Amphetamine sensitization animal model for schizophrenia and drug addiction: study of learning, memory and spontaneous behaviours

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Abstract

Amphetamine sensitization induces neuro-adaptations in the cortico-limbic-striatal circuit. Consequently, it disrupts reward processing and incentive salience attribution to behavioural stimuli. It is used as a model for aspects of drug addiction and positive symptoms in schizophrenia. However, it is unclear whether it models cognitive impairment and negative symptoms, which often represent an unmet medical need.

Amphetamine sensitization was confirmed by testing the mice used on locomotor response to a 2.5mg/kg amphetamine challenge 4 or 9 months post withdrawal from a repeated saline or amphetamine (2.5 mg/kg or 5 mg/kg) regimen.

Our first aim was to expand previous findings showing attenuated cue but spared spatial learning for rewards in amphetamine sensitized mice dependent on basolateral amygdala and hippocampus respectively (Ito and Canseliet, 2010). The same dual learning paradigm in the t-maze used in that study was used here but we regularly rotated the maze in order to prevent mice from relying selectively on intra-maze cues to solve the task. Spatial learning was not acquired by mice. Cue learning was spared in amphetamine sensitized mice, showing that the latter can proceed normally under conditions that spatial learning is not acquired.

Our second aim was to study appetitive spatial reference memory acquisition (acquisition phase), performance as well as spatial working memory (testing phase) and spatial pattern separation (both phases) in a within subjects, within trials radial maze task. No group differences were found. This is consistent with unaffected spatial learning shown by Ito and Canseliet (2010). Latency to acquire all rewards in the acquisition phase was reduced in the combined amphetamine sensitized group.

Our third aim was to study spontaneous behaviours. This is the first study that shows

impaired social interaction of freely interacting pairs of mice 2 months post d-amphetamine withdrawal. No elevated anxiety was found on elevated plus maze or open field test. Social approach in the non-reciprocal sociability test as well as novel object recognition were unaffected. There was a non-significant trend for a reduced discrimination ratio for the novel conspecific in amphetamine sensitized mice with 5mg/kg in the social recognition test. Finally, an object-in-place test was used but didn't work for control mice.

Overall amphetamine sensitization does not seem to impair familiarity recognition or novelty preference. Thus, it does not model short term habituation deficit in schizophrenia. Also, it does not seem to impair learning and memory under conditions that the task does not involve simultaneous cue and spatial learning acquisition. Amphetamine sensitization might model social withdrawal selectively under conditions that involve reciprocal social interaction, but not cognitive impairment or anxiety in schizophrenia. This adds to previous evidence that amphetamine sensitization has poor validity as a model for cognitive impairment in schizophrenia. Also, it shows that it may have some validity as a model for social behaviour. Further research is required to clarify these findings.

Chapter 1: General Introduction

1.1 Sensitization of the dopaminergic system: drug abuse

Sensitization results from repeated intermittent exposure to a stimulus which leads to a progressive and long-lasting augmentation of the response to it (for review Rahn et al, 2013). Sensitization was initially shown in the Aplysia californica (Pinsker et al, 1973). It was found that pre-training for four days with a brief noxious stimulus once per day led to facilitation of a withdrawal reflex triggered upon application of a weak tactile stimulus to its siphon. This effect lasted for 3 weeks.

One example of sensitization is the sensitization to drugs of abuse such as amphetamine. Sensitization, also known as reverse tolerance, leads to a progressive increase in drug effects (Segal, 1975, Kalivas and Stewart, 1991, Robinson and Becker, 1986). Therefore, the dose of a drug required to produce a particular response decreases with sensitization. Alternatively, the original dose will trigger more pronounced effects under conditions of a sensitized state. The opposite of sensitization is tolerance. In the case of tolerance the efficiency of a drug is progressively reduced (Jaffe, 1990). Therefore, higher doses are progressively required to produce the same effect.

Psychostimulants are well known to act on the mesotelecephalic dopaminergic system (Zetterström et al, 1983, Laruelle et al, 1995, Di Chiara and Imperato, 1988). For example, amphetamine has been shown to increase extracellular dopamine compared to physiological levels (Zetterström et al, 1983, Laruelle et al, 1995). Amphetamine sensitization in animals is induced by single or repeated intermittent administration of the drug (Robinson and Becker, 1986) and is expressed by increased behavioural responses, ie. locomotor activity (Klawans et al, 1975, Klawans and Margolin, 1975, Segal and Mandell, 1974, Leith and Kuczenski,

1982, Paulson and Robinson, 1995) and striatal dopamine release (Robinson and Becker, 1982, Robinson et al, 1988, for review Pierce and Kalivas 1997) in response to an acute amphetamine challenge after a period of abstinence from the original amphetamine regimen. The latter was shown by measurement of dopamine release in vitro from striatal tissue (Robinson and Becker, 1982, Robinson et al, 1988) and by in vivo microdialysis (Paulson and Robinson, 1995). This effect is known as behavioural sensitization (Kalivas and Stewart, 1991). In this respect, assessment of the locomotor response to psychostimulant challenge in animals pre-treated with psychostimulants has been widely used as a test to confirm behavioural sensitization in experimental studies.

Evidence of behavioural sensitization to psychostimulants has been found in experimental animals since the 1930's. Also, it has been shown in humans since the 1950's (for review Kalivas and Stewart, 1991). It should be noted that genes seem to play a role in the vulnerability to sensitization which varies between different subjects (Hooks et al, 1994, Alexander et al, 1996). Behavioural sensitization has been also shown to occur with various drugs including morphine (Bartoletti et al, 1983), cocaine (for review Kalivas and Stewart, 1991) and MDMA (Spanos and Yamamoto, 1989, Robinson and Berridge, 1993).

An accumulating body of evidence shows that there is cross-sensitization of the dopaminergic system and its behavioural expression between amphetamine and cocaine (Robinson et al, 1988, Akimoto et al, 1990, Horger et al, 1992), morphine (Vezina and Stewart, 1989, Cunningham et al, 1997), cannabis (Lamarque et al, 2001), as well as stress (Booij et al, 2016, Hamamura and Fibiger, 1993, Antelman et al, 1980, Sorg and Kalivas, 1991). For example, application of mild tail pressure, which is a stressful stimulus, can sensitize a rodent to the effects of future amphetamine exposure and vice versa (Antelman et al, 1980). Also, there is evidence of cross-sensitization between nicotine and cocaine (Hoger et al, 1992), and between nicotine and amphetamine (Celik et al, 2006), as well as between

cocaine and stress (Sorg and Kalivas, 1991). Therefore, rodents pre-exposed to a psychostimulant drug show enhanced locomotor responses and increased dopamine release in response to some stressors, or subsequent challenge with another psychostimulant, or other drugs. This indicates that these drugs as well as some stressors may be interchangeable in their potential to induce sensitization.

All drugs of abuse seem to act on the mesotelencephalic dopaminergic system (Di Chiara and Imperato, 1988). In particular, Di Chiara and Imperato (1988) showed by using brain dialysis (dialysis tubes were implanted in the accumbens and caudate) that the extracellular concentration of dopamine was enhanced in the nucleus accumbens septi (a limbic area) and the dorsal caudate nucleus (a subcortical motor area) in freely moving rats after exposure to opiates, ethanol, nicotine, amphetamine, or cocaine at low doses. This effect was also accompanied by hypermotility. The biochemical findings of this study show that enhancement of mesolimbic dopamine accompanied by hyperlocomotion result from exposure to all drugs that due to their rewarding properties can end up being abused.

The mesolimbic doaminergic system mediates the incentive salience attribution to rewards rather than the hedonic experience of them (Koob and Moal, 1997, Berridge et al, 1989, Berridge and Valenstein, 1991, Smith et al, 2011). In this respect, drug abuse results from the sensitization of mesolimbic dopaminergic system which mediates the sensitization of incentive motivational properties of drugs of abuse (Robinson and Berridge, 1993, 2001, 2008). This process may underlie increased drug craving in drug addiction (for review Wise, 1987, 1988).

Incentive salience is triggered by cues paired with the reward (Cornell et al, 1989, Caggiula et al, 2002). Thus, sensitization of incentive salience attribution to drug associated cues can induce compulsive drug seeking and taking triggered by these cues (for review see Berridge, 2012). In this respect, in cocaine addicts craving can be triggered by cues of the

environment that had been associated with cocaine taking (for review Kalivas et al, 1998). Likewise, cues that are known to be present just prior to drug presentation can cause relapse in drug addicts even after prolonged abstinence (Shaham et al, 2003, Bossert et al, 2013).

In line with this, Wyvell and Berridge (2001) provided evidence that pre-treatment with amphetamine increased incentive salience attribution to sucrose reward associated cues. Also, it has been shown that sensitization of the dopaminergic mesolimbic system facilitates encoding of incentive salience attribution to reward predicting cues by ventral pallidal neurons (Tindell et al, 2005). In this study electrophysiological recording of the activity of ventral pallidal neurons was conducted. It was shown that acute amphetamine activated mesolimbic dopamine in both rodents that had been sensitized to amphetamine and rodents that received amphetamine for the first time. As a result, the neuronal firing pattern was gradually shifted away from prediction signal coding reward prediction towards incentive coding. Thus, neuronal firing didn't increase for the first Pavlovian conditioned stimulus (tone). Instead neural firing in the ventral pallidal neurons increased for the conditioned stimulus that followed (feeder click) which predicted an unconditioned stimulus (sucrose reward).

Overall, it seems that sensitization of the mesolimbic dopaminergic system meditates key components of drug addiction including incentive salience attribution to reward predicting cues and consequently compulsive drug seeking and drug taking as well as relapse.

1.2 Sensitization of dopaminergic system: schizophrenia and amphetamine induced psychosis

Schizophrenia is a complex and heterogenous disorder. It is marked by positive and negative symptoms as well as by cognitive impairment. Positive symptoms, which mainly characterize an acute psychotic episode and which respond well to antipsychotic $D_{2/3}$ receptor-

blocking drugs, include delusions and hallucinations. In addition they include thought disorder as well as motor symptoms. Negative symptoms, often encountered outside acute psychotic episodes, include social withdrawal, poverty of speech as well as apathy. Negative symptoms are also frequently found outside of acute psychotic episodes. Unlike positive symptoms they are considered to be challenging to treat (APA, 2013). Finally cognitive impairment which includes deficits in attention, working memory, verbal learning and memory as well as executive functions also often represent an unmet medical need.

An accumulating body of evidence shows that the mesotelencephalic dopaminergic system in schizophrenic patients is over-reactive, as it is endogenously sensitized (Weidenauer et al, 2016, Ujike, 2002, Laruelle, 2000). The sensitization seems to be a maladaptive response to repeated exposure to stimuli or states that enhance dopaminergic activity (Ujike, 2002) such as stress (Corcoran et al, 2003). Stress selectively stimulates dopamine release in the mesolimbic projection (Horger and Roth, 1996). Exposure to high levels of stress is caused either by very high exposure to strong stressors early in life or due to a predisposal or hyper-responsiveness to stressors (Miller et al, 2001). In line with this, peripubertal administration of the anxiolytic drug diazepam in the developmental disruption model of schizophrenia that involves prenatal administration of the mitotoxin methyl azoxymethanol acetate (MAM), blunt hyperlocomotion in response to subsequent amphetamine challenge and thus blunt expression of amphetamine sensitization (Du and Grace, 2013). MAM rats in that study had shown high levels of anxiety before administration of diazepam compared to the control group.

It should be noted though that the hyperdopaminergic state is likely to be the final common end point in schizophrenia. Other possible causes for hyperdopaminergia apart from stress include obstetric complications, trauma, drug use, genetic factors or a combination of some of these factors (for review Howes and Kapur, 2009). Such factors appear to lead to a

hyperdopaminergic state through enhanced presynaptic striatal dopamine activity. This might be caused, for example, by glutamatergic dysfunction associated with NMDA receptor hypofunction (Lisman et al, 2010, Lisman et al, 2008, Coyle, 2006, Krystal et al, 1999).

The gradual development of the endogenous sensitization starts during the premorbid stage of schizophrenia. According to the neurodevelopmental model, in early adulthood vulnerable brain circuits gradually change due to genetic and environmental factors as well as their interactions, which cause deficits in neural regulation (Rapoport et al, 2005). Under these conditions, enhanced dopamine release makes the brain susceptible to sensitization. At some point the clinical picture of the disease becomes apparent when a threshold is exceeded, resulting in a pathological state of endogenous sensitization of the dopaminergic system (Lieberman et al, 1997). This leads to progressive development of schizophrenia (Laruelle, 1999, Seeman, 2011). The endogenous sensitization as well as the progressive development of the disease is also evident by the fact that after every new episode or exacerbation of the disease, or relapse in patients with chronic schizophrenia, recovery time increases (Ohmori et al, 1999). Exacerbation could happen if treatment is discontinued or if patients are exposed to elevated levels of stress, both of which lead to enhanced dopaminergic activity.

Also, the progressive development of schizophrenia is evident by findings that the number of relapses of chronic schizophrenic patients, as well as the total duration of these relapses, were found to be correlated positively with the antipsychotic dose needed in maintenance treatment (Ohmori et al, 1999). In line with this, longer duration and more psychotic episodes have been associated with poor response to antipsychotics and generally poorer outcome (Lieberman et al 1997). Also, endogenous sensitization in schizophrenia is shown by findings that stressful events predict relapse risk in schizophrenic patients that are under standard antipsychotic treatment (Nuechterlein et al, 1992). Overall, these studies

show that re-exposure to a state that is associated with enhanced dopaminergic activity such as stress leads to worsening of the severity of the disease.

Sensitization in schizophrenia has also been shown by increased dopamine release in schizophrenic patients compared to healthy volunteers in response to acute amphetamine challenge (Laruelle et al, 1996, Abi-Dargham et al, 1998, Breier et al, 1997) or stress (Mizrahi et al, 2012). More specifically, in the study by Laruelle et al (1996) and in the study by Abi-Dargham et al (1998) single photon emission computerized tomography was used to measure amphetamine-induced dopamine release. This was estimated by the amphetamine-induced reduction in dopamine D2 receptor availability. This was measured as the binding potential of the specific D2 receptor radiotracer [123I] (S)-(-)-3-iodo-2-hydroxy- 6-methoxy-N-[(1-ethyl-2-pyrrolidinyl) methyl]benzamide ([123I]IBZM). In the study by Breier et al (1997) the dopamine D2/D3 positron-emission tomography radioligand [11C]raclopride was used. In the study by Mizrahi et al (2012) dopamine release in response to psychosocial stress was studied using positron emission tomography assessing [(11)C]-(+)-PHNO binding.

Other evidence consistent with a sensitized dopaminergic system in schizophrenia is the fact that neuroleptic drugs treat psychotic symptoms by reducing dopaminergic activity (van Rossum, 1966, Seeman et al, 1975, Creese et al, 1976). Also, positive symptoms of schizophrenia seem to be exacerbated after amphetamine administration (for review Lieberman et al, 1987). Furthermore, psychotic symptoms seem to be induced after repeated amphetamine use in healthy volunteers (Ellinwood et al, 1973). Characteristic symptoms of amphetamine induced psychosis include anxiety, delusions of persecution and auditory hallucinations reminiscent of paranoid schizophrenia. These symptoms are accompanied by lack of insight and concentration as well as enhanced motor activity. Finally, thought disorganization is also a symptom of amphetamine induced psychosis (Bell, 1973, Jönsson

and Sjöström, 1970). While amphetamine psychosis is reminiscent of acute psychotic episodes, some studies have shown that in amphetamine induced psychosis visual hallucinations and grandiosity are more intense (Srisurapanont et al, 2003).

After chronic use of amphetamine (Ellinwood, 1967) or cocaine (Post, 1975) such psychotic symptoms can progress to chronic amphetamine psychosis. In case the exposure to psychostimulants is not chronic, paranoid symptoms tend to disappear after discontinuation of the drug, while symptoms tend to re-appear if the drug is restarted even in lower doses than those used before (Sato et al, 1983). The period during which the individual remains sensitized and therefore sensitive for symptoms to re-appear after re-exposure to the drug can vary from some months to several years (Sato et al, 1983, Ellinwood, 1967). Relapse can be triggered also by exposure to psychological stressors highlighting again that there is cross-sensitization between amphetamine and stress (Yui et al, 2002).

Overall these studies show that sensitization of the dopaminergic system mediates amphetamine induced psychosis as well as schizophrenia and vulnerability to relapse in both cases either due to exposure to stress, or due to exposure to psychostimulants or due to discontinuation of antipsychotic medication.

1.3 Amphetamine sensitization is developed incrementally and is long lasting

Following withdrawal from an amphetamine sensitizating regime hyper-responsiveness of the dopaminergic system in the mesencephalon to acute amphetamine is long-lasting. In particular, it has been shown to last for up to a year in rodents post-withdrawal from the sensitizing regimen (Paulson et al, 1991). This is almost permanent if one takes into account the short life span of rodents. In rhesus monkeys sensitization has been shown to last up to 28 months (Castner et al, 1999). These findings indicate that

sensitization is not a result of accumulation of amphetamine in the brain of the animal. Instead, it is a result of the susceptibility to relapse to abnormal neurochemical and behavioural patterns that have developed in the brain due to repeated pre-exposure to the drug.

Sensitization does not happen immediately after withdrawal from the amphetamine regimen. Instead it seems to be an incremental process (Kolta et al, 1985, Paulson et al, 1991, Paulson and Robinson, 1995). The exact mechanism of this delayed onset effect is not entirely clear. The ventral Tegmental Area (VTA) seems to be one site at which amphetamine sensitization is initiated. Following repeated cocaine exposure dopamine release is increased in the ventral tegmental area, which was shown by in vivo brain microdialysis in awake rats (Parson and Justice, 1993). Also, dopamine is enhanced in nucleus accumbens in the same study. The nucleus accumbens is involved in the expression of amphetaminesensitization (i.e. the hyperlocomotion in response to amphatamine challenge in amphetamine pre-treated animals) (Akimoto et al, 1990, Kalivas and Duffy, 1993b, Robinson et al, 1988). In line with findings from the study by Parson and Justice (1993) it was shown using microdialysis in the ventral tegmental area and medial substantia nigra, that the basal levels of extracellular dopamine increase one day after discontinuing daily treatments of cocaine compared to saline (Kalivas and Duffy, 1993b). This effect however had disappeared by day 14 post-withdrawal, indicating possibly a transient change in the way that somatodendritic dopamine release is regulated. Given that such effects were transient it seems that they may not be implicated in the long-term effect of behavioural sensitization. However, they may be implicated in the initiation of behavioural sensitization. Thus, amphetamine sensitization is a long term and progressive process with its initiation considered a distinct phase from its expression.

Evidence that the development of amphetamine sensitization following amphetamine withdrawal occurs progressively comes from both animal and human studies. A human study

using PET scanning provided evidence of an incrementally increased dopamine release in response to amphetamine challenge following withdrawal from an intermittent amphetamine regimen (Boileau et al, 2006). In line with this, in rodents amphetamine sensitization was shown at day 28 after withdrawal but not at days 3 and 7 (Paulson and Robinson, 1995), at day 15 but not at day 3 (Kolta et al, 1985, Paulson et al, 1991) and on days 10-14 but not on days 3 to 4 (Wolf et al, 1993). In the latter study autoreceptor subsensitivity in the ventral tegmental area was found on days 3 to 4 post-withdrawal which was no longer evident at days 10-14.

These findings suggest that autoreceptor subsensitivity happens early after amphetamine withdrawal and is transient. Reduction in presynaptic dopaminergic autoreceptor sensitivity has been hypothesized to lead to a reduction in the autoregulatory ability of dopaminergic neurons. This might lead to increases in dopaminergic neuronal activity and release (Martres et al, 1977, Muller and Seeman, 1979). This is because it is known that D2 autoreceptor stimulation reduces dopamine release after being stimulated (Cubeddu et al, 1990, Dwoskin et al, 1986).

White and Wang (1984) showed that dopamine agonists suppress the activity of the majority of A10 dopaminergic neurons by acting directly on somatodendritic dopamine autoreceptors. Thus, somatodendritic dopamine autoreceptor subsensitivity in amphetamine pre-treated animals presumably stems from the continual stimulation of autoreceptors by dopamine released during repeated amphetamine treatment (White and Wang, 1984).

The exact mechanism through which the transient autoreceptor subsensitivity in the early stages of amphetamine withdrawal is linked to the progressive development of amphetamine sensitization that is evident later is not entirely clear. Weiss et al (1992) provided evidence that increases in extracellular dopamine in the nucleus accumbens by cocaine is inversely related to basal dopamine levels. They also showed that the transient

autoreceptor subsensitivity coincides with the transient increase in basal extracellular dopamine following psychostimulant withdrawal (Weiss et al, 1992).

Collectively, all these studies show that the enhancement of amphetamine stimulated dopamine release is not evident in the early stages during which amphetamine sensitization is progressively developed. The somatodendritic regions of the A10/A9 dopamine neurons seem to play a fundamental role in the initiation of sensitization. However, it is not clear how the changes in the A10/A9 region are encoded to allow enhanced dopamine transmission. It might be that the dopamine neurons have become hypersensitive to pharmacological stimuli or that the mechanism of inhibitory regulation is desensitized. Alternatively, changes in cellular activity may have a dysregulating effect on the presynaptic mechanism that controls dopamine release.

1.4 Behavioural Expression of amphetamine sensitization

Amphetamine sensitization is expressed behaviourally by hyperlocomotion (Hirabayashi and Alam, 1981, Leith and Kuczenski, 1982) in response to acute amphetamine challenge after withdrawal from an amphetamine regimen (for review Robinson and Becker, 1986). Stereotypic behaviours are also enhanced (e.g. Klawans and Margolin, 1975, Leith and Kuczenski, 1982, Conway and Uretsky, 1982). Examples of stereotypic behaviours that are sensitized are sniffing, as well as head and limb movements.

While stereotypic behaviours and hyperlocomotion are classically used as indices to verify sensitization, there is evidence of sensitization of other behaviours too. These include sensitization of rotational behaviour (Echols, 1977, Robinson, 1984, Robinson et al, 1982) intracranial self-stimulation (Robertson and Mogenson, 1979), acoustic startle response (Kokkinidis, 1984) and tail pinch behaviours (Antelman et al, 1980). Also, other studies report sensitization of drinking behaviour and cage climbing (for review Robinson and

Becker, 1986). In non-human primates static posturing, hallucinatory-like behaviours and fine motor stereotypies were sensitized (Castner et al, 1999, Castner and Goldman Rakic, 2003). Such behaviours are potentially analogous to positive symptoms of schizophrenia, and include hypervigilance, checking, staring, tracking of non-apparent stimuli. In addition, grasping or manipulating of thin air and parasitotic-like grooming were reported.

Human studies have also provided evidence of behavioural sensitization of a series of behaviours. This was shown by progressive enhancement of behavioural responses after administration of an intermittent 0.25mg/kg d-amphetamine protocol of three single oral doses given at 48 hours intervals, alternating with a matched placebo (Strakowski et al, 1998), or two daily doses of 0.25mg/kg d-amphetamine separated by 48 hours that alternated with two daily doses of matched placebo (Strakowski et al, 1996). These studies showed that following each amphetamine challenge there was an incremental increase in spontaneous eye blink rate as well as in motor activity/energy and talkativeness. These symptoms were assessed by counting the total number of spontaneous eye blinks per 5 minute period and by administering the Young Mania Rating Scale (Young et al, 1978). Similar but less pronounced responses were found for elevated mood and subjective drug effects.

1.5 Amphetamine sensitization: neural and neurochemical mechanisms

Hyperdopaminergia in the striatum (Laruelle et al, 1996, Breier et al, 1997, Abi-Dargham et al, 1998) accompanied by hypodopaminergia in the prefrontal cortex (for review Davis et al, 1991, Abi-Dargham and Moore, 2003) appears to contribute to schizophrenia's positive and negative symptoms respectively, as well as to cognitive impairments. Such dopaminergic imbalance is partially caused by the disinhibition of dopaminergic activity in the striatum, which may reflect the reduced dopaminergic function in prefrontal cortex.

Suchdopaminergic dysregulation in the cortico-limbic-striatal circuit appears to mediate drug addiction (for review Koob and Volkow, 2016), as well as amphetamine sensitization (Robinson and Becker, 1982, Castner et al, 2005).

Neuroplastic alterations in the cortico-limbic-striatal circuit

The chronic dopamine dysregulation in the cortico-limbic-striatal circuit (the reward circuit) that results from amphetamine sensitization leads to persistent neuroplastic alterations in this circuit (Selemon et al., 2010, Nakagawa et al., 2011, Castner and Williams, 2007), including increased length, branching and overall spine density in medial prefrontal cortex (Castner et al., 2005, Selemon et al., 2007) and striatum, and increased number of spines in hippocampus. Also, increased length of dendrites, density of dendritic spines and numbers of branches of dendritic spines with output to prefrontal cortex and nucleus accumbens were found (Robinson and Kolb, 1997).

Re-organization of neural networks accompanying amphetamine sensitization is also evident by the increased expression of spinophilin. This is a protein proposed to link synaptic transmission to changes in spine morphology in striatum, septum, cingulate cortex, amygdala and hippocampus (Boikess and Marshall, 2008). Also, increased MAP kinase phosphatase 3 (MKP-3) in striatum, hippocampus and cortices as well as MKP-1 in striatum, thalamus and cortices have been shown after acute and chronic methamphetamine exposure (Ujike et al, 2002). This finding is important given the well-known role of MKPs in synaptic plasticity.

There is also evidence of increases in other markers of neuroplasticity. Thus, enhanced activity of regulated cytoskeleton associated protein (Arc) mRNA, which is an effector immediate early gene has been found in striatum, hippocampal CA1 and cerebral cortices after both acute and chronic methamphetamine exposure (Kodama et al, 1998, Ujike et al, 2002). Also, Hashimoto et al (1998) demonstrated that acute amphetamine exposure led

to expression of tissue plasminogen activator (tPA) mRNA in a group of prefrontal cortical neurons that project to the medial striatum.

Tissue plasminogen activator is a brain plasticity related molecule. There is evidence that it may play a role in axonal growth as well as in the re-arrangements of synaptic contacts (Sumi et al, 1992, Friedman and Seeds 1994, 1995). Given the well-known neuroplastic alterations induced by amphetamine it was suggested that this effect might have been caused by the activation of the dopaminergic system. In this respect it was suggested that it might be involved in the initial stage of neuroplastic alterations that underlie the expression of amphetamine sensitization.

In addition to the above changes induced by amphetamine exposure, structural alterations have been revealed too by postmortem examinations. More specifically there is evidence of structural impairments of dorsolateral prefrontal cortex, including decreased dendritic length and spine density of pyramidal neurons along the apical trunk from layers II/IIIs, in amphetamine pre-treated non-human primates compared to a control group. These non-human primates were killed after having withdrawn for three and a half years from a repeated, escalating dose, sensitizing amphetamine regimen for 6 or 12 weeks (Selemon et al, 2007). Other postmortem examinations of non-human primates that had withdrawn from an amphetamine sensitizing regimen revealed decreased dopamine turnover in prefrontal cortex (Castner et al, 2005).

In addition, augmentation of synaptophysin mRNA, a marker of synaptogenesis, and stathmin mRNA, a marker of neuritic sprouting, in hippocampus, striatum and cortices, and augmentation of synaptophysin mRNA in nucleus accumbens following acute methamphetamine exposure, have all been shown (Ujike et al, 2002). Consistent with these findings, increased synaptophysin was found in nucleus accumbens, prefrontal cortex and temporal cortices and increased stathmin mRNA in prefrontal cortex after acute but not after

chronic methamphetamine administration, indicating that such neuroplastic alterations may reflect the induction rather than the maintenance phase of amphetamine sensitization (Takaki et al, 2001). These findings are in line with evidence presented above showing that the development of amphetamine sensitization is progressive.

Also, an increased phosphorylation state of synapsin I and neuromodulin, a neural specific calmodulin binding protein associated with synaptic release, has been found in amphetamine sensitized rats, which are indicative of alterations in presynaptic signaling linked with amphetamine sensitization (Gnegy et al, 1993, Iwata et al, 1996). Such presynaptic alterations might reflect long lasting neuroplasticity changes underlying increased dopaminergic release that is characteristic of amphetamine sensitization.

Also, increased synaptotagmin IV has been found in striatum and nucleus accumbens following single methamphetamine exposure as well as following acute methamphetamine challenge in rats that had withdrawn from a repeated methamphetamine regimen. This effect was attenuated by administration of D1 antagonists (Isao and Akiyama, 2004). This finding further highlights that neuroplastic alterations in the cortico-limbic-striatal circuit underlie the expression of amphetamine sensitization. In line with this, enhanced neuroplasticity underlying amphetamine sensitization is further shown by increased neuroglycan C mRNA, a neural network arrangement related gene, in striatum, hippocampus and amygdala following repeated methamphetamine treatment compared to acute treatment (Ishikawa et al, 2006). Collectively, all these studies strongly demonstrate that amphetamine sensitization induces neural re-organization. It should be noted that these neuroplastic alterations underlie the behavioural sequalae that accompany amphetamine sensitization and which resemble symptoms of schizophrenia and drug addiction.

Cortico-limbic-striatal dopaminergic system dysregulation and behavioural sequalae

Altered neuroplasticity in the mesolimbic pathway leads to dysregulation in the balance of the dopaminergic system and consequently on behaviours and cognitive functions that depend on it including learning and memory systems as well as reward processing. In this respect dysfunctional integration of limbic and prefrontal glutamate inputs through D1 and D2 receptors in the nucleus accumbens has been found (Selemon et al., 2010). In particular, impaired medial prefrontal cortex input is unable to suppress accumbal dopaminergic activity, via its projections to GABA neurons in ventral tegmental area, while at the same time dopamine neuron population activity in nucleus accumbens is further augmented by glutamate inputs from ventral hippocampus (Grace, 1991). In line with this, electrophysiological evidence has shown that repeated amphetamine exposure enhances ventral hippocampal output. This in turn was found to increase the tonic dopaminergic activity in the nucleus accumbens (Lodge and Grace, 2008). Collectively these data highlight the imbalance in the cortico-limbic-striatal circuit induced by repeated amphetamine and provide a link with various behavioural studies showing impairment in behavioural and cognitive processes that depend on this circuit.

Repeated treatment with low dose of methamphetamine appears to reduce the intensity of NMDA receptor ligand binding levels of [^{3}H]MK-801, an NMDA receptor specific ligand, in the prefrontal cortex and hippocampal CA1, CA2 and CA3 subfields. These effects accompanied memory deficits in a passive avoidance test and a spontaneous alternation deficit in a Y maze task (Lee et al, 2011). Spontaneous alternation behaviour was defined as successive entries into the three arms in overlapping triplet sets. The impaired spontaneous alternation was interpreted as an indication of impaired spatial working memory based on previous studies showing that this Y-maze task is a measure of spatial working

memory (Kim et al, 2006). However, the suitability of this task in measuring working memory has been strongly criticized (Sanderson and Bannerman, 2012, Sanderson et al, 2010). It might be that impaired alternation in this test reflects impaired exploratory behaviour.

Findings of the study by Lee et al (2011) are important as NMDA receptor mediated plasticity is well known to mediate the induction but not expression of amphetamine sensitization (Karler et al, 1991). It is also implicated in learning and memory (Bliss and Collingridge, 1993). These facts indicate a potential role of reduced NMDA receptor neuroplasticity in amphetamine sensitization which could also underlie the memory deficit that was observed. Collectively, these findings too strongly support that altered mesocortico-limbic neural plasticity underlies amphetamine sensitization and that this is strongly related with behavioural and mnemonic changes observed.

Dopaminergic activity in the substantial nigra and ventral tegmental area signals salient and arousing events in the environment, irrespective of their motivational valence. Through this mechanism arousal is induced that initiates goal directed behaviour (for review Horvitz, 2000). Dopaminergic activity in the nucleus accumbens signals unexpected reward as well as sensory prediction errors and also incentive salience. The reward prediction error is a mechanism proposed to drive the learning of conditioned-unconditioned stimulus associations. In this respect learning is caused by the discrepancy between expectation and experience of an outcome (for review Robinson and Berridge, 2008).

Attribution of incentive salience to a conditioned stimulus means that this stimulus acts beyond the prediction made, gets increased motivational value and becomes ‘wanted’ (Robinson and Berridge, 2008). Incentive salience depends not only on Pavlovian learning and memory but also on the neurobiological state of an animal or human. This varies independently of learning and memory. Neurobiological states that can affect incentive

salience are drug states and appetite states relevant either to natural appetite (Berridge and Schulkin, 1989) or elevated mesocorticolimbic activity which can be induced by stress (Peciña et al, 2006) or drug addiction, mediated by sensitization (Wyvell and Berridge, 2000). Variation in the neurobiological state can lead to unlearned fluctuations in the motivation triggered by a cue that had been learned to be paired with reward. Therefore, fluctuations in the motivational state that is triggered by stimuli paired with reward can lead to substantial changes in incentive salience. Such changes can depart from acquired values of reward predicting cues. Therefore, instant motivation triggered by a cue that has been paired with reward can cause urges that are disproportionally strong compared to its already acquired value.

Given the well-known effects of amphetamine sensitization on the cortico-limbic-striatal dopaminergic system it is not surprising that withdrawal from an amphetamine sensitizing regimen is associated with altered reward processing (O'Daly et al, 2014). Reward prediction errors and incentive salience are aberrant in both drug addiction (Robinson and Berridge, 1993) and schizophrenia (White et al, 2013, Olney et al, 2018).

In particular, increased incentive salience attribution to rewarding stimuli appears to mediate drug addiction, while increased salience attribution to irrelevant stimuli appears to mediate the formation of delusions (Olney et al, 2018, Heinz and Schlagenhauf, 2010) as well as motivational deficits in schizophrenia (Kim et al, 2018). Also, increased incentive salience attribution to cues paired with reward has been found in amphetamine abstinence (Robinson et al, 2015).

A more detailed description of the behavioural and cognitive sequalae of amphetamine sensitization including its effects on learning and memory systems as well as on spontaneous behaviours is given in the section aims below as well as in the individual chapters.

1.6 Amphetamine sensitization as a model of schizophrenia and drug addiction

In light of the sensitized mesocorticolimbic striatal dopaminergic system described above, which underlies the effects of amphetamine sensitization, schizophrenia and drug addiction, amphetamine sensitization has been widely used as a model of aspects of schizophrenia and drug addiction as well as stimulant induced psychosis (Robinson and Becker, 1986).

Indeed, an accumulating body of evidence points to key consistencies between the neurochemical alterations in humans with schizophrenia and drug addiction, and with animals sensitized to amphetamine. Such alterations include, for example, an increase in dopamine transporter function (Mash et al, 2002), D3 receptor binding sites (Mash and Stanley, 1999) and NMDA GluN1 levels, in addition to non-NMDA GluR2/3 glutamate receptor subunits in ventral striatum (Hemby et al, 2005) in cocaine users.

In addition, there is strong evidence that schizophrenic patients share similar changes in their dendritic spine density and branching with those observed in amphetamine sensitization (see section above). Garey et al (1998) provided evidence of reduced dendritic spine density on cerebral cortical pyramidal neurons of schizophrenic patients. Later, the same finding was confirmed by Glantz and Lewis (2000) specifically in pyramidal neurons of prefrontal cortex. Consistent with this, abnormal dendritic outgrowth has been found in the prefrontal cortex of schizophrenic patients (Black et al, 2004) and reduced basilar dendritic system (Kalus et al, 2000, Broadbeit et al, 2002). Furthermore, post-mortem examination of rats that were self-administering cocaine for 14 days and withdrawn for 7 days showed reduced dendritic spine density and branching in the rat's prefrontal cortex (Rasakham et al, 2014). In line with this finding, Ferrer et al (1986) provided evidence of reduced dendritic spine number in pyramidal neurons of the cortex of alcoholics.

The overlap in the mechanism of amphetamine sensitization between animals and humans has led to the shift of research in animal models of amphetamine sensitization to study both its neural and neurochemical circuit as well as some of its behavioural and cognitive sequalae. Taken together, the above findings, in addition with the fact that sensitization of the dopaminergic cortico-limbic-striatal system underlies amphetamine sensitization, schizophrenia and drug addiction, described above, constitute the basis for amphetamine sensitization being used as an animal model for some aspects of schizophrenia and drug addiction.

Several different paradigms involving amphetamine exposure have been used as animal models, which can produce distinct neural and behavioural profiles (for review Robinson and Becker, 1986). The two most common ones are described below. The first one is used to model the effects of chronic or continuous amphetamine exposure and involves induction of high concentrations of the drug that are kept for days. This is achieved by continuous amphetamine exposure through subcutaneously implanted silicone pellets which contain slowly released d-amphetamine. This can also be achieved by a series of repeated injections of the drug at high doses. This model can produce neurotoxicity and may mimic acute psychotic episode. It also mimics the pattern of exposure to high quantities of the drug in 'runs' lasting up to a week. This pattern of drug taking leads progressively to behavioural disorganization and intoxication. It also leads to acute psychosis with pronounced paranoid symptoms (Ellison and Eison, 1983).

The second amphetamine paradigm involves repeated intermittent exposure to the drug with relatively low does which induce amphetamine sensitization. This is what is studied in this thesis. This models mainly the long lasting hypersensitivity to the psychotomimetic effects of the drug after prolonged abstinence. Therefore, it models the

escalating drug craving and relapse of drug addicts as well as relapse in schizophrenia and amphetamine induced psychosis (Robinson and Becker, 1986, Robinson and Berridge, 1993).

1.7 Aims

Given the fact that amphetamine sensitization is being widely used as an animal model for schizophrenia and drug addiction, an accumulating body of research is focused on whether it can model the behavioural and cognitive profile of these conditions. It is well-established that it can model the positive-like symptoms of schizophrenia (Castner, Goldman-Rakic, 1999, 2003). However, it is not entirely clear whether it can model cognitive impairment and negative symptoms of schizophrenia, which are mimicked by glutamate models of schizophrenia (Olney et al, 1999). Research in the neural and neurochemical mechanisms of these symptoms is particularly important given that these are less effectively treated by antipsychotics (APA, 2013). In this respect, a series of behavioural experiments were conducted on learning and memory systems as well as on spontaneous behaviours in order to address the following four questions relevant to the effects of withdrawal from an amphetamine sensitizing regimen.

It should be noted that reference to amphetamine sensitization or amphetamine withdrawal in this thesis, suggests that animals had been pre-treated with a repeated amphetamine regimen from which they had withdrawn when experiments were conducted. A locomotor sensitization test is expected to have been conducted at some point normally after experiments are completed. This is to verify that animals were indeed still sensitized during the period that the behavioural experiments were conducted. Therefore, unless otherwise specified, any experimental findings relevant to amphetamine sensitization will refer, not to the phenomenon of the expression of sensitization after acute amphetamine challenge but, to

the underlying condition of withdrawal from amphetamine sensitizing regimen. The following 4 questions are being addressed in this thesis:

1.7.1 Does amphetamine sensitization selectively facilitate hippocampus dependent appetitive contextual learning while simultaneously reducing basolateral amygdala dependent cue learning when they take place in parallel?

Hippocampus and amygdala glutamatergic inputs overlap into nucleus accumbens dopaminergic neurons (Pennartz et al, 1994, Mulder et al, 1998, French and Totterdell, 2003) and compete for the control of appetitive behaviour (Ito et al, 2006, White and McDonald, 1993). It is known that nucleus accumbens enhances the most behaviourally relevant and salient input, while suppressing the competing inputs. In this respect, Floresco et al (2001) demonstrated that hippocampus activity is enhanced while basolateral amygdala is suppressed, following co-activation of the glutamatergic hippocampus to nucleus accumbens pathway and the postsynaptic D1 receptor in the nucleus accumbens.

Consistent with this is that withdrawal from repeated amphetamine, which enhances ventral hippocampus output and consequently tonic dopaminergic activity of nucleus accumbens (Lodge and Grace, 2008), enhances hippocampus dependent appetitive place conditioning while attenuating basolateral amygdala dependent appetitive cue conditioning in a conditioned cue/place preference test that was conducted in a radial maze (Ito and Canseliet, 2010).

The facilitation of place conditioning in the conditioned cue/place preference test was positively correlated for each animal with the degree of locomotor sensitization in the locomotor test following acute amphetamine challenge (Ito and Canseliet, 2010). This indicates that they may share a common neural substrate. Thus, the enhanced dopaminergic neurotransmission mediating the effect of the expression of amphetamine sensitization

(Kalivas and Stewart, 1991, Henry and White, 1992) mediates also the facilitated place conditioning in the cue/place preference test.

The same study by Ito and Canseliet (2010) tested hippocampus dependent appetitively driven spatial learning and basolateral amygdala dependent appetitive cue learning in an elevated T-maze task in which they take place in parallel but without directly competing with each other. Amphetamine sensitization attenuated the cue learning. However, unlike the radial maze task, spatial learning was spared. Cue and spatial learning trials in this task were intermingled in a pseudorandom order. Mice were trained to predict which of the two goal arms is baited based on whether a wire mesh or white perspex start arm floor insert was present in the starting arm (spatial trials). Also, they were trained in the absence of starting arm floor insert to predict which goal arm is baited and which is non-baited based on which one is covered by sand paper insert and which by green felt insert (cue trials). The fact that spatial learning in this task is hippocampus dependent has been shown by an excitotoxic lesion study by Schmitt et al, (2004). Ito and Canseliet (2010) provided evidence that cue learning in this task is basolateral amygdala dependent, taking into account that basolateral amygdala lesions selectively impaired cue learning in this task. This effect however was transient, in the first stage of cue learning. In both t-maze and radial maze tests the cue learning was acquired faster than the spatial learning by the control group. It was suggested that the unaffected spatial learning in the T-maze task might indicate that withdrawal from repeated amphetamine does not directly affect the hippocampal control over acquisition of associative spatial learning in this task. This is in line with evidence that withdrawal from repeated amphetamine does not affect acquisition rate in the water maze (Russig et al, 2003). On the other hand, there is also evidence of compromised non-associative spatial learning after withdrawal from a repeated amphetamine regimen (Mandillo et al, 2003). Thus, further

research is needed to elucidate the findings in the T-maze experiment of the study by Ito and Canseliet (2010).

One of the goals of the present study is to further elucidate the effects of withdrawal from repeated amphetamine regimen on basolateral amygdala dependent cue learning and hippocampus dependent spatial learning for rewards when they take place in parallel in the simultaneous learning t-maze task that was used in Ito and Canseliet (2010) study. The attenuated cue learning in the T-maze experiment by Ito and Canseliet (2010) was not in line with other studies which have shown that withdrawal from repeated amphetamine enhances the acquisition of Pavlovian discriminative approach behaviour (Taylor and Jentsch, 2001). This is known to be dependent to some extent on the integrity of basolateral amygdala (Burns et al, 1993).

Also, given that the hypothesis of Ito and Canseliet (2010) that appetitive spatial learning would be increased in the T-maze task was not supported by the findings, our aim is to further investigate this. This is done by promoting the use of an allocentric strategy in the same T-maze experiment used by Ito and Canseliet (2010). This is achieved by regular rotation of the maze ensuring the use of a spatial strategy to solve the task. Thus, mice do not rely only on an egocentric strategy using the intra-maze cues to solve the task but also rely on extra-maze cues. It is expected that rotation of the maze will increase engagement of the hippocampus in the task. This might facilitate competition between basolateral amygdala and hippocampus for control of appetitive learning in amphetamine sensitized mice which might cause enhancement of spatial learning and attenuation of cue learning. Findings from this study will provide insights into how withdrawal from amphetamine sensitization differentially affects distinct learning and memory systems. This aim of the thesis is being addressed in chapter 3.

1.7.2 Does amphetamine sensitization facilitate incrementally acquired spatial reference memory for rewards? Does it impair while impairing spatial working memory and pattern separation?

The next aim of the project is to assess the face validity of amphetamine sensitization as a model of cognitive impairment in schizophrenia and drug addiction. Excitability alterations (Morimoto et al, 1987) as well as temporally-limited reductions in monoamines and neurogenesis in the adult rat dentate gyrus have been found following amphetamine exposure. In particular, newly generated cells were quantified in this study. This was done in order to estimate the proliferation of progenitor cells and neurogenesis. It was found that at four weeks, but not at 20 hours post amphetamine withdrawal, progenitor cell proliferation and neurogenesis were reduced compared to a saline pre-treated group (Barr et al, 2010).

Neurogenesis in the dentate gyrus is known to be associated with pattern separation (Niibori et al, 2012). Also, NMDA receptors in CA3, mediate both memory acquisition and the representation of novel information from one-trial experience (Nakazawa et al, 2003, Cravens et al, 2006) and spatial working memory (Gilbert and Kesner, 2006). Furthermore, there appears to be reduced [3H]MK-801 ligand binding in amphetamine sensitization (Lee et al, 2011). Pattern separation (Tamminga et al, 2010, 2012, Chen et al, 2012, Das et al 2014, Martinelli and Shergill, 2015) and working memory (Barch and Smith, 2008) are well known to be impaired in schizophrenia. The effect of amphetamine sensitization on pattern separation has not been investigated. Also, research into the effects of amphetamine sensitization on working memory has given mixed results (Lee et al, 2011, Nagai et al, 2007, Stefani and Moghaddam, 2002, Peleg-Raibstein et al, 2009, Featherstone et al, 2008). The aim of this study is to assess whether amphetamine sensitization impairs spatial working memory and pattern separation. It should be noted though that spatial working memory in rodents is not always analogous to human spatial working memory. Spatial working memory

in rodents may share some features of short-term habituation procedures (Sanderson and Bannerman, 2012).

