Adulthood Outcomes in Rats Following Repeated Adolescent Exposure to 1-Benzylpiperazine (BZP) and/or Ethanol.

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Abbreviations

BZP	1-Benzylpiperazine
CNS	Central nervous system
CPP	Conditioned Place Preference
DA	Doapmine
DAT	Dopamine transporter
DSM-IV	The Diagnostic and Statistical Manual of Mental Disorders
ED	Emergency Department
GABA	γ-aminobutyric acid
g/kg	Grams per kilogram
i.p.	Intraperitoneal injection
MDMA	Methylenedioxymethamphetamine (Ecstasy)
mg/kg	Milligrams per kilogram
ml/kg	Millilitres per kilogram
NA	Nucleus Accumbens
PFC	Prefrontal Cortex
PND	Post Natal Day
5-HT	Serotonin
TFMPP	1-(3-trifluromethylphenyl)piperazine
VTA	Ventral Tegmental Area
W	Kendall Coefficient of Concordance
WHO	World Health Organization

Abstract

In New Zealand, it is common for young people to mix 1-*benzylpiperazine* (BZP) containing 'party pills' and ethanol (drinking alcohol). However, there is no scientific literature which compares the individual and combined long-term effects of these substances. Therefore, the aim of this study was to provide a comparison of BZP and ethanol's individual and combined effects on adulthood behaviour following repeated adolescent exposure. To investigate this 40 male and 40 female adolescent rats received daily exposure (post natal days 41 – 50) to BZP (10 mg/kg) and/or ethanol (2 g/kg) or saline vehicle (1 ml/kg) via intraperitoneal injection. Animals were tested in a Y maze, light/dark emergence box, and an open field during early adulthood (PND 78 – 81) and again during mid-adulthood (PND 117 – 120). Results found females treated with alcohol ambulated less in the open field. Interestingly, no other behavioural differences between the treatment groups were observed. Overall, it appeared that adolescent exposure to BZP and/or alcohol did not have long-term behavioural consequences, at least in rats. This finding was most likely due to the narrow range of testing ages adopted in the study.

1.0 Introduction

1.1 General Overview

Adolescence is an exciting and vulnerable period when young people make the gradual transition from childhood to adulthood. During adolescence, there is an increase in risk-taking behaviour such as the initiation of drug use (Brecht, Greenwell, & Anglin, 2007; Spear, 2000). Consuming such substances during this period can have detrimental developmental effects later in life (Casey, Giedd, & Thomas, 2000). As a result, there is a need for research into the effects of adolescent drug exposure with an emphasis on the substances current young people are commonly taking.

In view of this issue, the use of 'legal party pills' is common amongst New Zealanders with one in five aged 13 – 45 years reporting having used them at least once and 38% of young adults aged 20 – 24 having used them in the past 12 months (Wilkins, Girling, Sweetsur, Huckle, & Haukau, 2006). Party pills are taken for their stimulant effects produced by the active ingredient 1-*benzylpiperazine*. Party pills are marketed as a safer legal alternative to the methamphetamine class of drugs (Johnstone, Lea, Brennan, Schnek, Kennedy, & Fitzmaurice, 2007). However, there is little known about the longer-term outcomes of exposure to 1-*benzylpiperazine* to support a harm reduction hypothesis. Therefore, research on the longer-term outcomes of adolescent exposure to party pills is required.

In addition to party pills, ethanol (drinking alcohol) is a drug regularly consumed by young New Zealanders. Stefanogiannis, Mason, and Yeh (2007) found the most frequent alcohol users were those aged 18 – 24 years. The World Health Organization (WHO) recommends males should consume no more than six standard drinks and

female's no more than four standard drinks on a single drinking occasion. Consumption in excess of this recommendation is considered drinking large amounts of alcohol. Unfortunately, one in two New Zealanders aged 18 – 24 and one in five aged 12 – 17 exceed the WHO recommendations on a usual drinking occasion. In view of this, research on the longer-term outcomes of adolescent exposure to alcohol is needed.

In New Zealand poly drug use of party pills and alcohol is common. Over 90% of party pill users have reported using both alcohol and party pills together at some point. In addition, one in three users consumes more alcohol when using party pills than they would otherwise (Wilkins et al., 2006). This poly drug use by New Zealand adolescence could have detrimental developmental effects.

Currently there is no scientific literature on the combined effects of 1-*benzylpiperazine* and alcohol. Therefore, the primary aim of this thesis was to provide an assessment of longer-term behavioural outcomes following adolescent exposure to 1-*benzylpiperazine* and alcohol both alone and in combination thereby making an original contribution to the scientific literature on the effects of these drugs.

1.2 Substance use and addiction

To provide an understanding of the possible consequences of repeated exposure to 1-*benzylpiperazine* (BZP) and alcohol, the diagnostic criteria for Substance Abuse and Substance Dependence disorder is provided below. However, for an individual to become an abuser or an addict of these substances they must first go through an experimental phase. Therefore, the behavioural and physiological mechanisms of Substance Abuse and

Substance Dependence are briefly presented. These disorders can lead to significant personal, occupational, social, and interpersonal impairment.

Substance Abuse and Substance Dependence are two types of Substance Use Disorder. The differences between these disorders need to be clearly defined. The most prominent tool for diagnosing Substance Use Disorders is *The Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV; APA, 1994). The DSM-IV defines Substance Abuse as a maladaptive pattern of substance use manifested by recurrent and significant adverse consequences related to repeated use of the substance. Associated impairments include (but are not limited to) intoxication at work or school and the continued use of the substance in hazardous situations such as driving. To meet the criteria for Substance Abuse disorder the symptoms must not meet the criteria for Substance Dependence disorder. Additionally, the individual in the past 12 months must present one or more of the four symptoms described in the manual.

In comparison, the DSM-IV defines Substance Dependence disorder as a cluster of cognitive, behavioural, and physiological symptoms indicating that the individual continues use of the substance despite significant substance related problems. To meet the criteria for Substance Dependence disorder an individual must meet three or more of the seven symptoms outlined in the manual. Such symptoms include having developed tolerance to the substance and withdrawal symptoms when discontinued use of the substance occurs (see the DSM-IV for a full description of all symptoms; APA, 1994). Importantly, before an individual can develop a substance use disorder they must first experiment with the drug.

1.2.1 Initiation of Drug Use

The factors which contribute to the initiation of drug use need to be discussed to understand why young New Zealanders with no history of drug use often experiment with BZP and/or alcohol. The reasons why an individual experiments with a substance are varied and complex. Genetics and family environment are biological factors that are important aetiological variables which contribute to the initiation of substance use (Weinberg, Rahdert, Colliver, & Glantz, 1998). Studies of monozygotic and dizygotic twins have found both genetics and the family environment play an important role in the use and clinical diagnosis of substance abuse/dependence (Maes et al., 1999; van den Bree, Johnson, Neale, & Pickens, 1998). Recent evidence from twin studies (e.g. Xian et al., 2000) suggests the major genetic predisposition to drug use and dependence is heritability. Interestingly, this predisposition appears to be stronger with males than females (Wagner & Anthony, 2001; Xian et al., 2000).

In addition to genetic risk factors, there are several important environmental factors which contribute to young people experimenting with drugs. These risk factors include peer pressure, association with antisocial peers, substance availability, individual expectations of the substance being used, and economic factors (Andrews & Bonta, 2003; Crombag & Robinson, 2004; van den Bree et al., 1998). Exposing adolescents to one or more of these risk factors increases their likelihood of experimenting with drugs. Many young adults in New Zealand experiment with BZP and alcohol. Once substance experimentation has been initiated individuals can develop an addiction. To understand the aetiology of substance use disorders, the mechanisms of addiction will be discussed in the next section.

1.2.2 Mechanisms of Addiction

The mechanisms which initially lead to substance use disorders are behavioural. For an individual to become dependent on a drug the substance must first reinforce the individual's behaviour (Cardinal & Everitt, 2004; Carlson, 2004). At first, the reinforcing effects of drugs are mediated via positive reinforcement. When behaviour (e.g. substance use) is immediately followed by a reward (e.g. pleasurable feelings, gratification, relief of stress), that behaviour is reinforced (Kazdin, 2001; Robbins & Everitt 1999). For example, nicotine rapidly provides pleasurable feelings to the user thus reinforcing the person's smoking behaviour (Coe et al., 2005; Keating & Siddiqui, 2006). For drugs of abuse, the mechanisms which reinforce substance use behaviour are neurobiological.

The positive reinforcing properties of drugs of abuse are mediated by inducing changes in the neural circuits utilized for the reward values of natural reinforcers such as food, water, and sex (Cami & Farre, 2003). This neural system provides individuals with a motivation to obtain such rewards (Crombag & Robinson, 2004; Chiara, 1995). The dopamine hypothesis of addiction suggests most (if not all) addictive substances exert their psychoactive actions (either directly or indirectly) on the neurotransmitter dopamine (DA, Cardinal & Everitt, 2004; Robbins & Everitt, 1999). Increased DA levels within the mesolimbic DA system have been associated with the euphoric and addictive properties of drugs (Carlson, 2004; Julien, 2001). The mesolimbic DA system originates in the ventral tegmental area (VTA) of the midbrain and projects to forebrain structures such as the nucleus accumbens (NA) and the prefrontal cortex (PFC). The VTA and its associated neural circuitry has been shown to play a critical role in reinforcement. For example, when nicotine binds with $\alpha_4\beta_2$ receptors in the VTA the DA reward pathway is activated.

This activation results in increased mesolimbic DA levels. This increase in DA levels provides the smoker with pleasurable feelings which in turn reinforce smoking behaviour (Jorenby et al., 2006; Rollem et al., 2006; Tsai et al., 2007). In addition, Bozarth and Wise (1981) found rats would self-administer morphine. In their study, subjects could self-administer morphine directly into the VTA by pressing a lever. They found subjects response rates were stable and exceeded those of 'yoked' controls. Self-administration was blocked by the μ-opioid receptor antagonist naloxone. This finding suggests morphine's reinforcing properties are mediated via activation of μ-opioid receptors in the VTA. This activation causes an increase in mesolimbic DA levels which is common with drugs of abuse.

Even though increased DA levels appear to be primarily responsible for the reinforcing effects of drugs (see Chiara, 1995 for a review), serotonin (or 5-hydroxytryptamine, 5-HT) may also be important. Rocha et al. (1998a & 1998b) reported two studies with cocaine which challenged the dopamine hypothesis. Cocaine acts by blockading dopamine transporter's (DAT) that are responsible for the re-uptake of DA from the synaptic cleft. Therefore, cocaine blockade of DATs leads to increased extracellular DA. This effect is considered by many to be the primary cause of the reinforcing and addictive properties of cocaine (Rocha, 1998a). Therefore, animals lacking DATs would have high levels of extracellular DA and should not receive any reward or reinforcement from cocaine administration.

In their study, Rocha et al. (1998a) investigated whether or not mice genetically lacking the presynaptic DAT would self-administer cocaine. Interestingly they found cocaine self-administration occurred in these subjects at levels considered to be

reinforcing. Mapping of cocaine's binding sites suggested 5-HT was responsible for cocaine's reinforcement.

