlier treatment elicited anxious-like behaviour in the open-field but not the EPM, regardless of sex, and enhanced ethanol intake in female treated rats. Male treated rats made fewer crossings of the centre of the OF than female treated rats, and all treated rats made less crossings of the centre of the OF than control rats and female rats were significantly more active than male rats. There were significant sex effects in the EPM, with female rats spending more time in the open arms and making more closed arm entries than male rats. Similarly, female treated rats had higher ethanol intake than saline-treated females, and female rats had a higher ethanol preference than male rats. Overall, Vendruscolo et al. (2008) concluded that MPH treatment during adolescence induced persistent changes on emotionality, but these effects were dependent on the sex and behavioural tests utilised.

In addition to the above mentioned behavioural changes, cognitive processes may also be effected by adolescent MPH exposure. Scherer, Cunha, Matte, Schmitz, Netto and Wyse (2010) treated male rats (PND15-45) daily for 30 days with 2.0 mg/kg of MPH. At PND45, treated rats displayed impairment in spatial learning/memory, as assessed in the Morris water maze and a reduced efficiency for finding the platform position in a working memory task. These impairments were reported to have occurred in absence of any motor deficits, as revealed by an open field measure and swim speeds. LeBlanc-Duchin and Taukulis (2009) found similar impairment in recognition (14 days after drug exposure) and spatial memory (21 days after drug exposure) in adult rats treated with MPH (5 mg/kg and 10 mg/kg). However, Zhu, Weedon & Dow-Edwards (2007) found improved spatial learning and memory in MP-treated male and female rats treated with 3.0 mg/kg from PND22 to PND39 as displayed in a radial arm maze, seven days after drug exposure. Using similar doses to Zhu et al. (2007) and the radial arm maze, Dow-Edwards, Weedon & Hellmann (2008) found improved performance on the maze during the first week of testing, however prolonged dosing did not continue to improve performance. They concluded that 3 mg/kg MPH treatment (PND22 to PND59)

improved performance on a spatial cognitive task, only early in treatment, with female rats only displaying this improvement under conditions of increased motivation. In a recent investigation on sequential learning in adulthood, after adolescent (PND25) MPH exposure (20.0 mg/kg, 5 x a week for 5 weeks), Rowan et al. (2015) found adolescent treated rats had impaired learning for some aspects of the pattern learning in the training phase and learning for the violation element in the transfer phase. They concluded that earlier MPH treatment produced multiple cognitive impairments in male rats that persisted into adulthood.

Bethancourt, Camarena and Britton (2009) found transient effects on object recognition memory and contextual fear memory after adolescent exposure (PND27 to PND71) in rats tested 18 days after drug exposure, yet no cognitive impairment. They concluded that MPH treatment had modified the hippocampal-sensitive learning and Gray et al. (2007) found MPH chronically administered to juvenile rats affected brain areas involved in cognition, motivated behaviour, appetite and stress.

Research into the long-term effects of MPH treatment on the developing brain has focused on alterations to the PFC (Arnsten & Dudley, 2005), the caudate putamen, nucleus accumbens (Adriani, Canese, Podo & Laviola, 2007), and hippocampus (Bethancourt et al. 2009). (See Marco, Adriani Ruocco, Canese, Sadile and Laviola, 2011, for a full review). Ruocco et al. (2010) was interested in the long-term effects of MPH treatment on later adult behaviour and forebrain neurochemical functioning. They administered MPH (1.0 mg/kg) once daily to male rats from PND29 to PND42. Behavioural testing in a Lat maze (a measure of 'behavioural arousal to a novel environment) and radial maze occurred at PND70-75 and the neurochemical results were subsequently conducted. MPH exposure reduced horizontal activity, although to a different extent in the two behavioural measures (i.e. 39% in the Lat maze and 16% in the radial maze) and increased non-selective attention as assessed by higher duration of wall rearing. The neurochemical results found decreased DA, 5-HT and their metabolites in the PFC and dorsal striatum, decreased NE in the PFC, decreased DA and its metabolites in the ventral striatum and decreased NE, 5-HT and its metabolite, 5-HIAA in the hippocampus. They hypothesised that the reduction in 5-HT and NE in both the PFC and hippocampus may affect mood control in adulthood after adolescent MPH exposure. Overall, the published research into long-term effects of MPH treatment during earlier development suggests numerous complex behavioural and brain adaptations, that endure into adulthood.

### *Long-term Outcomes of Adolescent Exposure to Methamphetamine (MA) in Animals*

There is evidence that the adolescent brain is protected against the neurotoxic effects of MA on the DA systems compared to adult brains. Miller, O'Callaghan and Ali (2000) treated PND30 mice with MA (4 x 10 mg/kg) and found a 50% reduction in striatal DA levels 72 hours after drug exposure, whereas mice treated to the same dose at 12 months had a 80% reduction in striatal DA levels. Similarly, PND40 rats exposed to the same treatment regime displayed no changes in DA uptake, DAT binding, DAT activity, and tyrosine hydroxylase activity in the striatum 7 days after drug treatment, whereas the same doses administered to PND90 rats had reduced DA measures (Kokosha, Fleckenstein, Wilkins & Hanson, 2000; Riddle, Kokoshka, Wilkins, Hanson & Fleckenstein, 2002). However, Kokosha et al. (2000) did find reductions in striatal DA uptake and DAT activity one hour after MA exposure in both adolescent and adult treated rats, yet this effect mitigated more rapidly in adolescent brain.

Thuong et al. (2005) found high doses of MA (4 x 10 mg/kg) administered at PND40, moderately reduced vesicular DA uptake one hour after exposure, whereas the reduced vesicular DA uptake was considered to be severe in PND90 MA treated rats, with the PND40 administration effects not evident one week later yet still present in the PND90 treated rats. Absence of significant DA alterations have also been found in rats treated with neurotoxic MA (10 mg/kg or 20 mg/kg x 4, 2 hour apart) at PND20 (Pu & Vorhees, 1993) and prior to PND21 (Vorhees et al. 2005), which may be attributed, in part, to the immature thermoregulatory system. Cappon, Morford and Vorhees (1997) suggest that from PND40 hyperthermia is required to exhibit neurotoxicity in MA treated rats. However, although early adolescent MA treated rats may not have the same depletion of the DA system as seen in older animals (Iman & Ali, 2001) they still display hyperthermia induced from MA treatment. To summarise, it appears that after PND40, is the transition age, where rats' become susceptible to MS-induced hyperthermia, monoamine reductions and glial fibrillary acidic protein increases. On PND40 they are still resistant to MS-induced neurotoxicity, however if they are administered MA in a warmed environment then they can display adult-like MA neurotoxicity..

Serotonin depletion has also been implicated in MA affects in adolescent animals (Cappon et al., 1997). Kokoshka et al's (2000) study showed that the effect of earlier MA exposure on the 5-HT system (measured by reductions in tryptophan hydroxylase) were present in both the adolescent and adult rats at one hour after drug exposure and these reductions remained seven days later. Similarly, Zombeck, Lewicki, Patel & Rhodes (2010) found MA exposure to

PND30-35 and PND69-74 resulted in comparable increases in cFos activity in a variety of brain regions between the adolescent and adult treated mice. Overall these findings suggest adolescent MA exposure causes indiscriminate impairments in 5-HT functional activation, in contrast to potential age-specific effects displayed by MA on the DA system.

MA administered to adult rats has resulted in a broad array of learning and memory impairments; however there is little published work on the effects of MA on later cognitive functioning when administered to an adolescent brain. Ye, Pozos, Phillips and Izquierdo (2014) investigated the long-term effects of MA exposure in adolescent rats on visual discrimination and reversal learning and whether MA exposure in adolescence affected later MA use in adulthood. Rats were administered either MA (0.3 mg/kg, escalating in 0.3 mg/kg increments per day) or saline from PND41 -50. Behavioural testing occurred on PND55 (Visual discrimination and reversal learning) and a drug choice of either saline, MA or a bitter substance was conducted during adulthood. The authors reported that low escalating exposure to MA during “late” adolescence period led to a generalised learning impairment which was correlated with the degree of MA intake in adulthood. They concluded that this period may serve as a critical temporal window in which the adolescent brain is vulnerable to the neuroplastic alterations caused by MA exposure. Additionally, they found that adolescent treated rats consumed significantly more MA during adulthood than adult-treated rats

Williams, Morford, Wood, Wallace, Fukumura, Broening and Vorhees, (2003) administered MA (5.0, 10.0, 15.0 mg/kg) four times daily, to male and female rats from PND11 until PND20. Significant weight loss was later observed in both sexes from PND16 until PND59. Thereafter, these were no significant changes. However, six out of the eight animals in the highest dose treatment group died between PND14 and PND16. Rats were then tested from PND50 in a variety of spatial learning tasks. They concluded that all MA groups showed impaired spatial memory but not deficits in working memory. However, Moenk and Matuszewich (2012) found male rats treated with a low dose (2 mg/kg) of MA for 15 days, from PND20 to PND34 displayed improved spatial performance in the Morris water maze when tested 45 days after treatment, compared to rats treated with MA from PND70-84. Using the same drug dosing and developmental age period as Moenk and Matuszewich (2012), McFadden and Matuzewich (2007), found MA exposed rats had long-term effects on spatial memory in a gender specific manner. Female rats displayed improved acquisition and performance in the Morris water maze compared to control females. The male treated rats also displayed improved acquisition compared to control male rats, but this improvement was only

observed during the first day of acquisition. Female rats also located the hidden platform in a novel quadrant more efficiently than control females. It is possible that the improved spatial performance seen after PND20 MA treatment may be due to confounded by sex differences.

Vorhees et al. (2005) administered MA (1.25 – 10.0 mg/kg) for ten days to either PND21-30, PND31-40, PND41-50 or PND51-60 to male and female rats and tested them in a variety of measures after a thirty day abstinence period. Rats treated with MA (2.5, 5, or 10 mg/kg x 4 dose per day), PND31-40 (1.25, 2.5, 5, or 7.5 mg/kg x 4 doses per day), or PND51-60 (1.25, 2.5, 3.75, or 5 mg/kg x 4 doses per day) did not show any changes in spatial learning/reference memory and sequential learning compared to saline- treated rats. However, the highest dose of MA (6.25 mg/kg) administered to PND41-50 rats, displayed impairment in spatial and sequential memory as observed in the Morris water maze and Cincinnati water maze. They concluded that PND41-50 is the vulnerable stage of brain development in rodents and a period of susceptibility for MA-induced cognitive deficits.

However, Joca and colleagues (2014) treated male and female mice with 2 days of 4 x 7.5 mg/kg MA on PND30 and 31, and then tested them either at PND41-52 or PND62-73 in an open field, novel object recognition, social interaction, a forced swim test and the Morris water maze. The researchers reported that MA exposure increased depression-like behaviour in the forced swim test for both male and female adolescent-treated mice. Additionally, the researchers found that male-treated mice displayed a decrease in the number of vasopressin-immunoreactive neurons in the paraventricular nucleus compared to sex-matched saline-treated rats, yet no impairment of memory in either the novel object recognition, Morris water maze test or social interaction measures for either male or female mice (Joca, Zuloaga, Raber & Siegel, 2014). North et al. (2013) treated male mice (PND28-42) to daily 24 mg/kg MA for 14 days and after a 7, 14 and 21 day wash-out period, were tested in a novel object recognition and spatial recognition memory tasks. These researchers found no change in short-term memory when measured 14 days after last drug administration, however at all three later testing periods they found a deficit in spatial memory. They concluded that the cognitive consequences of earlier MA exposure may manifest and persist long after drug abstinence.

Although, there is research into the long-term effects of MA when administered in adulthood, on later anxiety-like behaviour (Clemens et al., 2004), there is limited studies into the long-term effects on emotionality after adolescent treatment. Although, Joca et al's., (2014), found increased depression-like behaviour, most studies on earlier adolescent exposure of MA focus

on neurotoxicity of high doses and/or subsequent cognitive functioning in adulthood. This gap in the literature is important to address, as there is evidence of human adolescent MA users experiencing anxiety long after discontinuation (London et al., 2004; Rawson et al., 2002) and an animal model is not contaminated by genetic or environmental variables that a human population presents with.

### ***Long-term Outcomes of Adolescent Exposure to Benzylpiperazine (BZP) in Animals***

There is one published study into subsequent behavioural effects following adolescent BZP treatment. Aitchison and Hughes (2006) treated male and female rats on PND45, with 10 mg/kg of BZP, for ten days. Behavioural testing in a Y-maze, SI test and a light/dark emergence box began on PND72. In the Y-maze, BZP treated rats entered the novel arm less, spent less time in it and also entered both arms significantly less than control rats. Female rats entered both arms significantly more than male rats. In the social interaction test, BZP-treated rats reared less in both the outer and inner squares of the open-field than saline-treated rats. Female rats entered significantly more squares than male rats. BZP-treated rats spent less time engaged in social interactions than control rats and female BZP treated rats spent a longer time restraining a partner rat than saline-treated females. In the light/dark box BZP-treated rats took a longer time to emerge into the illuminated arena than control animals, and deposited fewer fecal boluses in the darkened start box. Aitchison and Hughes (2006) concluded that that BZP treatment in adolescent rats led to higher levels of anxiety-like behaviour in adulthood. They proposed that this may have occurred from interference with the development of anxiety-related forebrain mechanisms operated by 5-HT.

In summary, the published research suggests that adolescent animals are less susceptible to neurotoxic effects of stimulant drugs, than adult animals. However, PND40 appears to be a transitional period when detrimental effects become more pronounced. 5-HT is affected earlier in development than the DA system and may account for later changes in emotionality. Female rats are generally more active than male rats and appear to not be as susceptible to learning deficits as male adolescent treated rats. Adolescent exposure to most stimulants results in sensitization and cross sensitization in adulthood, however low doses of AMP and MP are reported to improve performance in learning tasks. Overall there are many discrepancies within the different stimulant treated rats and between the different stimulant drugs. This may reflect differences in the stimulant treatment doses, duration, the exposure period during development, time between treatment and behavioural testing and behavioural

measures used. To add to the limited long-term studies on later anxiety behaviours, one of the aims of this thesis is to supplement the work of Vorhees et al. (2005) by expanding behavioural testing into old age (PND200), and Aitchison and Hughes (2006) research by further examining the effects of BZP comparable to MA, using three different non-toxic doses of each drug and both male and female animals.

## **Chapter 2: Aims and Method**

### ***Aims***

Since there is limited research on any long-term effects of adolescent BZP and MA exposure on subsequent adulthood behaviour, the main aim of this study was to compare possible differences in long-term outcomes between saline-, BZP- and MA-treated groups of rats. Anxiety (or emotionality) was principally investigated because, as in addition to affecting DA transmission, BZP may affect 5-HT levels in the brain, which are implicated in anxiety disorders (Carlson, 2001). Primarily, the current study sought to expand on the results of Aitchison and Hughes (2006) research, which found increased emotionality in adult rats who had been treated with BZP during adolescence. Additionally, long-term behavioural effects of MA was included as a comparative stimulant drug, and on the assumption that BZP was a safe alternative for MA use. As it is unclear whether any neurochemical changes that may occur at different developmental stages continue or redeem themselves over-time, this study measured the rats' emotionality at three different developmental stages up until middle age. Finally, since, both male and female humans take stimulant-based drugs, the study aimed to determine whether exposure to a psychoactive substance at different developmental periods affected the sexes differently.

### ***Method***

#### ***Subjects***

The subjects were 210 male and 210 female PVG/C Hooded rats, from the breeding colony at the University of Canterbury, New Zealand. When 30 days old, the pups were weaned and housed in 525 x 330 x 230mm plastic cages, in groups of three or four of the same sex, for the duration of the experiment. They were housed in a controlled environment (rh 22°C ± 2° and 48% ± 10% humidity), and maintained on a 12 hour light/dark cycle (lights on at 8.00am) with free access to food (commercial rat pellets) and water. Two females and one male rat died of unknown causes, prior to behavioural testing. All procedures were approved by the Animal Ethics Committee of the University of Canterbury.

The subjects were randomly allocated to one of 21 experimental groups. Each group had 10 males and 10 females. In the drug treatment phase animals were i.p exposed daily (for 10 consecutive days) to either saline, BZP (5 mg/kg, 10 mg/kg or 20 mg/kg) or MA (0.5 mg/kg, 1.0 mg/kg or 2.0 mg/kg). All doses were administered at approximately 09.00 hours. To

encompass the total adolescent stage of development in rats (Spear, 2000), rats were treated during either between PND31-40, PND41-50 or PND51-60. Subsequently, all rats were tested at three different developmental periods, firstly; 30 days after last drug dose, secondly at PND120 and finally at PND200. Test phase one (30 day wash-out period) was to ensure no drug effect was evident and occurred when all animals were adults (Spear, 2000). Test phase two occurred when all animals were 120 old and test phase three occurred when all rats were 200 days old. See Figure 2 for a comparison between stages of rat versus human development and experimental procedure.

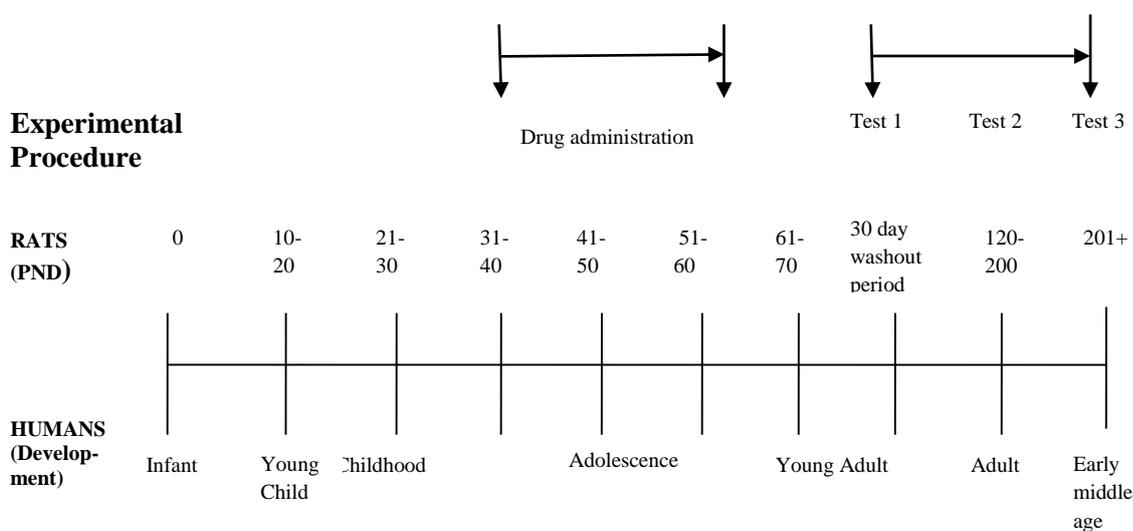


Figure 2: Experimental procedure and a relative comparison of ages and stages of rat versus human development (Adapted from Andersen, 2003)

### *Drugs and Rationale for Doses*

1-Benzylpiperazine (BZP) was purchased from ABCR GmbH & Co (Karlsruhe, Germany), and mixed daily in sterile 0.9% saline to produce doses of 5 mg/kg, 10 mg/kg and 20 mg/kg, and administered in a volume of 1 ml/kg. MA had been donated as a pure crystal form of the drug by Environmental Science & Research Ltd (ESR, Wellington, New Zealand). It was crushed and then dissolved in 0.9% saline to give doses of 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg. Previous research had demonstrated that these doses of both drugs were behaviourally effective in rats (Brennan et al., 2007a [BZP]; Hughes and Greig, 1976 [MA]) and Campbell et al., (1973) reported that AMP is approximately ten times more potent than BZP. All animals received a daily intraperitoneal injection (i.p.) of saline, BZP or MA for ten consecutive days either on PND31-40, PND41-50 or PND51-60 (See Table 1 below). Although human users

typically take BZP orally, or MA intranasally, an i.p route of administration was chosen for ease of drug delivery. According to the allometric scaling principles ("dose human [mg/kg] = dose animal [mg/kg] x animal weight/human weight<sup>¼</sup>) and the suggestion that a dose of 10 mg/kg MA in an adolescent rat is approximately equivalent to 117 mg in a 50 kg adolescent human, the doses used in this study can be considered small. The rationale for this was to examine long-term effects following adolescent recreational substance use as opposed to acute neurotoxic effects. The body weights and health of animals were monitored throughout drug treatment and all behavioural testing.

**Table 1: Days of treatment with saline (S), BZP, or MA for a total of 210 male and 210 female hooded rats**

	Juvenile (PND 31-40)	Adolescence (PND 41-50)	Early Adult (PND 51-60)
Control	S	S	S
Dose 1	BZP 5 mg/kg	BZP 5 mg/kg	BZP 5 mg/kg
Dose 2	BZP 10 mg/kg	BZP 10 mg/kg	BZP 10 mg/kg
Dose 3	BZP 20 mg/kg	BZP 20 mg/kg	BZP 20 mg/kg
Dose 1	MA 0.5 mg/kg	MA 0.5 mg/kg	MA 0.5 mg/kg
Dose 2	MA 1.0 mg/kg	MA 1.0 mg/kg	MA 1.0 mg/kg
Dose 3	MA 2.0 mg/kg	MA 2.0 mg/kg	MA 2.0 mg/kg

National Chemicals Inspectorate (2004) report that BZP's LD50 in both male and female rats is 2,600 mg/kg, therefore the BZP doses used in this study can be considered small. A typical 'small' dose of amphetamine for rats is 1 mg/kg (Robinson & Kolb, 2004). As there are no longitudinal studies looking at earlier administration of BZP this study used three different

doses of each drug to assess any differences within the class of drug and between BZP and MA.

The three periods of administration of the two different drugs took into account that, in rats, adolescence can endure from 28 to 55 days after birth (Spear 2007). However, for ease of comprehension, the PND31-41 period was called "juvenile", the PND41-50 period was called "adolescent" and the PND51-60 period was called "early adult", as suggested by Vorhees et.al. 2005. Rats are usually regarded as adults from PND 60 onwards (Marco, Macri, & Laviola, 2011), therefore this study spanned the adolescent developmental period of male and female animals.

### ***Behavioural Testing Rationale***

All rats were tested in a drug-free state on a battery of anxiety tests at three independent time periods. Firstly, 30 days after the last dose of saline, BZP or MA, secondly at PND 120 and finally at PND 200. Ideally, the first behavioural testing phase would be at PND60, when the adolescent brain had matured to adult levels, however in this study that would mean the behavioural testing would begin straight after drug treatment for PND51-60 and twenty days after treatment for the PND31-40 treated rats. The 30-day washout period was chosen to ensure all rats had the same recovery interval before behavioural testing (Vorhees et al., 2005). Therefore, PND31-40 treated rats were 70 days old, PND41-50 were 80 days old and PND51-60 were 90 days old, at the first testing phase. It is proposed that sex differences occur from PND60 (Imhof, Coelho, Schmitt, Morato and Carobrez, 1993) and most research suggests that performance on behavioural measures does not differ as a function of age after PND50. The second behavioural measures occurred when all rats had reached PND120 and the third measure at PND200.

### ***Apparatus and Behavioural Measures***

Emotionality, or emotional reactivity, in the rat is regarded as the animal equivalent to human anxiety. The experience of anxiety in humans is a normal phenomenon and has been suggested to be valuable in improving performance (Andrews, Creamer, Crino, Hunt, Lampe & Page, 2003). However when anxiety impacts on everyday functioning it can be detrimental to the individual experiencing it. There are different clinical classifications of different types of anxiety, such as generalized anxiety GAD), phobias, social anxiety, panic and post-traumatic stress disorders (Graeff, Netto, & Zangrossi, 1998). Anxiety in humans is characterized by unrealistic, unfounded fear and emotion (Andrews et al., 2003), typically exhibited as escape,

avoidance, non-verbal vocalisation and/or hypervigilance, whereas anxiety in animals is defined by changes in behaviour (Handley, 1995) and it is now recognised that both species may share common affective states (Palanza, 2001). This is important, as the assessment of anxiety in animals is based on the assumption that human and animal anxiety is similar (Ohl, 2003).

Four sets of apparatus were used and the behaviour measured probably reflect the equivalent of human GAD (Morley et al., 2001; Ohl, 2003; Samyai, Sibille, Pavlides, Fenster, McEwen, & Toth, 2000). All apparatus are empirically supported measures of anxiety-like behaviour (File & Seth, 2003; Millan, 2003; Ohl, 2003). In the Y-maze a more anxious animal will enter a novel arm less, spend less time in it and make fewer entries of both the novel and familiar arms, than a less anxious animal. In the social interaction test a more anxious animal will display less rearing behaviour (outer squares and inner unprotected squares) and spend less time in social interaction compared with a less anxious animal and this apparatus may also measure social anxiety (File & Seth, 2003). In the light/dark box more anxious animals will take longer to emerge from the start chamber than less anxious animals, and in addition to GAD may measure the human equivalent of panic disorder (Graeff, et al., 1998). In the EPM a more anxious animal will avoid the open arms more readily than a non-anxious animal (Pellow, Chopin, File & Briley, 1985). All four types of apparatus were present in the same experimental room and these ethologically appropriate tests are based on a rodent's innate curiosity to explore novel areas (Samyai et al., 2000) and do not require learning or any positive or negative reinforcers..

The experimental room was kept at 20°C, with 38% humidity and dim (47 lux) fluorescent room lighting. All testing took place between 10.00 and 15.00 hours during the light phase of the rats' light/dark cycle. At each of the three different testing periods each animal was tested in the Y-maze four times, elevated plus maze once or social interaction test once (randomly determined), and then placed in the light/dark box (times six trials per testing period). The order of trials was random, however every animal completed six separate trials per testing phase and the averages of all of their trials in each test setting were determined. There was a break of at least two days between each series of tests, to control for habituation effects.

### **1. Responsiveness to Change in the Y-Maze**

The Y-maze taps into rats' innate tendency to explore novel areas (Hughes, 2001; Hughes & Neeson, 2003; Samyai, et al., 2000) and normal animals will enter the novel arm more than the

familiar arm, whereas anxious animals will show less of a preference for novelty.

Additionally, the Y-maze is a test of short-term spatial memory for brightness change (Gray & Hughes, 2015). The clear Perspex enclosed clear-varnished wooden Y-maze sat on a 700mm-high table. The maze consisted of two 45-cm long arms and a 30-cm stem. The arms and the stem were 10cm wide and 14cm high, and the angle between them was 120°. One arm of the maze contained a removable black insert, and the other contained a white insert. Each insert occupied the width, height and 40-cm of the length of the arm. The animals were placed in the first 15cm of the stem and allowed to freely roam the entire maze for 6 minutes (acquisition trial). The rat was then removed and kept in a covered holding cage (530 x 330 x 230) while the white and black inserts were replaced with two clean black inserts, and the entire maze cleaned with a solution of 20% Powerquat blue and 80% water. The rat was then returned to the first 15 cm of the stem and allowed to freely roam the entire maze for 3 minutes (retention trail).

Arm entries were recorded by the experimenter using a PC keyboard, computer and suitably developed event recording software. The dependent variables measured in the retention trials were: (1) percentage of time spent in the novel arm for three minutes ( $\text{novel arm time} / \text{in seconds} / [\text{novel} + \text{familiar arm time in seconds}] \times 100$ ); (2) the percentage of entries made into the novel arm for three minutes ( $\text{novel arm entries} / [\text{novel} + \text{familiar arm entries}] \times 100$ ); and (3) the total entries of both changed arms for the three minute trials. The entry to an arm was defined as all four paws in the arm.

At the three periods of testing all animals were tested in this maze four times. The novel arm was on the left for half the trails and the right for the other half (randomly determined). The average of the four trials became the representative score for each rat on each measure.

## **2. Light/dark Emergence Test**

After testing in either the Y-maze, social interaction test or the EPM all animals were then observed in the light/dark emergence apparatus. This test is based on rodents' innate aversion to bright illuminated areas. In response to mild stressors, that is, bright light and a novel environment, a rodent faces a natural conflict between the tendency to explore and the innate tendency to avoid the unfamiliar (Bourin & Hascoet, 2003). This apparatus is quick and easy to use and does not require food or water deprivation, shock administration or prior training. The apparatus comprised a 20 x 15 x 20-cm high darkened start box, which was painted flat black inside and could be opened (via a sliding wooden door) to a larger 50 x 40 x 20-cm

illuminated arena. It was constructed from wood apart from the floor and the ceiling of the arena. The floor consisted of translucent white Perspex that was illuminated from underneath by two 16-lux fluorescent tubes. The light measurements were, 80 lux in the centre of the illuminated area at the roof level and 172 lux on the Perspex floor. The roof of the arena was made of fine wire mesh.

The animals were placed in the smaller darkened box for 60 seconds, and then a sliding door to the illuminated arena was opened. By means of a hand-held stopwatch the experimenter timed how long it took for the animal to fully emerge (all four paws) into the illuminated arena. If the animal did not emerge within five minutes, the trial was terminated and the rat assigned a score of 300 seconds. All animals were tested this way seven times with an interval of at least two to three days in between each test. The average emergence time was calculated for each rat on each of the three testing periods.

