

**Subsequent behavioural development of offspring exposed to methadone during
gestation, lactation or both**

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Abstract

Previous research into the subsequent effects of prenatal methadone exposure has primarily focused on neurological changes and short term physical development. While there have been some studies of behavioural development, only short term effects have been investigated. The present research therefore aimed to assess longer term behavioural development of offspring exposed to methadone gestationally, lactationally or both. Methadone was provided in the drinking water of drug-treated rat dams during gestation (2.39mg/kg/day) and lactation (2.86mg/kg/day). The four conditions were: non-exposure/control (N = 24), gestational-exposure (N = 20), lactational-exposure (N = 24), and combined-exposure (N = 21). As well as several measures of pregnancy characteristics, offspring postnatal physical development was assessed at 30, 60 and 120 days after birth. Behavioural assessments were also made at these ages by means of an open-field, Y maze and emergence apparatus. There were no significant differences in physical development. Maternal methadone exposure during gestation reduced the number of rat dams that became (or remained) pregnant. In the offspring, there was increased activity in lactationally-exposed rats through into adulthood. Anxiety was increased in the combined-exposure condition, primarily in adolescent males. The significant longer term effects of earlier methadone on the rats' behavioural development supported the need for more research into this hitherto relatively neglected area. More information about effects of methadone exposure on anxiety and activity, as well as on social functioning and motor coordination could be useful for understanding potential risk factors in the ever growing methadone-exposed population, and thus suggesting best practice for methadone maintenance programmes.

Section 1

Introduction

The general aim of the current research was to assess subsequent developmental effects on a number of behavioural variables (through to adulthood) of pre- and postnatal exposure to methadone. The following introduction discusses the increasing prevalence of opioid use, both in the general population and specifically in women of childbearing age. Following this, previous research findings will be summarised that concern potential pregnancy effects and the offspring's postnatal physical and behavioural development. Methadone effects on different areas of functioning will be assessed through the detailed discussion of past research in both clinical and animal samples. Shorter term outcomes will include acute drug effects on parenting ability, possible environmental influences during the prenatal period, the implications of the Neonatal Abstinence Syndrome (NAS), birth measurements, and the consequences of exposure to methadone through maternal milk during breast feeding. Then, long term behavioural effects will be discussed, including changes in activity levels, attention, cognitive development and environmental risk factors. Animal studies that suggest neurobiological impacts of prenatal methadone exposure on the offspring will also be considered. After discussion of previous research, the specific focus of the present study will be addressed.

1.1 Statistics of use

United Nations International Drug Control Programme (UNIDCP, 2001) have conservatively estimated that 80 million people worldwide abuse heroin and other opioid substances (Wilens et al, 1995). In terms of the use of opioid-type drugs, in

Oceania, these drugs accounted for approximately 33% of all drug use in 2006 (WDR, 2007). The only other drug that had a higher incidence of use was cannabis. In New Zealand, an Illicit Drug Monitoring System (IDMS) was established in order to assess drug-users' opinions of trends in illegal drug-use in the community. In 2007, frequent injecting drug users named methadone as their drug of choice as opposed to heroin (IDMS, 2008). The reason for this could be the availability and affordability of methadone compared with heroin. The transition from heroin to methadone use among drug users could potentially raise the incidence of pregnancies occurring while on methadone, highlighting the importance of developing a better understanding of the teratological effects of methadone.

Brown et al (1998) reported an estimate of 5 million women of childbearing age used illicit drugs in the United States, with opioid use among pregnant women ranging from less than 1% to as high as 21%. One of the features of chronic opioid use is the disruption to the menstrual cycle, potentially resulting in the users' assuming that contraception is unnecessary (Kakko et al, 2008). This may in turn increase the likelihood of unplanned pregnancy among this subpopulation. Each year more than 7,000 infants are born to heroin or methadone users (Dashe et al, 2002).

Along with the risk of individuals being addicted to narcotics during pregnancy, there is also the risk of direct effects on the foetus as well as the longer term risks associated with children being raised by drug-addicted parents. It is estimated that 12.5% of Americans are raised by an addicted parent, increasing the risk that they themselves may abuse drugs (Wilens et al, 1995).

1.2 *Heroin*

Dihydromorphine (heroin) is an opiate drug that is manufactured using the resin found in the opium poppy. Of all the opiate drugs abused worldwide, heroin is the most common (Carlson, 2007), with intravenous injection of heroin being the primary opioid of abuse for most of the 20th century (Hans and Jeremy, 2001). Heroin has analgesic properties and reduces stress and anxiety in the user. Heroin also produces euphoric effects, which is the primary reason it has become a drug of abuse.

Heroin is currently primarily cultivated in Afghanistan. Production in Afghanistan increased by nearly 50% in 2006, bringing opium production to a record high (World Drug Report, 2007). The WDR (2007) reported that the main problem class of drugs in the world to date is opiates, primarily heroin. From 2005 to 2006, global heroin production increased by 43%.

This extensively abused drug has a number of potential negative effects on the user. As heroin is taken more often, the user becomes increasingly tolerant to the effects, requiring more and more of the substance to achieve intoxication levels. The need for higher doses of heroin can influence the activities they are involved in to gain the drug as their habit becomes more expensive. Drug-seeking behaviours can include criminal activity, prostitution and increased risk of human immunodeficiency virus (HIV) through shared needle use (Carlson, 2007). A sub-population of heroin users is women of child bearing age. Effects of heroin use during pregnancy include spontaneous abortion, premature delivery, infections and neonatal withdrawal (Hagopian et al 1996).

1.3 *Methadone Background*

Methadone (6-dimethylamino-4, 4-diphenyl-3-hepatone-hydrochloride) is used to treat heroin-addiction as well as chronic pain (Sharpe and Kuschel, 2004). Methadone is a synthetic opioid introduced by Dole and Nyswander in 1965 as a treatment for heroin dependence (Rosen and Johnson, 1993). It is now estimated that more than 200,000 Americans are receiving methadone treatment at any one time (Wilens et al, 1995). Methadone is chemically different from heroin and morphine, but works on the same opiate receptors producing similar effects (Carlson, 2007). It reduces the cravings associated with heroin, blocks the euphoric effects, prevents withdrawal and reduces hunger (Kaltenbach et al, 1998). While the patient is still addicted to methadone, there is less intoxication and the individual is able to gain control over their drug seeking behaviours and reduce their drug-related activities (Amato et al, 2005). Methadone maintenance includes the prescription of methadone in combination with professional services. Methadone doses are determined on a case by case basis, with attempts to administer the lowest dose possible in order to reduce the severity of any withdrawal symptoms that might develop. It is, to date, the best practice for heroin addicted pregnant women, with the programme being administered in a number of different cultures and countries, including the United States, Thailand, Sweden, the United Kingdom and Oceania (Farrell et al, 1994). There are a variety of professional services and programmes that are offered in combination with methadone. These along with the delivery of services and policies differ between countries. The more common additions to treatment with methadone are counselling, mental health services, education programmes, treatment for other substances and prenatal care in pregnant individuals (Farrell et al., 1994; Wilson et al, 1994).

Opiates are δ and μ opioid receptor agonists. Methadone is a full μ -receptor agonist and is excreted in bile and urine. Methadone acts on the central nervous system (CNS) and typically crosses the placenta quite easily (Chasnoff et al, 1984). This ability to cross the placenta with ease increases the risk of methadone-related effects on foetal development, such as physical abnormalities, foetal growth retardation and neonatal withdrawal (Long and Marks, 1969; Myren et al, 2007). Methadone has a longer elimination half life than other opiates with the elimination half life averaging approximately 24 hours, with peak levels after 2-4 hours (Rosen and Johnson, 1993). This increase in half life means that the foetus goes through the harmful effects of withdrawal in the womb less often. In chronic pain sufferers it also means that patients require less medication daily.

While there are estimates of the average peak levels and drug half-life, there are still individual differences in the metabolism of methadone. Changes in pregnant women's metabolism rates through pregnancy result in changes in plasma levels in the 3rd trimester. These changes mean that there is a faster metabolism of methadone, with the elimination half life of methadone being reduced from 10 hours at 26 weeks to 8.2 hours at 38 weeks (Berghella et al, 2003; Rosen and Johnson, 1993). Because of this tendency, higher doses of methadone may be required throughout pregnancy to avoid maternal and foetal distress.

1.4 *Methadone versus Heroin*

Amato et al (2005) reviewed the findings of 52 studies assessing the effectiveness of the methadone maintenance programmes. Methadone was significantly more effective than no treatment at retaining patient compliance. These results were especially significant when methadone was administered at a flexible

dose, with patients' dosage being monitored and modified weekly to treat fluctuations in the individual's needs. More compliance during pregnancy is associated with better prenatal care, nutrition and less illicit-drug use.

Little was known about the potential effects of methadone until the late 1970s (Rosen and Johnson, 1993). Since then it has been found that compared to heroin, methadone has a decreased incidence of negative physical outcomes in the new born. It has now been found that, compared to heroin, methadone improves prenatal care, increases birth weight and decreases still birth (Kandall et al, 1976; Rosen and Johnson, 1993).

As well as methadone-exposed infants having better physical outcomes at birth compared to heroin-exposed infants, there is also the potential for improvement in maternal behaviour through professional services provided in the programme during the pre- and postnatal period. It has been suggested that methadone maintenance programmes are successful treatments for heroin because of the support systems put in place as part of the treatment package, such as drug counselling and prenatal care, as opposed to just the drug treatment itself. However, double blind studies of methadone versus placebo with and without social support services found that methadone had an effect on reducing criminal activity, illicit drug use and number of deaths in the groups (Farrell et al, 1994). These effects occurred over and above social support treatments, thereby providing support for the efficacy of methadone maintenance programmes per se.

1.5 Pregnancy Effects

While methadone appears to reduce maternal risk behaviours, methadone maintenance has been shown to increase the likelihood of a number of negative

outcomes on the developing foetus and infant during prenatal development. In part, methadone is associated with an increased risk in premature birth ranging from twice (Boer et al, 1994) to as high as four times (Arlettaz et al, 2005) the rate amongst non-users. However, within studies reporting increased prematurity rates, there is the potential confounding effect of poly-drug use. Boer et al (1994) reported increased prematurity rates but failed to distinguish between heroin-exposed vs. methadone-maintained women. Therefore, the reported effects could potentially be due to the prematurity risk associated with heroin, or other drugs the mothers were using during pregnancy not known to the researcher, (Finnegan et al, 1977; Thangappah, 2000) as opposed to methadone.

Methadone maintenance has also been reported to increase mortality rates in the unborn child (Boer et al, 1994). Higher mortality rates have also been reported in rats prenatally exposed to high doses of methadone (Barr et al., 1998). The increased mortality rate was assessed from still birth through to post natal day (PND) 7. It was suggested that the reason the rat pups died after birth may have been due to the pups' inability to nipple attach and suck, which could be attributed to withdrawal (Barr et al., 1998). It should also be noted that in studies where the prenatally exposed pups are fostered to untreated mothers, these mortality rates significantly reduced to a rate that did not differ from control pups (Hutchings et al., 1992; Hutchings et al., 1993). Another animal study was conducted by Kunko et al (1996) where they administered methadone to rats during gestation, lactation or both and found that methadone exposure during gestation reduced the litter size, particularly the number of males. It can be concluded that methadone exposure during pregnancy appears to affect the mortality rate across both animal and clinical samples, with the potential risk of sudden infant death being reduced by environmental factors such as parental care and

responsibility, as highlighted by the Hutching et al (1992) and Hutching et al (1993) studies.

While there have been studies that report an increased rate of prematurity and mortality, there have been just as many studies that have failed to find any significant differences in these pregnancy outcomes (Chasnoff et al., 1982; Kakko et al, 2008). Methadone is seen as a healthier alternative to heroin during pregnancy as it has been found to have fewer of these negative effects than heroin (Kandall et al, 1999; Rosen and Johnson, 1993).

1.6 Outcomes at Birth

At birth there are a number of adverse clinical outcomes associated with exposure to methadone during pregnancy. Methadone-exposed infants are frequently born with lower birth weights and height, increased duration and severity of Neonatal Abstinence Syndrome (NAS) and an increased incidence of microcephaly.

In contrast, no significant differences have been found between methadone-exposed infants and control infants in the number of congenital abnormalities. Even when methadone use was combined with the use of other drugs, the physical abnormalities found in the exposed group were still within the normal range of the general population (Burns et al., 1995). In their review of the effects of prenatal methadone exposure, Rosen and Johnson (1993) concluded that there was no increase in the rate of physical abnormalities in methadone-exposed babies.

1.6.1 Birth Weight

Birth weight in methadone-exposed infants is often significantly lower than in non-methadone-exposed control populations (Arlettaz et al, 2005; Bada et al, 2002;

Chasnoff et al, 1982; Chasnoff et al, 1986; Hagopian et al, 1996; Kaltenbach and Finnegan, 1987; Rosen and Johnson, 1993). To illustrate this, Arlettaz et al (2005) found that, of the 86 babies investigated in a Swiss methadone maintenance programme, 27% were growth restricted. The incidence of decreased birth weight in children prenatally exposed to methadone is nine times greater than that of children not exposed to the drug. Lower litter weights have also been reported in rats exposed to methadone during gestation (Kunko et al., 1996). Lower birth weights in clinical samples have been linked to mild problems in cognition, attention, neuromotor functioning, vision and hearing (Dombrowski et al., 2007; Hack et al., 1995; Synder et al., 2007; Vohr et al., 2000). These developmental deficits are negatively correlated with birth weight, and persist into adolescence (Hack et al, 1995; Hack et al, 2002).

Although birth weight can be significantly lower in methadone-exposed infants, there are additional factors that may exert more of an influence than methadone exposure alone. For example, Boer et al (1994) found that birth weights were lower primarily in infants requiring treatment for NAS, thereby supporting other research in which no effects were observed when NAS was controlled for (Kuschel et al, 2004). However, NAS has such high prevalence rates in methadone-exposed infants that assessing methadone effects independent of NAS would not be appropriate. Regardless of whether the decrease in birth weight is due to NAS rather than direct drug exposure, the two have such a high co-occurrence rates that even the indirect impact of NAS on methadone-exposed children is a large risk factor. It has also been shown that birth weight is affected more by cigarette smoking than by methadone. This could account for lower birth weights in these samples as cigarette smoking is four times more prevalent in drug users than in the rest of the population (Boer et al, 1994).

Maternal methadone dose is another factor that could potentially affect birth weight. There have been conflicting findings regarding the relationship between maternal dose and subsequent birth weight. In some, lower weights have been shown following prenatal exposure to high doses (Rosen and Johnson, 1993), while in others a reverse relationship has been observed with reduced birth weight following a lower dose (Dashe et al, 2002). Varying explanations have been suggested for the relationship between birth weight and prenatal dose. Research indicating that higher doses of methadone increase infant birth weight suggests that it is due to the reduction of withdrawal and the risk of prematurity, thus increasing the birth weight. On the other hand, there is research that concludes lower doses of methadone during gestation increases birth weight. It could be argued that this is because lower doses decrease negative effects on the growth of the foetus which in turn lead to increased birth weight. The causal influences behind the dose, birth weight relationship is unclear.

1.6.2 *Head Circumference*

As with birth weight, head circumference is also smaller in methadone-exposed infants. For example, Arlettaz et al (2005) observed a four fold increase in the risk of microcephaly in their cohort of 86 babies born in a Swiss methadone maintenance programme. Consistent with this finding, several other studies have also demonstrated reduced birth weight and smaller head circumference at birth in methadone-exposed infants (Boer et al, 1994; Brown et al, 1998; Chasnoff et al, 1986; Hagopian et al., Kaltenbach and Finnegan; 1987; 1996; Rosen and Johnson, 1983; Rosen and Johnson, 1993). This is of some concern given that a smaller cranial size has been shown to predict later cognitive development, attention, reasoning ability,

and to the incidence of attention-deficit hyperactivity disorder (ADHD) (Butz et al., 2005; Lahti et al., 2006; Peterson et al., 2006; Rosen and Johnson, 1993; Walker et al., 2007). However, it is difficult in clinical studies to control for environmental factors that may also influence head circumference, such as maternal nutrition, alcohol use and smoking, all of which have been shown to reduce cranial size (Barker, 1997; Lumeng et al., 2007; Salihu and Wilson, 2007).

1.7 Neonatal Abstinence Syndrome

1.7.1 Background

Neonatal Abstinence Syndrome (NAS) is a withdrawal syndrome that occurs several days after birth when the transplacental drug transfer stops but the infant continues to metabolise and excrete the substance, thereby causing it to go through withdrawal (Bada et al, 2002). The child therefore experiences a rapid decline in methadone availability which decreases levels of the drug in brain tissue, thereby increasing the availability of opiate receptors and levels of neurotransmitters. This results in changes in the ion channels, increasing neuronal excitability (Doberczak et al, 1993). Neuronal excitability increases the firing potential of the neuron, which leads to CNS excitation. Most of NAS onset occurs within 72 hours after birth, but can occur for as long as 2 weeks afterwards (Kaltenbach et al., 1998). NAS can last anywhere from 12 weeks to 6 months in humans and 20 to 25 days in rats (Hutchings, 1982).

Infants who suffer from NAS experience symptoms of CNS excitation (Bada et al, 2002; Hutchings, 1982; Rosen and Johnson, 1993). CNS-related physiological symptoms include changes in sucking rate, disturbed sleeping patterns, decreased interactive behaviours, decreased ability to self soothe and focus attention, tremors,

decreased motor maturity and poor visual habituation (Rosen and Johnson, 1993; Bada et al, 2002). As many as 70% of infants who suffer NAS have CNS irritability which may progress to seizures if left untreated (Dashe et al, 2002). Effects on the autonomic nervous system (ANS) include symptoms such as sweating, sneezing, tachycardia and hyper- or hypothermia (Bada et al, 2002).

Withdrawal symptoms in newborn rats are not well documented. Behavioural assessment of withdrawal in the adult rat may not be adequate to assess withdrawal in the newborn, as the behaviours are not age appropriate (Barr et al, 1998). Withdrawal symptoms in the rat pup are more subtle than symptoms in the adult rat. In the newborn rat, withdrawal symptoms include increased motor activity, wall climbing, decreased time spent with litter mates, changes in distress calls and head and paw movements (Barr et al., 1998; Barr and Wang, 1992; Jones and Barr, 1995). The subtle changes in behaviour can make the assessment of withdrawal in the newborn rat difficult.

Withdrawal in clinical samples is measured by assessing the number of NAS behavioural characteristics the infant presents with, the onset of NAS, and the need for and duration of treatment (Burns and Mattick, 2007; Fischer et al, 2006; Kakko, Heilig and Sarman, 2008). Pharmacological treatment may be required to prevent severe withdrawal symptoms, such as seizures. The need for treatment can have an impact on early parent-infant bonding, especially in the most extreme cases where NAS lasts as long as 6 months (Hutchings, 1982). Insecure early attachment has been linked to problems with later psychosocial development (Thompson, 2000) and social cognitive ability (Belsky and Fearon, 2002). However, continuity of attachment style is dependent on the stability of the child's environment. Therefore, when the infant's

treatment ceases, the mother's ability to develop a secure bond with her child is enhanced (Thompson, 2000).

1.7.2 *Occurrence and Severity*

There have been various estimates of the incidence of NAS in methadone-exposed infants. NAS has been reported from as few as 30-50% cases of methadone-exposed newborns (Burns et al, 1995; Chasnoff et al, 1982; Hagopian et al, 1996; Kuschel et al, 2004; Malpas et al, 1995), to as high as 70-90% (Doberczak et al, 1993; Kaltenbach and Finnegan, 1987; Rosen and Johnson, 1983; Rosen and Johnson, 1993). When compared with heroin-exposed infants, methadone-exposed babies have an increased risk of NAS and severity of symptoms (Rosen and Johnson, 1993).

The relationship between maternal methadone dose during pregnancy and the severity of NAS in the newborn has been difficult to establish. Observational studies have been used to assess the potential relationship, with most research considering the correlation between maternal methadone levels at birth and postnatal NAS symptomology in the infant. In some studies high maternal methadone levels at delivery have been associated with greater severity of NAS symptoms in the infant (Dashe et al, 2002; Hagopian et al, 1996; Malpas et al, 1995), while in others lower doses at birth were related to more severe NAS (Kushel et al, 2004). However, in other studies, no relationship between maternal dose during gestation and NAS severity in the offspring has been found (Berghella et al, 2003; McCarthy et al, 2005; Rosen and Johnson, 1993), making it difficult to draw firm conclusions from this research.