In a further study, Rocha et al. (1998b) investigated cocaine self-administration behaviour in mice lacking the postsynaptic 5-HT$_{1B}$ receptor. They found the effects of the drug in these subjects were greater than in control animals, and they were more motivated to self-administer. This occurs as 5-HT acts through many receptors to modulate the mesolimbic DA circuit. The 5-HT$_{1B}$ receptor is expressed on the terminal buttons of γ-aminobutyric acid (GABA) striatal neurons. These GABA neurons project to the VTA. GABAs role is to inhibit the release of DA in this area. Therefore, the lack of 5-HT activation of GABAergic striatal neurons causes additional stimulation of DA releasing neurons during cocaine use. Accordingly, this finding suggests 5-HT can also antagonize the reinforcing effects of cocaine. In summary, the DA reward system is shown to be the primary contributor to the acquisition of drug addiction, but some studies have shown the 5-HT system to also be involved.

Thus far positive reinforcement has been argued as the mechanism which leads to drug addiction. However, once an addiction has been acquired negative reinforcement is responsible for maintaining the behaviour. When a behaviour (e.g. drug taking) alleviates negative symptoms (e.g. relief of stress and anxiety) that behaviour is then reinforced as it has provided relief (Kazdin, 2001). For an individual to experience negative side effects during a period of substance abstinence they must have developed tolerance to the drug and then manifested withdrawal symptoms.

Tolerance is a state of progressively decreasing responsiveness to a drug. When a substance is repeatedly administered larger doses are required to generate the original

effect (e.g. euphoria, relief of stress) which the original smaller dose produced (Julien, 2001). This occurs as drug use causes a decrease in receptor sensitivity known as down regulation. Down regulation is the brain's compensatory mechanism for counteracting the drug's effects (Carlson, 2004). For example, as use of morphine progresses, the μ-opioid receptor becomes less sensitive to the drug. Therefore, increasingly larger doses of the substance are required to produce its pharmacologic effects (Bozarth & Wise, 1981). Once an individual has acquired substance tolerance they will experience withdrawal symptoms during a period of abstinence (Swann, Jack, Valdes, Ring, Ton, & Curry, 2007).

Withdrawal symptoms essentially reflect the opposite acute effects of the drug (Piper, et al., 2008). For example, cocaine produces feelings of pleasure, calmness, and euphoria. However, when an individual goes through cocaine withdrawal they experience anxiety and dysphoria. These symptoms lead to cravings for the drug to alleviate the discomfort they produce (Julien, 2001). When the user re-administers the drug the negative symptoms are relieved and the behaviour is reinforced (Lopez-Pation, Yu, Cabral, & Zhdanova, 2008).

Withdrawal symptoms for stimulant drugs are probably a result of low DA levels arising from the brain's compensation for the effects of the substance. The sudden absence of the substance means the required level of DA is no longer being released in the brain. This lack of DA produces withdrawal symptoms. Withdrawal symptoms are argued to be a key contributor to relapse during abstinence attempts (Keating & Siddiqui, 2006). Importantly, withdrawal from depressants can be different from stimulant withdrawal. For example, the rewarding properties of alcohol are mediated via release of

DA in the NA (Littleton, 1998). However, alcohol also interacts with other neurotransmitters (discussed in the *Ethanol* section). Alcohols down regulation of these neurotransmitters can lead to hallucinations, seizures and even death during a withdrawal episode if left untreated (Bayard, McIntyre, Hill, & Woodside, 2004) As a result, neurotransmitter systems additional to DA play a role in the negative reinforcing properties of depressants such as alcohol. Therefore, the role of negative reinforcement is to maintain drug addiction to alleviate the negative symptoms associated with withdrawal.

In summary, substance abuse and dependence are debilitating conditions which can be avoided by understanding the consequences of drug use. When an individual experiments with a substance they risk developing a substance use disorder. Once drug use has been initiated positive reinforcement via the DA reward pathway and possibly the 5-HT system can lead the individual to a substance use disorder. When addiction has been acquired negative reinforcement via tolerance and withdrawal maintain drug use. The long-term outcomes of drug use could have detrimental effects later in life. Therefore, I argue substances which are currently being taken by young people that have the potential for abuse should be thoroughly investigated.

1.3 Neurodevelopment: The adolescent point of vulnerability

Adolescence is defined as a period of gradual transition from childhood to adulthood. This period involves undergoing developmental challenges to attain the skills necessary to survive independently of parental caregivers (Spear, 2000). Importantly, the adolescent period extends well beyond puberty. Puberty is the onset of sexual maturation

and is sometimes considered the beginning of the adolescent period. Once sexual maturation is acquired the brain continues to develop (Spear, 2000). The duration of adolescent brain development (known as the periadolescent period) is unclear. The onset of the periadolescent period begins about age 12 in humans and can extend to as late as 20 – 25 years of age. During this phase the brain continues to undergo maturational changes (McFadyen, Brown, & Carrey, 2002; Spear, 2000). Therefore, the periadolescent stage of development is a 'brain in transition' period. This section will argue that interference with the brain during this transitional period can have consequences later in life.

The adolescent period is a time when individuals engage in risky sensation and novelty seeking behaviours. Engaging in such behaviours provides the opportunity to explore new situations and reinforcers. Taking part in such behaviours with peers promotes independence from parental caregivers (Spear, 2000). Participation in risky behaviours is considered a normal part of adolescent development. Such behaviour includes minor criminal behaviour, and use of drugs. Therefore, some experimentation with drugs is considered a normal part of adolescent development (Arnett, 1992).

The periadolescent period is characterised by regionally specific neurodevelopment. During this stage the brain undergoes a large amount of maturation. There are changes in the brain's neurochemistry and neuroendocrine control (Smith, 2003). Cellular events include an overproduction of synapses and receptors and increased myelination. Synapses that fail to make strong connections are pruned via apoptosis. This reduction process results in a loss of up to 40% of the synapses (Andersen & Navalta, 2004). Additionally, the brain experiences maturation of specific anatomical structures.

Structures which experience notable development are the nucleus accumbens (NA), amygdala, hippocampus, and the prefrontal cortex (PFC, Carlson, 2004). Each structure serves a specific function. In particular, the NA is involved with motivation, reward, and addiction. The amygdala regulates emotional reactions. The hippocampus is responsible for the formation of memories, and the PFC is involved in abstract reasoning and making judgements (Cassey, et al. 2000; Carlson, 2004; Chambers, Taylor, & Potenza, 2003; Spear, 2000). In addition, some sex differences in neurodevelopment are observed. For example, although females reach peak levels of cortical gray matter earlier than males, total cerebral volume is 9% larger in males than in females. The left amygdala of male brains increases significantly whereas female's experience a considerable increase in the size of the right hippocampus. Many of the anatomical differences observed in adolescence are still present in adulthood (Andersen, 2003; Cassey et al., 2000, Smith, 2003). In other words, adolescents undergo sex-specific neurodevelopment.

From observations of normal adolescent development it is seen why young people experiment with drugs, and also the potential consequences of such experimentation. Firstly, young people are motivated to seek new experiences as part of acquiring independence from their parents. Experimenting with substances (e.g. cigarettes, alcohol) is an example of such an experience. Some experimentation is considered normal. However, as argued earlier experimenting with substances can lead to Substance Use Disorders. Fortunately, only a minority of youth who experiment with substances develop a substance-related disorder (Spear, 2000). Secondly, the developing brain has an immature inhibitory mechanism. The continued development of the PFC means a mature level of judgment has not been reached. As a result, adolescents' combination of

immature judgement and motivation to seek new experiences means they are more likely than other age groups to experiment with substances (Chambers et al., 2003).

As discussed earlier, drugs exert their actions via the DA reward pathway. This pathway includes the NA and the PFC which both undergo development during the periadolescent period. Interference with the brain's normal development can lead to regionally specific deficits (Andersen & Navalta, 2004). For example, an immature PFC can result in poor judgment and difficulty processing complex information (Cassey et al., 2000). Therefore, drugs which interfere with brain maturation can have specific consequences latter in life. In addition, deficits could affect one sex more than the other due to differing rates of brain maturation in males and females. The following sub-section will discuss a theoretical approach to this phenomenon.

1.3.1 Neuronal Imprinting Theory

Neuronal imprinting occurs when the effects of drug exposure outlast the drug itself (Andersen & Navalta, 2004). Specifically, drug exposure during adolescence modifies the maturation of brain regions where the substance is active (Andersen, 2003). The brain alteration demonstrated by adolescents is different from what would be experienced by adults as studies of both humans and animals suggest the adolescent brain is more sensitive than the adult brain to the longer lasting effects of substances (Smith, 2003).

Drugs such as neuroleptics, marijuana, nicotine, alcohol, and stimulants have provided evidence for neuronal imprinting (see Andersen, 2003 for a review). For example, Graham & Diaz-Granados (2006) repeatedly administered alcohol to adult and

adolescent rodents. The subjects' conditioned taste aversion to the drug was tested six weeks after the final administration. Adolescent mice showed an attenuated response to alcohol taste aversion whereas adults did not. This finding suggests exposure to alcohol on the developing brain can lead to long-term changes in the aversive properties of alcohol.

Given there is evidence that exposure to drugs during adolescence can lead to behavioural changes in adulthood, it is likely adolescent exposure to BZP alone and/or alcohol will have consequences in adulthood. Any differences observed during adulthood would be consistent with the neuronal imprinting account of adolescent drug exposure.

In summary, adolescence represents a vulnerable period for the enduring effects of drugs. Substance experimentation is most likely to occur during adolescence and early adulthood. To complicate matters, the brain is very sensitive to the effects of drugs during this period. Given adolescents are susceptible to the long-lasting effects of substances and are most likely to experiment with them they deserve special attention. Therefore, researchers should pay particular attention to the periadolescent period when studying the developmental effects of drugs.

1.4 Methylenedioxymethamphetamine (Ecstasy)

Methylenedioxymethamphetamine (MDMA, 'Ecstasy') is an illicit amphetamine derivative which is popular with young people (Cassel et al., 2005; Wilkins, Girling, Sweetsur, & Butler, 2005). In humans, MDMA acts as a central nervous system (CNS) stimulant. It produces euphoria, enhanced well-being, and cognitive alterations (Peroutka,

Newman, & Harris, 1988). Understanding the mechanisms of action of MDMA is important as they are similar to those of BZP.

MDMA causes a release of 5-HT and DA in the brain via carrier release and/or reuptake inhibition of these neurotransmitters (Cassel et al., 2005; Fone, Beckett, Topham, Swettenham, Ball, & Maddocks, 2002). MDMA can elicit potentially fatal hyperthermia in rodents, primates, and humans. Chronic exposure results in depletion of 5-HT in the cortex, hippocampus, and striatum as these structures are highly sensitive to MDMA (Cassel et al., 2005, Piper, 2007). Finally, using MDMA can result in 5-HT toxicity. 5-HT toxicity (also know as the serotonin syndrome) is caused by excessive 5-HT release. Symptoms include (but are not limited to) confusion, hallucinations, coma, shivering, diarrhoea and seizures. 5-HT toxicity is potentially life threatening if left untreated (Piper, 2007; Clemens, Cornish, Hunt, & McGregor, 2007). These symptoms are a result of acute MDMA exposure. However, there are possible longer-term consequences of ecstasy use.