### **3. Elevated Plus Maze (EPM)**

The EPM is currently one of the most popular in vivo animal tests of anxiety (Carobrez & Bertoglio, 2005). There are two underlying assumptions for the EPM; firstly that it is an 'ethological' animal model of anxiety (Rodger, 1997) that has ecological meaning for the rat, and secondly, that anxiety (which can be regarded as agoraphobic) is induced by the unfamiliarity of the test (Carobrez & Bertoglio, 2005). Research suggests that rats prefer the secure closed arms of the maze to the more threatening open arms (Pellow, et al., 1985; Flint, 2003). This preference would be greater in more anxious animals. The EPM does not require any training procedures or the use of shock, or food or water deprivation.

The apparatus was elevated from the floor, consisted of two open arms (with 25 cm high clear Perspex walls) and two closed arms constructed of black painted wood. Each arm was 50 cm long and 15 cm across. Similar arms were opposite each other and there was a central zone (15 cm x 15 cm), in which the rat was placed facing a closed arm.

The animals were placed in the centre of the enclosure facing one of the closed arms and allowed to roam freely for five minutes. An infrared camera was mounted on the ceiling (200 cm) above the EPM and connected to a video recorder to record the animals' behaviour for later analysis. The measures recorded were (1) percentage of time spent in the open arms for the five minutes ( $\text{open arm time in seconds} / [\text{open} + \text{closed arm time}] \times 100$ ); (2) percentage of entries made into the open arms ( $\text{open arm entries} / [\text{open and closed arm entries}] \times 100$ ); and (3) the total entries of the closed arms over the five minute testing phase. Flint (2003) suggests that

these measures give a better indication of anxiety-like behaviour in the rat than the more sophisticated measures (i.e. grooming, rearing or stretch-attend postures). Cruz, Frei & Graeff (1994) suggests that entries of the closed arms is the most appropriate measure of activity in the EPM as it is not contaminated by anxiety. The entry to an arm was defined as all four paws in the arm. Animals were tested once at each of the three different periods as research suggests the EPM can produce habituation effects if tested within two weeks after first trial (Bessa, Oliveira, Cerqueira, Almeida & Sousa, 2005)

#### **4. The Social Interaction Test (SI)**

At all three testing periods pairs of unfamiliar animals were tested once in the social interaction test, which is a test of anxiety and curiosity (Wills, Wesley, Moore & Sismore, 1983). As with the other tests, it does not require training and thus the use of shock or deprivation. The animals were weighed on the day of the social interaction test and the weights of each pair did not differ by more than 10 grams. The apparatus consisted of a wooden open field (OF) 60 x 60 x 25-cm, that was placed on a table 700mm high in the same experimental room described above. The apparatus floor was painted flat black and divided into a 4 x 4 grid of 16 identical squares (twelve peripheral and four central), each measuring 15 x 15-cm. The wooden walls were 25-cm high and painted flat black. Uncertainty (and thus anxiety) is increased by placing animals in an unfamiliar environment, and by altering the light levels (Garau, Marti, SL& Balada, 2000). In this case, the light level was low (47 lux) and the test arena was unfamiliar, producing low to moderate anxiety (File & Seth, 2003). An infrared video camera was mounted on a single wooden arm 85-cm above the OF. The camera was connected to a video recorder, to record the animals' behaviour for later analysis. The tests were videotaped for 5 minutes and all testing was carried out between 10.00 and 15.00 hours. The OF was washed (20% Powerquat blue and 80% water) and dried between each pair of animals. Behaviour was scored manually by a research assistant blind to the conditions of the experiment.

Unfamiliar same-sexed rat pairs, matched on the basis of body weights and treatment conditions were placed in the middle of the OF facing each other for the start of the social interaction test. They were allowed to freely roam the entire OF together for five minutes. The animals were assessed for both their individual behaviours and their social interaction behaviours. The individual measures in the OF for each animal for the recorded five minutes were: (1) the total number of lines crossed (locomotion) defined as all four paws crossing the line; (2) the total rearing in the outside 12 squares (outer rearing); (3) the total rearing in the

inside four squares (centre rearing). The social interactive behaviour for each pair of animals was the total time spent in social interaction (e.g. sniffing, grooming, mounting, and crawling over, crawling under or following the partner). Because the behaviour of one animal affects that of the other, each pair of rats was treated as a unit (File & Seth, 2003) and only one score per pair was used for measures of social interaction behaviour. Lower levels of social interaction are considered to be indices of higher anxiety (Baranvi et al., 2005).

It has been suggested that the reciprocal neural circuits linking the PFC, the amygdala and hypothalamus are directly involved in fear and anxiety (Gonzalez, Rujano, Tucci, Paredes, Alba, & Hernandez, 2000; Voigt, Hortnagl, Rex, Van Hove, Bader, & Fink, 2005). Since the introduction of benzodiazepines, 5-HT has been recognised as playing a crucial part in anxiety-like behaviours (File & Seth, 2003). The current view is that human anxiety is related to insufficient 5-HT. Anxiety and drug abuse are inter-connected, possibly through the functioning of the 5-HT system (Gingrich & Hen, 2001). One premise of the present study is that 5-HT connections to the immature PFC will cause a change in anxiety observed in adulthood.

### **Chapter 3: Statistical Analyses and Results**

#### ***Statistical analyses***

The major focus of this study was to examine longer-term outcomes following earlier exposure to either BZP or MA with the expectation that animals exposed to either BZP or MA in adolescence would subsequently demonstrate higher levels of anxiety (when in a drug-free state) than the control animals. To facilitate interpretation, each response was subjected to a dose x sex ANOVA for each drug separately by means of the StatView programme for Macintosh computers, followed by Fisher PLSD post hoc tests ( $P < .05$ ) when appropriate. These analyses were conducted for testing that occurred at each of the three testing periods (30-day washout, PND120, PND200) following treatment at PND31-40, PND41-50 and PND51-60. Sex differences were taken into account because of the possibility that males and females may have differed in their responsiveness to the treatment (as has frequently been demonstrated with other treatments, Hughes, 2007). For the Y-maze, one-sample *t*-tests were also computed for % of novel time and % of novel entries compared with a mean expectancy of 50%. In view of the possibility that the use of Fisher post hoc tests may have increased the probability of Type 1 errors occurring, more rigorous Scheffe tests were also performed which confirmed the results of the original analyses.

#### ***Results at 30 day wash-out period***

All animals were tested in the Y-maze, emergence box, EPM and Social Interaction test 30 days after last administration of either saline, BZP or MA. The 30 day wash-out period ensured that all animals were tested in a drug-free state. Full ANOVA results are included in Table 2 and significant sex effects are included in Table 3.

Table 2. F ratios for dose and sex effects for each measure recoded at three periods following treatment with BZP or MA at three stages of adolescence

	BZP	MA	Sex (BZP)	Sex (MA)
<u>30 day washout testing</u>				
<i>Treatment @ PND31-40</i>				
Y-maze percent novel arm entries	F(3,72) = 1.03	F(3,72) = 2.28	F(1,72) = .66	F(1,72) = .06
Y-maze total arm entries	F(3,72) = 5.19**	F(3,72) = 1.76	F(1,72) = 20.14***	F(1,72) = 5.44**
Y-maze percent novel arm time	F(3,72) = 1.73	F(3,72) = 3.57**	F(1,72) = 1.28	F(1,72) = 1.90
Light/dark emergence	F(3,72) = 1.43	F(3,72) = 3.67*	F(1,72) = 1.28	F(1,72) = 2.86
EPM percent open arm entries	F(3,72) = 5.04**	F(3,72) = .49	F(1,72) = 4.27*	F(1,72) = .18
EPM percent open arm time	F(3,72) = 17.97***	F(3,72) = 1.62	F(1,72) = .20	F(1,72) = 2.83
EPM closed arm entries	F(3,72) = .48	F(3,72) = 2.49	F(1,72) = 3.92	F(1,72) = 2.83
SI squares entered	F(3,72) = 6.91***	F(3,72) = 2.36	F(1,72) = 15.38***	F(1,72) = 7.76**
SI outer rears	F(3,72) = 4.08**	F(3,72) = .96	F(1,72) = .54	F(1,72) = .48
SI centre rears	F(3,72) = 2.29	F(3,72) = 1.11	F(1,72) = 1.84	F(1,72) = .36
SI interaction time	F(3,36) = .51	F(3,36) = 1.55	F(1,36) = .60	F(1,36) = .24
<i>Treatment @ PND41-50</i>				
Y-maze percent novel arm entries	F(3,72) = 2.23	F(3,72) = 1.72	F(1,72) = .11	F(1,72) = .87
Y-maze total arm entries§	F(3,72) = 9.34***	F(3,72) = 2.38	F(1,72) = 25.28***	F(1,72) = 25.36***
Y-maze percent novel arm time	F(3,72) = 3.93**	F(3,72) = 2.07	F(1,72) = .01	F(1,72) = 1.60
Light/dark emergence	F(3,72) = 4.26**	F(3,72) = 2.66	F(1,72) = 13.70***	F(1,72) = 34.68***
EPM percent open arm entries	F(3,72) = 3.40*	F(3,72) = 1.75	F(1,72) = .76	F(1,72) = 3.70
EPM percent open arm time	F(3,72) = 7.60***	F(3,72) = 1.19	F(1,72) = 7.48**	F(1,72) = 12.55***
EPM closed arm entries	F(3,72) = 1.04	F(3,72) = 2.53	F(1,72) = 13.86***	F(1,72) = 14.08***

SI squares entered	F(3,72) = 2.29	F(3,72) = .35	F(1,72) = 22.99****	F(1,72) = 13.49****
SI outer rears	F(3,72) = .79	F(3,72) = 1.78	F(1,72) = .54	F(1,72) = 2.24
SI centre rears	F(3,72) = 1.66	F(3,72) = 2.73	F(1,72) = .66	F(1,72) = 1.18
SI interaction time	F(3,36) = .85	F(3,36) = .41	F(1,36) = .20	F(1,36) = .78

*Treatment @ PND51-60*

Y-maze percent novel arm entries	F(3,72) = 1.05	F(3,72) = .87	F(1,72) = 3.93	F(1,72) = 7.90**
Y-maze total arm entries	F(3,72) = 42.91****	F(3,72) = 43.35****	F(1,72) = 31.88****	F(1,72) = 11.86***
Y-maze percent novel arm time	F(3,72) = 1.14	F(3,72) = 1.37	F(1,72) = 2.19	F(1,72) = 7.12**
Light/dark emergence	F(3,72) = 2.43	F(3,72) = 2.59	F(1,72) = 16.95****	F(1,72) = 9.94**
EPM percent open arm entries§	F(3,72) = 5.76***	F(3,72) = 2.05	F(1,72) = 11.62***	F(1,72) = 12.92***
EPM percent open arm time§	F(3,72) = 3.90**	F(3,72) = 7.84****	F(1,72) = 10.38***	F(1,72) = 22.58***
EPM closed arm entries	F(3,72) = 1.47	F(3,72) = 2.18	F(1,72) = 7.29**	F(1,72) = 10.52***
SI squares entered	F(3,72) = 3.75**	F(3,72) = 5.49***	F(1,72) = 32.30****	F(1,72) = 13.43***
SI outer rears	F(3,72) = 3.67*	F(3,72) = 4.34**	F(1,72) = 1.75	F(1,72) = 3.32
SI centre rears	F(3,72) = 2.14	F(3,72) = 1.24	F(1,72) = .12	F(1,72) = 1.16
SI interaction time	F(3,36) = .16	F(3,36) = .79	F(1,36) = 6.51**	F(1,36) = 8.51**

Testing @ PND120

*Treatment @ PND31-40*

Y-maze percent novel arm entries	F(3,72) = 1.51	F(3,72) = .64	F(1,72) = .03	F(1,72) = 1.04
Y-maze total arm entries	F(3,72) = 1.05	F(3,72) = 6.50****	F(1,72) = 64.79****	F(1,72) = 43.09****
Y-maze percent novel arm time	F(3,72) = 3.95**	F(3,72) = .17	F(1,72) = .15	F(1,72) = 2.24
Light/dark emergence	F(3,72) = 5.48***	F(3,72) = 5.34**	F(1,72) = 29.25****	F(1,72) = 13.90***
EPM percent open arm entries	F(3,72) = 3.06*	F(3,72) = 3.41*	F(1,72) = .03	F(1,72) = 1.50

EPM percent open arm time	F(3,72) = 3.45*	F(3,72) = 1.12	F(1,72) = .02	F(1,72) = 3.13
EPM closed arm entries	F(3,72) = .26	F(3,72) = .88	F(1,72) = 26.47****	F(1,72) = 24.47****
SI squares entered	F(3,72) = 8.23****	F(3,72) = 7.95****	F(1,72) = 83.59****	F(1,72) = 54.18***
SI outer rears	F(3,72) = 5.80***	F(3,72) = 3.58**	F(1,72) = 9.18**	F(1,72) = 9.33**
SI centre rears	F(3,72) = 8.22****	F(3,72) = 5.22***	F(1,72) = 2.33	F(1,72) = 2.54
SI interaction time	F(3,36) = 1.02	F(3,36) = .45	F(1,36) = 4.30*	F(1,36) = 7.88*
<i>Treatment @ PND41-50</i>				
Y-maze percent novel arm entries	F(3,72) = 3.09*	F(3,72) = 4.30**	F(1,72) = .95	F(1,72) = 3.48
Y-maze total arm entries§	F(3,72) = 8.08****	F(3,72) = 16.17****	F(1,72) = 36.73****	F(1,72) = 24.81***
Y-maze percent novel arm time	F(3,72) = 2.51	F(3,72) = 2.84*	F(1,72) = .02	F(1,72) = 3.65
Light/dark emergence	F(3,72) = 13.44****	F(3,72) = 8.08****	F(1,72) = 6.85**	F(1,72) = 14.75***
EPM percent open arm entries	F(3,72) = 1.35	F(3,72) = .62	F(1,72) = 2.59	F(1,72) = 3.85
EPM percent open arm time§	F(3,72) = 1.29	F(3,72) = .34	F(1,72) = 1.13	F(1,72) = 4.70*
EPM closed arm entries	F(3,72) = .05	F(3,72) = 3.65*	F(1,72) = 21.77****	F(1,72) = 23.75****
SI squares entered	F(3,72) = 1.78	F(3,72) = .18	F(1,72) = 83.89****	F(1,72) = 125.80****
SI outer rears	F(3,72) = .87	F(3,72) = .84	F(1,72) = 27.95****	F(1,72) = 30.06****
SI centre rears	F(3,72) = 2.43	F(3,72) = .21	F(1,72) = 4.66*	F(1,72) = 2.16
SI interaction time	F(3,36) = .40	F(3,36) = 1.54	F(1,36) = .36	F(1,36) = 1.29
<i>Treatment @ PND51-60</i>				
Y-maze percent novel arm entries	F(3,72) = .72	F(3,72) = 1.05	F(1,72) = .29	F(1,72) = .30
Y-maze total arm entries	F(3,72) = 5.70**	F(3,72) = 14.59****	F(1,72) = 55.67****	F(1,72) = 24.38****
Y-maze percent novel arm time§	F(3,72) = 1.42	F(3,72) = 1.37	F(1,72) = .20	F(1,72) = .96
Light/dark emergence	F(3,72) = 4.64**	F(3,72) = 1.67	F(1,72) = 3.19	F(1,72) = 10.27**
EPM percent open arm entries§	F(3,72) = 3.51*	F(3,72) = 10.60****	F(1,72) = 3.42	F(1,72) = 9.40***

EPM percent open arm time	F(3,72) = 3.10*	F(3,72) = 6.93***	F(1,72) = 13.10***	F(1,72) = 17.68****
EPM closed arm entries	F(3,72) = .61	F(3,72) = 3.97**	F(1,72) = 18.76****	F(1,72) = 13.51***
SI squares entered	F(3,72) = 5.41**	F(3,72) = 2.54	F(1,72) = 18.59****	F(1,72) = 69.93****
SI outer rears	F(3,72) = 5.91**	F(3,72) = 1.54	F(1,72) = 13.36***	F(1,72) = 18.11****
SI centre rears	F(3,72) = 3.61*	F(3,72) = 1.45	F(1,72) = .30	F(1,72) = .52
SI interaction time	F(3,36) = .57	F(3,36) = 1.92	F(1,36) = .25	F(1,36) = .01

### Testing @ PND200

#### *Treatment @ PND31-40*

Y-maze percent novel arm entries	F(3,72) = 5.22**	F(3,72) = 1.13	F(1,72) = .03	F(1,72) = .03
Y-maze total arm entries	F(3,72) = 2.60	F(3,72) = .03	F(1,72) = 83.55****	F(1,72) = 80.62****
Y-maze percent novel arm time	F(3,72) = 3.95*	F(3,72) = .88	F(1,72) = .46	F(1,72) = .02
Light/dark emergence§	F(3,72) = 8.08****	F(3,72) = 6.95***	F(1,72) = 30.56****	F(1,72) = 18.25****
EPM percent open arm entries	F(3,72) = 3.71**	F(3,72) = 2.13	F(1,72) = .77	F(1,72) = 11.48**
EPM percent open arm time	F(3,72) = 3.32*	F(3,72) = 4.86**	F(1,72) = .15	F(1,72) = 9.75**
EPM closed arm entries	F(3,72) = 6.21***	F(3,72) = 2.28	F(1,72) = 43.44****	F(1,72) = 29.56****

#### *Treatment @ PND41-50*

Y-maze percent novel arm entries	F(3,72) = .24	F(3,72) = .58	F(1,72) = .95	F(1,72) = 3.47
Y-maze total arm entries	F(3,72) = 3.27*	F(3,72) = 8.79****	F(1,72) = 73.75****	F(1,72) = 40.78****
Y-maze percent novel arm time	F(3,72) = 2.35	F(3,72) = .71	F(1,72) = .43	F(1,72) = 1.25
Light/dark emergence	F(3,72) = 20.96****	F(3,72) = 18.93****	F(1,72) = 13.28***	F(1,72) = 12.64***
EPM percent open arm entries	F(3,72) = 1.23	F(3,72) = 1.40	F(1,72) = 6.78**	F(1,72) = 4.79*
EPM percent open arm time	F(3,72) = 2.02**	F(3,72) = 3.18*	F(1,72) = 5.60*	F(1,72) = 5.29*
EPM closed arm entries	F(3,72) = .69	F(3,72) = 1.03	F(1,72) = 22.89****	F(1,72) = 3.10****

*Treatment @ PND51-60*

Y-maze percent novel arm entries	F(3,72) = 1.36	F(3,72) = 2.27	F(1,72) = 3.31	F(1,72) = 7.18**
Y-maze total arm entries§	F(3,72) = .03	F(3,72) = 8.72****	F(1,72) = 80.62****	F(1,72) = 53.85****
Y-maze percent novel arm time	F(3,72) = .55	F(3,72) = 2.50	F(1,72) = 6.70*	F(1,72) = 3.51
Light/dark emergence	F(3,72) = 9.06****	F(3,72) = 5.07**	F(1,72) = 15.19***	F(1,72) = 14.56***
EPM percent open arm entries§	F(3,72) = 1.09	F(3,72) = 4.74**	F(1,72) = 6.64**	F(1,72) = 19.96****
EPM percent open arm time§	F(3,72) = .74	F(3,72) = 1.89	F(1,72) = 1.57	F(1,72) = 13.83***
EPM closed arm entries	F(3,72) = 3.81**	F(3,72) = 4.98**	F(1,72) = 39.20****	F(1,72) = 24.17****

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§Dose x sex interaction significant (see text); \*P<.05; \*\*P<.01; \*\*\*P<.001; \*\*\*\*P<.0001.

Table 3: Means  $\pm$  SEMs for sex differences that were significant for each measure at three periods following treatment with BZP or MA during three stages of adolescence

30 day washout testing

<i>Treatment @ PND31-40</i>	<b>BZP</b>		<b>MA</b>	
	<b>Male</b>	<b>Female</b>	<b>Male</b>	<b>Female</b>
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	1.78 $\pm$ .14	2.58 $\pm$ .13	2.45 $\pm$ .18	3.04 $\pm$ .19
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	124.54 $\pm$ .62	109.7 $\pm$ 3.08	<i>ns</i>	<i>ns</i>
EPM percent of open arm entries	36.33 $\pm$ 2.33	41.41 $\pm$ 1.18	<i>ns</i>	<i>ns</i>
EPM percent of open arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
EPM closed arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI squares entered	105.62 $\pm$ 2.45	119.67 $\pm$ 2.32	106.93 $\pm$ 3.12	119.18 $\pm$ 3.10
SI outer rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI centre rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI interaction time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
<i>Treatment @ PND41-50</i>				
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	1.39 $\pm$ .12	2.28 $\pm$ .18	1.68 $\pm$ .16	2.86 $\pm$ .19
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	146.64 $\pm$ 4.12	124.97 $\pm$ 4.67	143.1 $\pm$ 6.45	114.25 $\pm$ 5.93
EPM percent of open arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
EPM percent of open arm time	29.03 $\pm$ 2.61	37.85 $\pm$ 2.43	31.39 $\pm$ 2.94	44.83 $\pm$ 2.34
EPM closed arm entries	5.87 $\pm$ .28	7.64 $\pm$ .38	5.26 $\pm$ .37	7.22 $\pm$ .40
SI squares entered	92.07 $\pm$ 3.20	113.26 $\pm$ 3.13	101.78 $\pm$ 2.98	119.38 $\pm$ 3.64
SI outer rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI centre rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI interaction time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
<i>Treatment @ PND51-60</i>				
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	43.6 $\pm$ 3.04	54.56 $\pm$ 2.48
Y-maze total arm entries	1.87 $\pm$ .21	2.92 $\pm$ .22	1.89 $\pm$ .21	2.57 $\pm$ .25
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	41.32 $\pm$ 3.19	52.86 $\pm$ 2.91
Light/dark emergence	136.20 $\pm$ 4.25	107.69 $\pm$ 5.59	132.01 $\pm$ 4.77	121.53 $\pm$ 5.41
EPM percent of open arm entries	34.98 $\pm$ 2.09	43.41 $\pm$ 1.73	39.01 $\pm$ 2.01	47.09 $\pm$ 1.26
EPM percent of open arm time	28.75 $\pm$ 2.75	40.00 $\pm$ 2.44	34.95 $\pm$ 2.73	48.25 $\pm$ 1.87
EPM closed arm entries	6.19 $\pm$ .39	7.38 $\pm$ .33	6.67 $\pm$ .32	8.18 $\pm$ .35
SI squares entered	98.15 $\pm$ 3.44	121.22 $\pm$ 2.57	95.93 $\pm$ 3.34	112.7 $\pm$ 3.79

SI outer rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI centre rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI interaction time	116.45 ± 5.23	98.67 ± 4.36	120.3 ± 6.06	96.9 ± 6.96

### Testing @ PND120

<b>Treatment @ PND31-40</b>	<b>BZP</b>		<b>MA</b>	
	<b>Male</b>	<b>Female</b>	<b>Male</b>	<b>Female</b>
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	3.51 ± .20	5.88 ± .22	2.95 ± .19	4.90 ± .21
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	154.29 ± 7.07	117.51 ± 6.09	154.94 ± 4.64	130.99 ± 4.45
EPM percent of open arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
EPM percent of open arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
EPM closed arm entries	5.43 ± .30	7.64 ± .36	6.07 ± .28	8.08 ± .30
SI squares entered	80.10 ± 2.37	110.35 ± 2.38	78.95 ± 3.29	110.67 ± 3.51
SI outer rears	36.04 ± .88	40.42 ± 1.07	35.50 ± 1.12	40.67 ± 1.36
SI centre rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI interaction time	107.31 ± 4.36	91.83 ± 3.81	109.70 ± 6.37	88.05 ± 4.79

### **Treatment @ PND41-50**

Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	3.15 ± .20	4.88 ± .25	2.96 ± .21	4.30 ± .28
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	140.93 ± 6.67	121.53 ± 6.35	138.47 ± 4.61	108.84 ± 5.61
EPM percent of open arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
EPM percent of open arm time	<i>ns</i>	<i>ns</i>	41.88 ± 3.35	50.12 ± 2.01
EPM closed arm entries	5.20 ± .31	7.30 ± .31	6.22 ± .37	8.69 ± .37
SI squares entered	72.65 ± 3.21	113.36 ± 3.20	73.18 ± 2.90	122.57 ± 3.28
SI outer rears	34.70 ± 1.27	44.41 ± 1.34	33.90 ± 1.35	44.62 ± 1.38
SI centre rears	1.05 ± .27	2.13 ± .42	<i>ns</i>	<i>ns</i>
SI interaction time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>

### **Treatment @ PND51-60**

Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	2.95 ± .19	5.10 ± .21	2.61 ± .20	3.87 ± .24
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	<i>ns</i>	<i>ns</i>	138.27 ± 5.00	114.36 ± 5.63
EPM percent of open arm entries	<i>ns</i>	<i>ns</i>	42.12 ± 2.02	48.22 ± 1.26
EPM percent of open arm time	36.02 ± 2.55	47.45 ± 2.01	41.58 ± 2.47	525.67 ± 1.70
EPM closed arm entries	6.27 ± .53	8.10 ± .44	6.27 ± .30	8.10 ± .28
SI squares entered	80.55 ± 3.29	99.70 ± 3.43	73.00 ± 2.54	108.60 ± 3.57

SI outer rears	35.88 ± 1.24	42.53 ± 1.57	33.12 ± 1.12	40.17 ± 1.21
SI centre rears	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
SI interaction time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>

Testing @ PND200

	<b>BZP</b>		<b>MA</b>	
<b><i>Treatment @ PND31-40</i></b>	<b>Male</b>	<b>Female</b>	<b>Male</b>	<b>Female</b>
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	3.00 ± .16	5.28 ± .19	2.76 ± .17	5.10 ± .19
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	155.33 ± 4.06	117.51 ± 7.26	155.78 ± 4.96	132.55 ± 7.09
EPM percent of open arm entries	<i>ns</i>	<i>ns</i>	43.41 ± 2.53	49.09 ± 1.24
EPM percent of open arm time	<i>ns</i>	<i>ns</i>	34.74 ± 2.99	45.97 ± 2.42
EPM closed arm entries	4.98 ± .29	7.84 ± .37	5.36 ± .29	7.69 ± .32
<b><i>Treatment @ PND41-50</i></b>				
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Y-maze total arm entries	2.79 ± .17	5.00 ± .21	2.72 ± .18	4.31 ± .22
Y-maze percent of novel arm time	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
Light/dark emergence	115.09 ± 6.37	128.13 ± 6.89	149.35 ± 6.09	123.76 ± 7.11
EPM percent of open arm entries	39.67 ± 2.75	47.40 ± 1.13	42.22 ± 2.24	47.90 ± 1.30
EPM percent of open arm time	35.86 ± 3.40	47.90 ± 1.76	35.66 ± 3.26	44.70 ± 2.59
EPM closed arm entries	5.70 ± .37	8.03 ± .3	5.50 ± .29	7.75 ± .27
<b><i>Treatment @ PND51-60</i></b>				
Y-maze percent of novel arm entries	<i>ns</i>	<i>ns</i>	54.93 ± 2.65	63.30 ± 1.60
Y-maze total arm entries	2.76 ± .19	5.1 ± .19	2.21 ± .14	3.83 ± .22
Y-maze percent of novel arm time	46.69 ± 2.42	54.03 ± 1.40	<i>ns</i>	<i>ns</i>
Light/dark emergence	157.81 ± 4.47	130.38 ± 6.89	157.60 ± 4.59	129.79 ± 6.26
EPM percent of open arm entries	41.96 ± 2.15	48.20 ± 1.15	40.58 ± 1.97	49.83 ± 1.34
EPM percent of open arm time	<i>ns</i>	<i>ns</i>	38.71 ± 3.49	53.43 ± 2.30
EPM closed arm entries	5.10 ± .18	7.41 ± .16	5.68 ± .29	7.89 ± .38

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### 1. Y-Maze results

Each animal was tested in the Y-maze four times and averages of the four trials were used in each of the analyses. See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods.