Another common method for examining the relationship between maternal methadone dose during pregnancy and NAS in the offspring is to use the average

methadone dose over the course of the pregnancy (as opposed to the dose at birth). Determining the dose at birth may not be as accurate, with individuals going into labour at varying times after their last methadone treatment. The dose recorded may also not be an adequate measure of the dose the infant has been exposed to in utero. Research findings amongst the different methods for measuring maternal dose levels are mixed, with some indicating that lower maternal doses decrease NAS severity and others suggesting that higher maternal doses may be beneficial. Dashe et al (2002) reported that higher methadone doses during pregnancy were associated with more severe withdrawal in the infants. Sixty-five babies were divided into 3 different dose groups, high (>40mg/day), medium (20-39mg/day) and low (<20mg/day). The authors found that 46% of all the babies had to be treated for withdrawal. The percentage of babies within each group requiring treatment for withdrawal ranged from 12% in the low dose group, to 90% in the high dose group (Dashe et al, 2002). These findings supported other research describing a positive correlation between maternal methadone dose and severity of withdrawal symptoms (Hagopian et al, 1996).

Despite different methodologies being employed, most research has failed to show any relationship between maternal methadone dose levels during gestation and the severity of NAS in the new born (Berghella et al, 2003; McCarthy et al, 2005). For example, Berghella et al (2003) investigated two groups – a high dose (over 60mg/day) and low dose (under 60mg/day). There were no significant differences between them in either NAS severity or treatment duration. Even when dose ranges were varied to assess any potential dose effect at varying cut offs, significant differences still failed to occur.

One factor that may account for variability across studies might be individual differences in methadone metabolism. When beginning methadone maintenance, the individual is given sufficient quantities of the drug to prevent the occurrence of any withdrawal symptoms. As women who tend to metabolise methadone faster will be those accordingly taking higher doses during pregnancy, they may therefore not actually transfer the same high dosage to their foetuses, because of their higher methadone metabolism rate (Berghella et al, 2003).

There are individual differences in metabolism of methadone for both mothers and their foetuses (Rosen and Johnson, 1993). This was demonstrated by Kushel et al (2004) who tested 25 methadone-exposed babies for NAS severity and levels of methadone in the umbilical cord as well as concentration in their blood, 48 hours after birth. It was found that babies with lower levels of umbilical cord methadone were more likely to require treatment for NAS. It was also found that if an infant had undetectable amounts of methadone in its blood ($<.000007\text{mg/ml}$) 48 hours after birth, it was also more likely to require treatment. Infants who metabolised methadone quicker presented with more severe NAS symptoms (Malpas et al, 1995; Rosen and Johnson, 1993). This illustrated the influence of metabolism rates of both the mother and infant in determining the severity of withdrawal at birth. It is therefore clear that NAS severity is not related to the dose of methadone administered during pregnancy. It is also clear that metabolism rates need to be taken into account when assessing the magnitude of any short and long-term teratological effects.

1.7.3 *Confounding Variables*

One of the challenges in the investigation of pregnant methadone-maintained women is the inability to control for poly-drug use. There is the possibility that

outcomes for infants may be due, not only to methadone exposure, but to the effect of other drugs and the interactions between drugs. In a study by McCarthy et al. (2005), 77% of mothers in the methadone maintenance programme were also tobacco smokers. Tobacco use during pregnancy has also been linked to negative postnatal outcomes for children (Choo et al, 2004). Tobacco use in conjunction with methadone during pregnancy can influence the timing and severity of NAS (Choo et al, 2004). A problem with methadone research is the difficulty in controlling for poly-drug use, namely the use of a combination of drugs as opposed to methadone alone. Poly-drug use that is reported in research designed to assess the impact of dose has ranged from 31% (Dashe et al, 2002) through to 38% (McCarthy et al, 2005). Because of the combination of various drugs, it is difficult to draw any casual links between methadone use and teratological outcomes. There have been mixed findings as to whether most use of other illicit drugs occurs in the lower methadone dose groups (McCarthy et al, 2005) or in higher dose groups (Dashe et al, 2002). Because the possible effects of this confounding variable are found amongst all methadone dose groups, it is one of the most difficult to control within a clinical population.

1.8 *Lactational Exposure*

Up until September 2001, the American Academy of Pediatrics (AAP) recommended that mothers' on methadone doses above the cut off of 20 mg/day be advised against breast feeding (Philipp et al, 2004). As the maternal methadone dose is increased over pregnancy and through lactation (Hodge and Tracy, 2007) this cut off restricted the majority of methadone maintained mothers' from nursing. However, in New Zealand in 1997, it was reported that 73% of methadone maintained mothers' breast fed (Malpas et al, 1993). In September 2001, the AAP reviewed their

recommendation and removed the restrictions on methadone maintained mothers' breast feeding (Philipp et al, 2003). The increase in the number of babies each year that would then be exposed to methadone lactationally sparked research in the area examining the relationship between maternal dose and breast milk concentration, as well as the potential therapeutic effects of breast milk on infant NAS.

Breast feeding mothers who are ingesting methadone excrete the unchanged drug through their breast milk. While breastfeeding has been suggested to improve the bonding between mother and child (Anholm, 1986; Petryk et al, 2007; Philipp and Merewood, 2004) as well as providing protection against such conditions as asthma, obesity and otitis media (Dewey, 2003; Jansson et al., 2004; Oddy et al., 2002; Saarinen, 1982), exposure to methadone in breast milk during the early prenatal period may have implications for the child's physical and behavioural development therefore need to be considered carefully.

Another important consideration in lactational exposure to methadone is assessment of the relationship between maternal dose and concentration of methadone in the mother's milk. McCarthy and Posey (2000) obtained breast milk samples from 8 breast feeding women taking varying doses of methadone. Maternal methadone dose ranged from 25 to 180 mg/day (mean: 102 mg/day). The results showed no correlation between the maternal intake and concentration of methadone in the breast milk. The range of methadone in the breast milk was .000027 to .000260 mg/ml (average: .000095 mg/ml). Based on the average daily intake of milk by a newborn, it was calculated that the daily intake of methadone through breast milk was 0.05 mg/day (McCarthy and Posey, 2000). These results are consistent with other studies that found concentration ranges as low as .000011-.000070 mg/ml (Pond et al, 1985) up to .000110-.000250 mg/ml (Greaghty et al, 1997). However, in one earlier study

higher levels of methadone in breast milk had been reported, with the concentration reaching as high as .000570 mg/ml (Blinick et al., 1975). At this concentration the infant ingests approximately 0.27 mg/day which is substantially higher than the 0.01-0.05 mg/day reported in most subsequent studies (Greaghty et al, 1997; McCarthy and Posey, 2000; Pond et al, 1985). The extreme differences in concentration levels could have been due to Blinick et al's (1975) rather outdated research methodology, compared with the more rigorous current screening processes. Current research typically screens for other risk factors, such as poly-drug use, nutrition and gestational age. However, it is noted that even at a daily dose of 0.27 mg/day, the infant is exposed to just under the lowest dose that infants being treated for NAS receive. "A 3kg neonate with mild withdrawal could be treated with 0.3 mg/day of methadone" (McCarthy and Posey, 2000; 118). Even at the highest reported concentration of methadone in human milk it is not at a level that is considered toxic or harmful to the newborn.

There seems to be a contradiction between the reported minimal dose transferred to the infant through breast milk and the ability for breast feeding to affect the severity of NAS. On the one hand it has been concluded that the low concentrations of methadone in breast milk that an infant is exposed to would not be adequate to prevent or reduce withdrawal symptoms (Begg et al, 2001; Kuschel et al, 2004). However, on the other hand, studies looking directly at the relationship between breast feeding and NAS have shown a relationship between the two. Amongst a New Zealand sample of infants that required treatment for NAS, those that were breastfed were discharged from hospital an average of 8 days earlier than those that were not (Malpas et al, 1993). Ballard (2001) also found that breastfed infants

had less severe symptoms, required a shorter treatment time and were discharged 8 to 29 days earlier than formula fed infants.

Even with the low concentration of methadone present in the breast milk, Kunko et al (1996) found traceable amounts of methadone in the rats exposed lactationally. The amount of methadone in the offspring decreased with age. These results from animal research support the findings in clinical samples that suggest breast feeding affects the severity of NAS in infants. Even though the concentration levels of methadone in lactational milk are lower than would be thought to have any treatment benefit for NAS, offspring still have traceable amounts of methadone in their system after lactational exposure and offspring that suffer from NAS appear to benefit from being breastfed.

1.9 Maternal Behaviour

Methadone exposure during pregnancy and lactation, through breast feeding, may present as a risk factor for the infant, but the environment in which the child is raised can also potentially exacerbate any risks (Chasnoff et al, 1986). In their review, Rosen and Johnson (1993) reported that prenatal methadone exposure increases the likelihood that children will be affected by negative environmental influences. One reported factor that contributed to the outcome of methadone-exposed children was the quality of the home environment, with higher incidence of family dysfunction presenting as a risk factor for developmental outcome. The increase in negative effects can also be attributed to increases in other risk factors in the child's environment. For methadone-exposed infants, there is also an increased likelihood of poverty, single parent homes, domestic violence, homelessness and lower education being involved (Kaltenbach et al, 1999).

However, methadone is related to improved maternal behaviour when compared to heroin (Burns et al., 1995; Rosen and Johnson, 1993). Methadone-maintained mothers who spend more time on prenatal care, report greater appreciation of social support groups, and reduce their drug-seeking behaviour (Caplehorn et al, 1993; Jones and Prada, 1975; Kandall et al, 1977; Soepatmi, 1994). These aspects of maternal behaviour are important predictors of developmental success, with increased time spent on prenatal care being predictive of increased birth weight and length (Boer et al, 1994). This highlights another positive aspect of methadone versus heroin exposure during pregnancy. However, when compared to matched controls, 49% of methadone-maintained woman start prenatal care later than 20 weeks compared to only 13.8% of controls (Boer et al, 1994). Although prenatal care was improved in the methadone population compared to the heroin-exposed population it still does not reach the prenatal care rates of control samples.

Another risk factor in maternal behaviour involves possible continued drug-use after pregnancy, which can have an impact on the parents' ability to care for the child postnatally. Burns et al. (1995) reported that just under half of methadone-exposed children's fathers were using opiates through to 7 years of age. Previous studies have associated drug abuse with child neglect and abuse (Burns et al, 1995; Chaffin et al, 1996; Magura, 1996). Continued maternal drug-use has also been reported to decrease the quality of maternal behaviour. In rats exposed to opioids during nursing, the drugs affect the way mothers interact with their young. Yim (2006) conducted a study assessing different maternal behaviours based on the timing of administration of morphine. Morphine was administered to rat dams during gestation, lactation and both. Maternal behaviour was measured by removing the rat pups, then returning them and noting the time taken by dams to retrieve the pups,

group them and nurse them. Rats that were being treated with morphine during lactation took significantly longer to exhibit any maternal behaviour, and dams that were treated with morphine during both gestation and lactation did not engage in any maternal behaviour within the first 30 minutes (Yim, 2006). Morphine administration during lactation altered maternal rat behaviour decreasing their initiation and quality of interactions with their pups. These findings can be compared to the findings in clinical samples that indicate parental drug-use increases the likelihood of child neglect (Burns et al, 1995; Chaffin et al, 1996; Magura, 1996).

In a clinical study, which again highlights the risk of continued parental drug use, Wilens et al (1995) compared different personal and environmental risk factors to assess their potential effects on child behaviour. There were three experimental groups involved: one group comprised parents that were opioid-dependent (their children had not been prenatally exposed to opioids); another was a control group; and the third was a group of males who had been diagnosed with either Attention Deficit Hyperactivity Disorder (ADHD) or Conduct Disorder (CD). All children were between the ages of 4-18 years and were assessed by means of the parent completed Achenbach Child Behaviour Checklist (CBCL). The opioid group scored significantly higher on the social scale related to delinquent behaviour and thoughts and attentional problems compared to the control group. They also scored at similar levels to the ADHD/CD group on school functioning, but scored lower in social activities, suggesting psychosocial impairments. These differences in social and behavioural development in the opioid-dependent parents illustrates the potential impact of family environment independent of prenatal methadone-exposure. Compared to control children, those living with parents who were opioid dependent scored higher on scales of delinquent behaviour, thought problems and attention problems (Wilens et al,

1995). A limitation of the study was the fact that the groups consisted only of males, so that potential sex differences in environmental risks could not be assessed.

Although children living with their biological parents are at risk for negative environmental influences, there have also been studies of outcomes for prenatally exposed children living in foster care. Arlettaz et al (2005) reported that child protective services were involved in 56% of the 86 cases involved in their study, with 42% of infants having to be placed outside the home. Burns et al (1995) compared methadone-exposed children to matched controls from 3-7.5 years. Significantly more control children had father figures in their lives, and parental employment levels were higher in this group. Soepatmi (1994) also reported that 36% of methadone-exposed children were living with their biological parents. It was also noted that fostered children tended to have worse outcomes on a developmental neurological examination, with lower neurological optimality scores and parental self reports on the health of the child. Other studies of outcome variables in children prenatally exposed to illicit substances have shown that deficits in development are present regardless of whether the children had remained with their biological parents, or were placed in foster care (Bunikowski et al, 1998; Eriksson et al, 2000). Soepatmi's (1994) observation that fostered children do less well than children living with their biological parents could have been due to the self-report method that was employed. For example, foster parents may have higher expectations of children than biological parents. It is also possible that the increased likelihood of the biological parents abusing drugs increases the likelihood of neglect and reducing the emotional investment in the child. A final possible explanation is that, if the biological parent was still using methadone, the stress relieving effect of the drug may have reduced any anxiety about the child's developmental progress. The majority of the results in

human research may contradict the theory that developmental delays in methadone-exposed children are due primarily to decreased parental ability in drug-abusers.

In animal studies of more subtle behavioural deficits, there appears to be effects of maternal behaviour. Such studies are not focused on teratological effects of illicit drugs but rather consider the effects fostering has on behavioural traits in a particular strain of rats. Strains of rats can be bred for the presence of certain genetically determined characteristics, such as hypertension and elevated hypothalamo-pituitary adrenal (HPA) responses to stress (which will be discussed in more detail later). Studies have shown that, when rat pups bred for increased stress reactions are fostered to control dams, their stress responses reduce to control levels (Francis and Meaney, 1999; McCarty et al, 1992). This contrasts with conclusions described above that, fostering in humans does not decrease the negative developmental outcomes for the child. Maternal behaviour may alter subtle behavioural trends in methadone-exposed children but the cognitive and social development of the child does not appear to improve with fostering.

Schneider and Hans (1996) conducted a study that again highlighted the importance of maternal capabilities in predicting outcomes of at risk children. The authors assessed the focused attention of methadone-exposed two year olds using observations of mother-child interactions in a laboratory setting. Mothers were required to guide the infant in completing tasks over a 30-minute period which were video taped and subsequently coded. Tasks included such activities as reading a book together, the child playing alone, the mother teaching the child how to use a toy and free time. The focus of the study was on the free play, where the child's focused attention was assessed as the mother's ability to teach their child how to use a new toy. No significant differences were found between methadone-exposed children and

control children in their focused attention. The only predictors of focused attention in children were maternal teaching ability and maternal IQ. This suggests that, through training and the use of social support networks, well educated mothers with effective parenting skills may be able to lessen predicted negative outcomes for their children.

Animal studies have also found that another important factor affecting the behavioural development of offspring is the behavioural characteristics of the mother. There have been a number of studies of nongenomic transference of behavioural traits from rat dams to their offspring. The most researched area of environmental regulation of behavioural responses in rats is through 'handling' research (Francis and Meaney, 1999). Most 'handling' research involves taking rat pups out of their nest during the first week of life for approximately 3-15 minutes per day. Post-natally handled rats typically show decreased responsiveness to stress that persists into adulthood, compared with non-handled rats (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). Stress responses have been measured in these animals by means of open-field and elevated plus-maze testing, as well as via neurological developmental indices that are related to stress hormones. In handled rats, the hypothalamo-pituitary adrenal (HPA) axis is altered by this early post-natal influence (Vallee et al, 1997). The duration of corticosterone secretion when exposed to stress is reduced in handled rats (Francis and Meaney, 1999).

However, the results of this 'handling' research are explained, not primarily through the physical handling of the pups, but through the alterations in the maternal behaviour that follows. The lower stress responses in the rat pups are mediated by changes in maternal behaviour, thus showing the influence of early environmental factors on behavioural and neurological development that persists into adulthood (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). Rat mothers' that

have had their pups removed from the nest to be handled may alter the type of maternal behaviour they engage in. Although they may spend the same overall time with their pups as mothers that do not experience this intrusion, the quality of the mother-pup interaction may be superior. For example, they generally have shorter, but more frequent, incidences of nursing. They also spend more time licking/grooming their offspring, and when nursing, adopt an arch-backed pose more often. These behaviours decrease stress for the pups compared to pups that are groomed less often and that are nursed in a 'blanket-type' pose (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). Such findings highlight the importance of maternal characteristics in shaping an offspring's behavioural characteristics. More fearful, anxious mothers appear to be less maternally responsive, which is documented through the reduction in licking/grooming and arched-backed nursing of their offspring. This reduction in maternal responsiveness has been found to result in fearful and anxious offspring, thereby suggesting behavioural transmission of the dams' characteristics to their litters (Francis and Meaney, 1999). Stress responsiveness in both animal and human offspring typically mirrors that of the parent. This suggests that teratological effects may be highly influenced by maternal factors. That is, stressed mothers tend to raise stressed children.

1.10 Post Natal Physical Development

1.10.1 Weight, Height and Head Circumference

Prenatally methadone-exposed infants show greater weight loss in early infancy and slow catch-up weight gain during childhood (Rosen and Johnson, 1993). Chasnoff et al (1986) found that opiate-exposed infants had a significantly reduced weight and height at both 3 and 6 months of age, but showed catch up by 12 months.

Hagopian et al. (1996) suggested that the reason for the accelerated catch-up after this 6 month period is because NAS symptoms lessen at 4-6 months, thus eating and sleeping patterns improve thereby enhancing weight gain.

Unlike many studies that have documented catch-up in weight and length, Soepatmi (1994) found that in opiate-exposed infants, height was still significantly reduced in children from 3.5 years through to 12 years of age. Soepatmi's (1994) research is the longest follow-up study of birth weight and length, and contrasts with the more consistent finding that birth weight and height show catch-up (Lee and Chiang, 1985; Lifschitz et al., 1985; Zuckerman and Bresnahan, 1991). However, in Soepatmi's (1994) research, the experimental groups included one group of infants that were prenatally-exposed to heroin and another group exposed to both heroin and methadone. Therefore, the enduring effects on height could be attributed to heroin, methadone or the combination of the two.

Another common assessment of physical development at birth is the circumference of the infant's head. Head circumference has been shown to be smaller at birth in prenatally methadone-exposed infants (Boer et al, 1994; Brown et al, 1998; Chasnoff et al, 1986; Hagopian et al., Kaltenbach and Finnegan; 1987; 1996; Rosen and Johnson, 1983; Rosen and Johnson, 1993). Infants born with a head circumference below the 3rd percentile of the normal range, remained significantly smaller through to 18 months of age (Rosen and Johnson, 1983). Rosen and Johnson (1993) replicated their findings in another sample and found that, at 24 months of age, 63% of children who were born with small head circumferences showed no catch-up to normal head size. These results are supported by other research showing that head circumference does not show catch-up to the average age related range (Chasnoff et al, 1986). Infants that are born with head circumferences below the 3rd percentile

maintain a smaller head circumference compared to their peers through childhood (Chasnoff et al, 1986). Measurements of head circumference through to adulthood have not been conducted.

1.10.2 *Neurological and Neuromotor Abnormalities*

Rosen and Johnson (1983) assessed prenatally methadone-exposed infants through to 18 months of age and noted a number of neurological problems that were more common than in the normal population. Methadone-exposed children had a higher rate of abnormal eye functioning, inflammation of the middle ear and muscle tone discrepancies. Tone discrepancies in prenatally methadone exposed infants are characterised by either hypertonia, namely, the increase in tightness in muscle tone, or by hypotonia i.e., the decrease in muscle tension resulting in lack of muscle strength (Rosen and Johnson, 1983). Development of motor coordination has been reported to be affected by prenatal methadone exposure. Previous studies have found deficits in fine motor development through to 18 months, as well as poorer motor coordination (Rosen and Johnson, 1983; Rosen and Johnson, 1993). Contradictory results have arisen from use of the Bayley Scales of Infant Behavior (BSIB). For example, Chasnoff et al (1984) found no significant differences in the scales between methadone-exposed and control infants. However, it has also been reported that methadone-exposed infants score significantly lower on mental and motor indices of the BSIB (Rosen and Johnson, 1983). The lack of significant differences between methadone-exposed and control infants in the Chasnoff et al (1984) study could have been due to the low maternal dose of methadone.