Some research suggests MDMA exposure can increase the likelihood of developing depression. The rationale for this statement comes from findings of drug treatments which decrease depression by increasing 5-HT levels. Given MDMA depletes 5-HT it is possible it will have the opposite effect by increasing depression amongst users (Piper, 2007). Although reports of consistent major depression following MDMA use are mixed, one longitudinal study of 14 – 24 year olds has found evidence of persistent mild depression following MDMA exposure (Lieb, Schuetz, Pfister, von Sydow, & Wittchen, 2002). This finding suggests MDMA use can have consequences which outlast the acute effects of the substance.

Research on adolescent MDMA exposure has found longer-term behavioural outcomes. For example, Fone and colleagues (2002) administered adolescent rats either MDMA or saline. When tested during adulthood up to 29 days after the last injection they found significant attenuation of social interactions and enhanced sub-threshold conditioned place preference (CPP) to cocaine compared to saline controls. A major strength of this study was the use of a relatively low dose of MDMA that resulted in adult behavioural differences in the absence of neurotoxicity. This suggests that even exposure of the developing brain to low doses of MDMA can have negative behavioural outcomes in later life.

In a later study, Achat-Mendes, Anderson, and Itzhak (2003) exposed adolescent mice to MDMA to assess its effects on cocaine-induced reward in adulthood. During adulthood, MDMA and saline subjects exhibited similar levels of CPP to cocaine. However, two weeks following extinction of CPP and withdrawal from cocaine, a small priming dose of cocaine reinstated CPP at a significantly higher level in MDMA-treated mice compared with controls. The authors concluded that adolescent exposure to MDMA induced long-lasting neuronal adaptations which sensitized subjects to the rewarding properties of cocaine. This finding further supported adolescents being particularly vulnerable to the long-lasting effects of MDMA.

Overall, there is a relative lack of research into the long-term effects of periadolescent exposure to MDMA. However, results to date suggest that such exposure can have negative behavioural outcomes later in life.

1.5 1-Benzylpiperazine

Party pills, containing the psychoactive ingredient 1-*benzylpiperazine* (BZP), are regularly taken by young New Zealander's for their subjective psychoactive effects (Johnstone et al., 2007). BZP acts on the CNS producing feelings of euphoria, wakefulness, and increased vigilance. The average duration of these effects is 6 – 8 hours (EACD, 2004). BZP alone and combined with other piperazine derivatives such as 1-(3-trifluromethylphenyl)piperazine (TFMPP), produces effects very similar to MDMA (Baumann, Clark, Budzynski, Partilla, Blough, & Rothman, 2005; de Boer et al., 2001; Fantegrossi, Winger, Woods, Woolverton, & Coop, 2005). For this reason, BZP containing pills have become popular within the dance party scene. They are regarded as a safer legal alternative to drugs such as MDMA. However, little is known about their longer-term effects (EACD, 2004; Johnstone et al., 2007). Party pills are readily available to those 18 years or older from specialty stores such as Cosmic Corner and Herbal Heaven. In 2007, an estimated five million party pills were sold in New Zealand (Gee & Fountain, 2007). The combination of BZP's ready availability with the impression it is safe, means more research is required to clarify the possible long-term effects of adolescent exposure to BZP.

Interestingly, there is a popular view that BZP was originally developed as a potential antihelminthic agent (a treatment for internal parasites) for cattle (EACD, 2004; Fantegrossi et al., 2005). BZP's parent compound *piperazine* was developed for this use, but BZP was not (EMCDDA, 2008). Therefore, the idea that BZP is a cattle drench is inaccurate. In 1971, BZP's potential as an antidepressant was evaluated as it was found in rats to reverse the sedative effects of tetrabenazine, a drug used to treat hyperkinetic

movement disorders (unpublished work by Miller, Green, & Young, 1971, cited in Fantegrossi et al., 2005). However, it was never marketed as an antidepressant possibly because of its similarity to the amphetamines (as will be discussed later).

In New Zealand, prior to 2005 BZP was available for purchase by anyone regardless of age. Currently BZP is a restricted substance in New Zealand. Its sale is limited to those 18 years of age or over. In addition, the advertisement of *piperazine* containing party pills is prohibited from major media sources such as television, radio, and newspapers (Wilkins et al., 2006). A ban outlawing the sale, possession, distribution, importation, and manufacture of BZP and other *piperazine's* (e.g. TFMPP) was due to take effect in December 2007. However, delays with Parliament passing the bill meant this law did not take effect until April 1st 2008. Persons possessing party pills now have six months from this date to use or dispose of their supplies.

Staff members from Christchurch Hospital's emergency department (ED) have reported the effects of BZP from 61 people who presented themselves to the ED. Gee, Richardson, Woltersdorf, & Moore (2005) found mild symptoms included insomnia, anxiety, nausea, and palpitations. Serious cases included toxic seizures, epileptic attacks and severe respiratory and metabolic acidosis. Two cases were admitted to the hospital's intensive care unit. It was concluded that BZP products had a narrow safety margin possibly due to intrinsic pharmacodynamic properties, dosage variability, and/or genetic factors. This study provided seemingly persuasive evidence for the negative effects of BZP. However, the conclusions made cannot be held with certainty. Due to the nature of the study, it was not possible to control for extraneous factors such as poly drug use and

doses taken. Therefore, experimental studies are needed to establish cause and effect relationships thereby confirming any observations made in clinical settings.

The principle mechanisms of action of BZP are similar to those of the amphetamines (Aitchison & Hughes, 2006; Baumann et al., 2005; Hashimoto, Maeda, & Goromaru, 1992). *In vivo* microdialysis studies have shown BZP administration causes a parallel rise in extracelluar DA and 5-HT dialystate levels in the NA with the dopaminergic effect predominant (Baumann et al., 2005). *In vitro* studies have shown that blockade of DAT reuptake attenuated BZP's release of the DAT substrate [3H]MPP. This suggests BZP's release of DA is mediated via the DAT (Baumann et al., 2005). Interestingly, BZP had no *in vitro* effect on 5-HT release from the synaptosomes (Baumann et al., 2005). The inconsistencies between *in vivo* and *in vitro* studies are similar to early observations of methamphetamine (Baumann et al., 2002). This finding may be a sign of *in vivo* interaction between DA and 5-HT release. Essentially, BZP's facilitation of DA and 5-HT is shared with MDMA. Therefore, there is little to distinguish the mechanisms of action of BZP from those of the amphetamines (Baumann et al., 2005; Johnstone et al., 2007; Yarosh, Katz, Coop, & Fantegrossi, 2007).

Importantly, BZP has abuse potential at levels similar to the amphetamines. Like the amphetamines, BZP has been shown to induce conditioned place preferences, to elicit self-administration of the drug, and to produce sensitization and cross sensitization to other stimulants (as will be illustrated below).

Studies of the rewarding properties of BZP have shown it will induce conditioned place preferences in rats (Meririnne, Kajos, Kankaanpaa, & Seppala, 2006). This place preference was attenuated by pharmacological blockade of DA D1-like receptors and 5-

HT3 receptors thereby suggesting that BZP has rewarding properties mediated via the dopaminergic and serotonergic systems. As noted earlier, these systems are involved in the rewarding properties of MDMA (Baumann et al., 2005; Glennon, Young, Rosecrans, & Anderson, 1982).

Fantegrossi et al. (2005) investigated the reinforcing effects of BZP and TFMPP in rhesus monkeys previously trained to self-administer cocaine and found that BZP acted as a reliable reinforcer whereas TFMPP did not. The subjects self-administered BZP at levels equal to or higher than what they did for cocaine. This finding suggested that BZP has abuse potential similar to that of amphetamine, whereas TFMPP does not. In addition, Campbell, Cline, Evans, Lloyd, and Peck (1973) reported a group of amphetamine addicts who liked the effects of both 10 mg dexamphetamine and 100 mg BZP. From subjective reports of the drugs' effects, participants were unable to accurately discriminate between the two but preferred the effects of BZP. The difference in dosage used suggests BZP can produce an amphetamine-type response by taking ten times the recommended dose

BZP also has the potential to act as a gateway drug for other stimulants such as methamphetamine. In a recent study, repeated exposure to BZP produced behavioural sensitization and cross-sensitization to methamphetamine (Brennan, Johnstone, Fitzmaurice, Lea, & Schenk, 2007). This finding suggests that BZP can act as a gateway for amphetamine-type drugs possibly because of dopaminergic neuroadaptations which would sensitize users to other stimulants of abuse. In addition, in their survey, Wilkins et al. (2006) found 13.5% of respondents reported starting using party pills before they mainly used other illegal drugs. Together these findings suggest that BZP has the

potential to act as a gateway for other illicit drugs. Therefore, research into such gateway effects is warranted.

To date research on the longer-term outcomes of repeated adolescent exposure to BZP is very limited. However, local research by Aitchison & Hughes (2006) has provided some preliminary results of interest. In their study, adolescent rats were administered 10 mg/kg BZP daily for ten days. When tested during adulthood 17 days later, behavioural differences were observed between BZP treated rats and saline controls. Specifically, they found BZP-treated rats displayed heightened anxiety during adulthood. Interestingly, neuronal imprinting was demonstrated as the effects of exposure to the BZP outlasted the effects of the drug itself. This was possibly due to interference with the maturation of anxiety-associated forebrain mechanisms operated by 5-HT.

In summary, BZP is a substance regularly consumed by New Zealand young people. This is a major societal concern as there is little knowledge about the long-term outcomes of BZP exposure. BZP has the potential to be abused at levels similarly observed by the amphetamines. Currently, there is little to distinguish the mechanisms of action of BZP from MDMA. Additionally, BZP has the potential to act as a gateway drug to harder illicit substances. Therefore, research which elucidates the effects of repeated adolescent exposure to BZP is required.

1.6 Ethanol

Ethanol (drinking alcohol) is one of the most widely used psychoactive substances in the world. It is a recreational substance taken for its depressant effects on the CNS. Ethanol is rarely consumed in its pure form. Instead, it is mixed with other

liquids to produce varying concentrations e.g. 12% in wine, 4% in beer (Julien, 2001). In New Zealand, alcohol containing beverages are available for purchase by those aged 18 years or older. Those under 18 can drink at a private home or function, in supervised areas of pubs or restaurants, or in a public place when their parent or legal guardian supervises them (ALAC, 2008).

At moderate to high doses ethanol impairs motor coordination and cognitive abilities such as the speed of information processing and memory recall (Sircar & Sircar, 2006). At moderate doses the effects of ethanol consumption can result in pleasurable feelings (positive reinforcement) and/or anxiolysis and stress reduction (negative reinforcement). These effects can serve as a reinforcing mechanism for people who consume alcohol (Eckardt et al., 1998).

Ethanol's mechanisms of action are complex and varied. Moderate consumption of ethanol selectively affects the function of γ-aminobutyric acid via type A receptors (GABA$_A$), glutamatergic, serotonergic, dopaminergic, cholinergic, and opioid neuronal systems (Cami & Farre, 2003; Thanos, Dimitrakakis, Rice, Gifford, & Volkow, 2005). It affects the brain by either directly acting on these systems or through interactions between them (Sircar & Sircar, 2006). The pharmacological effect of ethanol is the sum of its interactions with these systems. For example, some ethanol doses produce sedation whereas others are stimulatory. Therefore, the specific behavioural effect observed depends on the dose and how it interacts with these systems (Eckardt, et al., 1998).