Whether amphetamine sensitization maladaptively facilitates appetitively driven spatial long-term memory will also be addressed. Gradually acquired spatial reference memory is a form a long-term memory that will be assessed here, and contrary to spatial working memory described above, is CA3 synaptic plasticity independent (Nakazawa et al, 2002). Long term memory is partially dependent on CA1 long term potentiation (Tsien et al, 1996), although the involvement of CA1 NMDA receptor synaptic plasticity in long term memory is not a consistent finding (Bannerman et al, 2012, 2014). Long term potentiation in CA1 is highly regulated by dopamine, release of which is enhanced in amphetamine sensitized animals (Kalivas and Stewart, 1991, Henry and White, 1992).

The hyper-responsitivity of the dopaminergic system has been shown to stem from a pathologically enhanced drive from ventral hippocampus in an animal model for schizophrenia. In this study prenatal methylazoxymethanol acetate was administered. That led to a significantly greater number of spontaneously firing dopaminergic neurons in ventral tegmental area compared to the control group. This phenomenon was reversed by ventral hippocampus inactivation which also normalized the hyperlocomotion induced by acute amphetamine administration (Lodge and Grace, 2008). In this respect, spatial reference memory for rewarding stimuli, which is dependent on the hippocampus and the dopaminergic system, is hypothesized to be enhanced by amphetamine sensitization.

There is also evidence of memory generalization in contextual locomotor sensitization induced by amphetamine (Engelke et al, 2017). In particular, it was found that after three days of withdrawal hyperlocomotion in response to acute amphetamine challenge was not evident in a novel context. Instead it was only evident in the context that amphetamine had been administered during pre-treatment. That context was, at the time of pre-treatment, a

novel context. However, 28 days post-withdrawal hyperlocomotion in response to acute amphetamine was shown in both contexts. This indicates that memories associated with amphetamine administration generalize after prolonged withdrawal. This generalization effect was not shown in rodents that had been pre-treated with amphetamine in their home cage. These findings suggest that memory generalization may play a role in amphetamine sensitization and thus may play a role in the process of addiction. However, it could be argued that such memory generalization might be due to an inability of mice to discriminate between similar memory traces (i.e. impaired pattern separation). In this respect, it will be of interest to study whether withdrawal from an amphetamine sensitizing regimen will impair pattern separation in mice.

In order to assess simultaneously appetitive spatial reference memory, spatial working memory and pattern separation, a radial maze paradigm was selected. In accordance with the paragraph above, acquisition of spatial reference memory for rewarding stimuli will be assessed (which is hypothesized to be facilitated). After mice acquire the reference memory component of the task, the second phase of the experiment follows in which reference memory is tested. Furthermore, while in the acquisition phase mice are not allowed to make spatial working memory errors, by using doors which block entrance to previously visited arms, the doors of the maze are not used in the second phase and thus working memory is also assessedin addition to spatial reference memory. In both phases spatial pattern separation is also assessed, i.e. the ability of mice to discriminate between adjacent arms. This experiment is being presented in chapter 4 of this thesis.

1.7.3 Does amphetamine sensitization have good face validity as a model of social withdrawal in schizophrenia and drug addiction?

Social isolation is a hallmark of schizophrenia and drug addiction (APA, 2013). It is found that methamphetamine sensitization (Liao et al, 2018), as well as continuous d-amphetamine (Gambill, Kornetsky, 1976, Sams-Dodd, 1998) and methamphetamine (Utena, 1966) administration reduce social interaction in rodents. However, the protocols used in these studies are different from the protocol of withdrawal from d-amphetamine regimen with intermittent low doses used in the present thesis. In these studies however, rodents were under the effect of the drug at the time of testing. There is also evidence that withdrawal from repeated methamphetamine (Janetsian et al, 2015b) and MDMA (van Nieuwenhuijzen et al., 2010) reduces social interaction. However, these are different drugs from d-amphetamine that we used in the present thesis. It is therefore of interest to investigate whether the paradigm that we used produces social withdrawal.

Methamphetamine abstinence appears to disturb the social neuropeptide systems (Georgiou et al, 2016). There is evidence that the social brain network is disturbed by psychostimulants (Sarnyai, Kovács, 1994, Steketee, 2003). Whether withdrawal from an amphetamine sensitizing regimen induces social anhedonia, remains unknown. This will be investigated using a social interaction paradigm by placing together two unfamiliar mice in a familiar open field, at 2 months post amphetamine withdrawal.

Through this test a variety of social interaction behaviours will be assessed including the duration and frequency of social contacts (anogenital and mouth sniffing, body contacts, following, time spent together). Additionally, measuring time and frequency of avoidance/flee behaviours, will be informative of whether the mice are afraid of each other. Finally, time spent immobile or in stereotypic behaviours, or exploring the cage are also measured. These measurements, will provide more insight into whether potential avoidance

of social contact is associated with fear/anxiety of the unknown animal (i.e. avoidance/flee), or with over-engagement in stereotypic behaviours.

In order to further confirm if there is an impaired sociability phenotype in amphetamine sensitized mice a second sociability test will be performed. This test is different from the reciprocal social interaction test, as in this test free reciprocal interaction is prohibited given that only one mouse is free and the other is constrained within a chamber. Therefore, time spent exploring an empty chamber in a known open field compared to time spent exploring a chamber that contains a novel mouse will be calculated. Immobility and stereotypic behaviour will also be measured in order to give more insight into the nature of the reduced sociability (if reduced sociability is found).

In case reduced sociability is found, then it will be assessed if it is associated with increased anxiety or with increased stereotypic behaviour in order to gain better insight into whether it reflects mostly social anhedonia, or social anxiety or an inability to switch to a social mode of behaviour (i.e. to disengage from i.e. repetitive stereotypic behaviour). In order to further confirm any potential effects of amphetamine sensitization on anxiety, the unconditioned, ethological elevated plus maze anxiety test will also be performed. This aim is being addressed in chapter 5 of the thesis.

1.7.4 Does amphetamine sensitization model aberrant novelty processing in schizophrenia and drug addiction?

Novelty detection facilitates behavioural adaptation by attributing more salience and attention to novel stimuli in the environment. There is evidence that novelty detection is aberrant in schizophrenic patients (Weiss et al, 2004) which is associated with deficits in habituation and consequently aberrant salience attribution. Such an impairment appears to be

mediated by the mesolimbic dopaminergic system (Heinz, Schlagenhauf, 2010) which mediates novelty detection (Pierce et al, 1990).

There is evidence that repeated ketamine administration in rats induces aberrant novelty processing (Schumacher et al, 2016). This is interesting given that it is well known that ketamine like amphetamine induces sensitization and models schizophrenia in animals (for review Nowacka, Borczyk, 2019). In the study by Schumacher et al (2016) rats were administered an associative mismatch detection task to examine their ability to detect novel configurations of familiar audio-visual sequences. Also, a sequential novel object and novel object location exploration task was performed. Rats pre-treated with repeated low dose of ketamine exhibited locomotor sensitization to acute amphetamine challenge. They also exhibited increased novelty detection in novel object location, as measured by both the degree to which ketamine pretreated mice explored the objects that were in novel spatial locations compared to the saline group and compared to the objects that remained in the familiar locations. They also exhibited increased novelty detection in the mismatch detection task compared with control group. This was measured by percentage of orientation responses to a visual stimulus. On the contrary rats pre-treated with high dose of ketamine showed reduced locomotor response to amphetamine and reduced novelty preference.

Findings on novel object recognition test in amphetamine pre-treated rodents are mixed with other studies showing spared (Che et al, 2013), impaired (Janetsian et al, 2015a, Kamei et al, 2006, Belcher et al, 2006, Bisagno et al, 2002, Belcher et al, 2008, Peleg-Raibstein et al, 2009, Lee et al, 2011, Tenn et al, 2003) or enhanced novel object recognition (Peleg-Raibstein et al, 2009). MDMA pretreated rats with moderate level of sensitization also appeared to have reduced novel object recognition (van Nieuwenhuijzen et al, 2010).

In this respect, the aim of the present study is to screen amphetamine sensitized mice across a variety of recognition tests which include novel object recognition (given that

research on that test is inconclusive), object in place (in light of findings of Schumacher et al, 2016), and social recognition. In addition, exploratory behaviour in the open field will be assessed in the habituation phase of each of these tests separately. The effects of amphetamine sensitization on recognition tests will help assess whether amphetamine sensitization models aberrant salience, impaired novelty processing and short term habituation deficits reminiscent of schizophrenia and drug addiction. This aim is being addressed in chapter 5 of this thesis.

Chapter 2: General Methods and Validation of Amphetamine Sensitization Protocol

2.1 General methods

Subjects

37 C57BL/6 female mice (18-22g) were group-housed in 5 cages. The first set of mice was group-housed in two cages. The first cage contained 9 mice and the second contained 10 mice. The second set of mice, were group-housed in three cages, with each cage containing 6 mice. All mice were group-housed at 21 C^{o} degrees, with water and food available ad libitum (unless otherwise specified), under a 12 hour light-dark cycle (lights on from 07.00 – 19.00).

Drug injections

Injections were administered intraperitoneally in a distinct room than the room that the mice were grouped-housed, once daily for 7 successive days. Mice were injected with 2.5 mg/kg (n=13), 5 mg/kg (n=13) D-amphetamine sulphate or saline vehicle (n=11). Allocation of mice to groups was random and was counterbalanced across cages in both sets of mice. Unlike the second set of animals, in the first set, the number of mice was different between treatment groups. Also, unlike the second set there were not equal numbers of mice from each treatment group in each cage. This was because the sample size of each one of the three groups was an even number, while the cages were two. The order that groups were drug injected was counterbalanced across cages.

Behavioural experiments

After withdrawal from the repeated amphetamine or saline regimen a series of behavioural experiments was conducted in the mice. The chronological order that these experiments were conducted in is presented in figure 1.1, while details about the experimental procedures and the subjects that participated in each one of these are presented in Table 2.1 below. There were no differences between sensitized and saline pre-treated mice in body weights during the period the experiments were conducted.

Experiment	time post-treatment (withdrawal)	saline n	amph 2.5mg/kg n	amph 5mg/kg n	set of mice	Food deprivation (mice kept at 85% of their ad libitum weight)	Habituation (free exploration ofapparatus) before test in the testing room ie. the room were the experiment would be conducted *(t)* or distinct room than the room that the experiment would be conducted in *(d)*
locomotion sensitization test	4, 9 months	11	13	13	Second set at 9 months and first at 4 months	no	no
radial maze	4 months	11	13	13	both	yes	10 min./day for a week drinking condensed milk (50% water) from steel wells of all arms' ends *(d)*
T-maze	15 days	6	6	6	second	yes	2 min./day for 4 days ~~(~~milk put in thewells of the 2 goal arms~~)~~ *(d)*
social interaction	2 months	6	6	6	second	no	10 min/day for a week *(t)*
novel object recognition	8 months	9	7	5	both	no	10 minutes 1 hour before test *(t)*
social recognition	8 months	9	11	10	both	no	10 minutes 1 hour before test (t)
elevated plus maze (pilot study)	3 months	4	4	4	Subgroup of the second	no	no
open field	8 months (45 minutes priorsocial recognition test)	11	11	11	both	no	no
object in place	8 months	9	8	7	both	no	5 minutes 1 hour before test *(t)*

Table 2.1. Timetable of all the experiments conducted after withdrawal from the amphetamine regimen including information about sample size and information about experiments involving food deprivation during testing and/or habituation.

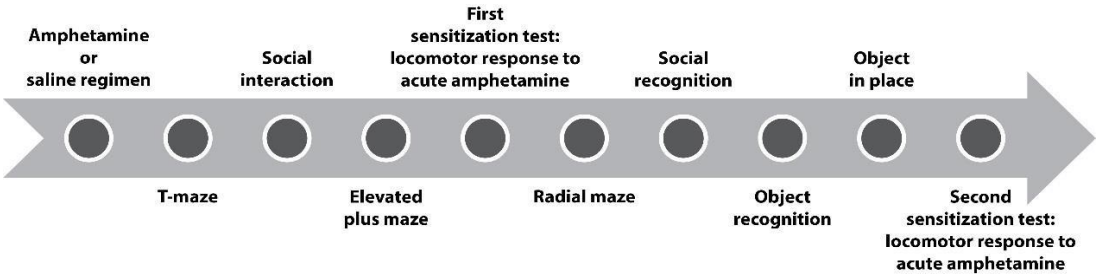


Figure 2.1. Timeline. Chronological order of all procedures conducted

2.1.1 Data analysis

All data were analyzed using the SPSS statistical package. Two analyses were conducted for each set of data. One was with two groups, the first one being the saline pre-treated mice and the second being all the amphetamine pre-treated mice. The second analysis was conducted with three groups, the first one being the saline pre-treated mice, the second group was the amphetamine pre-treated mice with 2.5mg/kg and the third one being the amphetamine pre-treated mice with 5mg/kg. The first analysis ensured a larger sample size, while the second analysis offered us the possibility to check whether there was a particular effect with one of the two different doses that were used. More importantly, the first analysis gave us the broader picture of the effects of the drug independent of the dose on the variables that were assessed. It should be noted that in the study by Ito and Canseliet (2010) the two groups pre-treated with amphetamine showed consistent performance in many of the assessed variables but not in all of them.

Data were tested for normality as well as for homogeneity of variance. Homogeneity of variance was tested using Levene's test. In case repeated measures ANOVA was used, then Mauchly's test of sphericity was used. In cases sphericity was violated then degrees of freedom and p value were corrected (Greenhouse-Geisser correction). Assumption of normality was tested unless repeated measures ANOVA had to be conducted. In case data

were not normally distributed logarithmic transformation or square root transformation were tried by trial and error in an attempt to correct positive skewness, if that was the cause of deviation of normality. There were no negatively skewed data. In case data tended to zero a constant was added to all of the data before transformation was conducted. When transformation did not solve the problem then non-parametric statistics were used.

The expected effect sizes were based on priorly published experiments. However, for reasons explained, it was not possible to extract data from all our subjects in some experiments. The sample sizes used in the study by Paulson et al (1991) and Paulson and Robinson (1995, 2007) exhibited enough power to detect statistically significant group differences in crossovers after amphetamine challenge in the locomotor sensitization test. The same applied for correct responses in appetitive learning maze tasks (Ito et al, 2006, Ito and Canseliet, 2010, Schmitt et al, 2004) spatial working memory errors (Sanderson et al, 2007, Schmitt et al, 2003, Stefani and Moghaddam, 2002), duration of social investigation (Qiao et al, 2001, Belforte et al, 2010, White et al, 2009), preference index as far as duration of investigation in the novel object recognition test (Belcher et al, 2005, 2008, Noda et al, 2010) and time spent in the center in the open field (Ageta et al, 2008, Matsuo et al, 2010, Kanai et al, 2009). As resources were limited and since similar research protocols had enough power to detect statistically significant differences we decided to recruit a similar number of animals for our study, expecting that effect sizes would be similar as well.

The outcomes indicated that this was already enough in some of our primary outcomes. Statistically significant differences were detected concerning the crossovers in the locomotor sensitization test as well as in the duration of social contacts in the reciprocal social interaction test. Post hoc power analyses were conducted by g*power on some of our outcomes in which no significant group differences were found. Those outcomes were selected because they were more important or relevant to our key research questions or there

were more indications supporting that group differences would be found. It must be noted that the estimates were based on a rather conservatory approach regarding effect size. Therefore the power estimations appearing are a lower bound of the true power.

Our post hoc power analysis regarding correct responses in cue learning, (power=0.80 for both 2 and 3 group analysis) and spatial learning (power=0.85 for 3 group analysis and 0.83 for 2 group analysis) in the t-maze experiment, reference memory errors during acquisition phase (power=0.80 for both 2 and 3 group analysis), working memory errors (power=0.80 for 3 groups analysis and 0.81 for 2 groups analysis) and reference memory errors during testing phase (power=0.81 for 3 groups analysis and 0.80 for 2 groups analysis) in the radial maze experiment showed that our samples were large enough. Therefore, if an effect existed we had an >80% likelihood of detecting it. This level of power is a standard adopted in a number of studies.

On the other hand, in other outcomes the sample size was found to be small. In particular, 42 mice would be required in the object recognition test in the discrimination ratio (duration) to achieve a power of 0.81 (3 groups analysis) and 38 to achieve a power of 0.82 (2 group analysis). In the discrimination ratio in the social recognition test 64 mice would be required to achieve a power of 0.81 (2 group analysis) and 42 to achieve power of 0.82 (2 group analysis). In the duration of social approach in the sociability test 63 mice would be required to achieve power of 0.81 in the 3 group analysis and 70 mice in the 2 group analysis to achieve a power of 0.80. Finally, in the open field test in the duration of time spent in the center, we had a power of 0.82 in the 3 group analysis, while 58 mice would be required to achieve a power of 0.80 in the 2 group analysis.

2.1.2 Assessment of locomotor activity in response to acute amphetamine

Apparatus

Individual transparent plastic cages were used to assess locomotor activity. The size of each one was 27x15x17cm. Two sets of infrared photo beams 14cm apart, and 1.5cm above the cage's floor, monitored the locomotor activity of the mice.

Procedure

Locomotor activity was assessed at either 4 or 9 months post-amphetamine withdrawal. The first set of animals was tested at 4 months post-withdrawal and the second set at 9 months post-withdrawal.

Locomotor activity (measured as crossovers between the front and back part of the cage) was assessed during a four hour test session (48 x 5 minute- time bins). After a two hour assessment of baseline spontaneous locomotor activity, all mice were injected intraperitoneally with 2.5mg/kg amphetamine and placed back into the same activity cages. Spontaneous locomotor activity and the locomotor response to amphetamine were analyzed separately.

2.2 Results

2.2.1 Locomotor response to acute amphetamine challenge to test sensitization to amphetamine

Below is reported the statistical analysis of the sensitization test in which the locomotor response to acute amphetamine challenge was assessed 9 months post amphetamine withdrawal, thus at the end of the behavioural testing. This test was done to

verify that animals were indeed sensitized during the period when the behavioural experiments of this thesis were conducted. The data of the same test that was conducted in the remaining animals at 4 months post amphetamine withdrawal are not reported here given that these data had been collected by Ito and Canseliet and are presented in the study by Ito and Canseliet, 2010. The mice used in present study to which the locomotor sensitization test was conducted at 4 months post amphetamine withdrawal were a subgroup of the cohort of mice from the study by Ito and Canseliet (2010). Sensitization in these mice is evident in figure 8 of that published study.

2.2.2 Locomotor response to amphetamine test at 9 months post amphetamine withdrawal

Summary of results

Baseline locomotor activity progressively decreased for all mice at the same rate (see figure 2.2). Following amphetamine challenge locomotor activity enhanced for all mice. Amphetamine sensitized animals compared to saline showed hyperlocomotion in response to amphetamine challenge (see figure 2.2 and figure 2.3). This indicates that amphetamine pre-treated mice remained sensitized to amphetamine 9 months after withdrawal from the repeated amphetamine regimen.

2.2.3 Spontaneous locomotor activity

Separate analysis for baseline locomotor activity based on crossovers for mice tested 9 months post-amphetamine withdrawal indicated the following results for an analysis, repeated measures ANOVA which included 3 groups of mice (saline, 2.5 mg/kg amphetamine and 5 mg/kg amphetamine) as between subjects factor and bin of 5 min as

within subject factor. There was a significant main effect of time bin, (F(23, 345)=9.448, p<.001), indicating that as time passed locomotor activity decreased for all mice (see figure 2.2) and a non-significant trend towards a main effect of group, (F(2, 15)=4.10, p=.067). The groups by time bins interaction was non-significant, (F(46, 345)= .635, p=.970).

Similarly, the two groups analysis (with the two amphetamine groups now combined into a single group) provided the following results. There was a significant main effect of time bin, (F(23, 345)=8.382, p<.001), a non-significant main effect of group, (F(1, 15)=.820, p=.378). The groups by time bins interaction was also non-significant, (F(46, 368)=.547, p=.585).

2.2.4 Locomotor response to amphetamine challenge

Repeated measures ANOVA was conducted with groups as between subjects factor and time bin as within subject factor. Results indicated that the locomotor response to an amphetamine challenge was greater in the mice that had been pre-treated with amphetamine 9 months earlier, indicating that the sensitisation effect was still present at this time point (see figures 2.2-2.3). Inparticular, separate analysis of crossovers for mice tested 9 months post-amphetamine withdrawal indicated the following results for locomotor activity post-amphetaminechallenge.

In the three group analysis there was a significant main effect of time bin, (F(23, 345)=5.816, p<.001), indicating that as time passed locomotor activity increased for all mice. Also, there was a significant main effect of group, (F(2, 15)=5.497, p=.016) indicating that amphetamine pre-treated mice were sensitized (see figure 2.2 and 2.3). Pairwise comparisons using least significant difference for confidence interval adjustment indicated that locomotor activity in amphetamine pre-treated group with 2.5mg/kg was significantly higher compared to saline, (mean difference= -20.076, standard error=6.069, p=.005). There was a non-

significant trend for amphetamine pre-treated mice with 5mg/kg to differ from the saline group, (mean difference=11.243, standard error=6.069, p=.084). Amphetamine (2.5mg/kg)- and amphetamine (5mg/kg) groups did not differ, (mean difference=8.833, standard error=6.069, p=.166). The groups by time bins interaction was non-significant, (F(46, 345)=1.551, p=.180).

Similarly, in the two group analysis (saline versus combined amphetamine group) there was a significant main effect of time bin, (F(23, 368)=4.022, p=.011), indicating that as time passed locomotor activity increased for all mice. A significant main effect of groups was found, (F(1,16)=8.296, p=.011), indicating that locomotor activity was enhanced in amphetamine pre-treated mice compared to saline pre-treated mice. There was a non-significant group by time bin interaction (F(23,368)=1.745, p=.167).

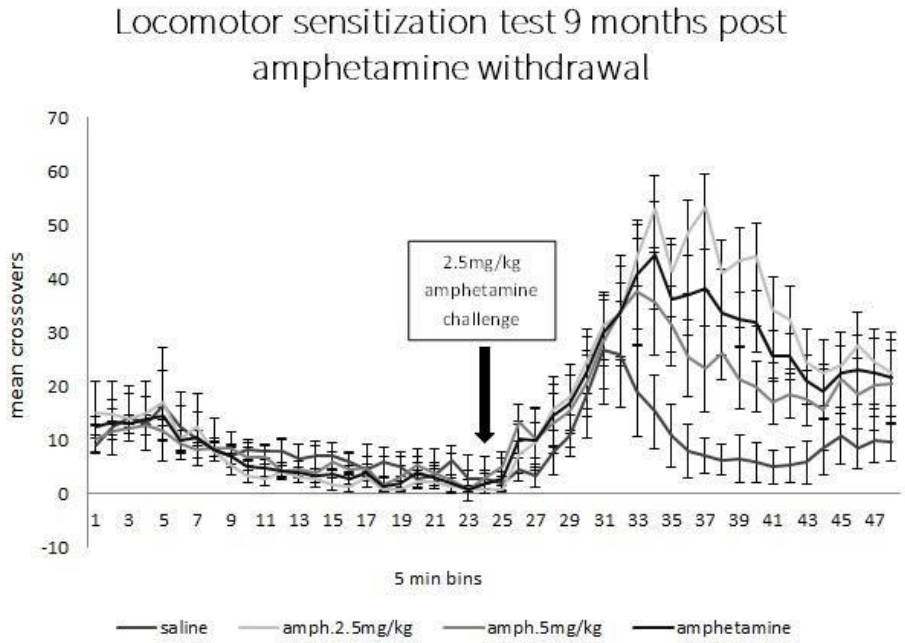


Figure 2.2. Mean (±SEM) crossovers before and after amphetamine challenge 2.5mg/kg (time bin24, see arrow) for saline (n=6), amphetamine 2.5mg/kg (n=6), amphetamine 5 mg/kg (n=6), and combined amphetamine group (n=12). The arrangement of the horizontal axis is the same as in figure 8 from the study of Ito and Canseliet (2010) so that the graphs can be comparable.

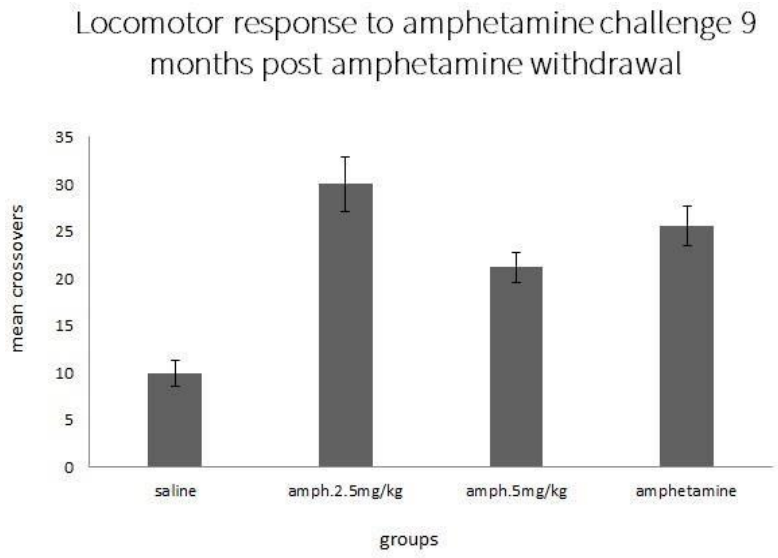


Figure 2.3. Mean (±SEM) total crossovers in the 2 hr post-injection after amphetamine challenge for saline (n=6), amphetamine 2.5mg/kg (n=6), amphetamine5 mg/kg (n=6) and for the combined amphetamine group (n=12).

2.3 Discussion

Overall, results are in agreement with the well-established findings that amphetamine sensitization is expressed by hyperlocomotion to acute amphetamine challenge following withdrawal from an amphetamine regimen (Klawans et al, 1975, Klawans and Margolin, 1975, Segal and Mandell, 1974, Leith and Kuczenski, 1982, Paulson and Robinson, 1995). Findings confirmed that mice remained sensitized to amphetamine at 4 and 9 months following withdrawal from repeated amphetamine regimen. These findings verify that all the experiments conducted in the present thesis assessed indeed how withdrawal from an amphetamine sensitizing regimen affects the behaviors that were examined. These findings are also in agreement with findings by other studies which show that amphetamine sensitization is long lasting (Paulson et al, 1991).

It should be noted that findings from the 9 month post-amphetamine withdrawal test showed clearly that sensitization was evident in the 2.5mg/kg group. In particular, pairwise

comparisons showed that the 2.5mg/kg group had significantly increased locomotor activity in response to amphetamine challenge compared with saline. The 5mg/kg group had a non-significant trend for increased locomotor response to amphetamine challenge compared to saline. However, when both groups were integrated into one group and were compared to saline, they had significantly increased locomotor response to amphetamine challenge. This indicates that mice were sensitized to amphetamine.

It should be noted that acute amphetamine 2.5mg/kg dose was administered in all groups to test locomotor response to amphetamine. The same acute dose was administered in all mice as in the study by Ito and Canseliet (2010) in which the two amphetamine groups did not differ in the locomotor response to 2.5mg/kg amphetamine challenge. In our study too pairwise comparisons showed no difference between the two amphetamine pre-treated group in the locomotor response to amphetamine challenge. In general, lower doses induce mostly hyperlocomotion while higher doses are more likely to induce stereotypic behavior in amphetamine pre-treated rodents given that different doses affect different groups of dopaminergic neurons. Stereotypic behavior is known to result from stimulation of dopaminergic system of basal ganglia, corpus striatum (Munkvad et al, 1968). Stereotypic behavior can compete with locomotor activity (Segal and Mandell, 1974).

It is well known that sensitization progressively enhances the effects of amphetamine and thus the dose needed to cause a particular effect decreases if mice are sensitized (Segal, 1975, Kalivas and Stewart, 1991, Robinson and Becker, 1986). The administration of 2.5mg/kg amphetamine challenge in the locomotor sensitization test has also been used in other studies in which rodents had been pre-treated with 5mg/kg amphetamine (Kolta et al, 1985, Ito and Canseliet, 2010). In another study 2.6mg/kg was given as a challenge dose in rodents that had been pre-treated with an escalating amphetamine regime 1 to 10 mg/kg (weight of the salt) (Paulson et al, 1991). The same amphetamine regimen was used in the

study by Paulon and Robinson (1995), where the challenge dose in the locomotor sensitization test was 0.5mg/kg (weight of the salt). Other studies have provided evidence that the challenge dose of 2.6mg/kg is the highest possible dose that produces mainly hyperlocomotion and not focused stereotypies. Therefore, with higher challenge doses qualitative assessment might be required as a behavioral change (Segal, 1975, Paulson et al, 1991). Such measurement might be more complex and have reduced validity and reliability compared to assessment of locomotor activity.

Also, results from this chapter indicate that there is no significant difference in the baseline locomotor activity of mice from different groups. This finding is important first of all because it shows that the group differences in locomotor activity following amphetamine challenge were indeed caused by the administration of acute amphetamine challenge. However, in the study by Ito and Canseliet (2010) the amphetamine pre-treated group with 5mg/kg had reduced baseline locomotor activity compared to saline (Ito and Canseliet, 2010). The reason for the discrepancy in this finding with our study is not clear. It should be noted that other studies have given saline challenge injection in the first phase of the locomotor sensitization test, while it was shown that it has a negligible effect (Camp and Robinson, 1988, Paulson and Robinson, 1995). Instead, baseline measurements were found to be equally good to be compared with measurements after amphetamine challenge as measurements after saline injection. Also, the finding that no group differences were found in the baseline locomotor activity and that all groups progressively had reduced locomotor activity as time passed is useful in the interpretation of results from other experiments of the thesis ie. spontaneous behaviours, in which a potential difference in the baseline locomotor activity between groups of mice could have affected results. This finding is in agreement with findings from the novel open field and the elevated plus maze and the habituation phase

of the reciprocal social interaction test, as far as the measures of locomotor activity is concerned.

One limitation of the present test was the fact that the mice that were tested at 4 months post drug treatment were not tested again at 9 months, while experiments were still conducted till 9 months post drug withdrawal. One cannot exclude the possibility that by 9 months the sensitization might not have been still present in some of the mice that had been tested in the locomotor sensitization test at 4 months post drug treatment. However, the finding that the groups of mice that were tested at 9 months were still sensitized, as well as findings that sensitization can last up to a year (Paulson et al, 1991) do not support this possibility.

Chapter 3: Amphetamine Sensitization Effects on Simultaneous Spatial and Cue Learning for Reward

3.1 Introduction

3.1.1 Amygdala and hippocampus dysfunction: key role in aspects of schizophrenia and drug addiction

Dysregulation in the hippocampal-nucleus accumbens pathway is a hallmark of both schizophrenia and drug addiction (Grace, 1991, Grace, 2010). Likewise, ventral hippocampus hyperfunction in a developmental animal model for schizophrenia leads to enhanced dopaminergic activity (Lodge and Grace, 2007). Wolff et al (2018) also found that ventral hippocampus overactivity, which was induced by optogenetic activation of excitatory neurons, leads to hyperlocomotion. The hyperlcomotion that is produced by acute amphetamine challenge following withdrawal from a repeated amphetamine regimen follows a similar pattern of gradual development as the development of psychotic symptoms in amphetamine psychosis in humans (Ellinwood, 1967, Ellinwood et al, 1973). Thus, hyperlocomotion in response to acute amphetamine and positive symptoms shizophrenia may represent a manifestation of the same underlying mechanism. In the same study it was found however that ventral hippocampus overactivation also leads to short term memory deficits in the spatial novelty preference test, which were shown to be independent of the hyperdopaminergic state that could be induced by amphetamine administration. This finding is potentially consistent with the resistance of cognitive impairment in schizophrenic patients to antipsychotic medication. Overall, these studies show that hippocampal overactivity underlies at least some of the psychotic symptoms in schizophrenia.

In line with this, hippocampal hyperactivity has been suggested to form the basis for

the facilitated formation of maladaptive associations observed in schizophrenia, through enhanced dopaminergic neurotransmission in striatum, which is, in turn, facilitated by hippocampal hyperactivity (for review Tamminga et al, 2010). On the other hand, it has been suggested that reduced hippocampal neuroplasticity leading to impaired short-term habituation could underlie aberrant salience attribution and consequently lead to the formation of maladaptive associations in schizophrenia (for review Barkus et al, 2014). Overall, impaired hippocampal function appears to be strongly implicated in the generation of schizophrenic symptoms.

In addition to dysregulated hippocampal function (for review Lieberman et al, 2018, Grace and Gomes, 2019), dysfunctional amygdala activation has also been observed in schizophrenic patients. There is evidence of amygdala inactivity during emotional processing, underlying a potential impairment of emotional processing in schizophrenia (Schneider et al, 1998, Gur et al, 2002). Takahashi et al (2004) also provided evidence that reduced activation of medial prefronal cortex and right amygdala underlies impaired emotional behaviour in schizophrenic patients. On the other hand, other evidence suggests increased amygdala activation during the initial presentation of fearful and neutral facial expressions in schizophrenia (Holt et al, 2006). Collectively, these studies show that amygdala function is disrupted by schizophrenia although the direction of effect for amygdala activity seems to vary and that this may be crucial to impaired emotional processing.

In addition to findings about altered activity of the amygdala, dysfunctional connectivity between medial prefrontal cortex and amygdala in schizophrenic patients, as already described above, appears to underlie impaired emotional perception (Takahashi et al, 2004, Bjorkquist et al, 2016). Mukherjee et al (2016) showed that such dysfunctional connectivity is associated with a greater deficit and worse functioning in schizophrenic patients. Finally, impaired connectivity between prefrontal cortex and amygdala in

schizophrenic patients was found in an fMRI decision making task, selectively in a condition in which processing of negative distractors was required. Such impaired connectivity predicted negative symptom severity (Anticevic et al, 2012), providing evidence of impaired cognitive processing of emotional cues in schizophrenia. Overall, these studies highlight the key role of altered amygdala function in psychotic symptomatology.

Furthermore, Hernandez et al (2015) showed that in a neonatal ventral hippocampal lesion animal model of schizophrenia, the basolateral amygdala signalled abnormally for salience during learning of an odor-guided discrimination task. In line with this, Uliana et al (2018) provided evidence that in a methylazoxymethanol acetate (MAM) developmental disruption model of schizophrenia, fear extintion was disrupted, indicative of impaired emotional processing and learning. This correlated with compromised medial prefrontal cortex basolateral amygdala plasticity. This finding further highlights that an impaired prefrontal-amygdala connection underlies altered emotional processing in schizophrenia, which contributes to both positive and negative symptoms.

Altered amygdala function is not only implicated in schizophrenia but also in drug addiction. Drug abuse is well known to involve maladaptive reward learning and thus the basolateral amygdala likely plays a key role. Prefrontal cortex-basolateral amygdala pathways mediate cue induced reinstatement (Everitt and Wolf, 2002, Weiss et al, 2001). It is well known that after psychostimulant exposure the cost of drug taking is devalued while the reward is over-valued. This effect is mediated partially by the basolateral amydgala (Everitt and Wolf, 2002). Pelloux et al (2013) indicated that basolateral amygdala-lesioned rats are unable to suppress their cocaine seeking behaviour in light of intermittent punishment.

Collectively, these studies highlight that amygdala and hippocampus are dysfunctional in schizophrenia and drug addiction which is related to various aspects of the disease such as dysfunctional appetitive behaviour and emotional processing. Dysregulation in these systems

by amphetamine sensitization is linked to abnormal manifestations, including cognitive impairment and symptoms reminiscent of positive symptoms of schizophrenia (Castner and Goldman Rakic, 2003). Given the accumulating body of evidence that amygdala and hippocampus function is impaired in schizophrenia and drug addiction it is interesting to study the effects of amphetamine withdrawal on behavioural and cognitive tests that depend on these structures.

3.1.2 Sensitization to psychostimulants and appetitive learning

An accumulating body of evidence indicates that sensitization to psychostimulants, through increased dopaminergic neurotransmission in the mesolimbic-striatal circuit, maladaptively facilitates various types of learning for rewarding stimuli. In particular, Harmer and Phillips (1998) showed that sensitization to D-amphetamine enhances Pavlovian conditioning for reward associated stimuli in rats. Accordingly, repeated intermittent administration of D-amphetamine or cocaine appears to accelerate learning for appetitively motivated cues (Taylor and Jentsch, 2001).

Findings from studies with infusion of psychostimulants into the nucleus accumbens consistently support their facilitating role in appetitive learning. Wyvell and Berridge (2000) demonstrated that amphetamine infusions into the nucleus accumbens potentiated instrumental responses for sucrose predicting cues in rats. Taylor and Robbins (1984) also showed that conditioned reinforcers' control over appetitive behaviour appears to be enhanced by infusion of d-amphetamine into the nucleus accumbens of rats. In line with this, withdrawal from a cocaine sensitizing regimen enhances responses for conditioned reward, an effect that is potentiated by intra-accumbens amphetamine infusion (Taylor and Horger, 1999). Also, discriminative approach acquisition was facilitated by intra-amygdala d-amphetamine microinjections post-training (Hitchcorr et al, 1997). These findings highlight

the role of mesolimbic dopamine in psychostimulant sensitization mediating facilitated appetitively driven cue learning.

Other forms of associative learning dependent on mesolimbic dopamine also appear to be facilitated by repeated amphetamine exposure, including habit formation (Nelson and Killcross, 2006, Nordquist et al, 2007) and the conditioned freezing response to a tone previously paired with shock (Pezze et al, 2002). This depends on the integrity of basolateral amygdala (Phillips and LeDoux, 1992). Also, increased sign tracking was found in animals that had been sensitized to amphetamine (Doremus-Fitzwater and Spear, 2011, Robinson et al, 2015). Overall, there is evidence that appetitive cue learning that is dependent on mesolimbic dopamine is facilitated by amphetamine sensitization.

3.1.3 Sensitization to psychostimulants: Hippocampus and the role of context

Associative learning between the context in which the drug is taken and its rewarding effects appears to be a main component of addiction (for review O' Brien et al, 1992, White, 1996, Haracz et al, 1999, Kutlu and Gould, 2016). The hippocampus is well known to be involved in contextual processing (Hirsh, 1974). Also, the hippocampus appears to be implicated in the behavioural sensitization to amphetamine. In particular, the reversible inhibition of dorsal but not ventral hippocampus by lidocaine, a local anaesthetic that blocks sodium channels and so also silences fibers of passage (contrary to muscimol in the study by Riaz et al, 2017), blocked the expression of amphetamine sensitization in animals well-habituated to the drug associated context (Degoulet et al, 2008). This finding is potentially consistent with the fact that human addicts show increased craving and relapse when they are exposed to contextual cues that are associated with drug taking (Shaham et al, 2003, Bossert et al, 2013) suggesting that drug addiction can be maintained by exposure to contextual cues

that are associated with drug abuse. The key role of the hippocampus in amphetamine sensitization is further supported by the study of Lodge and Grace (2008) who demonstrated that augmented activity in ventral hippocampus leads to an increased number of spontaneously active mesolimbic dopaminergic neurons and thus could contribute, at least partially, to behavioural sensitization to amphetamine. Collectively, all these data show that there is a commonality in the neural substrates of contextual learning, memory and amphetamine sensitization, and that the hippocampus plays a key role in all of them.

Furthermore, the context that is paired with drug taking interacts with the process of sensitization (Anagnostaras and Robinson, 1996, Badiani et al, 1995, Browman et al, 1998, Fraioli et al, 1999). Thus, through contextual conditioning incentive salience is attributed to contextual stimuli that have been paired with drug taking. This is a main component in the progression to addictive behaviour (Anagnostaras and Robinson, 1996). In this respect, the context associated with cocaine has been shown to facilitate conditioned cocaine responses in sensitized animals, and thus facilitate addiction to the drug (Carey and Gui, 1998).

Amphetamine when administered in novel environments to which rodents had not been habituated facilitates the induction of amphetamine sensitization. More specifically, acute amphetamine challenge to amphetamine pretreated rodents led to enhanced locomotor responses in the group that had been pre-treated with amphetamine in a novel environment compared to the group that had been pre-treated in a familiar environment. This finding potentially indicates that contextual novelty serves as a conditioned stimulus (Crombag et al, 2001).

Increased attention and processing of the context by psychostimulants is associated with induction of c-fos mRNA expression in the neocortex, caudate nucleus and nucleus accumbens (Uslaner et al, 2001). Cocaine-induced increases in spine density in the nucleus accumbens core but not shell have also been demonstrated when the drug is administered in a

novel context. In this study, the degree of sensitization was associated with the structural plasticity in nucleus accumbens core and medial prefrontal cortex (Li and Kauer, 2004).

This finding might show that neuroplasticity in these structures is associated with the facilitating effect of contextual novelty on the process of sensitization. In addition, the strong associative effect of context on behavioural sensitization and addiction, is demonstrated by the findings that different contexts act as occasion setters, either inducing or inhibiting expression of sensitization dependent on whether or not they induce drug expectation (Anagnostaras et al, 2002). Also, amphetamine sensitization facilitates conditioned place preference (Shen et al, 2006, Ito and Canseliet, 2010). Overall, these findings support the common substrate between contextual learning and behavioural sensitization.

Previous studies on the effects of amphetamine sensitization on spatial learning have given mixed results. In particular, no effect of withdrawal from amphetamine sensitization has been found on the acquisition of a water maze task, which is dependent on the hippocampus (Russig et al, 2003). On the other hand, impaired spatial learning after repeated amphetamine exposure has been shown in an object in place learning task that assessed animal's reactivity to object displacement and object substitution (Mandillo et al, 2003). These findings are inconsistent with those by Gelowitz et al (1994) who showed enhanced spatial learning acquisition in the water maze in rats previously exposed to repeated amphetamine. However, in that study rodents had also received acute amphetamine before each session during training. Further research is required to clarify the effects of amphetamine withdrawal on spatial learning and memory.

3.1.4 Neural and neurochemical mechanisms of appetitively-driven contextual and cue learning and memory in behavioural sensitization

Cortico-limbic-striatal circuit regulating goal-directed behaviour

According to the most popular theoretical framework the hippocampus encodes contextual cues while basolateral amygdala encodes elemental cues (Moses et al, 2002). However, one should bear in mind that there is evidence for hippocampus affecting processing of elemental cues (Marshall et al, 2004, Honey et al, 1998, Honey and Good, 2000) and evidence of deficit in context fear conditioning with amygdala lesion (Phillips and LeDoux, 1992). The amygdala is well known to regulate associations between emotional experiences and discrete cues (Philips and LeDoux, 1992, Fanselow and LeDoux, 1999, Amorapanth et al, 2000, Huff and Rudy, 2004). Also, it mediates learning involving information about the rewarding or aversive properties of cues by signalling reward history, incentive salience and the reward outcomes predicted by stimuli (for review see Wassum and Izquierdo, 2015) as well as drug seeking elicited by reward predicting cues (Fuchs et al, 2005).

Both basolateral amygdala and hippocampus have glutamatergic outputs to nucleus accumbens and prefrontal cortex where they overlap with dopaminergic input from the ventral tegmental area (Pennartz et al, 1994, Mulder et al, 1998, French and Totterdell, 2003). Mesoaccumbal dopamine responds to convergent afferent inputs from hippocampus and basolateral amygdala by amplifying one signal while suppressing the other in an activity dependent manner (Floresco et al, 2001). Thus, hippocampus and amygdala are key components of a cortico-limbic-striatal circuit that controls goal directed behavior (Cardinal et al, 2002). In this respect, Ito et al, (2006) provided evidence of compromised acquisition of conditioned place preference by hippocampal lesions, which on the other hand enhanced

acquisition of cue conditioning. In the same study, basolateral amygdala lesions had the reverse effects to hippocampal lesions. Thus, amygdala lesions compromised acquisition of cue conditioning, while leaving the acquisition of conditioned place preference unaffected. These results supported previous findings showing that amygdala and hippocampus compete for the control of appetitively driven goal directed behaviour (White and McDonald, 2003).

Convergent and mutually inhibitory inputs by basolateral amygdala and hippocampus are also received by infralimbic and prelimbic subdivisions of the rodent prefrontal cortex (Ishikawa and Nakamura, 2003). Accordingly, prelimbic and infralimbic dopamine have opposing effects on place and cue conditioning known to be mediated by hippocampus and basolateral amygdala respectively. Thus, infralimbic dopamine opposes prelimbic dopamine's facilitating effect on place conditioning (Hayen et al, 2014).

Likewise, nucleus accumbens core and shell dopamine have opposing roles in regulating place and cue conditioning. This is shown by evidence that infusion of amphetamine into nucleus accumbens core impaired appetitively driven spatial learning (Ito, Hayen, 2011). The hippocampus-nucleus accumbens shell pathway mediates spatial context conditioning for rewarding stimuli. Nucleus accumbens shell lesions selectively impaired the acquisition of conditioned place preference and the use of spatial information to retrieve information about a discrete cue. The same findings were shown by disconnection of the hippocampus from the nuclues accumbens shell using unilateral, asymmetric lesions of each structure (Ito et al, 2008).

This finding is in line with evidence that projections from ventral medial prefrontal cortex to nucleus accumbens shell mediates reinstatement of heroin seeking (Bossert et al, 2012) and that nucleus accumbens shell mediates the influence of contexts on conditioned ethanol-seeking (Chaudhri et al, 2010). Similarly, repeated amphetamine infusions in nucleus accumbens shell before training, facilitated spatial learning for rewarding stimuli (Ito and

Hayen, 2011). On the contrary, nucleus accumbens core is required selectively for acquisition of appetitively motivated discrete cue conditioning but not contextual conditioning (Ito et al, 2008).