1.10.3 *General comments*

Effects of prenatal methadone exposure on physical development, pregnancy and birth measures have been inconsistent. There have been a variety of methods for assessing these physical outcomes, and both clinical and animal samples have been studied. Whether due to prenatal methadone exposure, or a combination of factors, the research to date suggests a number of outcome risks. Methadone has been associated with premature births, decreased birth weight, decreased head circumference, increased severity and duration of NAS in a large proportion of infants, and associated environmental risk factors during development. Research has suggested that there are alterations in maternal behaviour due to acute drug effects, as well risk factors in the home when being raised by an addict. While the majority of these findings have been replicated in a number of studies, there are still some limitations that make it difficult to draw firm conclusions about the drug's developmental effects. These limitations are discussed below.

1.10.4 *Limitations in Research*

The majority of the studies to date have been conducted on small sample sizes and have assessed only short term impacts of prenatal methadone-exposure. One of the limitations of the research to date is the small pool of investigations of physical outcomes in school-aged children, and a lack of longer term studies ranging through adulthood. In those studies involving school-aged children, the methadone-exposed group was confounded by combined exposure to heroin (Soepatmi, 1994). In the most recent review of potential teratological effects of methadone (Farid et al., 2008), drew attention to the limited number of studies that have assessed long term development, with the majority of studies involving follow-ups to 24 months of age only. Another

obvious limitation highlighted by Farid et al (2008) is that the majority of the studies of postnatal development were conducted many years ago. Most of the studies reviewed were published and written in the 1970s and 1980s, with a few exceptions in the early 1990s. The differences in best practice of methadone maintenance programmes, with regard to dose, dose increases during pregnancy, prenatal care programmes and breastfeeding protocols, have the potential to markedly influence the postnatal development of prenatally exposed infants. Therefore, more research is needed into effects on postnatal development, particularly long term behavioural, cognitive and physical development.

1.11 *Behavioural Development*

Behavioural development of children prenatally exposed to methadone has been little studied. Of those studies that do exist, the majority focus on immediate or short term effects up to 2 years of age. There is currently no research into behavioural development through to adulthood and limited research into behavioural development in adolescence. Behavioural development within the methadone-exposed population has been conducted through follow-up studies and a small number of longitudinal studies. Follow-up studies in drug-exposed populations have the potential for selection bias, with more concerned mothers, and better adjusted mothers and families, staying involved in studies (Kaltenbach, 1996). The broader range of behavioural deficits associated with prenatal drug exposure may thus be underestimated or over-estimated due to sample bias and attrition. Therefore, the research fails to more accurately assess risk factors related to prenatal drug exposure. There have been a number of longitudinal studies assessing children to approximately 5 years of age (Hans and Jeremy, 2001, Kaltenbach and Finnegan, 1987; Schneider

and Hans, 1996). As with other research into effects of prenatal methadone exposure, generally there have been few longitudinal studies carried out since the 1980s (Kaltenbach, 1996).

There is also very limited evidence concerning the effects of prenatal methadone on anxiety, stress reactions, memory and attention. While animal research has primarily focused on neurobiological impacts (Robinson et al., 1996a; Robinson et al., 1996b; Robinson et al., 1996c; Rosen and Johnson, 1993; Yim et al., 2006), clinical research to date has largely been concerned with short term physical outcomes, such as head circumference and birth weight. There has been a shift during the recent decade to consider behavioural development to a greater extent, but most studies have primarily involved the assessment of social, cognitive and motor coordination changes.

1.11.1 *State Control and Focused Attention*

Poor state control in infancy is a consistent finding among infants that have been prenatally exposed to narcotics. Symptoms of poor state control include decreased ability to self regulate irritability and poor self-quieting (Gupta, 1999). One such study that assessed state control in newborns was conducted by Chasnoff et al (1986), who assessed the effect of prenatal methadone and heroin exposure on behavioural development at 2 days of age using the Brazelton Neonatal Behavioral Assessment Scale (BNBAS). The exposure decreased the infants state control ability, with methadone-exposed infants being tenser, more irritable, jerkier and more active.

As well as not being able to self-soothe after birth, methadone-exposed children also show a decreased ability to engage in activities that involved focused attention (Hutchings, 1982; Rosen and Johnson, 1993). In a review of the effects of

prenatal methadone exposure on behavioural development from birth through to 2 years, Hutchings (1982) concluded that methadone-exposed infants had a shorter attention span. These deficits are also found in later research that was reviewed by Rosen and Johnson (1993). They reported that attention span was especially reduced when the child was required to complete a series of set tasks.

This review supported previous research that also found methadone-exposed children to have deficits in focused attention (Hans and Jeremy, 2001). However, a study conducted by Schneider and Hans (1996) found no difference in focused attention between methadone-exposed and non-exposed children. These authors assessed focused attention of prenatally exposed 2 year old children by observing mother and child interactions for 30mins in a laboratory setting with tasks to complete. There was a problem with the fact that parenting and child ability were not assessed independently of each other. However, they concluded that the focused attention of the child was related more to the mother's instructional abilities as opposed to methadone exposure. Mothers were more successful teachers when they used instructions involving demonstrating and labelling objects while pacing the teaching to the child's learning ability; reviewing concepts the child was unable to grasp while not going too slow that the child loses interest and focus.

1.11.2 *Activity Levels*

As with state control and attention, activity levels have been reported to be altered in prenatally methadone-exposed children, with heightened activity, increased impulsivity and hyperactivity (Hutchings, 1982; Rosen and Johnson, 1993). Clinical studies have also found alterations in activity levels in methadone-exposed children. This is clear from a review of behavioural development from birth to 5 years of

children prenatally exposed to methadone. He found that methadone exposure heightened activity levels and increased impulsivity (Hutchings, 1982). It was also noted that methadone exposure produced impairments in motor inhibition while performing tasks. In a later review, Rosen and Johnson (1993) concluded the same increase in hyperactivity in prenatally-exposed children at various follow-up periods.

In animal studies, activity levels are typically assessed through open-field testing and results have highlighted the influence of timing of methadone exposure on activity levels (Kunko et al, 1996). An animal study assessing the impact of methadone exposure during gestation, lactation and both found that rat pups exposed over both periods presented with significantly lowered levels of spontaneous locomotor activity (Kunko et al, 1996). In contrast, higher activity levels were found in the experimental group that was exposed during lactation only.

The results of animal studies are somewhat contradictory to human clinical findings which consistently suggest that prenatal methadone exposure is associated with the risk of hyperactivity. Animal studies suggest a decreased level of activity when offspring are exposed to methadone during both gestation and lactation. In a study by Kunko et al (1996), activity levels were only increased when the offspring were exposed to methadone during lactation. The inconsistencies between animal and human research findings might suggest that, while prenatal methadone exposure could be a risk factor for hyperactivity, there may be a more important influence of confounding variables such as post natal care, parental skills and other environmental influences. When environmental factors are not controlled for, as they are in the animal population, it is hard to separate the effect of methadone from other factors correlated with methadone use that may also place children at elevated risk of later behavioural problems. This could suggest that a combination of drug effects and

environmental factors alter the presentation of behavioural alterations, as opposed to drug effects alone.

1.11.3 *Cognitive Development*

There has been some suggestion from past studies that prenatal methadone exposure is associated with certain cognitive impairments. Early human cognitive development is typically measured through assessment tools, such as the Bayley Scales of Infant Development (BSID). In one such study that involved this scale for assessing prenatally methadone- and heroin-exposed children, the methadone/ heroin group scored significantly lower on the Mental Development Index (MDI) of the BSID compared to controls at 6 and 12 months of age (Chasnoff et al, 1986). Chasnoff et al's (1986) findings were replicated more recently by Hans and Jeremy (2001). These authors also reported that these cognitive deficits persisted with age, and actually increased in severity through childhood. Opioid-exposed children's scores on the MDI decreased with age relative to age appropriate norms. However, once covariates such as other drug use, social-environmental risk and birth weight were taken into account, opioid exposure no longer had a significant effect on MDI scores. The only factor that was still predictive of reduced scores on the MDI was birth weight. However, the analysis in itself may have been flawed with the covariates of drug use, birth weight and social-environmental risk factors being potential mediators for methadone-exposure and cognitive outcomes as opposed to covariates.

Amongst research conducted to date, there are still a number of studies in which there were no adverse methadone effects on cognitive development. One such study was conducted by Kaltenbach and Finnegan (1987) who compared 141 methadone-exposed infants to 127 matched controls at 6 months of age and found no

differences in development on the MDI. A number of other studies have also reported no significant deficits in social and cognitive functioning in methadone-exposed children, up to 5 years of age, compared to control samples (Goodman et al, 1999; Lifschitz et al, 1985; Strauss et al, 1979)

The variability in findings across cognitive and behavioural development in prenatally methadone-exposed populations highlights the need for further research. The variations also suggest that studies using different methodologies also lead to the presence of differing numbers of confounding variables. This is especially problematic in clinical studies of behavioural outcomes in which family dynamics, poverty, social support and potential abuse and neglect can influence outcomes.

1.12 Neurobiological Effects

Many of the previously discussed behavioural outcomes of pre- and postnatal exposure to methadone may have been, in part, due to alterations in brain chemistry. In animal samples, there have been a number of neurobiological effects attributed to methadone exposure. These neurological changes raise the important questions about the potential mechanisms behind the long term alterations seen in prenatally methadone-exposed humans. The areas of neurological change in the current discussion focus on three specific areas 1.) Cyclin-dependent kinase 5; 2.) cholinergic development; and 3.) the involvement of nerve growth factor.

1.12.1 Cyclin-Dependent Kinase 5

Cyclin-dependent kinase 5 (Cdk5) is structurally related to the kinases that controls the cell cycle, but does not play a role in cell division. It has been shown to help control neuronal migration and neurite outgrowth. Cdk5 has also been shown to

influence a number of critical processes in neural development, such as cell migration and dendrite spine outgrowth. Decreased Cdk5 has been reported in adult brains of opioid users (Bhat et al, 2006). Decreasing these functional abilities may produce long lasting effects within the nervous system.

In their assessment of the potential deficits in Cdk5 activity due to prenatal opioid exposure, Bhat et al (2006) exposed rats prenatally to saline, cocaine, morphine or cocaine and morphine combined. The rats were sacrificed at PND 1, 7, 14 and 28 for dissection and analysis of Cdk5 activity. Cdk5 levels remained constant over time in the control group. In contrast, morphine exposure decreased Cdk5 exposure, with the lowest levels of activity occurring at approximately PND 14, then tended towards normal control levels.

While Bhat et al. (2006) did not specifically investigate methadone, their findings provided some insight into the potential effects of opioid exposure in utero on critical processes in neural development. These deficits early in life could have an impact on later subtle behavioural and cognitive development. Due to the testing occurring within a time frame in which withdrawal could potentially have an effect, further research into the area is needed before assumptions of opioid effects on Cdk5 activity can be made. Another area of interest to inform current research into prenatal methadone exposure's effect on neurological development would be to test different areas of the brain for Cdk5. This could provide further support for any possible influences the decrease in Cdk5 may have and enable linkages of these neurological changes to specific behavioural deficits.

1.12.2 *Cholinergic Development*

It has also been found that opioids could also potentially affect cholinergic neurons in specific brain regions (Robinson et al., 1996a; Robinson et al, 1996b; Robinson et al, 1996c). Cholinergic neurons are operated by the neurotransmitter acetylcholine (Ach), which plays an important part in synaptic plasticity and excitability. Damage to cholinergic systems in the brain has been reported to be associated with deficits in memory. Ach is also involved in the parasympathetic nervous system (PNS) and is the transmitter responsible for the initiation of voluntary motor activity at the level of the neuromuscular junction (Carlson, 2007).

Comprehensive research into effects on cholinergic development of pre- and postnatal methadone exposure has been carried out by Robinson and colleagues (Robinson, 2000; Robinson et al, 1996a; Robinson et al, 1996b; Robinson et al, 1996c). Rats were exposed to 9mg/kg/day of methadone during gestation, lactation or both. After birth, litters were cross fostered, then at PND 21 cholinergic activity was measured through turnover rate of Ach in different brain regions. Prenatal methadone exposure disrupted cholinergic activity through increased Ach turnover at PND 21. These changes occurred in both sexes and postnatal exposure did not influence the increase in Ach turnover after prenatal exposure, ruling out any potential withdrawal effects (Robinson et al, 1996c).

The brain region primarily affected by the decrease in Ach turnover, due to methadone-exposure, is the striatum (Robinson et al., 1996a; Robinson et al., 1996b; Robinson et al., 1996c). The striatum is associated with planning and modulation of physical movement and cognitive processes involving executive functions. Striatal cholinergic activity is significantly reduced in prenatally-exposed rats independent of post natal methadone-exposure.

Robinson (2000) summarized the changes in cholinergic development and attempted to explain the causal mechanisms behind the persistent alteration in Ach activity. After prenatal exposure to methadone, cholinergic turnover and the expression of choline acetyltransferase protein (ChAT) are reduced. ChAT-positive-striatal neurons have a reduced size and are less reactive in the early postnatal period. There is a reduction in the expression of ChAT protein. ChAT protein levels return to normal levels, but cholinergic neural activity levels remain elevated at levels almost double the normal Ach turnover rate. However, most cholinergic development in rats occurs postnatally. This means that prenatal exposure to methadone would not affect cholinergic neurons to the levels observed in the current study, particularly as postnatal exposure produces no further alterations to Ach activity. Therefore, it is probable that prenatal methadone-exposure alters cholinergic development indirectly through another type of cell. Robinson (2000) suggested that Nerve Growth Factor (NGF) may potentially mediate the disruption of cholinergic development caused by opioid exposure.

1.12.3 *Nerve Growth Factor (NGF)*

NGF is known to stimulate cholinergic activity in the striatum. NGF content also decreases in the striatum due to opioid exposure (Wu et al, 2001). Based on NGF functioning in relation to opioids and cholinergic neuron development, research into NGF as a mediating variable for methadone-exposure and increased Ach turnover is warranted.

Robinson (2000) suggested that opioid exposure reduces NGF, which in turn delays and disrupts cholinergic development particularly in the Striatum. After this disruption, there is a reduction in the expression of ChAT protein. Early in the

postnatal period ChAT protein levels return to normal levels. However, the cholinergic neurons remain disrupted as reflected in increased Ach turnover.

In their investigation of NGF content following opioid exposure, Wu et al (2001) exposed rats to methadone or buprenorphine prenatally, postnatally or both, after which their brains were dissected at PND10. Exposure to either of the opioids at any time decreased the striatal NGF content from 40-50% of that of controls. The fact that there was no difference between the two opioids suggests that the mechanism behind the cholinergic and NGF disruption was primarily due to μ -opioid receptors. Exposure to opioids at any time affects NGF. The reduction of NGF influences the development of cholinergic neurons, even into the postnatal period. Cholinergic neurons may be altered due to the reduction of NGF in the striatum, caused by opioid exposure during either the pre or postnatal period, thereby contributing to future alterations in behavioural and cognitive functioning in the offspring (Wu et al, 2001).

1.13 *Current Research Aims*

The current research was conducted in order to provide more information about the causal pathways of physical and behaviour alterations seen in methadone-exposed samples. As the literature to date lacks any research into long term methadone effects, one of the main research aims in the current study was to assess the developmental trajectory from birth through to what is considered adulthood in the rat. There have been a number of clinical studies assessing the behavioural development of methadone exposed samples through childhood. These clinical studies frequently have methodological limitations arising from the lack of control for environmental influences on behavioural development. Therefore, an important aim of the current study was to provide more information about direct drug effects on

behavioural development. There were three explicit aims of the current research project, which were as follows:

Research Aim 1.) To assess any changes during pregnancy in methadone-exposed dams and effects at birth in their offspring. There have been observations in clinical samples of reduced maternal weight gain, increased prematurity rates, reduced birth weight and increased risk of miscarriage and sudden infant death. One of the aims of the current study was to assess the changes in these areas while controlling for confounding environmental factors, such as nutrition and differences in housing conditions.

Research Aim 2.) To measure any long term changes in physical development. There is limited coverage of long term physical development in current published research although it has been suggested that children with low birth weights show catch up rates which bring them to normal peer levels early in childhood. While some physical abnormalities have been reported, these still fall within the levels found in the general population. There has not been research to date that assesses the physical development of methadone-exposed infants through into adolescence and adulthood. Therefore, the current research aimed to assess long term physical development in rats, specifically weight gain, physical abnormalities and stress reactions.

Research Aim 3.) To assess long term behavioural development. As with physical development, behavioural development has been limited to childhood and the studies have been plagued with a vast number of confounding environmental influences. The major aim of the current research was to focus on the causal influence of methadone exposure on long term behavioural development while controlling for environmental influences as much as possible. Specific behavioural phenomena that

were assessed were activity levels, anxiety and memory. The methods through which these assessments were made are outlined in the following section.

Section 2

Method

2.1 *Subjects*

Forty-eight Wistar albino female rats, approximately 100 days old, were housed in individual plastic cages with stainless steel tops (width, length, depth = 35 x 55 x 21cm). They were kept on a 12-h light-dark cycle at an ambient temperature of $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $48\% \pm 10\%$ humidity. Groups of 3-4 female rats were paired with an individual male rat for 72 hour in order to mate.

After the female rats had been mated, 24 were randomly assigned to an experimental group and the remaining 24 were randomly assigned to the foster mother group. Of the experimental mothers, 12 received pure drinking water throughout pregnancy and the other 12 received drinking water containing methadone at a daily dose of approximately 2.5 mg/kg/day (see section 3.1.1). All 24 foster mothers received pure drinking water during their pregnancy.

Within 24 hours of birth, the litters of the foster mothers were culled and the offspring of the experimental litters were reduced to 4 males and 4 females per litter where possible. All litters were fostered to the non-methadone-treated foster mothers. Once fostering had taken place, 12 foster dams received pure, unadulterated drinking water and 12 foster dams received methadone in their drinking water. This ensured that these pups were exposed to methadone via their foster mothers' milk. Prenatal-methadone exposed litters were divided evenly between the two lactational conditions, as were the prenatally non-exposed litters. Litters were randomly assigned to foster dams depending on the timing of birth and the availability of litters. After birth, methadone-exposed litters were paired with the first available dam from the

foster dam group. After fostering there were four experimental groups. These groups were as follows: 1) the non-exposure group (WgWl); 2) the prenatal exposure group (MgWl); 3) the lactational exposure group (WgMl); 4) prenatal + lactational exposure group (MgMl) (the fostering procedure can be seen in figure 1).