#### BZP and MA treated: Percentage of Entries of the Novel Arm of the Y-maze at 30 day wash-out period.

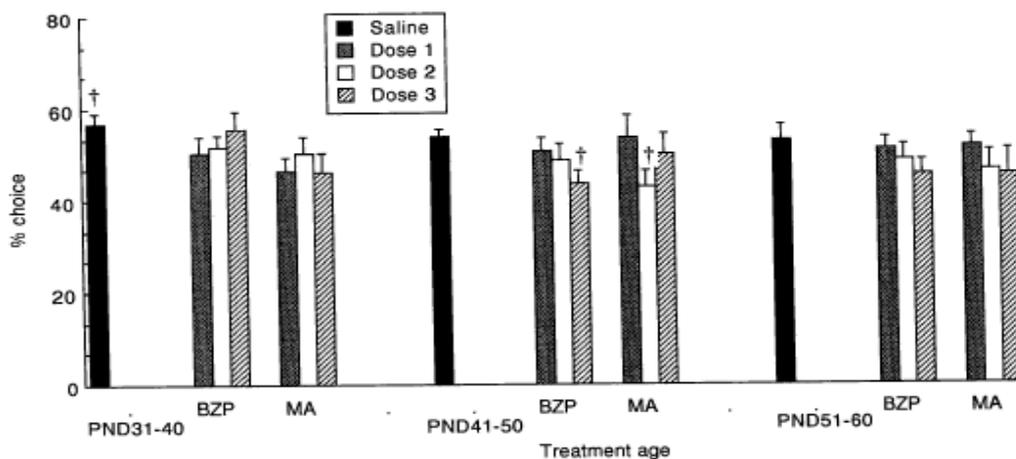


Figure 3: Mean ( $\pm$ S. E. M.) percent of novel entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test).

Figure 3 illustrates that although the dose effect was not significant for either drug, rats treated with saline during PND31-40 displayed a significant preference for entering the novel arm over the familiar arm and rats treated during PND41-50 with 20 mg/kg BZP or 1.0 mg/kg MA displayed significant preference for entering the familiar rather than novel arm. There was one sex effect for treatment at PND51-60: female MA-treated rats made significantly more entries of the novel arm at than male MA-treated rats.

**BZP and MA treated: Total Entries of both arms of the Y-maze at 30 day wash-out period.**

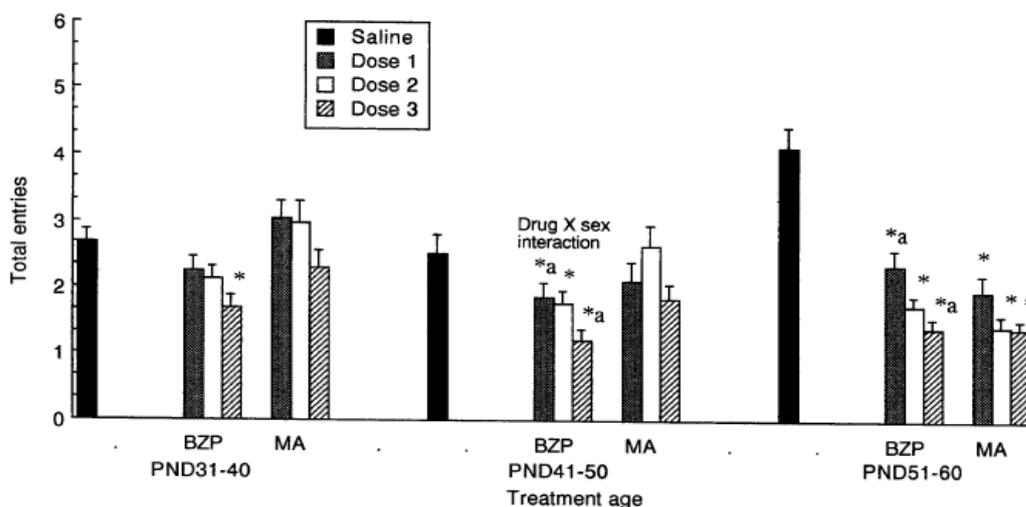
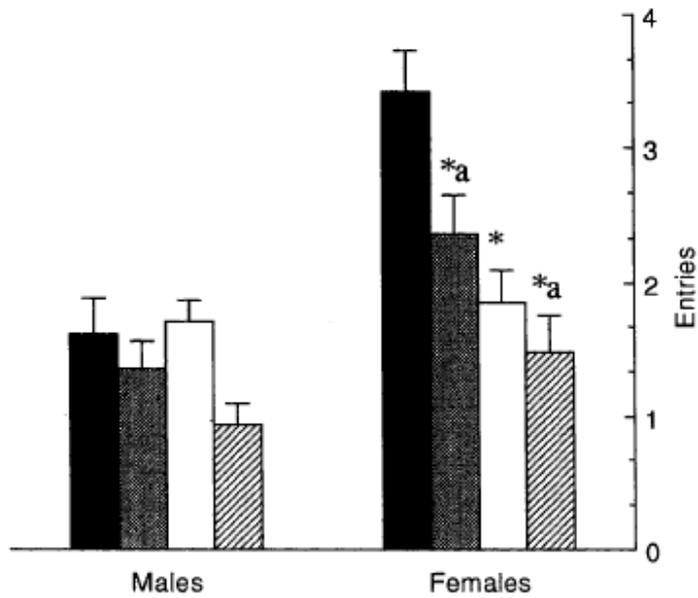


Figure 4: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 4 illustrates that PND31-40 BZP-treated rats made significantly fewer entries of both arms of the Y-maze than saline treated rats of the same age. PND 41-50 and PND51-60 BZP-treated rats made significantly fewer entries of both arms of the Y-maze than saline treated rats of the same age. For both PND41-50 and PND51-60 BZP-treated rats, there was a significant difference between the lowest and highest doses, indicating that as the BZP dose increased, treated rats became less active. However, in the former case, a significant dose x sex interaction outlined in Figure 5 showed that the effect applied to female but not male rats. Only the PND51-60 MA-treated rats made significantly fewer entries of both arms of the Y-maze compared to saline-treated rats of the same age. All treated female rats made significantly more total arm entries than similar treated male rats. Overall, the pattern of results suggests that as dose of BZP increased, regardless of administration age, animals became less mobile. This outcome only typified rats treated with MA during later adolescence.

**Drug x sex interaction for PND41-50 BZP treated rats: Total Entries of both arms of the Y-maze at 30 day wash-out period.**



**Figure 5: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), BZP PND41-50 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex. a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.**

**BZP and MA treated: Percentage of Time spent in the Novel Arm of the Y-maze at 30 day wash-out period.**

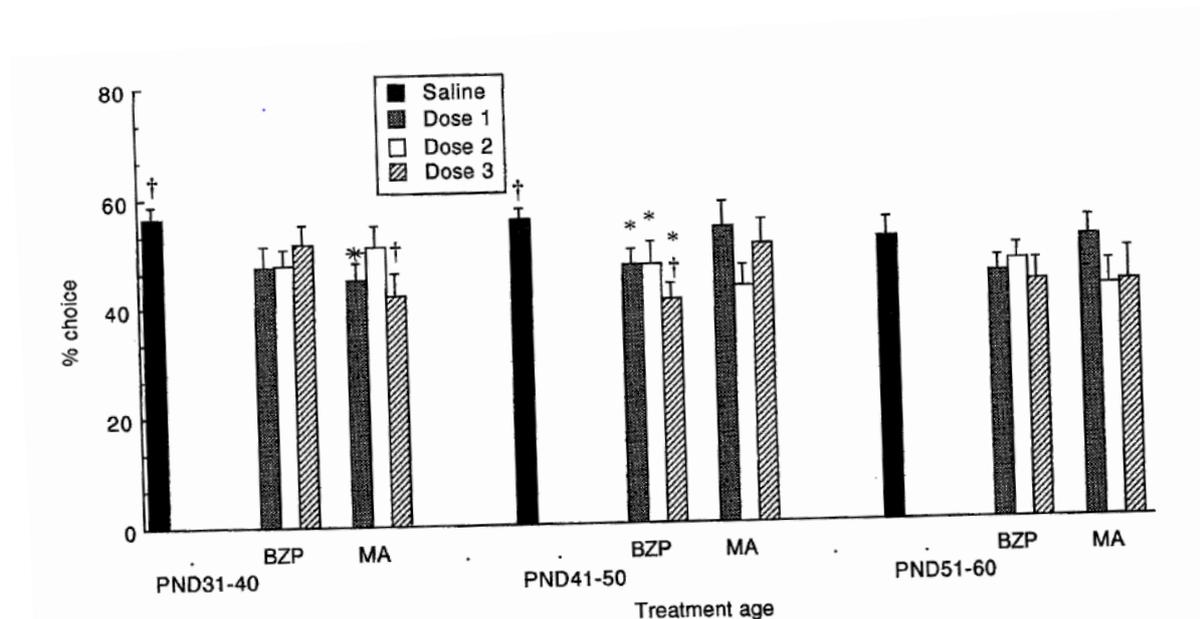


Figure 6: Mean ( $\pm$ S. E. M.) percent of time spent in the novel arm of the Y-maze for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug; † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 6 shows that, at PND31-40 and PND41-50, saline-treated animals preferred to spend time in the novel arm of the Y-maze over the familiar arm, compared with a mean expectancy of 50%. However, PND31-40 highest MA-treated and PND41-50 highest BZP-treated rats preferred to spend significantly more time in the familiar arms of the Y-maze, compared with a mean expectancy of 50%. Rats treated with 2.0 mg/kg of MA at PND31-40 spent a significantly less percentage of time in the novel arm, compared to saline-treated rats. At PND41-50, BZP-treated rats spent a significantly less percentage of time in the novel arm of the Y-maze compared with age matched controls. There was one significant sex effect for MA-treated rats at PND51-60; females spent significantly more time in the novel arm of the Y-maze than their male counterparts.

***Summary of Y-maze results for 30 day wash-out period.***

The Y-maze taps into rats' innate tendency to explore novel areas (Hughes, 2001; Hughes & Neeson, 2003; Samyai, et al., 2000) and normal animals will enter the novel arm more than the familiar arm, whereas anxious animals will show less of a preference for novelty.

Additionally, the Y maze is a test of short-term spatial memory for brightness change (Gray & Hughes, 2015). The results showed that there were systematic differences in measures of emotionality similar to Aitchison and Hughes (2006) research. That is, as adults, the rats that had been exposed to BZP as adolescents displayed more emotionality than control rats. This was most evident in the PND41-50 developmental period. However this may also be attributed to differences between male and female treated rats, with the female treated rats being more affected by dose increases in BZP compared to male rats. In regards to earlier MA administration, the results suggest that that administration of MA during the later developmental period of adolescence (PND51-60) affects male rats significantly more than females, with male rats possibly displaying more anxiety in the Y-maze than females. Overall, the results of adult testing of adolescence BZP and MA administration imply that, 30 days after drug treatment, there are dose-related changes in Y-maze behaviour that could be indicative of changes in anxiety.

## ***2. Light/dark emergence results***

As each animal was tested in the light/dark apparatus seven times on each of the three different testing times, averages of the seven trials were used in each of the analyses. See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods.

### BZP and MA treated: Emergence latency time from the Light/dark apparatus at 30 day wash-out period.

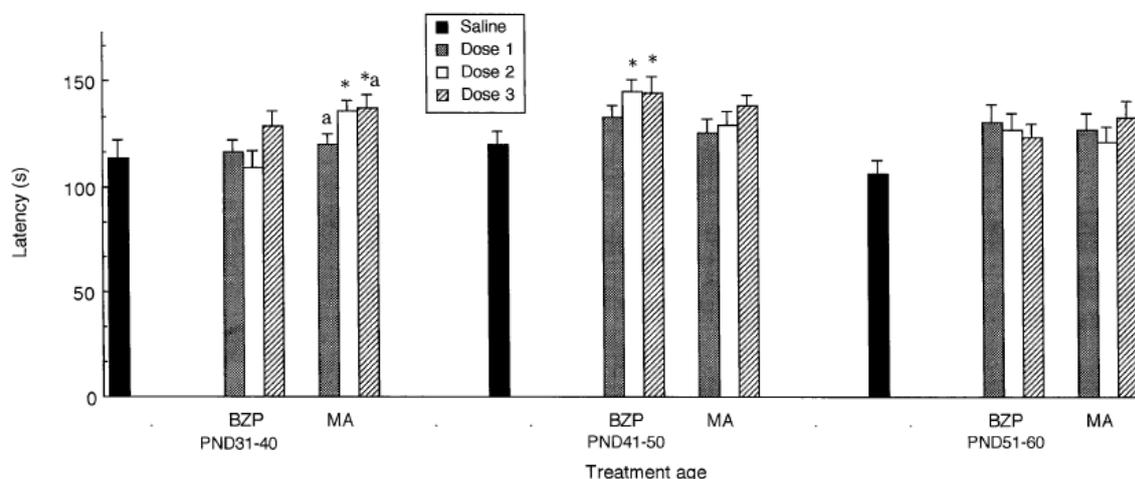


Figure 7: Mean ( $\pm$ S. E. M.) emergence latency from the Light/dark box for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); . \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 7 illustrates that at PND31-40, latencies to emerge from the dark following treatment with the two higher doses of MA were significantly different from same aged saline treated rats. Additionally, there was a significant difference between the doses, with the higher MA dosed rats slower to emerge than those treated with the lowest dose. Similarly, PND41-50 rats administered both 10 mg/kg and 20 mg/kg BZP took longer than saline-treated matched controls to emerge from the dark compartment. Neither drug produced significant effects after treatment at PND51-60.

There were five significant sex effects in the light/dark box at 30 day wash-out period, with female rats in all conditions being faster to emerge than male rats, with the exception of male and female rats treated with MA during PND31-40 which were comparative.

#### Summary of Light/dark box emergence at 30 day wash-out period.

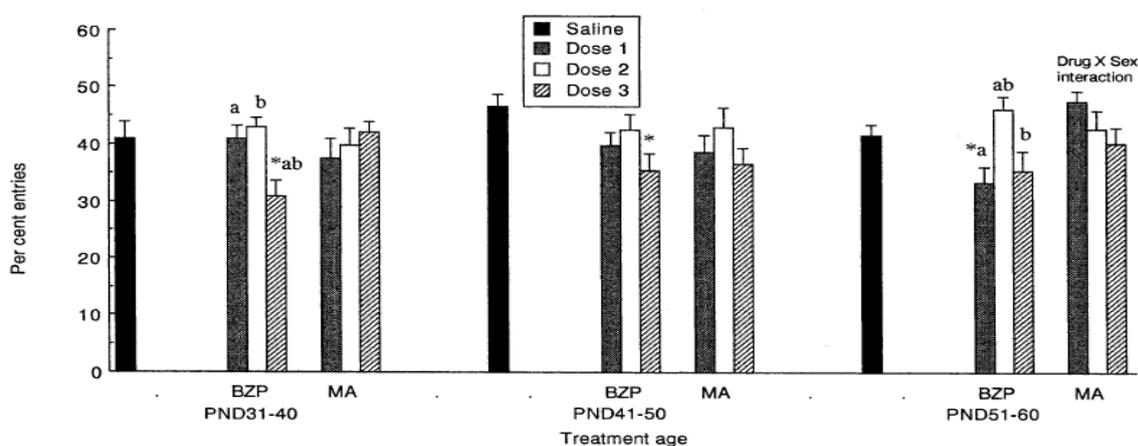
The light/dark box is based on rodents' innate aversion to bright illuminated areas. In response to stressors, that is, bright light and a novel environment, a rodent faces a natural conflict between the tendency to explore and the initial tendency to avoid the unfamiliar (Bourin & Hascoet, 2003). As MA dose increased for PND 31-40 treated rats and BZP dose increased for PND 41-50 treated rodents, the rats were slower to emerge from the light/dark box, suggesting

an increase in anxiety for these conditions. This implies differences in drug administered and adolescence developmental periods have different outcomes on latency to emerge from the light/dark box. However, with the exception of PND 51-60, (which for both BZP and MA treated rats there was a longer latency to emerge, but no obvious dose-related pattern), both drugs administered during PND 31-40 and 41-50 showed increasing anxiety with increased doses. Additionally, consistent with most research using both male and female rats: female rats generally are more active than male rats (Bridges & Starkey, 2004; Hughes & Neeson, 2003; Palanza, 2001), which was supported in this measure. Female rats (in all conditions, except MA treatment administered during PND31-40) generally emerged faster than males.

### 3. EPM results

Each animal was tested once at each of the three testing periods in the EPM, as research suggests the EPM can produce habituation effects if tested within two weeks after first trial (Bessa et al., 2005). See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods.

#### **BZP and MA treated: Percentage of Entries of the open arms of the EPM at 30 day wash-out period.**



**Figure 8:** Mean ( $\pm$ S. E. M.) percent of open arm entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 8 illustrates that administration of BZP during PND31-40 significantly affected percentage of entries of the open arms of the EPM, with the highest dose significantly different

from saline and the two lower doses. Likewise, the highest BZP dose administered during PND41-50 was significantly different from saline controls, but not from the other two BZP doses. However, it was the lowest BZP dose administered during PND51-60 that was significantly different from control animals. There are also differences between the doses of BZP administered during PND51-60, with the lowest dose (5 mg/kg) significantly different from the middle dose (10 mg/kg) and the middle BZP dosed animals significantly different from the highest BZP (20 mg/kg) treated rats in the percentage of entries of the open arms of the EPM. With the exception of MA- treated females during PND31-40 and BZP and MA- treated during PND41-50 all other female treated rats made significantly more entries of the open arms of the EPM than similar treated male rats.

A significant dose x sex interaction for PND51-60- MA-treated rats ( $F(1, 72) 2.92, P < .0397$ ) outlined in Figure 9 revealed that the two lower doses of the drug increased this response for female but not male rats. However, males treated with 1.0 mg/kg made fewer entries of the open arms than those treated with 0.5 mg/kg. Female rats treated with 2 mg/kg scored lower on this response than those treated with 1 mg/kg.

**Drug x sex interaction for PND51-60 MA treated rats: Percentage of Entries of the open arms of the EPM at 30 day wash-out period.**

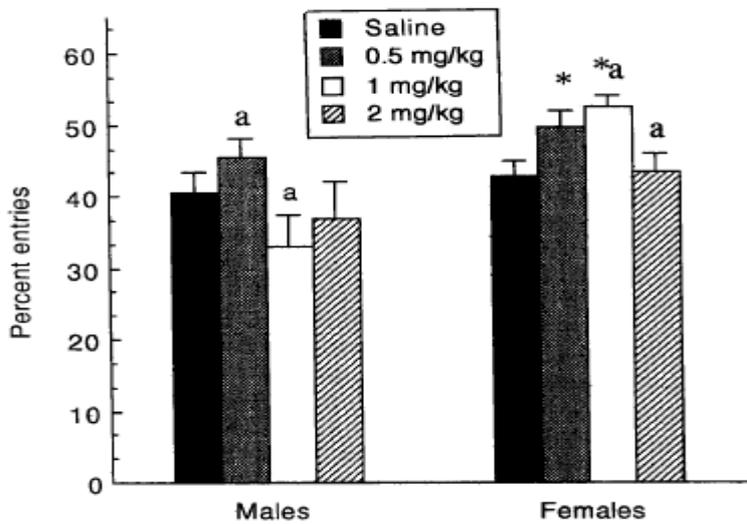


Figure 9: Mean ( $\pm$ S. E. M.) percentage of open arm entries for control (Saline), MA PND 51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex. a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

**BZP and MA treated: Percentage of time in the open arms of the EPM at 30 day wash-out period.**

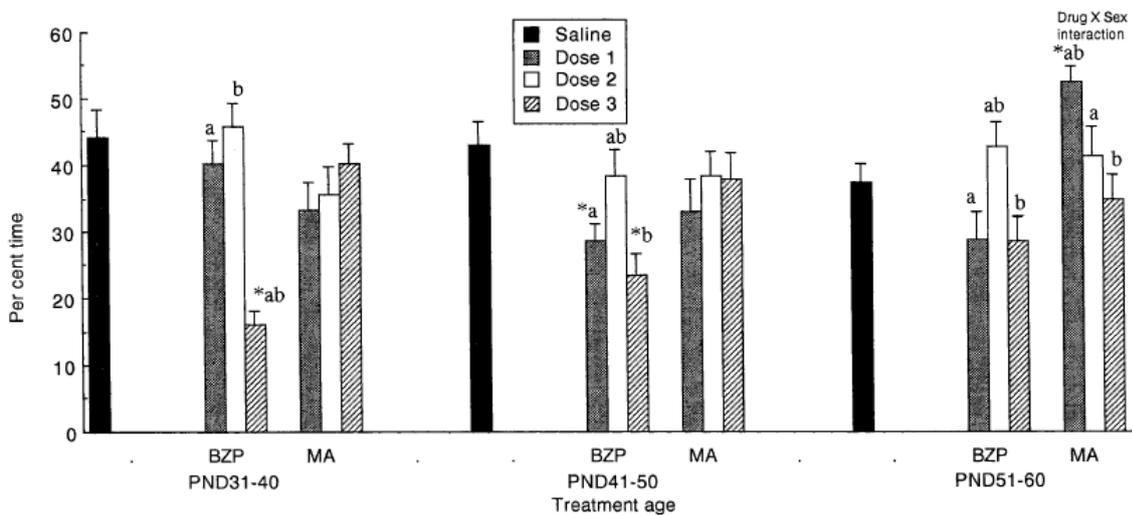


Figure 10: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 10 illustrates that PND31-40 BZP treated rats spent significantly less percentage of time in the open arms of the EPM compared to saline controls and the 5 mg/kg and 10 mg/kg treated rats. Rats from both the lowest and the highest PND41-50 BZP treatment groups were significantly different from controls, and 10 mg/kg BZP treated rats. For PND51-60 the BZP 10 mg/kg treated rats spent significantly more time in the open arms of the EPM compared to the higher (20 mg/kg) and lower (5 mg/kg) treated rats, but results were not statistically different from control rats. Although the lowest MA dosed rats (0.5 mg/kg) spent significantly more time in the open arms than control animals, and the two MA-treated groups, a significant dose x sex interaction ( $F(3,72) 4.51, P < .0059$ ) outlined in Figure 11 revealed that this response was increased by the two lower doses for females only. Although no male group differed significantly from saline, both higher doses significantly reduced time in the open arms compared with the lowest dose.

Similar to the results for total closed arm entries, there was a significant difference between the sexes for both BZP- and MA-treated PND41-50 and 51-60 rats, with female treated rats spending a significant longer percentage of time in the open arm, than male treated rats.

**Drug x sex interaction for PND51-60 MA treated rats: Percentage of time spent in the open arms of the EPM at 30 day wash-out period.**

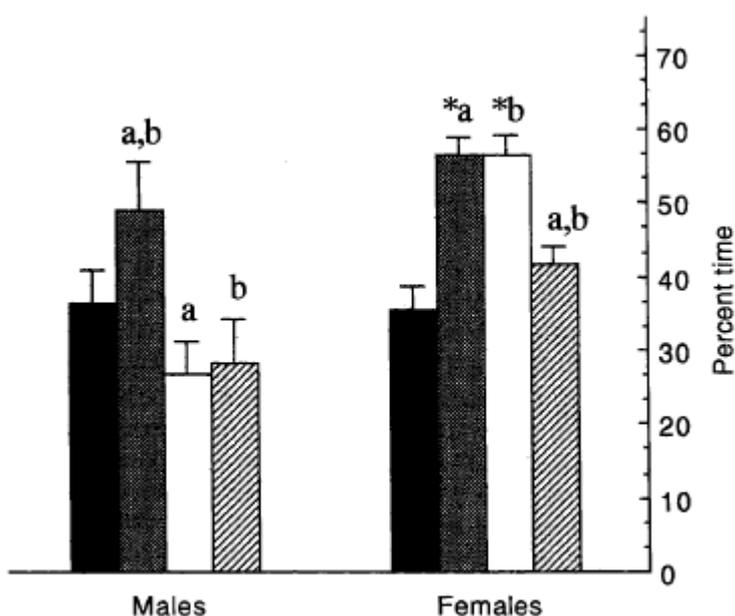


Figure 11: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms for control (Saline), MA PND 51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex. a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

### BZP and MA treated: Entries of the closed arms of the EPM at 30 day wash-out period.

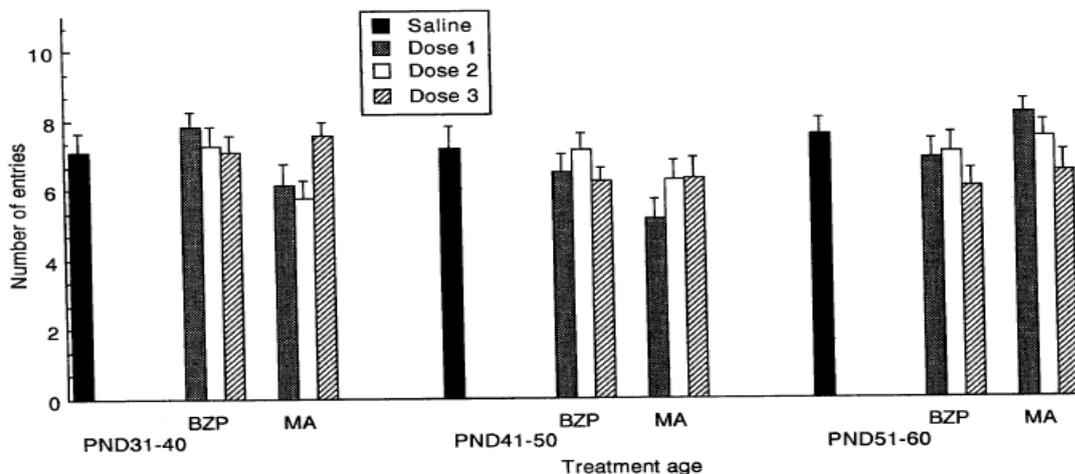


Figure 12: Mean ( $\pm$ S. E. M.) entries of the closed arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats.

Figure 12 illustrates that there were no significant dose effects for all treatment groups on entries of the closed arms in the EPM at the 30 day wash-out period. However, again there were sex effects for both BZP- and MA-treated rats at both PND41-50 and 51-60, with female treated rats making more closed arm entries than male treated rats.

### Summary of EPM results for 30 day wash-out period.

The EPM is currently one of the most popular *in vivo* animal tests (Carobrez & Bertoglio, 2005) and research suggests that rats prefer the closed arms of the maze to the open arms and this preference is caused by fear or anxiety (Pellow et al., 1985; Flint, 2003). The pattern of results suggests that the highest dose of BZP administered during the PND31-40 and 41-50 affected the rats' tendency to enter and spend time in the open arms of the EPM. Inconsistent with a dose-related increase in avoidance of the open arms, the lowest BZP dose group administered during PND51-60 made significantly fewer entries of the open arms of the EPM. Consistent with the results found in the Y-Maze and light/dark emergence box, female rats were more active than male rats. However, this effect was only significant for animals treated during PND41-50 and 51-60.

While there appears to be a general pattern of increased avoidance with escalating BZP dose, in the EPM for rats administered BZP during PND31-40 and 41-50, the results for BZP and MA administered during PND51-60 are not as clear. The lowest MA dose administered at PND51-

60 resulted in a significantly longer percentage of time spent in the open arms, indicative of decreased anxiety compared to saline treated rats. PND51-60 female rats administered either 0.5 mg/kg or 1.0 mg/kg MA made significantly more entries and spent a longer percentage of time in open arms compared to saline treated female rats. Interestingly, the female rats administered the highest MA dose (2.0 mg/kg) were comparable to the saline treated female rats in percentage of entries, with the saline treated rats and MA 2.0 mg/kg female rats choosing to enter the open arms of the EPM 43 percent of the time. Likewise, the saline treated female rats and MA 2.0 mg/kg spent a similar amount of time, 38 percent and 41 percent retrospectively in the open arms. It appears that female rats administered MA during PND51-60 may be less than or equally anxious to untreated animals.

#### 4. Social Interaction test.

Each animal was paired with an unfamiliar animal of a similar weight ( $\pm 10$  grams) and tested once at each of the two testing periods in the Social Interaction test. As already mentioned, PND200 data was destroyed in the February 2011 Christchurch Earthquake. See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods.

#### BZP and MA treated: Total squares entered in the Social Interaction test at 30 day wash-out period.

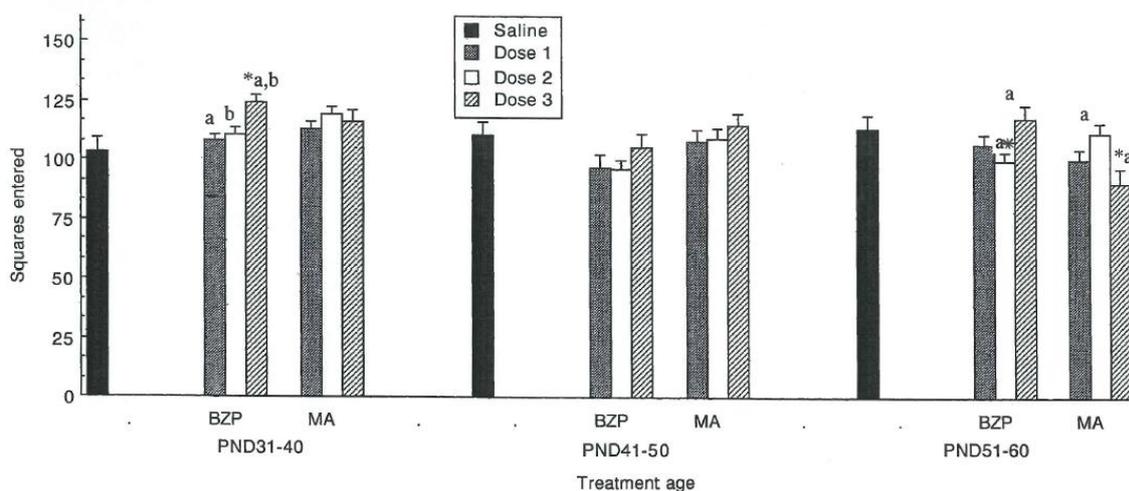


Figure 13: Mean ( $\pm$ S. E. M.) total squares entered in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. . \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug. .