Disturbance of the balance of cortico-limbic-striatal circuit by amphetamine withdrawal

Amphetamine sensitization increases hippocampal glutamatergic output to nucleus accumbens (Karler et al, 1991, Karler et al, 1994, Pierce et al, 1996) and subsequent increased ventral tegmental area- nucleus accumbens tonic dopaminergic activity (Lodge and Grace, 2008). Consequently, hippocampal drive is further enhanced through ventral tegmental area dopaminergic afferent projections, which regulate long term potentiation in CA1 (Frey et al, 1990, Frey et al, 1991) through D1 receptor activation (Frey et al, 1991, Granando et al, 2008). Also, hippocampal drive may be further enhanced due to prefrontal cortex dysfunction and subsequent loss of control in the regulation of hippocampal dopaminergic activity (Selemon et al, 2007, Castner et al, 2005, Lodge and Grace, 2008). Thus, hippocampus increases phasic dopaminergic activity for reward predicting contextual stimuli to which 'incentive salience' is attributed (Grace, 1991, Robinson and Berridge, 1993).

In light of a possibly hyperfunctional hippocampus, the balance between hippocampus versus basolateral amygdala dependent information processing may be disturbed as a consequence of amphetamine sensitization, when they compete for the regulation of appetitive behaviour (Ito and Canseliet, 2010). In particular, in mice that had withdrawn from repeated amphetamine and which were shown to be sensitized, basolateral amygdala dependent cue learning was reduced while hippocampus dependent place conditioning was enhanced in a simultaneous conditioned cue and place preference task in a six-arm radial

maze. Accurate performance in this task was shown to be dependent on the integrity of basolateral amygdala (cue learning) and hippocampus (place conditioning).

In the same study, however, in an experiment conducted in an elevated T-maze, basolateral amygdala dependent cue learning was attenuated only in the amphetamine sensitized group with 2.5mg/kg and not in the amphetamine sensitized group with 5mg/kg, while hippocampus dependent spatial learning was unaffected in both groups. Contrary to the first experiment of the study in which basolateral amygdala cue and hippocampus dependent place conditioning compete for control of appetitive behaviour (i.e. mice were tested on preference for either cue or place arm of the maze), in the second experiment the two forms of learning were taking place in parallel to each other and thus independently of each other (i.e. not simultaneously). Thus, mice did not have to make a choice between reward encoded by either spatial or elemental cues; therefore, it could be argued that the task as it was did not promote competition between hippocampus and amygdala in the control of goal directed behaviour as happened in the radial maze experiment of the same study in which hippocampus dependent place learning was competing with basolateral amygdala cue learning. These findings provide evidence that alteration of mesocorticolimbic dopamine by amphetamine sensitization, leads to alterations in the balance of hippocampal versus basolateral amygdala competition over the control of goal directed behaviour but only under the conditions in which the two learning processes directly compete with one another.

Attenuated cue learning in the T-maze task by Ito and Canseliet (2010) was suggested to be consistent with compromised activity of the amygdala, a putative characteristic of schizophrenia (Schneider et al, 1998, Calder et al, 2001, Gur et al, 2002, Takahashi et al, 2004). Also, it was suggested it might be linked to reduced dopaminergic tone in the medial prefrontal cortex after repeated amphetamine exposure (Paulson and Robinson, 1995, Pierce and Kalivas, 1997, Hedou et al, 2001) which could have augmented responsiveness to

glutamate and thus glutamatergic activity of prefrontal cortex (Peterson et al, 2000, Hedou et al, 2001). This could have led to excitatory input to the ventral tegmental area and nucleus accumbens. According to neurophysiological findings this could lead to inhibitory interneurons being recruited and suppressing basolateral amygdala activity (Rosenkranz and Grace, 2001).

However, it should be added that reduced basolateral amygdala dependent cue learning that was found in the t-maze task was inconsistent with findings showing that amphetamine exposure facilitates other forms of appetitively driven cue learning for rewards (i.e. Harmer and Phillips, 1998, Taylor and Jentsch, 2001, Wyvell and Berridge, 2000) (see 3.1.2 subheading: Sensitization to psychostimulants and appetitive learning). Based on the discrepancy between findings of these studies and findings of the t-maze task of the study by Ito and Canseliet (2010) it was suggested that repeated amphetamine exposure might change basolateral amygdala function dependent on the task and on which other brain structures connected with it are engaged in the task.

Findings by Stalkaner et al (2007) are in line with this hypothesis. What was shown in that study is that repeated cocaine pretreatment leads to persistent responses by basolateral amygdala neurons to outdated associative cue information during reversal learning of odor cue-go, no go discriminations (Stalkaner et al, 2007). This was contrary to the fact that this process had been established to be dependent on the integrity of the orbitofrontal cortex and not on basolateral amygdala (Schoenbaum et al, 2003, Izquierdo and Murray, 2007, Stalkaner et al, 2007). Therefore, persistent encoding of outdated associative information in basolateral amygdala in that study could have delayed reversal learning by interfering with normal processing in other structures with which it is connected. Such an effect has been previously shown in cocaine pretreated rodents (Schoenbaum et al, 2004, Jentsch et al, 2002) as well as in non-human primates with bilateral amygdala lesions (Izquierdo and Murray, 2007).

The finding of unaffected spatial memory in the t-maze is in line with findings from another spatial learning test (Russig et al, 2003) but not in line with evidence of decreased spatial memory (Mandillo et al, 2003) (see subheading 3.1.3 ‘Sensitization to psychostimulants: Hippocampus and the role of context’ above). It might be thus interesting to test wether withdrawal from amphetamine will enhance spatial learning in the same task if this is conducted in a way that engages more the hippocamppus through regular rotation of the maze. This will help clarify the effects of amphetamine sensitization on spatial learning. Also, it will provide more clear insight into whether the facilitated spatial learning found by Gelowitz et al (1994) in the Morris water maze was due to the fact that rodents that had withdrawn from amphetamine were also receiving acute amphetamine challenge before each training session. Finally, it will be of interest to check whether the attenuated cue learning found by Ito and Canseliet (2010) in the t-maze task will be found.

3.1.5 Hypothesis

We used a version of T-maze task for the simultaneous assessment of cue learning and spatial learning for natural rewards that involves regular rotation of the maze. This is done to promote an allocentric strategy in order to increase the hippocampal engagement in the task relative to the egocentric version of the T-maze task used in the Ito and Canseliet study. Through this we are checking whether spatial learning will be enhanced in amphetamine sensitized mice as was the case in the conditioned cue and place preference test in the radial maze in Ito and Canseliet (2010) study.

While in the Ito and Canseliet study the T-maze had a fixed position throughout the whole experimental procedure (egocentric version of the task), in our study the maze was rotated 180 degrees periodically (allocentric version of the task). This was done to ensure that

mice do not rely only on an egocentric strategy based on body turns.

It has been consistently reported that the hippocampus is critically involved in place navigation based on extra pool-cues, but not cued-responding, whereas the dorsolateral striatum is engaged during intra-pool cue responding, but not place navigation (McDonald and White, 1994, Rice et al, 2015). It should be noted though that the integrity of the hippocampus is required in tasks involving alternation driven by an egocentric strategy (Praag et al, 1994, Brito and Brito, 1987). Also, there is evidence that the processing system for distal cues in spatial learning tasks is mediated by the entorhinal cortex while for proximal visual cues is mediated by parietal cortex. Both systems converge to the hippocampus (Save and Poucet, 2000; Parron et al, 2004). Also, tissue-type plasminogen activator (tPA)-dependent N-methyl-D-aspartate receptor signaling pathway in the entorhinal cortex plays a key role in the processing of distal but not proximal cues (Hébert et al, 2016). It seems that competition can occur between distal and proximal cues with the former overshadowing the latter, but not vice versa, when both are distributed at 360 degrees across the environment (Hébert et al, 2017). Also, there is evidence of increased integration and cooperation throughout the hippocampal dorsoventral axis when the training protocol involves allocentric learning and memory (Ramos, Morón, 2022). In particular, results from this study showed a retrograde impairment in both groups of ventral hippocampus and dorsal hippocampus lesioned rats compared to control group but only with regard to allocentric information. Also, other evidence suggests that a flexible use of stored allocentric information in order to reach a goal destination, even from a location never explored during training, induces a shift from fronto-striatal to hippocampal circuits (Rinaldi et al, 2020). Flexible use of allocentric information is expected to occur under conditions that the maze is rotated at regular but random intervals as in being conducted in this study. Thus, it is expected that the allocentric version of this task will increase the engagement of the hippocampus. Therefore, competition

between distal versus proximal cues for the control of goal-directed behavior and consequently increase the control of extra maze cues versus intra-maze cues is expected to occur.

Packard and White (1987) provided evidence for an increase in the involvement of thehippocampus in learning maze tasks relative to the competing dorsal striatum in a space with heterogeneous visual cues. Accordingly, the relative prevalence between place and response learning depends on the amount of variability of visual stimulation that the maze paradigm provides (Restle, 1957). In an allocentric version visual stimulation is increased given that themaze is periodically rotated and thus the animals see more of the extra maze cues given that tend to overshadow proximal cues as explained above. These studies overall show that the more variation mice have in terms of the visual cues relevant for the task, as happens in allocentric maze tasks, the more the hippocampus is engaged. Consequently, it is expected that the level of engagement of the hippocampus is much higher in an allocentric task compared to an egocentric version of the same task.

The prediction we make is that amphetamine sensitization increases hippocampal dependent learning for rewards. The logic behind the selection of an allocentric version of the T-maze task is to explore the possibility that in Ito and Canseliet's study (2010) the fact that the T-maze task ~~was~~ had a fixed position might have contributed to the fact that contextual learning was not enhanced in amphetamine sensitized mice. While this does not explain the differences between the direct cue competition (ie. the conditioned cue/place preference test in Ito and Canseliet study of 2010) and no cue competition designs (ie. the t-maze test used in both Ito and Canseliet 2010 study and here) it might show that if in the no direct competition design the engagement of the hippocampus in the task is increased (i.e. by turning the task into a more allocentric one), then similar effects with the competition task might also be observed. This task will thus provide more insight into the effects of withdrawal from

amphetamine sensitization on tasks that involve simultaneous acquisition of cue and spatial learning for rewards.

Thus, the prediction was that amphetamine sensitization would increase hippocampus dependent contextual learning. This assumption is based on the fact that the hippocampal involvement is expected to be increased relative to the egocentric condition. Also, attenuated cue learning for rewards is expected, which is also what was found in the study by Ito and Canseliet (2010).

3.2 Methods

Subjects

Groups were: AMPH.2.5 mg/kg (n=6), or AMPH.5 mg/kg (n=6), and saline (n=6). Food restriction was maintained until completion of the experiment, involving approximately 2 gr lab chow daily per mouse, equivalent to maintain mice at 85% of their free feeding weight.

Apparatus

An elevated wooden T maze consisting of three 35 x 10 cm arms, surrounded by a 10 cm raised wall was placed in a novel room with prominent, extramaze allocentric spatial cues (i.e. wall posters and lab equipment). Steel wells were placed at the ends of the goal arms (Ito and Canseliet, 2010).

Habituation

Mice were habituated for four successive days 2 minutes per day in the maze to drinking condensed milk from both goal arms, in a different room to the one in which the

experiment was to be conducted.

3.2.1 Assessment of spatial and cue learning

Spatial learning involved learning to associate a particular floor insert (white Perspex or wire mesh) covering the start arm, with either the east or west baited goal arm, irrespective of the direction of the maze (see figure 3.1). Thus, when the white Perspex floor insert was present in the start arm of the maze the reward was located in the East goal arm, irrespective of whether trials started in the North or South. The orientation of the maze was changed and the start arm was rotated 180 degrees at random intervals to increase reliance on a place (allocentric) versus response (egocentric) strategy.

Each mouse was trained in 2 spatial conditions, with the first one being the white perpex floor insert in the starting arm and the second being the wire mesh in the starting arm. Given that the maze was regularly rotated the mice were trained to turn always west or east, depending on the floor insert present in the start arm irrespective of the orientation of the maze.

Non-spatial cue learning involved learning to associate a reward with one of two floor inserts (either rough kaoutsuk or sandpaper), each one covering one of the goal arms. The pairing of the reward for each mouse with one of the two floor inserts was consistent throughout training for each mouse. Across trials the 2 floor inserts were regularly exchanging positions between the two goal arms (i.e. East vs. West), so the mouse could not associate the reward with East or West but only with the discrete cue provided by the floor insert. A conditional discrimination task was used to assess spatial learning given that this was a dual experiment the aim of which was to assess indirect competition effects when cue and spatial learning take place in parallel. Therefore, a similar procedure was followed in spatial trials as in the cue trials. This was achieved by conducting both types of trials in the T-

maze using floor inserts. In addition, the goal of this study was to test whether rotation of the maze will generate different results in the spatial learning than those shown in the same experiment conducted by Ito and Canseliet (2010), therefore the same spatial task was conducted as in that study.

Trials were arranged in a pseudorandom order with equal numbers of the different combinations in each session. Spatial and non-spatial cue learning trials were intermingled. Pairing of floor inserts with baited arms was counterbalanced across groups. Each session consisted of four cue learning trials, and eight spatial learning trials. Half of the spatial learning trials were with one floor insert and the remaining were conducted with the other. Following completion of 144 cue learning trials, spatial learning trials continued without any more extra cue learning trials taking place in parallel. Mice received in total 144 non-spatial cue learning trials and 360 spatial learning trials. The maximum number of consecutive trials arranged with the same combination/reward contingency was three. Interval between trials was around 5 minutes.

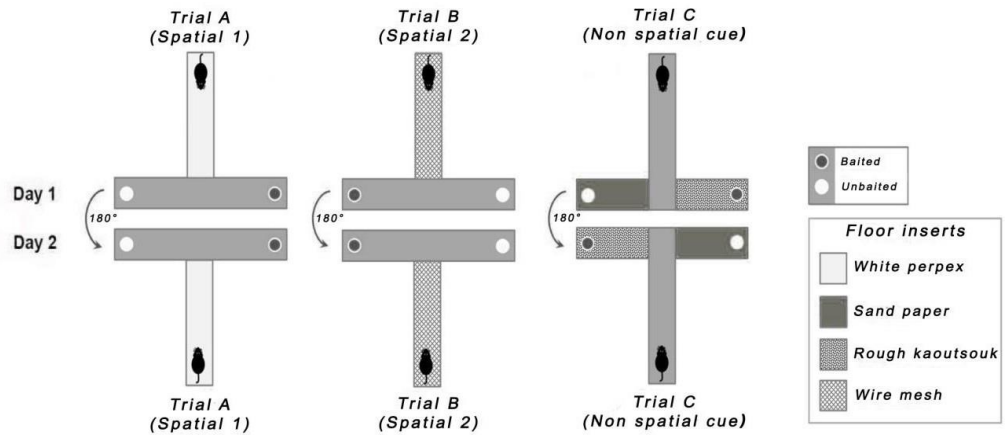


Figure 3.1. Simultaneous allocentric spatial (trials A, B) and non-spatial, cue (trial C) learning.

Outcome measures

Data were analysed in 9 blocks. The learning index used was the percentage of correct responses over 40 trials for spatial trials and over 16 trials for the cue version of the task.

3.3 Results

Summary of results

All mice acquired the non-spatial cue memory task, at the same rate (see figures 3.2 and 3.3). However, there was no evidence of spatial memory acquisition in any of the mice and no group differences in the number of errors were found (see figures 3.4 and 3.5).

3.3.1 Non-spatial cue learning

Mauchly's test indicated that the assumption of sphericity had been violated, $\chi^2(35)=83.215$, $p<.001$, therefore degrees of freedom and p value were corrected using Greenhouse Geisser estimates of sphericity ($\varepsilon=.488$). Results for the three group analysis indicated a significant main effect of blocks ($F(3.904, 58.563)=20.717$, $p<.001$), a non-significant main effect of groups ($F(2, 15)=1.602$, $p=.234$) and non-significant groups by blocks interaction ($F(7.808, 73.563)=1.220$, $p=.340$). These results indicate that mice acquired the task but that there were no significant group differences (see figure 3.2).

When the two amphetamine pre-treated groups were integrated into one single group and compared with the saline pre-treated group Mauchly's test again indicated that the assumption of sphericity had been violated, $\chi^2(35)= 84.932$, $p<.001$, therefore degrees of freedom and p value were again corrected using Greenhouse Geisser estimates of sphericity ($\varepsilon=.502$). A significant main effect of blocks was again found ($F(4.018, 64.286)=17.744$, $p<.001$) indicating that all mice acquired the cue learning component of the task. A non-significant group by block interaction, ($F(4.018, 64.286)=1.865$, $p=1.270$) and a non-significant trend towards a significant main effect of groups ($F(1, 16)=3.402$, $p=.084$) were reported, indicating that groups did not differ significantly in the acquisition of cue learning (see figure 3.3). As seen in this figure the non-significant trend for groups to differ was due to

amphetamine pre-treated mice making slightly less errors than saline pre-treated mice.

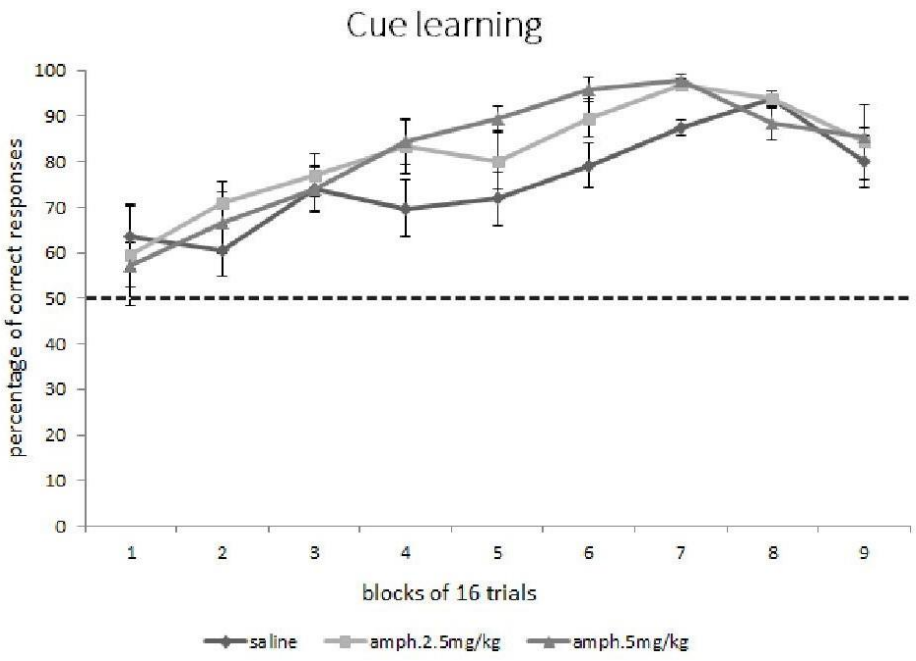


Figure 3.2. Cue learning for reward. Mean (±SEM) percentage of correct responses for saline (n=6), amph.2.5mg/kg (N=6) and amph.5mg/kg (n=6). Error bars represent standard error of the mean. Dashed line equals chance levelperformance.

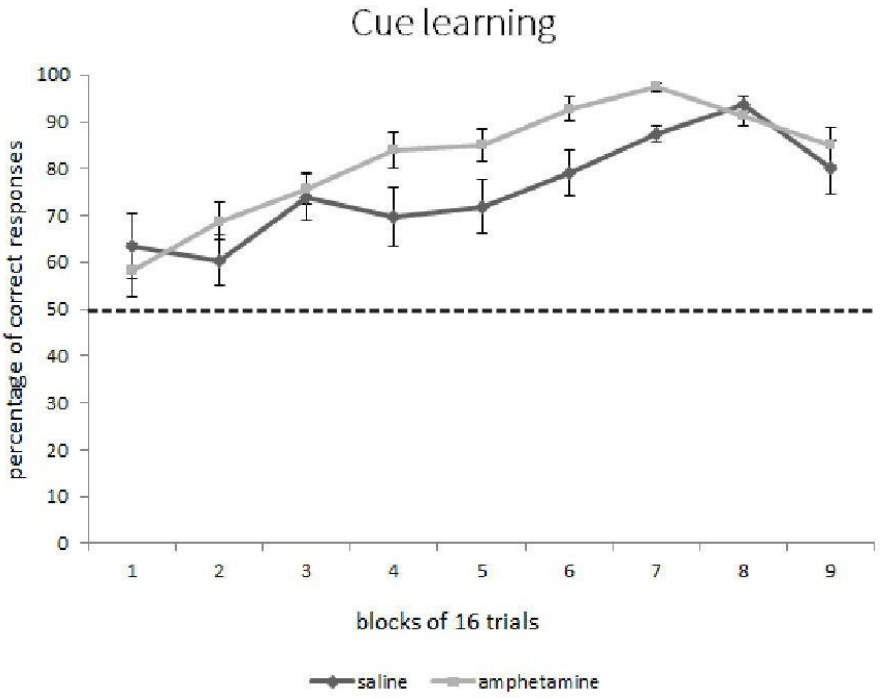


Figure 3.3. Cue learning for reward. Mean (±SEM) of percentage of correct responses for saline (n=6), and amphetamine (n=12). Error bars represent standard error of the mean. Dashed line equals chance level performance.

3.3.2 Spatial learning

Repeated measures ANOVA revealed a significant main effect of blocks, (F(8, 120)=2.718, p=.009), a non-significant main effect of groups, (F(2, 15)=2.354, p=.129) and a non-significant groups by blocks interaction, (F(16, 135)=1.369, p=.169). However, the significant main effect of blocks did not reflect acquisition of the task (see figure 3.4).

When amphetamine pre-treated groups were integrated into one combined group the main effect of blocks again was significant, (F(8, 128)=3.325, p=.002), the groups by blocks interaction appeared to be marginally significant, (F(8, 144)=1.880, p=.069) andthe main effect of groups appeared to be non-significant, (F(1, 16)=.3340, p=.568). Again, however, the significant main effect of blocks did not reflect acquisition of the task (see figure 3.5).

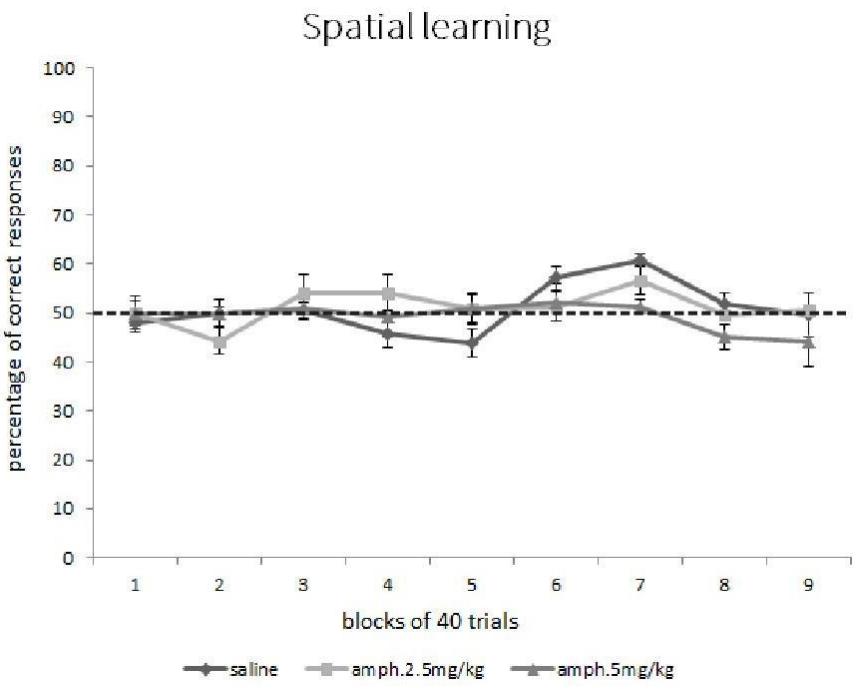


Figure 3.4. Spatial learning for rewards. Mean (±SEM) percentage of correct responses for spatial learning for saline (n=6), amph.2.5mg/kg (n=6) and amph.5mg/kg (n=6). Error bars represent standard error of the mean. Dashed line equals chance level performance.

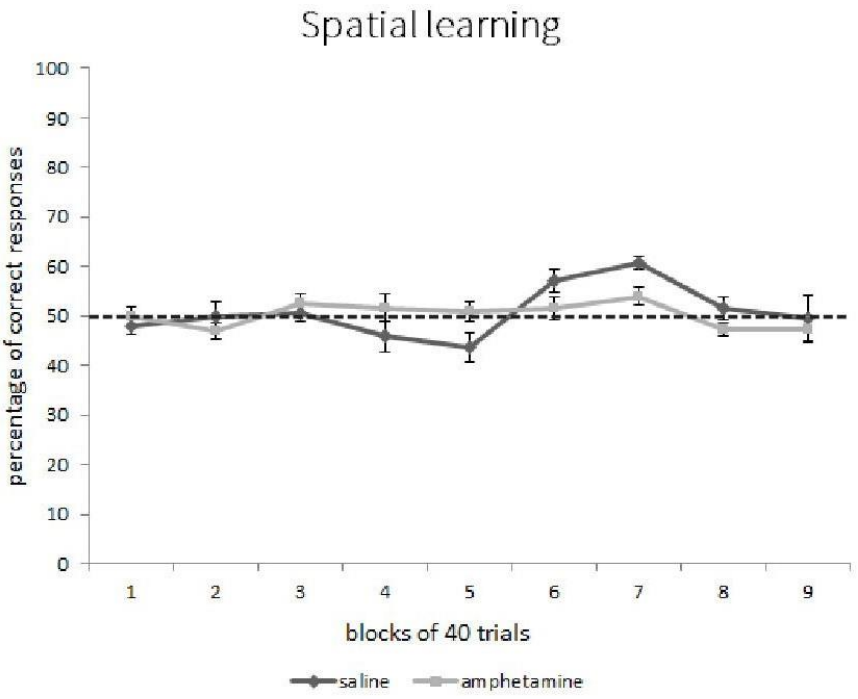


Figure 3.5. Spatial learning for rewards. Mean (±SEM) percentage of correct responses for saline (n=6), amphetamine combined group (n=12). Error bars represent standard error of the mean. Dashed line equals chance level performance.

3.4 Discussion

Overall, results indicated that basolateral amygdala dependent cue learning for reward was unaffected by repeated amphetamine withdrawal, while hippocampus dependent spatial learning was not acquired by the saline group. These results collectively with the results by Ito and Canseliet (2010) provide evidence that cue learning for rewards can proceed normally in amphetamine sensitized mice under conditions when spatial learning is not acquired simultaneously.

Mice did not acquire the spatial component of the task and therefore no conclusions can be made about the effect of amphetamine sensitization on acquisition of spatial learning under conditions when it takes place simultaneously with cue learning and when the maze is rotated. According to post hoc power analysis our sample size was large enough (see section

2.1.1). Further research with more trials could be informative. However, it is also worth pointing out that there was little, if any, evidence of spatial learning in the present study, even after 360 trials. Thus, it seems unlikely that having conducted more trials would have caused animals to learn because the number of trials performed was already high. It is worth mentioning that the Ito and Canseliet (2010) T-maze task acquisition of spatial learning was also limited, not exceeding 70% of correct responses by the end of the 224 trials that were conducted. However, it should be noted that in another study when the spatial version of the T-maze experiment in our study was conducted the control group acquired the task very well (Schmitt et al, 2004). However, unlike our study, in that study the maze was not rotated. Also, unlike both our study and the study by Ito and Canseliet (2010) the T-maze task in that study was not a dual task. Thus, mice were trained only in spatial leaning and not in spatial learning in parallel with cue learning.

One possibility could be that the current allocentric version of the task was too complicated for mice to acquire. In that respect the same experiment could be conducted with basolateral amygdala and hippocampus dependent learning trials less intermingled (i.e. run in blocks of a specific trial type such that mice could do every few days only spatial learning and every few days only cue learning instead of every day doing both). Also, different types of cues could be used instead of the floor inserts in the spatial component of the task. Also, one could argue that the floor inserts that were used were too similar and thus one could try floor inserts that are more dissimilar and discriminable than those used in this study.

Finally, one should test whether mice can acquire the spatial component of the task on its own in the absence of training for cue learning. It would be of interest to test whether amphetamine abstinence facilitates spatial learning in that task if cue learning does not take place in parallel and whether there is a difference in its effect dependent on whether or not the starting arm of the maze is rotated. This is of interest given evidence that the integrity of

hippocampus is required for acquisition of this task (Schmitt et al., 2004) as well as evidence of the hippocamppus-nuccleus accumbens pathway being overactive following withdrawal from amphetamine sensitization (Lodge and Grace, 2008). Thus, rotation of the maze might increase engagement of the hippocampus which might cause an enhanced acquisition rate in the amphetamine group. Also, given that Ito and Canseliet (2010) showed that the control group acquired cue learning in this task much faster than spatial learning one could try doing first the spatial task and then introduce the cue learning component of the task once, and only if, mice have acquired the spatial component to a sufficient degree.

The results of this study with regards to cue learning were not in line with the results from the Ito and Canseliet study (2010) that found attenuated cue conditioning. In the present study, a non-significant trend for improvement in cue learning was shown for amphetamine mice. According to post hoc power analysis our sample size was large enough (see section 2.1.1). The spared cue learning in our study where spatial learning was not acquired indicates that under conditions that the two forms of learning do not take place in parallel, withdrawal from repeated amphetamine regimen does not impair cue learning.

Spared cue learning in our study might indicate that the attenuated cue learning in the study by Ito and Canseliet (2010) was a result of some sort of interference between the two forms of learning that took place in parallel. In line with the attenuated cue learning found by Ito and Canseliet (2010), they also found attenuated cue conditioning as well as facilitated spatial conditioning in a combined cue and place preference task. It was suggested that under conditions of direct competition between basolateral amygdala and hippocampus for the control of appetitive behaviour, amphetamine sensitization causes an imbalance in the competitive interactions between these structures (White and McDonald, 1993, Floresco et al, 2001).

Thus, there might be two levels of imbalance being caused by amphetamine

sensitization when hippocampus and basolateral amygdala are both engaged simultaneously in a dual appetitive task that involves acquisition of cue and spatial learning. The first one is caused by direct competition as in the conditioned cue/place preference task that facilitated spatial learning while attenuated cue learning in amphetamine pre-treated mice. The second one is caused by some sort of interference enough to cause impairment in basolateral amygdala but without facilitating spatial learning in amphetamine pre-treated mice. Further research should be done to elucidate the distinct neural and neurochemical mechanism underlying the effects of amphetamine sensitization on neural processes underlying these two separate levels of imbalance.

One should take into account that unlike the Ito and Canseliet (2010) study in which the performance of saline treated mice in cue learning was close to 100% by the end of training, our control group was only close to 80% by the end of training. By looking at our cue learning graph and that of the Ito and Canseliet (2010) study one can see also that our amphetamine group performance was comparable to the amphetamine group of Ito and Canseliet (2010). Thus, it might be that the different cue learning findings between our study and that of Ito and Canseliet (2010) are due to the attenuated performance of the control group in our study. The reason for the compromised performance of the control group is not clear. That could be because of changes to the paradigm we used involving rotation of the maze. That might have made the task more complex for mice to learn.

Hippocampus gates prefrontal cortex-nucleus accumbens output (O'Donnell and Grace, 1995), as accumbens neurons cannot enter a depolarized state without hippocampal input. Also, activation of prefrontal cortical inputs is prevented from evoking spike firing in accumbens neurons unless nucleus accumbens neurons are active. Thus, perhaps lack of spatial learning acquisition meant that hippocampus was not overactive thus letting nucleus accumbens core select inputs from prefrontal cortex-basolateral amygdala pathway in

amphetamine sensitized mice instead of selecting hippocampal inputs, as would have happened if the hippocampus had been overactive. Further research is needed before any conclusions are drawn.

Given that spatial learning was not acquired it was not possible to test the hypothesis that cue learning would be reduced in amphetamine sensitized mice as a result of simultaneous acquisition of cue learning with spatial learning. Thus, the unaffected cue learning in the absence of spatial learning should be interpreted within the framework of studies using non-dual tasks to test cue learning. While we found unaffected cue learning other studies show enhanced appetitively driven cue learning (see subheading 3.1.2 'Sensitization to psychostimulants and appetitive learning' above).

Thus, our results show that, at least under conditions when spatial learning is not acquired, basolateral amygdala dependent cue learning can proceed normally in amphetamine sensitized mice. It could be that the discrepancy of cue learning findings between our study, Ito and Canseliet (2010) and other studies might be due to different involvement of other brain structures connected with basolateral amygdala.

It would be of interest to study the distinct involvement of prelimbic and infralimbic cortices as well as that of nucleus accumbens core and shell in this task, given findings thatthey have crucial role in regulation of the balance between hippocampus and basolateral amygdala when they are simultaneously engaged in an appetitive task (Ito and Hayen, 2011, Hayen et al, 2014). Also, further research is required to provide more clear insight into how amphetamine sensitization affects appetitively driven basolateral amygdala dependent cue learning and hippocampus dependent spatial learning in general.

Conclusions

Overall, these results do not necessarily strengthen findings by Ito and Canseliet

(2010). This is mainly because the basolateral amygdala cue learning was not reduced as in Ito and Canseliet's study. On the contrary there was a trend for facilitation which did not reach significance. Also, given that spatial learning did not occur in the saline group one cannot draw any conclusions. The hypothesis that the allocentric version of the task would facilitate spatial learning in amphetamine mice could not be verified because the spatial component of the task did not work. Also, because the spatial learning component did not work we were unable to assess context/cue competition effects. What can be concluded from the present study is that cue learning can proceed normally in amphetamine sensitized mice under conditions when spatial learning is not acquired and therefore is not competing with cue learning. This conclusion further strengthens the notion that the reduced basolateral amygdala dependent conditioning observed in Ito and Canseliet (2010) radial maze experiment was indeed a result of direct competition with hippocampus dependent spatial conditioning. Additionally, the reduced cue learning in the T-maze experiment of the same study was a result of interference with the spatial learning that was taking place in parallel.

Chapter 4: Amphetamine Sensitization Effects on Appetitively Motivated, Spatial Reference Memory, Working Memory and Pattern Separation

4.1 Introduction

4.1.1 Effects of amphetamine sensitization on the cortico-limbic-striatal circuit

Amphetamine sensitization enhances the hippocampal drive onto nucleus accumbens dopaminergic neurons (Lodge and Grace, 2008, Yoshikawa et al, 1991). In line with this, evidence suggests that following cocaine sensitization limbic and prefrontal glutamate inputs are dysfunctionally integrated in the nucleus accumbens through D1 and D2 receptors. These are activated by enhanced tonic and phasic dopaminergic neurotransmission during activation of the hippocampus. Such activation of D1 and D2 receptors shifts the balance of information flow in the nucleus accumbens from prefrontal cortex to hippocampus (Goto and Grace, 2005).

Consequently, the prefrontal cortex is unable to suppress via its GABA projections to the ventral tegmental area the enhanced accumbal dopaminergic activity. Lesions in the hippocampus- nucleus accumbens pathway block behavioural sensitization to methamphetamine (Yoshikawa et al, 1991). This indicates that this pathway is crucial in the neural mechanisms of amphetamine sensitization. In summary, hippocampus overactivity increases hippocampal output to nucleus accumbens and consequently increases baseline dopaminergic activity.

Thus, incentive salience is attributed to hippocampal inputs. Mesoaccumbal dopamine is well-known to be implicated in incentive salience attribution to reward predicting cues

(Robinson and Berridge, 1993, 2001). Hippocampus is well known to mediate spatial memory (Morris et al, 1982). In this respect it would be worthwhile to investigate the effects of withdrawal from repeated amphetamine on different forms of appetitively driven spatial memory.

4.1.2 Appetitive spatial reference memory

Overview of studies on the effects of withdrawal from amphetamine sensitizing regimens on appetitive learning

Consistent with the above is the evidence that amphetamine exposure is associated with increased incentive salience attribution to reward predicting stimuli (Wyvell and Berridge, 2001, Tindell et al, 2005). This effect is mediated by mesolimbic dopaminergic hyperactivity. Incentive salience attribution to stimuli is a fundamental component of emotional learning. This is attributed to the fact that it enhances attention paid to stimuli and facilitates goal directed behaviour (Berridge and Robinson, 1998). In this respect, certain forms of appetitive learning appear to be maladaptively facilitated following amphetamine exposure.

For example, appetitive conditioning appears to be enhanced (Harmer and Philips, 1998). In particular, it was shown that amphetamine pre-treated rodents had enhanced acquisition of conditioned responding selectively to reward predicting stimuli compared to saline treated rodents and not to stimuli that were negatively correlated with the availability of reward.When the conditioned instrumental efficacy of (S+) relative to (S-) was assessed by stimulus availability being made contingent on a novel level pressing response there was no group differences in the preference of S+ versus S-. Consistent with this are the findings by Taylor and Jentsch in 2001 who provided evidence that repeated intermittent amphetamine administration in rats facilitates acquisition of Pavlovian approach behaviour.

Nelson and Killcross (2006), also demonstrated that amphetamine sensitization accelerates formation of habitual responses. This was also replicated later by Nordquist et al (2007) who demonstrated that this effect is associated with amphetamine sensitization increasing the incentive salience of reinforcers. The involvement of mesolimbic dopamine, which also mediates the effects of amphetamine sensitization (Paulson and Robinson, 1995, Pierce and Kalivas, 1995), in these forms of learning is well known. Furthermore, there is evidence that facilitation of habitual learning by repeated methamphetamine use is mediated by reduced glutamate receptors and glutamate vesicular proteins in dorsomedial striatum (Furlong et al, 2018). These proteins may contribute to goal-directed behaviours. Reduction in proteins necessary for goal directed behaviour may lead to a shift towards habitual responding. Indeed, these proteins were increased in dorsolateral striatum, which is known to be involved in the formation of habits.

Conditioned place preference (Lin et al, 2007, Ito and Canseliet, 2010), conditioned motor sensitization and operant conditioning serving drug self-administration (Vezina, 2004) are also facilitated by amphetamine. Neuroplastic alterations that accompany learning, including elevation of TrkB, a tyrosine kinase receptor for a neurotrophic factor in the hippocampus and nucleus accumbens pathway, mediate amphetamine induced conditioning (Shen et al, 2006).

More specifically, elevation of TrkB-like immunoreactivity specifically in CA3/dentate gyrus was found after repeated AMPH (0.3 mg/kg)-induced conditioned place preference. Also, elevated TrkB in the CA3/DG, as well as in the nucleus accumbens shell, was found to accompany conditioned place preference and conditioned motor sensitization after a repeated higher AMPH dose administration (1.0 mg/kg). The development of both conditioned behaviours was blocked by intra-CA3/DG infusion of the Trk inhibitor K-252a. These results indicate that the Trk system in the hippocampus-nucleus accumbens pathway

mediates neural adaptations necessary for the amphetamine induced conditioned place preference and conditioned motor sensitization. Thus, the neurotrophic activation in the hippocampus is required for the acquisition of conditioned learning and the development of a conditioned motoric response to amphetamine. This may reflect the fact that contextual cues that have been paired with drug administration become salient and strengthen its addictive properties.

In line with these findings Ito and Canseliet (2010) provided evidence that amphetamine sensitization enhances hippocampus dependent, appetitively driven, place conditioning while it impairs appetitively driven cue conditioning dependent on the basolateral amygdala in a conditioned cue and place preference test that involved direct competition between the two forms of conditioning. It was suggested that nucleus accumbens dopamine mediated these effects. Dopaminergic activity in nucleus accumbens may regulate the balance of the competing basolateral amygdala and hippocampus inputs by strengthening the one while suppressing the other in an activity dependent manner (Floresco et al, 2001). Amphetamine withdrawal, as shown by electrophysiological data, augments ventral hippocampal output which, in turn, increases dopaminergic activity in nucleus accumbens (Lodge and Grace, 2008). This could have led to aberrant salience attribution to hippocampal inputs at the expense of basolateral amygdala inputs and thus facilitation of place conditioning at the expense of cue conditioning (for more details see chapter 3).

However, in the same study amphetamine withdrawal did not enhance appetitively driven contextual memory in a dual task that involved simultaneous spatial and cue learning. Also, there is evidence provided by infusion studies that amphetamine sensitization through elevated mesolimbic dopamine facilitates appetitive learning. Taylor and Robbins (1984) showed that intra-accumbens d-amphetamine infusion to rats that had been sensitized to cocaine facilitated the control that conditioned reinforcers had over their behavioural

responses. Accordingly, there is evidence of increased responding for conditioned reward in cocaine sensitized rodents in which d-amphetamine was infused into the nucleus accumbens (Taylor and Horger, 1999). Wyvell and Berridge (2000), demonstrated that intra-accumbens amphetamine increased the incentive salience attribution to sucrose reward without increasing the liking for it.

Effects of withdrawal from an amphetamine sensitizing regimen on other forms of learning including aversively driven learning

It has been demonstrated that amphetamine sensitization in mice does not affect other forms of learning known to be dependent on dopaminergic-glutamate interactions in the striatum, including motor learning on an accelerating rotarod, Pavlovian conditioning (learning that a lever cue was associated with food availability, without havingto press the lever to get the reward) and aversively driven cue based learning in a U water maze in which mice had to find an escape platform (Eldred and Palmiter, 2013). The lack of effect of amphetamine sensitization on Pavlovian conditioning in this study is not consistent with findings of enhanced appetitive conditioning described earlier (ie. Harmer and Philips, 1998). It might be that amphetamine sensitization can either enhance or leave Pavlovian conditioning unaffected dependent on the conditioning cues and species that are used in an experiment. Also, different amphetamine sensitization regimens that were used in these studies might have accounted for the discrepancy in the findings. In the same study by Eldred and Palmiter (2013) amphetamine sensitization failed to restore learning in those paradigms in mice lacking NMDA receptor signaling in striatal medium spiny neurons. These findings highlight the fact that even though amphetamine enhances extracellular dopamine in striatum, where dopamine-glutamate interactions are known to mediate learning, amphetamine sensitization does not affect multiple learning paradigms used in this study. This indicates

that the synapticalterations that occur in response to amphetamine sensitization are different from thoseinvolved in learning in the paradigms used.

Likewise, Featherstone et al (2008) demonstrated that amphetamine sensitization does not alter long term memory assessed in a standard fixed-platform location water maze task, an aversively motivated, hippocampus-dependent spatial memory task. Russig et al (2003) also showed that withdrawal from repeated amphetamine does not affect acquisition rate in the water maze. On the other hand, another study demonstrated that amphetamine sensitization impairs learning and memory in the passive avoidance test (Lee et al, 2011), which is a test of aversively driven hippocampus dependent memory (Cimadevilla et al, 2007). Also, Mandillo et al (2003) showed that non-associative spatial memory is impaired in mice that have withdrawn from repeated amphetamine. Collectively, these studies show that withdrawal from repeated amphetamine does not facilitate aversively driven learning and memory and other forms of learning that are not reward driven and may even impair them in some situations.

Conclusions

Overall, many studies provide evidence for facilitation of appetitively driven learning by amphetamine sensitization which appears to be mediated by elevated mesolimbic dopamine and by enhanced neuroplasticity in hippocampus and nucleus accumbens. However, this is not a consistent finding. Further research is required to elucidate the effects of amphetamine sensitization on different forms of appetitively driven learning. Appetitively driven, incrementally acquired spatial reference memory on maze tasks has not been studied in amphetamine sensitized rodents. It is hypothesized that spatial reference memory for rewards might be enhanced in rodents that have withdrawn from an amphetamine sensitizing regimen. This hypothesis is based on evidence showing overactive hippocampus-nucleus

accumbens pathway (Lodge and Grace, 2008, Kalivas and Stewart, 1991, Henry and White, 1992), as well as facilitated goal directed behavior and appetitive learning by amphetamine withdrawal (Taylor and Jentsh, 2001, Harmer and Phillips, 1998). It is known that hippocampus nucleus accumbens pathway mediates acquisition of appetitively driven spatial conditioning (Ito et al, 2008). Also, spatial reference memory is dependent on the hippocampus (Pothuzen et al, 2004).

Clinical relevance with schizophrenia and drug addiction

The hippocampus, which is important for learning and memory systems, including spatial and declarative memory (Jarrard, 1993) also regulates the rewarding effects of drugs of abuse through its direct projections to nucleus accumbens and ventral tegmental area (Keleta and Martinez, 2012). The firing rate of neurons in the nucleus accumbens appears to be dopamine-dependent and reflect the timing of cocaine seeking behaviour in rats on a progressive ratio schedule of reinforcement (Nicola, Deadwyler, 2000). These results might reflect that the reinforcing signals of drugs of abuse are mediated by hippocampus-nucleus accumbens pathway. This is possible given evidence that dorsal hippocampus may have an involvement in cocaine seeking (Werner et al, 2020, Ramirez et al, 2009). This is explained by the fact that contextual memory which is regulated by the hippocampus is a key component of drug seeking. Also, it is well established that nucleus accumbens receives glutamatergic input from hippocampus, which is involved in appetitively driven contextual learning and memory (Burns et al, 1993, Ito et al, 2006). In addition, the ventral hippocampus-nucleus accumbens pathway is critical for aversive conditioning to context, but not to cue (tone) (Riedel et al, 1997).