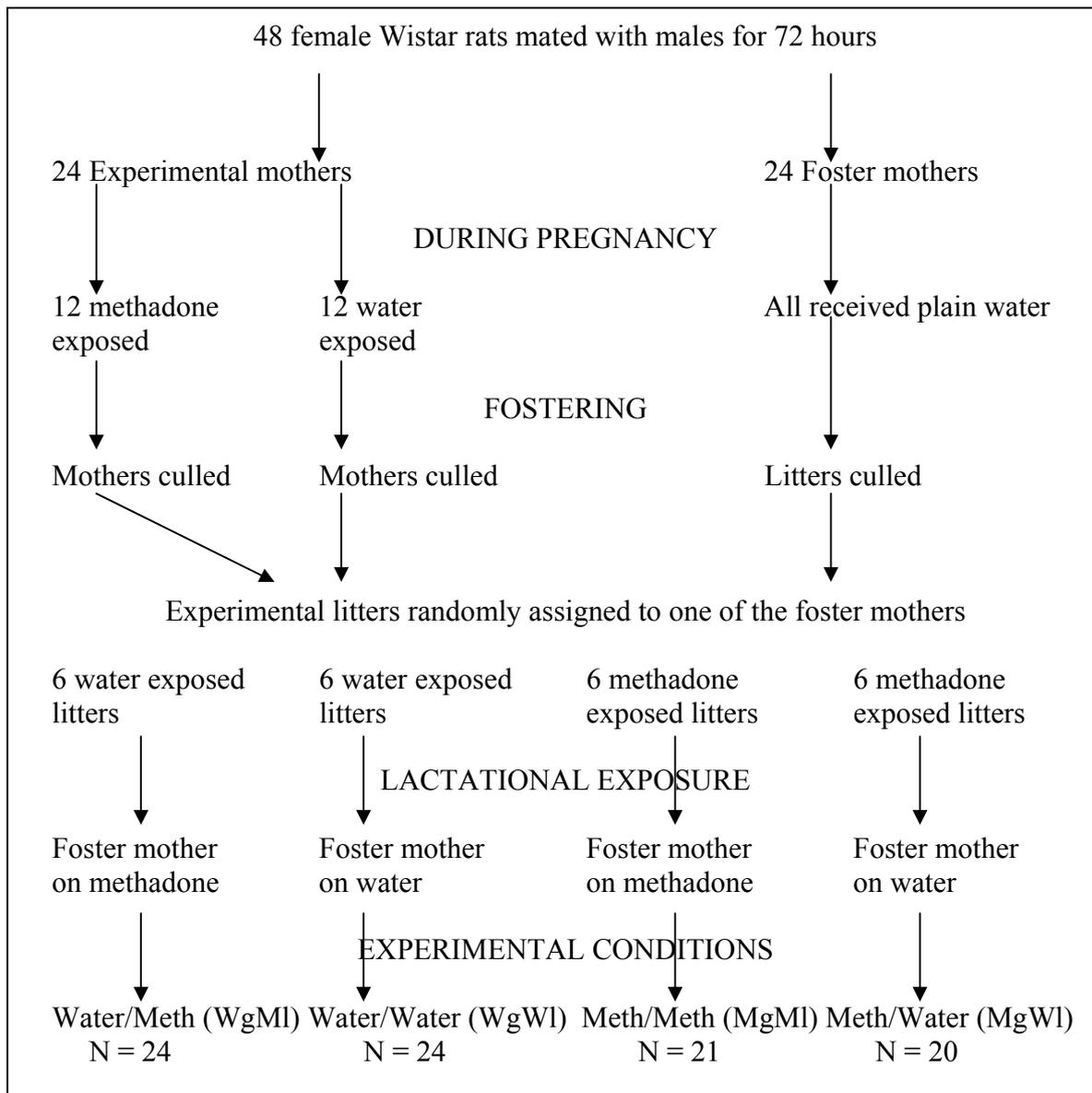


Figure 1: Flowchart of Cross Fostering Procedure and Resulting Experimental Conditions

Weaning occurred at PND 28, at which time 2 males and 2 females from each litter were randomly selected for later testing (the remainder were culled). The only

exception to this was the MgWl and MgMl groups. As there were limited numbers of litters that were born to the experimental mothers receiving methadone-treated water, all rat pups were kept in order to maintain sufficient numbers of subjects.

Due to low numbers of pregnancies in the experimental methadone group of dams, rebreeding was necessary. Of the rats that had not been exposed to methadone and had either not given birth, or had eaten their babies shortly after birth, 10 were used for this rebreeding process. Methadone-exposed rats were excluded from rebreeding. The rats went through the same breeding process outlined previously. After 72 hours of confinement with a male, the female rats were housed individually, with 5 rats in the experimental group (exposed to methadone) and 5 rats in the foster-mother group that received pure drinking water. During their pregnancy, the rebred rats and their offspring were treated in the same way as rats in the original breeding programme.

The number of rats in each of the four experimental conditions varied slightly as a result of litter numbers in the prenatal methadone exposure group. The overall numbers were 24 in both the WgWl and WgMl groups, (12 females and 12 males), 21 in the MgMl condition (10 females and 11 males) and 20 in the MgWl group (12 females and 8 males).

2.2 Drugs and Rationale for Administration

Liquid methadone was purchased from CDC Pharmaceuticals Ltd, Christchurch, as a 10mg/ml solution. Methadone concentrate was mixed with plain tap water once a week and stock-piled in a locked safe. Based on the rats' normal water intake and with a target dose of 3.0 mg/kg/day in mind, concentrations of 0.2ml/L were provided for the appropriate rats during pregnancy. This dose was

selected for use because it fell within the range of methadone doses shown to have prenatal effects on rats in previous research (Chipkin and Rosecrans, 1978; Pierce et al., 1992). The concentration of methadone was reduced to 0.1ml/L during lactation because of increased consumption of water by the dams while nursing (Godbole et al, 1981).

Administration of the methadone-treated water was by means of free-access water bottles attached to the rats' cages. The bottles were changed once a week and topped up in between times when needed. Orally is the usual form of administration in methadone maintenance programmes (Strang et al, 1996). For this reason, oral administration of methadone to dams was thought to be the most appropriate way of assessing methadone-related behavioural development that could be relevant to clinical populations.

2.3 Materials

2.3.1 Pregnancy, Lactation and Birth

Weights of the water bottles and rat mothers during pregnancy and lactation, as well those of the offspring at birth and throughout post natal development were measured with Mettler PR3000 scales.

2.3.2 Open Field Apparatus

The Perspex open field comprised 60 X 60 X 30cm high transparent walls with the black floor divided into 16 equal-sized squares by means of a grid of white lines.

2.3.3 *The Emergence Apparatus*

The emergence apparatus consisted of a darkened chamber measuring 20 X 15 X 20cm high covered by a wooden, hinged lid that opened out into a 50 X 40 X 20-cm high arena which was brightly lit with fluorescent tubes beneath the translucent floor. Access to the arena was enabled by withdrawing a guillotine slide from between it and the darkened compartment.

2.3.4 *The Y-maze*

The clear-varnished wooden Y-maze comprised two arms, 45cm long, and a 15-cm-long stem. The angle between the arms was 120 degrees. The arms and stem were 10cm wide and 14cm high. The top of the Y-maze was covered with a clear Perspex lid. Black and white painted aluminum inserts were placed in the Y-maze arms to subsequently assess responsiveness to brightness change (see 2.4.5.3). The inserts occupied the width, height and 40cm of the length of the arm. Each insert covered the end, two sides and floor of the arm. A computer program was developed in order to record observations of the movement of each rat.

2.4 *Procedure*

2.4.1 *During Pregnancy*

After they had been mated, the dams were individually housed in conditions previously outlined. Each cage contained some tissue paper for the rat to nest in thereby alleviating some of the potential stress associated with isolation. Each day during their pregnancy, the water bottles were weighed between 13:00 and 16:00 to calculate the amount of individual fluid intake and the methadone dose for each of the 12 methadone-exposed rats. The pregnant rats were also weighed every two days,

with odd numbered subjects being weighed on one day, and even numbered the next. Rat body weights were used to calculate both the daily dose intake of the methadone-exposed subjects (mg/kg/day) as well as their weight gain. When litters' were fostered to non-methadone-exposed rats, the foster mothers' tissue nests were left in the cages to attempt to reduce any olfactory differences between litters. The cages were not changed for up to a week after birth to reduce any possible stress reactions in the mothers.

2.4.2 *At Birth*

The litters of rat pups from all mothers were weighed within 48 hours of birth whenever possible. Six of the 30 litters had to be excluded from the overall birth weight calculations as they were born during a weekend and well outside of the 48 hours timeframe because of the unavailability of relevant personnel. Whole litters were weighed and the average weights in grams per rat pup were calculated. The numbers of rat pups per litter were also calculated and each pup was sexed in order to assess the sex ratio. The offspring of methadone-exposed dams were then fostered to water-exposed dams after their own litters had been removed.

2.4.3 *Lactation*

As occurred during pregnancy, water bottles were weighed and refilled when necessary each day between the hours of 13:00 and 16:00. The mothers were not weighed during their first week of lactation to reduce stress after birth. In order to calculate the methadone consumption during this period average weight losses after birth were calculated. Week one weights were calculated by taking an average of the PND 7 measurement and the last pregnancy weight for each dam. This could have

potentially increased the estimated weights of the dams, thereby leading to an under estimation of individual daily doses. However, removing the dams for weighing during the first week of life may have been harmful for the fostering process or potentially altered maternal behaviour if separated from the litter for too long (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). During the next two weeks of lactation the rat mothers were weighed at the start of each week (PND7 and PND 14) and that one weight was used to calculate each individual daily dosage. Unnecessary handling was avoided to attempt to promote successful cross-fostering.

2.4.4 Physical assessment

Within 48 hours of birth all rat pups were weighed, and litter sizes and numbers of each sex per litter noted. Animal technicians were present to help assess any physical abnormalities in the rat pups. Physical development of the offspring was also assessed at PND 30, 60 and 120. All rats were individually weighed and physical abnormalities noted (in their legs, tails and eyes). Casual observations of stress behaviours and symptoms were also made which included hypersensitivity to touch, increased levels of vocalisation, fur thinning from over-grooming and redness around the eyes (chromodacryorrhea). All of these symptoms have been documented as valid measures of chronic anxiety (Cloutier, and Newberry, 2008; Kalueff and Tuohimaa, 2005; Ross, 1994; Sanchez, 2003; Van Erp et al, 1994; Windle et al, 1997).

2.4.5 Behavioural Assessment

All behavioural testing was conducted on PND 30, 60 and 120 (+/- 5 days). All rats were tested between the hours of 09:00 – 13:00 in the emergence apparatus and between 11:00-17:00 in the open-field. Y-maze testing took place from 9:00

through to 17:00. The testing took place during the light phase of the rats' light/dark cycle. All behavioural tests were conducted in the same room with temperature and light intensity unaltered across trials. The rats were tested on a maximum of two behavioural tests a day, excluding the Y-maze. No additional testing was carried out in between the Y-maze exposure (acquisition trial) and testing 24 hours later (retention trial). Each animal ended up being testing in each apparatus 3 times.

2.4.5.1 *Open-Field Testing*

At PND 30, 60 and 120 (+/-5 days) each rat was tested in the open field to assess general motor behaviour. The open field enables the assessment of activity levels as well as other forms of behaviour.

Each rat was placed in the centre of the Perspex open field. Every 5 seconds for 5 minutes, the rat's location was noted as well as what behaviour they were engaging in. Behaviours coded were walking, rearing up on hind legs (against the Perspex wall or free-standing), grooming or other. Ambulation scores were calculated by counting the number of times the rat was located in a different square than the square noted 5 seconds previously. After the 5 minutes was up the number of faecal boli were counted. Higher levels of anxiety/emotionality in rats produce increased defecation and decreased ambulation (Mohanty and Mishra, 1987; Renard, Rivarola and Suarez, 2007; Roth and Katz, 1979).

2.4.5.2 *Emergence Task*

All rat pups were tested on the emergence task at PND 30, 60 and 120 (+/- 5 days). The emergence apparatus is a measure of anxiety in the rat. Rodents have a natural tendency to explore novel environments (Alvarez and Alvarez, 2008; Carey et

al, 2008; Hughes, 2001; Hughes and Neeson, 2003; Vago and Kesner, 2008) but also have an aversion to bright light (Ballaz, Akil and Watson, 2007; Costel et al, 1989). The tendency to explore combined with an aversion to the larger, open, well-lit area is used to measure anxiety levels in the rat, with more anxious rats taking longer to emerge into bright light (Costell et al, 1989; Rodgers and Dalvi, 1997).

Each rat was placed individually in the darkened chamber of the apparatus. The barrier that blocked the entrance was removed and the time taken to enter the illuminated area was recorded. Emergence was recorded once all of the rat's four feet were out of the darkened chamber. If after 5 minutes it had still not emerged, the trial was recorded as "failed to emerge". For statistical analyses, emergence latencies for rats that failed to emerge were recorded as 300 seconds. After the trial, the rat was returned to its home cage.

2.4.5.3 Responsiveness to Brightness Change

At PND 30, 60 and 120 (+/-5 days) all rat pups were assessed for their reference-memory-related responsiveness to brightness change. The Y-maze is used to measure reference memory by exploiting rats' natural curiosity and tendency to explore novel environments (Alvarez and Alvarez, 2008; Carey et al, 2008; Hughes, 2001; Hughes and Neeson, 2003; Vago and Kesner, 2008). Typically, a rat will enter the changed (or novel) arm of the Y-maze more often than the unchanged (or familiar) arm. Anxious/fearful rats typically avoid novel environments (Ballaz, Akil, and Watson, 2007; White et al, 2007) and may spend more time in the familiar rather than novel arm of the Y-maze.

For an acquisition trial, one arm of the Y-maze contained a black insert and the other contained a white insert. The location of the white arm was randomly

determined for individual rats with equal numbers of subjects within each group experiencing it on the left or right. Each rat was placed in the stem of the Y-maze and allowed to freely explore the maze for a period of 2 hours. The rat was then removed and returned to its home cage. All of the maze was washed with 20% Powerquat, 80% water, and towel-dried. Twenty four hours later (+/- 1 hour) the rat was again placed in the Y-maze after both arm inserts had been replaced with clean black ones. In addition to providing an arm that had now changed from white to black, replacement of both inserts ensured the removal of any odour cues that may have been left by the rat during its acquisition trial and thus may have guided its behaviour, rather than the brightness change. The number of entries of, and time spent in each arm was recorded over 3 minutes using a computer program specifically designed for recording observations of the rats' behaviour. It was then possible to subsequently calculate five dependent variables namely, the arm first entered (i.e. novel/changed vs. unchanged), total time spent in the novel arm, total time spent in the unchanged arm, percentage of time spent in the novel arm and percentage of time in the unchanged arm.

Section 3

Results

Throughout this results section, the four experimental conditions will be referred to as follows: 1) WgWl = water-exposed during gestation, water-exposed during lactation (control condition); 2) MgWl = methadone-exposed during gestation, water-exposed during lactation (gestationally-exposed condition); 3) WgMl = water-exposed during gestation, methadone exposed during lactation (lactationally-exposed condition); 4) MgMl = methadone-exposed during gestation, methadone-exposed during lactation (combine-exposure condition).

3.1 *Pregnancy and Birth Measurements*

3.1.1 *Fluid Intake and Methadone Dosage*

During pregnancy, daily measurements of individual rat's fluid intake were made. The means for all groups, including the re-breed, for each week can be seen in Table 1.

Table 1: Weekly fluid intake of pregnant rats (mls)

Group	Week 1	Week 2	Week 3
Group 1	53.47	57.07	62.34
Group 2	49.36	54.27	61.84
Group 3	55.24	61.29	67.46
Group 4	32.57	35.69	36.36
Group 5	50.12	55.26	59.02
Group 6	32.90	37.86	43.06

Group 1: Water-exposed foster mother; Group 2: Water-exposed foster mother; Group 3: Water-exposed experimental mother; Group 4: Methadone-exposed experimental mother; Group 5: Re-breed water-exposed foster mother; Group 6: Re-breed methadone-exposed experimental mother.

For the analyses, all prenatal water-exposed groups were treated as one group and methadone-exposed groups as another. The fluid intake for both the prenatal

water and prenatal methadone groups increased significantly during pregnancy, $F(2,106) = 24.973, p < 0.01$. The rats receiving water prenatally drank significantly more fluid than the rats that received methadone in their water, $F(1,106) = 225.945, p < 0.01$.

From the daily fluid intake and rat weight measurements (discussed in the next section), a mean daily dose of methadone was calculated (see Table 2).

Table 2: Methadone-exposed rats' mean daily dose (mg/kg/day) for all rats (original and re-breeds combined) during pregnancy (brackets = \pm SEM).

Average Daily Dose	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Week Average
Week 1	1.57 (0.83)	2.09 (0.31)	2.08 (0.13)	2.06 (0.44)	2.30 (0.44)	2.62 (0.52)	2.33 (0.76)	2.15 (0.32)
Week 2	2.19 (0.28)	2.50 (0.44)	2.30 (0.53)	2.66 (0.56)	2.65 (0.39)	2.94 (0.57)	3.16 (0.93)	2.63 (0.34)
Week 3	2.48 (0.36)	2.35 (0.27)	2.47 (0.29)	2.50 (0.10)	2.41 (0.33)	2.15 (0.89)	4/5 given birth	2.39 (0.13)

The mean daily dose of methadone ranged from 1.57-3.16 mg/kg/day during pregnancy. The mean dose consumed by all rats increased from week 1 to 2, and then decreased slightly in week 3. The overall mean dose of methadone consumed during pregnancy was 2.39 (SEM = \pm 0.28) mg/kg/day.

3.1.2 Maternal Weight Gain and Duration

During pregnancy the rats were weighed every second day (odd numbered rats one day, even numbered the next) in order to determine doses of methadone consumed, and maternal weight gain. Rats that did not become pregnant were excluded from this exercise. Rats that received pure drinking water during their pregnancy exhibited a mean percentage weight gain of 23.50 grams (SEM = \pm 1.39),

with a range of 7.89 to 47.86 grams. Rats that received methadone in their drinking water had gained an average of 22.91 grams (SEM = ± 1.56) by the end of their pregnancy, with these gains ranging from 16.67 to 26.42 grams. According to a t-test for independent samples, the difference in the percentage of maternal weight gain between methadone-exposed and water-exposed dams was not significant $t(34) = 0.18, p > 0.8$).

The dams that received pure, unadulterated drinking water during their pregnancy had a mean pregnancy duration of 22.08 days (SEM = ± 1.62). Those that received methadone in their drinking water during pregnancy had a slightly lower mean pregnancy duration of 20.80 days (SEM = ± 0.84). However, as shown by a t-test, this difference was not statistically significant ($p = 0.10$).

The numbers of rats that became pregnant, and maintained their pregnancy to term, were noted. There was a difference between the methadone and water-exposed rats in the percentage that either failed to become pregnant or possibly reabsorbed their foetuses. Dams that received water during pregnancy had a pregnancy rate of 73.17%, with 30 out of the 41 rats maintaining their pregnancy. Methadone-exposed dams had a pregnancy rate of 35.29%, with only 6 out of the 17 maintaining their pregnancy. Using a chi-square analysis, the difference in the number of successful pregnancies was found to be significantly lower in methadone-exposed compared with water-exposed dams, $\chi^2(1) = 7.32, p < 0.01$.

A small number of rats ate their litters after birth. One methadone-exposed and 3 water-exposed mothers ate their litters shortly after birth. Using a chi-square analysis, the difference between the two groups was not statistically significant $\chi^2(1) = 0.2, p > 0.6$.

3.1.3 Birth Measurements

At birth, measurements were taken of the pups' bodyweight, numbers of still births, sex ratios, litter numbers and physical abnormalities. The results of this analysis are summarised in Table 3.

Table 3: Summary of birth measurements for offspring of water- and methadone-exposed dams i.e., mean litter sizes, sex ratios, birth weights (g) and numbers of still births (brackets = \pm SEM).

Group	Mean Litter Size	Number dead in group	Overall Ratio Male:Female	Average rat pup weight (weighed under 48hrs)
Prenatal Water (n=26)	9.19 (0.66)	4	119:104	7.29 (1.22)
Prenatal Methadone (n=5)	9.6 (0.93)	1	20:27	6.90 (1.31)

As shown, there were no obvious physical abnormalities in any of the individual rat pups apart from one litter of the methadone-exposed rats appearing to be noticeably redder in colour than usual.

The mean litter sizes for both the prenatal water and prenatal methadone groups were calculated. There was no significant difference between the litter size of methadone-exposed and water-exposed groups $t(29) = 0.26, p > 0.8$. There was also no difference in the number of stillbirths between the two conditions, with water-exposed dams having 4 stillbirths and methadone-exposed dams having one stillbirth, $\chi^2(1) = 0.03, p < 0.86$. There was also no significant difference in the ratio of male to female offspring between methadone and water-exposed dams $\chi^2(1) = 1.82, p > 0.1$.

Each rat litter was weighed as soon as possible after birth. Rats that were weighed within 48 hours after birth were included in the calculation of the mean rat pup weight. Six litters of rat pups (all in the water-exposed condition) were excluded as they gave birth at the beginning of a weekend when the animal technicians were

unable to assist with the task. Nevertheless, 32 litters of the water-exposed pups were available for meaningful comparisons to be made. The mean weight of rat pups in the prenatal water groups was 7.29 grams (SEM = ± 0.28) and that of the prenatal methadone-exposed rat pups was 6.90 grams (SEM = ± 0.59). This difference between the two conditions was not statistically significant ($t(22) = 0.64, p > 0.5$).

3.2 Lactational Measures

3.2.1 Fluid Intake and Methadone Dose

The fluid intake of all rats during lactation was recorded and the mean ingested dose of methadone calculated. The average fluid intake for all four groups during the 4 weeks of lactation is shown in Table 4.

Table 4: Mean Fluid intake across rats during lactation (mls, brackets = \pm SEM).