The reinforcing properties of ethanol have been assessed using conditioned place preference (CPP) tests. This test involves training an animal to associate a drug with a particular environment. In the absence of the substance, reinforced animals will prefer the

environment associated with drug administration. Several studies have found evidence of ethanol CPP in the rat (e.g. Bechtholt & Cunningham, 2005; Cunningham, Niehus, Malott, & Prather, 1992). These findings confirm that ethanol has abuse potential.

Currently there is little research on the longer-term outcomes of adolescent exposure to ethanol. Research conducted by DeBellis et al., (2000) measured the hippocampal volumes of alcohol-dependent adolescents. Hippocampal volumes were significantly smaller in adolescents with alcohol use problems compared to controls. In addition, Spear (2002) suggests that females are more sensitive to the effects of ethanol than males. This could be partly due to females having shorter drinking histories than males (see Spear, 2002 for a brief review). In comparison to human studies, some animal studies have shed light on adolescent exposure to ethanol.

As mentioned earlier, adolescent exposure to ethanol leads to long-term changes in its aversive properties in rats (Graham & Diaz-Granados, 2006). Additionally, adolescent exposure has led to long-term impairment of spatial memory and learning (see Sicar & Sicar, 2005). This finding suggested that ethanol led to cognitive dysfunction of the developing brain. Further findings by Pascula, Blanco, Cauli, Minarro, & Guerri (2007) have supported this conclusion. In their study, they intermittently exposed adolescent rats to ethanol. When tested during adulthood they found impaired performance on cognitive tasks such as object recognition and discrimination learning. The impaired performance was most likely due to increased cell death in the neocortex, hippocampus, and cerebellum. Together these findings suggest adolescent exposure to ethanol can lead to long-term changes in behaviour and cognitive performance.

Therefore, more research into the longer-term behavioural and cognitive consequences of adolescent exposure to ethanol is required.

1.7 Combined Exposure to MDMA and Ethanol

As discussed earlier there is little to distinguish BZP's neurochemical and behavioural effects from MDMA. Both substances have demonstrated long-term behavioural outcomes following adolescent exposure. In addition, ethanol has also shown long-term detrimental effects when administered during adolescence. Given BZP and MDMA are similar in their effects if combined exposure to MDMA and ethanol has long-term consequences it is likely combined exposure to BZP and ethanol will have long-term effects.

To date there is only one study which addresses the long-term effects of combined exposure to MDMA and ethanol in experimental animals. In their study, Cassel et al. (2005) administered rats MDMA and/or ethanol or saline over four consecutive days. Starting four days after the last injection they monitored the animals' behaviour and neurochemistry. In the long-term the combined exposure effects on behaviour were similar to those observed by MDMA alone. However, neuropharmacological results found combined exposure had specific long-term effects on presynaptic modulation of 5-HT release in the hippocampus. They concluded that the psychological problems reported by MDMA users who concurrently drink alcohol are possibly not an exclusive result of MDMA alone. Because combined exposure to MDMA and ethanol resulted in specific long-term consequences, combined exposure to BZP and ethanol might also be expected to have long-term consequences.

1.8 The Present Study

The present study investigated the long-term behavioural effects of adolescent exposure to BZP and ethanol alone and in combination. On post natal days (PND) 41 – 50, male and female rats were treated daily with BZP (10 mg/kg) and/or ethanol (2g/kg) or saline vehicle. Subjects were tested during early adulthood (PND 78 – 81) and again during mid-adulthood (PND 117 – 120) in a Y maze, light/dark box, and an open field. Differences in behaviour at each testing age were examined to assess possible additional deterioration from early to mid-adulthood. Sex differences in behaviour were also recorded because of sex differences in brain maturation (Andersen, 2003). Given males and females brains develop differently, exposure to these substances is likely to affect the two sexes differently in the long-term.

2.0 Aims and Hypotheses

The aim of research described in this thesis was to provide an original contribution the scientific literature regarding the long-term effects of adolescent exposure to BZP and ethanol both alone and in combination. To date there has been little research into the long-term effects of adolescent exposure to these substances alone. But more importantly, there has been no research at all into the effects of adolescent exposure to these drugs when administered in combination. Because of the lack of literature on the combined effects of these substances, it was not possible to make any specific predictions. However, it was not felt unreasonable to expect there might be similar outcomes to those observed with combinations of ethanol and MDMA discussed above (Cassel et al., 2005).

3.0 Method

3.1 Subjects

The subjects were 40 male and 40 female hooded rats from the Animal Facility, Department of Psychology, University of Canterbury, New Zealand. On post natal day (PND) 30, rats were weaned and caged in 475 x 280 x 230 mm plastic cages in small groups (2-4) of the same sex for the period of the experiment. The animals were housed in a humidity-controlled (48% ± 10%) environment on a 12 hr light/dark cycle (lights on at 0800). The ambient temperature was 22˚C (± 2˚C). All rats had free access to food and water for the duration of the study. The total number of subjects and all procedures used were approved by the University of Canterbury Animal Ethics Committee (see Appendix A).

On PND 40, the rats were randomly assigned to four experimental conditions. Each group had 10 males and 10 females. From PND 41-50 subjects experienced daily exposure to either 1-Benzylpiperazine (BZP), Ethanol (EtOH), BZP + EtOH, and/or saline. All doses were freshly prepared and administered during the afternoon of the animal's normal light/dark cycle. The age of drug exposure was selected to represent the comparative human periadolescent stage of neurodevelopment in rats (Andersen, 2003). The rats were behaviourally tested on measures of timidity, anxiety, and general activity during early adulthood (PND 78 - 81) and mid adulthood (PND 117 – 120). Testing four weeks after drug administration permits us to examine the longer term consequences of adolescent exposure to these drugs. The chosen testing periods allow the rats to reach adulthood. Any behavioural differences observed during adulthood are therefore not ascribable to the acute effects of the drugs (Andersen & Navalta, 2004).

3.2 Drugs and Rationale for Doses

1-Benzylpiperazine was purchased from ABCR Gmbh & Co (Karlsruhue, Germany). Ethanol was purchased from BioLab (Auckland, New Zealand). BZP and EtOH were mixed with 0.9% isotonic saline to produce solutions of 10 mg/kg BZP and 2g/kg EtOH. Solutions were administered in a volume of 1 ml/kg. During adolescence (PND 41 to PND 50), all rats received daily exposure to the drugs and/or saline via intraperitoneal injection (i.p.) in a volume of 1ml/kg. BZP animals received an injection of 10 mg/kg BZP and one of saline. Alcohol subjects were administered 2g/kg EtOH and saline. The combined group received 10mg/kg BZP and 2g/kg EtOH daily. Finally, control rats were administered two injections of 1 ml/kg saline (Table 1 shows each animal's treatment regime and total exposure to BZP and EtOH). Two injections were required to control for the combined groups dual drug exposure. All animals received their two injections within five minutes of each other. The order of drug/saline presentation was counterbalanced so for drug-exposed animals their respective drug/s was administered both first and second on an equal number of treatment days. Intraperitoneal administration was the preferred method of delivery for its ease of use and accurate control of the quantity of drug administered. Body weights were recorded on each treatment day and doses adjusted accordingly.

BZP has approximately a tenth the potency of methamphetamine (Campbell, Cline, Evans, Lloyd, & Peck, 1973). For this reason, doses of 10 mg/kg were chosen as this is ten times the dose of methamphetamine shown to have mild stimulant effects on rats (Hughes & Greig, 1976). In addition, similar past research has used a 10 mg/kg dose (Aitchison & Hughes, 2006). For EtOH, a dose of 2 g/kg was chosen because this (or

close to) has been used in investigations of the long-term effects of adolescent exposure to alcohol in rats (Abreu-Villaca, Medeiros, Lima, Faria, Filgueiras, & Manhaes, 2007; Graham & Diaz-Granados, 2006; Sicar & Sircar, 2006).

Table 1.

Days of treatment with BZP (mg/kg), EtOH (g/kg) and Saline (S) from post natal day 41 for 40 male and 40 female rats.

Group	Days of BZP exposure	Days of EtOH exposure	1	2	3	4	5	6	7	8	9	10	Total BZP exposure	Total EtOH exposure
Control (Males, 10)	0	0	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	0 mg/kg	0 g/kg
Control (Females, 10)	0	0	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	S+S	0 mg/kg	0 g/kg
BZP (Males, 10)	10	0	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	100 mg/kg	0 g/kg
BZP (Females, 10)	10	0	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	BZP (10) + S	100 mg/kg	0 g/kg
Alcohol (Males, 10)	0	10	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	0 mg/kg	20 g/kg
Alcohol (Females, 10)	0	10	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	EtOH (2) + S	0 mg/kg	20 g/kg
Combined (Males, 10)	10	10	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	100 mg/kg	20 g/kg
Combined (Females, 10)	10	10	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	BZP (10) + EtOH (2)	100 mg/kg	20 g/kg

3.3 Apparatus and Behavioural Measures

In this study, three acknowledged tests of anxiety-like behaviour were used: responsiveness to change in a Y maze, an emergence test, and behaviour in an open field. Each of these tests were simple to use and did not require prior training of the animals or any form of deprivation or shock. In the Y maze, a more anxious rat will enter the changed arm less often, and spend less time in it than a less anxious animal (Atchison & Hughes, 2006). In the emergence test, a more anxious animal will take longer to emerge from the dark into the light than a less anxious animal (Hascoet, Bourin & Dhonnchadha, 2000). Finally, in the open field, a more anxious animal will display less rearing behaviour, occupy the centre squares less and the corner squares more often, will ambulate less and groom more often, and defecate more often than a less anxious animal (Prut & Belzung, 2003; Walsh & Cummins, 1976).

The experimental room was 22˚C ±2˚C, with humidity control 48% ±10% and dim lighting (44 lux). All testing took place between 1000 and 1900 hrs during the light phase of the animals' normal light/dark cycle. At each of the four-day testing phases, all animals were tested twice in the Y maze and once each in the open field and the light/dark emergence box. Each of the animals completed only one test a day. To control for habituation effects in the Y maze, the order of testing was arranged to ensure there were at least two days between each of these experiences. Every possible order which satisfied this requirement was determined, and each order was administered to an equal number of subjects at both testing phases. Averages of the two Y maze trials at each testing phase were calculated.

3.3.1 Responsiveness to Brightness Change in the Y-Maze

The Y maze exploits rat's tendency to explore novel areas. An anxious animal will explore the novel areas of the Y maze to a lesser extent than normal animals (Atchison & Hughes, 2006; Hughes, 2001; Hughes & Neeson, 2003).

The wooden apparatus sat on a 700-mm-high table and was illuminated overhead by dim (44 lux) fluorescent lighting 1, 390 mm above the maze. The arms of the maze were 45 cm long with a 120° angle between them. The stem of the Y was 30 cm long with a wooden barrier inserted to give a shorter stem of 15 cm. Both the stem and arms were 10 cm wide and 14 cm high. One arm contained a black and the other contained a white painted metal insert which occupied the width, height, and 40 cm of the length of the arm. A clear Perspex lid covered the entire apparatus.

In the Y maze each animal experienced a pair of acquisition and retention trials. Individual animals were initially placed in the 15 cm stem and allowed to freely roam the maze for six minutes (acquisition trial). During the acquisition trial, one arm was black and the other white. After this trial the rat was returned to its normal cage. Immediately the inserts were replaced with two clean black inserts and the entire apparatus washed and disinfected with a 20% Paraquat blue solution. Rats were returned to the stem of the maze and allowed to freely roam for three minutes (retention trial). For the retention trial, one arm had been changed from white to black (novel) while the other remained black (familiar).