Interestingly, maladaptive hyperactivity in the hippocampus-ventral tegmental area loop, which was induced in rats by prenatal administration of methylazoxymethanol acetate

(MAM) appeared to underlie amphetamine's sensitization potency, replicating symptoms reminiscent of schizophrenia (Lodge and Grace, 2007). Hyperdopaminergia in this study was shown by a greater number of spontaneously firing ventral tegmental dopamine neurons in these MAM treated rats and was considered to be a consequence of excessive hippocampal activity. This is because inactivation of ventral hippocampus completely reversed the hyperdopaminergia and also normalized the augmented amphetamine-induced hyperlocomotion. Therefore, hyperdopaminergia seems to be associated with hippocampal dysfunction. Both of them have been linked to hyperlocomotion in response to acute amphetamine challenge in animal models of psychosis. The role of the hippocampus-VTA loop in such phenomena is further highlighted by evidence of conditioned locomotion. This involves pairing of the context where drug is administered with the rewarding effects of the drug, leading to the former being sufficient to induce increased locomotion even in the absence of the drug (for review Bardo and Bevins, 2000).

Aberrant salience attribution is a hallmark of schizophrenia, and is mediated by increased mesolimbic dopamine release (Kapur, 2003, Heinz and Schlagenhauf 2010). Furthermore, aberrant incentive salience is well known to mediate drug addiction (Robinson and Berridge, 1993, 2001). Salience attribution is a component of appetitive learning. Remarkably, increased drug self-administration and intensified reward seeking have also been consistently shown in sensitized animals and seems to model compulsive drug seeking (Robinson and Berridge, 1993). Acquisition of spatial reference memory for rewarding stimuli is thought to involve the progressively increased associative strength of spatial cues and thus increased salience of certain locations in the environment.

Thus, it might be of relevance to such impaired information processing in schizophrenia and drug addiction. Therefore, it is of potential interest and should be studied. Dopaminergic activity in nucleus accumbens, which is elevated in schizophrenia, drug

addiction and amphetamine sensitization, is known to signal reward prediction errors and incentive salience, and to mediate appetitively driven associative learning (Day et al, 2007). Increased incentive salience attribution to reward predicting stimuli is expected to facilitate acquisition of spatial reference memory in amphetamine sensitized mice. Therefore, enhanced spatial reference memory is hypothesized in amphetamine pre-treated mice.

4.1.3 Spatial working memory

Common neural circuits underlie working memory and amphetamine sensitization

Amphetamine sensitization is associated with neuronal morphological alterations (Lu et al, 1997, Peterson et al, 2000, Selemon et al, 2007) and reduced dopaminergic activity in the prefrontal cortex (Wolf et al, 1995, Castner et al, 2005, for review Pierce and Kalivas, 1997). Dopaminergic activity through D1 receptors in the prefrontal-hippocampal circuit, mediates both the effects of amphetamine sensitization (Ujike et al, 1989) and working memory (Williams and Goldman-Rakic, 1995, Sawaguchi and Goldman-Rakic, 1991), but also the integration of recently encountered information with executive function (Seamans et al, 1998). Ventral CA1/subiculum connections with prefrontal cortex mediate spatial working memory performance in rodent tasks with a delay, which require retrieval of information encountered before that delay (Floresco et al, 1997). Ishikawa et al in 2005 provided evidence for a D1 receptor mediated impairment of long-term potentiation (LTP) in the hippocampal-prefrontal cortex pathway in methamphetamine sensitized rodents.

Furthermore, a D1 antagonist reversed this working memory impairment and the accompanying atrophy of pyramidal dendrites in the dorsolateral prefrontal cortex in nonhuman primates caused by amphetamine sensitization (Selemon et al, 2010). Also, it is known that reduced dopamine turnover in prefrontal cortex is accompanied by impaired

working memory in nonhuman primates (Castner et al, 2005). Repeated administration of Methylphenidate (MPH), an amphetamine derivative, impairs hippocampal LTP. This is mediated by a decreased number of AMPA-GluA1 receptor subunits in the plasma membrane (Carvallo et al, 2018). GluA1 deficient mice have impaired spatial working memory performance in the working memory component of the radial maze (the same experiment that is presented in this chapter), which is thought to reflect impaired short-term habituation (i.e. Schmitt et al, 2003, Sanderson and Bannerman, 2012, Sanderson et al, 2010). All of the above findings indicate that there might be an overlap in the neural circuits underlying amphetamine sensitization and spatial working memory, which mainly involves the prefrontal -hippocampal pathway.

Overview of rodent studies on the effects of amphetamine sensitization on working memory

Given the common neural circuits underlying both amphetamine sensitization and working memory, studying this form of memory after amphetamine sensitization is of interest. Methamphetamine sensitization has been shown to impair spatial working memory in the spontaneous alternation Y maze paradigm, accompanied by reduced NMDAR ligand binding in CA1, CA2 and CA3 hippocampal subfields, and in prefrontal cortex (Lee et al, 2011). Spontaneous alternation in that study was tested during the days of drug administration and not following drug withdrawal. Also, spontaneous alternation in the continuous Y maze may be a flawed task for assessing working memory. This is because animals can show high levels of alternation due to stereotypical behaviours (i.e. always turning left into the next arm) rather than as a result of spatial working memory.

Rodents treated with high dose of d-amphetamine immediately before each of the two sessions of a spatial cone field task had selectively impaired spatial working memory but not spatial reference memory. In this task a large open field was used that included 4 baited and

12 non-baited cones, and both forms of memory were simultaneously assessed (Blokland et al, 1998). The dissociable effect that amphetamine had on reference and working memory in this task indicates that the impairment in working memory may not be related to a deficit in memory per se.

Withdrawal from repeated methamphetamine in rats caused deficient spatial working memory for at least 14 days after withdrawal. This deficit was found in a spatial win-shift task in a radial arm maze when the acquisition phase of the test was separated by a delay of 30 minutes from the testing phase, No deficit was found when the delay was 5 minutes. This delay-dependent working memory deficit was accompanied by abolishment of the hyperphosphorylation of ERK1/2 in the hippocampus that was observed in saline pretreated rodents immediately after training (Nagai et al, 2007), and which has been shown to play a critical role in spatial working memory (Nagai et al, 2006). The working memory deficit was improved by clozapine but not haloperidol. These findings indicate that the spatial working memory deficit in mice after withdrawal from methamphetamine might be mediated by dysfunctional ERK1/2 pathway in the hippocampus. These findings indicate that this might be a good model for the study of specific aspects of cognitive impairment in schizophrenia known to be improved by clozapine.

In line with these results, catha edulis, a psychostimulant, appears to impair selectively spatial working memory in rodents, but not spatial reference memory in a radial maze paradigm (Alfadly et al, 2014). In line with the study of Blokland et al (1998) described above, this result shows that there is no general memory deficit in psychostimulant pretreated rodents. Amphetamine administration, both within the retention interval of the radial maze and before the to-be remembered event, impairs rodents' spatial working memory performance in a radial maze paradigm. In this paradigm rats were required to learn a win-shift food-searching strategy and avoid arms previously visited. However, the impairment in

that study was suggested by the authors to be a result of altered appetite and motor activity caused by amphetamine rather than a memory impairment per se (Beatty and Rush, 1983).

The distinction between the effects of the drug on memory from the effects on performance (i.e. caused by other reasons such as motor activity or appetite) was achieved by drug administration both during the retention interval and before the to-be-remembered event. Rats tested at a 5 hours retention interval treated with 2mg/kg d-amphetamine had a retention deficit. This deficit however was shown only when drug was administered half an hour or 4.5 hours after the to-be-remembered event. Thus, rodents did not have a retention deficit when given drug 0 or 2 hours after or 3 hours before the event. Treatment with 3 mg/kg leads to retention deficit only if given 2 hours after the event. However, at 7 hours delay 3mg/kg given 2 hours after the event did not lead to a retention deficit.

Overall results from this study show compromised performance in the radial maze in amphetamine pre-treated rodents under some conditions only. Thus, they do no support that this is due to a general memory deficit in amphetamine pre-treated rodents. However, the impairment of working memory is not a consistent finding. Using a delayed alternation task dependent on the integrity of prefrontal cortex, Stefani and Moghaddam (2002) demonstrated that repeated amphetamine exposure produced a transient working memory deficit. This was evident only before the 10th day post- withdrawal from the treatment. No locomotor sensitization test was performed in that study. Therefore, it has not been confirmed that animals were actually sensitized. Also, phencyclidine was administered to all mice following withdrawal from the amphetamine regimen, before behavioural testing. Given that mice were always administered an NMDA receptor antagonist before testing, interpretation of results of this study with regards to the effects of amphetamine sensitization is complex and no clear conclusions can be drawn. In another study in which amphetamine sensitization was demonstrated in rodents, working memory was enhanced in Fischer but not Lewis rats

following withdrawal (Peleg-Raibstein et al, 2009). Other evidence that amphetamine sensitization does not disrupt working memory performance comes from Featherstone et al (2008) study of working memory in an operant delayed non-match to position task. However, it should be noted that unaffected working memory performance in that task might be due to animals adopting mediating strategies to accurately respond as has been shown in both rats (Dunnett, 1985, Chudasama and Muir 1997) and pigeons (Blough, 1959).

Overall, the literature with regards to the effects of amphetamine sensitization on working memory, are mixed. While there is some evidence that working memory might be impaired by amphetamine sensitization, in most studies it appears that this may not be due to a memory deficit but to impaired performance which might be caused by motor deficits or appetite, both of which are well known to be affected by amphetamine sensitization.

Clinical relevance with schizophrenia and drug addiction

Spatial working memory in rodents has potentially limited translational potential for the classical form of spatial working memory observed in humans and thus it should be seen as an index of executive function and cognitive impairment observed in schizophrenia and drug addiction with caution (for review Castner and Williams, 2007). However, it is of interest to study spatial working memory as it will give more insights into the effects of amphetamine sensitization on prefrontal-hippocampal circuits, described above, which are crucial in schizophrenia and drug addiction.

4.1.4 Pattern separation

Pattern separation is thought to be mediated by the dentate gyrus-CA3 auto-associative network which dissociates overlapping memory traces in order to prevent interference, which causes overgeneralization (O'Reilly and McClelland, 1994, Gilbert and

Kesner, 2006, for review Miller and Sahay, 2019). Likewise, several studies support the role of newly generated dentate gyrus neurons in pattern separation (Aimone et al, 2009, for review Deng et al, 2010). Reduced neurogenesis in the dentate gyrus has been shown to be associated with pattern separation deficits when animals had to differentiate between very similar contexts (Nibori et al, 2012, Kheirbek et al, 2012, Nakashiba et al, 2012, Clelland et al, 2009).

On the other hand, Groves et al (2013) showed that rats lacking adult neurogenesis were not impaired in distinguishing between adjacent or distant maze arms in a radial maze paradigm. The discrepancy of these findings might suggest that neurogenesis might be required for behavioural tasks that involve greater levels of interference, which was not the case in the radial maze paradigm used by Groves at al (2013) study. Temporarily reduced neurogenesis in dentate gyrus compared to a saline pretreated group has been found in rodents that withdrew from chronic amphetamine for one month but not 24 hours (Barr et al, 2010).

Neurogenesis in the dentate gyrus in the latter study was measured by quantification of newly generated neurons, four weeks after withdrawal from a chronic amphetamine regime, compared to rodents withdrawing from saline pretreatment. NMDA receptors in CA3, are thought to mediate spatial pattern separation (for review Gilbert and Kesner, 2006). This is conducted through rapid and automatic formation of representations of unified contexts (Cravens et al, 2006). NMDA receptors seem to exhibit reduced ligand binding in amphetamine sensitization after repeated but not acute drug treatment (Lee et al, 2011).

In that study ligand binding was examined in mice that were sacrificed the same day that the last injection of the amphetamine regimen was administered and locomotor sensitization was assessed. Also, there is evidence that acute amphetamine potentiates the population excitatory postsynaptic potential (EPSP) at the perforant path-granule cell

synapse. Maybe this is mediated by amphetamine induced increases in monoaminergic transmission, which do not involve changes in the population spike. Long term amphetamine exposure on the other hand, reduces long term potentiation of the population spike excitability in the dentate gyrus without changing LTP of the EPSP. This is possibly mediated by long lasting catecholaminergic supersensitivity (Morimoto et al, 1987). Notably, the effects of amphetamine sensitization on behavioural pattern separation have not been studied yet.

Clinical relevance with schizophrenia and drug addiction

Reduced neurogenesis has been associated with both schizophrenia and drug addiction (for review Yun et al, 2016). There is evidence of reduced cell proliferation and differentiation in drug addiction (Bayer et al, 2015, for review Yun et al, 2016). Also, in schizophrenia, there is evidence of reduced proliferation in anterior hippocampus (Allen et al, 2016, Reif et al, 2006, for review Yun et al, 2016) and differentiation in the hilus region of the hippocampus (Bardeau et al, 1995, for review Yun et al, 2016). These deficits are improved by antipsychotic treatment in the case of schizophrenia and abstinence from cocaine self-administration in the case of cocaine addiction. Neurogenesis impairments in schizophrenia have been associated with impaired pattern separation (for review Tamminga et al, 2010, 2012). Further studies also confirmed the link between impaired pattern separation and schizophrenia (Chen et al, 2012, Das et al, 2014, Martinelli and Shergill, 2015). In this respect, the study of pattern separation in animal models of schizophrenia is of great interest.

4.1.5 Selection of a behavioural paradigm

In light of the effects of amphetamine sensitization on neural circuits affecting distinct forms of learning and memory described above, a radial maze task was selected. This task

simultaneously studies incrementally acquired appetitive spatial reference memory, spatial working memory and pattern separation. It was hypothesized that one possibility would be that amphetamine sensitization will disrupt selectively spatial working memory and pattern separation. Another possibility would be that it would facilitate spatial reference memory.

In this respect, aberrant salience attribution as a result of amphetamine sensitization will be studied. In particular, increased salience attribution to the contextual cues that predict reward (i.e. the locations where food is found) and thus increased salience attribution to certain locations in the environment is hypothesized. Another possibility is that impaired performance in the working memory component may be found in amphetamine sensitized mice well as impaired ability to distinguish between similar memory traces leading to impaired pattern separation. .

Neural circuits engaged in the behavioural paradigms

The spatial working memory performance of mice in the retrieval phase of the radial maze experiment conducted in the present study appears to depend on hippocampal NMDA receptors (Bannerman et al, 2008, Niewoehner et al, 2007). NMDA receptor subunit GluN2A, which is required selectively for spatial working memory performance in the radial maze experiment used here, but not in the reference memory component (Bannerman et al, 2008), is required for the development of behavioural sensitization to methamphetamine (Miyamoto et al, 2004) and for keeping the dopaminergic activity under control (Miyamoto et al, 2001), which in amphetamine sensitization is increased. Simões et al (2007) showed that repeated methamphetamine administration reduced NR1 subunit expression in the hippocampus. This indicates that NMDA receptor function was reduced.

In addition, the involvement of NMDA receptors in the hippocampus in the spatial working memory component of the radial maze task (see Niewoehner et al (2007) suggests

that neuroplasticity in the dentate gyrus, which as indicated above is altered by amphetamine sensitization, is required for the working memory but not for the reference memory component of the task. Also, as indicated above, both hippocampal NMDA receptors and dentate gyrus neurogenesis may be required for pattern separation (McHugh et al, 2007). Collectively, findings from these studies indicate that the neural circuits selectively engaged in the spatial working memory component of the radial maze and for pattern separation, but not in the reference memory component of the radial maze experiment conducted here are altered by amphetamine sensitization. Therefore, they provide evidence that spatial working memory and pattern separation in the radial maze maybe selectively compromised in amphetamine sensitized mice.

4.2 Methods

Subjects

The same three groups of mice were used including vehicle pretreated mice, n=11, amphetamine 2.5mg/kg pretreated mice, n=13, and an amphetamine 5mg/kg pretreated group, n=13.

Apparatus

A radial maze was used in a room with distinct extra-maze cues (i.e. wall posters, pieces of equipment). The maze consisted of a circular 18 cm diameter centre area. The centre area was surrounded by a transparent Perspex cylinder from which 6 equally spaced arms (60x7 cm) extended. At the entrance to each arm there was a door which could be raised by strings by the experimenter. At the end of each arm there was a steel well. At the start of each trial three of the wells contained sweetened, condensed milk diluted in 50% water. The

rest of the wells were empty. Two of the three baited arms were adjacent to each other and the third baited arm was between two non-baited arms. The location of each arm was fixed and defined relative to the extra-maze cues. Four different combinations of baited arms were used, counterbalanced across experimental groups.

Experimental Procedure

The radial maze experiment was conducted 4 months post drug treatment. Mice were trained 6 out of 7 days per week whenever possible and kept at > 85% of their ad libitum weight. The maze was regularly rotated at random intervals to avoid the animals using local intra-maze cues. Trials started with the mouse placed in the central platform and ended when all three rewards were acquired and consumed. Between trials the maze was cleaned with 70% alcohol. Habituation took place before the experiment started for 10 minutes per day for a week. For this habituation, the apparatus was placed in a distinct room (i.e. not the testing room). Mice were left free to explore the maze and to drink condensed milk diluted with 50%water from the steel wells at the end of all 6 arms.

4.2.1 Acquisition of the spatial reference memory phase

Mice were trained to discriminate between the baited and non-baited arms (see figure 4.1). Each time a mouse returned to the central platform after an arm visit all doors were closed. The mouse was confined in the central platform for 5 seconds following which only the doors of non-visited arms were re-opened. Thus, arm re-visits were prevented at this stage of the experiment (Schmitt et al, 2003). This prevented the mice from making working memory errors during this stage of the experiment as previous studies have shown that working memory errors can impact on reference memory performance (Schmitt et al, 2003). 8 blocks of 16 trials each were conducted for each mouse (128 trials in total). Trials were

conducted for one mouse after another without gaps. Therefore, the inter-trial interval for each mouse was the time that was required for all the remaining mice to complete a trial (approximately 90 min). Mice were trained in the same order every day. Therefore, as training and acquisition was progressing the inter-trial interval for each mouse was getting smaller, given that fewer errors were made. The experiment was conducted 6 days per week. On average 2-4 trials per day were conducted for each mouse.

4.2.2 Simultaneous spatial working memory and spatial reference memory assessment phase

Once the mice had acquired the spatial reference memory component of the task, spatial working and reference memory were then assessed simultaneously. The same procedure was applied as above with the major difference that arm revisits were now no longer prevented (i.e. it was now possible to make working memory errors). 8 blocks of 3 trials were conducted for each mouse (24 trials in total). As with reference memory acquisition, trials were conducted for every mouse one after the other without gaps. Therefore, the inter-trial interval for every mouse was the time that was required for all the remaining mice to complete a trial. Mice were trained in the same order every day. The experiment was conducted 6 days per week. On average 2-4 trials per day were conducted for each mouse. The same combination of baited/non-baited arms was used for each mouse as in the acquisition phase, with baited and non-baited arms being counterbalanced across mice.

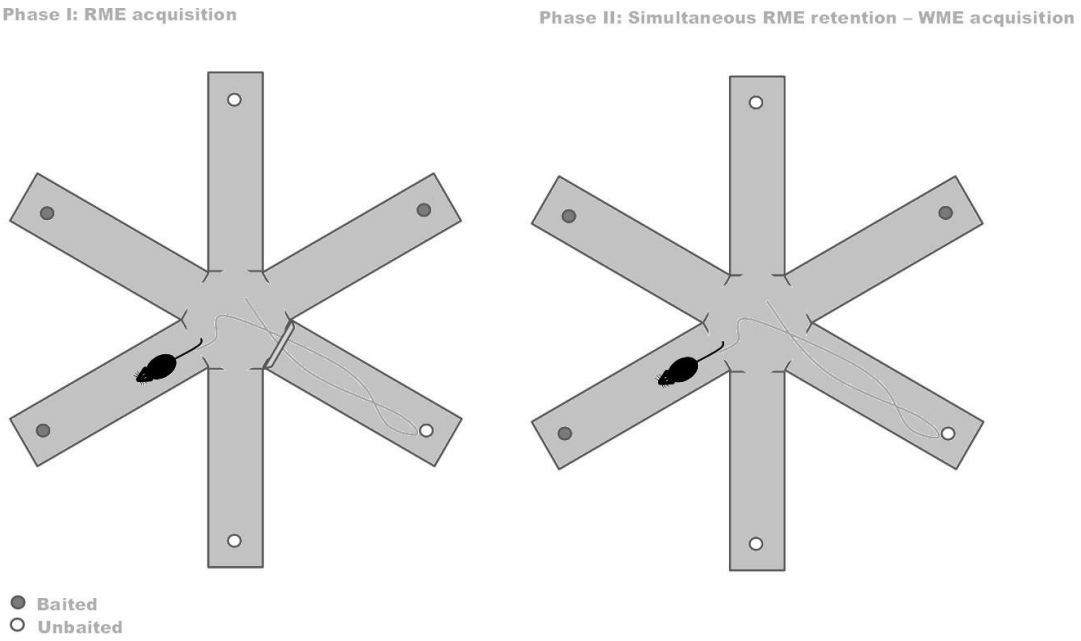


Figure 4.1. Spatial reference memory and spatial working memory assessment in the radial arm maze.

Outcome Measures

The following parameters were assessed (each one was assessed in a different analysis): reference memory errors to collect all three rewards (i.e. number of entries into a non-baited arm; maximum 3 errors), latency to complete a trial (time from being first placed in the maze until consuming the last reward but excluding the time spent in the central platform with closed doors after each arm visit), mean speed of running per arm (latency to complete a trial divided by total number of arm entries).

In the second phase of the experiment working memory errors (revisits to a baited arm) were also compared between groups. In addition to the total number of working memory errors, the number of working memory errors (i.e. revisits) into baited and non-baited arms were compared separately between groups.

Spatial pattern separation was also assessed to determine whether a reference error was more likely to occur in an un-baited arm that was between two baited arms (i.e. single

arm; figure 4.2). Accordingly, whether a working memory error was more likely to occur in a baited arm that was adjacent to another baited arm was also assessed. Error rates were adjusted for the 2:1 ratio of adjacent to single arms.

Mixed ANOVA was used with groups as the between subject factor (3 groups). In addition, for some analyses the two amphetamine groups were combined to provide a single experimental group with a larger n. For some analyses testing block was the repeated measure (within-subject factor). Independent sample t-tests (for 2 groups) or ANOVA (for three groups) were used to compare the percentage of errors in single and adjacent arms (divided by 2 to adjust for 2:1 ratio) between groups. Also, the percentage of trials in which the first spatial reference memory error was made in the single arm out of the total number of trials in which at least one spatial reference memory occurred was compared between groups using independent sample t-tests for two groups and ANOVA for three groups. The number of trials in which the first spatial reference memory error occurred in any of the 2 adjacent arms was divided by 2 to adjust for 2:1 ratio.

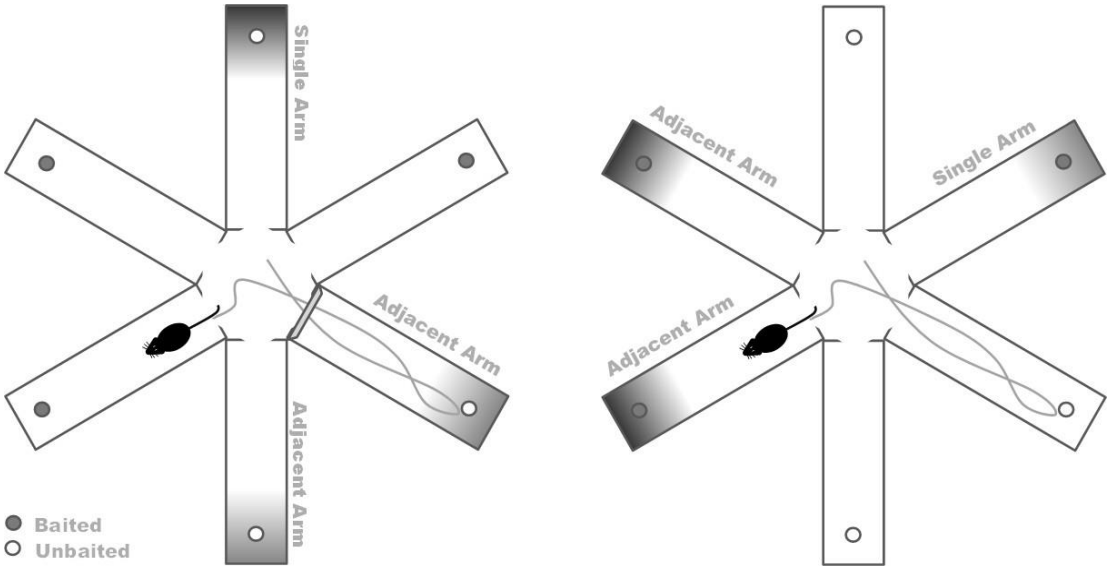


Figure 4.2. Spatial pattern separation assessment. Increased likelihood of error distribution in darkending arms indicates impaired spatial pattern separation.

4.3 Results

4.3.1 First phase: Reference memory acquisition

Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=48.538$, p=.007. Therefore, Greenhouse-Geisser ε=.708 was applied to correct degrees of freedom and p value. All of the mice acquired the spatial reference memory radial maze task at the same rate (see figure 4.3). Mixed ANOVA with 3 groups as a between subjects factor and 8 blocks as a within-subjects factor revealed that all mice learned the spatial reference memory task at the same rate and made progressively less errors. The main effect of block was significant, F(4.935, 168.399)= 91.350, p<.001), but the main effect of group was not, F(2, 34)=.846, p=.438). The groups by blocks interaction was also non-significant, (F(9.906, 168.399)=1.34; p=.256).

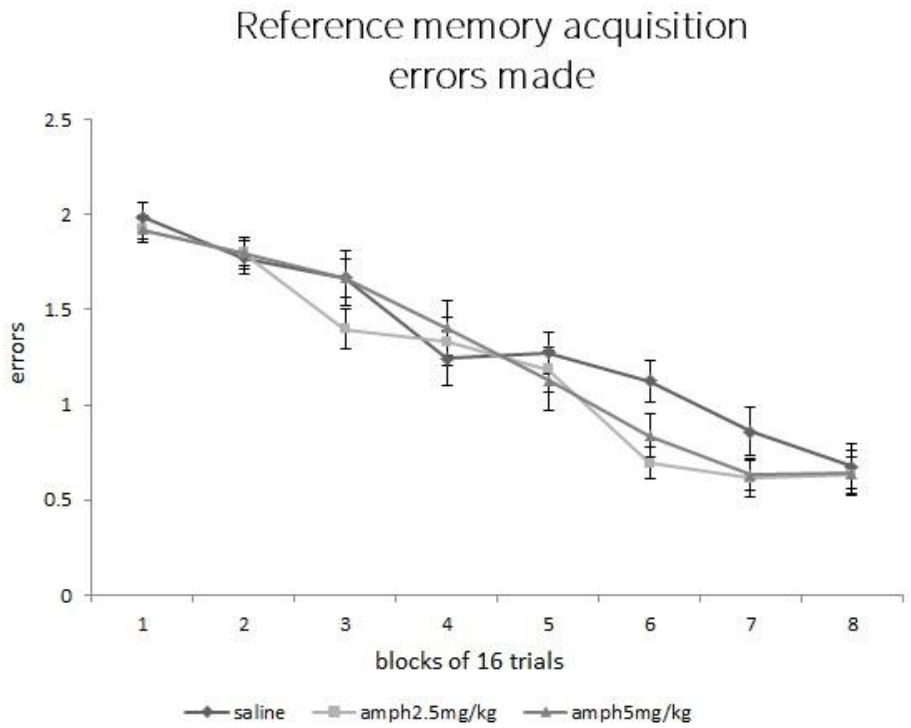


Figure 4.3. Acquisition Phase. Mean (±SEM) spatial reference memory errors, for saline pre-treated group (n=11), amphetamine 2.5mg/kg pre-treated group (n=13) and amphetamine 5mg/kg pre-treated group (n=13).

In the same analysis for 2 groups (with the amphetamine treated subjects now combined into a single experimental group) Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=47.883$, p=.008. Therefore, Greenhouse-Geisser ε=.721 was applied to correct degrees of freedom and p value. Mixed ANOVA revealed a significant main effect of block, (F(5.046, 176.613)=74.049, p<.001), a non-significant main effect of group, (F(1, 35)=1.361, p=.251),and no groups by blocks interaction (F(5.046, 176.613)=1.812, p=.112), indicating that all mice acquired the task at the same rate and made progressively less errors (see figure 4.4).

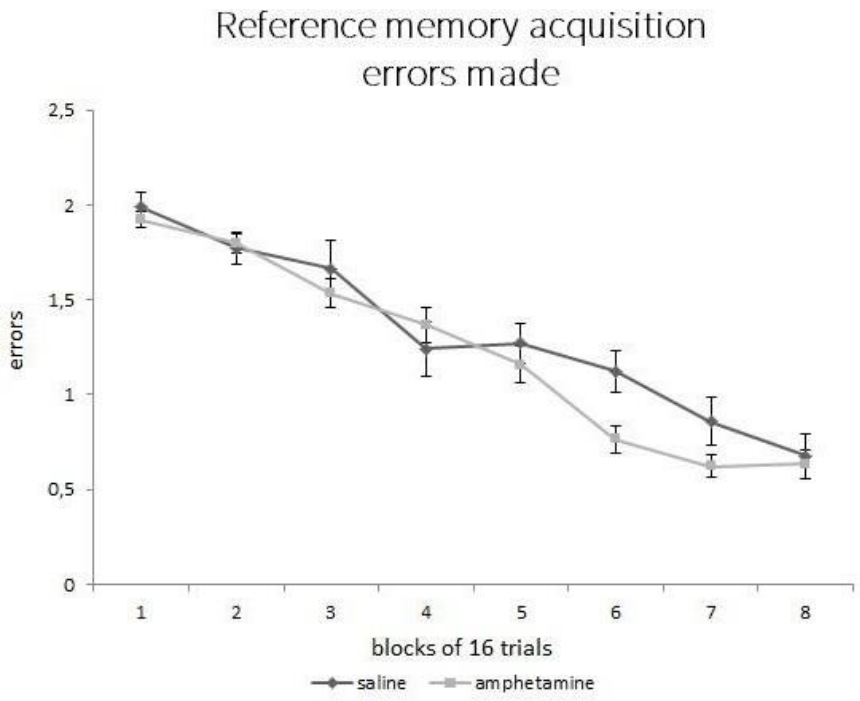


Figure 4.4. Acquisition Phase. Mean (±SEM) spatial reference memory errors, for saline pre-treated group (n=11), amphetamine pre-treated group (n=26). (N.B. these are the same data as figure 4.3 but replotted with a single amphetamine group).

4.3.2 Latency to acquire all three rewards

Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=110.891$, p<.001. Therefore, Greenhouse-Geisser ε=.504 was applied to correct degrees of freedom and p value. There were no differences between groups in terms of latencies to collect all three rewards, while latency to acquire all rewards progressively decreased for all mice (see figure 4.5). Mixed ANOVA with 3 groups as a between subject factor and 8 blocks as a within subject factor revealed that latency to collect all three rewards reduced for all mice as training progressed, (the main effect of blocks was significant, F(3.528, 119.949)=134.190, p<.001). Latency reduced at the same rate for the three groups with the main effect of group, F(2, 34)=2.020, p=.148) and groups by blocks interaction both non-significant, (F(7.056, 119.949)=1.294, p=.259).

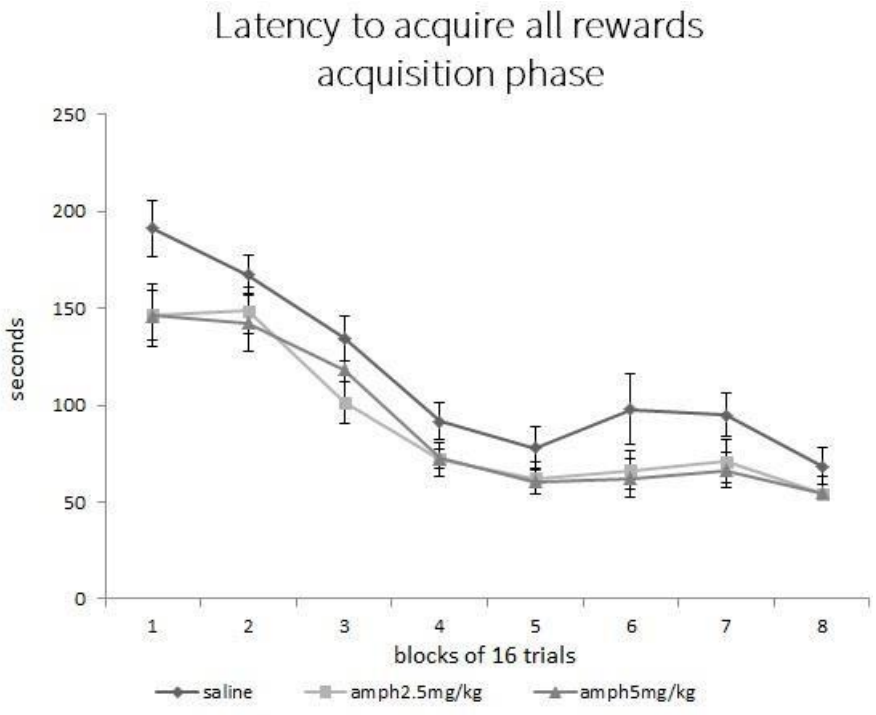


Figure 4.5. Mean (±SEM) latency in seconds until all three rewards are consumed during the acquisition phase for the saline pre-treated group (n=11), amphetamine 2.5mg/kg pre-treated group (n=13), amphetamine 5mg/kg group (n=13).

Likewise, in the same analysis for two groups (with the amphetamine subjects combined into a single group) Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=111.644$, $p<.001$. Therefore, Greenhouse-Geisser $\varepsilon=.516$ was applied to correct degrees of freedom and p value. Mixed ANOVA indicated a significant main effect of block, ($F(3.615, 126.512)=119.356$, $p<.001$). However, the groups did differ (main effect of group, $F(1, 35)=4.158$, $p=.049$), due to the amphetamine group showing reduced latencies to collect all the rewards. The groups by blocks interaction was non-significant, ($F(3.615, 126.512)=1.808$, $p=.138$). Thus, as training progressed all mice acquired all three rewards progressively quicker, while amphetamine pre-treated mice acquired all three rewards quicker compared to saline pre-treated mice (see figure 4.6).

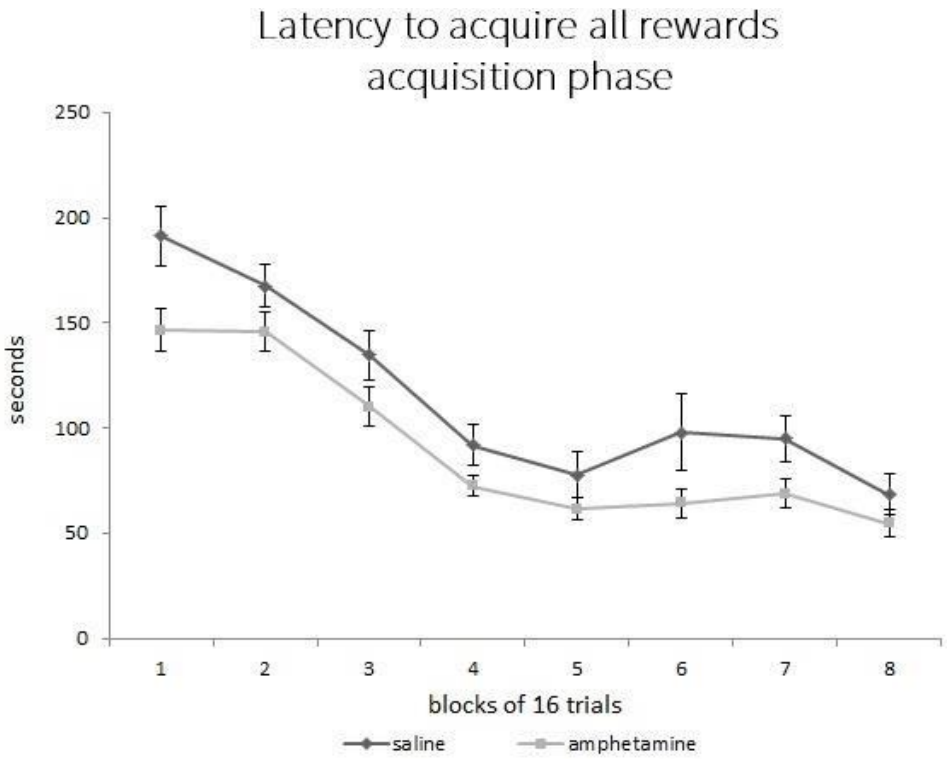


Figure 4.6. Mean (±SEM) latency in seconds until all three rewards are consumed during the acquisition phase for the saline pre-treated group (n=11), and combined amphetamine pre-treated group (n=26). (N.B. these are the same data as figure 4.5 but replotted now with a single amphetamine group).

4.3.3 Mean latency per arm

Because the latency to complete a trial will be affected by the number of errors made and hence the total number of arms entered, a mean latency per arm measure was also calculated by dividing the latency to complete the trial by the total number of arms entered. Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=95.519$, $p<.001$. Therefore, Greenhouse-Geisser $\varepsilon=.527$ was applied to correct degrees of freedom and p value. Mixed ANOVA with 3 groups as a between subjects factor and 8 blocks as a within subject factor indicated a decrease in mean latency per arm for all mice as training progressed, (i.e. the main effect of block was significant, $F(3.689, 125.432)=62.642$, $p<.001$). Mean latency per arm was not different between groups of mice, (main effect of group, $F(2, 34)=1.372$, $p=.267$) and there was no groups by blocks interaction, ($F(7.378, 125.432)=.577$, $p=.798$) (see figure 4.7).

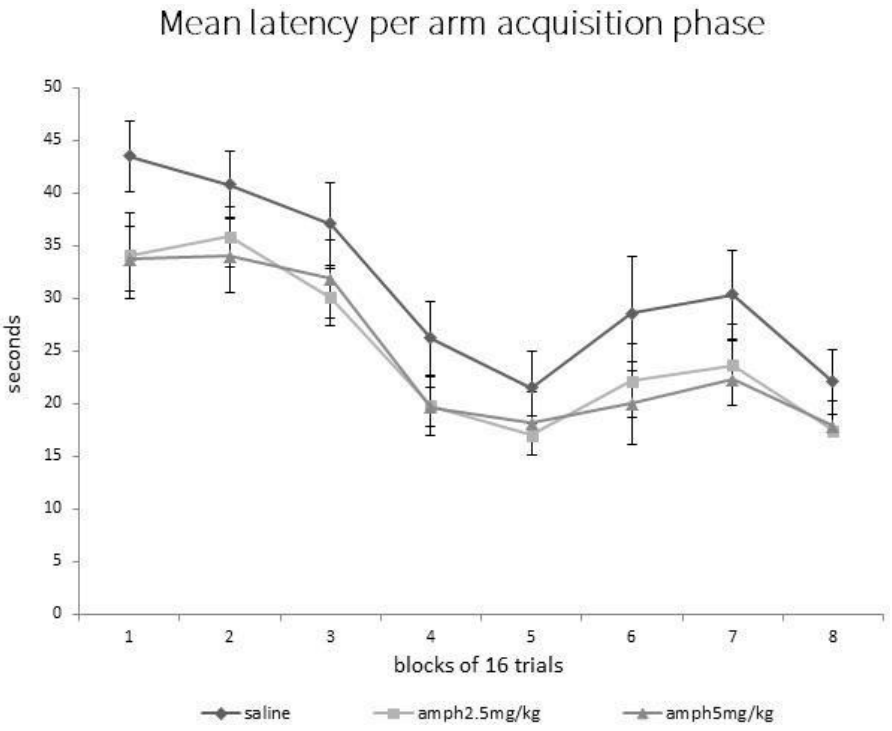


Figure 4.7. Mean (±SEM) latency per arm during acquisition (8 blocks of 16 trials), for saline pre-treated group (n=11), amphetamine 2.5mg/kg pre-treated group (n=13) and amphetamine 5mg/kg pre-treated group (n=13).

In the same analysis for 2 groups Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=95.709$, p<.001. Therefore, Greenhouse-Geisser ε=.534 was applied to correct degrees of freedom and p value. Mixed ANOVA indicated a significant main effect of block, (F(3.740, 130.916)=56.142, p<.001) but there was no significant main effect of group, (F(1, 35)=2.819, p=.102,) and no groups by blocks interaction, (F(3.740, 130.916)=.751, p.551). Therefore, amphetamine had no significant effect on mean latency per arm (see figure 4.8).

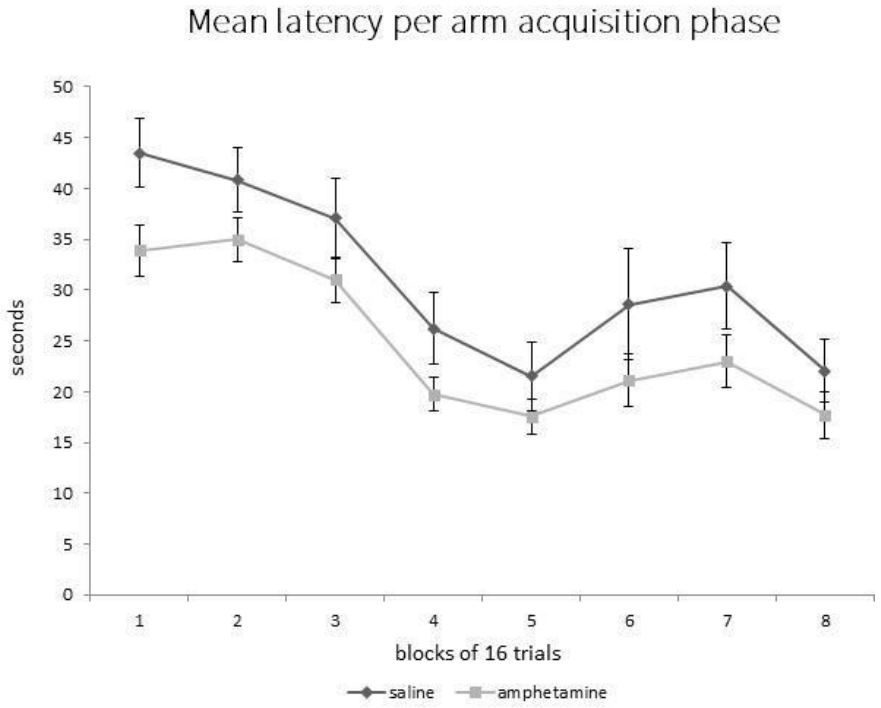


Figure 4.8. Mean (±SEM) latency per arm during the acquisition phase (8 blocks of 16 trials), for saline pre-treated group (n=11), amphetamine pre-treated group (n=26). (N.B. these are the same data as figure 4.7 but now replotted with a single amphetamine group).

4.3.4 Reference memory acquisition: Total errors in single versus adjacent non-baited arms

Mice made more reference memory errors into the single, non-baited arm compared to the average for the adjacent non-baited arms. There were no group differences (see figure 4.9). In particular, mixed ANOVA with 3 groups as a between subject factor and 2 arm types as a within subject factor revealed that errors in the single arm were more than in adjacent arms for all mice, [i.e. the main effect of arm was significant, ($F(1, 34)=20.182$, $p<.001$)]. There were no significant differences between the groups [main effect of group, ($F(2, 34)=.728$, $p=.490$), groups by arms interaction, ($F(2, 34)=2.149$, $p=.132$)]. The findings from the analysis for 2 groups revealed a similar pattern. The main effect of arm was significant ($F(1, 35)=12.760$, $p=.001$), while the main effect of group, ($F(1, 35)=.576$, $p=.453$) and group by arm interaction, ($F(1, 35)=2.099$, $p=.157$) were non-significant.

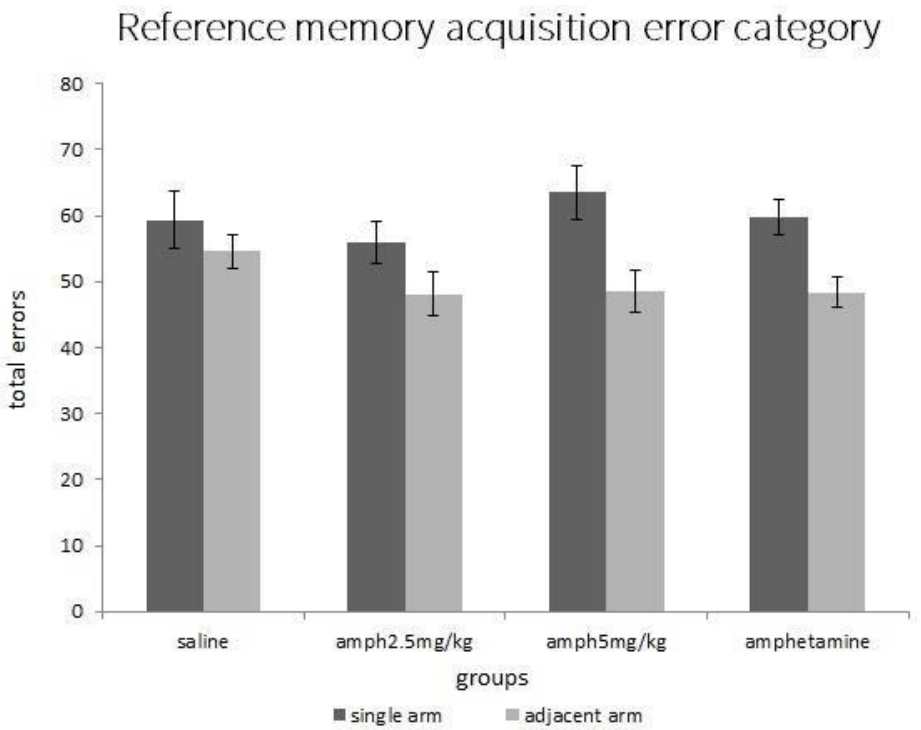


Figure 4.9. Mean of total (±SEM) collapsed across blocks reference memory errors in adjacent (divided by 2 to adjust for 2:1 ratio) versus single arms for saline (n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13), amphetamine combined group (n=26).