Condition	Week 1	Week 2	Week 3	Week 4
Wg/Wl (n=5)	86.20 (11.79)	129.93 (15.96)	179.02 (24.28)	227.33 (22.27)
Mg/Wl (n=3)	73.98 (18.63)	101.14 (15.79)	141.56 (23.69)	187.82 (17.58)
Wg/Ml (n=5)	57.25 (12.10)	83.44 (9.56)	117.68 (17.31)	176.94 (24.71)
Mg/Ml (n=3)	65.37 (13.20)	95.68 (10.36)	116.4 (18.69)	174.72 (30.52)

For all groups, there was an increase in the amount of fluid consumed from week 1 to week 4. During all four weeks there was a significant difference in the amount of fluid consumed between dams drinking pure drinking water and those with methadone in their drinking water. At each week, dams drinking plain water had a significantly higher fluid intake than methadone-exposed dams ($t(110) = 6.79, p < 0.00001$; $t(109) = 5.87, p < 0.00001$; $t(110) = 7.18, p < 0.00001$; $t(39) = 2.15, p < 0.04$). By applying a Bonferroni post hoc analysis for each week of fluid intake, it was shown that the WgWl group drank significantly more fluid than MgWl rats during week 1, 2, 3, but not week 4 ($p = 0.0404$; $p = 0.00062$; $p = 0.00020$; $p = 0.14$

respectively). WgWl consumed more fluid than both WgMl and MgMl during all four weeks (WgMl, $p < 0.000001$; $p < 0.000001$; $p < 0.000001$; $p = 0.011$; MgMl, $p = 0.000045$; $p = 0.000031$; $p < 0.000001$; $p = 0.013$). The two lactationally methadone-exposed groups (MgMl and WgMl) did not significantly differ in their fluid intake during any week.

The mean daily dose of methadone consumed by both the MgMl and WgMl dams was calculated from their mean weekly intake of fluid. The results can be seen in Table 5.

Table 5: The mean daily weight (mg) of methadone consumed during lactation (brackets = \pm SEM)

Condition	Week 1	Week 2	Week 3	Week 4
MgMl (n=3)	1.78 (0.45)	2.58 (0.64)	3.15 (0.92)	4.46 (1.02)
WgMl (n=5)	1.47 (0.36)	2.12 (0.52)	2.99 (0.89)	4.32 (1.44)

Rats in both conditions showed an increasing consumption of methadone during the weeks of lactation. Rats in the WgMl condition had a slightly lower consumption during each of the four weeks. There were positive correlations between dose and litter size, which reached significance for weeks 2, $r = 0.8$, $p < 0.05$, and 3, $r = 0.95$, $p < 0.05$, but not for either week 1 or 4, $r = 0.56$ and $r = 0.63$ respectively, indicating that the higher doses consumed by certain dams during lactation may have been primarily due to the number of rat pups being nursed.

3.3 Physical Development of Offspring

At each of the three testing ages (PND 30, 60 and 120), all rats were weighed, assessed for any physical abnormalities, and behavioural/physical symptoms of stress recorded. The results for both female and male rats are shown in Tables 6 and 7. For each outcome, the results were as follows.

Table 6: Summary of physical development in female rats at 30, 60 and 120 days after birth i.e., weight (g), stress reactions and physical abnormalities (brackets in weight = \pm SEM); (brackets in stress reactions and physical abnormalities = percentage of rats affected).

Weight (g)	WgWl (n=12)	MgWl (n=12)	WgMl (n=12)	MgMl (n=10)
Day 30	100.0 (11.14)	99.82 (6.76)	93.92 (5.13)	102.94 (15.03)
Day 60	234.10 (16.41)	232.21 (19.11)	229.82 (18.33)	243.47 (36.98)
Day 120	303.97 (21.45)	307.36 (25.05)	307.26 (25.23)	320.90 (44.91)
Stress Reactions				
Day 30	0	0	0	0
Day 60	0	0	0	1 (10%)
Day 120 (total)	3 (25%)	0	3 (25%)	2 (20%)
Physical Abnormalities				
Day 30	0	0	0	0
Day 60	0	0	1 (8.33%)	0
Day 120 (total)	0	0	1 (8.33%)	0

Weight= grams

Table 7: Summary of physical development in male rats at 30, 60 and 120 days after birth i.e., weight (g), stress reactions and physical abnormalities. (brackets in weight = \pm SEM); (brackets in stress reactions and physical abnormalities = percentage rats effected).

Weight	WgWl (n=12)	MgWl (n=8)	WgMl (n=12)	MgMl (n=11)
Day 30	105.15 (15.16)	109.16 (8.45)	106.19 (7.92)	110.36 (12.91)
Day 60	351.65 (33.73)	357.25 (29.36)	363.95 (27.96)	345.78 (28.17)
Day 120	505.51 (54.77)	498.46 (37.67)	514.03 (44.28)	500.4 (37.23)
Stress Reactions				
Day 30	0	0	0	0
Day 60	0	0	0	0
Day 120 (total)	0	0	0	0
Physical Abnormalities				
Day 30	0	0	0	0
Day 60	0	1 (12.5%)	0	1 (9.09%)
Day 120 (total)	2 (16.67%)	1 (12.5%)	1 (8.33%)	2 (18.18%)

Weight= grams

3.3.1 Weight

There were no significant differences produced in a repeated measure ANOVA in the postnatal weight gain at any of the 3 testing ages for either male or

female rats. For the females, rats in the MgMI had both the highest and lowest weight at each testing age (PND 30 = 65.4-113.1(g); PND 60 = 181.3-292.5(g); PND 120 = 245.4-375.0g).

3.3.2 *Stress Reactions*

As can be seen in Tables 6 and 7, stress reactions were restricted to female rats. These included stress reactions around the eyes, (an appearance of redness around the eyes called chromodacryorrhea), fur thinning and the casual observation of hypersensitivity to touch (vocalisations and struggling while handling). Stress reactions did not appear before 60 days of age. The only condition in which rats did not display any signs of physical stress was the MgWl condition. However, the frequencies of total stress reactions in all four conditions were so low that statistical analyses were neither appropriate nor necessary. It was therefore clear that methadone effects on physical manifestations of stress were negligible.

3.3.3 *Physical abnormalities*

Physical abnormalities were also noted at each testing age. Abnormalities included a number of deformed bone growth in the tail, resulting in a 'kinked' look, and early blindness. As can be seen in Tables 6 and 7, only one female had a physical abnormality, which was in the WgMI condition. Males had a higher rate of physical abnormalities than females. No males presented with any physical abnormalities at day 30. One male out of each of the MgWl and MgMI groups had developed a physical abnormality by day 60. All groups had at least 1 physical abnormality by day 120. But as for stress reactions, the frequencies of physical abnormalities were too

low to warrant valid statistical, analyses. It can therefore be assumed that the effects of methadone treatment on physical abnormalities were again negligible.

3.4 Behavioural Testing

Each rat experienced a series of behavioural tests at 30, 60 and 120 (\pm 5days) days of age.

3.4.1 Open-Field Testing

The rats' behaviour in the open-field apparatus was coded at each testing age. ANOVAS for each coded response can be seen in Table 8 which outlines main effects for methadone treatment, sex and age on each measure.

Table 8: Mean (\pm SEM) responses emitted by (1) rats in conditions WgWI (n=24) MgWI (n=20) WgMI (n=24) and MgMI (n=21), and by (2) each sex and (3) by all rats at each testing age.

Condition	WgWI	MgWI	WgMI	MgMI	F (3, 81)	P
Ambulation	38.33 (1.79)	41.67 (1.47)	43.42 (0.88)	36.30 (1.91)	5.31	.002
Walking	8.83 (0.61)	8.70 (0.59)	10.63 (0.40)	6.91 (0.43)	9.15	<.001
Rearing	16.08 (3.30)	14.58 (0.83)	16.56 (0.96)	13.76 (1.14)	0.5	>.6
Grooming	1.90 (0.20)	1.72 (0.23)	1.83 (0.25)	2.16 (0.35)	0.41	>.7
Boluses	0.10 (0.06)	0.77 (0.25)	0.85 (0.29)	1.43 (0.44)	3.97	.011

Sex	Male (n=43)	Female (n=46)	F (1, 81)	P
Ambulation	35.85 (1.06)	43.83 (0.93)	38.74	<.001
Walking	7.96 (0.40)	9.65 (0.38)	11.17	.001
Rearing	12.00 (0.57)	18.44 (1.69)	11.66	.001
Grooming	2.09 (0.18)	1.72 (0.18)	1.96	>.1
Boluses	1.20 (0.26)	0.36 (0.13)	11.07	.001

Age	Day 30	Day 60	Day 120	F (2, 162)	P
Ambulation	34.02 (1.41)	45.10 (0.77)	40.80 (0.96)	41.87	<.001
Walking	11.28 (0.57)	8.34 (0.31)	6.88 (0.31)	37.86	<.001
Rearing	9.96 (0.66)	19.73 (2.48)	16.29 (0.68)	11.36	<.001
Grooming	2.45 (0.30)	1.33 (0.14)	1.92 (0.20)	6.63	.002
Boluses	1.01 (0.21)	0.79 (0.19)	0.49 (0.13)	4.25	.016

3.4.1.1 Ambulation

Females exhibited significantly higher ambulation rates than males at all testing ages. There was a significant age effect on ambulation, with the least amount of activity taking place at PND 30 and the highest at PND 60.

There was a significant methadone treatment effect on ambulation, as seen in Figure 2. According to Newman-Keuls post hoc tests, WgMI condition produced a significantly higher rate of ambulation than WgWI ($p < 0.05$). Both the MgWI and

WgMI conditions produced significantly higher levels of ambulation than the MgMI condition ($p < 0.05$ and $p < 0.05$ respectively).

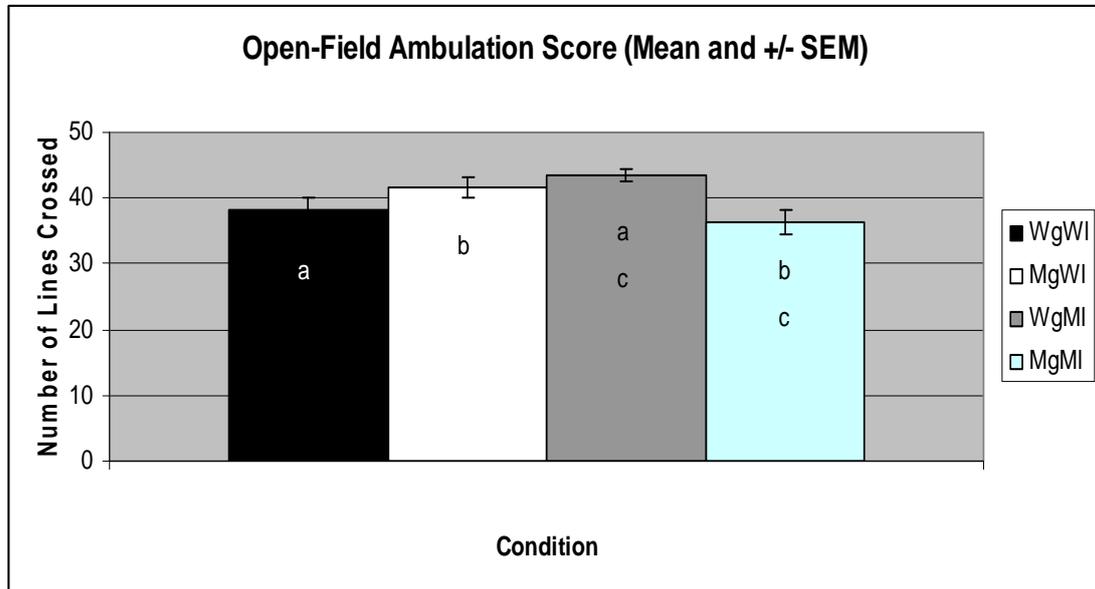


Figure 2: Mean frequency of ambulation for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) at all ages. Matching subscripts indicate significant differences between the conditions indicated ($p < 0.05$, Newman-Keuls).

3.4.1.2 Walking

Walking frequency was significantly higher in females than males for all testing ages. An age effect was also present, with levels of walking progressively reducing as the rats aged. A significant main effect of methadone exposure was found, as can be seen in Figure 3.

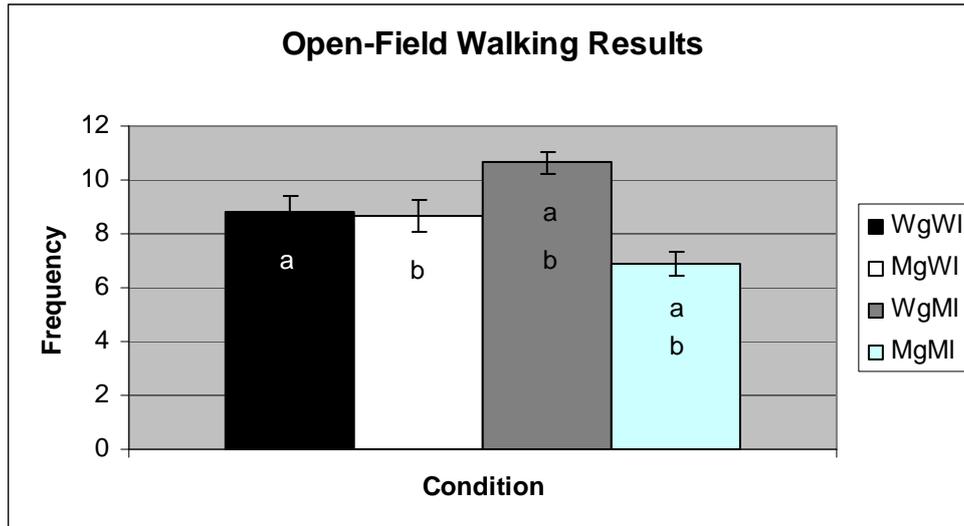


Figure 3: Mean frequency of walking for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) across all ages. Matching subscripts indicate significant differences between conditions at $p < 0.05$ (Newman-Keuls).

Post hoc analyses showed that WgMI rats exhibited a higher frequency of walking than rats in any of the other three conditions ($p < 0.05$). The MgMI rats had significantly lower levels of walking than rats in any of the other three conditions ($p < 0.05$).

3.4.1.3 Rearing

Females reared significantly more often than males for all testing ages combined. Methadone treatment had no significant effect on this response. There was a significant age effect, with the highest frequency of rearing occurring at PND 60 and the lowest at PND 30. There were no significant interactions.

3.4.1.4 Grooming

There were no significant sex differences in grooming. There was, however, a significant age effect on this response. The highest levels of grooming occurred at PND 30 and the lowest levels at PND 60. There were no significant methadone effects or interactions.

3.4.1.5 Defaecation

A significant methadone effect on defaecation occurred. The frequency of defaecation increased progressively from the control group through to the MgMI group, as shown in Figure 4.

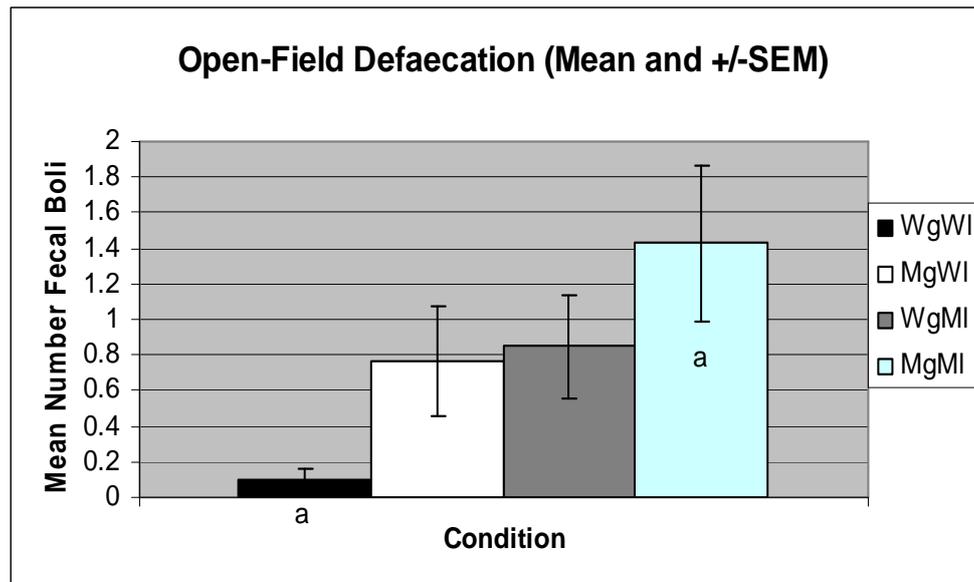


Figure 4: Mean number of faecal boluses for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) for all ages. Matching subscripts indicate significant differences between the conditions indicated ($p < 0.05$, Newman-Keuls).

As shown by Newman-Keuls post hoc tests, there was a significant difference between the control condition (WW) and the MgMI condition, with MgMI rats defaecating more frequently ($p < 0.05$).

There was also a sex effect, with males defaecating significantly more often than females. A significant age effect on defaecation occurred, with the mean number of faecal boluses reducing progressively as the rats became older. An interaction between age and sex was shown to be statistically significant $F(2,162) = 5.37$, $p < 0.006$, as outlined in Figure 5. This was due to a significant sex effect occurring at PND 60 but not 30 or 120.

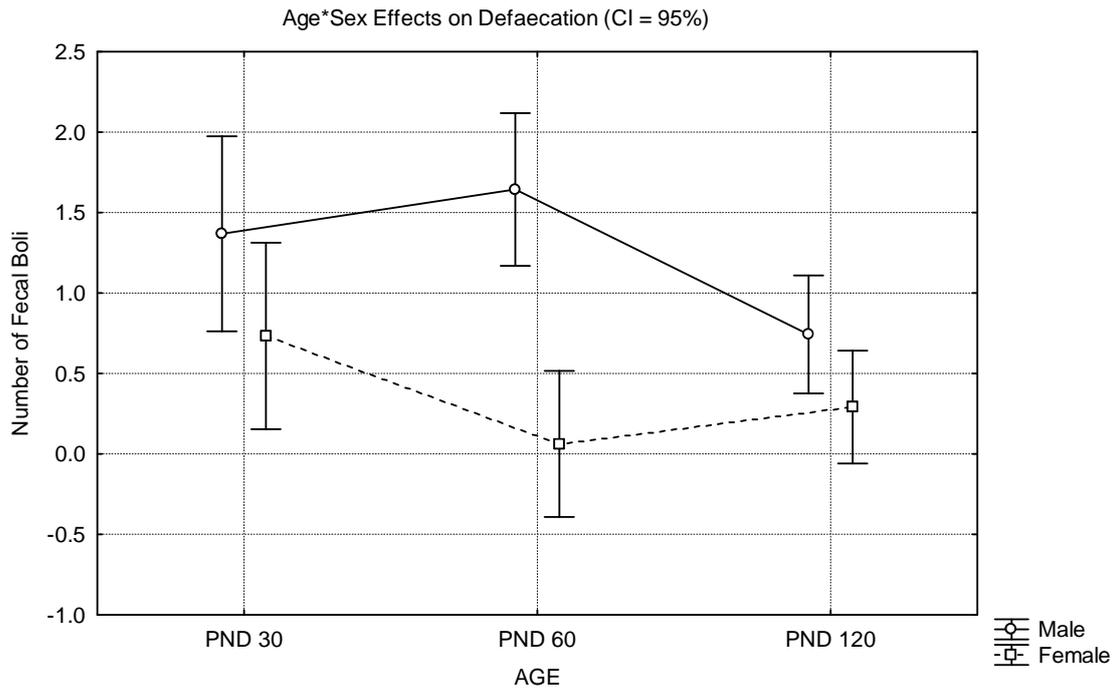


Figure 5: Mean number of faecal boluses for males (n=43) and females (n=46) across all testing times.

Both male and female rats showed significant variations in levels of defaecation across time (males $p < .02$; females $p < 0.01$). At PND 60 there was a significant sex difference, with rates of defaecation in males being significantly higher than those for females $t(87) = 4.21$, $p < .01$. Sex differences at day 30 and 120 were not significant.

3.5 Emergence Testing

Each animal was tested on their speed to emerge at each of the three testing times. The repeated measure ANOVA results can be seen in Table 9.

Table 9: Mean (\pm SEM) emergence latencies emitted by (1) rats in conditions WgWI (n=24) MgWI (n=20) WgMI (n=24) and MgMI (n=21), and by (2) each sex and (3) by all rats at each testing age.

Condition	WgWI	MgWI	WgMI	MgMI	F (3, 88)	P
Emergence Latency	168.05 (12.70)	163.43 (19.54)	153.83 (19.52)	149.45 (17.57)	0.59	>.6
Sex	Male		Female		F (1,81)	P
Emergence Latency	207.80 (10.23)		115.81 (9.83)		40.92	.001
Age	Day 30	Day 60	Day 120	F (2,162)		P
Emergence Latency	243.45 (10.93)	126.25 (12.57)	111.06 (12.21)	54.14		<.001

There was no significant main effect of methadone treatment on emergence latencies, but a significant sex effect occurred with females emerging faster than males. There was also a significant age effect, with rats emerging progressively faster as they got older. There was no significant interaction between methadone treatment and sex. However, an interaction between methadone treatment and age was shown to be significant $F(6,162) = 2.62, p < 0.02$. As can be seen in Figure 6, this interaction can be accounted for by rats in the WgWI, WgMI and MgMI conditions showing a progressive decrease in the amount of time taken to emerge as they became older, with rats in the MgWI condition failing to show a similar significant trend.