Timing for the retention trial began when the subject fully entered (all four paws) an arm. For exactly three minutes, the experimenter recorded via keyboard and appropriate PC software, (1) the percentage of entries of the novel arm, (2) the percentage of time spent in the novel arm, (3) the total entries of both arms, and (4) the total time spent in both arms.

Subjects were tested in the Y maze twice at each testing phase with the novel arm randomly appearing left on one trial and right on the other for each subject. The two trials at each testing phase were averaged to represent a subject's score for this measure.

3.3.2 Light/dark Box Emergence Test

The animals were exposed to the light/dark emergence box once at each testing phase. This paradigm takes advantage of rodents' innate aversion to brightly illuminated areas. By applying mild stressors (in this case a novel environment and bright light), a natural conflict occurs between the spontaneous tendency to explore and the initial tendency to avoid bright light (Hascöet et al., 2000). One third of the apparatus consisted of a 200 x 150 x 200-mm high darkened start box (painted black on the inside) which opened into an illuminated arena via a sliding door. The illuminated arena comprised two thirds of the apparatus and was 500 x 400 x 200-mm high. The start box and the walls of the arena were constructed from wood. The roof was made of fine wire mesh and the floor of the arena constructed from translucent Perspex. The floor of the arena was illuminated from underneath by two 16-lux florescent tubes. The light intensity was 80 lux in the centre of the arena and 172 lux on the floor. The centre measurement was taken 100 mm above the centre of the floor.

Each rat was placed in the darkened start box for 60 seconds before the sliding door to the arena was opened. By means of a hand-held stopwatch, the time it took to fully emerge (all four paws) from the dark into the light was recorded. If a subject did not emerge within five minutes, the trial was terminated and the rat assigned a score of 300 seconds. The apparatus was washed and disinfected with 20% Paraquat blue between subjects.

3.3.3 General Activity in the Open-Field

The open field measures rats' anxiety generated by removing them from their social group and exposing them to a novel environment (Prut & Belzung, 2003; Walsh & Cummins, 1976). The plastic open-field was 600 x 600 x 305 mm high and placed on a 700 mm high table. The walls comprised clear Perspex to allow easy observation of each subject's behaviour. The floor consisted of black Perspex divided into a numbered 4 x 4 grid of 16 equal sized (150 x 150 mm) squares. Four squares were in the centre and twelve occupied the periphery of the field. Dim (44 lux) fluorescent lighting illuminated the apparatus 1,225 mm overhead.

A time-sampling procedure was used to measure the animals' behaviour. Individual rats were placed in the centre of the open-field and after a six second delay, time-sampling of their behaviour began. This consisted of recording their behaviour every three seconds for exactly five minutes. The time samples were determined by means of a 3-second audio "beeper" delivered to the experimenter's ear via headphones. At each interval the experimenter recorded each rat's location and whether it was rearing or grooming. Location was determined by the position of the rat's rear paws. If they were located in two different squares, a half score was assigned to each position. Rearing occurred when the animal was on its hind haunches with both front paws off the floor, either unsupported or supported by a wall. Grooming was defined as any nibbling, licking, or scratching of its body, and was preferentially recorded over rearing if the animal needed to rear to groom parts of its body. In addition, ambulation (transitions) was measured by counting the total occurrences the subject had been observed to be in a different square from its previous location within the field.

For each rat the dependent variables for this test were (1) the total rearing occurrences observed, (2) the total grooming occurrences observed, (3) total centre

occupancy observed, (4) total corner occupancy observed, (5) total transitions, and (6) total faecal boluses deposited. All animals were tested once in the open field at each testing phase. After each test the apparatus was washed and disinfected with 20% Paraquat blue.

A possible confounding variable of behavioural sampling is experimenter error i.e., observations of the behaviour emitted and when it was emitted could be inaccurate. Therefore, to rule out this source of error, a post hoc inter-observer reliability study was performed (see below).

3.4 Inter-observer Reliability Study

This study was designed to assess the possible presence of experimenter error during observations of animals in the open field. To achieve this, two additional observers independently rated the animals' behaviour.

3.4.1 Method

The subjects were four male and four female hooded rats from the saline treatment group who had completed all of the experimental test phases. The apparatus and procedure were the same as outlined above with the exception of two additional observers who independently rated the animal's behaviour. All three observers received their 3-second tones from the same audio beeper via means of multiple headphones and a multi-plug. This ensured the time sampling interval was exactly the same for each observer. Prior to commencement the additional observers were told how to code the responses. They were given practice trials to ensure their competency with the procedure. The animals in the practice trials were different from the eight animals used in the control study.

Each animal was tested once for five minutes. All dependent variables were the same as previously except faecal boluses were not counted. The apparatus was washed and disinfected with 20% Paraquat blue after each subject. The experimenter coded all data sheets and calculated the overall inter-observer reliability.

4.0 Statistical Analyses

All raw data were analysed via the statistical programme *Statistica* 8.0. Each measure was treated to a separate 4 (treatment group) x 2 (sex) x 2 (age) factorial repeated measures ANOVA. Post hoc Tukey tests were carried out when a main effect or interaction was significant ($p<.05$). Differences in behaviour at each testing age were examined to observe any additional deterioration from early to mid-adulthood. Sex differences in behaviour were tested for due to sex differences in brain maturation (Andersen, 2003). Given males and females brains develop differently, exposure to these substances is likely to affect the two sexes differently in the long-term.

For the inter-observer study, the Kendall Coefficient of Concordance (W) was calculated to assess the degree of agreement between observers.

5.0 Results

5.1 Open-Field Results

In the open field, each animal was tested once at each testing age. For main effects of treatment and sex, averages of both trials were used in the analyses. For the main effect of age, averages of each individual trial were used. The results are presented in Table 2.

Table 2.
Means (standard deviations) for transitions, corner occupancy, centre occupancy, rearing, grooming, and total defecations for saline (n=20), BZP (n=20), ethanol (n=20), and combined (n=20) treatment groups for male (n=40) and female (n=40) rats at early (n=80) and mid (n=80) adulthood, and results of F tests.

Treatment group	Saline	BZP	Ethanol	Combined	F (3, 72)	P
Transitions	53.13(16.15)	53.50(12.43)	51.23(12.82)	54.80(13.03)	0.44	>.7[a]
Corner Occupancy	40.34(14.78)	38.18(8.77)	42.53(10.91)	42.86(11.08)	1.17	>.3
Centre Occupancy	14.63(8.30)	14.84(6.97)	13.00(6.73)	12.09(6.28)	1.14	>.3
Rearing	25.40(9.19)	26.05(8.16)	26.08(9.08)	24.58(7.26)	0.27	>.8
Grooming	2.03(2.85)	1.88(1.99)	2.23(1.79)	1.95(2.14)	0.14	>.9
Defecation	3.60(2.50)	3.24(2.45)	2.33(2.44)	2.88(2.72)	1.40	>.2

Sex	Male (n=40)	Female (n=40)	F (1, 72)	P
Transitions	48.78(14.18)	57.58(11.53)	16.16	<.01[a]
Corner Occupancy	41.74(12.98)	40.21(10.14)	0.57	>.4
Centre Occupancy	14.38(7.73)	12.89(6.46)	1.45	>.2
Rearing	23.19(8.06)	27.86(8.13)	11.54	<.01
Grooming	1.75(1.58)	2.29(2.69)	1.74	>.1
Defecation	2.56(2.46)	3.49(2.59)	4.13	<.05

Age	Early adulthood	Mid adulthood	F (1, 72)	P
Transitions	55.34(11.78)	51.01(15.00)	6.43	<.05
Corner Occupancy	42.47(9.93)	39.48(13.02)	3.40	.069
Centre Occupancy	14.16(7.39)	13.10(6.88)	1.17	>.2
Rearing	25.96(7.73)	25.09(9.06)	0.79	>.3
Grooming	2.39(2.38)	1.65(1.98)	6.36	<.05
Defecation	3.33(2.54)	2.73(2.57)	3.38	.075

[a] Treatment group x Sex interaction significant ($F(3, 72) = 3.79$, $p <.05$), see text and figures 1 and 2.

5.1.1 Transitions

There was no significant treatment effect for transitions. However, there was a significant sex x group interaction. This was likely due to a significant difference between saline and ethanol for females ($p < .05$, see Figure 1). The male rats did not show any statistically significant differences (see Figure 2).

Mean Transitions for
Females
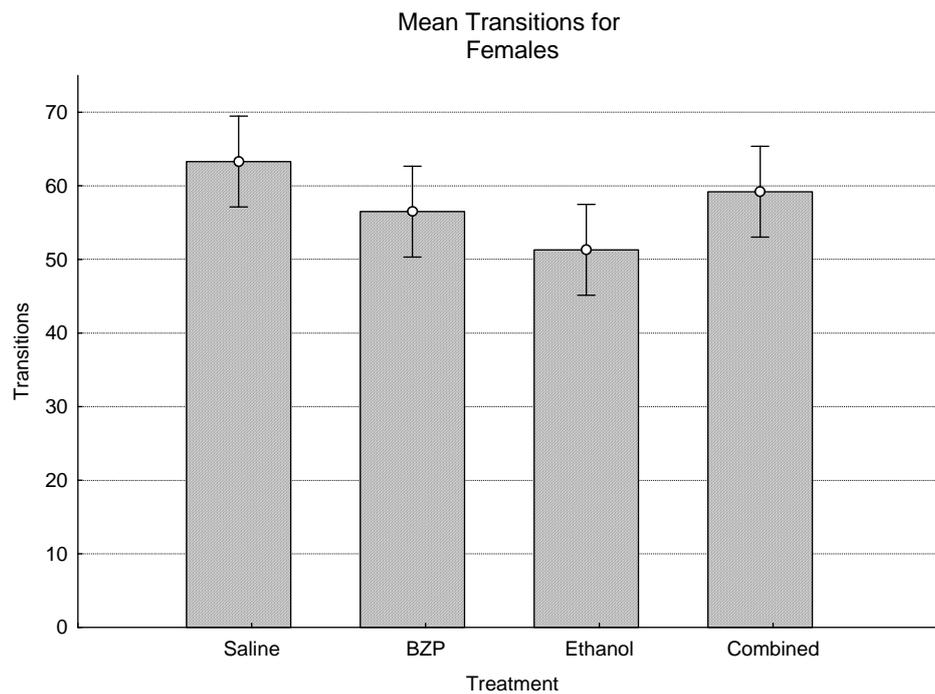
Figure 1. Mean transitions for female rat's as a function of treatment. Vertical bars denote the 0.95 confidence interval.