4.3.5 Reference memory acquisition: Likelihood of first error to occur in single non-baited arm

The percentage of trials in which the first error was into the single, non-baited arm, was also calculated. ANOVA with 3 groups as a between subject factor and percentage of trials (out of a total of 128 trials) in which the first error was made in the single non-baited arm as the dependent variable, indicated that the likelihood of making the first error in the single arm did not differ between groups, (main effect of group, $F(2, 34)=2.284$, $p=.117$). Likewise, the same result was obtained from an independent sample t-test comparing just the 2 groups (saline vs the amphetamine groups combined, $t(35)=-.986$, $p=.330$ (two-tailed). Results are shown in figure 4.10.

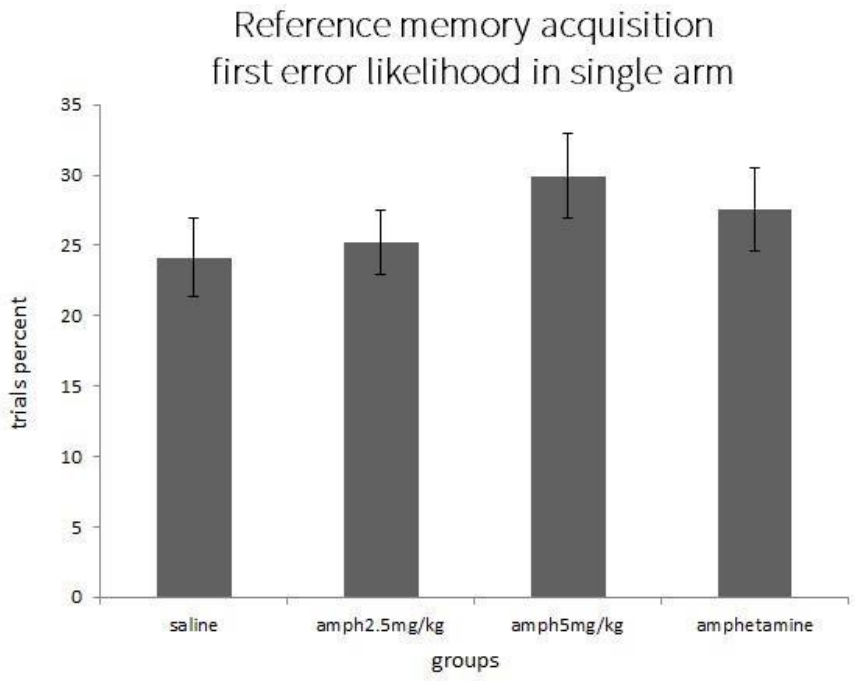


Figure 4.10. Mean percentage (±SEM) (out of a total of 128 trials) of first reference memory errors in the single non-baited arm for saline (n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13), amphetamine groups combined (n=26).

4.3.6 Second phase: Simultaneous assessment of spatial working memory performance and spatial reference memory

Summary of results

Amphetamine treated mice showed a trend for increased reference memory (srm) compared to saline injected animals, although this did not reach statistical significance (see figure.4.11 and figure 4.12). Groups did not differ in the number of spatial working memory errors that were made (see figure 4.15).

4.3.7 Spatial reference memory test

Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=53.637$, p=.002. Therefore, Greenhouse-Geisser ε=.658 was applied to correct degrees of freedom and p value. A mixed ANOVA with 3 groups as a between subject factor and 8 blocks as a within subject factor indicated that spatial reference memory errors for all mice did not differ across blocks [main effect of block, (F(4.607, 156.649)=2.153, p=.067)]. For this analysis, there was also no difference between groups (main effect of group, F(2, 34)=1.528, p=.232), and no groups by blocks interaction (F(9.215, 156.649)=.631, p=.773). See figure 4.11. It should be noted that in the acquisition phase results were organized into 8 blocks of 16 trials. However, in the second phase the total number of trials was only 24 and therefore results were arranged into blocks of 3 trials in order to be consistent with the acquisition phase as far as the total number of blocks is concerned.

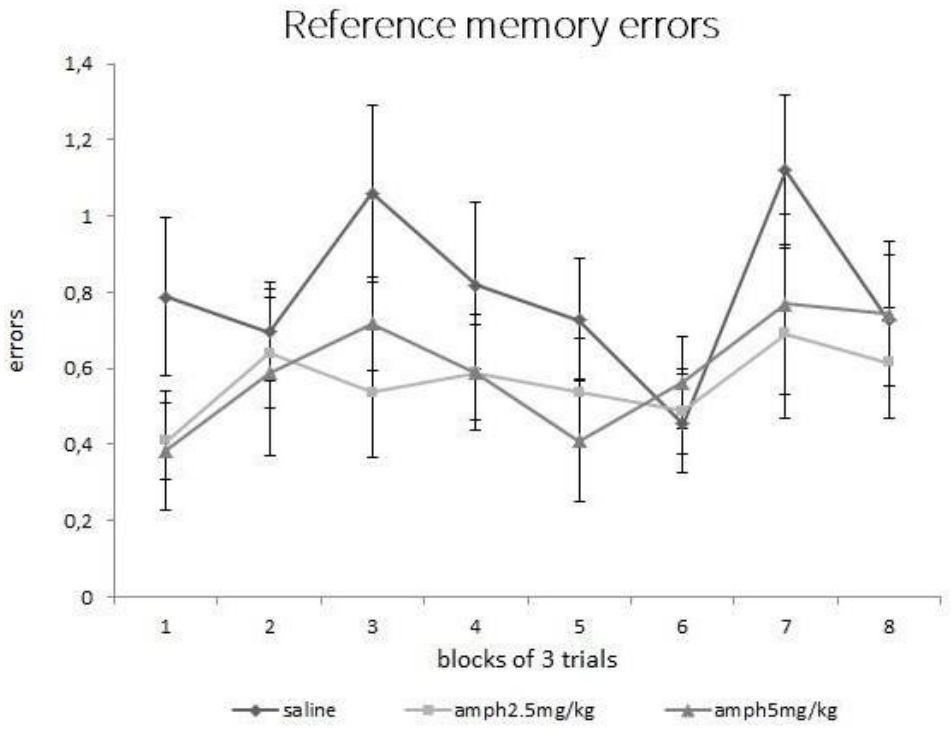


Figure 4.11. Mean (±SEM) reference memory errors for saline, (n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13), across eight 3 trial blocks.

A separate 2-tailed independent t-test (between subject factor: groups), in which the data from the two amphetamine groups were combined, revealed that there was a nearly significant difference between the groups in spatial reference memory, t (35)=1.753, p=.088 (2-tailed), reflecting the trend for increased memory in amphetamine mice.

However, this group effect did not quite reach statistical significance and so should not beover-interpreted (see figure 4.12). Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=55.502$, p=.001.Therefore, Greenhouse-Geisser ε=.659 was applied to correct degrees of freedom and p value. A mixed ANOVA with 2 groups as a between subject factor and 8 blocks as a within subject factor indicated that spatial reference memory errors for all mice differed across blocks [main effect of block, (F(4.614, 161.493)=2.393, p=.044). There was also no difference between groups (main effect of group, F(1, 35)=3.085, p=.088), and no groups by blocks interaction (F(4.614, 161.493)=1.028, p=.400). See figure 4.12.

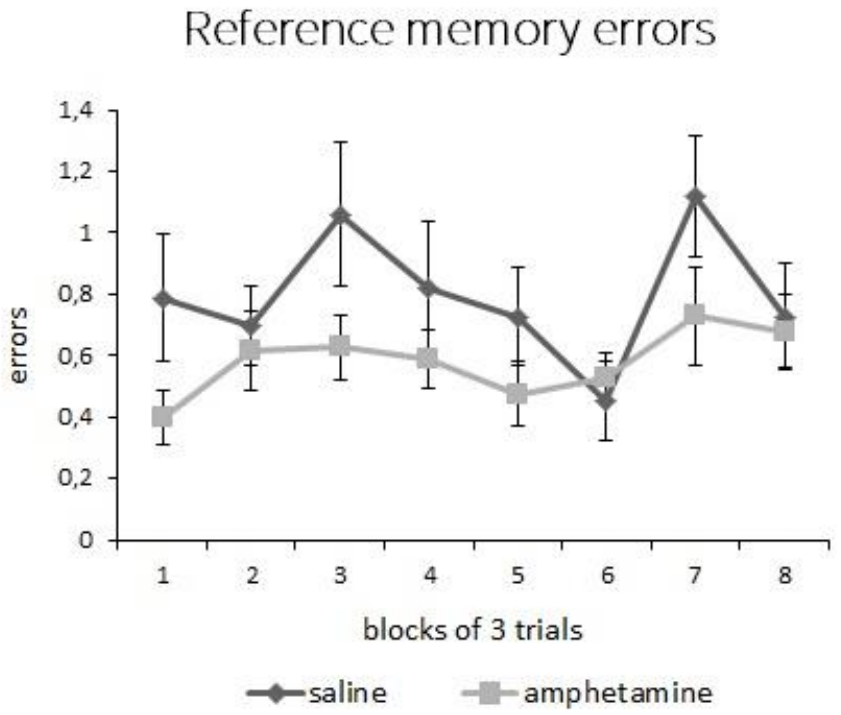


Figure 4.12. Mean (±SEM) spatial reference memory errors across eight 3 trials blocks. Saline (n=11), amphetamine (n=26).

4.3.8 Spatial pattern separation: Reference memory errors in single versus adjacent non-baited arms

To assess spatial pattern separation performance during testing of spatial reference memory, the number of errors into the single and average, adjacent non-baited arms were investigated (correcting for the fact there are two adjacent non-baited arms). Although the mice were more likely to make errors into the single, non-baited arm, there were no reliable differences between the groups.

Mixed ANOVA with the 3 groups as a between subject factor and 2 categories of error types as a within subject factor indicated that errors were more likely to occur in single compared to adjacent arms for all mice [(main effect of error type, $F(1, 34)=4.890$, $p=.034$), (main effect of group, $F(2, 34)=1.066$, $p=.356$), (groups by blocks interaction $F(2, 34)=.591$, $p=.599$)]. Analysis for 2 groups (with the amphetamine groups combined) indicated that

errors in single and adjacent arm show a trend to differ for all mice, which did not reach significance, (main effect of error type, $F(1, 35)=3.316$, $p=.077$). The number of errors did not differ between groups, (main effect of group, $F(1, 35)=2.148$, $p=.152$), and there was no groups by error type interaction, ($F(1, 35)=.410$, $p=.526$). See figure 4.13.

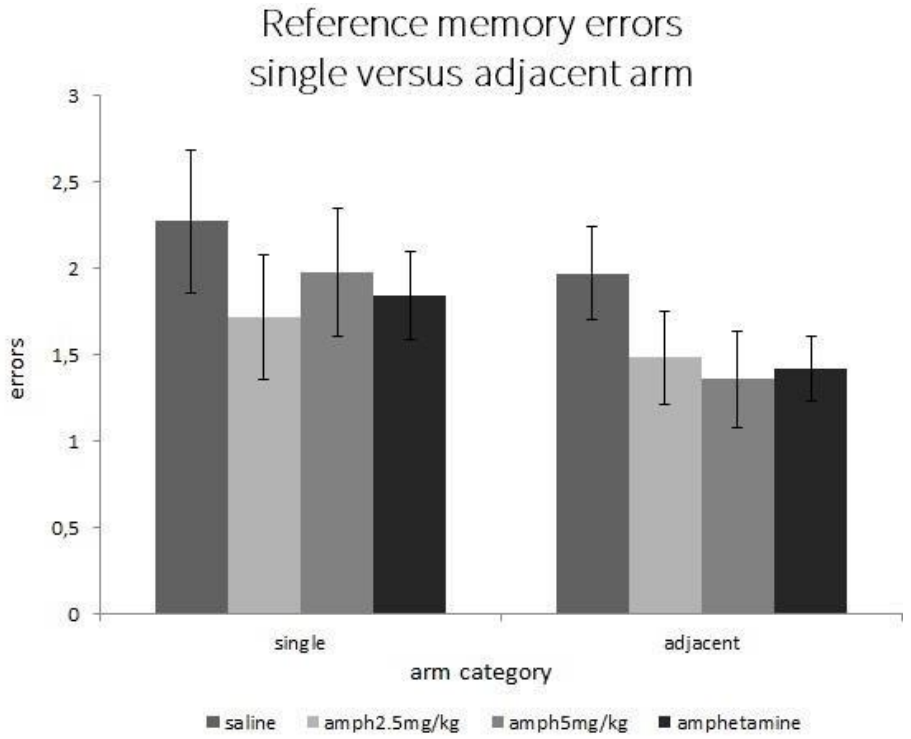


Figure 4.13. Mean (±SEM) reference memory errors in single and adjacent arms (after correcting for the fact there are two adjacent non-baited arms) for saline, (n=11), combined amphetamine group (n=26), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13).

4.3.9 First reference memory error in the single non-baited arm

I also calculated the number of trials on which the first reference memory error was made into the single non-baited arm. One-way ANOVA with 3 groups as a between subject factor and percentage of trials as the dependent variable indicated a trend for groups to differ in the likelihood to make the first reference memory error in the single arm, (main effect of groups, $F(2, 34)=3.199$, $p=.053$), with saline having lower likelihood to make the first

reference memory error in the single arm. A 2-tailed independent t-test also revealed a trend for increased percentage of first reference memory errors in the single arm for the combined amphetamine group compared to saline, t (35)=1.843, p=.074). However, these effects did not reach statistical significance and therefore should not be over-interpreted. See figure 4.14.

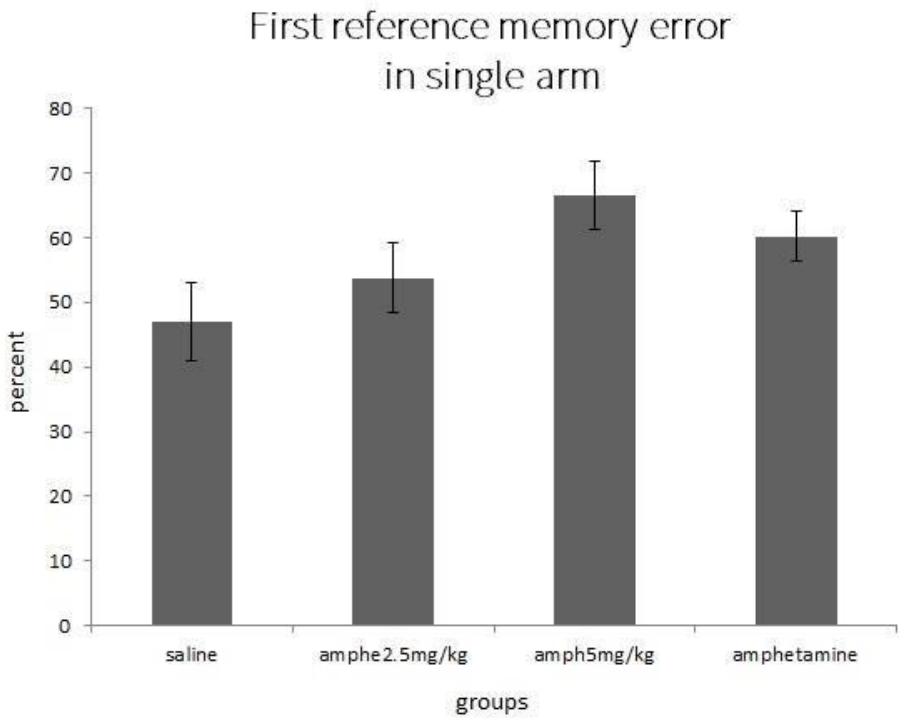


Figure 4.14. Mean percentage (±SEM) of first arm error in the single arm (out of sum of first error in single andadjacent arm divided by 2 to adjust for 2:1 ratio).

4.3.10 Spatial working memory

A mixed ANOVA with 3 groups as between subjects factor and 8 blocks as within subject factor analysis indicated a significant main effect of blocks, F(7, 238)=13.126, p<.001, a non-significant main effect of groups, F(2,34)=.426, p=.656 and a significant group by block interaction, F(14,238)=4.262, p<.001. In the 2 group analysis, the Mauchly test indicated that sphericity was violated, $\chi^2(27)$ =42.859, p=.028. Therefore, Greenhouse-Geisser ε=.711 was applied to correct the degrees of freedom and p value. Results indicated a

significant main effect of block, F(4.977, 174.204)=6.939, p<.001, a non-significant main effect of group, F(1,35)=.859, p=.360 and a significant group by block interaction, F(4.977,174.204)=6.944, p<.001. See figure 4.15

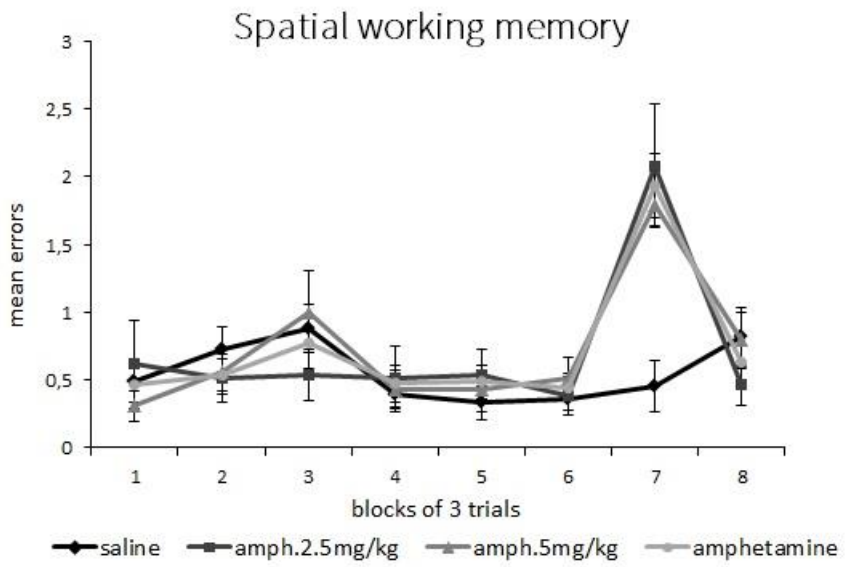


Figure 4.15. Mean (±SEM) number of working memory errors for saline,(n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg, (n=13), and the combined amphetamine group (n=26).

4.3.11 Working memory errors in baited compared to non-baited arms

It is possible for mice to make two kinds of working memory error. They can re-enter a previously baited arm (working memory correct errors; see Jarrard, 1983, 1993, Schmitt et al, 2003) and they can re-enter a never baited arm (working memory incorrect errors). Not surprisingly, mice made more working memory errors into previously baited arms and the pattern was similar for all of the groups (see figure 4.16).

Mixed ANOVA with the 2 types of error as a within subject factor and the 3 groups as a between subject factor indicated that all mice made more errors in previously baited arms, (the main effect of error type was significant, F(1, 34)=88.400, p<.001). However, there was

no difference between groups (no main effect of group, F(2, 34)=.010, p=.990), and no groups by arms interaction (F(2, 34)=.315, p=.732).

Consistent with these results are the findings of the same analysis for 2 groups (with the two amphetamine groups combined). There was a main effect of error type which was significant, F(1, 35)=80.578, p<.001, but no main effect of group, F(1, 35)=.019, p=.890, nor error type by groups interaction, F(1, 35)=.533, p=.470).

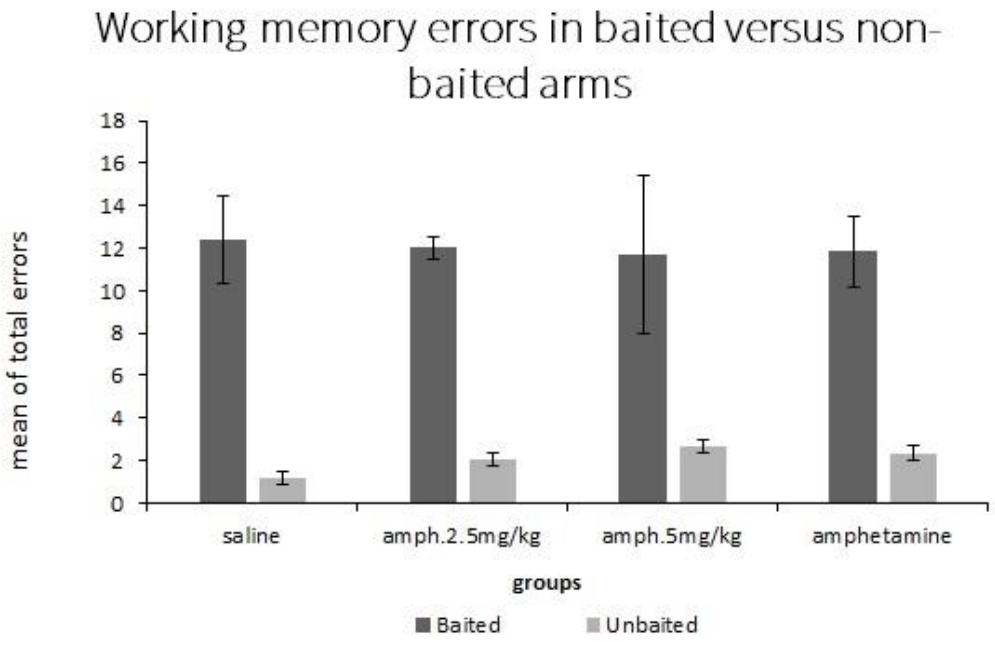


Figure 4.16. Mean of total (±SEM) working memory errors in baited and non-baited arms for saline, (n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13) and the combined amphetamine group (n=26).

4.3.12 Sum of errors in the first 3 choices

As an alternative measure of performance, the total number of errors (reference and working memory errors) made in the first three choices was also examined. Again, there were no differences between groups (see figure 4.17).

A mixed ANOVA with 3 groups as a between subject factor and 8 blocks as a within subject factor revealed that mice did not improve in terms of the total number of errors they

were making in the first 3 choices as training progressed as the number of errors did not differ across blocks (main effect of block, F(7, 34)=1.460, p=.182). Mice of all groups were making errors at the same rate. Both the main effect of group, and groups by blocks interaction were non-significant F's < 1; p's > 0.50.

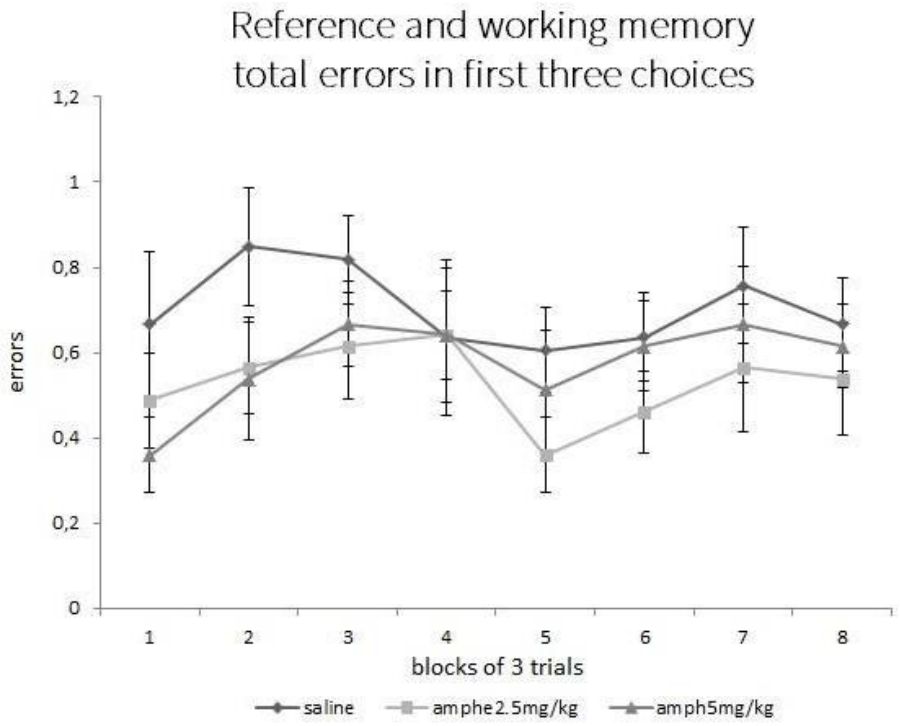


Figure 4.17. Mean total (±SEM) number or errors in the first three choices for saline, (n=11), amphetamine 2.5mg/kg(n=13), and amphetamine 5mg/kg (n=13).

Results from analysis with 2 groups (with the two amphetamine groups combined) were in agreement with the analysis for 3 groups (see figure 4.18). The main effect of block was not significant, (F(7, 35)=1.234, p=.285). Also, the main effect of group, (F(1, 35)=2.809, p=.103) and groups by blocks interaction, (F(7, 35)=.488, p=.843) were not significant.

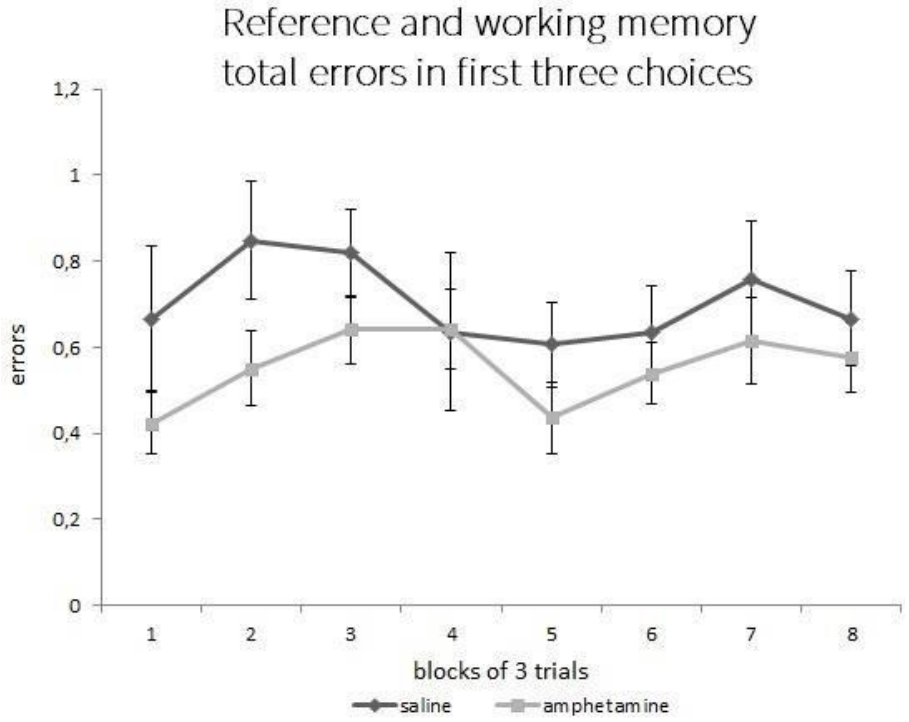


Figure 4.18. Mean total (±SEM) number or errors in the first three choices for saline (n=11), and combined amphetamine group (n=26). (N.B. these are the same data as figure 4.17 but now replotted with a single amphetamine group).

4.3.13 Mean latency per arm

Differences in the mean latency per arm (see methods for definition of difference between latency to complete a trial and mean latency per arm) could impact on their working memory performance, as mean latency per arm will determine the interval between choices. Therefore, mean latency per arm was calculated. Importantly, there were no differences in the mean latency per arm between groups.

Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=51.162$, p=.004. Therefore, Greenhouse-Geisser ε=.726 was applied to correct degrees of freedom and p value. A mixed ANOVA with the 3 groups as a between subject factor and 8 blocks as a within subject factor revealed that mean latency per arm did not change across blocks for all mice, (main effect of block, F(5.080, 172.736)=1.858, p=.103) and that the groups of

mice did not differ in the mean latency per arm, (both the main effect of group, F(2, 34)=.170, p=.845), and groups by blocks interaction were non-significant, (F(14, 34)=1.345, p=.209). See figure 4.19.

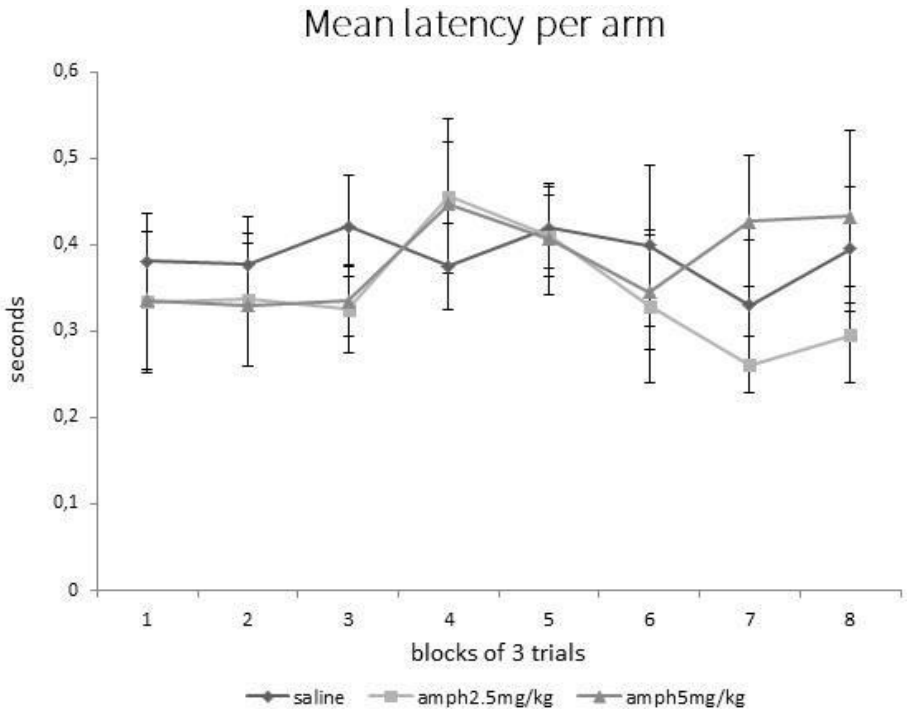


Figure 4.19. Mean (±SEM) latency per arm for choices for saline, (n=11), amphetamine 2.5mg/kg (n=13), and amphetamine 5mg/kg (n=13).

Mauchly's shericity test indicated that sphericity was violated $\chi^2(27)=59.993$, p<.001. Therefore, Greenhouse-Geisser ε=.704 was applied to correct degrees of freedom and p value. Results from analysis with 2 groups (with the two amphetamine groups combined) were in agreement with findings for 3 groups [(main effect of block, F(4.926, 172.410)=1.139, p=.341, main effect of group, (F(1, 35)=.110, p=.742), groups by blocks interaction, (F(4.926, 172.410)=1, p=.399)], in revealing no differences in mean latency per arm. See figure 4.20.

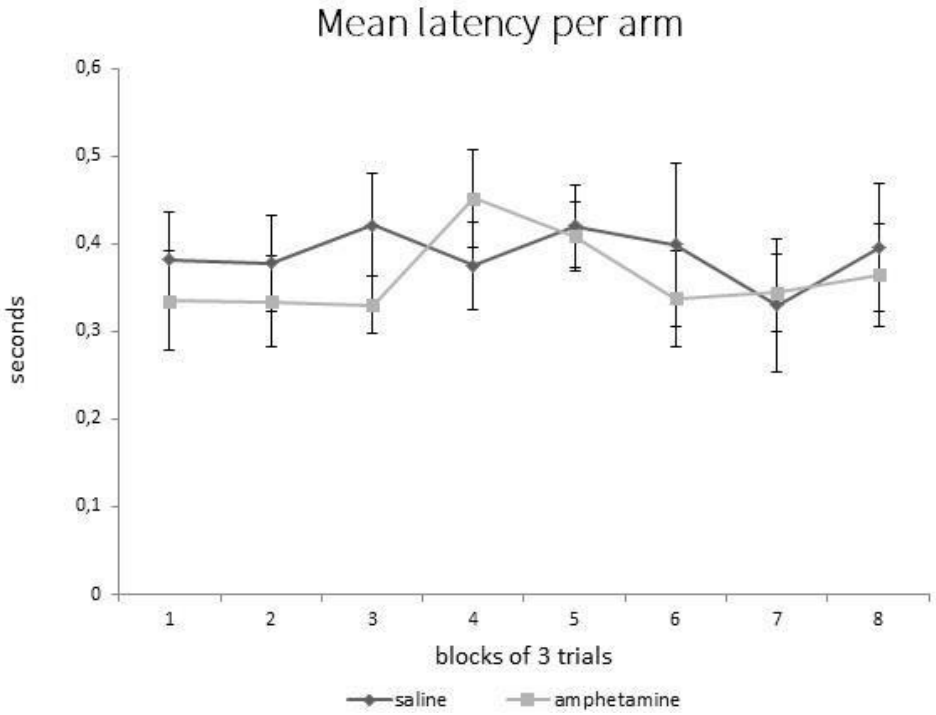


Figure 4.20. Mean (±SEM) latency per arm for choices for saline, (n=11), and combined amphetamine group (n=26). (N.B. these are the same data as figure4.19 but now replotted as a single amphetamine group).

4.3.14 Spatial pattern separation: Working memory errors in single baited versus adjacent baited arms (divided by two)

Mixed ANOVAs with 3 groups as a between subject factor and 2 categories of error types as a within subject factor indicated that errors in the single arm were as likely to occur as into adjacent arms divided by two for all mice (see figure 4.21).

In the three group analysis, there was no significant main effect of error type, ($F(1, 34)=.267$, $p=.609$). Performance did not differ between groups, (main effect of group, $F(2, 34)=.061$, $p=.941$). Also, there were no groups by error types interaction ($F(2,34)=.184$, $p=.833$). The same pattern of results was obtained when the two amphetamine groups were combined into a single group (no main effect of error type, group or group by error type interaction; all $F's < 1$; $p's > 0.50$).

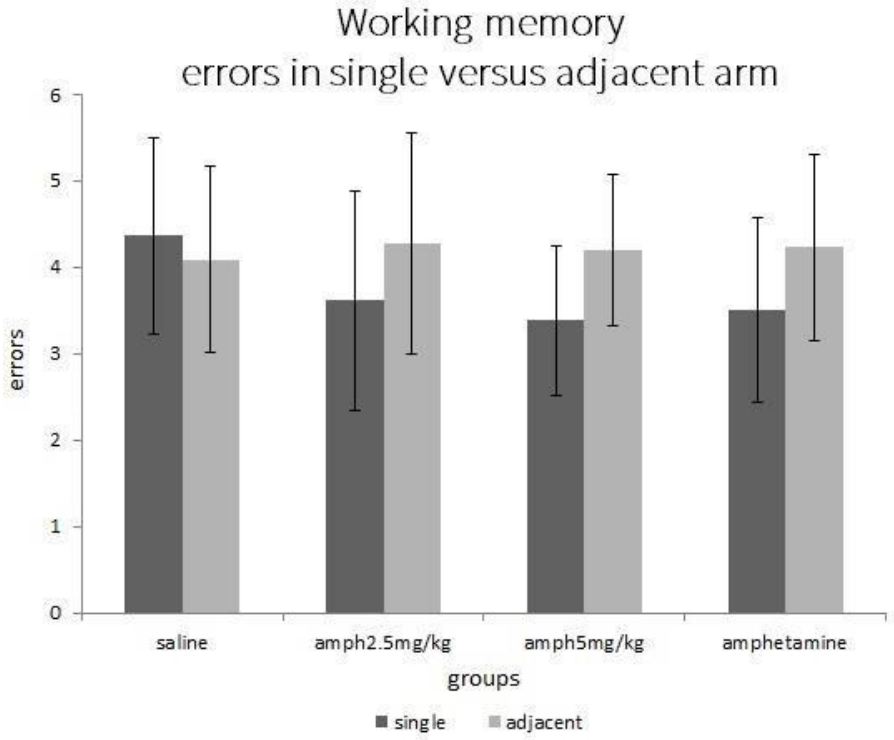


Figure 4.21. Mean working memory errors into single and adjacent (divided by 2 to adjust for 2:1 ratio) arms for saline, (n=11), amphetamine 2.5mg/kg (n=13), amphetamine 5mg/kg (n=13) and amphetamine combined group (n=26).

4.4 Discussion

Most previous experiments have indicated that withdrawal from amphetamine sensitization facilitates appetitively driven learning (Harmer and Philips, 1998, Taylor and Jentsch, 2001, Ito and Canseliet, 2010). On the other hand, findings on the effects of amphetamine sensitization on spatial working memory have given mixed results. The present study was the first study that examined the effects of withdrawal from amphetamine sensitization on incrementally acquired spatial reference and working memory on the radial maze. The present findings do not show facilitation of spatial reference memory acquisition for rewarding stimuli in the radial maze. A marginally significant increased retrieval of spatial reference memory was found for amphetamine sensitized mice in the testing phase. This however did not reach significance. Spatial working memory was not impaired in

amphetamine sensitized mice. Finally, this was the first study that tested the effects of withdrawal from amphetamine sensitization on pattern separation. Collectively, results do not support the hypothesis that withdrawal from amphetamine sensitization impairs spatial pattern separation.

4.4.1 Amphetamine withdrawal does not affect appetitive spatial reference memory

Our first result that withdrawal from amphetamine sensitization does not facilitate incrementally acquired spatial reference memory does not support our initial hypothesis. This hypothesis was based on the fact that amphetamine exposure maladaptively facilitates incentive salience attribution to reward predicting stimuli (Wyvell and Berridge, 2001, Tindell et al, 2005). The current finding is consistent with the study of Eldred and Palmiter (2013). This study showed that Pavlovian conditioning, which constitutes another form of appetitively driven learning, was unaffected by amphetamine sensitization. However, it is not consistent with other studies showing facilitated appetitively driven learning (Harmer and Philips, 1998, Taylor and Jentsch, 2001). Differences between drug protocols used and differences between tasks that were used might have contributed to this discrepancy. It should be noted that the discrepancy of findings between studies might be due to the use of different species. This is because in the above studies that rats were used, enhanced appetitive driven learning was found (Taylor and Jentsch, 2001, Harmer and Philips, 1998). On the other hand, mice were used in the study that found unaffected appetitive driven learning (Eldred and Palmiter, 2013). Further research is required on the effects of amphetamine sensitization on other forms of appetitivelydriven long-term memory.

Also, with regards to our finding of a non-significant trend for amphetamine sensitized mice to have facilitated spatial reference memory performance in the testing phase

further research is required with more trials in the testing phase to investigate further this potential finding. However, these findings are in agreement with the findings from the Ito and Canseliet (2010) study which provided evidence that contextual memory is not facilitated by withdrawal from amphetamine sensitization when contextual and cue learning for rewarding stimuli take place simultaneously but without competing with each other, but is facilitated when these forms of learning are in direct competition. According to post hoc power analysis our sample size was large enough (see section 2.1.1).

Collectively, our findings from the radial maze experiment presented in this chapter combined with the Ito and Canseliet (2010) findings in the T-maze, which showed unaffected spatial memory, show that hippocampus dependent appetitive spatial learning and memory is not enhanced per se by amphetamine sensitization. It seems that it is only under conditions in which basolateral amygdala and hippocampus compete for control over appetitive behaviour that spatial conditioning is enhanced. Thus, in the conditioned cue/place preference test by Ito and Canseliet (2010) the hippocampus dependent place conditioning is enhanced.

4.4.2 Amphetamine withdrawal reduces latency to acquire all rewards for the combined amphetamine group

Results on latency to acquire all rewards were not significantly different between the three groups. Thus, they were in line with results on spatial reference memory. However, in the two group comparison amphetamine mice seemed to have reduced latencies to acquire all 3 rewards. Neither reference memory nor mean latency per arm which reflects locomotor activity and/or motivation, both of which affect latency to acquire all rewards were different between groups. One possibility is that the difference between groups in the latency to acquire all rewards might indicate increased incentive salience of non-spatial Pavlovian arm cues. Such an explanation is in line with incentive salience being attributed to cues paired

with reward which leads to them triggering Pavlovian approach. Such an effect is mediated by the mesolimbic dopaminergic pathway that is well known to be enhanced in mice that have withdrawn from repeated amphetamine (Kalivas and Stewart, 1991, Henry and White, 1992) and is a result of hippocampal overdrive (Robinson and Berridge, 1993, 2001). Further research is required to elucidate these findings.

4.4.3 Amphetamine withdrawal does not affect spatial working memory

Our second finding that withdrawal from amphetamine sensitization does not impair spatial working memory is not in line with findings from the study of Lee et al (2011). This study showed a spatial working memory impairment in the continuous spontaneous alternation Y maze paradigm, accompanied by reduced NMDAR ligand binding in CA1, CA2 and CA3 hippocampal subfields, and in prefrontal cortex (Lee et al, 2011). However, findings from that study could be due to effects on stereotypical behaviours rather than a spatial working memory impairment. Also, our findings are not in line with other findings (Blokland et al, 1998, Nagai et al, 2007, Beatty and Rush, 1983). These studies showed compromised working memory. However, as explained earlier in all those studies results seemed to be caused by other factors and not by reduced retention of information in spatial working memory. Such factors include impaired performance in terms of altered locomotion or appetite.

Our results are in line with other studies showing unaffected spatial working memory performance following amphetamine sensitization (Featherstone et al, 2008) when testing started 10 days following withdrawal (Stefani and Moghaddam, 2002). However, no locomotor sensitization test was performed in the latter study to confirm sensitization. Also, phencyclidine was administered to all mice following withdrawal from the amphetamine

regimen, before behavioural testing. This fact makes interpretation of results more complex and comparison of studies difficult. More studies should be conducted on the effects of withdrawal from amphetamine sensitization on spatial working memory on the radial maze. According to post hoc power analysis our sample size was large enough (see section 2.1.1).

There is evidence that medial prefrontal cortex in rodents mediates working memory (Kolb et al, 1974, Delatour and Gisquet-Verrier, 1996). It should be noted though that some lesion studies do not support the idea that the integrity of medial prefrontal cortex in rodents is required for working memory performance and the temporary on-line storage of information (Aggleton et al, 1995; Deacon et al, 2003, Gisquet-Verrier and Delatour 2006, Touzani et al, 2007) but only for the control of information required for the arrangement of an ongoing action, i.e. dealing with an interfering event or unexpected delay (Gisquet-Verrier and Delatour, 2006, Touzani et al, 2007).

Delatour and Gisquet-Verrier, (2000) indicated that medial prefrontal cortex is not involved in spatial working memory. Instead, it is involved in attentional processes and behavioural flexibility that is required for information processing in spatial working memory tasks. It should also be noted that the spatial working memory component in the radial maze experiment that is used in this chapter could instead reflect non-associative short-term habituation processes rather than spatial working memory, i.e. mice tend to prefer arms that are more novel compared to familiar ones, i.e. arms that were recently visited (for review Sanderson et al, 2010, Sanderson and Bannerman, 2012). It should also be noted that spatial working memory deficits in mice tested by other win shift spatial working memory tests, could also reflect short-term habituation deficits (Sanderson et al, 2010, Sanderson and Bannerman, 2012).

In light of the above, spared spatial working memory in this task might conceivably reflect that short-term habituation processes are not affected by withdrawal from an

amphetamine sensitizing regimen. This finding indicates that withdrawal from repeated amphetamine may not model short term habituation deficits that are a potential hallmark of schizophrenia and drug addiction, and which seem to be involved in aberrant salience attribution (for review Barkus et al, 2014). This is because a short term habituation impairment could lead to an increased amount of attention paid to recently encountered information which results in stimuli that have already been presented to be perceived as novel and salient and to grab a disproportional amounts of attention.

4.4.4 Amphetamine withdrawal does not affect spatial pattern separation

Finally, our last result that amphetamine sensitization does not impair spatial pattern separation does not support our initial hypothesis. This is the first study on the effects of amphetamine sensitization on spatial pattern separation in the radial maze. Therefore, further research should be done to verify these findings. The same tests should be conducted with larger sample sizes. Additionally, other experiments should be conducted assessing pattern separation differently i.e. under conditions in which the dissociation between similar memory traces is more difficult, and/or tests that use different protocols than the one we used. In this respect, different tests can be used such as those in which objects are used (Bekinschtein et al, 2013, van Goethem et al, 2018), or touch screen based tasks (Oomen et al, 2013). Overall, our results do not support that withdrawal from repeated amphetamine can be used as a model of impaired pattern separation deficit in schizophrenia (Tamminga et al, 2010, 2012, Chen et al, 2012, Das et al, 2014, Martinelli and Shergill, 2015).

Conclusions

Overall, results show that amphetamine sensitization does not impair spatial working memory or spatial pattern separation. Thus, they do not support the use of withdrawal from amphetamine sensitization as a model for cognitive impairment in schizophrenia. Also, our finding that spatial reference memory was not different between groups does not support the use of amphetamine sensitization as a model for the maladaptively facilitated formation of associations which is a hallmark of both schizophrenia and drug addiction.