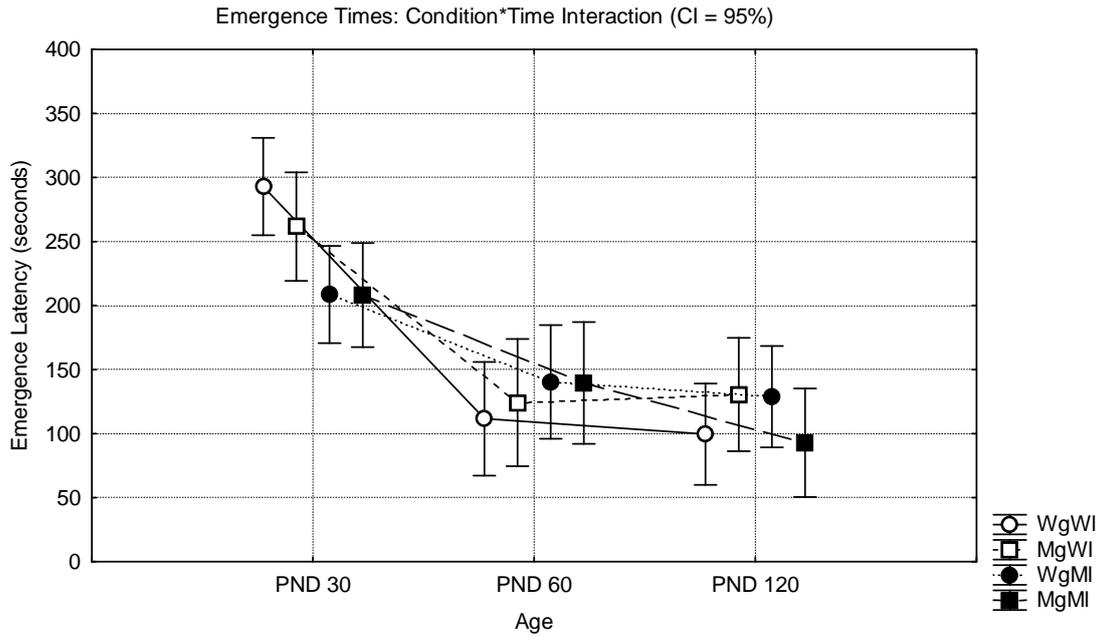


Figure 6: Mean emergence time for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) at PND 30, 60 and 120.

An interaction between sex and age was also significant $F(2,162) = 4.48, p < .02$. Females emerged significantly faster than males at every testing time, as can be seen in Figure 7, but this was not significant at PND 30.

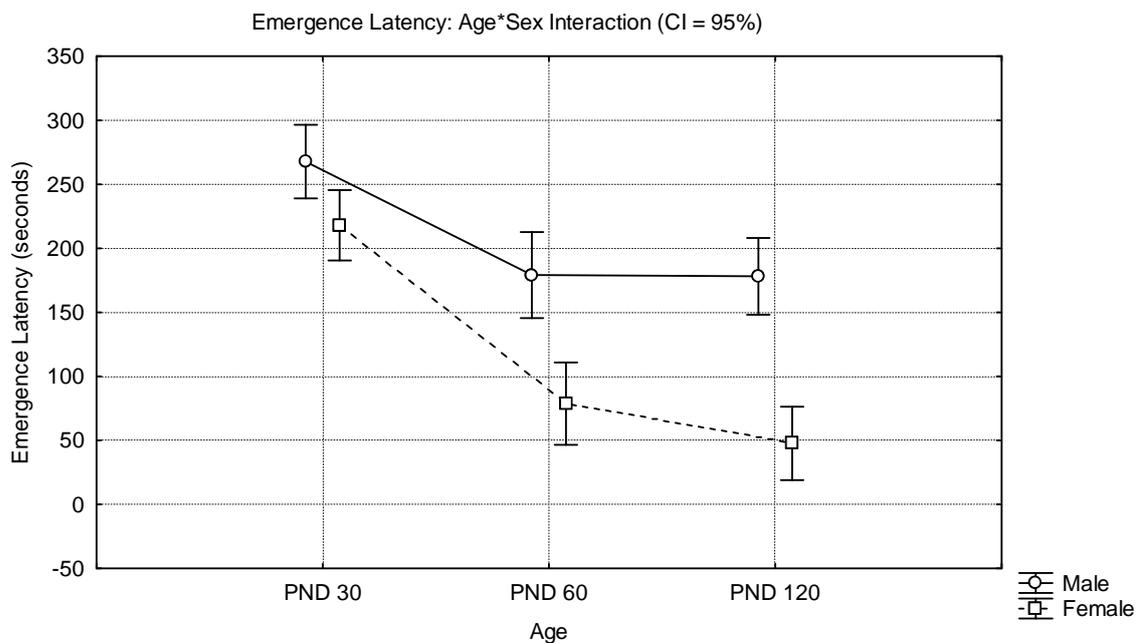


Figure 7: Mean emergence times for males (n=43) and females (n=46) at PND 30, 60 and 120.

The significant methadone treatment x age interaction revealed that PND 30 was the only testing time when a significant treatment effect occurred (see Figure 8). It took rats in the WgWI condition significantly longer to emerge into the lit area compared to those in both the WgMI and MgMI conditions ($p < 0.05$ and $p < 0.05$ respectively).

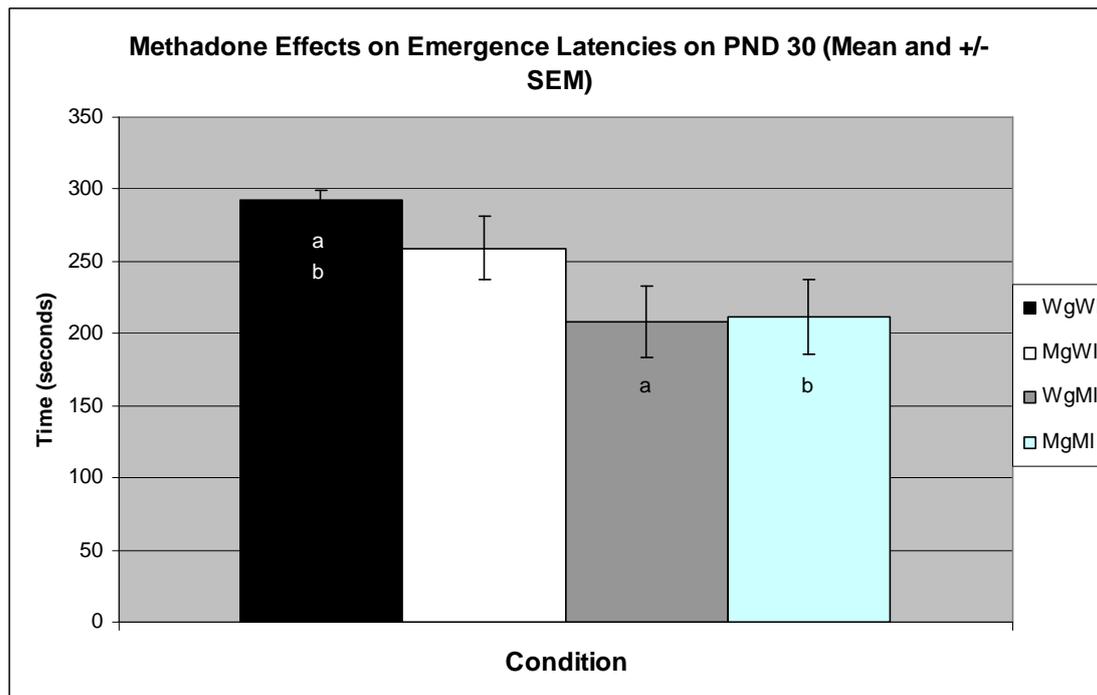


Figure 8: Mean time taken to emergence for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) at PND30. Matching subscripts indicate significant differences between the conditions indicated ($p < 0.05$).

3.6 Y-Maze

Group means for per cent time spent in and entries of the novel versus unchanged arm and total time spent in and entries of both arms were calculated. These plus results of separate ANOVAs for treatment, sex and age can be seen in Table 10.

Table 10: Mean (\pm SEM) of time spent in the novel arm, percentage of entries into the novel arm, total time in both and total entries by (1) rats in conditions WgWl (n=24) MgWl (n=20) WgMl (n=24) and MgMl (n=21), and by (2) each sex and (3) by all rats at each testing age.

Condition	WgWl	MgWl	WgMl	MgMl	<i>F</i> (3, 81)	<i>P</i>
% novel entries	51.74 (1.01)	51.72 (1.99)	52.23 (0.85)	50.63 (1.56)	0.28	>.8
% novel time	51.10 (2.15)	53.03 (2.16)	52.78 (1.58)	51.14 (2.05)	0.38	>.7
Total Entries in both	7.63 (0.36)	7.47 (0.31)	8.64 (0.38)	6.29 (0.46)	8.30	<.001
Total Time in Both	91.11 (2.91)	86.09 (2.62)	88.93 (2.74)	83.70 (3.68)	1.19	>.3

Sex	Male	Female	<i>F</i> (1, 81)	<i>P</i>
% novel entries	52.01 (1.02)	51.22 (0.89)	0.49	>.4
% novel time	52.64 (1.40)	51.40 (1.38)	0.52	>.4
Total Entries in both	6.58 (0.25)	8.45 (0.27)	32.54	<.001
Total Time in Both	88.06 (2.13)	87.25 (2.15)	0.61	>.6

Age	Day 30	Day 60	Day 120	<i>F</i> (2,162)	<i>P</i>
% novel entries	52.02 (1.27)	51.98 (0.96)	49.81 (0.92)	2.05	>.1
% novel time	51.22 (2.12)	51.88 (1.46)	52.90 (1.50)	0.29	>.7
Total Entries in both	7.15 (0.28)	8.14 (0.28)	7.36 (0.30)	4.79	.001
Total Time in Both	1110.28 (2.51)	84.14 (2.30)	68.52 (2.39)	87.34	<.001

3.6.1 Per cent Entries of the Novel Y-maze arm

There were no significant effects of methadone treatment, sex or age on per cent entries of the novel Y-maze arm. There were no significant interactions of any kind.

3.6.2 Per cent Time Spent in the Novel Y-maze Arm

As with per cent entries, the per cent time spent in the novel arm was not significantly affected by methadone treatment, sex or age.

3.6.3 Total Entries of Both Y-maze Arms

There was a significant methadone effect on total number of entries of both Y-maze arms which can be seen in Figure 9. Post hoc analyses showed that rats in the MgMI condition made significantly fewer entries of both arms than those in any of the other three conditions ($p < 0.05$). WgMI rats also made significantly more entries than MgWI rats ($p < 0.05$).

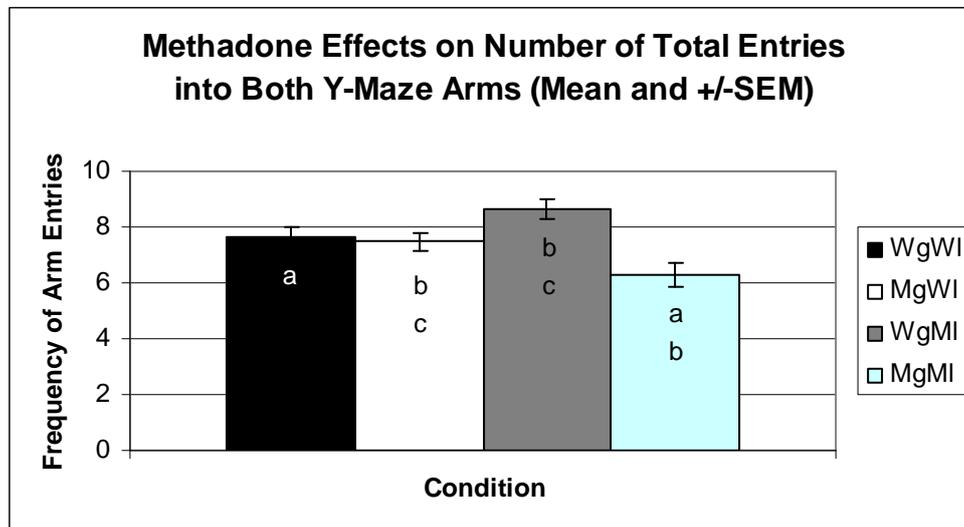


Figure 9: Mean total entries of both Y-maze arms for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) rats for all testing times combined. Matching subscripts indicate significant differences between the conditions indicated ($p < 0.05$, Newman-Keuls).

Females made significantly more entries of both Y-maze arms than males.

There was also a significant age effect for this measure, with most entries occurring at PND 60 and the least at PND 30. The variation across ages was found to be statistically significant. A significant interaction between sex and age occurred $F(2,162) = 3.57, p < .03$, as can be seen in Figure 10.

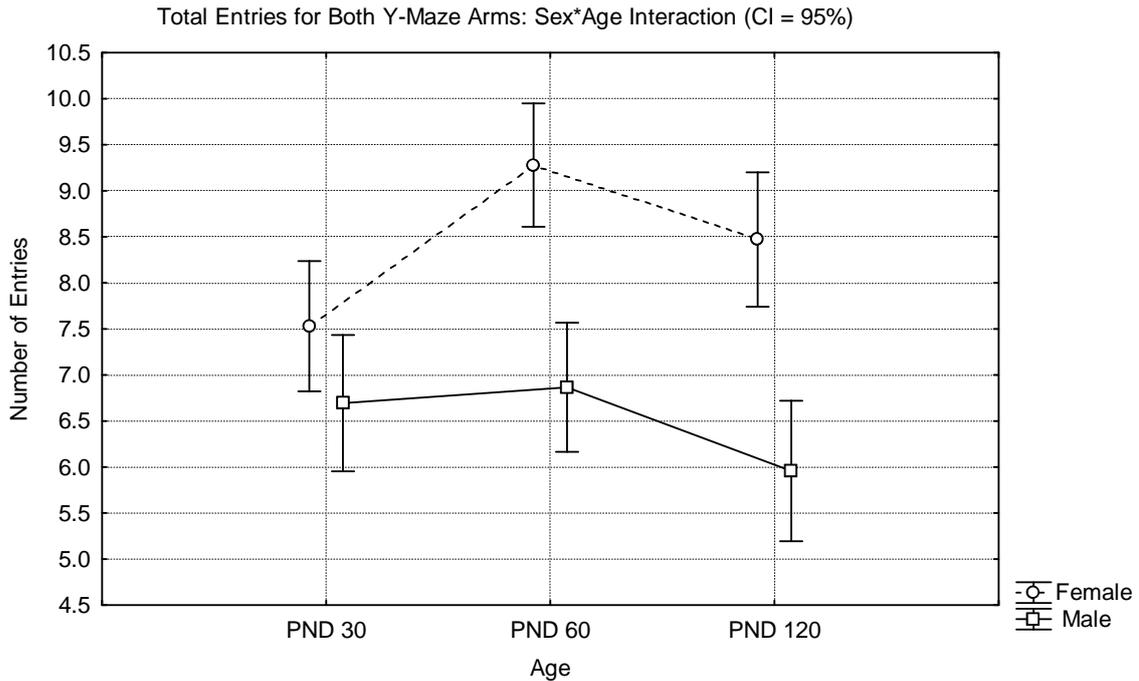


Figure 10: Mean (\pm SEM) total entries of both Y-maze arms for males ($n=43$) and females ($n=46$) at PND 30, 60 and 120.

Although entries of both arms did not vary significantly between the three testing ages for the male rats ($p > .2$), they did so for females, $F(2,140) = 6.96$, $p < .01$, with the lowest number occurring at PND30 and the highest at PND 60. The differences between males and females total number of arm entries was significant at PND 60 $t(87) = 4.83$, $p < .01$, and PND 120 $t(87) = 4.42$, $p < .01$.

3.6.4 Total Time Spent in Both Y-Maze Arms

There was no main effect of methadone treatment or sex on the time spent in both Y-maze arms. However, there was a significant age effect with the longest time being spent at PND 60. There was also a significant interaction between methadone treatment and age $F(6,162) = 2.11$, $p < .055$, as shown in Figure 11. Rats in all methadone treatment conditions spent progressively less time in both Y-maze arms as they grew older.

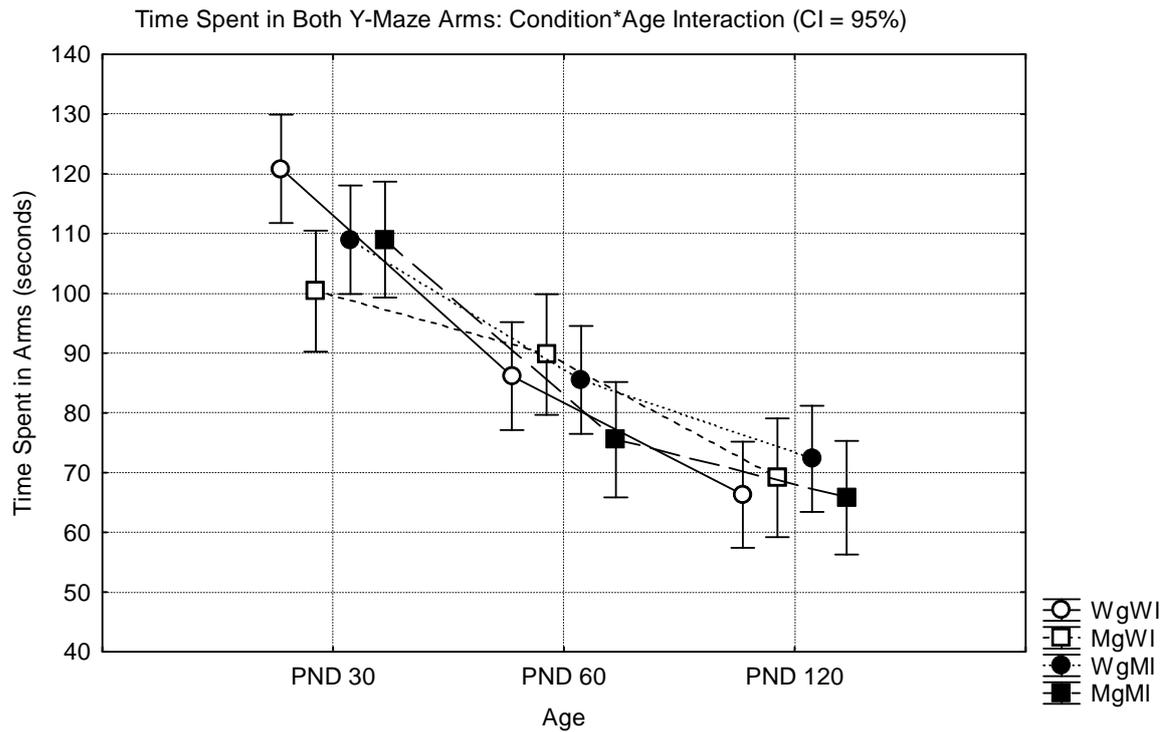


Figure 11: Mean (\pm SEM) total time spent in both Y-maze arms for each condition WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) at PND 30, 60 and 120.

The methadone treatment x age interaction was mainly due to a significant treatment effect only at PND 30 $F(3,88) = 3.12, p < .03$ (see Figure 12). MgWI rats spent significantly less time in both arms compared to those in the WgWI condition ($p < 0.05$).