Figure 13 illustrates that PND31-40 BZP (20 mg/kg) treated animals entered significantly more squares in the open field of the Social interaction test than saline, and other two BZP doses. Similarly, the highest dose of BZP administered during PND51-60 treated rats entered significantly more squares in the OF, compared to controls and were significantly different from the BZP- (10 mg/kg) treated rats. Conversely, the highest dose MA-treated rats entered significantly fewer squares than the same aged saline controls, and lowest MA-treated rats.

There were sex effects for all treated rats in total squares entered in the open field of the Social Interaction test, with female rats entering significantly more squares than similar treated male rats.

### BZP and MA treated: Outer Rears in the Social Interaction test at 30 day wash-out period.

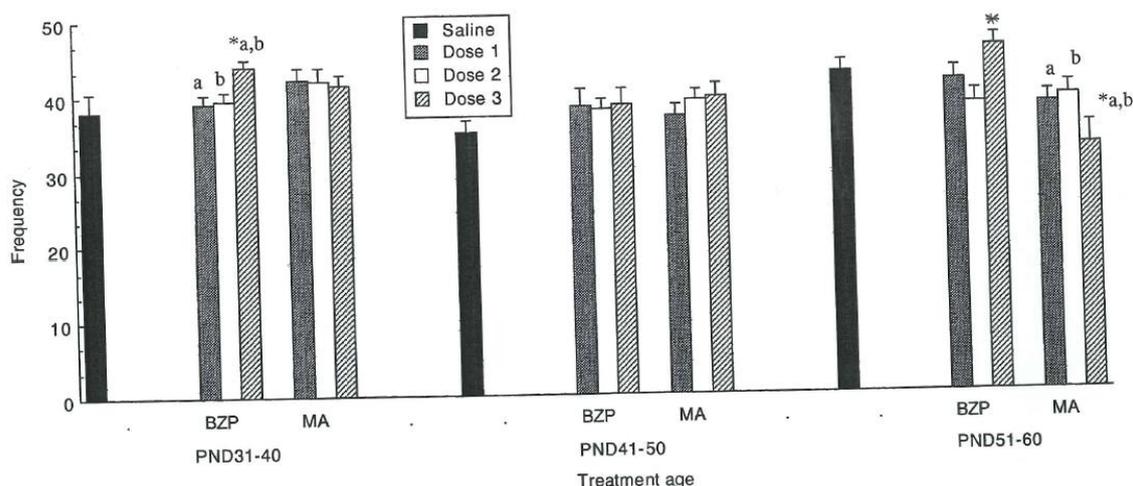


Figure 14: Mean ( $\pm$ S. E. M.) total outer rears in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 14 illustrates that the highest dose of BZP administered during PND31-40 and PND51-60 animals made significantly more outer rears in the open field of the Social Interaction test, than same aged control animals, and the two lower BZP doses. However the highest dose of MA administered during PND51-60 animals made significantly less outer rears in the Social interaction test than saline controls, and the two lower MA dosed groups. There were no significant sex effects.

**BZP and MA treated: Centre Rears in the Social Interaction test at 30 day wash-out period.**

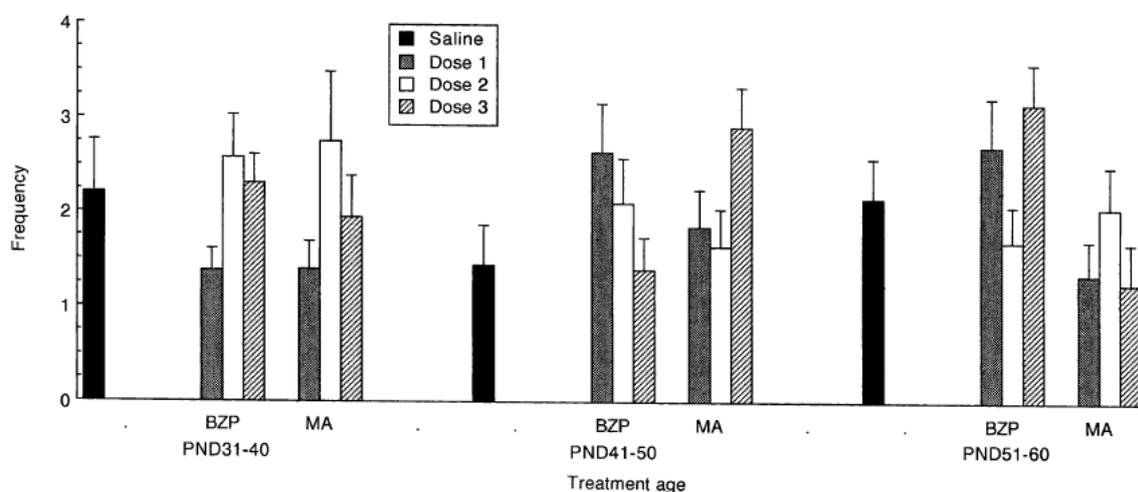


Figure 15: Mean ( $\pm$ S. E. M.) total centre rears in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats.

Figure 15 illustrates no significant results for any conditions in rearing in the middle of the open field of the Social Interaction test or any significant sex effects.

### BZP and MA treated: Interaction time in the Social Interaction test at 30 day wash-out period.

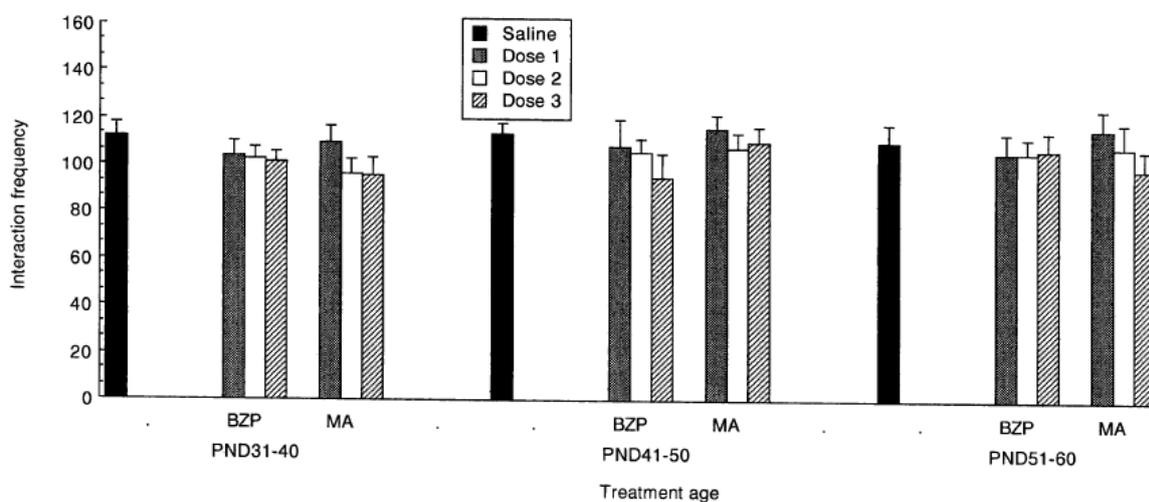


Figure 16: Mean ( $\pm$ S. E. M.) total interaction time in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats.

Figure 16 illustrates no significant dose effect for interaction frequency in the Social interaction test. However, there was a significant sex effect for both BZP and MA administered during PND51-60. PND51-60 BZP and MA male treated rats spent a significantly higher frequency of time in social interaction than PND51-60 BZP female treated rats.

### Summary of Social Interaction test results at 30 day wash-out period.

After a 30-day wash-out period pairs of unfamiliar animals from the same treatment condition were tested once in the Social Interaction test, which is a test of anxiety and curiosity (Wills, et al., 1983). Total squares entered is a measure of locomotion similar to that recorded in the Y-maze, Light/dark emergence Box and EPM. For this, as for those recorded on the other types of apparatus, female rats typically engaged in more locomotor activity than male rats. However, there was still a significant dose effect for the PND31-40 BZP treated animals, in that as the dose of BZP increased both male and females increased the amount of squares entered, with females making significantly more entries than male rats. Only the highest dose of MA administered during PND51-60 decreased locomotor activity for both male and female rats, with female rats still entering significantly more squares than male rats. The highest dose of BZP administered to PND31-40 increased rearing in the outer squares, whereas the highest dose of MA administered to PND51-60 decreased outer rearing compared to matched controls.

The significant results for the PND51-60 MA- and BZP- treated rats may be explained, to some extent, by the significantly more time spent in social interaction seen by the male treated rats, whereas they were more interested in the unfamiliar peers than exploring the novel environment.

### **Results at PND120**

All animals were tested in the Y-maze, light/dark box, EPM and Social Interaction test at PND 120. See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods.

#### **1. Y-Maze results**

As each animal was tested in the Y-maze four times the averages of the four trials were used in each of the analyses.

#### **BZP and MA treated: Percentage of Entries of the Novel Arm of the Y-maze at PND120.**

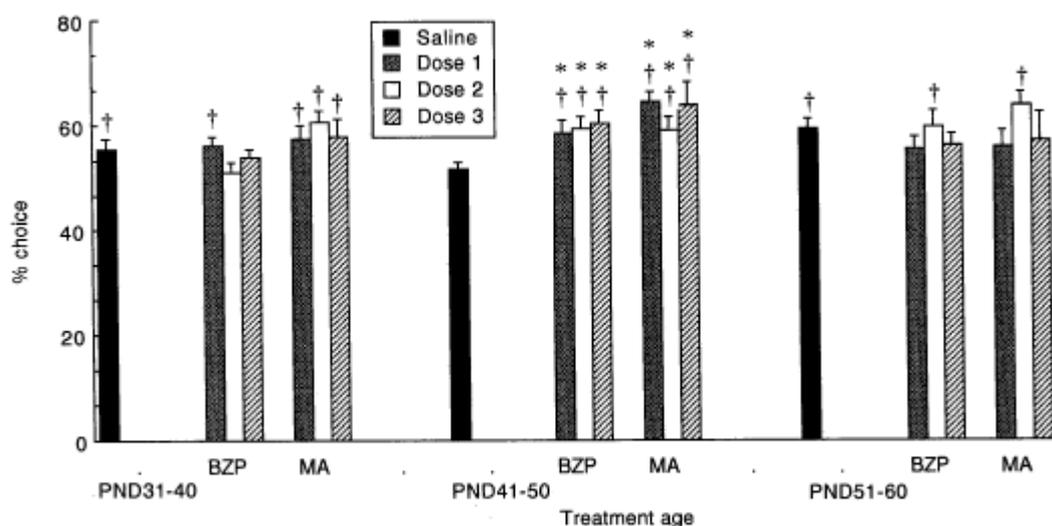


Figure 17: Mean ( $\pm$ S. E. M.) percent of novel entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); . \* significantly different ( $p < .05$ ) from saline for that particular drug.

Figure 17 shows a significant dose effect for all three BZP and MA doses administered during PND41-50, that were significantly different from same aged control rats in the percentage of entries of the Y-maze. Although, there were no other significant dose effects, rats treated with

saline during PND31-40 and 51-60, and rats treated with BZP during PND31-40 or all three doses of MA, and all three doses of BZP and MA during PND41-50 or 10 mg/kg BZP or 1.0 mg/kg MA during PND51-60 displayed significant preferences for entering the novel arm rather than the familiar arm. There were no significant sex effects.

### BZP and MA treated: Total Entries of both arms of the Y-maze at PND120.

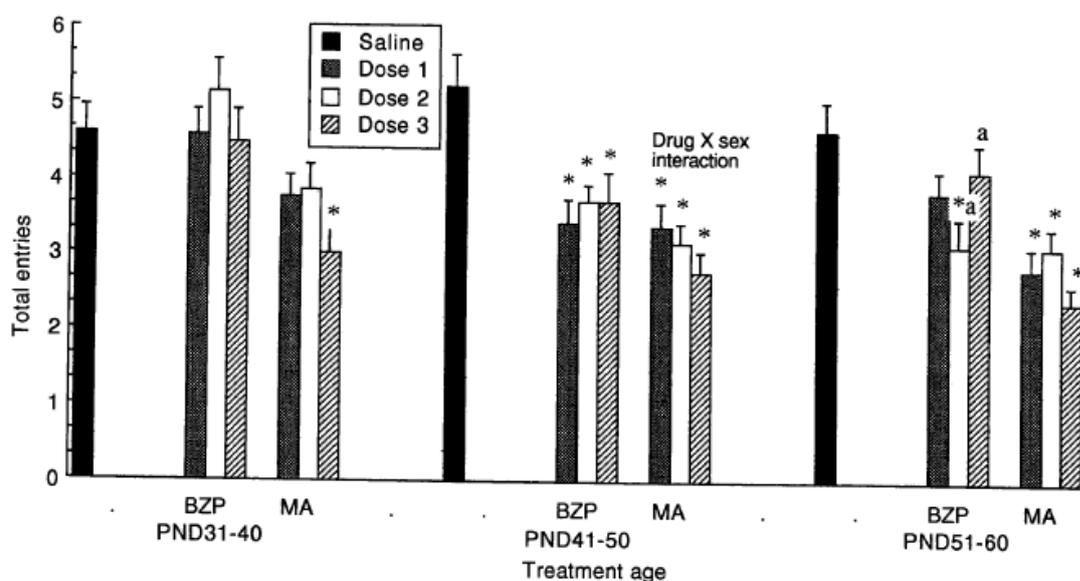


Figure 18: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 18 illustrates that rats treated with 2.0 mg/kg of MA during PND31-40, and rats treated with all three BZP and MA doses at PND41-50, and rats treated with 10 mg/kg of BZP during PND51-60, or all three MA doses, made significantly fewer entries of both arms than saline-treated rats. However, a significant dose x sex interaction ( $F(3, 72) 2.77, P < .048$ ) for effects of MA treatment at PND41-50 (outlined in Figure 19) revealed that the treatment effect was only typical of female rats. Rats treated with 10 mg/kg BZP during PND51-60 made significantly fewer entries of both arms than 5 mg/kg and 20 mg/kg rats.

Similar to the 30 day wash-out period, there were significant sex effects for all treated rats, with female rats making significantly more entries of both arms than male treated rats.

**Drug x sex interaction for PND41-50 MA treated rats: Total Entries of both arms of the Y-maze at PND120.**

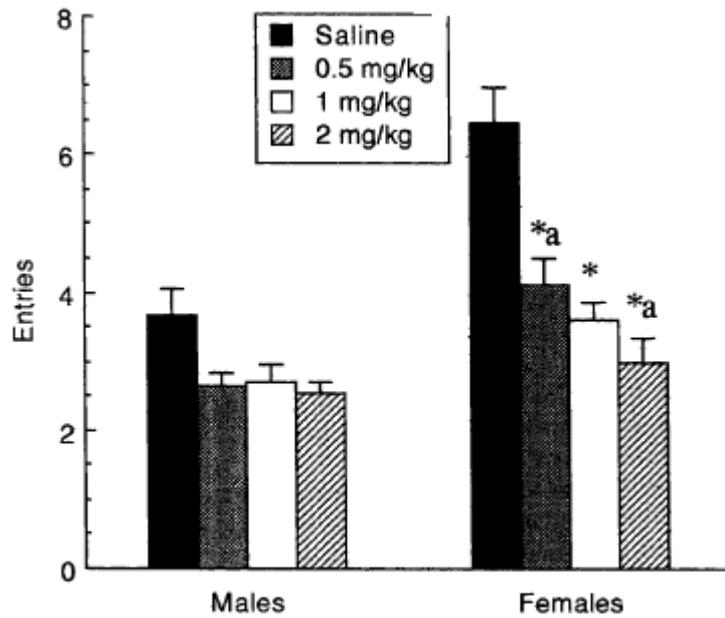


Figure 19: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), MA PND41-50 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex. a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

### BZP and MA treated: Percentage of Time spent in the Novel Arm of the Y-maze at PND120.

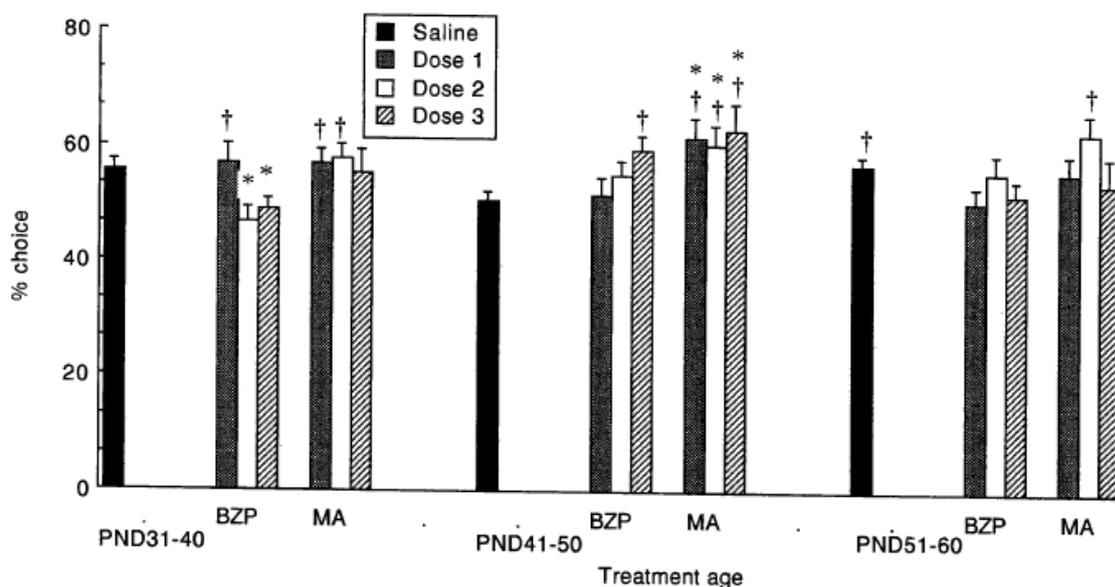


Figure 20: Mean ( $\pm$ S. E. M.) percent of time spent in the novel arm of the Y-maze for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug.

Figure 20 shows a significant dose effect for 10 mg/kg and 20 mg/kg BZP doses administered during PND31-40, and all three MA doses administered during PND41-50, with the BZP-treated spending lesser percentage of time in the changed arm while the MA-treated rats spent a significantly higher percentage of time in them. Although, there were no other significant dose effects, rats treated with 5 mg/kg BZP or 0.5 and 1.0 MA during PND31-40, and rats treated with 20 mg/kg BZP or all three doses of MA during PND41-50, saline at PND51-60 and 1.0 mg/kg MA during PND51-60 displayed significant preferences for spending time in the novel arm rather than the familiar arm. There were no significant sex effects.

#### Summary of Y-maze results at PND120

The general pattern of results in the Y-maze at PND120 suggest that rats treated with MA during PND41-50 had a significant tendency to enter and spend time in the novel Y-maze arm. PND41-50 BZP treated rats preferred to enter the novel arm over the familiar arm also, but it appears that once they entered the novel arm they did not prefer to spend time in it. It is therefore possible that the PND41-50 treated rats are more impulsive than saline-treated rats. Ueno et al. (2002) report impulsive behaviour to appear as increased time and entries in a novel

environment in relation to total arm entries. Total entries of both arms is a measure of general locomotor activity and female rats are consistently more active than male rats. However, female rats treated with MA during PND41-50 showed a decrease in locomotor activity in a dose dependent manner. Overall, it appears that rats treated with both BZP and MA during PND41-50 had decreased anxiety or possible impulsivity when tested at PND120, which was not contaminated by locomotor activity.

## 2. Light/dark emergence results

As each animal was tested in the light/dark box seven times and averages of the seven trials were used in each of the analyses.

### BZP and MA treated: Emergence latency time from the Light/dark box at PND 120.

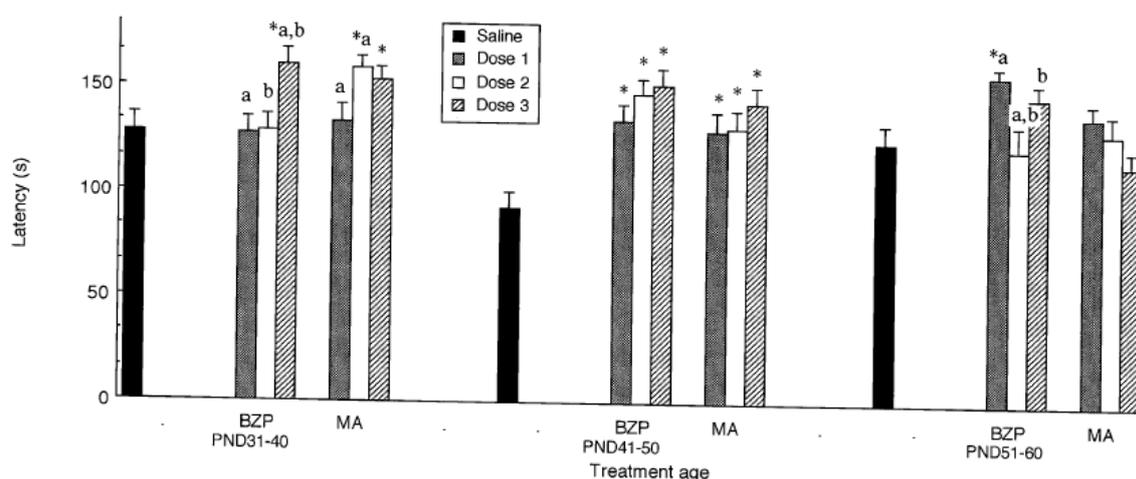


Figure 21: Mean ( $\pm$ S. E. M.) emergence latency from the Light/dark box for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 21 illustrates that rats treated during PND31-40 with 20 mg/kg BZP, or 1.0 mg/kg and 2.0 mg/kg MA, and all rats treated during PND41-50, and rats treated with 5 mg/kg BZP, during PND51-60 took significantly longer to emerge from the dark than saline-treated rats. Rats treated with 20 mg/kg BZP during PND31-40 had a significantly longer latency to emerge than the other two BZP doses and rats treated with MA 1.0 mg/kg, during the same period were significantly different from rats treated with MA 0.5 mg/kg. Also, rats treated with 5 mg/kg or

20 mg/kg of BZP during PND51-60 had a significantly longer latency to emerge compared to the 10 mg/kg BZP treated rats.

There were significant sex effects for all groups except for the rats treated with BZP during PND51-60, with female rats having a faster latency to emerge than male rats.

### **Summary of Light/dark emergence results at PND120**

The general pattern of results again suggest that rats treated with BZP or MA during PND41-50 were significantly different from saline-treated rats. However, whereas these rats possibly displayed decreased anxiety or impulsivity in the Y-maze, longer latencies to emerge from the darkened box into the illuminated arena are indicative of higher anxiety (Archer, 1975) suggesting that the treatment of BZP or MA during PND41-50, and highest dose BZP rats or 1.0 and 2.0 mg/kg MA treated rats at PND31-40 or lowest dose BZP PND51-60 are more anxious compared to control rats.

### 3. EPM results

As already mentioned, due to potential habituation effects, each animal was tested once in the EPM.

#### BZP and MA treated: Percentage of Entries of the open arms of the EPM at PND120.

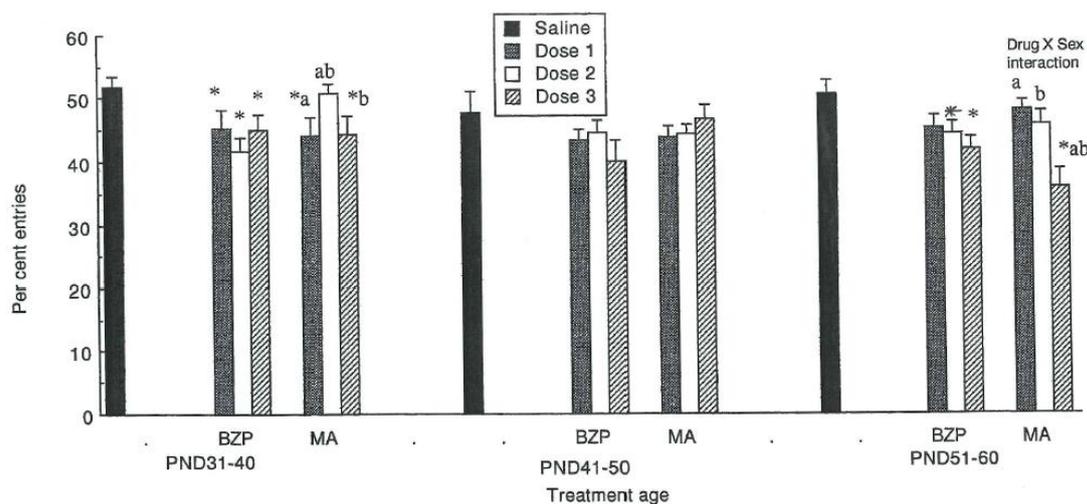
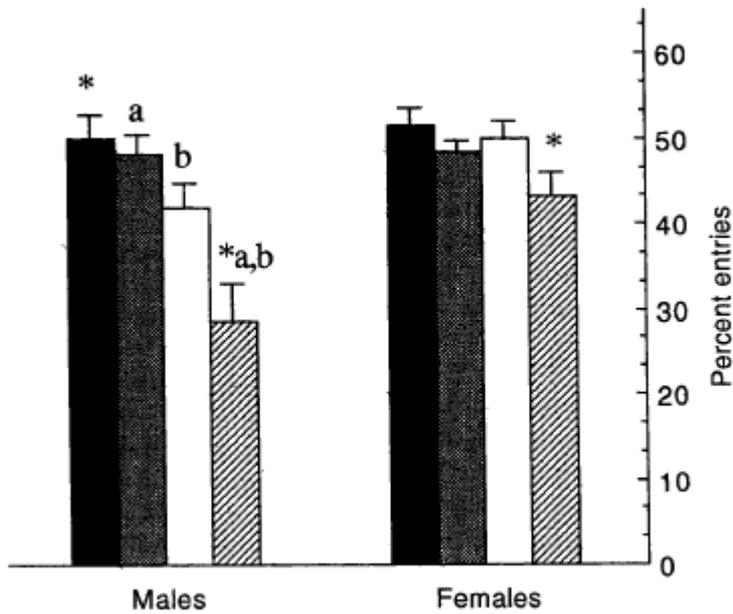


Figure 22: Mean ( $\pm$ S. E. M.) percent of open arm entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 22 illustrates that rats treated with all three doses of BZP during PND31-40, and rats treated with 0.5 mg/kg and 2.0 mg/kg MA, during PND31-40 had a significantly lower percentage of entries of the open arms of the EPM than saline-treated rats. Similarly, rats treated with 10 mg/kg and 20 mg/kg of BZP during PND51-60, and rats treated with 2.0 mg/kg MA, during the same developmental period had a significantly lower percentage of open arm entries than saline-treated rats. Rats treated with 1.0 mg/kg of MA during PND31-40 and rats treated with 2.0 mg/kg of MA during PND51-60 were significantly different from those administered the other two doses of MA during the same period.

There was one sex effect, with female rats treated with MA during PND 51-60 having a higher percentage of open arm entries than male treated rats. There was also a significant drug x sex interaction ( $F(3, 72), P < .05$ ) which, as outlined below in Figure 23, revealed that the drug effect following treatment with MA at PND51-50 was most obvious with male rats.

**Drug x sex interaction for PND51-60 MA treated rats: Percentage of Entries of the open arms of the EPM at PND120.**



**Figure 23: Mean ( $\pm$ S. E. M.) percentage of open arm entries for control (Saline), MA PND51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex. a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.**

This graph illustrates that both male and female rats treated with 2.0 mg/kg of MA during PND51-60 had a significantly lower percentage of entries of the open arms than saline-treated rats. However it appears that as the dose of MA increased, male rats were more affected, with the rats treated with 2.0 mg/kg being significantly different from those treated with either 0.5 mg/kg or 1.0 mg/kg.