Chapter 5: Amphetamine Sensitization Effects on Spontaneous Behaviours

5.1 Introduction

5.1.1 Amphetamine sensitization and preference for novelty

Changes in responses to novelty and salience processing in schizophrenia and drug addiction are well known (Kapur, 2003, Kapur and Mizrahi, 2005, Martinelli et al, 2018, Weiss et al, 2004). There is an intrinsic tendency for species to pay attention to novel and salient stimuli, due to the potentially biologically significant properties of these stimuli. Novelty detection facilitates behavioural adaptation. It involves attribution of salience and attention to novel stimuli in the environment. Indeed, novel stimuli generate vigor for approach and exploration in the absence of reward (Koster et al, 2016). Generally, salient and arousing events, including appetitive, aversive, high intensity, and novel stimuli, induce increased dopaminergic activity in the mesocorticolimbic striatal dopaminergic circuit (for review, Horvitz, 2000). It is known that the dopaminergic activity in the mesocorticolimbic and striatal pathways that mediate novelty detection mediate also amphetamine sensitization.

In particular, exposure to novelty (De Leonibus et al, 2006) and novelty detection (Rangel-Gomez et al, 2013, Bunzeck et al, 2014) are mediated through dopaminergic signaling. Preference for novelty is mediated through striatal activity (Wittmann et al, 2008). It is also known that forebrain dopamine depletion has a detrimental impact on novelty preference (Pierce et al, 1996).

In addition, other key brain regions, that are well known to mediate the effects of amphetamine sensitization, appear to be implicated in novelty detection including the

hippocampus (Honey et al, 1998, Lisman, Otmakhova, 2001). Accordingly, increased hippocampal dopaminergic activity which facilitates LTP in the hippocampal CA1 subfield has been demonstrated following exposure to spatial novelty (Li et al, 2003). More recent studies have also implicated the involvement of hippocampus in the processing of novelty (Murty et al, 2013, Howard et al, 2011, Kumaran and Maguire, 2009). The hippocampus, which has long been thought to play a role in contextual processing (Hirsh, 1974, 1978, Good and Honey, 1991, 1997, Honey and Good, 1993, Phillips and LeDoux, 1992) also seems to be implicated in the effects of amphetamine sensitization which is evident by the fact that psychostimulant sensitization appears to be context specific (Anagnostaras et al, 2002, Crombag et al, 2001, Ostrander et al, 2003). Also, Beveridge et al (2006), provided evidence that repeated cocaine exposure decreases metabolism in the hippocampus among other regions (for review Castner and Williams, 2007). Therefore, the effects of amphetamine sensitization on cognitive functions that depend on the hippocampus such as novelty processing is of interest and requires to be studied.

Impaired novelty processing might lead to attentional deficits in schizophrenia. Indeed, aberrant regulation of attention and salience attribution to internal and external stimuli is thought to be a hallmark of schizophrenia (Kapur, 2003). One possibility could be that abnormal dopamine responses and impaired novelty processing could be a consequence of deficits in non-associative, stimulus specific, short-term habituation (for review Sanderson and Bannerman, 2012, Barkus et al, 2014). Long-term exposure to methamphetamine has been shown to impair novelty salience and object recognition in rodents (Belcher et al, 2005, Reichel et al, 2011, Ito et al, 2007, Arai et al, 2009, Noda et al, 2010, Lu et al, 2010) which is restored by perirhinal cortex mglu5 receptor activation (Peters et al, 2016), which is known to be downregulated (Reichel et al, 2011). Repeated cocaine exposure has also been shown to impair object recognition (Briand et al, 2008). There is however evidence that abstinence

from a repeated amphetamine regimen has no effect on novel object recognition (Che et al, 2013). However, in that study there was no test to verify whether rodents were sensitized. Behavioural sensitization induced by repeated exposure to ketamine and expressed by acute amphetamine challenge differentially affects novelty processing dependent on the dose used (Schumacher et al, 2016). The effects of withdrawal from an amphetamine sensitizing regimen on behaviours which involve a temporary reduction of the attention paid to recently presented stimuli are not very clear. Indeed, findings from rodent studies on its effects on object recognition have given mixed results (Peleg-Raibstein et al, 2009, Janetsian et al, 2015a, Kamei et al, 2006, Belcher et al, 2006, 2008, Lee et al, 2011, Tennet al, 2003). The effects of amphetamine sensitization on novelty detection in other experimental settings and with other stimulus types (i.e. non-objects) have not been studied.

5.1.2 Does amphetamine sensitization induce social withdrawal and anxiety?

Whether withdrawal from an amphetamine sensitizing regimen mimics negative symptoms of schizophrenia (i.e. blunting of affect, anhedonia, reduced social drive, loss of motivation, lack of social interest, and inattention to social or cognitive input), is also not clear. Drug withdrawal is known to activate the body's hedonic homeostatic mechanisms, with a consequence that the previously hedonic effects of the drug are countered (Koob and Moal, 1997). Therefore, withdrawal from psychostimulants has been associated with anxiety and depressive like states (for review, Barr and Markou, 2005, 2002b, Kitanaka et al, 2008). More specifically, anxiety, anhedonia, depression and social isolation constitute the main characteristics of a broader dysphoric state that is observed in early amphetamine abstinence (Koob and Moal, 1997, Lago and Kosten, 1994).

Such a syndrome is consistent with findings of withdrawal from an escalating amphetamine regimen leading to suppression of adrenocorticotrophic hormone and corticosterone, a phenomenon that lasted for one month (Russig et al, 2006). In line with this, withdrawal from a fixed dose, repeated amphetamine regimen led to a reduction of the release of plasma adrenocorticotrophic hormone in rats after exposure to restraint stress (Swerdlow et al, 1991). This finding indicates that amphetamine withdrawal alters the hypothalamic-pituitary-adrenal axis response to stress. All these findings provide support for the hypothesis that neurochemical mechanisms that regulate emotionality are impaired by amphetamine withdrawal. This is in agreement with findings by London et al (2004) that in recent abstinent methamphetamine abusers there are metabolic abnormalities in brain regions implicated in mood disorders (i.e. anterior cingulate, insula, amygdala) which covaried either positively or negatively for different brain regions with self-reports of depression and anxiety. The literature is further extended by human studies showing that anxiety and depression follow methamphetamine withdrawal and are progressively resolved within 2 weeks (Mancino et al, 2011, McGregor et al, 2005).

Anhedonia studies

Anhedonia is considered to be a loss of the capacity to feel pleasure and is a hallmark of the negative symptoms of schizophrenia. There is evidence that during the first stage of withdrawal from psychostimulant drugs depressive symptoms have been found in humans. These have been modelled in animals and seem to be reversed by antidepressant (Barr et al, 2002b). In rodent studies, insights into anhedonia can be obtained through tests that measure responding to rewarding stimuli. It seems that withdrawal from a repeated d-amphetamine regimen reduces responding for natural rewards as measured by a progressive ratio schedule of reinforcement (Barr and Phillips, 1999). Also, evidence has been provided that early

amphetamine withdrawal is associated with reduced intracranial self-stimulation, indicating a decrease in the reward value of the stimulation and thus reflects a drug-induced anhedonic state (Leith and Barrett, 1980, Kokkinidis et al, 1986, Wise and Munn 1995). Lin et al (1999) provided evidence that this effect is proportional to the duration and dose of pre-withdrawal amphetamine administration. This effect was reversed by administration of repeated electroconvulsive shocks (Barr et al, 2002c) and diminished by fluoxetine, a selective serotonin reuptake inhibitor, an effect which was increased by co-administration of p-MPPI, a serotonin-1A receptor antagonist (Harrison et al, 2001). Reduced intracranial self-stimulation after amphetamine withdrawal was also mitigated by the tricyclic antidepressants imipramine and amitriptyline (Kokkinidis et al, 1980). These findings may support the use of early amphetamine withdrawal as a model of drug-induced anhedonia.

Psychomotor/behavioural depression studies

Withdrawal from chronic amphetamine treatment has also been shown to increase the duration of immobility in a forced-swim test (Kokkinidis et al, 1986, Che et al, 2013, Valvassori et al, 2019), which is thought to model behavioural despair and loss of motivational behaviour which is a key negative symptom of schizophrenia. In the early phase of withdrawal from a non-neurotoxic sensitizing amphetamine regimen, but not in the later phase, psychomotor depression was observed in rodents while the enhanced locomotor response to amphetamine challenge had not yet been evident. Psychomotor depression was evident as a reduction of spontaneous nocturnal locomotor activity (Paulson et al, 1991). This was considered analogous to a "distress syndrome" seen in human amphetamine withdrawal, which is associated with depressive like symptoms. Importantly it differs from persistent behavioural sensitization, which is analogous to the long-lasting hypersensitivity to the effects of amphetamine. In non-human primates persistent behavioural depression, shown by

some animals for up to 20 months after withdrawal from repeated amphetamine regimen. Such an effect could also be considered analogous to the psychomotor poverty syndrome, a key negative symptom of schizophrenia (Castner and Goldman-Rakic, 1999). Further research is needed to clarify whether behavioural depression exists after the early post-amphetamine withdrawal phase.

Anxiety studies

Increased anxiety has also been found in methamphetamine addicts that are abstinent for 7 days (Su et al, 2017). Consistent with this human study are several rodent studies. Barr et al (2010) provided evidence that withdrawal from chronic amphetamine in rats induced anxiety, both at early and late stages of withdrawal. After one month of withdrawal, but not earlier, there was a reduction of survival of newly generated neurons in the dentate gyrus. It was suggested that this may contribute to the maintenance of anxiety-like behaviour at later post-withdrawal time points. In line with this finding, it has been shown that withdrawal from repeated amphetamine induced anxiety in rodents, as indicated in an elevated plus maze experiment (Vuong et al, 2009, Reinbold et al, 2014, Valvassori et al, 2019). Barr et al (2002a) provided evidence of a sensitized hypothalamic pituitary adrenal (HPA) axis response to 30 minutes restraint stress in rats which had withdrawn from a sensitizing regimen of amphetamine for two weeks. Consistent with this, is the finding that amphetamine withdrawal potentiates stress-induced corticosterone in the ventral hippocampus.

Such an effect plays a role in the enhanced sensitivity to stress following withdrawal from amphetamine sensitization (Bray et al, 2016), although no sensitization test was conducted in these studies. Che et al (2013) found anxiety in rodents in the open field test after withdrawal from a repeated amphetamine regimen. A more recent study indicated that abstinence from a single amphetamine injection also induced anxiety in rats (Rincon-Cortes

et al, 2018). Peleg-Raibstein et al (2009), on the other hand showed that there was no anxiety in the open field test in rats that had withdrawn from a sensitizing, escalating amphetamine regimen.

Increased anxiety is known to be a symptom of both schizophrenia and drug addiction. Assessment of anxiety as well as exploratory behaviours in amphetamine sensitized mice is important, not only to assess whether it mimics anxiety in schizophrenia and drug addiction, but also because such assessment is important for the interpretation of findings of other experiments conducted in these mice such as the social interaction experiment. This is because anxiety might interfere with their social interaction (Bannerman et al, 2002b, Lukas and Wöhr, 2015) in tests of spontaneous exploratory behaviour.

Social behaviour studies

Social withdrawal is a core negative symptom of schizophrenia. Also, impairments in social cognition, shown by impaired emotion recognition and understanding of the social context is also a hallmark of schizophrenia. These symptoms respond poorly to antipsychotic medication (Swartz et al, 2007, Keefe et al, 2007) and are well known to lead to chronic functional disability. In this respect, further research is needed in the study of social deficits in schizophrenia. The mesolimbic dopaminergic system is known to mediate interplay between drugs of abuse and social behaviour (for review Young et al, 2011). This is in line with evidence of alterations in oxytocin, which is a social neuropeptide, accompanying social interaction deficits in rodents after repeated methylenedioxymethamphetamine (MDMA) exposure (van Nieuwenhuijzen et al, 2010) and impairment in pair bonding in rodents that were sensitized to psychostimulants (Gobrogge et al, 2009, Liu et al, 2010, for review Young et al, 2011).

Also, it is in line with evidence of reduced social interaction in rats, whose mesolimbic dopaminergic system was electrically sensitized (Glenthøj et al, 1993). All the above findings suggest that there might be a strong link between sensitization of the mesolimbic dopaminergic system and impairment of social behaviour in schizophrenia. In this respect, research on the effects of amphetamine sensitization on social behaviour in rodents might provide insights into whether this can be used as a model to study social impairment in schizophrenia.

The social interaction test is the most popular test to assess social impairments in rodents. Like humans, rodents are social species and social interaction is therefore rewarding for them. On the other hand, social interaction can be aversive for some rodents (it is often accompanied by aggression and fighting) and therefore is also anxiogenic. Thus, tests that assess social interaction can generate a classic approach/avoidance conflict, conflict between social affiliation/exploration and fear of potential social threat (Kirlic et al, 2017). The hippocampus, is well known to be implicated in the effects of amphetamine sensitization, and also plays a key role in classic approach/avoidance conflict (Gray and McNaughton, 2000, Bannerman et al, 2004, for review Ito and Lee, 2016).

Studies on the effects of amphetamine on social behaviour are very limited and with mixed results (for review Gururajan et al, 2010). Chronic exposure to an escalating amphetamine regimen reduced social interaction and increased aggressive behaviour, only in subordinate hooded rats of a social colony, which became hypervigilant (defensively rearing or boxing or fleeing if the other animal was approaching) and defensive (they withdrew to locations that permitted easy defence) during the period of d-amphetamine administration. This model was therefore proposed as a model to study paranoid symptoms in schizophrenia. However only two pairs of rodents were administered with repeated amphetamine and thus the sample size was small (Gambill and Kornetsky, 1976). Therefore, further research is

needed. In line with this finding, Ellison et al (1978) found social withdrawal 4 days after the onset of an intoxicating, repeated amphetamine regimen (48 mg over 10 days). Thus, as in the previous study, the animals were under the effect of the drug at the time of testing. The social withdrawal effect was followed by an increase in aggressive behaviour.

With regards to acute amphetamine exposure it seems to reduce social behaviours, some of which (side threats, mounting, crawling under) are reversed by the antipsychotic drug haloperidol but not clozapine (Steinpreis et al, 1994), and to suppress play fighting (Beatty et al, 1984). On the other hand, other studies report no changes in social interaction, after both repeated and acute amphetamine exposure (Sams-Dodd, 1995), or even enhanced social interaction after acute amphetamine exposure (Guy and Garder, 1985). In all the studies mentioned above, which provided mixed results on the effects of amphetamine on social interaction, the animals were under the effect of amphetamine at the time of testing.

On the other hand, with regards to the effects of amphetamine withdrawal on social interaction it has been found that withdrawal from chronic amphetamine in rats induced anhedonia as found by a test that assessed the motivated response for sucrose reward (Deravakian and Markou, 2014), but does not induce reduced social interaction in the reciprocal social interaction test. However, no locomotor sensitization test was performed in that study to verify that the animals were indeed sensitized. Therefore, further research is required to elucidate whether withdrawal from amphetamine sensitization impairs social interaction in rodents. In line with this, another study found no differences in reciprocal social interaction 24 h or 30 days following the induction of amphetamine sensitization, while acute injection in non-sensitized animals impaired social interaction and induced avoidance (Janetsian et al, 2015b). Overall, research on the effects of withdrawal from amphetamine sensitization in rodents is very limited.

While the reciprocal social interaction test measures the free interaction of pairs of mice, the sociability test measures the propensity of a mouse to spend time with/close by another mouse (kept isolated in a small chamber), as compared to time spent alone in an identical but empty chamber, and thus it is potentially less anxiogenic than the reciprocal social interaction test (Moy et al, 2004). The social recognition test is based on the innate preference of the mouse to explore novel stimuli (including novel social stimuli) and measures time spent with a familiar versus time spent with a novel conspecific within the same test session and arena.

Reduced sociability and social recognition were found in rodents exposed to a sensitizing methamphetamine regimen. The rodents withdrew from the sensitizing regimen of methamphetamine for 10 days and were then exposed daily for three days to a low dose of ketamine regimen. 24 hours later they underwent a sociability and social recognition test, exhibiting reduced performance on both measures (Liao et al, 2018). A more recent study indicated that abstinence from acute amphetamine injection reduced social approach in the sociability test (Rincon-Cortes et al, 2018). While both the first and second study mentioned above report impaired sociability, in the first study ketamine was also administered while in the second one, an acute and not repeated amphetamine regimen was administered. It would be of interest therefore to explore whether withdrawal from amphetamine sensitization, which is a putative model of certain aspects of schizophrenia, provides similar results.

5.1.3 Description of tests that were selected and on what basis they were chosen

The exploration of novel stimuli and contexts is an intrinsic characteristic of rodents (Hugh, 2007). The tests used in this thesis are based on this innate tendency of rodents to prefer novelty and to reduce attention to stimuli that have become familiar as a result of

habituation. Since these tests measure spontaneous behaviours they have high ecological validity. Therefore, there is no need to induce strong motivations either positive or negative with appetitive or aversive training stimuli. Thus, these tests may induce less stress, than in other memory tests (e.g. fear conditioning and Morris water maze). Also, they do not involve prolonged training and can be done in sequence in the same cohorts of animals.

Amphetamine sensitized mice may have compromised performance in these tests, either due to the fact that these mice may have short term recognition memory impairments (if amphetamine sensitization models cognitive impairment in schizophrenia), or due to the fact that novel stimuli are potentially less rewarding for these mice, for example if they have anhedonia (given that there is at least some evidence that amphetamine sensitization models negative symptoms of schizophrenia), or both. Therefore, there are two separate reasons for hypothesizing compromised performance in these tests. These tests were the last tests that were conducted in the cohort of mice that were used throughout the present thesis. Results from previous experiments in this cohort of animals did not show learning and memory impairments (i.e. radial maze reference and working memory, T-maze experiment cue learning). However, recognition tests based on novelty detection may measure a different type (or types) of memory.

A series of tests assessing recognition memory based on novelty preference were selected (novel object recognition, object in place, social recognition), in order to screen the mice more thoroughly, given that each test measures a different aspect of recognition memory, each of them engaging potentially different neural circuitry.

A battery of behavioural tests was used:

Recognition memory

These tests include novel object recognition memory, object in place memory and social recognition memory.

Anxiety

Tests were used that measure spontaneous behaviours driven by preference for novelty and exploration, but which also generate anxiety levels given the approach-avoidance conflict that arises when there is the choice to explore or avoid open exposed regions of the environment (open field and elevated plus maze). Studies which used benzodiazepines, drugs which are known to reduce anxiety in humans, provide validation that the elevated plus maze is a test for anxiety in rodents (Pellow et al, 1985). This was further validated by the reduction of anxiety in the elevated plus maze after serotonin depletion, which was induced by selective central lesions of the serotonin neurons in the forebrain (following 5,7-Dihydroxytryptamine injection into the cerebral ventricles of the rat brain (Briley et al, 1990)).

Social behaviour

The sociability test measures social affiliation/motivation, (i.e. the preference to approach and explore a conspecific placed in a chamber as compared to exploring an identical chamber that is empty). This test prohibits direct physical interaction. On the contrary, the reciprocal social interaction test assesses the social interaction of a pair of mice (that have never met before) that are freely moving and thus are free to have direct physical contact. In that test the behaviour of one mouse can have an effect on the behaviour of the other mouse. Therefore, the two tests may measure different aspects of social behaviour in

rodents. Also, the two tests generate different levels of anxiety given that in one test physical interaction is prevented. The social recognition test, which follows directly after the sociability test, assesses the preference to approach and explore a novel conspecific placed in a chamber (the chamber that was empty during the sociability phase of the experiment) as compared to exploring a familiar conspecific (the one the mouse had met during the sociability phase of the experiment) in an identical chamber. To test the hypothesis that amphetamine sensitization models social withdrawal in schizophrenia, both a reciprocal social interaction test and a sociability test were used. Thus, a more complete assessment of social behaviour is achieved.

To date only one study has been conducted which used methamphetamine sensitization instead of D-amphetamine (as used here), which found reduced sociability and social recognition. However, in that study low doses of daily ketamine injections were administered after amphetamine withdrawal (Liao et al, 2018). Therefore, the results of that study should not be interpreted as clear evidence of the effects of amphetamine sensitization on social behaviour. Instead they may have had to do with the potency of withdrawal from repeated amphetamine exposure to enhance the effects of ketamine on social behaviour.

5.1.4 Hypotheses

First hypothesis

It was hypothesized that withdrawal from the amphetamine sensitizing regimen will cause impaired novel object recognition and object-in-place recognition memory, as well as a social recognition memory deficit. It should be noted that if social recognition is deficient it is expected that this might lead to increased social interaction as mice might have deficits in habituation. If preference for social novelty is impaired this might lead to reduced social

interaction as mice will be expected to have reduced interest to interact with an unfamiliar conspecific. Our hypothesis that amphetamine sensitization will cause deficit in recognition memory tests is based on the fact that novelty detection and preference for novelty are mediated through corticostriatal dopaminergic signaling (Rangel-Gomez et al, 2013, Bunzeck et al, 2014, Wittmann et al, 2008, Pierce et al, 1996) which is altered in withdrawal from amphetamine sensitizing regimens. Also, the hypothesis is based on the fact that the hippocampus which is known to be involved both in novelty detection (Honey et al, 1998, Lisman and Otmakhova, 2002) and novelty processing (Murty et al, 2013, Howard et al, 2011, Kumaran and Maguire, 2009), appears to be implicated in the effects of amphetamine sensitization (Anagnostaras et al 2002, Crombag et al, 2001). Likewise, the hippocampus is compromised by repeated cocaine exposure (for review Castner and Williams, 2007).

The hippocampus seems to play a role in the three recognition tests included in this chapter (i.e. object in place, social recognition and novel object recognition, although for the latter the literature is mixed). More specifically, evidence has been provided that the hippocampus is required for object in place memory (Gilbert and Kesner, 2002, 2004, Barker and Warburton, 2011, Blue et al, 2013, Jo and Lee, 2010, Bachevalier and Nemanic, 2008). With regards to social recognition, it has been shown that selective cytotoxic lesions in the hippocampus (including of pyramidal cells in the CA subfields and of granule cells in the dentate gyrus) did not impair social recognition (Bannerman et al, 2001), while lesions in the retro hippocampal region (including subiculum and entorhinal cortex) induced a mild social recognition deficit, although lesioned rodents still showed some recognition for the conspecific (Bannerman et al, 2002b). Maaswinkel et al (1996) demonstrated that fimbria-transected rats had deficits in social recognition.

The discrepancy of these findings might show that different areas within the medial temporal lobe are involved as distinct parts of the recognition memory process, or that they

regulate different recognition memory systems. More recent studies have shown that CA2 is required for social recognition in mice (Hitti and Siegelbaum, 2014, Stevenson and Caldwell, 2014) and that hippocampal lesions in octodon degus impair social recognition (Uekita and Okanoya, 2011). In line with these findings, CA1 inactivation in golden hamsters induced a social recognition deficit (Lai et al, 2005). These findings are also potentially in agreement with evidence that the parvalbumin subtype of interneurons in the ventral hippocampus functions as a discriminator in social memory (Deng et al, 2019). Overall, it appears that the hippocampus, or at least selective parts of it, or the wider hippocampal formation, may be involved in social recognition.

With regards to novel object recognition most experiments with permanent hippocampal lesions in rodents conclude that the integrity of hippocampus is not required for the basic novel object recognition effect (Forwood et al, 2005, Winters et al, 2004, Bussey et al, 2000, Barker and Warburton, 2011, Langston and Wood, 2010 for review Cohen and Stackman, 2015). Likewise, Ainge et al (2006) showed that novel object recognition is not impaired by hippocampal lesions. On the other hand, most experiments in which the hippocampus was temporarily inactivated report impaired object recognition with delays longer than 3 hours between the sample and the choice phase, but not when the delay was 5 minutes (Baker and Kim, 2002, Hammond et al, 2004, Cohen et al, 2013). Given that novel object recognition is a time-dependent behavioural process, temporary inactivation of the hippocampus might be more appropriate than a permanent lesion.

However, other evidence suggests that it is not all about temporary versus permanent lesions, and that hippocampal integrity is, in any event, necessary for novel object recognition (Clark et al, 2000, Broadbent et al, 2009, for review Squire et al, 2007). It could be argued that spared novel object recognition, only when the lesion is permanent, might reflect a

compensatory mechanism potentially through a reorganization of the medial temporal lobe circuits (for review Cohen and Stackman, 2015).

Alternatively, it could reflect a redundancy of function within the medial temporal lobe memory system. Independence of different aspects of memory within the temporal lobe, at least to some extent, has been shown previously by Bussey et al (1999) who demonstrated that combined perirhinal and postrhinal cortex lesions impaired object recognition while leaving spatial memory completely intact. Finally, evidence of impaired object recognition memory in rodent models of methamphetamine abuse (Belcher et al, 2005, Reichel et al, 2011, Ito et al, 2007, Arai et al, 2009, Noda et al, 2010, Lu et al, 2010, Peters et al, 2016) and rodent models of cocaine addiction (Briand et al, 2008) further supports our hypothesis that mice that have withdrawn from amphetamine will have impaired performance in the object recognition test.

Second hypothesis

The second hypothesis to be tested in this chapter is that amphetamine sensitized mice will have impaired social interaction. This hypothesis is based on evidence of alteration of the social neuropeptide oxytocin by psychostimulant sensitization in rodents (van Nieuwenhuijzen et al, 2010), and impaired social bonding after repeated amphetamine exposure in rodents (Gobrogge et al, 2009, Liu et al, 2010, for review Young et al, 2011). Also, the hypothesis is based on evidence of reduced sociability after acute amphetamine injection in rats (see description of study in more detail above, Rincon-Cortes et al, 2018) as well as after withdrawal from repeated amphetamine that was followed by ketamine (see description of the study above, Liao et al, 2018), and reduced social interaction after electrical sensitization of the mesolimbic dopaminergic system (Glenthøj et al, 1993), which is well known to mediate the effects of amphetamine sensitization.

Third hypothesis

The third hypothesis of this chapter is that amphetamine sensitized mice will have increased anxiety. It should be noted that in case of increased anxiety social interaction might be reduced. This might subsequently cause reduced social recognition. Our hypothesis of increased anxiety is based on the fact that the majority of studies have shown increased anxiety levels in recently abstinent methamphetamine abusers (London et al, 2004, McGregor et al, 2005 Mancino et al, 2011) and mice that have withdrawn from cocaine (Barr and Markou, 2005). Also, it is based on evidence by Barr et al (2010), indicating that anxiety was evident 8 days (Valvassori et al, 2019) and 1 month after amphetamine withdrawal (Barr et al, 2010). We tested here whether anxiety will be found at longer withdrawal periods.

5.1.5 General methodological issues

In the following tests: reciprocal social interaction, open field, sociability, preference for social novelty test, elevated plus maze test, novel object recognition and object in place (see below) both duration and frequency measure time spent engaged in a given activity. However, duration is generally a more accurate index. Therefore, for reasons of simplicity in all the methods and results sections of this chapter, only duration will be reported, unless frequency does not mirror the duration results.

There are very small differences in the group numbers in some of the tests of this chapter. In tests others than the social interaction test, this is because a few of the trials were not videotaped properly due to technical reasons and thus some data were lost. As far as social interaction test is concerned, some mice were not tested because they were being used by another experimenter for another behavioural experiment.

5.2 Reciprocal social interaction test

5.2.1 Methods

Subjects

18 C57BL/6 female mice (18-22g), 6 in each of the three treatment groups were used.

Apparatus

Social behaviour was investigated using a 41.5x21x11 cm cage, the floor of which was covered with sawdust. Trials were video recorded.

5.2.1.1 Experimental Procedure

Habituation Phase

2 months post-withdrawal from amphetamine, mice were placed individually in this novel cage for 6 successive days, for 10 minutes each day (ITI of 24 hours), under moderate illumination. Only duration of immobility was measured. While the duration of activity is a more sensitive measure compared to the duration of immobility, this was not scored as sessions were not videotaped. However, we note that these measures are likely to be strongly correlated.

Testing Phase

24 hours after the last habituation day, mice were re-introduced to the cage under moderate illumination in pairs from the same treatment group. The two mice in each experimental pair had never met before. Social interactions between each pair were scored for 10 minutes. Each mouse was tested twice with two different mice from the same treatment

group. Therefore, there were in total 6 pairs of mice tested in each treatment group. Sessions were recorded and scored manually using an event recorder, by an experimenter blind to the treatment condition of the mice.

The total durations of the following behaviours were scored for each pair of mice: (a) social behaviours, in particular, anogenital sniffing, mouth/nose sniffing (directed at the head or snout), following (moving with the head towards the other mouse, or walking around the other mouse), body contacts (mice are side by side), (b) avoidance/fleeing behaviours (moving the anterior part of the body or head away from the other mouse), repeatedly circling when mouse approaches, walking away from the mouse, and (c) stereotypic or other non-social behaviours such as digging, rearing, grooming and repetitive head swaying from side to side (left to right or right to left).

5.2.2 Results

A Kruskal Wallis non-parametric test was applied as the data were not normally distributed for duration of immobility during the first day of habituation in the novel arena even after log, ln and square root transformation was applied. Results indicated that there was no significant difference between groups, neither in the analysis in which amphetamine 2.5mg/kg and amphetamine 5mg/kg were treated as two separate groups, $H(2)=4.172$, $p=.123$, nor in an additional analysis in which they were treated as a single group that was compared to saline, $U=15.5$, $p=.125$ (2-tailed) (see figure 5.1). These results indicate that all mice had similar levels of immobility in the novel arena during the first day of habituation. Results for the subsequent habituation sessions were not recorded. Therefore, we cannot completely exclude the possibility that any differences in the social interaction test could be attributed to different levels of immobility in the arena, but there was no evidence for this from the data that we were able to analyze.

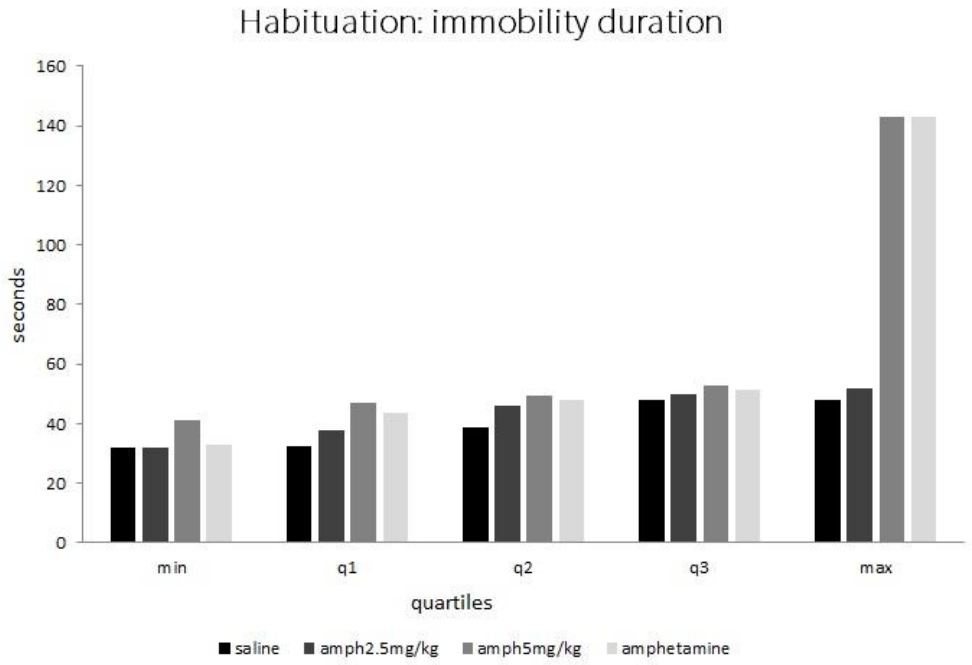


Figure 5.1. Quartiles for immobility duration during habituation. Q1= first quartile, q2=median, q3=third quartile, min=lower value, max=higher value. Saline (n=5), amphetamine 2.5mg/kg (n=6), amphetamine 5mg/kg (n=6), combined amphetamine (n=12).

Pairs of saline pre-treated mice interacted socially. Pairs of amphetamine pre-treated mice showed increased avoidance behaviours and reduced social contact (see figures 5.2a-c). Separate one-way ANOVAs were performed on the ln transformed data for time spent engaged in stereotypy and social contacts. Ln transformation was conducted because data were not normally distributed. Separate ANOVA was conducted for the duration of avoidance behaviors. There was a significant main effect of group for social contact, ($F(2, 15)=6.127$, $p=.011$). Bonferroni post hoc analysis indicated that there was a significant difference only between the saline group and the amphetamine 2.5mg/kg group, $p=.024$. The Saline group had increased duration of social contacts compared to amph2.5mg/kg group (see figure 5.2a). One-way ANOVA indicated also that there was a significant difference between groups in the duration of avoidance behaviours, ($F(2, 15)=5.217$, $p=.019$). Bonferroni post hoc analysis indicated that saline group differed significantly from amphetamine 2.5mg/kg

group, p=.020 due to more avoidance behaviours found in the amphetamine 2.5mg/kg group (see figure 5.2c).

There were no group differences for non-social behaviours including stereotypies, (F(2, 15)=2.004, p=.169).

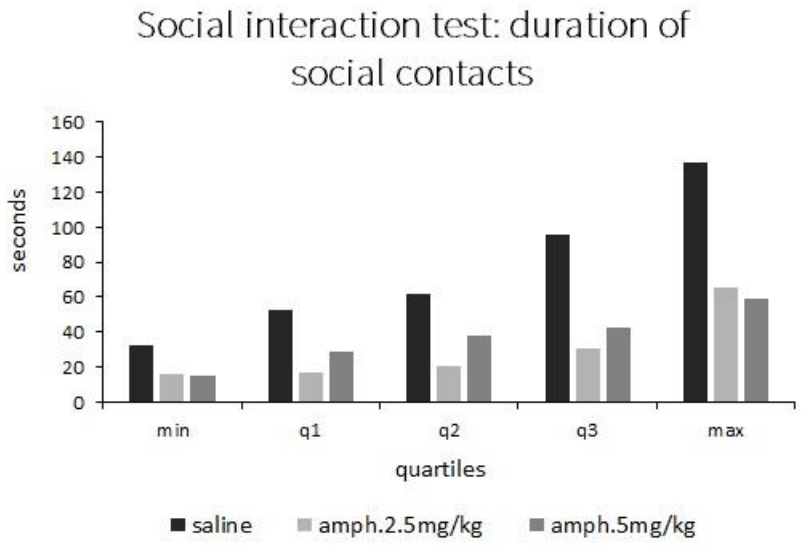


Figure 5.2a. Quartiles for duration (seconds) of social contacts. Saline (n=6), amphetamine 2.5mg/kg (n=6), amphetamine 5mg/kg (n=6).

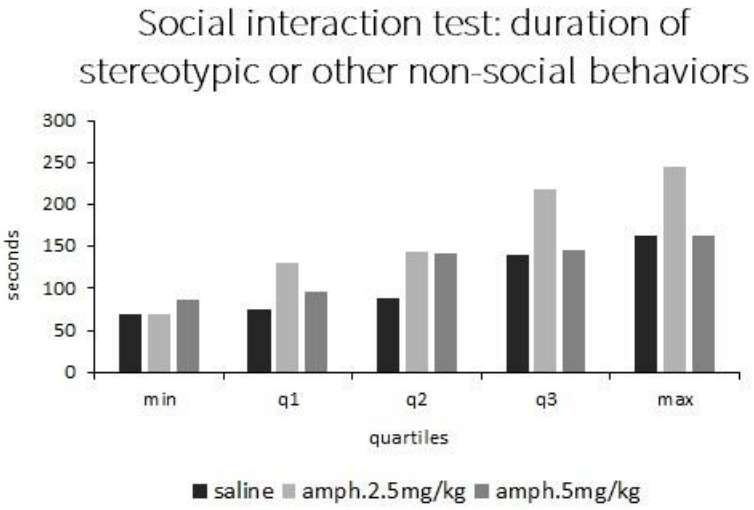


Figure 5.2b. Quartiles for duration (seconds) of stereotypic and other non-social behaviors. Saline (n=6), amphetamine 2.5mg/kg (n=6), amphetamine 5mg/kg (n=6).

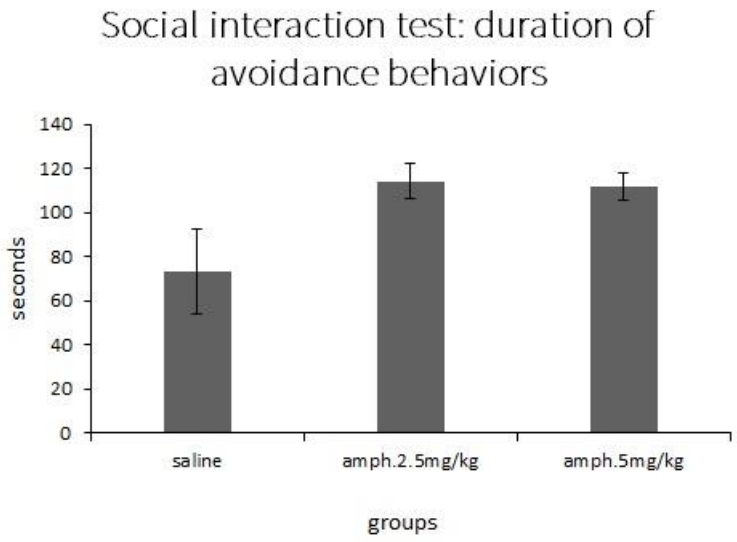


Figure 5.2c. Mean (±SEM) duration (seconds) of avoidance behaviours. Saline (n=6 pairs), amphetamine 2.5 mg/kg (n=6 pairs), amphetamine 5mg/kg (n=6 pairs).

Given the small group sizes involved, further analyses were run in which the two amphetamine groups were combined into a single experimental group (n=12; N.B. preliminary analysis showed that the 2.5 and 5 mg/kg AMPH groups did not differ from each other on any of these measures).

Saline and AMPH groups differed in the duration of social contacts, t(16)=2.570, p=.042 (2-tailed), reflecting the decreased social interaction in amphetamine pre-treated mice. The groups also differed in avoidance behaviours, t(16)=2.775, p=.014 (2-tailed), due to amphetamine pre-treated mice avoiding social interaction. There was no significant difference between groups in the non-social behaviours including stereotypies, t16=-1.682, p=.112 (2-tailed). See figure 5.3.

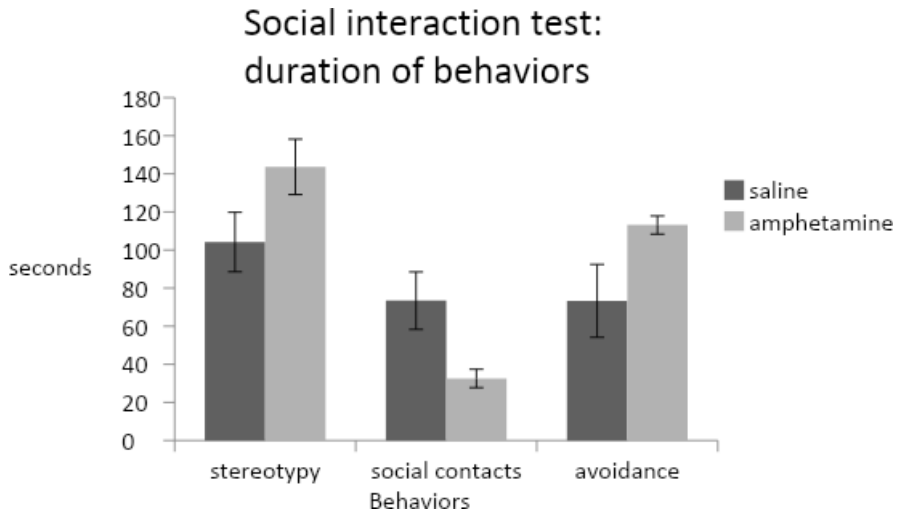


Figure 5.3. Mean (±SEM) duration (seconds) of **(a)** stereotypic or other non-social behaviour **(b)** social contacts and **(c)** avoidance. Saline (n=6 pairs), combined amphetamine groups (n=12 pairs).

5.3 Open field, sociability and social recognition test

5.3.1 Methods

The test had three phases: 1. Novel open field exploration, 2. Sociability and 3. Social recognition.

Subjects

For exploration of the open field, the saline group included 11 mice, the AMPH 2.5mg/kg group contained 11mice and the AMPH 5mg/kg group contained 11 mice.

For the sociability phase and the social recognition test, the saline group included 9 subjects, the AMPH 2.5mg/kg group included 11 subjects and the AMPH 5mg/kg group included 10 subjects.

Apparatus

During the first phase a novel plastic arena 50x50x50cm was used with a floor marked off in 10x10cm squares, and walls marked with 9cm thick stripes. The centre of the field was considered as the square 30x30cm area in the center of the arena (see photograph in figure 5.4).



Figure 5.4. Plastic arena used in novel open field, sociability and social recognition tests.

During the second phase, a pair of wire rectangular chambers 8x8x20cm, with a plastic top and bottom was placed in the plastic arena. The chambers were placed adjacent to two opposite walls, 10 cm away from the top left and the bottom right corners of the square arena respectively (see photograph in figure 5.5).

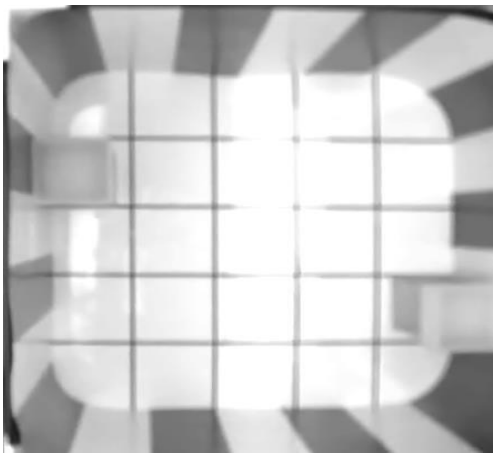


Figure 5.5. Plastic arena now with wire chambers.

5.3.1.1 Experimental Procedure

The experiment was conducted 8 months post-drug withdrawal. Sessions were recorded and scored manually by an experimenter using as event recorder, blind to the treatment condition of the mice. Between trials the arena, as well as the chambers, were cleaned with 70% alcohol. A mouse was considered to be exploring a stimulus when it touched or faced the stimulus at a 1 cm distance maximum and is pointing directly at it so that an elongated line from eyes to nose would hit the object.

5.3.1.1.1 Novel Open field exploration

Each mouse was individually introduced into the novel arena for 10 minutes with no stimulus mouse present.

Outcome measures

The number of lines crossed (as an index of distance travelled and thus exploratory behaviour) was measured. Also, the duration of the following behaviours was recorded: non-

social behaviours including stereotypies (i.e. rearing, grooming, and repetitive head swaying from side to side), immobility, visits to the center of the arena, defecation. In addition, stretching behaviour was also measured, as a measure of risk assessment (Blanchard et al, 1990) defined as a extension of the abdomen followed by an extension of the hind limbs.

5.3.1.1.2 Sociability test

Approximately 45 minutes after the open filed phase, mice were re-introduced for 10 minutes to the arena with the two wire chambers. A novel conspecific (C57BL/6 female mice, 18-22g) was placed in one of the two chambers. The spatial location of the chamber containing the conspecific (i.e. chamber placed by the left or right wall) was counterbalanced across groups. See figure 5.6.

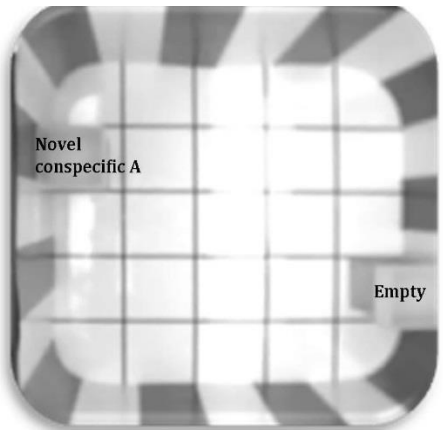


Figure 5.6. Placement of a conspecific in one of the two chambers.

Outcome measures

The duration of exploration of each chamber was recorded. The duration of time (in seconds) that was spent exploring the chamber that included the conspecific was used as an index of social approach.

5.3.1.1.3 Social recognition test

One hour later each mouse was re-introduced to the arena for a further 10 minutes. Now the previously encountered conspecific was placed in one of the chambers and a novel conspecific was placed in the opposite chamber. See figure 5.7.

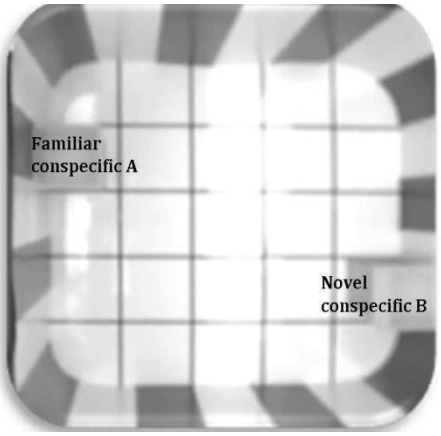


Figure 5.7. Placement of a novel conspecific in the chamber that was empty during the previous phase of the experiment. The conspecific that was used in the previous phase (familiar conspecific) was re-introduced in the same chamber that had been placed in the previous phase. Location of the two chambers varied between sample and choice phase.

Outcome measures

Again, the duration of exploration of each chamber was recorded. From this was calculated the total amount of chamber exploration and the preference for the novel social stimulus over the familiar social stimulus as a ratio (time spent with novel conspecific minus time spent with familiar conspecific divided by the sum of time spent with both conspecifics).

5.3.2 Results

5.3.2.1 Open field

Summary of results

Locomotor activity progressively decreased for all mice across the session, indicating that the mice habituated with continued exposure to the testing environment. There was a suggestion that the amphetamine group as a whole exhibited increased locomotor activity compared to the saline group, especially during the first minute of the test.