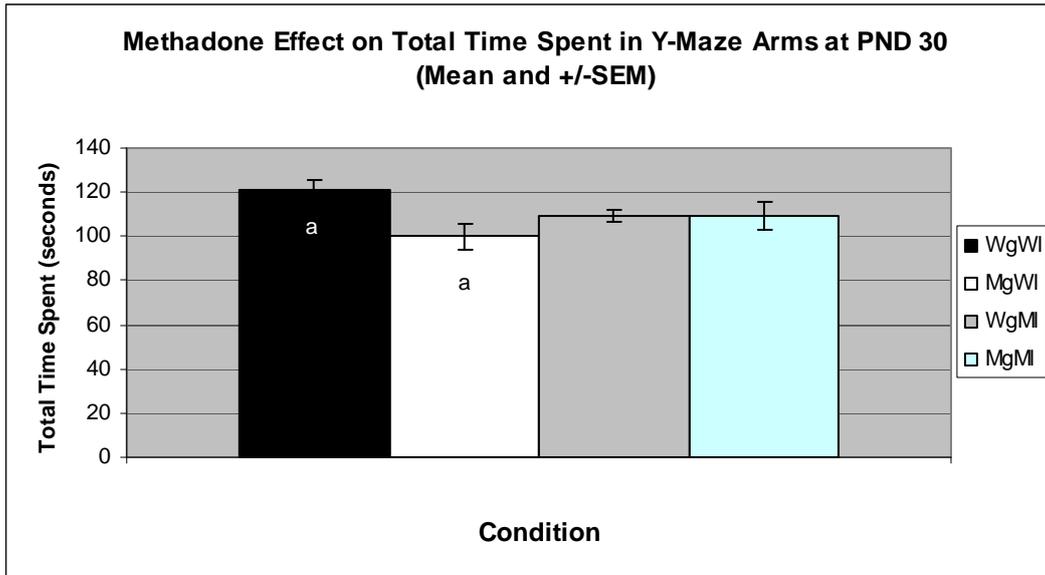


Figure 12: Mean total time spent in both Y-maze arms for WgWI (n=24), MgWI (n=20), WgMI (n=24) and MgMI (n=21) at PND30. Matching subscripts indicate significant differences between the conditions indicated (<0.05).

There was also a significant interaction between sex and age for total time spent in both Y-maze arms $F(2,162) = 4.01, p < .02$, as outlined in Figure 13. The only testing time at which the sex difference was significant was PND 30 $t(87) = 2.28, p < .025$, with males spending significantly more time than females in both arms.

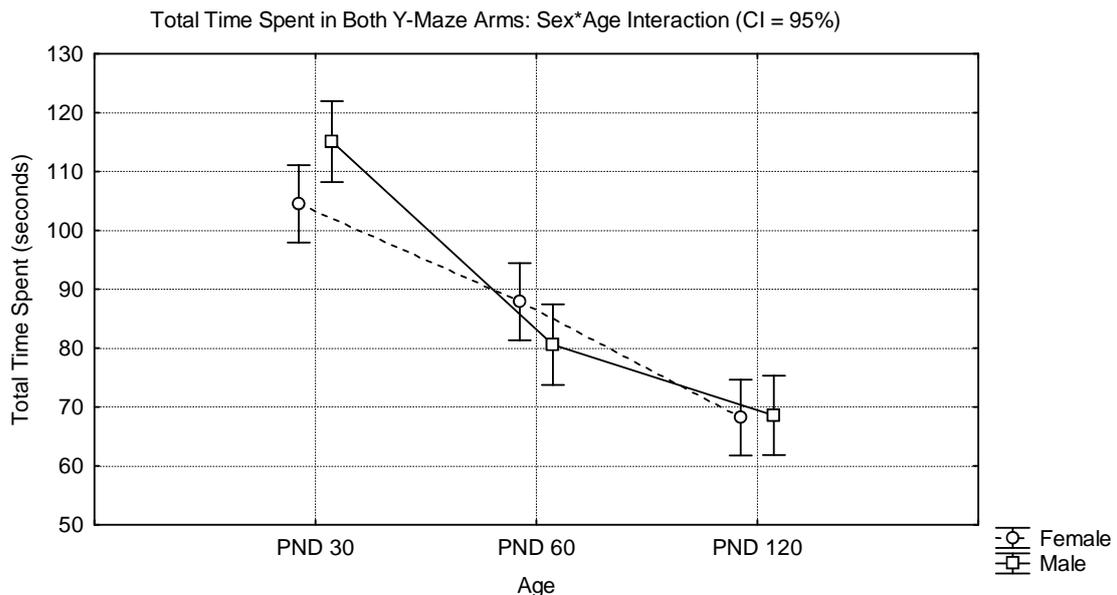


Figure 13: Mean (\pm SEM) total time spent in both Y-maze arms for males (n=43) and females (n=46) at PND 30, 60 and 120.

Overall, there were a number of sex differences in the measures recorded with females consistently weighing less and obtaining higher activity scores than males. There were also a number of age effects which in most cases reflected age-related changes in motor activity. But the most important outcomes were the significant methadone effects, and their interactions with either the age or sex of the rats. These methadone effects and interactions will be accounted for and related to and discussed with previous research in the following discussion section.

Section 4

Discussion

The research described in this thesis was in part concerned with effects on litter sizes and weights, and numbers of still births and birth abnormalities following exposure of pregnant rats to methadone in their drinking water. However, the main focus of the study was to assess long term physical and behavioural effects of methadone exposure during gestation, lactation or both consecutively. In this section, the findings will be discussed and compared to previous findings and theories in the area along with consideration of methodological limitations of the study, and suggestions for further research.

The research described that pregnant dams that received methadone in their drinking water consumed a dose of approximately 2.39 mg/kg/day that had been shown to produce addiction in the rat. Prenatal methadone exposure significantly reduced the number of dams that maintained pregnancy to term compared to dams receiving water. Prenatal methadone exposure did not significantly alter any other pregnancy or birth measure. There were no significant differences across the four conditions for post natal physical development. There were a number of behavioural changes recorded that differed depending on timing of methadone exposure. Activity levels were significantly increased in the lactationally-exposed condition across all three testing times for both sexes. This change in activity level was observed in both the open-field and the Y-maze. In the open-field, lactationally-exposed rats exhibited increased ambulation and walking scores, and increased entries into both arms of the Y-maze. The combined-exposure condition significantly reduced walking levels

through to adulthood, and the ambulation scores followed the same trend with decreased activity, but did not reach statistical significance. Faster emergence in the light/dark box emergence apparatus was seen in both lactationally exposed groups, WgMI and MgMI. This decrease in emergence latency was restricted to PND 30 - no significant differences amongst the groups were found in adolescence or adulthood. The amount of defecation in the open-field apparatus progressively increased in frequency, dependent on the timing of exposure. Defecation was significantly increased in the combined-exposure condition compared to control levels. These findings, combined with decreased walking scores suggested heightened anxiety. There was a sex-age interaction in amount of defecation. Males defecated significantly more often than females at all three testing times. The largest sex difference in number of faecal boluses occurred during adolescence, with males defecating significantly more often.

4.1 Pregnancy Effects

As described above, the current research produced no evidence of methadone effects on maternal weight gain or premature birth in the offspring. These findings do not fit with previous clinical studies that suggest an increased likelihood of prematurity in the prenatally methadone exposed child (Arlettaz et al, 2005; Boer et al, 1994). Rather, findings for the present study suggest that prematurity and maternal weight gain during pregnancy may have less to do with methadone than originally assumed by past clinical findings. There are confounding variables that are unable to be controlled for in the clinical population which may produce these variations during pregnancy, as opposed to any drug effect. For example, environmental factors such as

nutrition, poly-drug use and prenatal care may play a more important role than methadone per se in influencing these outcomes.

The only pregnancy effect that was found to be significant was the number of successful pregnancies, which was significantly different in the gestationally methadone-maintained dams compared to the water-exposed dams. There was a decreased number of pregnancies in the methadone-exposed group compared to the control group. In fact, there were so few pregnancies that a rebreed was required in order to produce adequate numbers of offspring that had been prenatally-exposed to methadone. It is probable that, rather than not being pregnant after mating, a number of the methadone-exposed dams miscarried or reabsorbed their foetuses. This assumption is based on the fact that the weights of a number of dams increased at the beginning of the gestation period, as the other dams did, but then decreased as pregnancy progressed. Therefore, it is possible that methadone during gestation increases the potential for miscarriage or reabsorption in rodents. This finding supports the current literature that suggests an increased risk of miscarriage and SIDS in the clinical population (Barr et al., 1998; Boer et al, 1994). Animal research also supports the current finding of decreased litter size (particularly in male numbers) when methadone is administered to pregnant dams (Kunko et al, 1996).

Therefore, it is important to note that some pregnancy risk factors are still present. Although methadone is reported to reduce these negative effects compared to heroin-use during pregnancy (Kandall et al, 1999; Rosen and Johnson, 1993), there is still an increased risk of reabsorption of the foetus in methadone-exposed rats compared to control samples possibly indicating an increased risk of miscarriage. While maternal weight gain and the risk of prematurity appear to be largely due to

environmental risk factors, such as nutrition and prenatal care, the risk of miscarriage is increased in methadone-maintained pregnancies.

4.2 Birth Effects

As with the birth measures, the current study failed to confirm many of the physical changes reported in past literature that occur following prenatal methadone exposure. No differences were found in any birth measure amongst the offspring of dams that received methadone during gestation compared to the offspring of dams that received unadulterated water. There were no differences in birth weight, litter size, gestational age or sex ratio. The failure to find any significant differences in terms of birth weight and litter size does not support animal literature that has previously reported smaller litter sizes, particularly reduced numbers of male rat pups (Barr et al., 1998). These differences in results may reflect methodological differences in the administration and doses of methadone. For example, in research assessing methadone use during gestation, either osmotic mini pumps or subcutaneous injections are typically used to administer the methadone to the dam (Bhat et al, 2006; McGinty and Ford, 1980; Robinson et al, 1996b; Yim et al, 2006). Also, the dose administered during gestation in past animal research was, in the majority of cases, approximately 9.0mg/kg/day, which is substantially higher than the average of 2.39mg/kg/day used in the current study. Differing methods of administration combined with a lower dose may explain why the present study did not replicate those findings obtained in previous animal studies. However, the dose used in the present study is known to produce addiction in adult rats (Chipkin and Rosecrans, 1978; Pierce et al., 1992), but may not be toxic enough to produce any negative birth effects such as decreased birth weight or litter size.

As with the previously mentioned birth measures, there was a lack of any significant differences in birth weight, which does not support the consistent finding in clinical samples that birth weight is significantly reduced in methadone-exposed infants (Arlettaz et al, 2005; Bada et al, 2002; Chasnoff et al, 1982; Chasnoff et al, 1986; Hagopian et al, 1996; Kaltenbach and Finnegan, 1987; Rosen and Johnson, 1993). As with maternal weight gain and prematurity, the lower birth weights of prenatally methadone-exposed infants may be primarily due to environmental factors, such as nutrition and prenatal care, rather than to any teratological effects of the drug. It may be the case that adverse effects, as seen in previous studies, are only found at higher dose levels.

The failure to find any significant decrease in birth weight in the current study supports the theory that gestational age (the age of the foetus at birth typically measured in the number of weeks of pregnancy), may mediate birth weight in clinical samples. Studies have reported that gestational age is related to the consistent finding of reduced birth weight in prenatally methadone-exposed infants (Hagopian et al., 1996). Methadone increases the risk of prematurity, which in turn reduces the birth weight of the infant. When gestational age is taken into account, birth weight is no longer significantly related to drug use during gestation. In the current study, gestational age did not differ across the dams that received methadone compared to the dams that received water during gestation. As the gestational age is suggested to have more of an impact on birth weight than methadone exposure, the fact that gestational age does not vary across experimental conditions in the current study would mean that birth weight should not vary either. This was the case, supporting the theory that gestational age may mediate birth weight in methadone-exposed populations. If there was a higher probability of premature births amongst methadone-

treated dams, as is reported in clinical samples (Arlettaz et al, 2005; Boer et al, 1994), then birth weight may have been affected. The findings add support to the view that there are mediating variables which can influence birth weight to a greater extent than the drug effect on its own; it is plausible that one of these variables is gestational age.

4.3 *Physical Development*

As with the impact of methadone-exposure on pregnancy measures, there have also been a vast number of studies assessing physical development in clinical samples. However, in the current study there were no significant differences amongst the four conditions in post natal development. These results also failed to replicate clinical findings which suggest height and weight are reduced in methadone-exposed children compared to their non-exposed peers. However, such children who are consistently smaller than their peers are typically also well below the age-related norms in infancy. Children that are only slightly below these norms show catch up by 12-18 months (Chasnoff et al., 1986; Lee and Chiang, 1985; Lifschitz et al., 1985; Zuckerman and Bresnahan, 1991). The current study failed to show any significant differences at birth which could account for why there were no observable differences through to adulthood. It would have been interesting to monitor individual growth of prenatally methadone-exposed rats that were significantly lighter at birth than other rats in order to see if they showed the same 'catch up' effect as seen in human clinical samples. Running statistical analyses across groups as a whole may produce a degree of data loss through averaging effects. In clinical samples, only those infants that are significantly smaller at birth show the same reduction in weight through different stages of development. It would be reasonable to expect that, if the clinical results are

directly related to methadone exposure, then individual rat pups should show the same trends.

The reason for this lack of support for previous research could be explained by potential confounding variables. Post natal physical development could be reduced in methadone-exposed children in clinical samples because of the likelihood of increased exposure to environmental risk factors. For example, methadone infants are more likely to live in poverty and suffer from abuse and neglect (Burns et al, 1995; Chaffin et al, 1996; Kaltenbach et al, 1999; Magura, 1996) which may be contributing to children's postnatal development to a larger extent than the drug effects suggested by clinical studies. These environmental factors were controlled for in the present study in which no causal links were found between prenatal methadone-exposure and postnatal physical development.

There was one area of physical assessment that supports past research. The present study was consistent with clinical studies in which there were no significant increases in physical abnormalities (Burns et al., 1995; Rosen and Johnson, 1993). There were no differences between the four experimental conditions in congenital abnormalities at birth, postnatal growth abnormalities or physical symptoms of stress. However, it was casually observed that the lactationally exposed group showed hyperactivity and an aversion to being handled. This characterised both males and females and was likely to have been due to stress rather than withdrawal since it was consistently observed right through into adulthood.

4.4 Activity Levels

Even though a number of the pregnancy and birth measures did not support previous research, due to failure to find any differences between methadone- and

water-exposed conditions, there were a number of significant behavioural changes in exposure groups. One of the more enduring alterations in methadone-exposed rats was in activity level. Activity levels were significantly altered compared to control rats depending on the timing of methadone exposure. Activity levels were increased in the lactationally-exposed group, with a higher frequency of ambulation and walking in the open-field apparatus compared to the control group (WgWI). This increase in the activity levels of the lactationally-exposed condition was also seen in the Y-maze testing, with the lactationally-exposed rats having more entries into each of the arms over the 3 minute period. These findings support other research involving the same experimental conditions which also showed an increase in activity levels in the lactational exposure group (Kunko et al, 1996). The increase in activity that was seen in the lactational exposure group can not be explained by withdrawal effects because it persisted into adulthood, long after any withdrawal effects would have ceased.

In contrast, the combined-exposure group showed significantly lower levels of walking than controls. There was a similar trend in the ambulation scores but the reduction in activity did not reach statistical significance. These findings support Kunko et al. (1996) who concluded that offspring that had been exposed to methadone during both gestation and lactation were characterised by lower levels of spontaneous motor activity than control rats.

In human samples, there is a large number of prenatally exposed breast fed infants that also receive methadone during lactation. Therefore, when assessing the effect of prenatal methadone exposure alone there is the potential confounding effect of exposure not being limited to the prenatal period, with combined prenatal and postnatal exposure, as with the combined-exposure group in the current study (MgMI). But, despite possible dual exposure during gestation and lactation, there is

no evidence to date to suggest that clinical samples are affected in the same way as rats were in the current study.

These present results are not consistent with previous clinical research that suggests prenatal methadone exposure increases hyperactivity and impulsivity in children (Hutchings, 1982; Rosen and Johnson, 1993). The activity of prenatally methadone exposed offspring in the current study was not significantly increased or decreased compared to controls. The current study controlled for environment factors that can not be controlled for in the clinical population, such as nutrition, poverty (relating to the provision of food and shelter for the offspring) and poly-drug use. Increased activity levels in prenatally methadone exposed infants in clinical samples could be primarily due to environmental influences that are difficult to control for fully in human studies.

The increase in activity levels in the lactational exposure group reported in the current research and Kunko et al's (1996) study could be linked to the reported increase in hyperactivity found in prenatally exposed clinical samples (Rosen and Johnson, 1993). Lactational exposure-induced increases in activity in rats could be more in line with similar increases for humans following prenatal exposure to the drug, because newborn rats have not reached the same relative degree of CNS development as newborn humans. For example, the growth spurt in brain neuronal proliferation begins midway through gestation in humans, but not until birth in the rat (Dobbing and Sands, 1973). Consequently, maximal development in this respect occurs after birth in the rat meaning that newborn rats may be more equivalent to foetal humans. So the lactational exposure effects seen in rat samples could be more like human exposure during the third trimester. It is then possible that there is a

critical period of exposure during lactation in the rat and during the third trimester in humans that accounts for the increase in activity levels.

However, the increase in activity levels in the lactational exposure group is confounded by maternal behaviour, because the dams were consuming methadone during the lactational period and were thus susceptible to its immediate effects. This means that, while changes in the offspring's activity could have been due to their exposure to methadone, the changes could also have arisen from the drug's direct effects on their foster mothers' maternal behaviour. Differences in maternal behaviour while taking opioids have been reported in humans, especially increased abuse and neglect (Burns et al, 1995; Chaffin et al, 1996; Kaltenbach et al, 1999; Magura, 1996). Methadone use also alters maternal behaviour in the rat, with the dam taking significantly longer to engage in any maternal behaviours such as nursing and grooming (Yim, 2006). Nursing and grooming by mother rats can decrease stress levels in their offspring (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). Therefore, the increased activity levels in the lactationally exposed rats could have been due to increased anxiety and hyper-vigilance resulting from their mother's methadone-related impairment of maternal care. Some indication of this might be obtained from observing precisely what type of activity was increased. For example, it may be that stress-related escape rather than curiosity was the motive for their behaviour.

4.5 *Anxiety*

One method of measuring anxiety levels in rats is through the use of open-field testing. More anxious rats tend to present with decreased ambulation levels and increased defecation. In the current study, defecation progressively increased with methadone exposure. These findings suggest that anxiety increases from control

levels, to lactational exposure, gestational exposure, and with the highest levels of anxiety being found in the combined exposure group. These results are combined across all three testing times, suggesting that the changes in anxiety levels persist through to adulthood. The combined-exposure condition defecated significantly more often than the control group. The combined-exposure rats also had significantly reduced rates of walking compared to control rats, with ambulation levels following the same trend but not reaching significance. The reduced rate of activity and increased levels of defecation suggest that methadone exposure during both the gestational and lactational period may increase anxiety.

Another measure of anxiety used in this study was the recording of emergence latencies in the dark/light box apparatus. More anxious rats typically take a longer time to emerge into the brightly lit area. Age related changes in the current study were seen with decreases in emergence latencies primarily from PND 30 to 60, and for three of the four groups from PND 60 to 120. This decrease in emergence latencies suggests that the rats concerned may have become less anxious as they grew older. Similar age-related changes have been reported by other authors e.g., Candland and Campbell, 1962; Hughes, 1968. Also in the current study, there were no significant differences across any of the experimental conditions in emergence latencies during adolescence and adulthood. The only time that groups significantly differed from the control condition was during childhood, PND 30. The lactationally-exposed and combined-exposure conditions emerged significantly faster from the darkened chamber when compared to control, non-exposure rats. These findings indicate that there was a significant change in anxiety levels in both groups lactationally exposed to methadone, with a decrease in anxiety levels being found in these two conditions when compared to controls.

Taking these two measures of anxiety together, methadone effects on anxiety appear to be mixed. The combined-exposure condition suggests increased anxiety levels in terms of increased defecation and decreased ambulation in the open field. However, in the emergence apparatus, there was a significant decrease in anxiety-like behaviour, with combined-exposure rats emerging faster than control rats. Another inconsistency across the two forms of anxiety assessment was the decrease in anxiety levels found during childhood in the lactationally-exposed condition in emergence testing, compared to no conclusive findings in the open-field for any anxiety-based differences (with an increase in ambulation, but also a decrease in defecation compared to controls). The different findings between the two measures of anxiety could be due to confounding variables present in one or both types of experimental apparatus.

Within the emergence apparatus, emergence latencies were only significantly altered for the lactationally-exposed groups. These significant differences are only present at PND 30, which suggests that there was either an alteration in anxious behaviour early in life that is corrected for with age, in influence of maternal/mothering effects, or that there was potentially a withdrawal effect or an acute drug effect that influenced the results. As behaviour changes related to anxiety are not restricted to childhood in the open-field, nor are they in the same direction, the results recorded in the emergence testing are most probably due to either withdrawal or acute drug effects.

Changes in anxiety levels in both conditions exposed to methadone in the lactational period (the lactationally-exposed and combined-exposure conditions) could potentially be explained through remaining methadone in the rats system. Opioids have analgesic properties and reduce stress and anxiety in the user. Decreased

emergence latencies may be due to decreased stress levels compared to control rats. However, if the decrease in emergence latencies were due to the reduction of stress because of immediate drug effects, then you would expect other measures of anxiety-like behaviour to be restricted to childhood also. This was not the case, since lactationally-exposed rats showed increased activity, indicative of reduced anxiety, right through to adulthood. The combined-exposure rats had reduced walking scores in the open-field through to adulthood. This suggests that the changes in emergence latencies in both lactationally-exposed conditions might be better explained, not by acute drug effects, but probably by withdrawal effects.