### BZP and MA treated: Percentage of time in the open arms of the EPM at PND120.

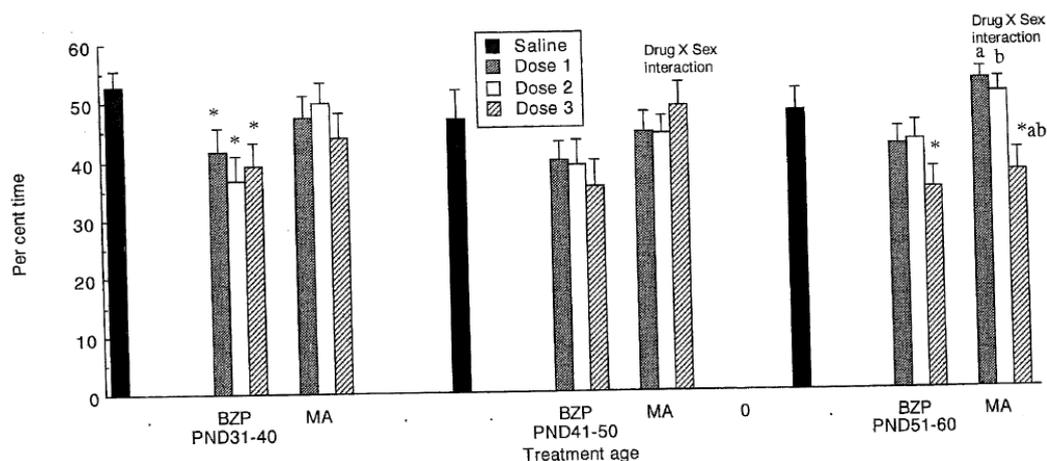


Figure 24: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

As depicted in Figure 24 rats treated with all three doses of BZP during PND31-40, 20 mg/kg of BZP during PND51-60, and rats treated with 2.0 mg/kg MA during PND51-60, spent a significantly lower percentage of time in the open arms of the EPM than saline-treated rats. The rats administered 2.0 mg/kg of MA during PND51-60 were significantly different from the other two doses of MA administered at that time. However, as shown in Figure 25, a significant drug x sex interaction ( $F(3,72)$ ,  $P < .009$ ) for rats treated with MA during PND 41-50 was due to a significant decrease in open-arm entries with 1.0 mg/kg for females only. Similarly, for rats treated with MA during PND51-60 a significant dose x sex interaction ( $F(3, 72)$ ,  $P < .0408$ ) revealed that the differences that appeared in the significant main effect applied to males but not to females (see Figure 26).

There was no significant sex effect for rats treated with either BZP or MA during PND31-40. However, female rats treated with MA during PND41-50, and Both BZP or MA during PND51-60 spent a significant percentage more time in the open arms than male treated rats

**Drug x sex interaction for PND41-50 MA treated rats: Percentage of time spent in the open arms of the EPM at PND120.**

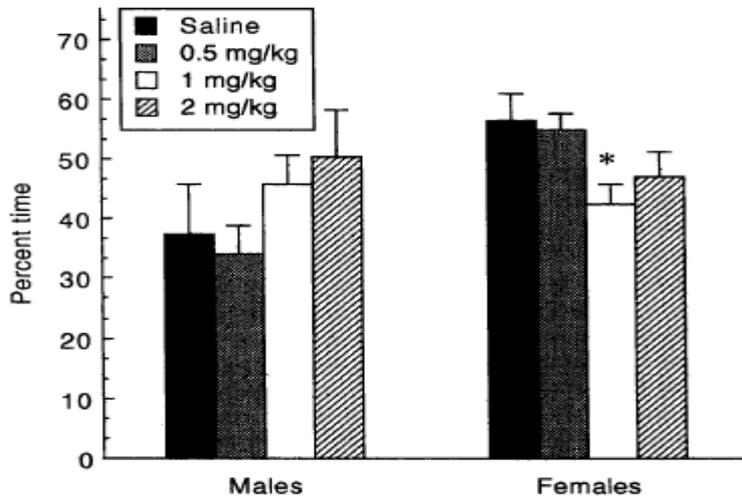


Figure 25: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms for control (Saline), MA PND 41-50 treated male and female rats. . \* significantly different ( $p < .05$ ) from saline for that particular drug.

**Drug x sex interaction for PND 51-60 MA treated rats: Percentage of time spent in the open arms of the EPM at PND 120.**

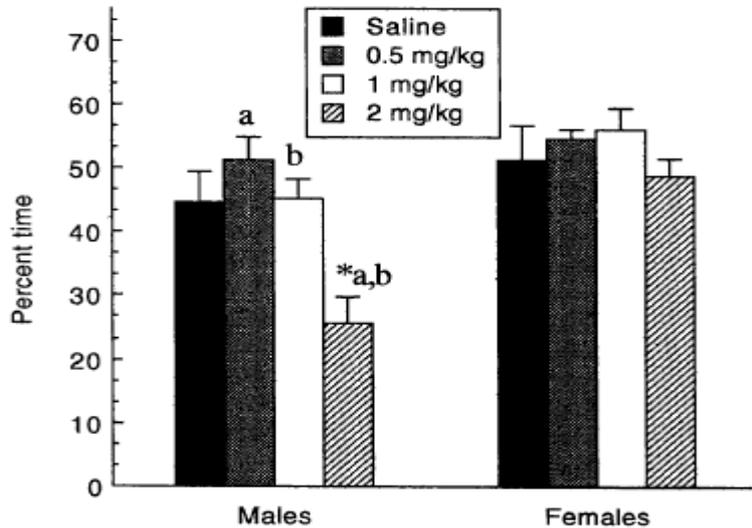


Figure 26: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms for control (Saline), MA PND 51-60 treated male and female rats. . \* significantly different ( $p < .05$ ) from saline for that particular sex; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular sex.

### BZP and MA treated: Entries of the closed arms of the EPM at PND120.

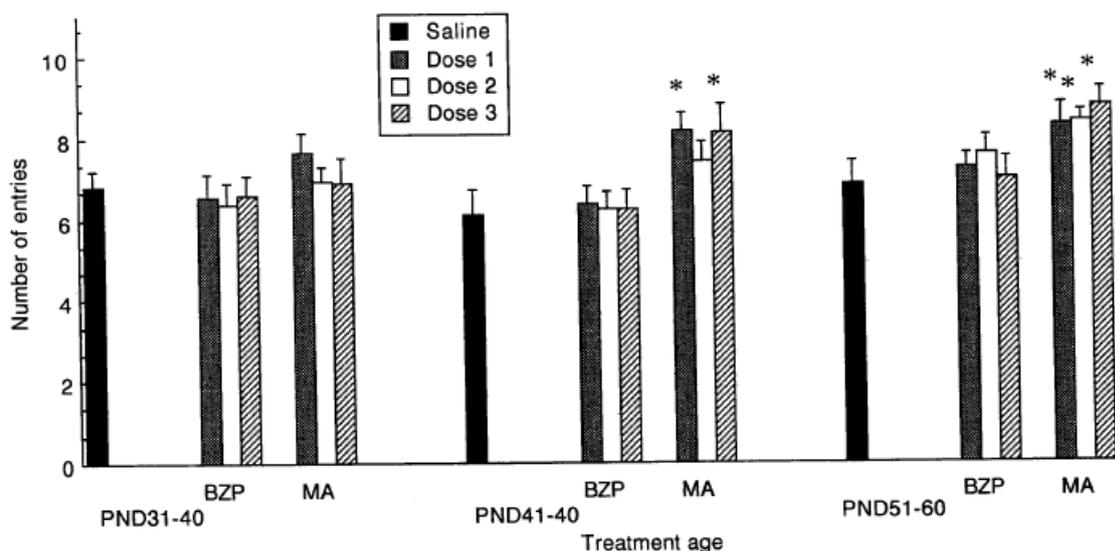


Figure 27: Mean ( $\pm$ S. E. M.) entries of the closed arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. . \* significantly different ( $p < .05$ ) from saline for that particular drug.

As illustrated in Figure 27, rats treated with 0.5 mg/kg and 2.0 mg/kg of MA during PND41-50 and rats treated with all three MA doses during PND51-60 made significantly more closed arm entries than saline-treated rats.

There were significant sex effects for all treated rats' at all three different stages of adolescence, with female treated rats making significantly more entries of the closed arms than male treated rats.

### Summary of EPM results at PND120

Rats treated with BZP during PND31-40 displayed increased anxiety in the EPM as evidenced by decreased percentages of entries of and time spent in the open arms. However, this may be attributable to general activity for female rats only, as there was no significant difference in entries of the closed arms for male rats compared with saline-treated rats. Similarly rats treated with 0.5 and 2.0 mg/kg of MA during PND31-40 displayed increased anxiety as shown by a decrease in percentage of open arm entries. However, the MA treated rats appeared to be less impulsive than the similar aged BZP treated rats as there was no significant difference in the percentage of time spent in the open arms, suggesting that although the PND31-40 MA rats have had a heightened reluctance to enter the open arms, once on them they displayed a

similar response to saline-treated rats. PND41-50 BZP and MA treated rats displayed emotional reactivity comparable to saline treated rats. However, although the highest dose BZP-treated rats entered the open arms equally as often as the saline-treated rats, once actually on them they probably found them aversive and thus preferred to not occupy them. Rats treated with 0.5 and 2.0 mg/kg of MA were more active than control rats, suggesting that MA treatment at PND41-50 impacts on later locomotor behaviour, primarily only for male rats as observed by the sex-related effects. Additionally, only the female rats treated with 1.0 mg/kg of MA during PND41-50 had a reduced percentage of time spent on the open arms of the EPM, suggesting increased emotionality for female rats treated with 1.0 mg/kg of MA compared with males and 0.5 and 2.0 mg/kg MA-treated females. Rats treated with BZP (5 and 20 mg/kg) during PND51-60 displayed increased anxiety as observed by a reduced percentage of entries into the open arms, which was not related to general activity. However only the high dose BZP-treated rats displayed increased anxiety in the percentage of time spent on the open arms and due to the significant sex effect, it is likely that male rats treated with BZP during PND51-60 were more anxious than similar aged females and control rats. Similarly, rats treated with the highest dose of MA (2.0 mg/kg) displayed increased anxiety as evidenced by reduced percentages of entries into and time spent on the open arms for both male and female rats. However, the male rats appeared more affected by the highest dose than the other two lower MA doses. All three MA-treated groups made significantly more entries of the closed arms, with female rats making significantly more entries than males. This suggests that earlier MA treatment impacted on later locomotor activity as opposed to increased anxiety, with the exception of male rats treated with 2 mg/kg of MA during PND51-60 displaying increased anxiety over the EPM measures.

#### ***4. Social Interaction test results***

Each animal was paired with an unfamiliar animal of a similar weight ( $\pm 10$  grams) and tested once on the Social Interaction test.

### BZP and MA treated: Total squares entered in the Social Interaction test at PND120.

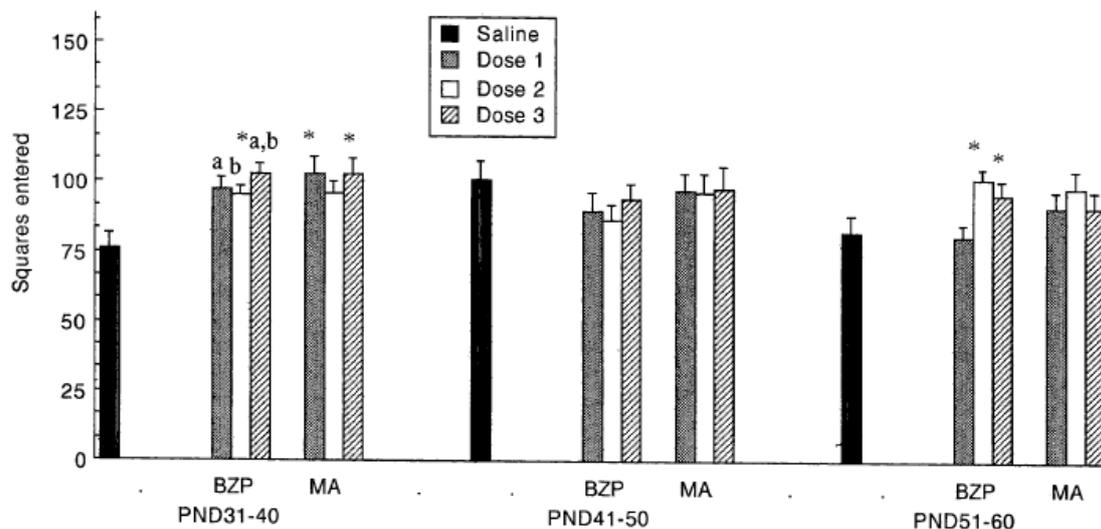


Figure 28: Mean ( $\pm$ S. E. M.) total squares crossed in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

As illustrated in Figure 28 rats treated with 20 mg/kg of BZP during PND31-40, or 0.5 mg/kg and 2.0 mg/kg of MA entered significantly more squares than saline-treated rats. Rats treated with 20 mg/kg were also significantly different from rats treated with 5 mg/kg and 10 mg/kg, during PND31-40. Additionally, rats treated with 10 mg/kg and 20 mg/kg during PND51-60 entered significantly more squares than saline-treated rats.

There was a significant sex effect for all conditions, with female treated rats entering significantly more squares in the open field than similar treated male rats.

### BZP and MA treated: Outer Rears in the Social Interaction test at PND120.

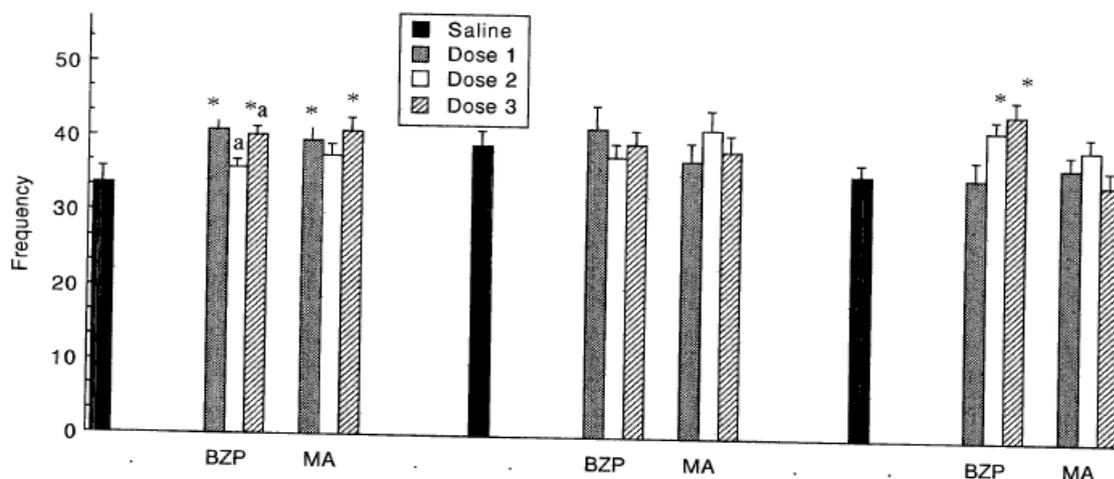


Figure 29: Mean ( $\pm$ S. E. M.) total outer rears in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - .5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

As illustrated in Figure 29 rats treated with 5 mg/kg and 20 mg/kg of BZP during PND31-40, or 0.5 mg/kg and 2.0 mg/kg of MA reared significantly more often than saline-treated rats in the outer squares of the OF. Rats treated with 10 mg/kg of BZP during PND31-40 were also significantly different from rats treated with 20 mg/kg of BZP. Rats treated with 10 mg/kg and 20 mg/kg of BZP during PND51-60 also reared significantly more often than saline-treated rats.

For all conditions there was a significant sex effect, with female treated rats rearing more often than male treated rats on the outer squares of the OF in the Social Interaction test.

### BZP and MA treated: Centre Rears in the Social Interaction test at PND120.

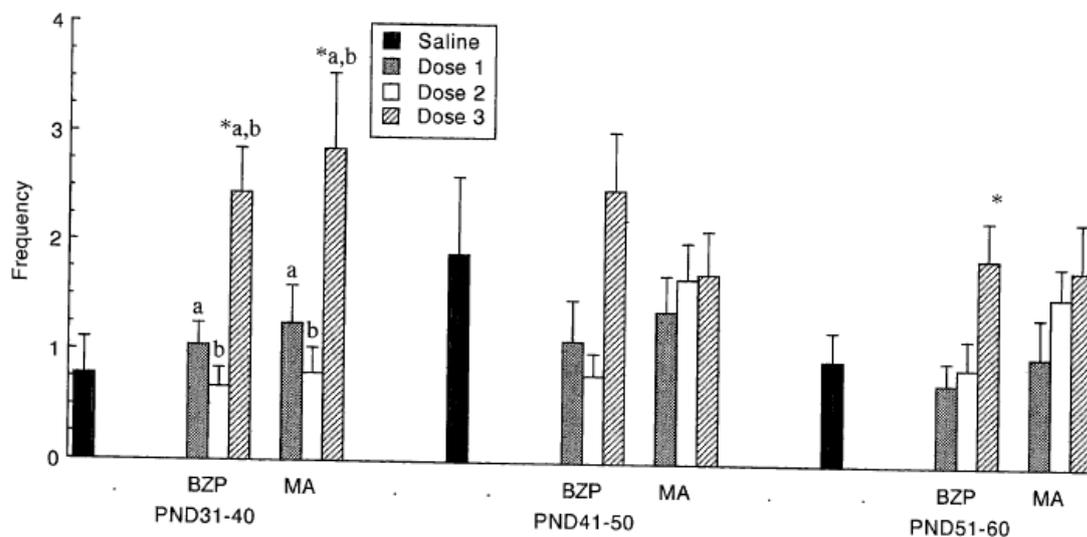


Figure 30: Mean ( $\pm$ S. E. M.) total inner rears in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 30 illustrates that rats treated with 20 mg/kg BZP or 2.0 mg/kg of MA during PND31-40, and rats treated with 20 mg/kg of BZP during PND51-60, reared significantly more in the inner squares of the OF than saline-treated rats. Additionally, there was a significant difference between rats treated with 20 mg/kg of BZP and 2.0 mg/kg MA during PND31-40, and the other doses of BZP and MA. Only the female rats treated with BZP during PND41-50 reared significantly more than comparative treated male rats in the centre of the open field.

### BZP and MA treated: Interaction time in the Social Interaction test at PND120.

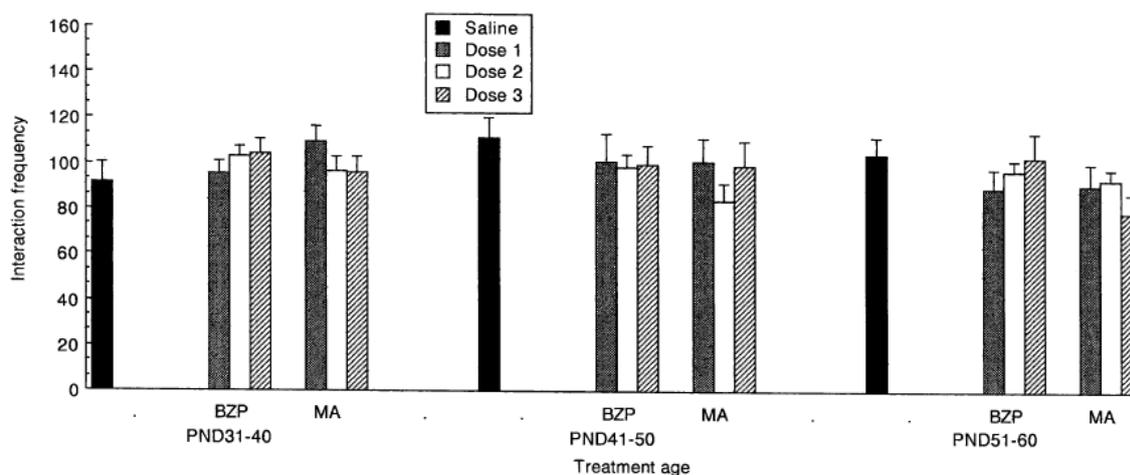


Figure 31: Mean ( $\pm$ S. E. M.) total interaction time in the Social Interaction test for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats.

As illustrated in Figure 31, there was no significant dose effects for treated groups in interaction time, however male rats treated with BZP and MA during PND31-40 spent significantly more time in social interaction than BZP treated female rats.

### Summary of Social Interaction results at PND120

With the exception of PND41-50 treated groups, and PND51-60 MA treated rats, the highest dose of either BZP or MA administered at either PND31-40 and BZP administered at PND51-60 significantly affected outer and inner rearing. This suggests that decreased anxiety increased vertical exploration, but may have also reflected increased locomotor activity, as evidenced by the treated animals entering more squares in the open field than saline treated rats. However female treated rats in all conditions had increased locomotor activity compared to male treated rats and a significantly higher frequency of rearing in the outer area of the open field than male treated rats, which may explain the differences found. Alternatively, increased inner rearing in rats treated with BZP during PND31-40 and 51-60, and in rats treated with the highest dose of MA during PND31-40 may not have reflected decreased anxiety but rather indicated scanning for an exit from the aversive environment, thereby suggesting increased anxiety and reduced risk assessment. Interestingly, and against the general pattern of results was the treated male rats during PND31-40 increasing their time in social interaction compared

to female rats. It is possible that for the male treated rats, the novelty of the unfamiliar peer was greater than the novelty of the testing apparatus, suggesting decreased anxiety.

### **Results at PND200**

All animals were tested in the Y-maze, light/dark emergence box and EPM at PND200. See Table 2 above for F ratios and Table 3 for significant sex differences, across the three testing periods. Unfortunately it was not possible to examine results of the social interaction test at this period because all video-recorded data for this test was destroyed in the devastating Christchurch earthquake of February 2011 before it could be analysed.

#### **1. Y-Maze results**

As each animal was tested in the Y-maze four times and the averages of the four trials were used in each of the analyses.

#### **BZP and MA treated: Percentage of Entries of the Novel Arm of the Y-maze at PND200.**

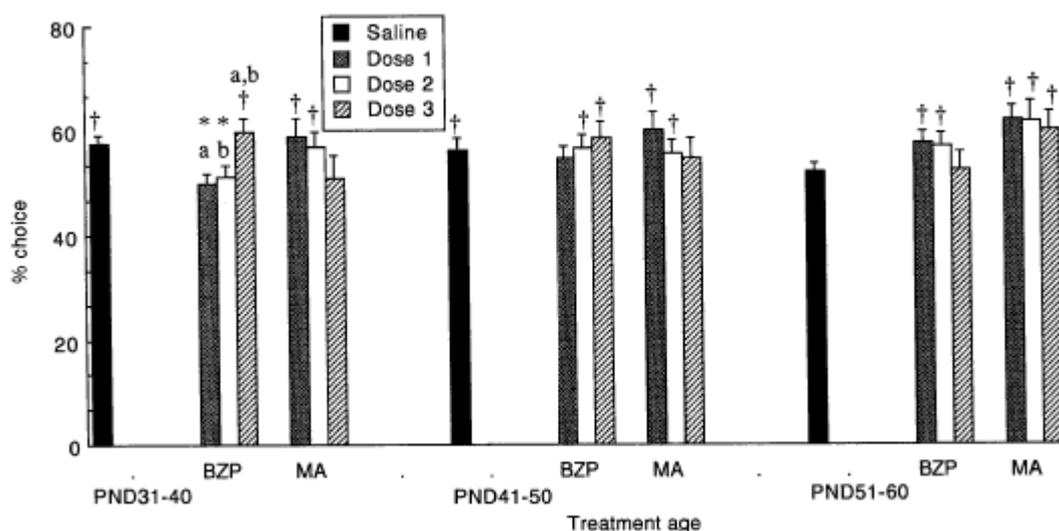


Figure 32: Mean ( $\pm$ S. E. M.) percent of novel entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 32 shows a significant dose effect for 5 mg/kg and 10 mg/kg of BZP during PND31-40. Rats treated with saline and 20 mg/kg of BZP during PND31-40, rats treated with saline and 10 mg/kg and 20 mg/kg of BZP or 0.5 mg/kg and 1.0 mg/kg of MA during PND41-50, and rats

treated with 5 mg/kg and 10 mg/kg of BZP or all three doses of MA during PND51-60 displayed significant preferences for entering the novel rather than the familiar arm. There was one significant sex effect, with female rats (mean = 63.305, SEM = 1.6) treated with MA during PND51-60 making a higher percentage of entries of the novel arm than male treated rats.

### BZP and MA treated: Total Entries of both arms of the Y-maze at PND200.

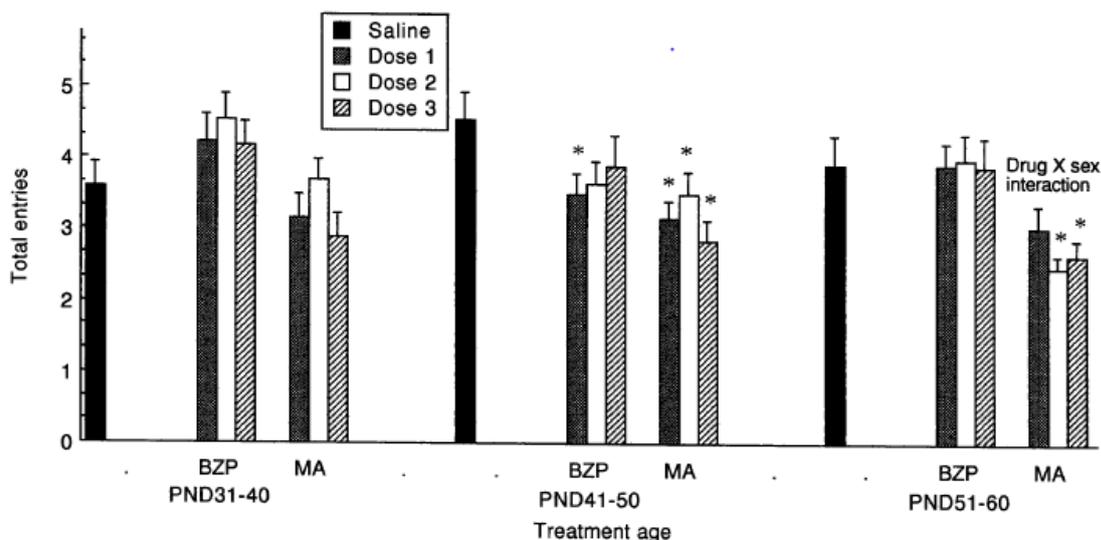


Figure 33: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug.

As depicted in Figure 33, rats treated with 5 mg/kg of BZP, or all three doses of MA, during PND41-50, and rats treated with 1.0 mg/kg and 2.0 mg/kg of MA, during PND51-60 made significantly fewer entries of both arms than saline-treated rats. However, a significant dose x sex interaction ( $F(3, 72) 3.30, P < .025$ ) outlined in Figure 34 showed this effect of MA to characterise female, but not male rats. Additionally, all treated female rats made significantly more entries of both arms than male treated rats.

**Drug x sex interaction for PND51-60 MA treated rats: Total Entries of both arms of the Y-maze at PND200.**

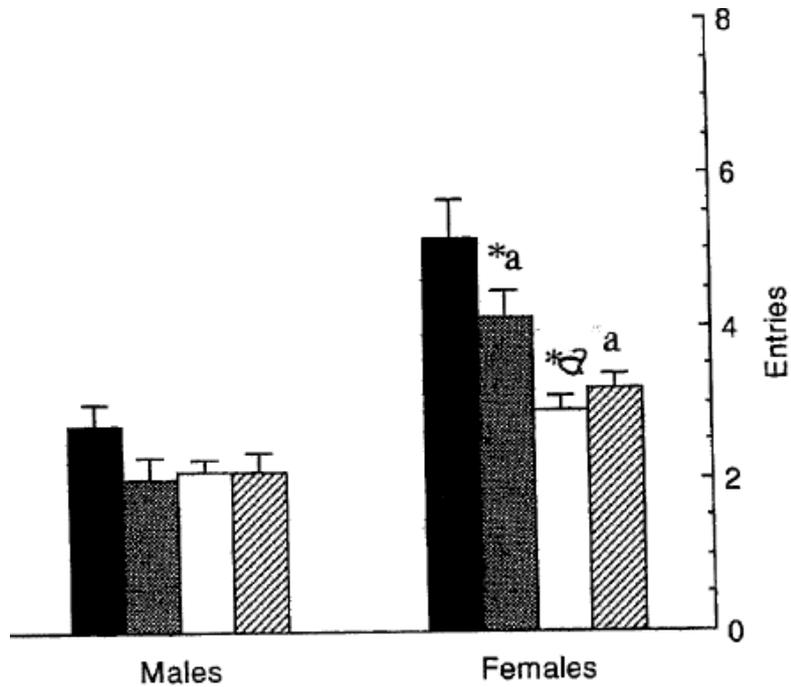


Figure 34: Mean ( $\pm$ S. E. M.) total entries of both arms for control (Saline), MA PND51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex; a difference between groups with superscript in common significant ( $p < .05$ ) for that particular sex.