5.3.2.1.1 Locomotor activity

Statistics

Repeated measures ANOVA for the number of lines crossed indicated that the sphericity assumption was violated. Greenhouse-Geisser ε=.759 was conducted to correct the degrees of freedom and p value. Results indicated that as time passed locomotor activity decreased for all mice (significant main effect of block, $F(3.037, 91.112)=49.919$, $p<.001$). There was no overall significant difference between groups (main effect of group, $F(2, 30)=1.500$, $p=.239$). There was a trend towards a groups by blocks interaction, which just failed to reach significance ($F(6.074, 91.112)=1.965$, $p=.078$). Groups were nevertheless well matched for locomotor activity levels by the end of the session. See figure 5.8.

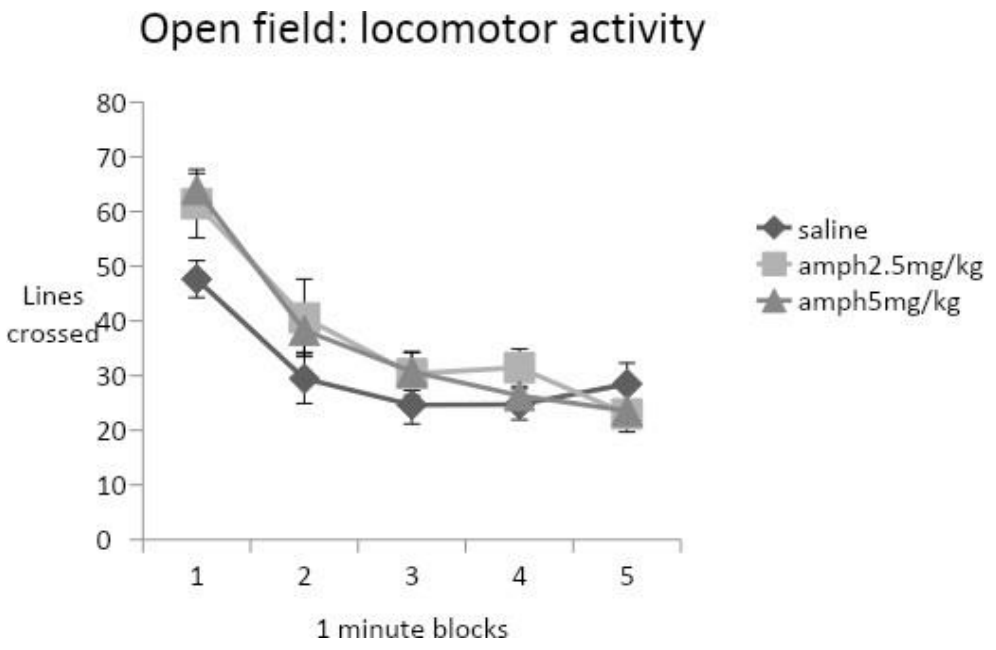


Figure 5.8. Mean (±SEM) lines crossed. Saline (n=11), amphetamine 2.5mg/kg (n=11), amphetamine 5mg/kg (n=11).

When the saline group was compared to the two amphetamine groups combined into a single group, sphericity was still violated. Thus, Greenhouse-Geisser ε=.769, was again used to correct the degrees of freedom and p value. As time passed locomotor activity decreased for all mice, (the main effect of blocks was significant, $F(3.075, 95.319)=38.523$, $p<.001$). There was a trend for groups to differ overall, which was not significant, (main effect of groups, $F(1, 31)=3.057$, $p=.090$). Furthermore, there was a significant group by blocks interaction, (groups by blocks interaction, $F(3.075, 95.319)=3.592$, $p=.016$) indicating that locomotor activity was affected differently by the passage of time as the test progressed depending on which of the two treatment groups a mouse belonged to. Bonferroni post hoc analysis indicated that the first block differs significantly from all others (all $ps<.001$). Additionally, the second block differs significantly only from the third one ($p=.048$). All the other pairwise comparisons were not found to be statistically significant. From the sign of the mean differences as shown by the graph below, it is obvious that as the time passes, the

locomotor activity was decreased (see figure 5.9).

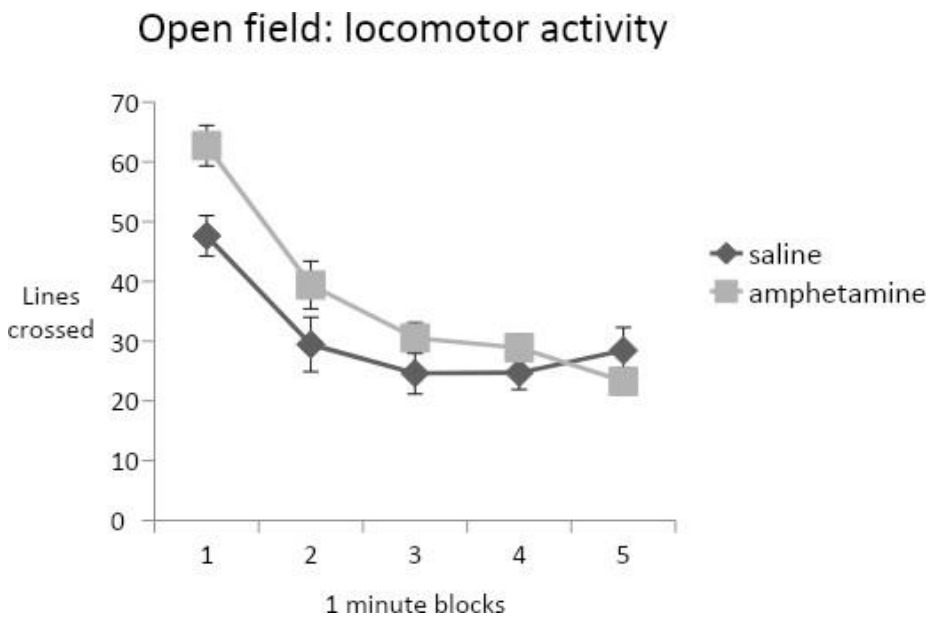


Figure 5.9. Mean (±SEM) lines crossed. Saline (n=11), amphetamine (n=22).

5.3.2.1.2 Immobility

Summary of results

Immobility gradually increased for all mice across the session. This increase was greater at the beginning of the test. There was a trend for groups to differ with saline mice showing a trend for increased immobility compared to the amphetamine groups, mirroring the results for locomotor activity.

Statistics

In the three group analysis, repeated measures ANOVA for immobility duration across 1 minute blocks, was performed. As time progressed immobility increased for all mice, potentially indicating that mice were habituating, (the main effect of blocks was significant, $F(4, 120)=42.219$, $p<.001$). Groups did not differ significantly, (main effect of groups, F(2,

30)=1.584, p=.222), and there was no groups by blocks interaction, (groups by blocks interaction, F(8, 120)=.701, p=.636). See figure 5.10.

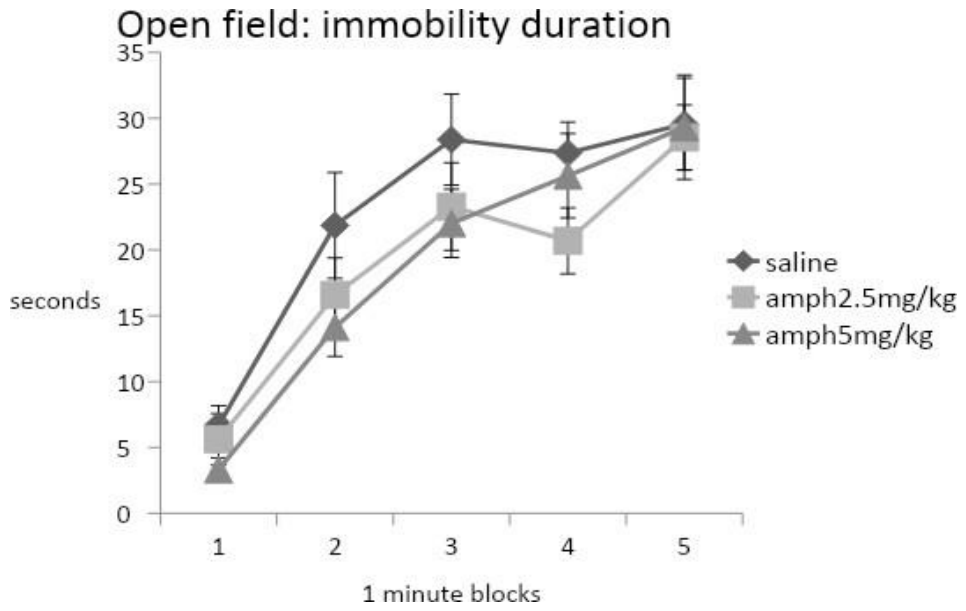


Figure 5.10. Mean (±SEM) immobility duration (seconds). Saline (n=11), amphetamine 2.5mg/kg (n=11), amphetamine 5mg/kg (n= 11).

The fact that as time progressed immobility increased for all mice, (main effect of blocks was significant, F(4, 124)= 42.219, p<.001) was also evident in the two group analysis. There was a trend for groups to differ which was not significant, (main effect of groups, F(1, 31)=3.273, p=.080). There was no significant groups by blocks interaction (groups by blocks interaction, F(4, 124)=.643, p=.575). See figure 5.11.

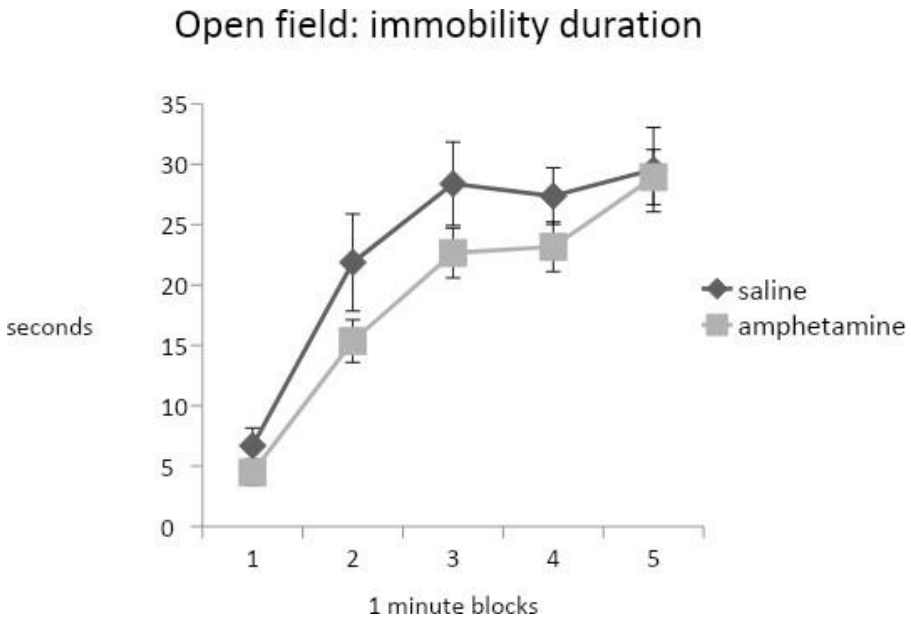


Figure 5.11. Mean (±SEM) immobility duration (seconds). Saline (n=11), amphetamine (n= 22).

5.3.2.1.3 Visits in the centre of the open field

Summary of results

The duration of visits to the centre of the open field progressively increased for all mice across the five 1-minute bins of the session. There were no group differences.

Statistics

Repeated measures ANOVA for duration of visits to the centre indicated that sphericity was violated and thus Greenhouse-Geisser correction was applied to p value and to degrees of freedom, ε=.752. All mice visited the centre more as the session progressed (the main effect of blocks was significant, $F(3.009, 90.258)=16.748$, $p<.001$). There were no differences between groups, (maineffect of groups, $F(2, 30)=.227$, $p=.798$), and no significant groups by blocks interaction (groups by blocks interaction, $F(6.017, 90.258)=1.472$, $p=.197$). See figure 5.12.

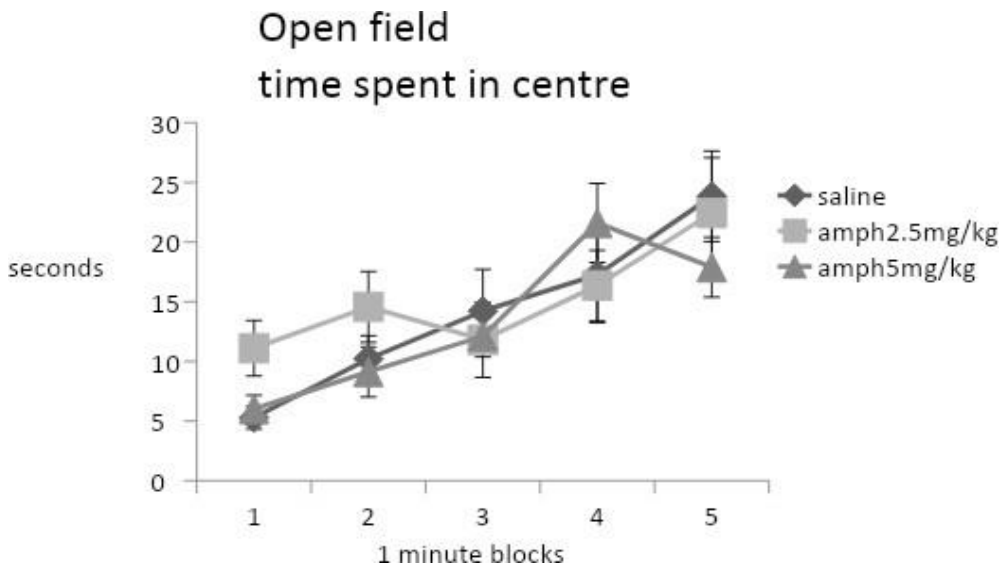


Figure 5.12. Mean (±SEM) time spent in the centre of the novel open field arena (seconds). Saline (n=11), amphetamine 2.5mg/kg (n=11), amphetamine 5mg/kg (n=11).

When the saline group was then compared to the amphetamine groups combined into a single group sphericity was again violated and thus Greenhouse-Geisser correction was used to correct p value and degrees of freedom, ε=.749. Again, as time progressed all mice visited the centre more (the main effect of blocks was significant, $F(2.995, 92.839)=16.209$, $p<.001$). Groups did not differ, (main effect of groups, $F(1, 31)=.003$, $p=.995$), and there was no groups by blocks interaction (groups by blocks interaction, $F(2.995, 92.839)=1.003$, $p=.395$). See figure 5.13.

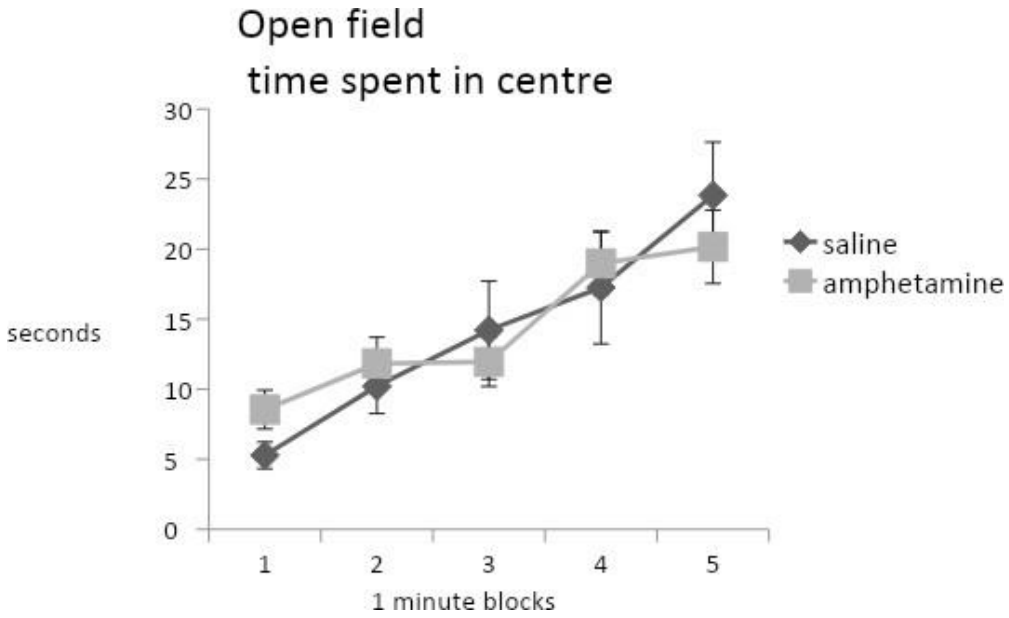


Figure 5.13. Mean (±SEM) time spent in the centre of the novel open field arena (seconds). Saline (n=11), amphetamine (n=22).

5.3.2.1.4 Defecation

Summary of results

All groups defecated during the test. There was no significant difference in the defecation between groups. See figure 5.14.

Statistics

Data for number of defecations were not normally distributed even after application of transformations and thus a non-parametric independent samples Kruskal-Wallis test was conducted to compare the total amount of defecation in the session for each group. This indicated that the number of defecations was the same across the three groups, H(2)=.651, p=.743 Likewise an independent samples Mann-Whitney U test, indicated that the number of

defecations was the same across the saline group and the combined amphetamine group, U=103, p=.442 (2-tailed).

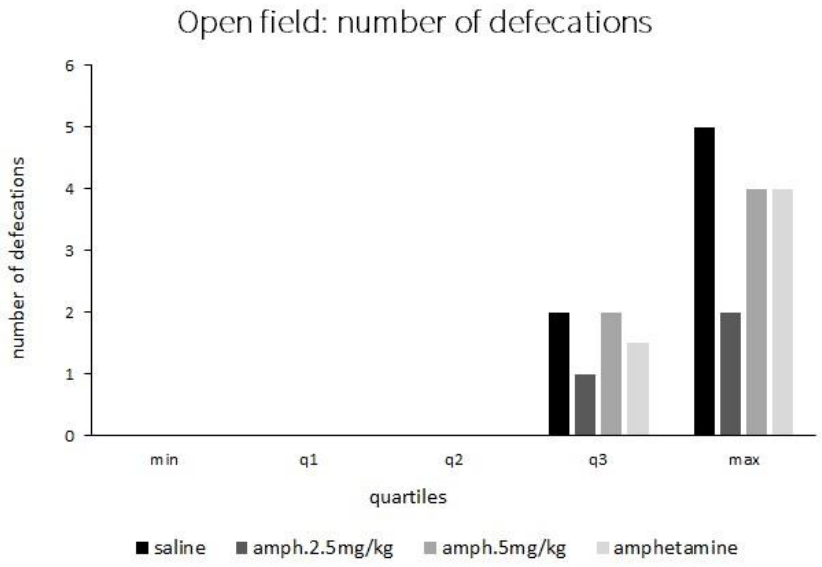


Figure 5.14. Quartiles for number of defecations. Q1= first quartile, q2=median, q3=third quartile, max=higher value. Saline (n=11), amphetamine 2.5mg/kg (n=11), amphetamine 5mg/kg (n=11), amphetamine (n=22).

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5.3.2.1.5 Duration of non-social behaviours including stereotypies: statistical analysis

Summary of results

Non-social behaviours including stereotypies gradually decreased for all mice across the session. There were no group differences.

Statistics

In the 3 groups analysis a repeated measures ANOVA for the duration of non-socialbehaviours, including stereotypies, across the 5 blocks of 1 minute in the session was conducted. Mauchly's test of sphericity, indicated that sphericity was violated, $\chi^2(9)$ =,178, p<.001, Greenhouse-Geisser ε=.499. Non-social behaviours including stereotypies reduced significantly as the test progressed for all mice, (main effect of blocks, F(1.996,

59.872)=21.251, p<.001). Groups did not differ in the time spent engaged in non-social behaviours including stereotypies (main effect of groups, F(2, 30)=1.065, p=.357), and there was no groups by blocks interaction, (F(3.991, 59.872)=1.143, p=.345). See figure 5.15.

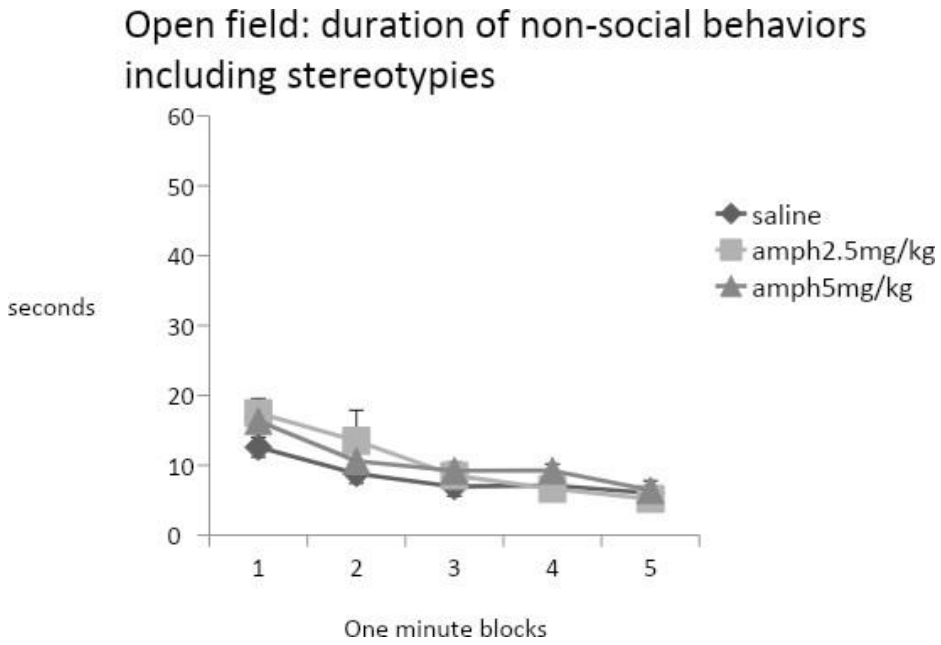


Figure 5.15. Mean (±SEM) duration of non-social behaviours including stereotypies (seconds). Saline (n=11), amphetamine 2.5 mg/kg (n=11), amphetamine 5mg/kg (n=11).

When the two amphetamine groups were then combined into one single drug group sphericity was again violated. Greenhouse-Geisser ε=.491 was conducted to correct the degrees of freedom and p value. Again non-social behaviours, including stereotypies, were seen to decrease as time progressed for all mice, (the main effect of blocks was significant, F(1.964, 60.888)=16.013, p<.001). Again, there were no significant differences between groups (main effect of groups, F(1, 31)=2.197, p=.148), and no significant groups by blocks interaction (F(1.964, 60.888)=1.100, p=.339). See figure 5.16.

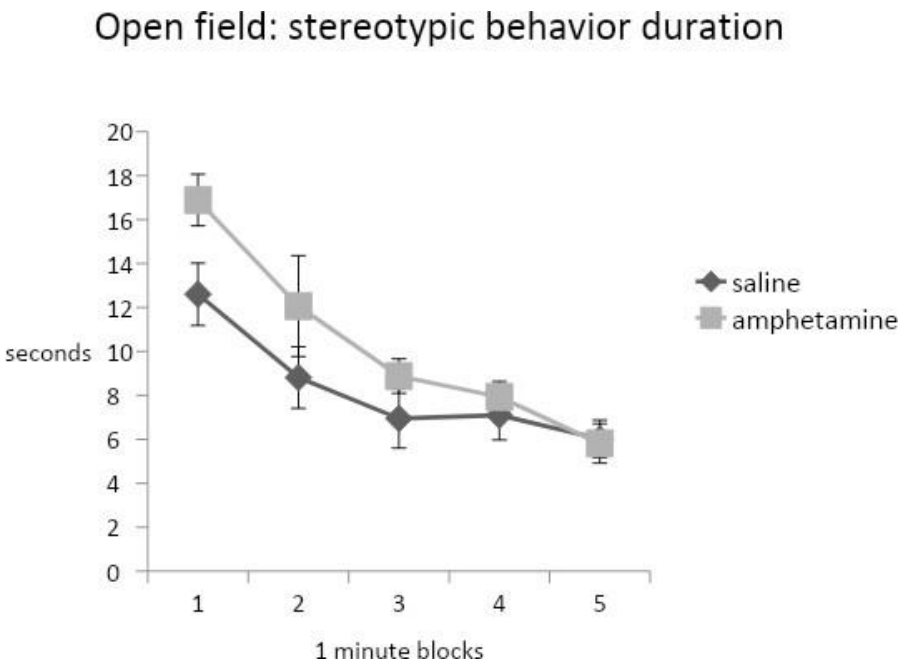


Figure 5.16. Mean (±SEM) duration of stereotypic or other non-social behaviours (seconds). Saline (n=11), amphetamine (n=22).

5.3.2.1.6 Stretching

Summary of results

The duration of time spent in stretching progressively increased for all mice as the session progressed (see figure 5.17). There was no significant difference between groups in the duration of stretching behaviour.

Statistics

Data for 3 group analysis were not normally distributed and thus the non-parametric Kruskal-Wallis test was applied. Results indicated that there were no significant differences between groups, H(2)=.474, p=.789. When amphetamine treated groups were combined into one group and compared to the saline group, data were again not normally distributed and thus Mann-Whitney test was conducted. Results indicated that there was no significant

difference between groups, U=103.5, (z=-.669), p=.516 (2-tailed). See figure 5.17.

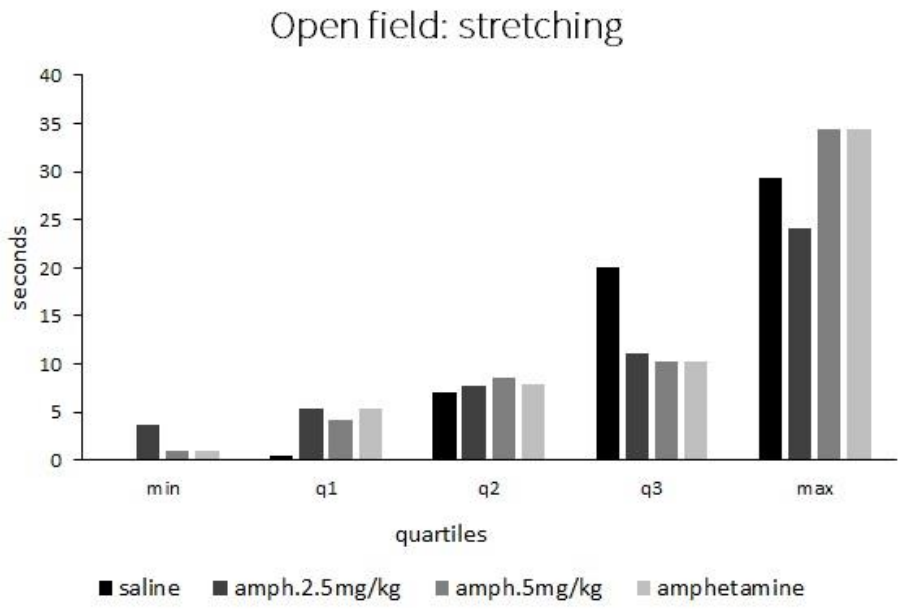


Figure 5.17. Quartiles for duration of stretching. Q1=first quartile, q2=median, q3=third quartile, min=lower value, max=higher value. Saline (n=11 mice), amphetamine 2.5 mg/kg (n=11 mice),amphetamine 5mg/kg (n=11 mice), amphetamine (n=22).

5.3.2.2 Sociability test

Summary of results

Although the amphetamine treated mice engaged in less social approach behaviour numerically, there were no significant group differences for sociability in this particular test.

Statistics

ANOVA reveled no difference between the three groups in the duration of social approach ($F(2, 27)=1.385$, $p=.267$). When the two amphetamine groups were combined, again the groups did not differ in the duration of exploration of the conspecific, $t(28)=1.629$,

p=.114 (2-tailed). See figure 5.18.

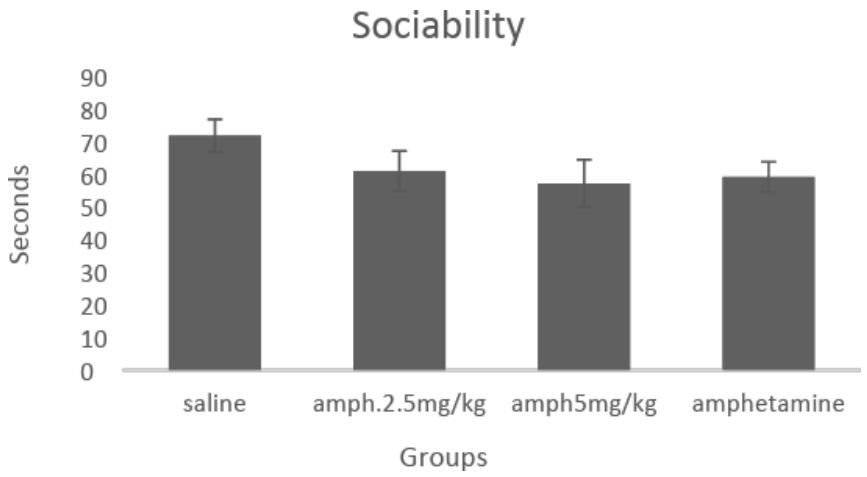


Figure 5.18. Mean (±SEM) duration of time that was spent exploring the chamber that included the conspecific (seconds). Saline (n=11), amphetamine 2.5mg/kg (n=11), amphetamine 5mg/kg (n=10), and combined amphetamine group (n=21).

5.3.2.3 Social recognition test

Summary of results

All mice had a similar total duration of social exploration of the two conspecifics during the recognition memory test phase. There was a non-significant trend for groups to differ as far as the discrimination ratio was concerned. However, the difference between the groups was not quite significant. It should be noted though that there was a high level of variability which may have precluded finding significant group differences.

Statistics

A strong but non-significant trend was found for the groups to differ in their preference for the novel mouse over the familiar mouse, potentially reflecting reduced social memory in the amphetamine treated animals (discrimination ratio for duration of social

investigation, (F(2, 27)=3.257, p=.054)). However, when the two amphetamine groups were combined into a single group there was less evidence of a significant difference between the groups, t(28)=1.629, p=.114. This reflected the fact that the reduced social interaction was much more pronounced in the 5 mg/kg group. See figure 5.19. Also, see table 5.2 which presents the raw data for duration spent with both familiar and novel conspecifics in the choice phase. Data for the duration of time spent with the conspecific during the sample phase are presented in figure 5.18.

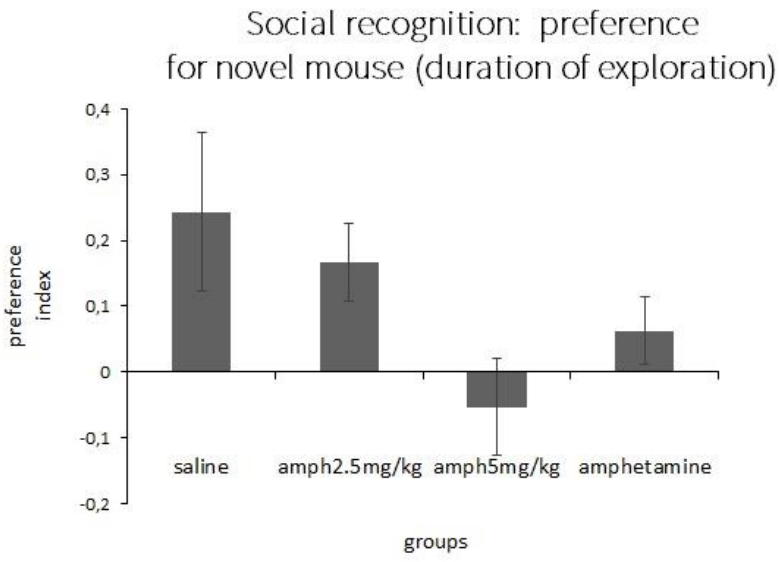


Figure 5.19. Mean (±SEM) preference for the novel mouse over the familiar mouse as a preference index (for duration of social investigation). Saline (n=9), amph 2.5mg/kg (n=11), amph 5mg/kg (n=10). Combined amphetamine group, n=21. Chance performance index = 0.

There was no difference between groups in the total duration of social approach to both conspecifics during the recognition test, either in the analysis for 3 groups (F(2, 27)=.752, p=.481), or with just the 2 groups t(28)=.647, p=.522 (amphetamine groups combined).

Additional one group t-tests were conducted to determine whether there was evidence of significant memory performance (comparisons against chance levels of performance).

Overall, the experimental cohort as a whole exhibited significantly above chance performance, indicating a social recognition memory effect (see table 5.1). The saline group had a trend to differ from chance level performance in the discrimination ratio for duration, which did not quite reach significance. However, the discrimination ratio for duration of social exploration differed significantly from chance level performance in the amphetamine 2.5mg/kg group, showing a preference for the novel conspecific. This was not the case for amphetamine 5mg/kg group which showed chance level performance.

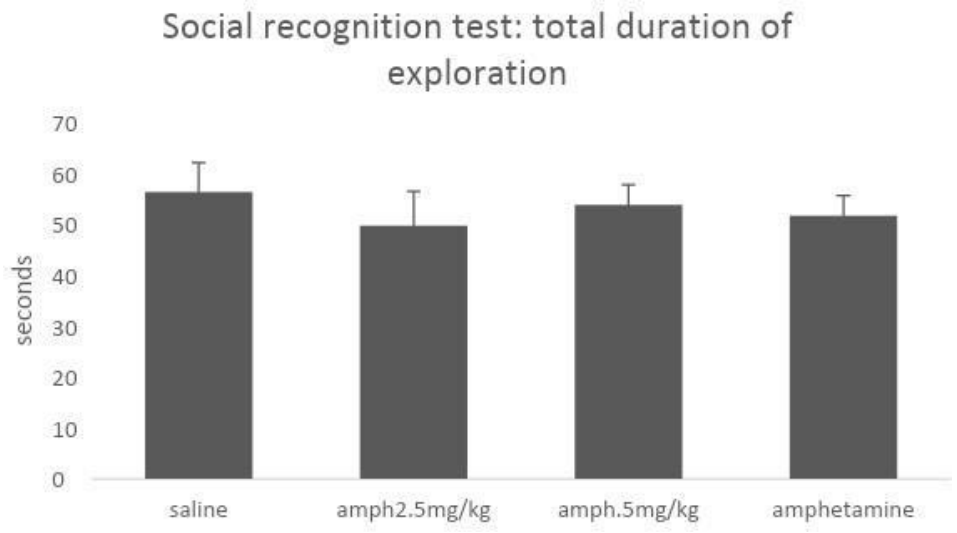


Figure 5.20. Mean (±SEM) total duration of social exploration of both conspecifics. Saline (n=9), amph 2.5mg/kg (n=11), amph 5mg/kg (n=10), and combined amphetamine group (n=21).

	preference for novel index (duration)
all groups (2-tailed)	t29=2.229, p=.034
saline (2-tailed)	t8=2.011, p=.078
amphetamine combined (2-tailed)	t20=1.207, p=.242
amphetamine 2.5mg/kg (2-tailed)	t10=2.843, p=.017
amphetamine 5mg/kg (2-tailed)	t9=.732, p=.483

Table 5.1. Comparison of preference for the novel conspecific versus the familiar mouse against chance level performance.

Group	**saline**		**amph2.5mg/kg**		**amph5mg/kg**		**amphetamine**	
conspecific	**novel**	**familiar**	**novel**	**familiar**	**novel**	**familiar**	**novel**	**familiar**
mean	33.789	22.5	29.70909091	20.03636	26.01	27.82	27.94761905	23.74286
Standard deviation	11.332	13.36189	16.11064583	8.332739	10.00582608	7.080458	13.35711867	8.55246
standard error of the mean	3.7773	4.453962	4.857542487	2.512415	3.164120029	2.239037	2.914762255	1.8663

Table 5.2. Mean, standard deviation and standard error of the mean per treatment group for time spent (seconds) with novel and familiar conspecific in choice phase.

5.4 Novel object recognition test

5.4.1 Methods

Subjects

For object recognition, the saline group contained 9 subjects, the amph2.5 mg/kg groups contained 7 subjects and the amph5mg/kg contained 5 subjects.

Apparatus

A plastic chamber (35x27x40 cm) was used. Two pairs of identical objects (two wooden rectangular boxes (5x5x18 cm) and two plastic bottles (5x18cm) were used as stimuli. A camera above the chamber video-recorded the trials. See figure 5.21.

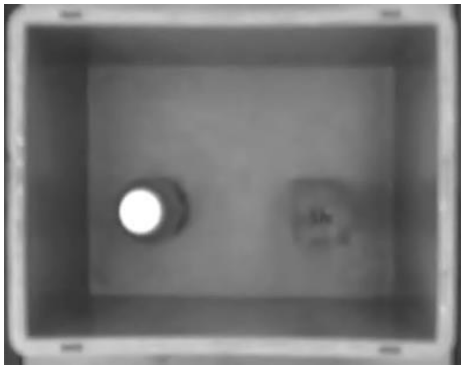


Figure 5.21. Plastic chamber with two objects, chosen from the two different pairs of identical objects. The figure refers to choice phase

5.4.1.1 Experimental Procedure

The experiment was conducted 8 months post drug treatment. Sessions were recorded and scored by an experimenter, using an event recorder, who was blind to the treatment condition of the mice. A mouse was considered to have explored a stimulus when it touched

or faced the stimulus at a 1 cm distance maximum.

Habituation

Each mouse was placed in the novel chamber for 5 minutes for one day under moderate illumination and was left free to explore. Habituation sessions were not recorded.

Sample phase

One hour later each mouse was reintroduced into the chamber for 5 minutes. A pair of identical objects had been placed in the chamber 12 cm apart from each other, 6 cm apart from the shortest middle line of the box (see photograph in figure 5.21 to see locations of objects). There were two different sets of identical objects used (see photograph in figure 5.21) counterbalanced across groups. Between trials the objects and the chamber were thoroughly cleaned with 70% ethanol.

Choice phase

One hour later each mouse was reintroduced for 5 minutes, with one familiar object being placed in the arena in the same place as before and the other one replaced by a different, novel object from the other pair of objects (see figure 5.21). The identity of the novel and familiar object, and the left or right position of the novel object was counterbalanced across groups.

Outcome measures

Duration was measured for each object's investigation, as well as total duration. A discrimination ratio for the two objects was also calculated for the choice phase by

subtracting the duration of investigation of the familiar object from the duration of investigation of the novel object and dividing the difference by the total duration of investigation of both objects.

5.4.2 Results

Summary of results

The total duration of object exploration was not significantly different between groups of mice, neither in the sample phase nor in the choice phase. There were also no significant group differences as far as the preference for the novel object is concerned. When the two amphetamine groups were integrated into a single group a trend for a reduced preference ratio for the novel object was found in the amphetamine group compared to saline group. Furthermore, the discrimination ratio of amphetamine group as a whole was not significantly different from chance levels.

Sample phase: total duration of exploration

ANOVA for total duration of object investigation during the sample phase indicated that data were not normally distributed and thus square root transformation was applied. Counterbalance (wooden rectangular box versus plastic bottle as sample object) was also added as a factor in the analysis. The groups did not differ significantly, (main effect of groups $F(2, 20)=.342$, $p=.716$). Also, there was no significant main effect of counterbalancing ($F(1, 20)=.094$, $p=.763$). Also, there was no significant group by counterbalance interaction, ($F(2, 20)=.264$, $p=.772$). Groups also did not differ significantly in the total duration of exploration of objects when the two amphetamine groups were combined into a single group, $t(19)=1.137$, $p=.540$ (2-tailed) (see figure 5.22a).

Choice phase: total duration of object exploration

Data for total duration of object exploration during the choice phase were also not normally distributed and thus again a square root transformation was applied. ANOVA was conducted and counterbalancing (wooden rectangular box versus plastic bottle as a choice object) was also included as a factor. The reason that in this particular experiment this was added as a factor was because no preference testing was done before the experiment to ensure that animals do not have an innate preference for any of the objects. Also, by visual inspection of the data it seemed that the condition that mice were assigned to might be a confounding factor (see graphs in appendix). Therefore, any potential effect that the type of objects might have on the results would be controlled. It was revealed that there were no significant group differences (main effect of groups, $F(2, 20)=.274$, $p=.764$). Also, there was no significant main effect of counterbalancing, ($F(1, 20)=1.766$, $p=.204$) and no significant group by counterbalancing interaction, ($F(2, 20)=.675$, $p=.524$). An independent sample t-test for the 2 groups analysis also indicated that there were no significant differences between groups, $t(19)=.901$, $p=.379$ (2-tailed). See figure 5.22b.

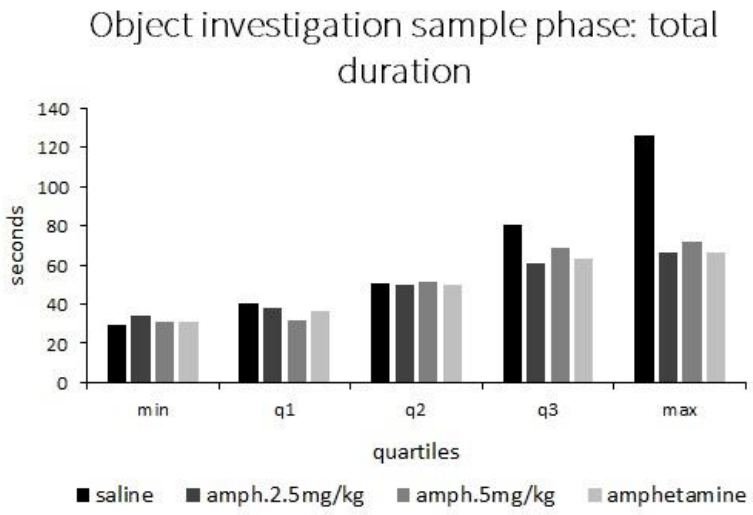


Figure 5.22a. Quartiles for total duration of investigation (seconds) of both objects in sample phase. Q1=first quartile, q2=median, q3=third quartile, max=higher value. Saline (n=9), amph.2.5mg/kg (n=7), amph.5mg/kg (n=5), amphetamine (n=12).

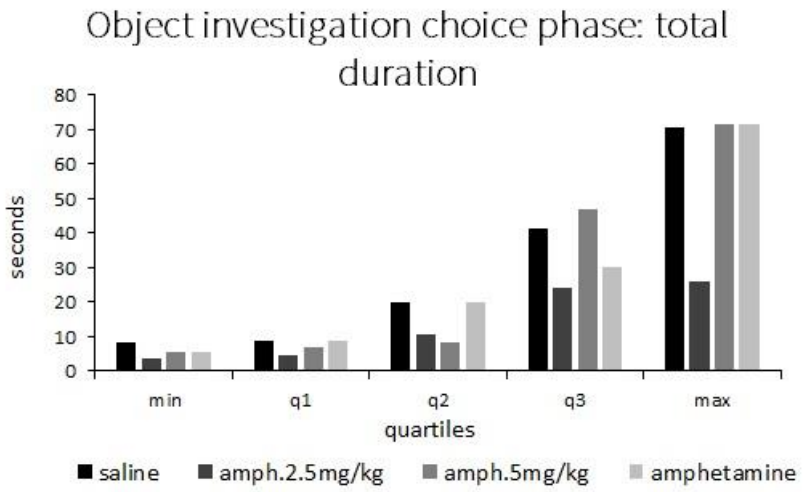


Figure 5.22b. Quartiles for total duration of investigation (seconds) of both objects in choice phase. Q1=first quartile, q2=median, q3=third quartile, max=higher value. Saline (n=9), amph.2.5mg/kg (n=7), amph.5mg/kg (n=5), amphetamine (n=12).

Choice phase: discrimination ratio for duration of object exploration

ANOVA for the discrimination ratio for duration of investigation of the novel object relative to the familiar object during the choice trial indicated that the groups did not differ significantly, (main effect of groups, $F(2, 20)=1.538$, $p=.247$). There was no significant main effect of counterbalancing, ($F(1, 20)=.648$, $p=.433$) and no significant group by counterbalancing interaction, ($F(2, 20)=1.436$, $p=.269$). When the two amphetamine groups were combined into a single group and compared to the saline group there was now a trend towards a significant difference between groups in the discrimination ratio $t(19)=1.895$, $p=.072$ (2-tailed). See figure 5.23. Also, see Table 5.4 for raw data on time spent with novel and familiar object in the choice phase.

Comparison of the discrimination ratio with chance level performance indicated that only the saline group differed from chance level performance, suggesting that amphetamine groups did not show a significant preference for the novel object (see Table 5.3).