For female subjects, all BZP, ethanol, and combined treatment animals made fewer transitions than saline controls. However, the only significant difference was observed between ethanol and saline. Ethanol-treated rats made significantly fewer transitions than saline controls.
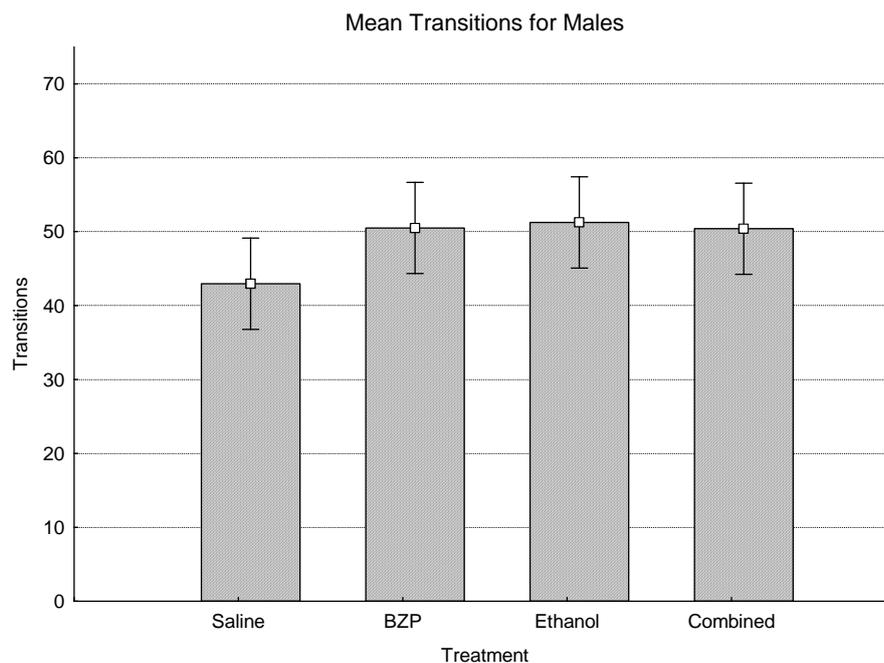
Figure 2. Mean transitions for Male Rat's as a function of treatment. Vertical bars denote the 0.95 confidence interval.

The difference observed for females was not evident for males. All treatment groups made more transitions more than saline controls, but these differences were not statistically significant ($p=.26$). Additionally, as shown in table 2 females made significantly more transitions than males, and all rats made significantly more transitions when they were younger then when older. No other interactions were statistically significant.

5.1.2 Corner Occupancy

For corner occupancy there was no significant treatment or sex effect nor were there any significant interactions. However, there was a suggestive effect for age ($p=.069$) suggesting that the rats may have occupied the corners of the open field more often when they were younger than when they were older.

5.1.3 Centre Occupancy

There was no significant treatment effect for centre occupancy. In addition, there was no effect for sex or age nor were any interactions significant.

5.1.4 Rearing

All rats engaged in about the same amount of rearing behaviour regardless of treatment or age. However, female rats reared significantly more often than male rats.

5.1.5 Grooming

There were no significant treatment or sex differences for grooming nor were there any significant interactions. However, the rats groomed significantly more often when they were younger than when older.

5.1.6 Defecation

For defecation there was no significant treatment effect. However, there was a significant sex difference. In the open field females defecated more often than males. There was also a suggestive age effect ($p=.075$) indicating that the rats may have defecated more when they were younger than when older.

5.2 Light/dark Box Emergence Results

In the light/dark emergence test, each animal was tested once at each testing age. For main effects of treatment and sex, averages of both trials were used in the analyses. For the main effect of age, averages of each individual trial were used. The results are presented in Table 3.

Table 3.

Means (standard deviations) for emergence latencies in the light dark emergence box for saline (n=20), BZP (n=20), ethanol (n=20), and combined (n=20) treatment groups for male (n=40) and female (n=40) rats at early (n=80) and mid (n=80) adulthood, and results of F tests.

Treatment group	Saline	BZP	Ethanol	Combined	F (3, 72)	P
Emergence-latency (sec)	139.47(105.62)	124.71(102.60)	145.35(111.99)	142.08(114.65)	0.23	>.8

Sex	Male (n=40)	Female (n=40)	F (1, 72)	P
Emergence-latency (sec)	151.94(109.13)	123.86(105.84)	2.13	>.1

Age	Early adulthood	Mid adulthood	F (1, 72)	P
Emergence-latency (sec)	105.53(89.25)	170.28(115.86)	23.95	<.01

As shown in Table 3, for emergence latency there was no significant effects for treatment or sex. However, there was a significant effect for age. The results indicated that the rats took approximately one minute longer to emerge from the dark into the light when they were older than when younger. There were no significant interactions.

5.3 Y-maze results

Each rat was tested twice in the Y maze at each testing age. An average of the two scores was used to represent the animals overall score for that testing age. For main effects of treatment and sex, averages of both trials were used in the analyses. For the

main effect of age, averages of each individual trial were used. The results are presented

in Table 4.

Table 4.
Means (standard deviations) for time spent in and entries of both arms and the novel arm
for saline (n=20), BZP (n=20), ethanol (n=20), and combined (n=20) treatment groups
for male (n=40) and female (n=40) rats at early (n=80) and mid (n=80) adulthood, and
results of F tests.

Treatment group	Saline	BZP	Ethanol	Combined	F (3, 72)	P
Total time (sec)	61.78(15.59)	58.71(17.25)	60.57(19.21)	57.46(15.59)	0.46	>.7
% Novel time	56.95(13.50)	57.84(18.32)	54.43(16.72)	56.48(14.39)	0.45	>.7
Total entries	5.58(1.71)	5.15(1.86)	5.50(1.96)	5.50(1.78)	0.67	>.5
% Novel entries	58.10(11.69)	53.39(16.02)	57.10(14.39)	56.85(12.93)	0.80	>.4

Sex	Male (n=40)	Female (n=40)			F (1, 72)	P
Total time (sec)	54.42(17.80)	64.84(14.24)			13.29	<.01
% Novel time	57.01(18.42)	55.84(12.62)			0.59	>.4
Total entries	4.71(1.72)	6.15(1.64)			21.19	<.01[a]
% Novel entries	56.70(16.65)	56.02(10.38)			0.09	>.7

Age	Early adulthood	Mid adulthood			F (1, 72)	P
Total time (sec)	58.35(19.32)	60.91(14.07)			0.58	>.4
% Novel time	55.64(17.25)	57.22(14.16)			0.07	>.7
Total entries	5.36(2.01)	5.50(1.61)			0.61	>.4[a]
% Novel entries	54.69(14.89)	58.03(12.56)			2.38	>.1

[a]Sex x age interaction effect significant ($F(1, 72) = 10.15$, $p<.01$), see text.

5.3.1 Total Time

There was a significant effect for sex with females spending more time in both

arms of the Y maze than males. No other main effects or interactions were significant.

5.3.2 %Novel Time

For % time spent in the novel arm there were no significant main effects or

interactions.

5.3.3 Total Entries

There was a significant sex effect for total entries of both arms of the apparatus. Females entered the arms more often than males. Additionally, there was a significant sex x age interaction effect. This difference was most likely due to males entering the arms significantly more at mid adulthood (M = 5.10, SD = 1.61) than at young adulthood (M = 4.33, SD = 1.75), $F(1, 36) = 9.51$, $p<.01$. Females entered the arms more at young adulthood (M = 6.40, SD = 1.72) than at mid adulthood (M = 5.90, SD = 1.30). However, this difference was not significant, $F(1.36) = 2.17$, $P>.10$. No other main effects or interactions were significant.

5.3.4 %Novel Entries

For % entries into the novel arm there were no significant main effects or interactions.

5.4 Inter-Observer Reliability Study

Overall, the inter-observer reliability for behaviour in the open-field was very good. The raw data presented in appendix B show little differences in ratings between the judges. The Kendall Coefficient of Concordance (*W*) was calculated to assess the degree of agreement between the observers. For each behaviour, *W* indicates the extent of agreement between the observers. When *W* equals 1.0, all observers completely agreed about how much of a given behaviour a subject showed relative to other animals.

For transitions, corner occupancy, centre occupancy, and rearing *W* was 0.98 in each case, thereby indicating a very high degree of agreement between the observers. Since grooming rarely occurred (see appendix B), *W* was not calculated for this response.

These results suggest that reliability of the experimenter's observations had not been a major source of error during earlier open-field testing.

6.0 Discussion of Results

In this study, adolescent rats were administered BZP and/or ethanol, or saline vehicle. All animals received daily exposure to 10 mg/kg BZP, 2 g/kg ethanol, BZP + ethanol, or saline via i.p. injection during the periadolescent stage of development (PND 41 – 50, Andersen, 2003). Following the last injection, animals were tested on three measures of anxiety-like behaviour during early adulthood (PND 78 – 81) and again during mid-adulthood (PND 117 – 120).

6.1 Summary of Results

The results revealed a lack of behavioural differences between the drug-treated animals and saline controls. This means that, exposure to BZP and/or ethanol during adolescence did not influence the anxiety-like behaviour of the rats when they reached adulthood. Therefore, the expectation that there might be differences in behaviour between the treatment groups was not supported. The results of the three tests are summarized as follows.

In the open field, there were no differences between the treatment groups on measures of corner occupancy, centre occupancy, rearing, grooming, and defecation. However, for transitions, females treated with ethanol ambulated significantly less often than saline females. During observations of animals in the open-field the experimenter could have been a source of error. The inter-observer reliability study was designed to exclude this possibility. This revealed a high degree of agreement between all observers regarding the frequencies of each response emitted by each animal. Consequently, the experimenter can be excluded as a significant source of error during open field observations. In the light/dark emergence box there was no difference in time to emerge from the dark into the light between the treatment groups. Finally, in the Y maze there

were no treatment effects on measures of time spent exploring the arms, percent time in the novel arm, total entries of both arms, and percent entries of the novel arm. Together, with the possible exception of ethanol treated females, these results suggest adolescent exposure to BZP and/or ethanol did not have adulthood consequences on these measures of anxiety-like behaviour.

6.1.1 Sex Differences

Significant sex differences were observed in both the open field and the Y maze. In the open field females ambulated, reared and defecated more than male rats. Open-field studies using both adult female and male rats have found females ambulate and rear more than males (Andersen, 2003; Slob, Huizer, & Van der Werff Ten Bosch, 1984; Valle & Bols, 1976). In general, females are more active than males in the open-field. However, this difference is not observed until after puberty (see Slob et al., 1976). Interestingly, contrary to usual findings (Masur, 1972; Slob, Bogers, & Van Stolk, 1981), the females defecated more than males during open-field testing. This could mean that, in this particular study, the females were more anxious than the males. However, this is not likely as the difference was small and females also ambulated and reared more than males. Finally, males and females showed a difference in responsiveness to ethanol treatment. Female rats treated with ethanol ambulated less than female controls. Males did not show this difference. An explanation for this finding is that adult female increases in open-field ambulation are attributed to the organizing effects of adolescent gonadal steroid exposure (Slob et al., 1984). Ethanol exposure during this period could have interfered with this mechanism thus decreasing ethanol-exposed females' ambulatory behaviour in adulthood. This finding suggests adolescent ethanol exposure might affect females more than males. Further research is required to substantiate this possibility.

In the Y maze, females made more entries of the arms and spent more time in them. This finding is consistent with most previous research on sex differences in Y-maze performance (Hughes & Neeson, 2003; Palanza, 2001). Together the results from the open field and Y maze are consistent with the view that females show more exploratory activity than males (Andersen, 2003; Slob et al., 1984; Valle & Bols, 1976).