### BZP and MA treated: Percentage of Time spent in the Novel Arm of the Y-maze at PND200.

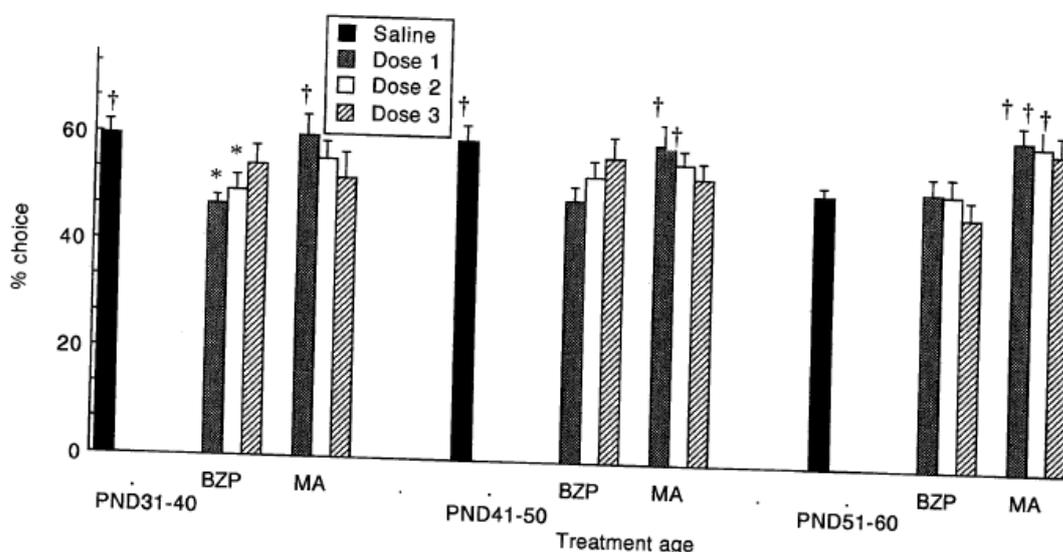


Figure 35: Mean ( $\pm$ S. E. M.) percent of time spent in the novel arm of the Y-maze for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. † differs significantly ( $p < .05$ ) from chance expectancy of 50% (one-sample t test); \* significantly different ( $p < .05$ ) from saline for that particular drug.

Figure 35 shows that rats treated with saline or .0.5 mg/kg of MA at PND31-40, and saline or 0.5 mg/kg and 1.0 mg/kg of MA at PND41-50, and all three doses of MA administered during PND51-60 displayed significant preferences for occupying the novel rather than the familiar arm. Rats treated with 5 mg/kg and 10 mg/kg of BZP during PND31-40 spent significantly less time than saline treated rats in the novel arm. There was one sex effect, with female rats treated with BZP during PND51-60 spending significantly more time in the novel arm than male treated rats.

### Summary of Y-maze results at PND200

Interestingly, the general pattern of results at PND200 in the Y-maze suggest that most treated rats prefer to enter and spend time in the changed arm over the familiar arm, which is not related to general activity for male rats only. Fairly consistent with the other two behavioural testing phases, there was significant sex differences in treated female rats displaying increased motor activity compared to male treated rats treated during PND31-40. However, for rats treated during PND41-50 general locomotor activity was decreased and this may account for why only male rats made significantly fewer total entries of both arms than females. In

contrast to female MA treated rats during PND51-60 who made significantly less entries of both arms of the Y-maze than male treated rats and BZP treated female rats who made significantly more entries of the changed arm than controls or male rats. However, female MA treated rats had a higher percentage of novel arm entries which as mentioned was not related to general locomotor activity.

## 2. Light/dark emergence results

As each animal was tested in the light/dark box seven times and the averages of the seven trials were used in each of the analyses.

### BZP and MA treated: Emergence latency time from the Light/dark box at PND200.

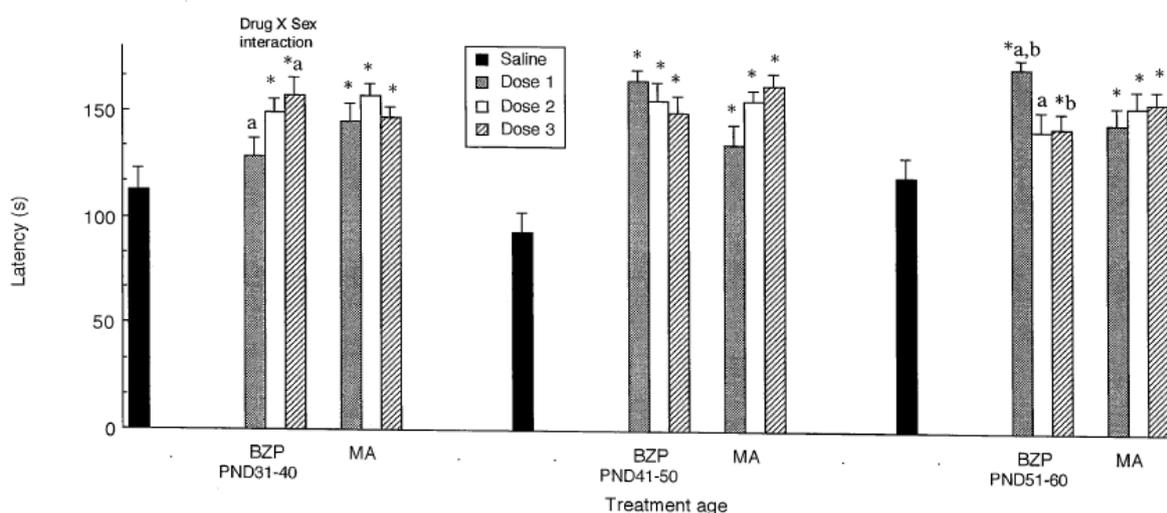


Figure 36: Mean ( $\pm$ S. E. M.) emergence latency from the light/dark box for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 36 illustrates that rats treated during PND31-40 with 10 mg/kg and 20 mg/kg of BZP, or all three doses of MA, and BZP and MA rats treated during PND41-50 and rats treated during PND51-60 with 5 mg/kg and 20 mg/kg of BZP, and all three doses of MA, took significantly longer to emerge from the dark than saline-treated rats. Rats treated with 20 mg/kg BZP during PND31-40 had a significantly longer latency to emerge than rats treated with 5 mg/kg of BZP. However, as revealed in a significant dose x sex interaction ( $F(3,72) 3.47, P < .0207$ ) for rats treated with BZP at this age, the effect only applied to females (see Figure 37). Also rats treated with 5 mg/kg of BZP during PND51-60 had a significantly longer latency to emerge compared to the 10 mg/kg and 20 mg/kg BZP treated rats.

There were significant sex effects for all groups, with all female rats having a faster latency to emerge from the dark compartment of the light/dark box, than male rats.

**Drug x sex interaction for PND31-40 BZP treated rats: Emergence latency from Light/dark box at PND200.**

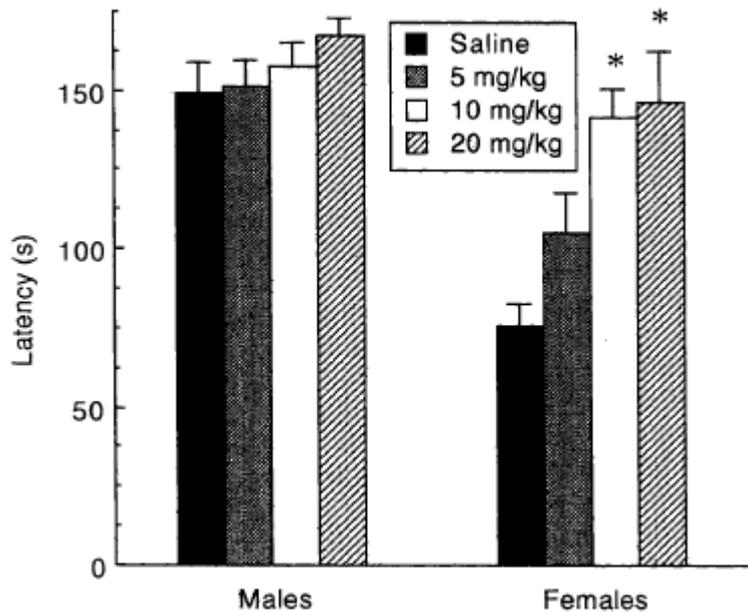


Figure 37: Mean ( $\pm$ S. E. M.) emergence latency from light/dark box for control (Saline), BZP PND 31-40 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex.

**Summary of Light/dark emergence results at PND200**

Consistent with the other behavioural testing periods, female rats in all groups showed a significantly faster emergence time than male rats. All treated groups with the exception of rats treated with BZP (10 mg/kg) during PND51-60 had a longer latency to emerge into the illuminated area compared to saline-treated rats. Of interest is the significant sex x drug interaction for PND31-40 BZP treated rats, with female treated rats increasing their emergence time in a dose-dependent manner.

### 3. EPM results

Each animal was tested once in the EPM.

#### BZP and MA treated: Percentage of Entries of the open arms of the EPM at PND200.

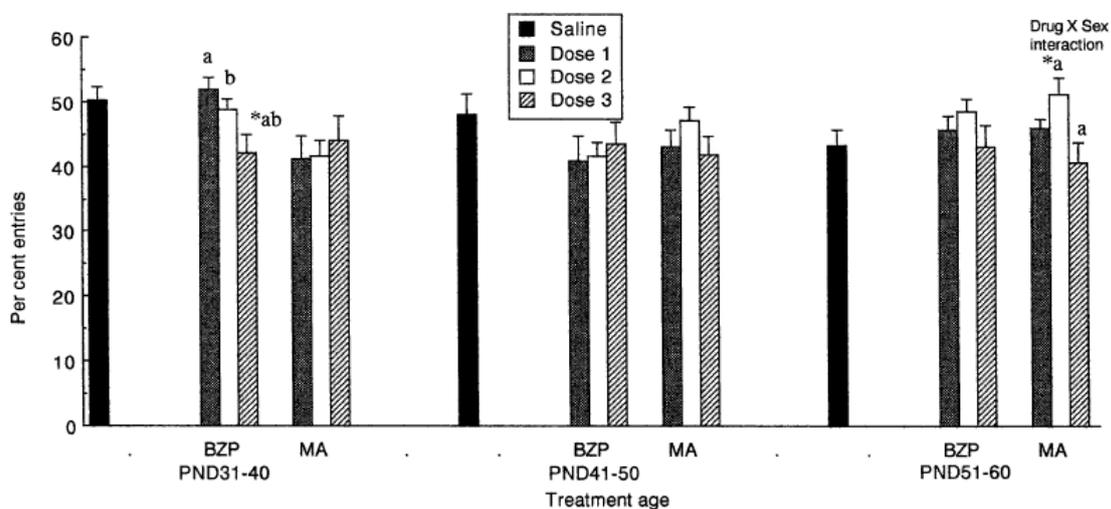


Figure 38: Mean ( $\pm$ S. E. M.) percent of open arm entries for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

Figure 38 illustrates that rats treated with 20 mg/kg of BZP during PND31-40, made a significantly lower percentage of entries of the open arms of the EPM than saline-treated rats. Alternatively, rats treated with 1.0 mg/kg of MA, during PND51-60 made a significantly higher percentage of entries of the open arms of the EPM than saline-treated rats. However, a significant dose x sex interaction ( $F(3, 72) 5.77, P < .0014$ ) for this latter result (illustrated in Figure 39) showed that this effect may be attributable to female rats treated with 1.0 and 2.0 mg/kg MA spending a greater percentage of time in the open arms than 2.0 mg/kg male treated rats who spent a significantly lower percentage of time in the open arms than the other two MA-treated males. Additionally, rats treated with 20 mg/kg of BZP during PND31-40 were significantly different from rats treated with 5 mg/kg and 10 mg/kg. Likewise, rats treated with 1.0 mg/kg of MA during PND51-60 were significantly different from rats treated with 20 mg/kg of MA.

With the exception of rats treated with BZP during PND31-40 there were significant sex effects for all other conditions, with female rats preferring to enter the open arms compared to male treated rats.

**Drug x sex interaction for PND51-60 MA treated rats: Percentage of Entries of the open arms of the EPM at PND200.**

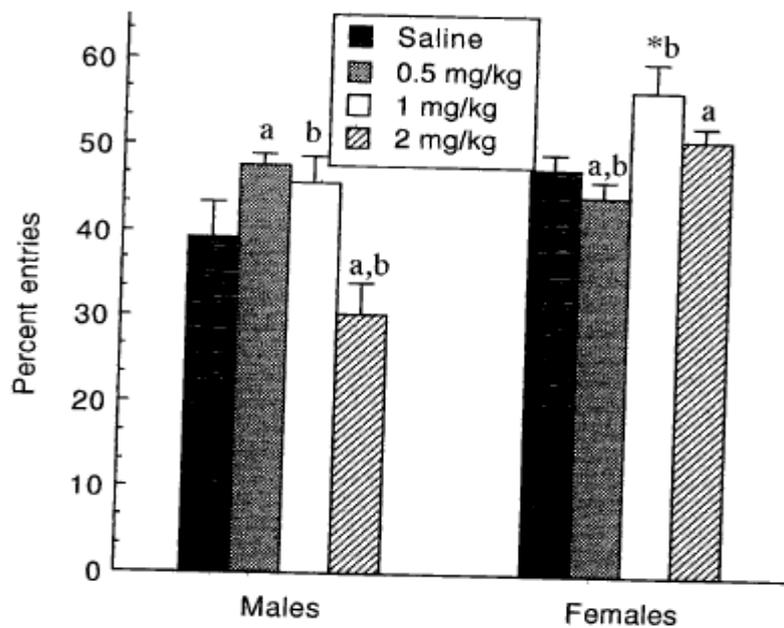
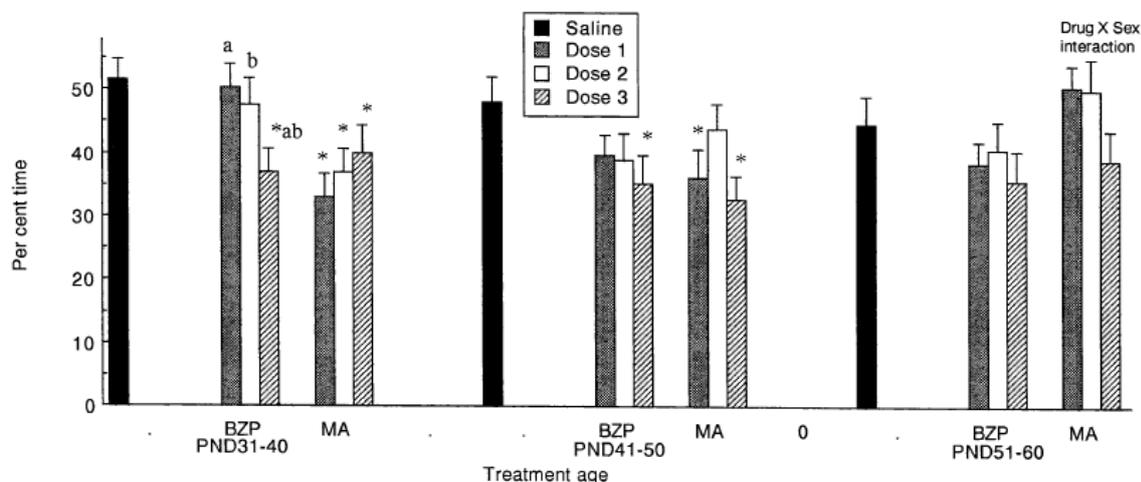


Figure 39: Mean ( $\pm$ S. E. M.) percentage of entries of the open arms of the EPM for control (Saline), MA PND 51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular sex.

### BZP and MA treated: Percentage of time in the open arms of the EPM at PND200.



**Figure 40:** Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND 31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

As depicted in Figure 40, rats treated with 20 mg/kg of BZP during PND31-40, and all three MA doses, 20 mg/kg of BZP during PND41-50, and rats treated with 0.5 mg/kg and 2.0 mg/kg of MA, spent significantly less time in the open arms of the EPM than saline-treated rats. The rats administered 20 mg/kg of BZP during PND31-40 were significantly different from the other two doses of BZP administered at that time. Although there were no significant drug main effects for rats treated at PND51-60, a significant dose x sex interaction ( $F(3,72) 3.02$ ,  $P < .0057$ ) for MA-treated rats at this age showed that, for males alone, treatment with 2.0 mg/kg significantly reduced this response following treatment with saline and both lower doses (see Figure 41).

With the exception of BZP- treated rats during PND31-40 and PND51-60, female treated rats spent a significantly longer percentage of time in the open arms compared to male rats.

**Drug x sex interaction for PND51-60 MA treated rats: Percentage of time spent in the open arms of the EPM at PND200.**

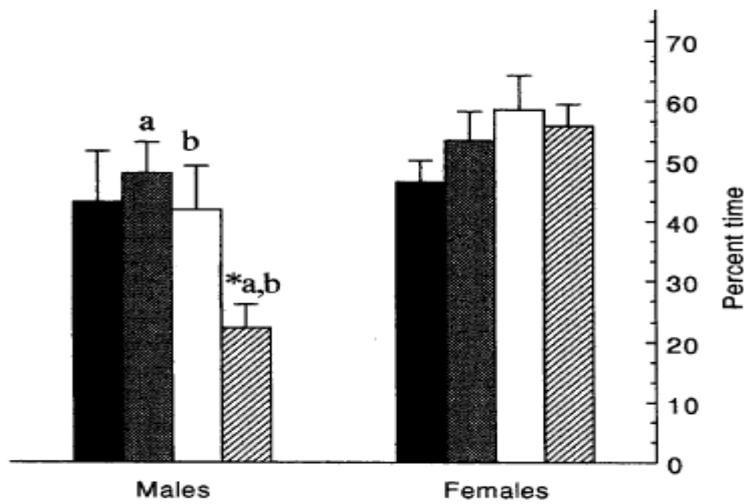


Figure 41: Mean ( $\pm$ S. E. M.) percentage of time spent in the open arms for control (Saline), MA PND51-60 treated male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular sex; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular sex.

**BZP and MA treated: Entries of the closed arms of the EPM at PND200.**

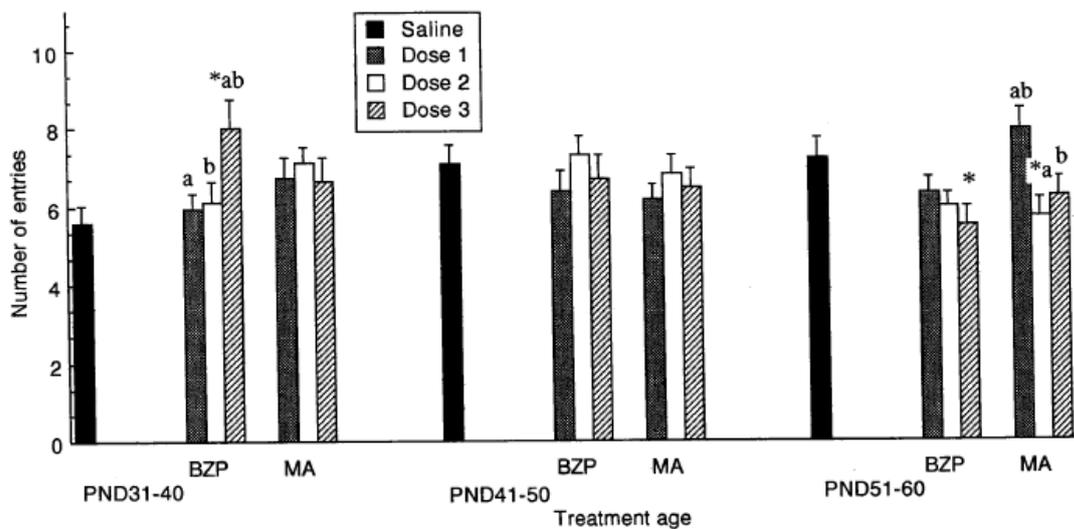


Figure 42: Mean ( $\pm$ S. E. M.) entries of the closed arms of the EPM for control (Saline), BZP (Dose 1 - 5mg/kg; Dose 2 - 10mg/kg; Dose 3 - 20mg/kg) and MA (Dose 1 - 0.5mg/kg; Dose 2 - 1.0mg/kg; Dose 3 - 2.0mg/kg) for PND31-40, 41-50 and 51-60 male and female rats. \* significantly different ( $p < .05$ ) from saline for that particular drug; a,b difference between groups with superscript in common significant ( $p < .05$ ) for that particular drug.

As illustrated in Figure 42, rats treated with 20 mg/kg of BZP, during PND31-40 made significantly more entries of the closed arms than rats treated with 5 mg/kg, 10 mg/kg or saline. However, rats treated with 20 mg/kg of BZP during PND51-60 made significantly fewer entries of the closed arm than saline-treated rats. Similarly, rats treated with 1.0 mg/kg of MA during PND51-60 made significantly fewer entries of the closed arms than saline-treated rats. Rats treated with 0.5 mg/kg of MA during PND51-60 made significantly more entries of the closed arms than rats treated with 1.0 mg/kg and 2.0 mg/kg of MA.

Consistent with other testing periods and across behavioural measures, there was significant sex effect for all conditions, with female rats making significantly more entries of the closed arms than male rats.

### **Summary of EPM results at PND200**

Rats treated with the highest dose of BZP during PND31-40 displayed increased emotionality in the EPM as evidenced by decreased percentage of entries into and time spent on the open arms. However, this could be explained by a higher frequency of closed arms entries, suggesting that the 20 mg/kg BZP may have increased locomotor activity rather than emotionality, but only for females. Rats treated with MA during PND31-40 spent less time in the open arms, with female rats making significantly more entries and spending more time in them than male treated rats. Therefore, it is possible that, although male rats chose to enter the open arms, they did not prefer to spend time in them. For the rats treated during PND41-50 highest dose of BZP- and MA-treated rats (0.5 and 2.0 mg/kg) had reduced percentages of time spent in the open arms, which again could be attributed to male treated rats primarily, as female treated BZP and MA treated rats had a significantly higher percentage of entries and time spent in the open arms than male treated rats.

Similar to other tests and behavioural testing times, the PND50-61 MA-treated rats displayed a complex behavioural profile. The rats treated with 1.0 mg/kg of MA during PND50-61 had a significantly higher percentage of entries of the open arms than control rats. However the drug x sex interaction suggests that this may be accountable for the female 1.0mg/kg treated rats who made significantly more entries than the other two MA doses and saline-treated rats. This was not due to general locomotor activity as observed in the decreased total entries of the closed arms for the 1.0 mg/kg treated rats. This suggests that female rats treated with 1.0 mg/kg MA during PND51-60 had decreased anxiety-like behaviour compared to males, females treated with 0.5 and 2.0 mg/kg of MA and saline matched controls. Additionally, the

male rats treated with 2 mg/kg of MA during PND51-60 spent a significantly lower percentage of time in the open arms than the other two lower MA dose and male controls and made fewer entries of the open arms than the other two doses, suggesting increased anxiety due to earlier MA treatment.

**Table 4:** Summary of the significant effects of BZP or MA treatment for each measure at three periods, on responses emitted by male and female rats in the Y-maze, Light/dark emergence, EPM and Social Interaction.

	PND31-40	PND41-50	PND51-60
<b>30 day wash-out testing</b>			
Y-maze percent novel arm entries	<i>No significant effects</i>	<i>No significant effects</i>	MA ♀ increased
Y-maze total arm entries	BZP dose 3 ↓, all treated ♀ ↑	BZP all doses ↓, ♀ only. MA ♀ ↑	BZP and MA all doses ↓ and all treated ♀ ↑
Y-maze percent novel arm time	MA dose 3 ↓	BZP all doses ↓	MA ♀ ↑
Light/dark emergence	MA dose 2&3 longer emergence BZP ♀ shorter emergence	BZP dose 2 & 3 longer emergence BZP & MA ♀ shorter emergence	BZP & MA ♀ shorter emergence
EPM percent open arm entries	BZP dose 3 ↓, BZP ♀ ↑	BZP dose 3 ↓	BZP dose 1 ↓, BZP & MA dose 1 & 2 ♀ ↑
EPM percent open arm time	BZP dose 3 ↓	BZP dose 1 & 3 ↓, BZP & MA ♀ ↑	MA dose 1 ↑, BZP & MA dose 1 & 2 ♀ ↑
EPM closed arm entries	<i>No significant effects</i>	BZP & MA ♀ ↑	BZP & MA ♀ ↑
SI squares entered	BZP dose 3 ↑, BZP & MA ♀ ↑	BZP & MA ♀ ↑	BZP dose 3 ↑ & MA dose 3 ↓. BZP & MA ♀ ↑
SI outer rears	BZP dose 3 ↑	<i>No significant effects</i>	BZP dose 3 ↑ and MA dose 3 ↓.
SI centre rears	<i>No significant effects</i>	MA dose 3 approaching significance (P<.0503)	<i>No significant effects</i>
SI interaction time	<i>No significant effects</i>	<i>No significant effects</i>	BZP & MA ♂ ↑
<b>Testing at PND120</b>			
Y-maze percent novel arm entries	<i>No significant effects</i>	All 3 BZP & MA doses ↑	<i>No significant effects</i>

Y-maze total arm entries	MA dose 3 ↓, BZP & MA ♀ ↑	All 3 BZP & MA doses ↓, BZP ♀ ↑. As MA dose ↑ ♀ ↓	BZP dose 2 and all 3 MA ↓. BZP & MA ♀ ↑
Y-maze percent novel arm time	BZP dose 2 & 3 ↓	All 3 MA ↑	<i>No significant effects</i>
Light/dark emergence	BZP dose 3 & MA dose 2 & 3 longer emergence, BZP & MA ♀ shorter emergence	All 3 BZP & MA longer emergence, BZP & MA ♀ shorter emergence	BZP dose 1 longer emergence, MA ♀ shorter emergence
EPM percent open arm entries	All 3 BZP & MA dose 1 & 3 ↓	<i>No significant effects</i>	BZP dose 2 & 3 & MA dose 3 ↓, MA ♀ ↑, however only MA dose 3 ♂ & ♀ significantly different from other MA doses
EPM percent open arm time	All 3 BZP ↓	MA ♀ i↑, however only MA dose 2 ♀ ↓ compared to ♂ or other MA ♀	BZP & MA dose 3 ↓, BZP & MA ♀ ↑, however only MA dose 3 ♂ impacted
EPM closed arm entries	BZP & MA ♀ ↑	MA dose 1 & 3 ↑, BZP & MA ♀ ↑	All 3 MA ↑, BZP & MA ♀ ↑
SI squares entered	BZP dose 3 & MA dose 1 & 3 ↑, BZP & MA ♀ ↑	BZP & MA ♀ ↑	BZP dose 2 & 3 ↑, BZP & MA ♀ ↑
SI outer rears	BZP & MA dose 1 & 3 ↑, BZP & MA ♀ ↑	BZP & MA ♀ ↑	BZP dose 2 & 3 ↑, BZP & MA ♀ ↑
SI centre rears	BZP & MA dose 3 ↑	BZP ♀ ↑	BZP dose 3 ↑
SI interaction time	BZP & MA ♂ ↑	<i>No significant effects</i>	<i>No significant effects</i>

### ***Testing at PND200***

Y-maze percent novel arm entries	BZP dose 1 & 2 ↓	<i>No significant effects</i>	MA ♀ ↑
Y-maze total arm entries	BZP & MA ♀ ↑	BZP dose 1 and all 3 MA doses ↓, BZP & MA ♀ ↑	MA dose 2 & 3 ↓, however only for ♀, BZP ♀ ↑
Y-maze percent novel arm time	BZP dose 1 & 2 ↓	<i>No significant effects</i>	BZP ♀ ↑
Light/dark emergence	BZP dose 2 & 3 & all 3 MA doses longer emergence, MA ♀ shorter emergence & as BZP dose increased ♀ had longer emergence	All BZP & MA longer emergence, BZP & MA ♀ shorter emergence	BZP dose 1 & 3 & all 3 MA longer emergence, BZP & MA ♀ shorter emergence

EPM percent open arm entries	BZP dose 3 ↓, MA ♀ ↑	BZP & MA ♀ ↑	MA dose 2 ↑, accountable for ♀ only, BZP ♀ ↑
EPM percent open arm time	BZP dose 3 & all 3 MA ↓, BZP & MA ♀ ↑	BZP dose 3 & MA dose 1 & 3 ↓, BZP & MA ♀ ↑	MA dose 3 ♂ ↓
EPM closed arm entries	BZP dose 3 ↑, BZP & MA ♀ ↑	BZP & MA ♀ ↑	BZP dose 3 & MA dose 2 ↓, BZP & MA ♀ ↑

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BZP dose 1 = 5 mg/kg; BZP dose 2 = 10 mg/kg; BZP dose 3 = 20 mg/kg, MA dose 1 = .05 mg/kg; MA dose 2 = 1.0 mg/kg MA dose 3 = 2.0 mg/kg ♂ = Male rats; ♀ = Female rats ↑ = increased ↓ = decreased

## Chapter 4: Discussion of Results

In the present study, at three ages (PND 31-40, PND 41-50 and PND 51-60), adolescent rats were exposed to either saline, BZP (5 mg/kg, 10 mg/kg or 20 mg/kg) or MA (0.5 mg/kg, 1 mg/kg or 2 mg/kg). All groups received one daily i.p injection for ten consecutive days. The rats were subsequently tested on measures of emotionality after a thirty day washout period, and then at PND120 and PND200, to assess any long-term effects.