Withdrawal effects present after the withdrawal of exposure to methadone, in the lactationally-exposed group this would occur after weaning. All rats were weaned at approximately PND 21 and the first round of testing, during their childhood phase, was conducted at PND 30. Withdrawal in rats has been documented to last up to 25 days after exposure (Hutchings, 1982). Thus, the lactationally exposed rats are well within the period of withdrawal. Both lactational groups presented with decreased emergence latencies and the significant differences were not found to persist into adolescence or adulthood. During withdrawal, rats presented with increased locomotor behaviour, distress signals and wall climbing (Barr et al., 1998; Barr and Wang, 1992; Jones and Barr, 1995). It could be the case that the decrease in emergence latency is rather a manifestation of increased spontaneous motor activity and/or escape behaviour that is characteristic of rats experiencing withdrawal. Behaviour after emergence was not recorded, but would have been useful for the interpretation of the decrease in emergence latency. Measuring behaviours such as freezing and other escape behaviours could have helped to clarify the changes in the lactationally exposed group.

The significant increase in anxiety found in the combined-exposure group reflected in increased defecation and decreased activity in the open field cannot be attributed to withdrawal as it persisted through to adulthood. This suggests a drug effect on anxiety levels when methadone is administered during both gestation and lactation. Sex differences were also present, with males being more anxious than females, as shown by their higher defecation rates and decreased ambulation in the open field. There was also a significant sex-age interaction effect. Males were significantly higher in emotionality during adolescence. It is therefore possible that males may be more affected by methadone exposure due to interactions of the drug with male hormone levels. This increased impact of methadone upon males is similar to findings that reductions in litter numbers in rats are primarily due to the reduction of male numbers (Kunko et al., 1996).

This sex difference across anxiety levels, as shown by number of faecal boluses in the open-field apparatus, decreased in males from adolescence through to adulthood, but increased in females. Assessing any sex-age effects in clinical populations would support the current findings that suggest methadone may affect mood across the sexes at various stages of development. Methadone-exposure-related increases in anxiety may have been due to an increase in arousal, thus supporting the idea of decreased levels of focused attention and task performance in childhood because of hyper-vigilance in the child (Hans and Jeremy, 2001; Hutchings, 1982; Rosen and Johnson, 1993). Increased anxiety could also be linked to clinical studies that found reductions in social interactions, attention levels and reduced cognitive capacity. Assessing future mood disorders, particularly in those prenatally exposed to methadone would provide insight into whether increased emotionality persists through to adulthood in the clinical population as it does in the current animal study.

Overall, withdrawal appears to have affected the emergence latencies in both conditions where rats were exposed to methadone during lactation. It would have been informative to document the type of behaviour the rats engaged in once emerging to assess for any freezing or escape behaviours. Changes in emergence latencies are most probably the result of withdrawal effects. Methadone exposure during both gestation and lactation combined increased anxiety. Anxious behaviour was also increased primarily in males, particularly during adolescent development. The current research suggests a need for further study with clinical samples of emotional reactivity in both adolescence and adulthood, taking into account possible sex differences. Methadone could potentially affect an individual's emotional state and thus increase the likelihood of the development of mood disorders compared to the normal population. However, these findings are not consistent with past clinical research that suggests breast feeding a prenatally exposed (MgMI condition) child may reduce negative outcomes (Ballard, 2001; Malpas et al, 1993). This reduction may be the case for short term NAS symptoms, but combined gestational and lactational exposure appears to worsen emotional, anxiety-like symptoms in the long term.

4.6 Neurobiological Correlates

The effects of methadone exposure on neurobiological variables were not directly measured in the current study. Subtle behavioural changes across the different conditions dependent on the timing of exposure can be used to support previous findings and theories behind neurological alterations.

Based on the timing of exposure and testing, the significant changes in emergence latencies, seen in both of the lactationally exposed conditions, can most probably be attributed to behavioural features of withdrawal. However, the findings

could potentially be related to methadone effects on cholinergic development. Cholinergic development occurs primarily after birth (Wu et al, 2001). It could be the case that the exposure to methadone during lactation, in the early postnatal period, disrupted cholinergic activity and development which in turn altered anxiety-like behaviour. However, these findings do not persist through to adulthood. This could suggest that the alterations on cholinergic development are not permanent. This conclusion does not fit with Robinson's (2000) suggestion that opioids permanently increase the turnover rate of Ach because the behavioural changes in the lactational group were restricted to childhood only.

No clear-cut support for opioid effects on cholinergic development can be seen in the present results. Past research found alterations in Ach activity after prenatal exposure independent of post natal development. The fact that cholinergic development occurs primarily postnatally favoured a mediating cell being involved in the neurobiological changes. Robinson (2000) suggested that NGF may be affected by opioid exposure which in turn affects cholinergic development, but evidence of this was not apparent in the current results. Had this been the case, behavioural changes would have occurred in both of the prenatally exposed conditions, MgMl and MgW. The combined-exposure condition had some changes in behavioural development, but the prenatally-exposed condition did not differ significantly from controls across the different behavioural assessments.

Sacrificing a certain number of offspring from each condition to measure for Cdk5 and cholinergic development would provide more support for any neurobiological correlates associated with the long term behavioural alterations seen in the current study. It would provide further insight into particular variables influencing behavioural alterations caused by methadone exposure. Previous research

reported changes in Cdk5 to return to normal functioning by PND 28 (Bhat et al., 2006). Because the first round of behavioural testing in the current study occurring at PND 30, changes in behaviour due to Cdk5 reductions could not be assessed. The potential long-term changes in the nervous system due to Cdk5 deficits were not seen in either prenatally-exposed condition. Therefore, the current research does not appear to support the long term changes that Bhat et al (2006) suggested could arise from early Cdk5 reduction.

The limited support for previous theories underlying neurobiological deficits caused by methadone exposure could have been due to dose differences. Previous studies assessing the implications of methadone on neurological functioning typically used a dose of around 9 mg/kg/day, over three times the dose used in the current study (Robinson et al, 1996a; Robinson et al, 1996b; Robinson et al., 1996c). Thus, it is possible that there could be a critical dose level that effects cholinergic development and NGF that was not reached in this study. Studies assessing various opioid doses would provide more insight into this possibility.

4.7 Limitations

While there were a number of significant findings in the current research, there were still a number of limitations that may have comprised some of the results. One limitation affecting the accuracy of the conclusions made in this study is the fact that, in order to reduce stress from the fostering process, during lactation dams were only weighed weekly instead of daily. This decreased the accuracy of the estimated daily dose of methadone consumed during lactation. Having more accurate dose information during both gestation and lactation would have strengthened the ability to attribute addiction to the dams based on previous research on dose levels. Also, more accurate measurements of individual dam levels of methadone during both exposure

periods would mean that assessment of litter effects could be made. Knowing the various doses that each litter is exposed to, and correlating dose with outcome variables, may provide insight into dose effects on behavioural teratology. It would also provide the distinction between the potential effect of various dosages over the gestational and lactational periods separately, highlighting any critical periods of high versus low methadone exposure. In future research, weighing dams every second day (as occurred during pregnancy), would permit more accurate calculations of the lactational dose. However, weighing of the dam in the first week should still be conducted as rarely as needed to reduce separation of the dam from the litter, as separation during this time can alter maternal behaviours (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997) thereby resulting in a confounding variable in any subsequent behavioural testing.

In the current study there was an added stress effect on pregnant dams from being housed individually. As rats are social animals that live in colonies, being housed alone has been documented to result in increased stress reactions (Ehlers et al, 1993; Kim and Kirkpatrick, 1996; Stanton et al; 1988). Each dam was housed individually during pregnancy to enable calculation of its methadone intake. To reduce this added stress effect during pregnancy, dams could be housed together while restricting which water bottle each rat could access. This could be achieved by housing dams in pairs with a wire mesh barrier along the centre of the cage. By doing so, the rats would still be able to smell each other and engage in some limited social activity, but would remain restricted to the water bottle on their side of the cage. This should reduce any anxiety generated by being housed separately, while still allowing for individual calculations of gestational methadone intake.

Addiction was not measured during pregnancy, but the levels of methadone consumed have been previously shown to produce addiction in adult rats. However, measurements of NAS were not taken and could have provided some interesting information about frequencies of occurrence after prenatal exposure. The assessment of NAS can be effected through behavioural coding as well as separating pups from their mother and measuring ultra sonic distress calls.

As with addiction during pregnancy, there was no testing for withdrawal effects in the rat pups that were lactationally exposed to methadone after weaning. Because withdrawal in the rat can last for up to 25 days after drug exposure, behavioural effects at PND 30 (after being weaned at PND 21) could have been due to this process. Testing for symptoms of withdrawal in the pups would allow for any behavioural findings at PND 30 to be attributed to drug effects as opposed to the potential confounding by withdrawal. Withdrawal in the rat pups could have been assessed by video-recording a number of randomly selected rat pups from each condition during a 5-10 minute period after weaning. Then the video records could have been coded for signs of withdrawal, such as tremors, increased licking, wall climbing and pacing (Barr et al., 1998; Barr and Wang, 1992; Jones and Barr, 1995).

The Y-maze methodology was not appropriate as too much time elapsed between acquisition and retention trials. The measure was therefore not effective for assessing memory, because even control rats were unable to detect the novel arm. The design could be improved by reducing the time between acquisition and retention to 2-4 hours. Or the Y-maze testing could be altered significantly to test for reference memory in rats and thus enable comparisons with reduced visual orientation, perceptual and organisational ability found in methadone-exposed children (Chasnoff et al, 1982; Chasnoff et al, 1984; Hutchings, 1982). Testing for reference memory

would be possible by allowing each rat access to the Y-maze with one white and one black arm and then, several minutes later, allowing it further access after the white arm had been changed to black and noting its preference for this changed arm (Hughes, 2001). There have been very few studies of memory in clinical samples. Changing the testing to assess more perceptual-based aspects of memory could be more relevant to the suggestion that there is a decrease in orientation and perceptual ability following exposure to methadone during early development (Hans and Jeremy, 2001; Hutchings, 1982; Rosen and Johnson; 1993).

A major confounding variable in the current study was the behaviour of the dams during lactation. Maternal behaviour was likely affected by the drug exposure, thus offspring behaviour in the lactational exposure group could be due to either drug effects or environmental, maternal behaviour during the nursing period. Alterations in maternal behaviour due to drug effects could be controlled by administering methadone postnatally via injection to rat pups. However, injecting methadone would not fit well with what characterises humans whereby methadone exposure during lactation is never independent from drug effects on maternal behaviour. Therefore, inclusion of possible methadone-induced changes in maternal behaviour during lactational exposure may not have been a major limitation. While causal links between drug exposure during lactation and behavioural development can not be made, the behavioural alterations that occurred in the current literature are more comparable to real life settings. The current results could still be used to suggest potential developmental risk factors, with specific parental training being employed to deal with altered developmental trajectories. Assessing lactational exposure to methadone independent of maternal behaviour, while being informative for direct

drug effects, would be unrealistic and not useful in comparisons with clinical samples as lactational exposure is never separated from maternal behaviour in this context.

4.8 Implications and Applications

The current research can be used to inform best practice of methadone prescription with pregnant women with an opioid addiction. The results also provide insight into potential risk factors and behavioural alterations that could be addressed through clinical and behavioural interventions. Also, the results do have further replication of, and extensions to, the current research if needed, the present results do have some implications for methadone-exposure during the pre- and postnatal period for humans.

In the current study, rats were exposed to a comparable dose of methadone for human usage in terms of ml/kg/day. However, because of their higher metabolic rate, rats typically metabolize drugs faster than humans. Consequently, the effects of exposure to methadone at the dose provided may have been comparatively less for the rats than would have been the case for humans. Nevertheless, in spite of this possibility, there were still significant behavioural effects. There has long been debate about the probability of potential negative outcomes associated with the administration of a high versus low dose of methadone during pregnancy. However, this distinction between high and low doses may be unimportant as even at close to the lowest levels of methadone known to produce addiction in the rat, there are clearly still long-term behavioural outcomes. These outcomes may be greater with higher doses, but it is important to note that even when the lowest dose possible for producing withdrawal symptoms is administered, there are still behavioural effects in the offspring. There is still uncertainty about the implications for NAS, but the present

results show that even when offspring exposure is minimal, there are still some long-term consequences for offspring, particularly with respect to emotional and behavioural development and adjustment.

These findings suggest that assessment of behavioural development through to adolescence and adulthood may also be important. Most current research focuses primarily on physical development, cognitive ability and measures of attention during the early post natal period. While these aspects of development are important, the present results also suggest a need for assessment of activity levels as well as anxiety-like behaviour that could affect the offspring's future personal and social development.

One of the more important conclusions from the current results is the potential for environmental factors to influence behavioural development in methadone-exposed children. The current research highlights the importance of environmental factors, particularly maternal behaviour. The interpretation of the current results suggests that immediate drug effects on maternal behaviour in the early post natal period may influence offspring behaviour, especially activity levels. Knowing the degree to which continued maternal methadone use affects parenting ability, thus exacerbating risk factors associated with methadone-exposure in the offspring, reinforces the potential importance of parent support training in infant care and parenting in the early postnatal period. Parenting training by professionals should be put in place for methadone maintained mothers and fathers. Altering the mother-child interactions early in the infants' development may lower the potential for long term adverse behavioural alterations to occur.

In the current study, NAS was not directly measured, so the results cannot assess the potential benefit or risk associated with breast feeding. It has been

suggested that breastfeeding has more benefits, such as mother-child bonding and an increase in the functioning of the immune system. However, there were some long-term behavioural deficits associated with lactational exposure identified. The confounding variable of maternal behaviour was not controlled for; therefore suggestions for best practice regarding breastfeeding cannot be made. Focusing on, and promoting, parental training in opioid-users may be of more benefit to the child than restrictions placed on breastfeeding.

4.9 Future Research Directions

Future research should consider motor coordination in methadone-exposed rats. This might be done by, for example, a rotarod or somersault test (Turner, 1965). Research in clinical samples has suggested a reduction in motor coordination in prenatally methadone exposed infants during childhood development (Chasnoff et al, 1982; Hans and Jeremy, 2001). Assessing motor coordination and development would help provide support for the current clinical findings that these deficits are due to methadone drug effects.

Another area of behavioural development, that has recently received more attention in the clinical population, is social functioning. Social functioning is reported to be reduced during childhood in prenatally methadone-exposed samples. Testing for these factors in controlled animal studies would help determine cause-effect relationships independent of environmental factors, such as parental ability and socio-economic status. Social interactions in rats could also be assessed by pairing same-sex pairs, as well as different-sex pairs in the same condition but from differing litters, and then coding social behaviour over a selected time frame. Behaviours such as time spent interacting, aggressive behaviour, avoidance and exploration could be coded in order to assess normative levels of social functioning (Fone et al, 2002).

The inclusion of physiological measures of stress in future research would also enhance findings with regard to emotionality and anxiety. This could be achieved by such measures as the duration of adrenal cortical secretion after exposure to environmental stress, or relative adrenal gland weight post mortem. Future results in these areas would be useful to compare with those in clinical populations, and to also determine whether or not male rats are more responsiveness as is the case with behavioural measures of emotional reactivity.

Another area that could provide more information about the dynamics of the interaction between maternal behaviour and drug effects is through documentation of maternal behaviour. At varying times during the day during the course of lactation, maternal behaviour could be recorded. The amount of time spent with the litter, grooming and nursing the pups, as well as the type of nursing pose adopted could be coded in each condition. Such observations might help clarify the limited past research that demonstrated problems in maternal behaviour after opioid exposure (Burns et al, 1995; Chaffin et al, 1996; Magura, 1996; Yim, 2006). The research would also allow more robust conclusions about whether or not the offspring's behavioural development is affected by maternal behaviour as well as drug effects.

The interaction between maternal behaviour and drug action could be further understood by incorporating handling research into the current research design. Handling of litters has been found to positively influence maternal behaviour, making the dam more attentive and thus encouraging the adoption of more successful nursing postures and more frequent grooming (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997). Each of the four conditions in the current study might have half the dams assigned to a 'no handling' group, and the other half to a 'handling' group involving separation from their litters for 5 minutes daily in the first week after birth.

Maternal behaviours would then be measured at specific times over the following 2 weeks of lactation. It would be expected from previous research (Caldji et al 1998; Francis and Meaney, 1999; Vallee et al, 1997), that even with the lactational exposure, dams being under the influence of opioids and thus possibly manifesting reduced maternal activity, handling of their pups would increase their maternal activity to approximately control levels. With such a study, it would be possible to assess if changes in behavioural development in the lactational exposure group were due to the drug exposure over this time, or due to the combined influence of drug effects and maternal behaviour. If the findings were to suggest that it is only a combination of maternal behaviour and drug exposure that impacts upon behaviour, and not drug exposure alone, then it would strengthen the relevance of parental training early in the post natal period as a means of positively influencing infant development.

Future research might also investigate a wider range of doses of methadone than the single dose used in the present study. There are a lot of contradictory results obtained with clinical samples regarding the benefits of high versus low methadone levels during pregnancy. Varied effects on foetal stability, gestational age, birth weight and severity of NAS have been reported with different doses. Controlling for confounding variables that are often reported in clinical samples would allow for any causal drug-dose effects to be conclusively established. This would also inform best practice for the prescription of methadone during pregnancy.

Direct comparisons between methadone and heroin within a single study would provide more accurate estimates of subtle, long-term behavioural changes that methadone produces compared to heroin. Although some past studies have compared the outcomes of the two drugs during pregnancy and at birth through the measurement

of miscarriage, prematurity, birth weight, head circumference and NAS, there have not been studies comparing their effects on long-term behavioural development. It might also be useful to include comparisons with prenatal buprenorphine in the same study since this drug is currently receiving a lot of research attention as an alternative opioid treatment programme during pregnancy. Comparing behavioural differences between all three would add more depth to the results of current research that has to date focused primarily on short term, physical effects.

4.10 Overall Conclusions

The current research failed to support many earlier reported effects of exposure to methadone during pregnancy on pregnancy and birth measures. The only significant effect on methadone-exposed pregnancy was a reduction in the number of viable pregnancies. None of the other birth effects reported in clinical research were supported, suggesting that environmental factors that often present concurrently with methadone maintenance may be influencing these results more than drug effects alone. The most significant findings of the current research were the modifications of long term behavioural development which were dependent on sex of the rats, and timing of methadone exposure. Activity levels were increased in lactationally exposed rats as shown in two types of testing apparatus. These changes in activity persisted into adulthood, meaning that withdrawal effects were not responsible. Combined gestational and lactational exposure decreased activity and increased defecation, suggesting an increase in anxiety in these rats. Increased anxiety also occurred primarily in males, particularly during adolescence, which suggests a potential interaction of methadone with male hormones in the production of these anxiety reactions. Some of the conclusions drawn about the physical and behavioural outcomes following methadone-exposure should therefore be made cautiously. It is

possible that the reason why the current study did not support past research that suggests methadone produces reduced birth weight and length was due to the lower dose used. Perhaps higher doses may be required to increase the likelihood of these abnormalities occurring. The methadone-related increase in activity in the lactationally-exposed rats may have been due to maternal factors. The results of the research presented in this thesis will hopefully inform best practice for the delivery of methadone during pregnancy, since even at low doses there is still the possibility of enduring behavioural changes. The possible influence of maternal behaviour emphasizes the importance of parenting training during the postnatal period to hopefully offset the risks of methadone exposure. The current research highlights the importance of assessing, not only short term behavioural and physical development, but also long term behavioural outcomes. Future research should aim to replicate the current findings as well as observe the effects of exposure to methadone on the development of memory, motor coordination and neurobiological mechanisms. Another interesting possibility for future research would be to combine handling research methodology in early development with methadone exposure in rats. Handling litters early after birth alters the maternal behaviour in the dam, resulting in more positive nursing and attentive behaviours (Stern, 1996). It would be interesting to assess if these positive alterations in maternal behaviour during lactation would reduce the changes in their offspring's behaviour, which was seen in lactationally-exposed pups. While prenatal methadone exposure may be a risk factor for a number of behavioural outcomes, knowing the potential to offset these negative outcomes through positive parenting abilities could highlight the importance of incorporating parenting training during early postnatal development that might act as a protective factor. Knowledge of the potential impact of such environmental factors could assist

the implementation of parental training within the methadone maintenance programme, thereby possibly offsetting potential long term behavioural changes of the sort that occurred in the present study.

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