6.1.2 Testing -age Differences

Significant testing-age differences were observed in each of the tests. In the open field, the rats ambulated and groomed less when they were older than when younger. Less ambulation when older is consistent with the notion of rodent age-related differences in emotionality. The findings suggest that, as rats get older they increase their anxiety-like behaviour (Boguszewski & Zagrodzka, 2002 Miyagawa, Hasegawa, Fukuta, Amano, Yamada, & Nabeshima, 1998). In the open field, older rodents show less locomotor activity than younger animals (Boguszewski & Zagrodzka, 2002). The decrease in grooming behaviour is interesting and difficult to explain. One study of female rats also found a decrease in grooming behaviour with age (Scimonelli, Marucco, & Celis, 1999). The generally accepted explanation is that rodents display less activity with age (Miyagawa et al., 1998; Scimonelli et al., 1999). In addition to these findings, there were nonsignificant suggestions of less corner occupancy and defecation in older rats. Less corner occupancy is consistent with decreases in activity with age. Less defecation is harder to explain. Findings suggest rodents defecate less over successive exposures to the open field. This is due to less anxiety because of familiarity with the apparatus (Walsh & Cummins, 1976). In summary, the rats were less active in the open field when older than when younger. This is consistent with previous research.

In the light/dark emergence box, the rats took longer to emerge from the dark into the light when older than when younger. Hascöet et al. (2001) argued that naïve rats usually take longer to emerge than rats that have had previous exposure to the test. However, this finding is for rodents within the same developmental period. Therefore, the increase in emergence time with age is consistent with older rats being less active and more emotional (Scimonelli et al., 1999).

In the Y maze, there was a significant sex x age interaction. Male subjects entered the arms more often at mid-adulthood than they did at young adulthood. This finding is inconsistent with rodents displaying less activity and more anxiety when older. Casual observations of the males' behaviour suggested that they were more cautious about entering the arms when they were younger. When younger, the males tended to visually inspect the arms more often than females before entering them. This observation is supported by suggestions that female rats have superior visual exploratory capabilities than males (Hughes, 2001). However, the behaviour was less evident at mid-adulthood and may have been a result of repeated testing. At this age the rats had already been tested at least twice in the Y maze. The anxiety induced by the Y maze's novel environment was probably reduced because of familiarity with the apparatus. In summary, several testing age differences were observed. However, no additional deterioration from early to mid-adulthood as result of adolescent treatment was evident.

6.2 Methodological Strengths

The methodological strengths and limitations of this study need to be discussed to address the extent to which these findings can be generalized. The current experiment has several strengths and advantages. The use of rats is advantageous as they develop more quickly than humans do. To detect the consequences of adolescent BZP and ethanol

exposure on adulthood behaviour in humans would take many years. However, rats reach adulthood approximately two weeks after the periadolescent period (Andersen, 2003, Spear, 2000). This means the result of such exposure on adulthood behaviour can be detected much more quickly in rats than in humans. Detecting any negative outcomes in rats means that some understanding of the implications for humans can be acquired before clinical data are available. The aim of this research was to provide a longer-term assessment of consequences following adolescent BZP and ethanol exposure. The 28 days between last drug treatment and first testing means any differences in behaviour observed would not have been a result of the acute effects of the substances.

Applying findings from animal studies to humans should be done with caution. Animal studies of long-term effects of drug exposure provide a model for the enduring effects of substances rather than an exact replication of the human condition (Smith, 2003). Therefore, the strength of pre-clinical studies is to provide evidence of the effects of substances for use in clinical assessments (Andersen & Navalta, 2004).

Human users of BZP and ethanol often use other drugs (e.g. nicotine, caffeine, MDMA) in addition to these substances. Such poly drug use can 'cloud' the accuracy of clinical assessments and human drug research. The limitations of human drug research can include the non-random assignment of participants to drug conditions, the deleterious effects of other psychoactive substances, and the possibility that adverse effects observed could be a result of drug user's susceptibility to psychopathology rather than the substance itself (Parrott, 2000). Therefore, the use of animals is useful for conducting experimental research. In this study, any differences observed (or the lack of in this case) is not a result of underlying psychopathology as subjects were randomly assigned to conditions. In addition, all subjects' experiences were the same as they were handled, weighed, treated, and tested in exactly the same manner. Poly drug use was not an issue

as the animals were only administered the substances appropriate to their treatment group. Therefore, this study addresses some of the limitations commonly encountered in human drug research.

Finally, the last major strength of this research was the inter-observer reliability study. The result of this study excluded the experimenter as a source of error during open-field observations. Therefore, observational errors were probably not a major confounding variable.

The aim of this research was to provide the first model of long-term behavioural outcomes following repeated adolescent exposure to BZP and/or ethanol. While even taking account of its methodological strengths, the findings of this study were intended to be preliminary. It was designed to facilitate additional research in this area by providing a credible starting point. Consideration of the methodological limitations will hopefully help direct future investigations in this area.

6.3 Methodological Limitations

During this study, several limitations became apparent. The acute effects of drug exposure were not measured. Casual observations of BZP's acute effects were interesting. Soon after treatment with BZP or BZP + ethanol, these animals displayed hyperactivity and excessive grooming. Importantly, these acute effects are typical of the acute effects of MDMA (Vorhees, Reed, Skelton, & Williams, 2004). These effects have been observed in rodents and rhesus monkeys (Lile, Ross, & Nader, 2005; Vorhees et al., 2004) Therefore, this study would have benefited from measurements of some of the acute effects of exposure to the substances as casual observations of BZP suggested it had similarities to MDMA in its behavioural effects.

The second limitation which became apparent was the relatively narrow range of testing ages. Four days of testing at each phase of adulthood was expected to be adequate to detect any behavioural differences. However, the length of each of these phases is longer than the four days the subjects were tested. The experiment may have benefited from testing over the entire duration of these periods. This limitation is discussed in detail in the *General Discussion* section.

This study would have benefited from neurochemical analysis of the rats' brains but such analyses were outside the scope of this thesis. Nevertheless, if it could have been arranged, neurochemical analyses of the brain regions where the substances are active would have been informative. For example, abnormal 5-HT activity has been implicated in both anxiety and depression (Graeff, 2002; Piper, 2007). In comparison, DA activity has been implicated in motor coordination as rats administered a D1 DA receptor antagonist display less locomotion and rearing behaviour (Hoffman & Beninger, 1985; Spina, Longoni, Mulas, Chang, & Chiara, 1998). Therefore, if differences in adulthood neurochemistry were found this would have indicated long-term consequences following adolescent drug exposure.

The use of intraperitoneal (i.p.) injection as the method of drug delivery could be considered a limitation. Human users of BZP and ethanol usually administer them orally. Rats will voluntarily take ethanol orally (e.g. Thanos et al., 2005). However, it is not known if this also applies to BZP. Consequently, i.p. injection was preferable as it ensured the subject received an accurate dose. Most importantly all animals were exposed to the same treatment procedures. This means the results are not attributable to differences in dosing procedures.

Finally, in the light/dark emergence test the presence of the experimenter could have distracted the animals. Supporters of the light/dark test argue that automated

versions of the apparatus are highly preferable as there is nobody present during testing (Bilkei-Gorzo, Gyertyan, & Levay, 1998; Hascöet et al., 2001). The use of an automated apparatus would have removed any extraneous influences arising from the presence of the investigator. However, in this study the same experimenter was present for all light/dark tests so that behavioural differences were at least not attributable to the presence of different observers.

In summary, although this study had several limitations, none of them were sufficient to completely invalidate the results. Therefore, it can be reasonably concluded that adolescent exposure to BZP and/or ethanol did not result in observable differences in adulthood behaviour. However, even though this particular study did not find behavioural differences this does not mean that adolescent exposure to these substances definitely has no longer-term outcomes. The remainder of the discussion will address this possibility.

6.4 Relationship to Previous Findings

One of the findings of the present study was that adolescent exposure to BZP alone did not have longer-term consequences. This was inconsistent with previous research.

Atichison and Hughes (2006) daily exposed adolescent rats to 10 mg/kg BZP during PNDs 45 – 55 and assessed their behaviour during early adulthood (PND 72 – 95). All rats were observed in the Y maze (four times), open-field social interaction test (twice), and the light/dark emergence box (six times). In the Y maze, treated animals entered the novel arm less often, spent less time in it, and entered and spent less time in both arms than saline controls. In the open field, treated subjects ambulated and reared less, and engaged in fewer social interactions than controls. Finally, in the light/dark emergence test, animals previously treated with BZP took longer to emerge than controls.

Together, their findings are consistent with treated rats displaying heightened anxiety during adulthood. Unfortunately, comparable results were not found in this experiment.

There is little research which investigates the longer-term effects of adolescents exposed to ethanol. Thus far studies have shown adolescent rats exposed to alcohol develop attenuated ethanol-induced conditioned taste aversions and impaired spatial memory and learning when adults (Graham & Diaz-Granados, 2006; Sicar & Sircar, 2005). In addition, adolescent but not adult ethanol exposure impaired tone conditioning in an auditory fear task in the long-term (Bergstrom, McDonald, & Smith, 2006). This finding suggests that adolescent ethanol exposure can impair adulthood fear learning. Each of these findings demonstrates longer-term consequences of ethanol exposure. However, the studies concerned did not address the consequences for anxiety-like behaviour.

In their study, Salimov et al. (1996) investigated the long-term outcomes of adolescent ethanol exposure on anxiety-like behaviours. They repeatedly treated adolescent rats with ethanol and subsequently tested their behaviour during adulthood. Subjects were tested on measures of novelty-induced anxiety and stress as a result of being exposed to an inescapable situation. Interestingly, they found adolescent ethanol treatment reduced the anxiety associated with the novel environment and the stress associated with the inescapable situation (Salimov, McBride, McKinzie, Lumeng, & Li, 1996). A comparable finding of long-term ethanol induced reduction of anxiety was not evident in this experiment.

Given that the present study was the first to investigate the longer-term effects of combined BZP and ethanol exposure on the developing brain, no direct comparisons can be made. Because Cassel et al. (2005) found combined exposure to MDMA and ethanol during adolescence resulted in specific long-term effects on presynaptic modulation of 5-

HT release in the hippocampus, it is possible that combined exposure to BZP and ethanol might have long-term effects on memory. In addition to measuring anxiety-like behaviour, the Y maze is useful for measuring short-term memory (Hughes & Neeson, 2003). However, results for animals in the combined treatment group did not suggest any impairment of short-term memory compared to controls. This was because of the lack of between-groups differences in percentages of time spent in and entries of the novel arm. These measures are indices of spatial memory in rats (Hughes & Maginnity, 2007). In summary, although some earlier research has suggested that adolescent exposure to BZP or ethanol can have long-term consequences for anxiety-like behaviour in rats, the present study did not produce comparable results for these substances alone or in combination.

7.0 General Discussion

The findings of this research did not support the expectation. When adults, adolescent rats exposed to BZP and/or ethanol were no different behaviourally from saline controls. The following section will discuss the theoretical significance of the results, provide an explanation for this finding and suggest improvements to this study. Finally, the implications of this research will be discussed.

7.1 Neuronal Imprinting

This study intended to demonstrate neuronal imprinting of the substances individually and in combination. In other words, it was anticipated that the effects of the drug exposure on the developing nervous system would have detrimental effects well into adulthood following a sustained period of abstinence. Contrary to expectations, neuronal imprinting was generally not demonstrated. However, ethanol-treated females are a

possible exception. These animals displayed less ambulation in the open field than saline females thereby suggesting that ethanol exposure during adolescence had enduring sex-related effects which outlasted acute effects of the substance. Therefore, it is possible that ethanol-treated females demonstrated neuronal imprinting. Since these subjects did not show any other differences it is difficult to determine if they were more anxious or if this finding was simply an aberration. Additionally, combined exposure females did not show this difference. However, it is not known if BZP attenuated this effect. Clearly, further research is required to clarify this outcome. In summary, this research did not find support for the neuronal imprinting account of drug exposure as there was a lack of behavioural differences during adulthood testing.