Overall, it appears that early exposure to either BZP or MA affected later behaviour and this persisted over time. However, although the general pattern of results is suggestive of increased emotionality the results are complicated by no clear dose response for either drug and compounded by sex differences. See Table 4 for a summary of significant effects across the three periods of adolescence and three periods of behavioural testing.

### *Summary of Results over the three testing phases*

#### ***1: PND31-40***

In the Y-maze, there were no significant effects of BZP on percentage of novel arm entries for the 30 day wash-out period or at PND120. Although, there was no significant results for percentage of novel arm entries at PND120, BZP- treated rats (10 & 20 mg/kg) once in the novel arm preferred to spend less time in it, compared to control rats. Rats treated with BZP (5 & 10 mg/kg) made significantly fewer novel arm entries at PND200 and spent less time in the novel arm than control rats. MA- treated rats (2.0 mg/kg) spent a significantly lower percentage of time in the novel arms at the 30 day wash-out period and made less total arm entries at PND120.

In the Light/dark emergence test, the general pattern of results were consistent over time, with rats treated with MA (1.0 & 2.0 mg/kg) having longer latencies to emerge from the dark compartment across all three testing periods. BZP- treated rats had longer latencies to emerge and by PND200 it was evident that as the dose of BZP increased treated rats decreased in their emergence from the dark compartment. All female treated rats emerged faster than comparable male treated rats.

On the EPM, BZP- (20 mg/kg) treated rats had decreased percentage of open arm entries and decreased percentage of time spent in the open arms, across the three testing periods, compared to control rats. However, at PND120 this could be explained by increased closed arm entries.

Additionally at PND120 rats treated with all three doses of BZP made fewer entries and spent a lower percentage of time in the open arms, similar to the and MA- treated rats (0.5 & 2.0 mg/kg), who also made fewer entries of the open arms, but once on the open arms did not differ from control rats. However, at PND200 all MA- treated rats spent a significantly lower percentage of time in the open arms, compared to control rats. Additionally, the BZP- treated rats (20 mg/kg) spent a significantly lower percentage of time in the open arms, compared to control rats at PND200.

On the SI test, rats treated with BZP (20 mg/kg) entered more squares of the OF, made more rears in the outer squares at the 30 day wash-out period and PND120 and significantly more centre rears at PND120. Likewise, rats treated with MA (0.5 & 2.0 mg/kg) entered more squares, made more outer rears and the highest dose of MA- treated rats made more centre rears at PND120. All treated female rats made significantly more outer rears at PND120, than male treated rats, however both drugs increased interaction time for male rats only at PND120. Overall, the general pattern of results suggests that rats treated with both drugs during PND31-40 displayed more anxiety-related behaviour compared to control rats and this effect increased with drug dose, primarily for male- treated rats.

For rats treated during PND31-40, female rats displayed greater locomotor activity, evidenced in the Y-maze total arm entries, EPM closed arm entries (with the exception of testing at the 30 day wash-out period), and squares entered in the OF of the SI, compared to treated male rats, across the three testing periods.

## **2: PND41-50**

In the Y-maze, there were no significant effects of BZP at the 30 day wash-out period and PND200 on percentage of novel arm entries, however both BZP- and MA- treated rats made significantly more novel arm entries at PND120, than control rats. All BZP- treated rats spent a lower percentage of time in the novel arms at the 30 day wash-out period, and all three doses of MA increased the percentage of novel arm time at PND120.

In the Light/dark emergence test, the general pattern of results was consistent over time, with all female rats having a shorter latency to emerge from the dark compartment, compared to male rats. At the 30 day wash-out period only the BZP - treated (10 & 20 mg/kg) rats had longer emergence than control rats. However, all BZP- and MA- treated rats had longer emergence times at PND120 and PND200, than saline-treated animals.

On the EPM, similar to treated rats during PND31-40, BZP- (20 mg/kg) treated rats showed a decreased percentage of open arm entries and decreased percentage of time spent in the open arms, at the 30 day wash-out period, compared to control rats. However, this could be explained by increased closed arm entries for BZP female treated rats. Additionally at PND200 BZP- (20 mg/kg) and MA- treated (0.5 & 2.0 mg/kg) also had a decreased percentage of time spent in the open arms, compared to control rats. BZP and MA treated female rats spent a longer percent of time in the open arms compared to male rats at the 30 day wash-out period. Interestingly, at PND120, although there were significant sex effects for all MA- treated females who spent a longer percentage of time in the open arms, compared to male treated rats. The drug x sex interaction suggests that only female rats treated with MA (1.0mg/kg) spent a significantly decreased percentage of time in the open arms compared to the male treated and other MA female treated rats. At PND200 all female treated rats made significantly more open arm entries and spent a longer percentage of time in the open arms than the male treated rats, however as already mentioned this may be accountable for female rats being generally more active than male treated rats as evidenced by the measures of locomotor activity.

On the SI test, there were no significant drug effects for any of the four measures at the 30 day wash-out period and PND120 for BZP- and MA- treated rats. However, both drugs administered to both BZP- and MA- treated female rats resulted in increased outer rears at PND120 and only the BZP treated female rats displayed significantly more centre rears compared to male treated rats at PND120. Overall, the general pattern of results over time, suggests that rats treated with both drugs during PND41-50 displayed increased anxiety-like behaviour compared to control rats and these effects again, were primarily for male-treated rats.

For rats treated during PND41-50, female rats displayed greater locomotor activity, evidenced in the Y-maze total arm entries (MA- treated females only at 30 day wash-out period and as the dose of MA increased at PND 120 female treated rats decreased their total arm entries), EPM closed arm entries, and squares entered in the OF of the SI, compared to treated male rats, across all testing periods.

### **3: PND51-60**

In the Y-maze, there were no significant drug effects for either BZP- or MA- treated rats on percentage of novel arm entries or percentage of time spent in the novel arms across the three different behavioural testing periods. However, of interest is the behaviour displayed by the

BZP- (10 mg/kg) and MA (1.0 mg/kg) treated rats, who preferred to enter the novel arm at PND120 over the familiar arm and this was significantly difference from chance. Similarly, at PND200, rats treated with BZP (5 & 10 mg/kg) and all three doses of MA preferred to enter the novel arm over the familiar arm, however only the MA- treated rats (1.0 mg/kg at PND120 and all 3 doses at PND200) preferred to spend time in it. Although this would suggest they displayed decreased anxiety-like behaviour, it is more likely that once in the novel arm they froze as evidenced by decreased closed arm entries by the all the rats mentioned. This implies that they had no deficits in spatial memory as they remembered from the acquisition trail which arm had changed in brightness and were actively looking for a means of escape and once they realised there was no way out of the apparatus froze, suggestive of increased anxiety-like behaviour.

In the Light/dark emergence test, the general pattern of results were consistent over time, with all treated female rats having a shorter latency to emerge from the dark compartment, compared to male rats, with the exception of BZP-treated rats at PND120, however this was approaching significance ( $P < .0781$ ). BZP-treated rats (5 mg/kg) displayed a longer latency to emerge at PND120 and PND200, and rats treated with BZP (20 mg/kg) or all three MA doses had a longer latency to emerge from dark compartment at PND200, compared to control rats.

On the EPM, BZP- treated rats (5 mg/kg) displayed a decreased percentage of open arm entries at the 30 day wash-out period, compared to control rats, with all BZP-treated females displaying a higher percentage of open arm entries than males. The drug x sex interactions illustrated that MA treated-females (0.5 & 1.0 mg/kg) made a higher percentage of entries and spent a longer percentage of time in the open arms of the EPM compared to male treated rats. At PND120, rats treated with BZP (10 & 20 mg/kg) or MA (2.0 mg/kg) made significantly fewer entries of the open arms and the sex x drug interaction suggests the both the male and female 2.0 mg/kg MA- treated rats made significantly fewer entries of the open arms, compared to controls or the other MA- treated male and female rats. However, the sex x drug interaction on percentage of time spent in the open arms illustrates that only the 2.0 mg/kg male MA- treated rats had a significantly less percentage of time spent on the open arms. These results suggest that the highest dose of MA treated male rats upon entering the open arms would flee significantly faster from the aversive environment back to the safety of the closed arms, compared with 0.5 and 1.0 mg/kg male and female MA-treated rats. This reflects that the male rats treated with the highest dose of MA during PND51-60 displayed more anxiety-like behaviour on this measure at PND120. At PND200, rats treated with MA (1.0

mg/kg) made significantly more entries of the open arms than control rats. However, the sex x drug interaction illustrates that this interaction effect was due to the MA- (1.0 mg/kg) treated female rats only. Although there were no significant dose effects for treated rats on percentage of open arm time, a sex x drug interaction illustrated that male rats treated with MA (2.0mg/kg) spent significantly less percentage of time in the open arms compared to the two smaller doses of MA- treated rats and female MA- treated rats. Both BZP- and MA- treated female rats made significantly more entries of the open arms than male treated rats.

In the SI test, the highest BZP dose (20mg/kg) treated rats entered significantly more squares in the OF and made more outer rears, compared to saline treated rats, conversely, the highest dose of MA (2.0 mg/kg) treated rats entered significantly fewer squares in the OF and made significantly fewer outer rears than saline controls, at the 30 day wash-out period.

Interestingly, at this first behavioural testing phase, male BZP- and MA- treated rats spent a significantly longer time in social interaction than similar treated female rats. Social interaction is proposed to measure decreased anxiety (File, 2003), however this does not appear to be consistent with the other behavioural measures and results displayed by male rats in the SI test. It is possible that the male-treated rats in this study found the novelty of the OF more aversive than interaction with another unfamiliar male rat. At PND120, BZP- treated rats (10 & 20 mg/kg) entered more squares in the OF, and made significantly more outer rears than saline-treated rats. The highest dose of BZP- (20 mg/kg) treated rats made significantly more centre rears than saline controls and BZP-treated rats (10 & 20 mg/kg) made more outer rears at PND120 than control rats. Overall, the general pattern of results is more complex than the other two adolescence periods, with MA- treated female rats displaying increased anxiety-like behaviours as the MA dose increases. And the highest doses of BZP administered to rats during PND51-60 displaying decreased emotionality, as evidenced by increased rearing behaviours, however this may be accounted for by an augmented need to scan the environment for an escape from the aversive novel environment.

The sex effects for rats treated during PND51-60, are not as clear as observed at the other adolescent dosing periods, possibly due to a greater number of sex x dose interaction effects for the PND51-60 MA-treated rats. Both BZP- and MA- treated female rats displayed greater locomotor activity, as evidenced in the Y-maze total arm entries, EPM closed arm entries, and squares entered in the OF of the SI, compared to treated male rats, across all testing periods. There were, however significant differences for MA-treated female rats, with female rats administered MA (1.0 & 2.0 mg/kg) making fewer entries of both arms of the Y-maze

compared to male MA-treated rats and female rats treated with 0.5 mg/kg of MA at PND120. Additionally, female rats treated with 20 mg/kg of BZP or 1.0 mg/kg of MA made significantly fewer closed arm entries on the EPM compared to male treated rats at PND200.

## Chapter 5: General Discussion

The results indicate that there were systematic changes in measures of emotionality in the predicted direction, in almost all of the behavioural tests used. That is, as adults, the rats that had been exposed to both BZP and MA as adolescents displayed more anxiety-like behaviour than control rats that persisted over time. This was most evident in the light/dark emergence test. However, the results for the other three measures (Y-maze, EPM and Social Interaction test) were generally more complicated, and depended to a greater extent on the drugs, the sex of the rats, the responses recorded and the age of testing.

### *Similarities between BZP and Methamphetamine and differences between drug administration periods*

Overall, the results of the current research suggest that there are more similarities between BZP and MA adolescent treatment, on anxiety-like behaviours in adulthood, than differences. BZP was introduced into New Zealand as a safe and legal alternative for the escalating MA problem that was occurring in the early 2000's (Bowden, 2004). However, there was no research to support this assumption. In the 1970's Bye et al. (1973) and Campbell et al. (1973) reported that BZP had approximately 10 percent of the potency of dexamphetamine. Therefore, the doses used in this study were based on the assumption that the same potency difference would also apply to MA and BZP.

The general pattern of results however, is suggestive of more detrimental effects of earlier BZP treatment than MA treatment and this was observed over the three testing phases.

Notwithstanding, MA- treated rats additionally differed significantly from controls, in the same direction. However, whereas dose-related increases were observed for the BZP-treated rats, the behavioural profiles for MA-treated rats differed, with conflicting results for the three doses, at the different adolescent treatment phases, on the different measures and over time. For example, for the rats treated during PND51-60, 0.5 mg/kg MA- treated rats at the 30 day wash-out period spent more time on the open arms of the EPM, whereas the 1.0 mg/kg MA- treated rats spent more time on the open arms at PND200. Similarly, all three MA treated rats during PND41-50 spent more time in the novel arm of the Y-maze at PND120, whereas there were no MA effects following treatment at PND31-40 or PND51-60 on this measure. On measures of general locomotor activity, MA- treated rats treated during PND31-40 with 2.0 mg/kg of MA made fewer entries of both Y-maze arms, but more entries of squares in the SI

test at PND120. It seems that the lack of consistency in this study for MA- treated rats may have been due to male rats being more affected by their earlier drug exposure.

Most sex x drug interactions occurred for rats treated during PND51-60 with MA. As mentioned above this treatment period was more sensitive for male rats and this was maybe due to the earlier maturation of the female brain compared with males. Therefore, it is possible that the female brain had matured enough that the vulnerability to long-lasting neuronal changes was less. This may have been due to neurotransmitter systems approaching adult levels for female rats, whereas for males they were still very immature and thus more vulnerable to effects of MA. Nevertheless, female rats administered MA during PND51-60 still displayed some increased anxiety compared with control rats.

### ***Neurodevelopment***

Although, this research study was purely a behavioural teratological study without complimentary neurochemical assessments, the possibility of alterations to the neurodevelopment during adolescence will be briefly discussed. During adolescence there are dramatic changes in the 5-HT neurotransmitter system, with the density of the 5-HT transporter (SERT) rising, and 5-HT levels in the CNS increasing between the juvenile period and puberty, followed by a decline in young-adulthood. Consequently, this developmental period may be especially sensitive to effects of exposure to a variety of drugs (Piper et al., 2005). Exposure to environmental variables that affect 5-HT during development might have caused 'miswiring' in the CNS (De Jong et al., 2006) that may have led to the increased anxiety-like behaviour seen in BZP- and MA- treated rats.

One of the premises of the current research was that adolescent stimulant use would interfere with the 5-HT system, producing behavioural change displayed as anxiety in later age. It was proposed that the 5-HT projections in the PFC would most likely be affected, as the PFC is the last brain region to mature (Olmstead, 2006). Whether the different developmental stages of adolescence would produce differences in anxiety-like behaviour due to synaptic plasticity is not possible to ascertain from the present results. All three adolescent developmental periods resulted in a change in behaviour in adulthood compared to control animals. Olmstead (2006) administered cocaine to adolescent and adult rats and found on a PFC-mediated task (simple discrimination set-shifting task) that synaptic plasticity within the PFC was altered by cocaine only in adult animals. They concluded that adolescence was a period where PFC mechanisms are protected against drug insult and that this protection may enhance the exploration of novel

environments, risk-taking and sensation seeking (typical of adolescent behaviours), which conversely may increase the likelihood of experimentation with substances. Whether or not the drug treated animals in this study had alterations to the PFC is unknown and will require further research.

DA involvement in drug addiction is more likely mediated by the structural and functional changes in the circuits that are modulated by DA, including the PFC (Goldstein & Volkow, 2002). Abnormalities in DA neurotransmission are also important factors contributing to wide range of psychiatric illnesses, including affective disorders (Rodríguez, Chu, Caron & Wetsel, 2004). DA also plays a pivotal role in psychostimulant reinforcement but contributions from the NE and the 5-HT systems are starting to be recognised (Pierce & Kumaresan, 2006). Brown and Molliver's (2000) found that mice that lack the DA transporter would self-administer cocaine, thereby challenging the DA hypothesis of addiction. They therefore concluded that 5-HT neurons may also contribute to the rewarding effects of stimulant drugs.

However, Fone et al. (2002) and McGregor et al. (2003) observed persistent behavioural changes after MDMA administration in the absence of any detectable 5-HT depletion. 5-HT is of interest due to its effect on anxiety-like behaviours (which this study investigated).

Although there is ample evidence for the effect of earlier administration of stimulant drugs on later DA functioning and effects of MDMA on the 5-HT system, there is limited information about the role of 5-HT after BZP treatment. Reports of the mechanisms of action involved are conflicting. For example, Baumann et al. (2005) found that BZP administration caused a rise in 5-HT dialysate levels in the nucleus accumbens of rats. However, Simmler et al. (2014) argued that BZP is an indirect DA and NE agonist without 5-HT properties. Research is suggesting that 5-HT also plays a role in behavioural change after MA treatment (Cappon et al., 1997; Kokoshka et al., 2000; & Zombeck et al., 2010). However, there is only one published study to date looking at the long-term effects of MA administration during the adolescent phase on later affective behaviours.

This one study involved assessments of long-term effects of adolescent MA treatment on later depressive behaviour in mice (Joca et al., 2014). The subjects received 2 days of MA treatment (4 x 7.5 mg/kg in 2-hour intervals) during PND30-31 and then randomly assigned to one of two later testing groups, defined as late adolescent (PND41-52) or adult (PND62-73). MA treatment increased depression-like behaviour in the forced swim test in both late adolescent and adult treated male and female mice. The authors suggested that their results

were due to something more than MA's effect on the DA transporters, possibly a dysregulation of the HPA axis affected primarily the male treated mice.

The HPA axis is the central mechanism in the brain's neuroendocrine response to stress. The hypothalamus, when stimulated, secretes the corticotropin-releasing hormone (CRH), which stimulates the pituitary gland to secrete the adrenocorticotrophic hormone (ACTH) into the bloodstream. In order to prepare the body to meet a challenging situation, ACTH then causes the adrenal gland to release cortisol. This system works on a feedback loop and it is reported that damage or disruption to this feedback loop can affect normal responses to stress and the ability to regulate emotions, such as anxiety or low mood (see Panagiotakopoulos & Neigh, 2014 for a comprehensive review).

McCormick (2010) investigated social instability stress in adolescence and the risk of later consumption of DOA. Rats were confined to a small ventilated container for one hour a day, then paired with a new peer in a new cage during PND30 - PND45, to produce social instability. Rats were then tested both for the immediate social interaction effects and HPA functioning effects (PND46-52) and again at PND70-76 to investigate any lasting effects. Additionally, rats were tested at both periods on locomotor activation to nicotine and AMP. Their results suggested that both male and female rats displayed increased anxiety-like behaviour and increased locomotor sensitisation to psychostimulants. However the behavioural effects were greater for male rats whereas behavioural responses to DOAs were greater for females. These effects continued into adulthood. Overall, male rats appeared more affected from the earlier administration of both BZP and MA in the current study. This may have been due to disruption of the HPA axis which is implicated in anxiety disorders.

### ***Anxiety and behavioural measures***

This research utilised a battery of behavioural measures of animal anxiety, which tapped into approach/avoid conflicts in rats. However, Ramos (2008) argues that emotionality in rats is multidimensional and different tests may be measuring different aspects of anxiety. The current study results were consistent for the light-dark box, with all treated rats predominately displaying increased anxiety-like behaviour that was maintained over time. However, the results of anxiety-like behaviours' displayed in the Y-maze, EPM and SI test were not as straightforward.

The Y-maze involves rats choosing to enter a novel environment (the changed arm) rather than a familiar one. This response has been associated with increased DA levels within the nucleus

accumbens (Adriani, Chiarotti & Laviola, 1998). Novelty is believed to be rewarding for rats. However, an alternative interpretation is that rats may be avoiding the familiar arm as it was associated with stress-related aversion (inability to escape) due to the 6 minute acquisition trails prior to testing. For the treated rats that spent a more time in and made more entries into the changed arm (all three BZP and MA doses and all three MA doses respectively, treated during PND41-50 and tested at PND120) it is possible that they displayed decreased fear of novelty or were reacting to their prior confinement. It seems more likely that for these treated rats the latter explanation is most likely as evidenced by their behaviour on the other measures.

In the EPM, it is known that rats prefer the secure closed arms of the maze to the more threatening open arms (Pellow et al., 1985; Flint, 2003) and this preference is greater in more anxious animals. Albrecht-Souza, Borelli and Brandao (2008) reported that performance of rats in the EPM differs between their first trial in the maze and subsequent trials. They suggested that on the first trial, the response is typically based on an approach-avoidance conflict. On subsequent trials, there is a qualitative shift in the emotional state of the rat with its behaviour being motivated more by the learned fear acquired from the initial trial. Therefore, with repeated trials the rat actively avoids the open arms and no longer seeks an escape route. However, the current study suggests that the rats treated with all three doses of BZP during PND31-40 showed significantly decreased open arm entries and time spent on the open arms over all three testing phases, which is inconsistent with Albrecht-Souza et al (2008) hypothesis. However, rats treated during PND41-50 with BZP displayed decreased open arm exploration at the 30 day wash-out period and no significant effects at PND120, which suggests for these rats that they may have been motivated more by the learned fear at the second testing period. Conversely, rats treated with BZP (5 mg/kg) during PND51-60 displayed decreased open arm entries whereas the 0.5 mg/kg dose of MA increased time spent in the open arms at the 30 day wash-out period. At PND120 it was only the higher doses of both drugs that produced decreased open arm exploration with no significant drug effects at PD200.

In the SI test, not only was the apparatus novel but the pairing of one rat with an unfamiliar peer would have added to the novelty. With the exception of increased social interaction shown by male rats treated with both BZP and MA during PND51-60 and tested at the 30 day wash-out period, and male rats treated with both drugs during PND31-40 and tested at PND120, the general pattern of results suggested increased anxiety-related behaviour that was more pronounced at the second testing period. Novel environments are rewarding (Cain, Smith & Bardo, 2004) so that rats prefer novel stimuli over familiar (Hughes 1992). While novelty

preference can be explained as avoidance of familiar stimuli, it possible that the increased social interaction with an unfamiliar peer shown for male rats provided an alternative reinforce to exploration of the novel environment. As male rats have been shown to have higher levels of harm avoidance compared to females rats (Ray & Hansen, 2005) it is possible that exploration of the novel environment could have been more aversive than interaction with another rat.

### *Sex Differences*

Most research continues to examine the effects of drugs in male rats exclusively (Hughes, 2007; Zucker & Beery, 2010). Beery and Zucker (2011) investigated sex bias in studies from ten biological disciplines for 2009 and reported that male bias was evident in eight of these. They also reported that women are diagnosed with anxiety disorder 2.25 times more often than men, but the majority of animal studies of anxiety and anxiolytic drugs use male rats exclusively. However, both female and males individuals use stimulant drugs, for varying reasons and any study of long-term behavioural change due to earlier stimulant use needs to address sex differences.

Females are twice more likely to be diagnosed with depression than men, and have more symptoms, higher symptom severity and more self-reported subjective distress (Seney & Sibille, 2014). Anxiety symptoms are almost always co-morbid with depression for women, whereas men are more likely to have co-morbid substance abuse. This suggests different coping strategies for females compared to males. Most literature suggests that males start experimenting with substances for the positive reinforcement that receive from them whereas; females are more likely to take drugs to reduce stress or psychological distress (Becker, Perry & Westenbroak, 2012).

In 2008, substance dependence or abuse among youth aged 12 to 17 was similar for males (8.0%) to that for females (8.1%) (Johnston, O'Malley, Bachman, & Schulenberg, 2008). Rates are also similar for stimulant use for those younger than 25. Generally, women report using stimulant substances at an earlier age (Becker et al., 2012), progress through the stages of addiction more quickly and have shorter periods of abstinence before relapse than males (Cummings, Gowl, Westenbroek, Clinton, Akil & Becker, 2011). Female rodents generally exhibit a higher motivation for cocaine than male rodents. However it is only with low doses that a significant difference between the sexes emerges (Cummings et al., 2011).

Female rats treated with both BZP and MA during the three different adolescent exposure periods displayed fewer anxiety-related responses and higher locomotion than males, and this sex difference endured over time. This was possibly due to the different brain maturation rates of male and female adolescent rats (Andersen & Teicher, 2000). During adolescence there are dynamic changes in the brains of both male and females, with the magnitude of alterations being greater for males. In the striatum, DA D1 and D2 receptors are overproduced during the juvenile and adolescent developmental period in the male rat's brain and subsequently eliminated during early adulthood. In the female rat's brain, D1 and D2 receptor numbers gradually increase to adult levels during adolescence, with no subsequent elimination (Andersen et al., 1997; Koenig et al., 2005; McFadden, Yamamoto and Matuszewich, 2011). Therefore, it is likely that since the male brain undergoes more dramatic developmental changes in the DA system, males may be more vulnerable to the long-term effects of stimulant drugs during the adolescent stage compared to females.

In humans, sensation-seeking, novelty-seeking, reactivity to novelty and impulsivity increase the likelihood of substance abuse (Cummings et al., 2011). Novelty has positive incentive capacity and exposure to novel environments increases DA release in the NAc (Rebec, Christensen, Guerra, & Bardo, 1997). Accordingly, it is conceivable that the hypoactivity displayed by the female treated rats was due to decreased sensitivity to the rewarding aspects of exploring a novel environment. On the other hand, novelty also has some aversive-stressful components (Bardo, Donohew & Harrington, 1996). Nonetheless, it is well established that novelty-elicited behavioural activation is largely dependent on the mesolimbic DA system (Bardo et al., 1996) and that the level of behavioural responding is correlated with activity of the ventral tegmental area DA neurons (Marinelli and White, 2000). Therefore, regardless of whether the treated rats were responding to a rewarding or stressful stimulus, it is likely that the reduced activity by male treated rats, in all mazes is an indication of deficits in the mesolimbic DA system resulting from earlier BZP and MA treatment.

However, since female rats generally show more locomotor activity than males (Becker, 1999), there was an unexpected lack of sex differences among rats treated at PND31-40 with both BZP and MA. At the 30 day wash-out period, for all measures there were only two significant sex differences for female rats treated at PND31-40 that were not confounded by locomotor activity. These differences occurred for rats treated with BZP i.e., females emerged faster in the light/dark box and made significantly more open arm entries in the EPM than males. The lack of sex differences may have been due in part to the younger age of the PND31-40 treated rats

at the 30 day wash-out period (PND70), as the literature suggests that rats become less anxious and more exploratory up until approximately PND90 (Ray & Hansen, 2005). There are relatively few sex differences reported in young rats. Alternatively, the lack of sex differences may be explained by immaturity of glutamatergic and DA systems which are still developing until after PND40 (Pu, Broening & Vorhees, 1996) and may be thus less likely to be affected by early drug treatment. The general pattern of results at the 30 day wash-out period suggests that as the age of adolescence treatment increases male rats are more affected, as suggested by male rats displaying more anxiety-related behaviour that is not influenced by locomotor activity.

At PND120, both BZP- and MA-treated females emerged faster and displayed more locomotor activity in the open field. This suggests that MA treatment during PND31-40 increases general activity in female rats. Additionally, all doses of BZP and MA affected outer rearing at PND120, yet only the highest doses of BZP and MA affected inner rearing, with female rats rearing significantly more than male rats. Significant drug x sex interactions occurred at PND200, with female rats increasing their latency to emerge from the light/dark box as the dose of BZP increased. However MA-treated female rats displayed decreased anxiety in the EPM as shown by increased entries into and time spent on the open arms. Therefore, it is hypothesised that the behavioural outcomes of MA administered to female rats during PND31-40, initially decreased their locomotor response, but over time also decreased their emotionality.

Conversely, female rats treated with MA during PND51-60 displayed decreased anxiety from the first testing period which was maintained over time. However, this appeared to be limited to the two lower doses of MA, with the female rats treated with 1.0 mg/kg displaying the least emotionality. For male rats treated with MA the general pattern of results suggests that as the dose of MA increases, male rats display more avoidance of the novel environments, suggestive of increased anxiety-like behaviour.

### ***Behaviours observed but not investigated***

Although not a focus of investigation, the serotonin behavioural syndrome (low body posture, splayed hind limbs and head weaving; Haberzettl, Bert, Fink & Fox, 2013) was observed in three female rats (PND51-60) administered the highest dose of BZP on the eighth day of dosing. Research suggests that this syndrome appears to result from the release of both 5-HT and DA and the blockade of the 5-HT transporter (Bull et al., 2003). This is important as Gee

et al. (2005) reported human equivalents of this behaviour (seizures, severe respiratory and metabolic acidosis) in his work at Christchurch Hospitals Emergency Department, in which he collected data from 61 patients admitted to hospital with BZP toxicity. Gee et al. (2005) stated that female patients had more severe effects than male patients. Similarly, Baumann et al. (2004) found administration of BZP and TFMPP (10 mg/kg) caused seizures in five out of seven rats that were treated with the combination of the two psychoactive substances. Although, there were no significant effects on weights for any of the treated rats compared to controls, the rats treated with BZP had constant diarrhoea, which was more evident in the higher doses and became more pronounced over time. After drug administration the MA-treated rats displayed some stereotypical behaviour, e.g. sniffing, rearing and grooming (Kitanka et al., 2010), in their home cage, which again was observed to be more evident in the higher doses of MA, however this behaviour decreased over the ten days. Another casual observation was that, by day three, a number of treated female rats were agitated (as reflected in their increased locomotor activity in home cage and hanging from the top of their home cages) when the researcher came to administer either BZP or MA. On the other hand, over the course of 10 days, male rats displayed avoidant behaviours, such as remaining huddled in the corner of their home cages.