7.2 Explanation and Suggestions for Improvement

Although the present research did not reveal behavioural differences in adulthood (thereby failing to support the neuronal imprinting theory), this does not mean that differences would never appear. Therefore, some explanation for why differences were not found seems justified especially in the context of how the study differed from previous studies.

In their study, Aitchison and Hughes (2006) exposed rats to BZP on PND 45 – 55. They selected this time of administration to represent exposure during the latter portion of adolescent development. In the current study, subjects were treated on PND 41 – 50. This period was selected to represent the mid to later stage of adolescent development (Andersen, 2003) as this period represents a time when young persons have a high level of experimentation with substances (Spear, 2000). Therefore, it is possible the differences in results could have arisen from the different periods of brain development when the drugs were experienced.

Interestingly, past studies on the long-term effects of adolescent exposure to drugs have frequently used varying exposure periods. For example, Achat-Mendes et al. (2003) administered MDMA or Ritalin to mice on PND 26 – 32. Fone et al. (2002) exposed rats to MDMA twice daily on PND 39 – 41. In their study, Sircar & Sircar (2006) treated rats daily with ethanol on PND 30 – 36. Finally, Salimov et al. (1996) repeatedly administered rat's ethanol 3 – 8 weeks post natal. Each of these studies found adulthood consequences following adolescent exposure. Given that previous research has demonstrated adulthood consequences following a variety of adolescent treatment periods with both stimulants (e.g. BZP, MDMA, Ritalin) and depressants (e.g. alcohol) it seemed likely that the selected period for the present study would yield adulthood consequences for the substances alone and in combination. However, this was not the case. Therefore, further research which targets different adolescent treatment periods is clearly required.

In addition to the treatment period, the period of adulthood testing varied between this study and Aitchison's and Hughes' (2006). In their study, animals received all adulthood testing during the 23-day period PND 72 – 95. In the present study, animals were tested over two separate four-day periods i.e. early and mid-adulthood. The four days of testing at each period were relatively narrow in comparison to their study and to the duration of the periods themselves. The early adulthood period extends from PND 70 to approximately PND 80. In comparison, the mid-adulthood period extends from PND 110 – 120 (Andersen, 2003). Since the current study only required four days of testing at each age, the end of the periods were chosen. The use of limited testing days at specific periods is the best explanation for why no differences were found.

During behavioural testing Aitchison and Hughes (2006) repeatedly tested subjects on each measure (e.g. six times in the light/dark box) throughout the 23 days.

Averages for each animal from each test were used to represent their performance (Cassel et al., 2005 provides another example of this method). In my opinion, this method provided a broader indication of the animal's behaviour during adulthood rather than a specific indication that was employed by the current project. As a result, their testing period encompassed an age range which was wide enough to detect adulthood differences in behaviour. Therefore, the use of a narrow range of testing ages reduces the chance of observing differences which could be apparent across the broader adulthood period. The use of broader testing periods would be an improvement on the current study as this would provide a better representation of adulthood as a whole. If changes resulting from BZP and/or ethanol exposure were to be found, the testing periods could be adjusted to specify when the deficits occur and for how long they persist.

Given that both BZP and ethanol affect DA and 5-HT transmission, this study would have benefited from neurochemical analyses of the brain regions where these substances are active (Baumann et al., 2005; Thanos et al., 2005). Differences in the functioning of these neurotransmitters might have indicated long-term consequences even without the presence of behavioural changes. For example, animals with damage to the 5-HT system display heightened anxiety compared to controls (Graeff, Viana, & Mora, 1997). In comparison, abnormal DA activity has been linked to motor control deficits and aggressive behaviour (de Almeida, Ferrari, Parmigiani, & Miczek, 2005; Spina et al., 1998). Therefore, if this study had found altered activity of 5-HT and/or DA in adulthood it would indicate that future research would likely find behavioural deficits when appropriate testing ages are used.

In summary, the use of a narrow range of testing ages is a possible explanation for the lack of adulthood behavioural differences. This research would have benefited from neurochemical analyses. This would have provided an additional indication of the long-

term consequences of BZP and ethanol exposure. Future studies should first employ wider ranges of testing ages to provide a broad indication of the animal's adulthood behaviour.

7.3 Implications

This is the first experimental research to assess the longer-term consequences of individual and combined effects of adolescent exposure to BZP and ethanol. Neuronal imprinting (i.e., the effects of drug exposure outlasting the drug itself) of these substances was not found. However, this outcome still has implications for New Zealand society given there is much media attention and public debate about the safety of party pills and their use with alcohol.

The most crucial implication of this research is its significance for young people who frequently use party pills and alcohol. As discussed earlier, even though the study did not reveal adulthood changes in anxiety-like behaviour, it does not mean that other differences do not exist. Future studies which address the limitations outlined above are more likely to detect any negative outcomes following adolescent exposure to these substances. Thus far, using party pills during adolescence has been linked with possible heightened anxiety during adulthood (Aitchison & Hughes, 2006). The stimulant drug MDMA ('ecstasy') which is very similar to BZP has also shown some long-term consequences. Young people who use ecstasy have increased chances of developing mood disorders latter in life. These include depression and generalized anxiety disorder (Fone et al., 2002; Lieb et al., 2002). Additionally, young persons who use ecstasy are likely to have an increased responsiveness to the rewarding effects of other drugs (e.g. cocaine) later in life (Achat-Mendes et al., 2003). In comparison, young people who frequently use alcohol are more likely to have learning difficulties and problems with

memory when they are adults (Pascual et al., 2007; Sircar & Sircar; 2005). The effects of frequent use of party pills and alcohol on the developing brain are likely to negatively impact on the quality of life of the users during adulthood. Therefore, the frequent use of these substances by young persons in New Zealand is a major societal issue. The public in general should take this and similar future research into consideration when evaluating party pills safety and legal status.

8.0 Future Directions

This study has indicated the direction future research should take. Firstly, the limitations of this experiment need to be addressed. Future studies should use broader testing age ranges to detect any behavioural differences. When these differences have been observed, additional studies could determine if these deficits are maintained through out the life span. Neurochemical analyses of brain tissue are required. These analyses will identify the neural mechanisms which underlie the deficits associated with adolescent exposure to BZP and ethanol.

Following BZP exposure, acute hyperactivity and excessive grooming were observed but not measured. Although increased ambulation, and head bobbing have been found soon after BZP treatment (Baumann et al., 2005) no other research has indicated the level of hyperactivity or grooming observed in this study. Additional research which investigates this casual observation is justified as such behaviour is stereotypical of the acute effects of MDMA exposure (Johnstone et al., 2007). Hypofunctioning of the mesolimbic and mesocortical DA system has been associated with the impulsive and hyperactive behaviour observed in attention deficit/hyperactivity disorder (Sagvolden, 2000). Given that BZP has major effects on mesolimbic DA levels (Yarosh et al., 2007), it is possible that a similar effect could account for the hyperactivity and excessive

grooming observed in the present study. Clearly further research is required to substantiate this possibility.

Finally, adolescence represents a period of special vulnerability to the enduring effects of drugs (Chambers et al., 2003). However, there is limited research on the long lasting effects of adolescent drug exposure. Fortunately, this line of research has gained momentum over recent years (e.g Achat-Mendes et al., 2003; Aitchison & Hughes, 2006; Sircar & Sircar; 2006). The adolescent period deserves special attention as this is when individuals are most likely to experiment with drugs (Spear, 2000). Therefore, researchers should continue to investigate the effects of periadolescent drug exposure.

9.0 Conclusions

Substance use by young people is inevitable. Some experimentation with substances (e.g. alcohol, nicotine) can be considered a normal part of a young person's development (Arnett, 1992). However, ensuring that young people avoid illicit substances is difficult. Legislation informs young people that illicit substances should not be taken as they have been deemed harmful and unsafe. However, educating our children about the dangers of drug use will always remain our most powerful weapon against adolescent substance use. Fortunately, in New Zealand school based programmes which address the dangers of drug use exist (e.g. DARE, YES Resources, 2008). These programmes should be encouraged by parents and teachers of children who are about go through the adolescent period of development.

The main limitation of the current study was the relatively narrow adulthood testing ages. The aim of this study was to demonstrate behavioural differences in adulthood following adolescent exposure to BZP and/or ethanol. However, with the possible exception of ethanol-treated females, adolescent rats treated with BZP, ethanol,

or both did not show behavioural differences in adulthood compared to controls. This finding was most likely due to the narrow range of testing ages adopted in the study.

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Yes Resources. *http://www.police.govt.nz/service/yes/resources/dare/.* Retrieved March 25th. 2008

Appendix A

AEC Ref: 2007/23R

19 September 2008

Mr James Perry
Psychology
UNIVERSITY OF CANTERBURY

Dear Mr Perry

I am pleased to inform you that the Animal Ethics Committee has approved your application entitled: "Long-term behavioural effects of benzylpiperazine and its interaction with alcohol on adolescent rats"

Approval has been granted:

(a)   for the use of a maximum of 80 hooded rats
(b)   for your research project to be undertaken over a period six months from 16 April to 31 October 2007. If you require an extension of this period please contact the AEC Secretary.

Please find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information.

Also enclosed is a copy of the MAF Animal Manipulation Statistical form (and list of Animal Type Codes and brief guideline notes) which you are required to complete and return to the Secretary of the AEC (Mrs Deborah Wekking, Level 6 Registry) 30 days after the completion of this project, or once every three years, which ever comes first. If no animals have been manipulated in your project please provide a "Nil" return.

Yours sincerely

Associate Professor Jim Briskie
Chair
Animal Ethics Committee

c.c.   Animal Ethics Committee

Appendix B

Transitions

Subjects

	1	2	3	4	5	6	7	8
Observer 1	58	50	6	34	0	34	24	25
Observer 2	58	52	7	38	0	37	26	24
Observer 3	56	52	8	36	0	34	24	25

Corner Occupancy

Subjects

	1	2	3	4	5	6	7	8
Observer 1	48.5	35.5	93	51.5	100	27	58	23
Observer 2	48	33.5	92.5	93	100	27.5	54	19.5
Observer 3	49	35.5	93	47	100	30	55	21.5

Centre Occupancy

Subjects

	1	2	3	4	5	6	7	8
Observer 1	7.5	18.5	2	5	0	21	6	1
Observer 2	6.5	20.5	2	4	0	22	6.5	1
Observer 3	7	20.5	2.5	4.5	0	21.5	7.5	1

Appendix B contd.

Rearing

Subjects

	1	2	3	4	5	6	7	8
Observer 1	24	31	7	27	0	9	14	5
Observer 2	22	29	10	26	0	12	20	6
Observer 3	24	28	9	26	0	11	19	5

Grooming

Subjects

	1	2	3	4	5	6	7	8
Observer 1	1	0	0	1	0	4	0	0
Observer 2	0	0	0	1	0	4	0	0
Observer 3	1	0	0	0	0	4	0	0