### ***Consistent and Inconsistent Findings***

As already mentioned, very few studies have been devoted to the particular issues explored in this study, and any studies to date examining the effect of adolescent psychostimulant exposure on later anxiety-like behaviours are scarce and largely ambiguous. One of the few efforts in this regard has been the work of the current researchers. We administered 10 mg/kg (BZP) over 10 consecutive days during PND45-55 to male and female rats. Seventeen days later, the subjects were evaluated for anxiety-like responses emitted in the Y-maze, light/dark box and a social interaction test. We reported evidence for the development of anxiety-like responses following BZP treatment in all three behavioural tests. Although the results of our 2006 study were generally consistent with the BZP results of the present investigation, they administered BZP during a later age period (PND45-55), which in the current study encompassed two different developmental periods (PND41-50 and PND51-60). In addition, the present study utilised the experimental procedure of Vorhees et al. (2005) on the assumption that, as per these authors, PND41-50 would be a critical period of drug administration for later behaviour change. In the present study BZP or MA administration during each of the three different stages of adolescent development affected later anxiety-like behaviour (albeit to different

degrees). This contrasts with Vorhees et al. (2005) who concluded that MA-treated rats during PND41-50 showed impaired cognitive functioning at a 30 day wash-out period unlike those treated at PND21-30, PND31-40 or PND 51-60. However, as the study of Vorhees et al. (2005) was a dose-response experiment which assessed earlier MA toxicity on later cognitive functioning, it is possible that the mechanisms of action were different, or that their behavioural apparatus assessed different effects.

Findings of this study support other research suggesting earlier stimulant drug use affects later behaviour. Regardless of the differences in drug or apparatus used or the abstinent period, similar increased anxiety-like behaviour has been found with cocaine (Estelles et al., 2007; Santucci & Maderia, 2008; and Santucci & Rosario, 2010), MDMA (Bull et al., 2004; Cox et al., 2014; Gurtman et al., 2002; Fone et al., 2002; Kolyaduke & Hughes, 2013[males only]; McGregor et al., 2003 and Morley et al., 2001), MP (Bolanos et al., 2003; Crawford et al., 2013; Vendruscolo et al., 2008), MA (Joca et al., 2014) and BZP (Aitchison & Hughes).

Other research has provided conflicting results. For example, Labonte et al. (2012) reported decreased anxiety in AMP-treated adolescent rats as displayed in the EPM and Piper and Meyer (2004) showed decreased anxiety in the EPM after adolescent MDMA treatment. However, Modi, Yang, Swann and Dafnyl. (2006), report many animal studies correlating earlier MDMA use with lasting cognitive and behavioural deficits, including impairments in spatial memory and learning, increased anxiety-like behaviour and a weakened ability to react to stress as adults. Similarly, Estelles et al. (2007) found the same results for adolescent cocaine-treated mice. Untangling these conflicting results is complicated and as no neurochemical analysis was used in the present study, inferences about causation must be tentative and drawn from previous work in which both behavioural and neurochemical data were reported.

One area of research that can be tentatively related to the current study, is the impact of adolescent stress on later adult behaviours. Drug use could be considered a stressor, as adolescence is a critical developmental period where life experiences can shape brain development either positively or negatively. For example, Adriani, Deroche-Gamonet, Le Moal, Laviola and Piazza (2006) found environmental enrichment can reverse the detrimental effects of early life stressors. However, most research suggests that early life stressors lead to increased anxiety in adulthood (McCormick, Mathews, Thomas & Walters, 2010). For

example, Avital and Richter-Levin (2005) exposed male Wistar rats (PND26-28) to an elevated platform and then measured their locomotor activity at PND90 and found decreased activity, which they accounted for as enhanced anxiety at adulthood, evidenced by decreased open field activity and an increased startle response. Schmidt et al. (2007) exposed PND28-84 CD1 male mice to group housing (four mice) changed twice weekly and found decreased OF activity at PND80. A similar result was reported by Sterlemann et al. (2008) with PND28-77 CD1 male mice, investigated in the OF at PND45, suggesting that effects of induced anxiety by early life stress can be maintained over time.

Results utilising rats' supports the above finding with mice. Wright, Herbert and Perrot-sinal (2008) exposed male and female Long Evans rats to predator odour on PND40, 41, 44, 47 and 48 and found decreased OF activity at PND60 and 61. However, Toth, Avital, Leshem, Richter-Levin and Braun (2008) found rats exposed to 23 hour food restriction, an acute swim test (forced to swim for 10 minutes) and an elevated platform (PND 30 and 90) did not differ from controls at PND90 on the OF test. Additionally, Ulys et al. (2006) found increased rearing in Sprague Dawley male rats that had been stressed at PND28, 35 and 60, when tested in the OF at PND68, and Toth et al. (2008) found decreased time in social interaction in a SI test, but no effect on number of encounters. .

There are some similarities to this previous research in effects of earlier drug administration on behaviour in the OF. In the current research, rats treated with BZP (20 mg/kg) at PND31-40 displayed more rearing behaviour at the 30 day wash-out period and PND120. Likewise both BZP and MA treated PND41-50 rats displayed more rearing behaviour at PND120, however only the PND51-60 BZP- treated rats demonstrated this effect at the first testing period and conversely, the MA- treated rats (2.0 mg/kg) made significantly fewer rears in the OF at the 30 day wash-out period. Female rats in all these conditions displayed significantly more rearing behaviour than similarly treated male rats. However, with the exception of Wright et al. (2008), the researchers mentioned above used male rats exclusively. In contrast, with the exception of rats treated during PND41-50, treated rats in the present study displayed increased OF activity compared to saline controls. Female rats again were more active than male rats in these conditions thereby supporting similar observations by Wright et al. (2008).

In the EPM, Schmidt et al. (2007) described decreased time spent on the open arms and Sterlemann et al (2008) reported decreased open arm entries by CD1 male mice. Ulys et al. (2007) found increased rearing in the EPM, in rats, supporting a similar result they obtained in

the OF. Pohl, Olmstead, Wynne-Edwards, Harkness and Menard (2007) exposed Long Evans male and female rats to either chronic mild stress or severe sporadic stress during PND23-51, and investigated their behaviour on the EPM at PND70-91. Males in the severe stress group showed exaggerated anxiety-like behaviour (e.g., jumping off the open arms), whereas females in the severe condition displayed other types of behaviour related to depression, such as decreased sucrose consumption. For the mild stress condition, only the female rats showed signs of anxiety or depression in the form of reduced burying behaviour, decreased sucrose consumption and an exaggerated corticosterone response to cold-water immersion stress. However, McCormick, Smith and Mathews (2008) found that female rats that were stressed by daily 1 hour isolation, followed by housing with a new peer in a new cage) during PND30-45 displayed increased anxiety on the EPM (time on open arms and open arm entries) at PND90 only when in diestrus, whereas male stressed rats were observed to exhibit increased anxiety-like behaviour continuously.

The results from the current study support the above-mentioned research in terms of entries into and time spent in the open arms of the EPM for all three adolescent developmental ages at the 30 day washout period, although this seemed to primarily characterise BZP- treated subjects. Rats treated with 0.5 mg/kg of MA during PND51-60 spent more time in the open arms than saline controls. At PND120, all three doses of BZP administered during PND31-40 decreased entries into and time spent in the open arms. There were no significant dose effects for rats treated with either BZP or MA during PND41-50 on percentage of entries or time spent on the open arms. However, for rats treated with both BZP and MA during PND51-60 the results suggested that, as the dose of each drug increased, treated rats displayed more anxiety-like behaviour. Similar results were observed at PND200.

Overall, there is a very limited amount of published research with which to compare the results of the present study. And only assumptions can be made about what central processes might have been involved in the modification of brain development induced by adolescent exposure to the two drugs that led to long-lasting increases in anxiety-related behaviour. In this latter respect, the present study supports and significantly extends the earlier findings of Aitchison and Hughes (2006) with BZP- treated rats, and of Joca et al. (2014) research with MA-treated mice.

### ***Methodological Limitations***

The use of an animal model for the present research is confounded by the fact that the drugs were administered by the researcher and not self-administered by the animal. As substance seeking is a key component of addiction this research did not address this and the neurobiological effects of BZP and MA may vary depending on whether the animal chooses to take the drug or not (Robinson & Kolb, 2004). As mentioned above, there is a difference between an animal seeking the drug (Andersen, et al., 2002) and actually being given it (Brandon, et al., 2001). However, in some brain regions both self-administration and experimenter administration produce the same changes. For example, AMP administered either way increases the spine density in the NAc and PFC (Robinson & Kolb, 2004), therefore, affecting behaviour in a similar manner. Studies have demonstrated a difference between the two methods of delivery in different behavioural and neurobiological effects. However, Joffe et al. (2014) argue that not all the effects of substances are dependent on contingent administration and both methods have been utilised in addiction research.

Another concern is the administration of both BZP and MA by way of intraperitoneal (i.p) injection. Human users of BZP typically take the dose orally; MA is initially smoked before individuals' progress in their addiction to intravenous injecting. In this study voluntary oral administration of both drugs, as with other DOA, would be the optimal route of administration (Carlezon & Konradi, 2004), but there is no research to suggest that rats will orally take BZP. However, studies indicate that there is no protective effect of oral, as opposed to other routes of administration, and the effects of i.p. administration of AMP are similar to the effects of oral administration in rats (MacCann & Ricutre, 2004). Moreover, i.p. administration is a relatively slow and gradual process similar to oral administration (Blanchard & Blanchard, 1999). It must be stressed that all groups experienced i.p. exposure to either saline, BZP or MA and therefore the results cannot be attributed to differences in experimental procedure, but rather to the drugs themselves.

A potential issue is the question of whether the behaviour that was observed was anxiety or something else. A current issue with animal tests of anxiety is that they are rarely validated, other than pharmacologically. The reason for this is that animal models of anxiety are complicated by no single behavioural or physiological measure reflecting anxiety alone (Pellow et al., 1985). We cannot ask the rat whether it is anxious or afraid. The results of this research were assumed to be assessing anxiety in the adult rat after adolescent MA or BZP

administration. However, anxiety as a subjective component had to be inferred from behavioural measures.

Other researchers utilising similar behavioural models report that animals experience stress, fearfulness, emotionality, suffering, reduced wellbeing or anxiety (Ramos & Mormede, 1998). The current research exposed the adult animal to psychologically aversive stimuli (novel environments, strongly illuminated areas, open spaces, social interactions) as opposed to physically aversive stimuli (electric shocks, food deprivation, submersion in water, extreme temperatures). Some measures (Y-maze, EPM and light-dark box) allowed the animal to avoid the aversive stimulus. The results of this study indicate that increased anxiety-like behaviour in adult rats followed BZP and MA exposure during the rat's equivalent period of adolescence. The behavioural measures used for this study utilised the natural approach/avoidance conflict that rats display (File & Seth, 2003). That is, a natural conflict between curiosity about a novel environment and avoidance of a potentially threatening environment. However, whether or not this type of conflict is relevant to humans is uncertain. One study that throws some light on this concern is that of Blanchard et al. (2001), in which 161 male and female undergraduate students were interviewed. The participants read a set of 12 scenarios based on defensive responding in rodents (e.g. magnitude of threat, the need to escape the situation, distance between the threat and the subject, ambiguity of the threat stimulus and the presence of a hiding place). The authors concluded that the patterns of defensive behaviour and emotional responding were similar for rodents and humans (Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001) with no significant differences between males and females. That is, humans (both males and females) will react in a similar way as rats to a present or potentially threatening conspecific or environment. This study was replicated by Shuama et al. (2008) utilising 324 medicine and psychology students of both genders and the authors supported the view that defensive behaviour is similar for humans and non-human mammals. Therefore, it is possible that the use of the animal model in this research can be extrapolated back to the human population namely, that the administration of BZP and/or MA during earlier development can impact on later anxiety-like behaviours.

This research was based on evidence that adolescent brain development is a critical developmental period in which drugs can have long-term behavioural consequences that persist into adulthood. However, the conflicting results of this study suggest that there are differences in outcome, dependent on exactly what stage a substance is administered. Future research assessing earlier stimulant use on later behaviour would benefit from neurochemical analyses.

Due to the inclusion of different treatment ages in the experimental design, the first testing period (after a 30 days washout period) inevitably meant that the rats were tested at slightly different ages. Therefore, it is difficult to conclusively determine if the results obtained at this period were due to the age of testing or to the drugs' effects. However, Vorhees et al. (2005) provide a sound rationale for adopting this approach namely, that after PND50, behavioural testing does not markedly differ as a result of age. The fact that the results for the different groups of animals remained largely consistent over time supported this rationale.

Another potential limitation of this study is the issue of handling. In the course of completing the present research, the author conducted a total of 16,380 trials, with each rat being handled (prior to and after trial) 39 times. It is well-established that prior handling has anxiolytic effects in several testing situations (Hogg, 1996). There is also the possibility that repeated testing may have reduced the anxiety-inducing effects of the test environments. However, as the control rats were exposed to the same experimental procedure it is unlikely that handling or repeated testing contributed significantly to the results.

### ***Methodological Strengths***

In humans, it is likely that a combination of experiences may lead to the development of psychopathologies, therefore making it difficult to establish causality. Therefore, animal models represent a useful strategy to understand how early life events may affect the functioning of the central nervous system in adulthood, in terms of behaviour and its underlying mechanisms (Neto et al., 2012). Furthermore, human studies are often retrospective and correlational (Wilkin et al., 2012). Human users may often self-administer other drugs in addition to party pills. In many human studies there is little control over the amount of drug used, the purity of it and any polydrug habits of the subjects. As BZP was the main base drug in most party pills the present research provides a starting point for further research involving different drug combinations. Therefore, this study addressed the long-term effects of BZP and MA in a way that avoids the complex issues of polydrug use, drug purity and any pre-drug psychopathology that can possibly compromise human drug research (Koenig, et al., 2005). The animal model used in this study controls for the history, experiences and environmental conditions of the subject. As it is usually impossible to control for all of these in humans, this study provides an example of relatively uncontaminated BZP and MA effects.

An advantage of this study in measuring behaviour is that, because rats develop rapidly; the thirty days between the last exposures to BZP or MA and rats reaching adulthood was sufficient time to ensure that there was no residual direct drug action still affecting their performance (Snyder et al., 1998). In addition, the increased anxiety-like behaviour that was displayed from the twelve treated groups was unlikely to be due to the handling, weighing or experimental procedures because the control animals were treated in exactly the same way.

Although direct extrapolation of findings from the present research to humans must be approached cautiously, many former animal models have been useful in predicting long-term responses in humans. For example, Andersen et al. (2002) showed that chronic treatment with typical neuroleptic medications can induce brain morphological changes that are similar in rodents and humans (Andersen et al., 2002). One study of the metabolism of BZP in male Wistar rats found that the same identified metabolites were found in the rats' urine as was found in human urine (Staack, & Maurer, 2005). Notwithstanding, the half-life of MA in humans is 8 to 12 hours, but in rats it is closer to 1 hour. Therefore if a person takes MA daily, the concentrations in plasma will gradually increase, however the same is not true for rats. Larger effects may have been displayed if the animals had received BZP or MA several times a day. Nonetheless, the results obtained in this study may be applicable to a human population, bearing in mind that they concern subsequent effects of BZP and MA specifically, and do not include effects of other drugs that human users might also take.

Although, this study did not use large doses or extended dosing regimens, this protocol was not intended to exactly replicate human consumption situations; rather it was intended to capture some of the aspects of regular dosing of three escalating doses of BZP and MA, during a critical period of adolescent brain development. A dose of 1.0mg/kg of MA is considered to slightly increase locomotor activity, whereas 3.0 mg/kg of MA produces much higher levels of motor activity without causing toxicity (Milesi-Halle et al., 2005). Davison et al. (2001) reported that research conducted in both human and non-human primates showed long-term losses in DA and 5-HT function after chronic MA administration, and this was found in adult brains, so it goes without saying that because the adolescent brain is still developing, the administration of a non-toxic dosing regimen may have impacted in similar ways on both DA and 5-HT activity. Notwithstanding, this research provides a valuable starting point for future research.

Additionally the purity of the drugs used in this study is a methodological strength. In real life scenarios it is rare to find a pure form of a drug as many recreational drugs are made with ingredients that are easy to procure. As already stated, in New Zealand MA can be synthesised from general household products and BZP is often combined with other psychoactive substances. The current study used pure research-grade BZP purchased from ABRC GmbH & Co, Karlsruhe, Germany and pure crystal form MA donated by Environmental Science & Research Limited (ESR, Wellington, New Zealand).

### ***Implications***

This research did not address potential cross-sensitization and the implications of this could be far reaching. Cross-sensitization is the increased response to a different drug due to early exposure to another drug (Landa et al., 2014). It is possible that the cohort of individuals who consumed what they considered to be legal highs may be more prone to the development of substance use disorders in later life.

There is little empirical information on how stimulant exposure during the developmentally critical period of adolescence influences adult functioning, following extended periods of abstinence. This is vitally important as a relapse can occur despite individuals maintaining long periods of abstinence and the fact that adolescent stimulant use has risen significantly in the last two decades. The general consensus is that stimulant exposure during adolescence represents a greater risk factor for addiction (Schramm-Sapyta, Pratt & Winder, 2004) than exposure during adulthood and this leads to a greater possibility of the development of psychosocial dysfunctions and psychiatric symptoms. For example, King et al. (2010) found that adolescents who had been abstinent from MA for an average of 4-11 months had increased social anxiety and depression, following an average of 2-3 years of MA use. Females under the age of seventeen years had scores significantly higher on the Symptom Checklist-90R (SCL-90) compared to female controls. Therefore, the authors concluded that young female MA users may be more susceptible to, and stressed by social situations. This is important as stress can further induce MA craving and use, followed by relapse after a period of abstinence.

The implications of this study for the greater population could be far-reaching, regardless of whether or not there is full agreement about the validity of animal models in studying drug-induced anxiety. The results support temporal ordering (i.e., substance abuse can precede mood disorders) and neuronal imprinting (i.e., effects of the drug are long-term) theories. BZP and

MA may interfere with the brain's ability to reorganize neural circuits and earlier drug exposure could interfere with the cognitive advances that are made through development (Robinson & Kolb, 2004). This is critical as alterations in cognitive functioning may interfere with quality of life (Carlezon & Konradi, 2004), possibly because of difficulties in social cognition and decision-making (Rogers et al., 1999). For example, in a clinical investigation, the decision-making of 18 AMP and 13 opiate abusers, 20 patients with PFC damage and 26 controls (matched for age and intelligence) was compared. They were assessed on decision-making behaviour. The results included increased deliberation times to make a choice in the AMP, opiate and PFC damaged patients, and poorer quality of decision-making by PFC-damaged patients and AMP abusers compared with controls. For the AMP abusers this was not due to increases in deliberation times, but was instead correlated negatively with the number of years of abuse. Interestingly, tryptophan depletion in the control group produced the same outcomes as shown by the AMP and PFC groups. Because tryptophan depletion reduces central 5-HT activity, the authors concluded that AMP abusers and patients with damage to the PFC were similarly affected, possibly because of deficits in the neuromodulation of 5-HT and its inter-connected systems (Rogers et al., 1999).

The ability to quickly respond to objects, events and threats in the environment is essential for the survival of humans and animals alike (Gawronski & Cesario, 2013). Although fear and anxiety are behaviourally very similar, anxiety is defined as a long-lasting state of apprehension that can become pathological if it becomes extreme, whereas, fear is prompted by imminent and potential real danger and serves to activate the individual to respond. Anxiety is a future-oriented mood state that is associated with arousal and vigilance (Davis et al., 2010) and behavioural avoidance is a central feature of anxiety disorders. In rats, anxiety induces avoidance behaviour that can reflect fear of novelty, whereas approach behaviour (e.g. exploration) reflects motivation to explore novelty. In the current research, the conflict between approach and avoidance was exploited. However, it is possible that they do not represent ends of a continuum, but instead exert independent biological functions (Green et al., 2012).

Commencing early in life, anxiety disorders are among the most common mental disorders with an estimated one in fourteen people meeting diagnostic criteria at any one point in time (Baxter et al., 2012). No single brain structure or neurotransmitter controls the anxiety response system. Instead, several interrelated systems operate together in complex ways to induce anxiety. As discussed, the HPA axis and the limbic system (particularly the amygdala) act as a

mediator between the brain stem and the cortex, the PFC and other cortical and subcortical structures to evoke behavioural responses to potential threats and fear (Chaby et al., 2015). Adolescent treated rats in this study may have undergone an alteration to the HPA which led to increased anxiety when adult.

Anxiety and cognition interact in a fundamental way, and several studies suggest that cognitive dysfunctions may be the primary presenting feature of anxiety (Ohl, 2003). There is also considerable research that suggests that the mechanisms that are involved in emotion are also involved in learning and memory (Conrad, Jackson, Wiczorek, Baran, Harman, Wright, Ryan and Korol, 2004; Wolf, Sun, Mangiavacchi, & Chao, 2004). As the results of this study suggest an increase in anxiety, the interrelationship between anxiety, cognition, learning and memory may lead to adolescent BZP and MA users experiencing more seriously adverse outcomes because their brain is not fully developed. Vorhees et al. (2005), found that when rats were treated with MA during PND41-50 they developed deficits in spatial learning/reference memory and sequential learning, whereas those treated before PND41 or after PND50 did not. Although, memory and learning were not directly measured in this study, DA and 5-HT are involved in emotion as well as learning and memory thereby suggesting some comparability with the findings of Vorhees et al. (2005) with respect to effects of MA, but also BZP.

In conclusion, evidence provided in this thesis for increased anxiety-related behaviour following adolescent exposure to MA or BZP illustrates that alterations in emotionality are among the long-term effects of both of these drugs. In particular, these results add to the limited literature indicating that stimulant exposure during adolescence has detrimental consequences for behaviour that extend and are maintained for long periods of time, possibly for the remainder of the rat's life span.

## **Chapter 6: Future Directions**

This study highlights some important issues worthy of further investigation. For example, does the use of BZP during adolescence predispose the individual to later experimentation with other drugs? In other words, are there young adults who starting using BZP as adolescents now using the very drug (MA) that BZP was supposed to reduce the harm of. The neuronal imprinting theory was supported in this study, suggesting that early exposure to BZP leads to subsequent changes in adult behaviour. However, the alternative self-medication hypothesis was not examined. To fully examine the long-term effect of the administration of BZP in adolescence, investigations are needed with individuals who experience underlying mood disorders and who are possibly self-medicating with substances to alleviate negative emotions they might be experiencing. Finally, the ordering of dual diagnosis or temporal ordering of what precedes what, needs to be addressed. This study supports the theory that substance use precedes psychiatric disorder. However, the animals used were not genetically bred for underlying mood disorders, so testing for the possibility that a mood disorder leads to self-medication was not possible. Additionally aggression has been associated with stimulant drug use (Kirilly, Benko, Ferrington, Ando, Kelly & Bagdy, 2006). Aggression, impulsivity and risk-taking are all related to 5-HT depletion in the PFC and interrelated systems (Dawe & Loxton, 2004; Wills, Wesley, Moore & Sisemore, 1983). To examine whether the increased anxiety-like behaviour displayed by rats in this study is possibly connected also to aggression a resident-intruder test could be conducted. For example, Homberg, Fattil, Janssen, Ronken, De Boer, Schoffelmeer and Cuppen (2007) found that homozygous SERT knockout rats displayed reduced aggression in the resident-intruder test. Likewise, Centenaro, Vieira, Zimmermann, Miczek, Lucion and Martins de Almeida (2008) found a decrease in aggressiveness in a resident-intruder test, after microinjections of 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptor agonists into the PFC. This suggests a possible relationship between anxiety and aggression and maybe due to the animals heightened state of arousal, involving the fight flight response (Cancela et al., 2001).

BZP was introduced into New Zealand under the harm reduction umbrella, yet there was no evidence to support this premise. It is possible that BZP may be useful for MA addicted individuals as the “agonist substitution” therapy involves administering substances that are less potent and less addictive than the DOA. This can be described as “normalisation” therapy, in which the administration of a less potent substance normalises deregulated neurochemistry

provoked by the DOA (Rothman & Baumann, 2003). However, the possibility that individuals will substitute MA for BZP needs to be further explored, possibly with a counter-balanced conditioned place preference design. Nonetheless, the results of this research suggest that both substances are potentially harmful for development of anxiety-like behaviour long after the drugs are removed from the system. Yet, Hashimoto et al. (1992) suggest that BZP inhibits the reuptake of 5-HT which has been implicated in the rewarding properties of stimulant-like drugs, normalization therapy may work as a substitute for MA-dependent individuals.

There is growing clinical evidence that the period of adolescence is a period of heightened vulnerability to the addictive properties of both legal and illegal drugs (Chambers et al., 2003). The results of this study support the idea of vulnerability to DOA during adolescence and, further research needs to continue to utilise adolescent male and female animals to answer questions that this research has raised. For example, the lack of sex effects for female rats treated with BZP or MA during PND31-40 or the conflicting seemingly protective effect of MA administration to female rats treated during PND51-60. Additionally, to assess cross-sensitisation or the possibility that individuals who consumed BZP during adolescence, are more vulnerable to other stimulant use in later adulthood, a conditioned place preference may again help in answering some questions this research has raised (Bardo & Bevins, 2000).

By using a counter-balanced conditioned place preference, the stage theory of addiction may be further examined. This could shed some light whether BZP may act as a gateway drug for further experimentation, with other DOA. Secondly, will animals with underlying initial anxiety prefer to spent time in chamber associated with either BZP or MA, to alleviate negative emotions? This would address the self-medication hypothesis. Thirdly, as mentioned above, will MA dependent animals prefer BZP as a substitute for MA? This would test the assumption that BZP can act as a replacement drug for MA dependence, thereby, supporting normalization therapy. Finally the lack of neurochemical analyses in this study needs to be addressed. This would help clarify whether or not the increased emotionality displayed by the animals in this research was due to 5-HT or 5-HTT modification in the brain, especially the undeveloped PFC.

## **Chapter 7: Conclusions**

Substance use is a reality (Resnicow, Smith, Harrison, & Drucker, 1999) and no amount of research into the detrimental effects of drugs can prevent this actuality. However, this study opens the way for future understanding of the undesirable effects that could be observed in the next generation of individuals reaching adulthood. The administration of BZP or MA during this period may have caused a functional change in DA and 5-HT levels by altering modulation of the neurotransmitter release and/or altering the functional development of the PFC input, thereby leading to an increased risk of higher anxiety during adulthood. This higher anxiety during adulthood may lead to self-medication with illegal drugs, or worse still, dependence on these substances. Therefore, these ontogenetic changes, along with the fact that adolescence is the period of development when drug use is initiated, provide a compelling reason for future research into the long-term effects of adolescent BZP and MA use. The main limitation of the present study is clearly the lack of neurochemical analyses of the brains of the rats exposed to both drugs. While it is hypothesised that the observed effects on anxiety relate to the neurotoxic action of BZP and MA on the brain's 5-HT systems, conclusive proof of this is lacking. Notwithstanding the lack of neurochemical analyses, and possible conflicting behavioural profiles displayed by the treated rats in different anxiety measures and over time, the general results of the present study are strikingly clear. Rats treated with BZP or MA in adolescence show increased anxiety-like behaviours in adulthood compared with untreated controls.

The results of this research conclude that BZP and MA administered during adolescence affects later emotional behaviour, supporting the neuronal imprinting theory. Due to New Zealand's geographical separation and distinctive drug taking practices (limited access to well-known DOA, for example cocaine, thereby resulting in individuals procuring substances with general household products), it appears that policy makers are playing an endless 'cat-and-mouse' game with manufactures and users. In that, as one new drug appears and becomes subject to legal controls, a new unrecognised and uncontrolled alternative substance is produced. New Zealand's clandestine chemists and manufacturers appear to be one step ahead of both the forensic scientists and the law, pushing further and further into the realm of untried and untested drugs. Collins (2007) summed up the consumption of BZP adequately, by reporting that 20% of the general population of New Zealand were "lab rats" for substances

that were not regulated, for which there had been no animal or clinical trials, and which are readily available at local corner stores and petrol stations.

Finally, this research provides further information that may assist in understanding the potential links from early substance use to later psychiatric and addiction problems. Mental health and addiction treatment providers will need to screen and comprehensively assess earlier substance use, including earlier use of so-called legal substance to inform their treatment options and provide effective management of individuals with SUD.

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