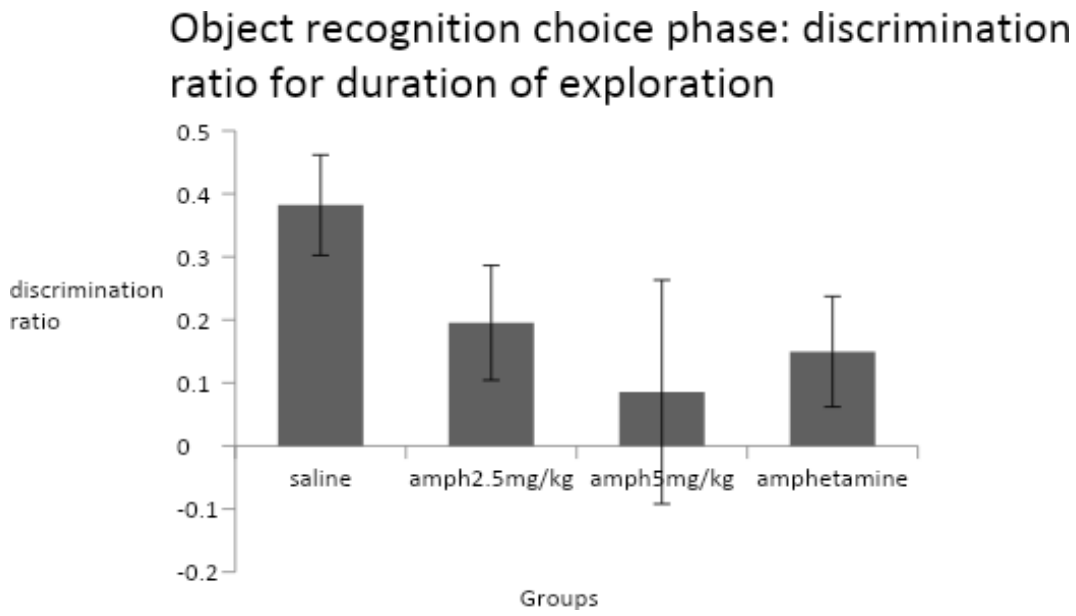


Figure 5.23. Mean (±SEM) Discrimination ratio for duration of exploration. Saline (n=9), amph.2.5 mg/kg (n=7) and amph.5mg/kg (n=5), combined amphetamine group (n=12).

	discrimination ratio duration
all groups (2-tailed)	t20=3.863, p=.001**
saline (2-tailed)	t8=4.796, p<.001***
AMPH (2-tailed)	t11=1.707, p=.116
AMPH2.5mg/kg (2-tailed)	t6=2.147, p=.075
AMPH5mg/kg (2-tailed)	t4=.482, p=.655

Table 5.3. Comparison with chance level performance for the discrimination ratio for duration of object investigation in choice phase for each group (one group t test vs a discrimination ratio of zero).

	saline		amph2.5mg/kg		amph5mg/kg		amphetamine	
object	**novel**	**familiar**	**novel**	**familiar**	**novel**	**familiar**	**novel**	**familiar**
min	5	1.1	4.4	2.8	3.3	1.9	3.3	1.9
q1	6.4	2.35	7.8	4.4	3.85	2.15	5.35	3.4
q2	12.3	5.3	13.1	10	6.3	4	10.75	8.95
q3	29.75	15.6	21.1	12.9	15	31.9	15.05	11.65
max	49.2	22.5	35.2	13	16.2	55.2	35.2	55.2

Table 5.4. Quartiles for each treatment group for time spent (seconds) with novel and familiar objects in choice phase.

5.5 Elevated plus maze

The elevated plus maze test was a pilot study with only a limited number of subjects (due to the fact that the mice were being used by another student for a different experiment).

5.5.1 Methods

Subjects

The saline group had 4 subjects, amphetamine 2.5mg/kg had 4 subjects and amphetamine 5 mg/kg had 4 subjects.

Apparatus

A white wooden plus maze was used with two open arms and two arms enclosed by grey painted walls of 21 cm height. The maze was elevated 70 cm above the floor. Each arm had a length of 35cm. The width of each arm was 6 cm.

5.5.1.1 Experimental Procedure

The test was conducted 3 months post-drug withdrawal. Each mouse was placed individually in the centre of the elevated plus maze. Its head was facing an open arm. Mice were left to explore the maze freely for 5 minutes.

Outcome measures

Outcome measures were the total distance travelled (pathlength), the number of entries into, and time spent in, the open and closed compartments, and latency to the first open-arm entry.

5.5.2 Results

Summary of results

All mice showed similar levels of activity in the maze, as indicated by the similar distance covered and similar number of entries into compartments between groups. All mice had similar latencies for their first visit to an open arm. While amphetamine mice did not differ from saline animals in the percentage of time spent in the open arms, the amphetamine pre-treated group as a whole had an increased percentage of entries into open compartments compared to the saline group, suggesting, if anything, lower levels of anxiety in these mice. Given that the sample sizes was too small for parametric statistics, results for total distance travelled, total number of entries, percentage of time spent in open arms and percentage of total entries into open arms must be interpreted with caution.

5.5.2.1 Total distance covered

One-way ANOVA was conducted to analyze the total distance that was covered during

the 5 min test. Results indicated no differences between the groups: (main effect of groups, F(2, 9)=.076, p=.927). Furthermore, an independent samples t-test for the 2 group analysis with the amphetamine treated animals now combined into a single group (n=8), also indicated that there was no significant difference between groups, t(10)=-.251, p=.243 (2-tailed). See figure 5.24.

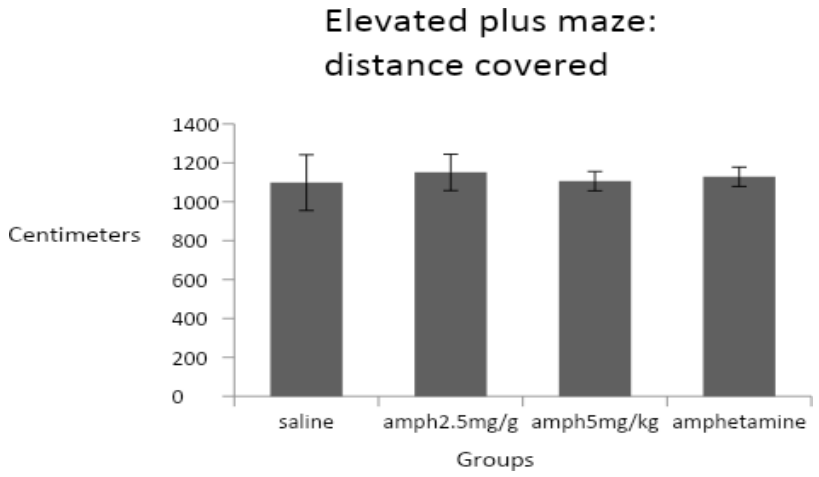


Figure 5.24. Mean (±SEM) total distance covered in cm. Saline (n=4), amph 2.5 mg/kg (n=4) and amph 5mg/kg (n=4), and the combined amphetamine group (n=8).

5.5.2.2 Total number of entries

One way ANOVA for data on total number of entries indicated that there was no significant difference between groups, (main effect of groups, F(2, 9)=1.376, p=.301). Likewise, an independent sample t-test for the 2 group analysis indicated that there were no significant differences between groups, t(10)=-.260, p=.800 (2-tailed). See figure 5.25.

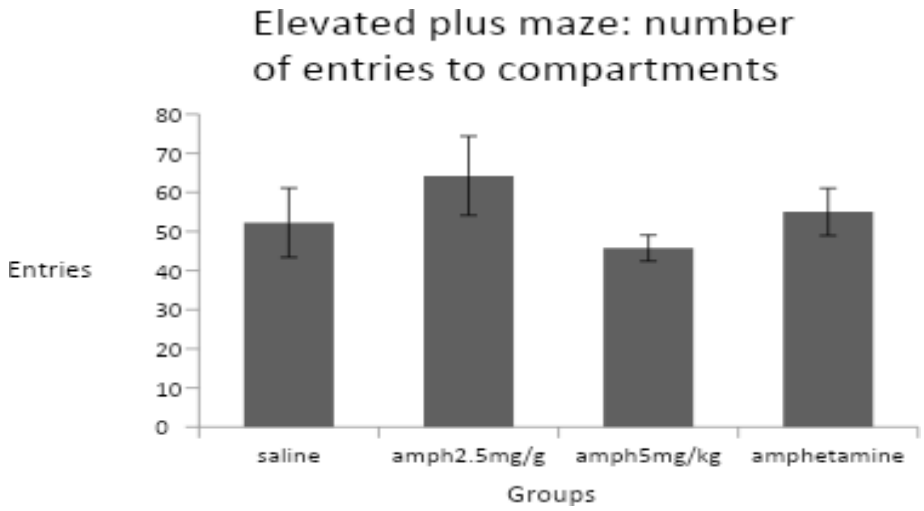


Figure 5.25. Mean (±SEM) total number of entries into compartments. Saline (n=4), amph 2.5 mg/kg (n=4) and amph5mg/kg (n=4), and combined amphetamine group (n=8).

5.5.2.3 Latency to first visit an open arm

Data for the latency to first visit an open compartment were not normally distributed even after ln, log and square root transformation and thus a non-parametric independent samples Kruskal Wallis test was conducted. Results indicated that groups did not differ in the latency to first visit an open compartment, H(2)=.177, p=.915. Furthermore, a Mann-Whitney test was conducted when the two amphetamine groups were combined into a single group and compared against the saline group. Results again indicated that there was no significant difference between thegroups, U=14.000 (z=-.404), p=.686 (2-tailed).

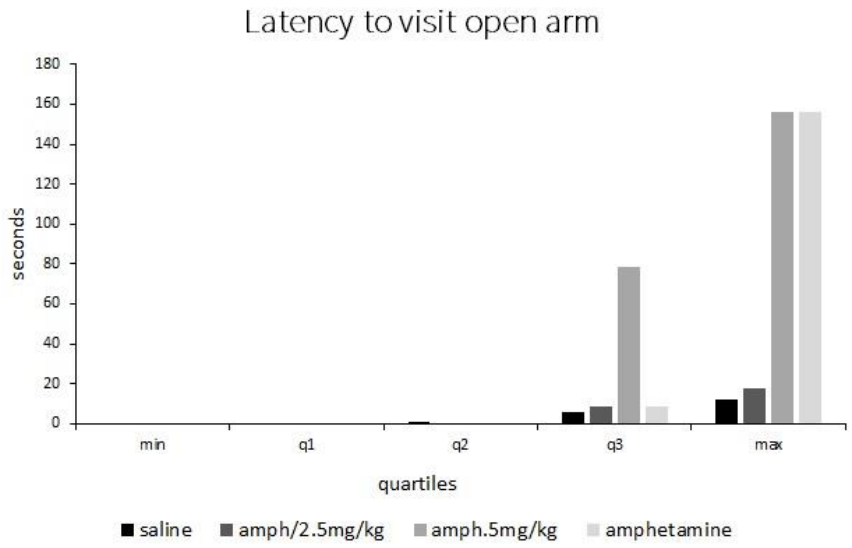


Figure 5.26. Quartiles of latency to visit an open arm. Q1=first quartile, Q2=median, Q3=third quartile, min=lower value, max=higher value. Saline (n=4), amph.2.5mg/kg (n=4), amph.5mg/kg (n=4), amphetamine (n=8).

5.5.2.4 Percentage of time spent in open arms

One way ANOVA for the percentage (%) of time spent in open arms indicated that there was no significant difference between groups [main effect of groups, ($F(2, 9)=.125$, $p=.884$)]. Furthermore, an independent samples t-test for the 2 groups analysis also indicated that there was no significant difference between groups [$t(10)=-.050$, $p=.205$ (2-tailed). See figure 5.27].

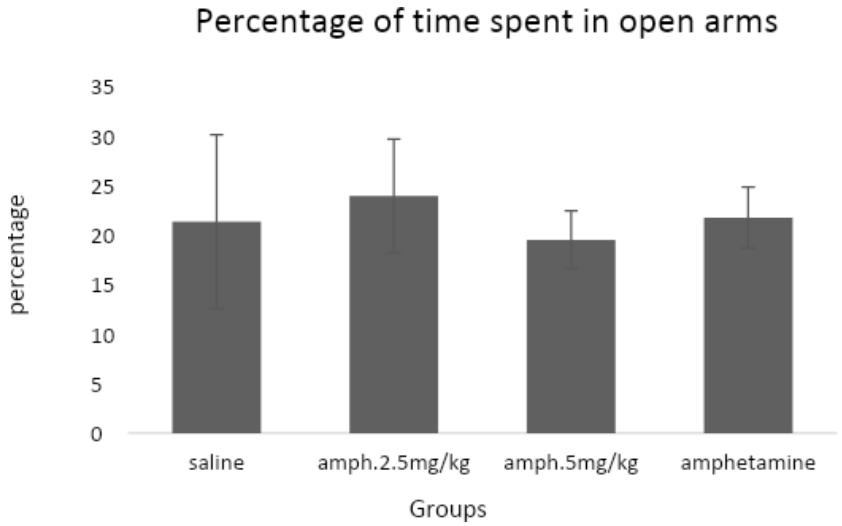


Figure 5.27. Mean (±SEM) percentage of time spent in the open arms out of the 5 minutes total duration of the test. Saline (n=4), amph.2.5 mg/kg (n=4) and amph.5mg/kg (n=4), and the combined amphetamine group (n=8).

5.5.2.5 Percentage of total entries into open arms

One way ANOVA for percentage of total entries made into the open arms indicated that there was no significant difference between groups [main effect of groups, (F(2 ,9)=2.210, p=.166)]. However, an independent samples t-test for the 2 groups analysis indicated that there was a significant difference between groups, t(10)=-.2.207, p=.046 (2-tailed), with amphetamine mice having a higher percentage of entries in the open arms which might suggest that they are less anxious. See figure 5.28.

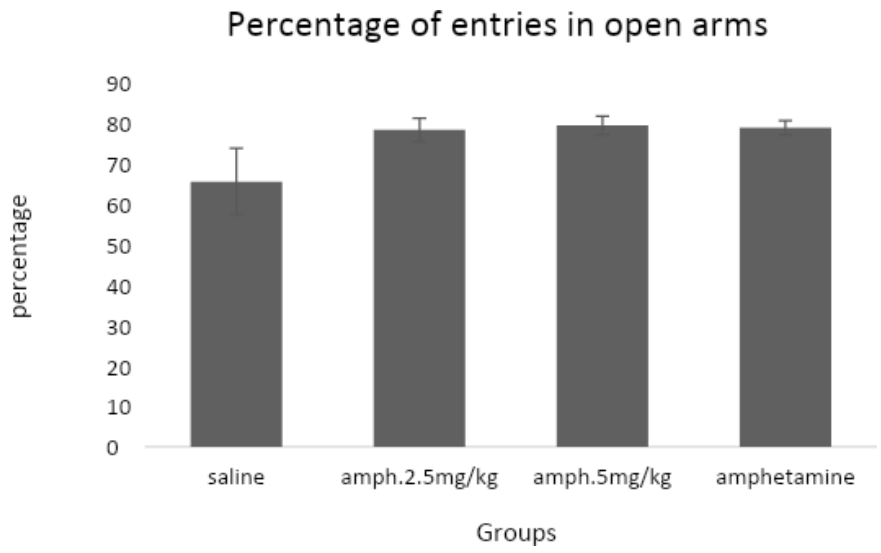


Figure 5.28. Mean (±SEM) percentage of entries into the open arms out of the total number of entries into closed and open arms. Saline (n=4), amph.2.5 mg/kg (n=4) and amph.5mg/kg (n=4), and combined amphetamine group (n=8).

5.6 Object in place test

5.6.1 Methods

Subjects

The saline group had 9 mice, the amphetamine sensitized group with 2.5 mg/kg had 8 mice and the amphetamine sensitized group with 5 mg/kg had 7 mice.

Apparatus

A plastic chamber 35x27x40cm was used. 4 metallic objects, 4x4x12 cm, were placed symmetrically with respect to the centre and corners. Pairing of objects with locations was counterbalanced across groups. See figure 5.29.



Figure 5.29. Object placement test: During the choice phase one of the four objects is shifted location.

5.6.1.1 Experimental Procedure

The experiment was conducted 8 months post drug treatment. Sessions were recorded and scored by an experimenter using an event recorder, blind to the group treatment condition. A mouse was considered to explore a stimulus when it touched or faced the stimulus at 1 centimeter distance maximum.

Habituation

Each mouse was placed in the novel chamber for 5 minutes under moderate, bright light illumination and was left free to explore.

Sample phase

One hour later each mouse was reintroduced into the chamber for 5 minutes with 4 metallic objects 4x4x12cm, of different shapes and colors. The four objects were placed near each corner of the box, at distance of 7cm from both adjacent edges. The objects were symmetrically placed with respect to the centre of the box.

Choice phase

One hour later each mouse was reintroduced for 5 minutes with one of the four

objects, counterbalanced across mice, rotated by 45 degrees translated by 4cm along the x-axis direction and 6cm along the y-axis direction, while the remaining objects were placed as before.

Outcome measures

Duration of object exploration was measured for each object separately in both sample and choice phases. Total duration of object exploration for each phase was calculated. A discrimination ratio was calculated in the choice phase by subtracting from the duration of investigation of the shifted object the average of the duration of investigation of the remaining three objects and dividing the difference by the total duration of investigation of all objects.

5.6.2 Results

Summary of results

There were no differences between mice in total object exploration, neither in the sample phase nor in the choice phase. Contrary to what was hypothesized, mice did not show a preference for the shifted object in the choice phase. Indeed, the amphetamine group as a whole, showed a preference for the familiar object.

5.6.2.1 Total duration of object exploration

Results indicated that groups did not differ in the total duration of object investigation neither in sample phase, ($F(2, 21)=1.822$, $p=.186$) (see figure 5.30), nor in the choice phase ($F(2, 21)=.402$, $p=.674$) (see figure 5.31). Results of the two groups analysis mirrored those for the 3 group analysis, both for the sample phase $t(22)=.151$, $p=.102$ (2-tailed) (see figure

5.30) and for the choice phase, t(22)=-.818, p=.422 (2-tailed) (see figure 5.31).

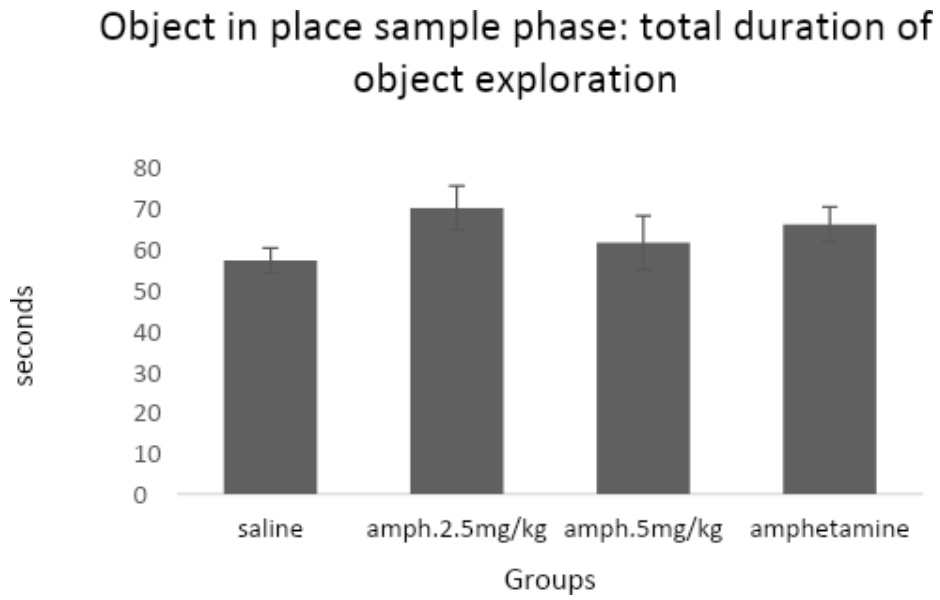


Figure 5.30. Mean (±SEM) total duration of investigation of objects in sample phase. Saline (n=9), amph.2.5 mg/kg (n=8) and amph.5mg/kg (n=7), combined amphetamine group (n=15).

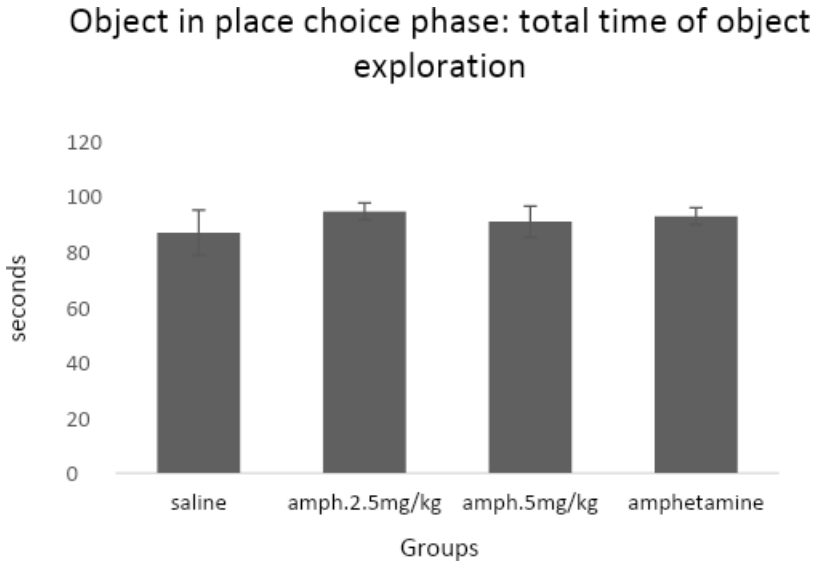


Figure 5.31. Mean (±SEM) total duration of investigation of objects in choice phase. Saline (n=9), amph.2.5 mg/kg (n=8) and amph.5mg/kg (n=7), combined amphetamine group (n=15).

5.6.2.2 Discrimination ratio

The discrimination ratio was not different between groups ($F(2, 21)=.718$, $p=.499$). In line with this finding, groups did not differ when the two amphetamine groups were integrated into a single group and compared with saline $t(22)=.195$, $p=.245$ (2-tailed). See figure 5.32.

Importantly, however, the discrimination ratio was at chance levels of performance for the saline group, $t(8)=-.085$, $p=.934$ (2-tailed), for amphetamine 2.5mg/kg, $t(7)=-1.619$, $p=.149$ (2-tailed), and for amphetamine 5mg/kg group, $t(6)=-1.526$, $p=.178$ (2-tailed), indicating that mice did not discriminate between the shifted object and the non-shifted objects. However, the amphetamine group as a whole did have a significantly different discrimination ratio from chance levels $t(14)=30.738$, $p<.001$ (2-tailed).

However, as shown in figure 5.32, the discrimination ratio had a negative value indicating that amphetamine pretreated mice spent more time with the non-shifted objects compared to the shifted object. Tables below present raw data for duration of investigation of each one of the 4 objects in the choice phase for the saline group (table 5.5), amph.2.5mg/kg group (table 5.6), amph.5mg/kg group (table 5.7) and the combined amphetamine group as a whole (table 5.8).

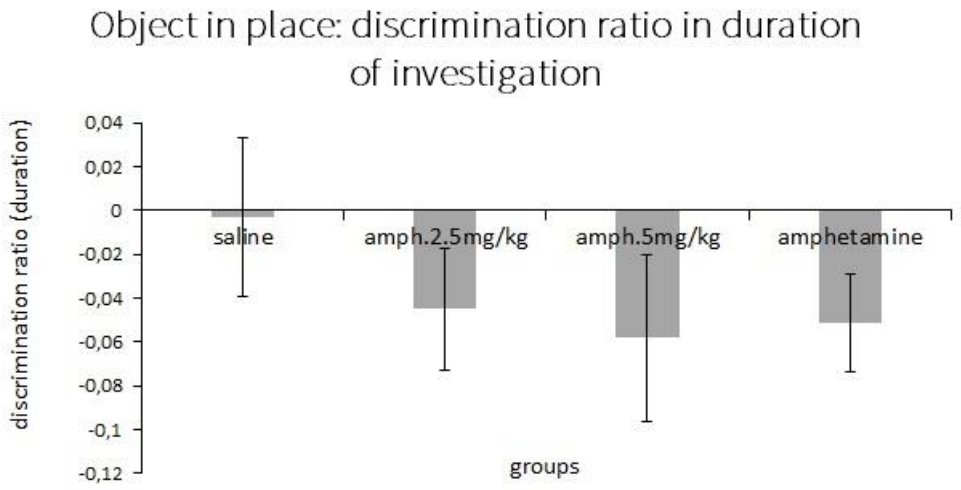


Figure 5.32. Mean (±SEM) discrimination ratio in duration of object investigation. Saline (n=9), amph.2.5 mg/kg (n=8) and amph.5mg/kg (n=7), combined amphetamine (n=15).

	shifted object	**object A**	**object B**	**object C**	**mean objects A-C**
average	205.5555556	186.8888889	292	181.6666667	220.1851852
standard deviation	66.41745085	66.10870677	157.916117	89.93330862	74.68621603
standard error of the mean	22.13915028	22.03623559	52.63870566	29.97776954	24.89540534

Table 5.5. Duration of object investigation for saline group, for each object separately.

	shifted object	object A	object B	object C	mean objects A-C
average	204.125	243.125	258.125	238.125	246.4583333
standard deviation	61.66832366	77.88533238	61.67990527	77.91834095	28.95148514
standard error of the mean	21.80304492	27.53662334	21.80713964	27.54829363	10.23589574

Table 5.6. Duration of object investigation for amph.2.5mg/kg group, for each object separately.

	shifted object	object A	object B	object C	mean objects A-C
average	185.5714286	180.2857143	258.8571429	281.8571429	240.3333333
standard deviation	72.01355692	60.38684814	49.95474142	123.2848038	51.03412076
standard error of the mean	27.21856609	22.82408323	18.88111752	46.59727591	19.28908456

Table 5.7. Duration of object investigation for amph.5mg/kg group, for each object separately.

	shifted object	object A	object B	object C	mean objects A-C
average	195.4666667	213.8	258.4666667	258.5333333	243.6
standard deviation	64.92948556	75.15906941	54.51456773	100.2973199	39.31036456
standard error of the mean	16.76472108	19.40598828	14.07560086	25.89665665	10.14989249

Table 5.8. Duration of object investigation for amphetamine group as a whole, for each object separately.

5.7 Discussion

Whether amphetamine sensitization impairs preference for novelty and/or the temporary reduction of attention paid to recently presented stimuli which are altered in schizophrenia and drug addiction (Kapur, 2003, Kapur and Mizrahi, 2005, Martinelli et al, 2018, Weiss et al, 2004) is not clear. In addition, no studies have screened the same cohort of rodents in a series of recognition tests across multiple stimulus domains (i.e. novel object recognition, object in place, social recognition) as used here. Also, the effects of withdrawal from a repeated amphetamine sensitizing regimen in social interaction have not been studied. This study is the first study to reveal reduced reciprocal social interaction after withdrawal from a repeated d-amphetamine regimen which induced sensitization. On the other hand, results in recognition memory tests used here do not support the hypothesis that withdrawal from amphetamine sensitization impairs recognition memory and/or preference for novelty.

5.7.1 Effects of withdrawal from repeated amphetamine on reciprocal social interaction and anxiety

More specifically, amphetamine sensitized mice with the 2.5mg/kg dose, as well as the amphetamine sensitized group as a whole, seemed to have impaired reciprocal social interaction. This is evident by the reduced duration of social contacts and increased duration of avoidance behaviours. No other factors appear to have contributed to this result, given that there was no significant group difference in non-social behaviours including stereotypies. It should be noted that we have data only for the first habituation day and not on the remaining habituation sessions, as well as that we have data only for duration of immobility and not on duration of activity which is a more accurate measure of habituation. Therefore, one could argue that results in the reciprocal social interaction test might have been due to increased

anxiety and thus reduced habituation to the context. This could have led to response competition as mice might have perceived the context as novel during the reciprocal social interaction trial and thus spent less time engaged in social interaction behaviours.

However, this seems unlikely as amphetamine pre-treated mice showed no significant differences with saline pretreated mice in the overall levels of exploratory behaviour in the open field test and in the levels of anxiety and exploratory behaviour in the elevated plus maze. It should be noted though that amphetamine mice showed an increased percentage of entries into the open arms in the elevated plus maze, which is an index of reduced anxiety levels compared to saline mice. However, reduced anxiety would have been expected to have led to increased social interaction in the reciprocal social interaction test not reduced. Also, other indices of anxiety in the elevated plus maze, including latency to the first visit to the open compartment and percentage of duration of time spent in the open arms were similar in all groups. Given that the elevated plus maze was just a pilot study, results should be treated with caution. While no increased levels of anxiety were found in the elevated plus maze one cannot rule out that the reduced social interaction could be due to context-specific socially driven anxiety.

A further possibility is that reduced social interaction might be driven by delusion-like or hallucinatory-like symptoms, that could lead mice to perceive a social context as extremely threatening. Such an explanation is in line with findings from non-human primates that were sensitized to amphetamine and which showed symptoms analogous to positive symptoms of schizophrenia including hypervigilance, checking, staring, tracking of non-apparent stimuli, grasping or manipulating of thin air and parasitotic-like grooming as well as fine-motor stereotypies (Castner and Goldman-Rakic 1999, Castner and Goldman Rakic, 2003).

It should be noted that there are two limitations in the reciprocal social interaction test, as a result of which one cannot exclude the possibility that reduced social interaction in

amphetamine pretreated mice was due to insufficient habituation. The first one is that only immobility was measured and not pathlength travelled which is a more sensitive measures of exploratory activity and hence of habituation. The second limitation is that only the first day of the six days of habituation was recorded and scored. Therefore, we cannot completely rule out the possibility that differences in social behaviours might have been due to different amounts of habituation to the context.

Impairment in the reciprocal social interaction could be interpreted as reduced ability to get pleasure from a social interaction, a phenomenon that resembles social anhedonia in schizophrenic patients. While this explanation is consistent with evidence of increased anhedonia in rodents that have very recently withdrawn from amphetamine sensitization (less than two weeks) (Leith and Barrett, 1980, Kokkinidis et al, 1986, Wise and Munn 1995), this test in the current study was performed at a much later withdrawal stage compared to other studies. Also, no specialized tests to measure anhedonia (i.e. ability to get pleasure from things that are supposed to be pleasurable for mice) were conducted in this cohort of mice, such as the hedonic taste reactivity test, sucrose consumption/preference test, conditioned place preference test, intracranial self-stimulation and sucrose administration. Performing these tests in mice that have withdrawn from the same regimen will provide evidence as to whether there is a general anhedonia problem caused by this protocol at this advanced point in time after amphetamine withdrawal.

5.7.2 Effects of withdrawal from repeated amphetamine on sociability and social recognition/preference for social novelty

This present study also provided evidence that amphetamine sensitized mice do not have impaired sociability under conditions when reciprocal social interaction is not allowed. This finding is potentially at odds with the impairment in reciprocal social interaction in the

previous experiment. Replication of tests with larger sample sizes should be done to verify these findings. In the case of the reciprocal social interaction test larger sample size is particularly important given that behaviour of pairs of mice is scored rather than the behaviour of individual mice. This has as a consequence that not only is there less data obtained but also the behaviour of one mouse might interfere with the behaviour of the other mouse of the pair. Therefore, some behaviours might be either magnified or minimized as a result of the interaction between mice.

The unimpaired sociability of amphetamine sensitized mice in the absence of direct physical contact, might indicate that the impairment of reciprocal social interaction found in the previous test is attributed to increased levels of socially driven anxiety in these mice. This is because unrestricted social interaction contexts are well known to be more anxiogenic compared to social contexts in which direct physical interaction is prohibited. This might explain the discrepancy of findings between the social interaction test and the sociability test. Also, in the reciprocal social interaction test, given that behaviour of pairs is measured rather than behaviour of individual mice, one could argue that the behaviour of one mouse mirrors the behaviour of the other and thus that any potential trends in behaviour are magnified (i.e. if a mouse is avoiding contact then the other mouse will stop trying to interact too). This could have also contributed to the discrepancy of findings between the reciprocal social interaction and the sociability test.

The social recognition test which constituted the second phase after the sociability test did not work properly. This is because the preference ratio for the novel conspecific in the social recognition test, was only marginally different from chance level in the saline group. It should be noted though that in case a 1-tailed t-test had been applied instead of 2-tailed, in light of the well-established finding about mice having an innate preference for novel over familiar conspecifics, the preference ratio for the novel mouse would have been statistically

significant. Therefore, replication of this study is required before any conclusions are drawn about the effects of amphetamine sensitization on social recognition.

5.7.3 Effects of withdrawal from repeated amphetamine on novel object recognition and object in place

Finally, it is potentially interesting that the amphetamine sensitized mice, contrary to saline treated animals, did not have a preference for novelty in the novel object recognition test. This does not seem to stem solely from either reduced time spent with the novel object or increased time spent with the familiar, but seems to result from a little bit of both (see table 5.4). Therefore, one could not conclude that the chance level performance in the amphetamine group in the choice phase is specifically due to impaired recognition or due to reduced preference for novelty. Also, it seems that this finding cannot be attributed to reduced object exploration by amphetamine pretreated mice in the sample phase, given that there were no differences in the total duration of object exploration between groups.

Also, it cannot be attributed to innate preference for one of the two objects or for one of the two locations given that the two different sets of identical objects that were used were counterbalanced across groups and given that the identity of the novel and familiar object and the left and right position of the novel object were counterbalanced across groups. No significant main effect of counterbalancing was found.

Replication of this study is required with larger sample sizes (see post hoc power analysis in section 2.1.1). Also, the same test could be re-done with more extended habituation of animals in the arena before testing. While a single habituation session was conducted here, the most common habituation protocol consists of three days habituation for 3 minutes twice daily, in order to ensure that stress and potential neophobia during choice phase are minimized and exploratory behaviour for the objects (and not the context) is

promoted (Leger et al, 2013). It should be noted that our finding of lack of difference between groups in the discrimination ratio in the choice phase is consistent with spared novel object recognition found in rodents which were abstinent from repeated amphetamine regimen (Che et al, 2013). On the contrary, this finding is inconsistent with long access to methamphetamine impairing performance in a novel object recognition test in rodents (Belcher et al, 2005, Reichel et al, 2011, Ito et al, 2007, Arai et al, 2009, Noda et al, 2010, Lu et al, 2010, Peters et al, 2016). However, these studies used different amphetamine regimens than the one used here.

Peleg-Raibstein et al (2009) findings showed enhanced spontaneous novel object preference in sensitized Fischer rats and impairment of spontaneous object recognition in sensitized Lewis rats. It is difficult to know how to interpret findings from this study compared with our finding of a lack of difference between groups in the discrimination ratio in the choice phase. However, one should bear in mind that this study was in rats rather than mice. Identification of the causes underlying such strain dependent effects is required. One possibility that was suggested was that intrinsic strain differences in the sensitivity of the dopaminergic system before sensitization might underlie the different results between strains not only as far as response to acute amphetamine challenge in non-sensitized mice is concerned (Di Chiara and Imperato, 1988) but also as far as the different effects after amphetamine withdrawal on memory that was found is concerned. It was suggested that the mesolimbic dopaminergic system may be more active in Fischer than Lewis rats. No baseline performance differences could have accounted for the discrepancy in findings between species.

Also, our finding is not in line with findings showing impaired novel object recognition in rodents after repeated methamphetamine (Janetsian et al, 2015a, Kamei et al, 2006, Belcher et al, 2006), short term neurotoxic methamphetamine regimen (Bisagno et al,

2002) and withdrawal from a single amphetamine dose (Belcher et al, 2008). However, discrepancy of our finding with findings from some of these studies might be attributed to different regimens producing different results. Overall, our findings are not sufficient to clarify the mixed literature on novel object recognition and thus further research is needed.

As far as object in place in concerned this test did not work, as saline mice did not show a preference for the shifted object, as expected. Replication of this test with larger sample size is required. Also, the test might be run differently i.e. with a larger arena so as there is a bigger distance between the four objects. The test should be also run with a squared rather than rectangular arena in order to ensure that there will be equal distance between all combinations of objects. Therefore, the mice are not expected to have a preference for one spatial arrangement versus another (i.e. objects that are close together or objects that are more far away). Also, shorter interval times (i.e. 5 minutes) between the sample and the choice phase should be tested as well as longer duration in sample and choice phases in an attempt to improve performance levels of the saline group. Also, multiple habituation sessions instead of the single one that was used here should be tried in order to ensure that stress and potential neophobia during choice phase are minimized and also that exploratory behaviour is promoted (Leger et al, 2013). Finally, the test could also be run differently, i.e., instead of one of the four objects being shifted in the choice phase, two of the four objects can exchange position in the testing phase. This will help because two out of the four objects will be in different location instead of one only and thus any effect might be enhanced. While the finding that amphetamine mice showed significant preference for the objects that were not shifted compared to the shifted object is potentially of interest and could reflect a form of sensitisation, it cannot be interpreted easily as evidence of impaired novelty preference given that the test did not work as expected for the saline group and given that no group differences were found.

Conclusions

In summary, amphetamine sensitization is well known to model positive symptoms of schizophrenia and amphetamine induced psychosis, while its potency to model negative symptoms and cognitive impairment has not been established. This is an important question as these symptoms are those that are less effectively treated by antipsychotic medication. Overall, the findings of the present study tentatively support the use of late withdrawal from amphetamine sensitization (i.e. after the early withdrawal period, which is considered to be after the first two weeks of withdrawal have passed) as a model for social withdrawal in schizophrenia. However, results do not support that anxiety and recognition memory deficits which are hallmarks of schizophrenia and drug addiction can be modeled by the withdrawal from amphetamine sensitization used in this study. Further studies are necessary to confirm these findings.

Chapter 6: General Discussion

The aim of the present thesis was to study the effects of amphetamine sensitization on spontaneous behaviours, and learning and memory, and to provide further insights into whether it can model aspects of cognitive impairment and negative symptoms in schizophrenia and drug addiction. This was achieved by testing mice that had been withdrawn from a repeated amphetamine regimen (and which were later confirmed to be sensitized in terms of their response to a subsequent amphetamine challenge) on social interaction, anxiety, recognition memory, preference for novelty, spatial working memory, spatial reference memory and pattern separation. Finally, simultaneously acquired (i) appetitive cue and (ii) spatial learning, which were previously studied by Ito and Canseliet (2010), were further investigated. Results tentatively suggest that amphetamine sensitization could potentially be used as a model for social withdrawal but not for anxiety and cognitive impairment in schizophrenia and drug addiction.

In the appetitive, simultaneous cue/place learning T-maze task (Ito and Canseliet, 2010) place learning was not acquired despite the 360 trials conducted. By the end of training there was no sign of leaning in any group. In the Ito and Canseliet (2010) study where the orientation of the T-maze was fixed, the extent of any spatial learning was also limited. It did not exceed 70% correct responses even after the 224 trials conducted. Possibly the protocol and the way the experiment was conducted might not be appropriate for spatial memory. Perhaps changes should be made such as using different types of cues instead of floor inserts or using more dissimilar wall inserts. Further studies could explore how the rotation of the maze might have prevented spatial learning acquisition. One possibility is that it might have made the task more complex. Also, one cannot rule out that mice were at chance level performance because they had been using a dorsal striatal dependent stimulus-response

strategy which would give them a reward on 50% of trials (Packard and Knowlton, 2002), thus relying on tactile or visual stimuli and turning left or right on every trial instead of developing cognitive maps to navigate. Such a strategy could have led to increased number of errors. Overall, no conclusions can be drawn about spatial learning in this task.

On the other hand, cue learning was acquired and, if anything, there was a non-significant trend for this to be improved in the amphetamine sensitized animals. On the contrary, Ito and Canseliet (2010) found attenuated cue learning. Our findings indicate that under conditions when spatial learning is not acquired, cue learning is spared in amphetamine sensitized mice. Normal cue learning in amphetamine sensitized micemight have been due to lack of interference between spatial and cue learning given that the former was not acquired. Also, results might be due to the attenuated performance of the control group compared with the control group in Ito and Canseliet study (2010). The performance of amphetamine mice was similar between our study and that of Ito and Canseliet (2010).

Normal cue learning in our study is inconsistent with evidence of enhanced appetitively driven cue learning in rodents exposed to repeated amphetamine tested using different types of paradigms than the one that we used here. Such tests did not involve simultaneous cue and spatial learning (Taylor and Robbins, 1984, Taylor and Horger, 1999, Wyvell and Berridge, 2000, Harmer and Philips, 1998, Taylor and Jentsch, 2001). Also, our findings do not support that amphetamine sensitization can model the impaired activity of amygdala in schizophrenia (Schneider et al, 1998, Calder et al, 2001, Gur et al, 2002, Takahashi et al, 2004). It might be that amphetamine sensitization alters the function of basolateral amygdala, dependent on the task and on which other brain structures are involved. Further research is required to elucidate the effects of amphetamine sensitization on spatial and cue learning for rewards when they take place simultaneously.

Overall, our results are not line with the results by Ito and Canseliet and do not

provide insights with regards to the effects of amphetamine sensitization on competition between hippocampus and basolateral amygdala when cue and spatial learning take place simultaneously. Finally, our results do not support the hypothesis that the rotation of the maze would increase spatial learning in amphetamine sensitized mice compared to the control group.

The study in chapter 4 was the first to examine the effects of amphetamine sensitization on incrementally acquired spatial reference memory for rewards and rapidly acquired spatial working memory, and the first to examine spatial pattern separation. Amphetamine sensitized mice had normal spatial working memory and pattern separation. Spatial reference memory acquisition was also normal, although there was a trend for subsequent retrieval of spatial reference memory to be facilitated. However, this was not significant. Overall, these results suggest that spatial learning and memory are normal in amphetamine sensitized mice. It should be noted that spatial working memory in mice may not be directly analogous to spatial working memory in humans, but rather could instead reflect short term habituation processes (for review Sanderson et al, 2010, Sanderson and Bannerman, 2012). Collectively, these findings together with findings from the T-maze experiment in chapter 3 do not support the use of amphetamine sensitization as a model of relevant aspects of cognitive impairment in schizophrenia. Also, results do not support the possibility that appetitively motivated incrementally acquired learning in amphetamine sensitized mice can be used as a model of facilitated (and potentially maladaptive) formation of associations between context and rewarding stimuli caused by aberrant salience attribution.

The fact that mice acquired the spatial reference memory task perfectly well possibly indicates that the lack of acquisition of the spatial component of learning in the T-maze task in chapter 3 (in which the same cohort of animals was used as in the radial maze) is a result of the particular T-maze protocol rather than anything to do with this cohort of mice. Spatial

learning was also not great in the Ito and Canseliet study (2010). There was no clear sign of learning (much above 50%) in any of the mice from the saline group of our study.

The normal acquisition of spatial reference memory for rewards in the radial maze is not in line with previous studies that showed facilitation of appetitively driven forms of learning in amphetamine sensitized animals, including conditioned place preference under conditions that cue and spatial learning compete with each other (Ito and Canseliet, 2010), Pavlovian approach behaviour (Taylor and Jentsch, 2001) and Pavlovian conditioning (Harmer and Philips, 1998). However, our finding is in line with another study showing that Pavlovian conditioning is normal in amphetamine sensitized animals (Eldred and Palmiter, 2013) and with the finding by Ito and Canseliet (2010) showing unaffected spatial learning by amphetamine sensitized animals in the T-maze under conditions when it competes with cue learning. Collectively, these findings show that amphetamine sensitization differentially affects appetitively driven learning dependent on the paradigm used.

It seems that when appetitively driven spatial learning does not directly compete with other forms of learning as in our radial maze experiment and in the T-maze experiment by Ito and Canseliet (2010), it remains unaffected. However, when it directly competes with cue learning, as in the radial maze experiment by Ito and Canseliet (2010) it can be enhanced. These findings should be seen collectively with findings showing unaffected (Russig et al, 2003, Ito and Canseliet, 2010) or impaired (Mandillo et al, 2003) spatial learning after withdrawal from a repeated amphetamine regime. In addition, facilitated spatial learning in the Morris water maze was shown only when withdrawal from repeated amphetamine is followed by acute amphetamine challenge before each session (Gelowitz et al, 1994). This indicates enhanced performance rather than enhanced acquisition. It is known that sensory, motor or motivational processes can affect performance in spatial learning tasks (Keith and Rudy, 1990, Cain et al, 1996, Cain, 1997). It might be that such processes might have been

affected by the allocentric version of the t-maze task that we used which might have led to impaired performance in the spatial learning trials in our saline group.

It seems based on both pharmacological studies using NMDA antagonists and glutamate receptor A knockout mice that neither impaired spatial learning acquisition nor impaired sensorimotor or motivational process can explain fully all data of impaired performance in spatial learning tasks (for review Bannerman et al, 2006). Performance can also be affected by hippocampal dependent plasticity which mediates flexible behavioral response selection based on contextual cues which can be trial specific (Schmitt et al, 2003, 2005). Such cues can be either spatial, temporal or related to internal states acting as occasion setters (Schmitt et al, 2004). Based on the above findings together with our findings, it seems that incremental acquisition of spatial learning which is not dependent on hippocampal NMDA, glutamate receptor A neuroplasticity (Bannerman et al, 1995, Good and Bannerman, 1997, Zamanillo et al, 1999, Schmitt et al, 2003) is unaffected by amphetamine sensitization. Therefore, neuroadaptations induced by withdrawal from repeated amphetamine increase hippocampus control over expression of behavioural output without facilitating the hippocampus dependent acquisition of associative spatial learning.

The unaffected spatial working memory for rewards that we found was consistent with some previous studies (Stefani and Moghaddam, 2002, Featherstone et al, 2008) but not with others (Nagai et al, 2007, Beatty and Rush, 1983). Differences between the tasks that were used to assess working memory in these studies, as well as differences in the length of delay intervals between different studies and differences between treatment protocols that were used, might have contributed to this discrepancy between findings.

Latency to acquire all three rewards in the radial maze was unaffected in amphetamine sensitized mice in the three group comparison in the first phase of the experiment (reference memory acquisition). However, in the two group comparison (i.e.

when the two amphetamine groups were combined) amphetamine mice seemed to have a reduced latency to complete a trial in the first phase of the experiment. This finding is unlikely to be an indication of facilitated memory acquisition given that the number of spatial reference memory errors was unaffected. Also, it cannot be seen as an indication of increased motivation given that mean latency per arm was not significantly different between groups. However, by looking at the graphs one can see that amphetamine mice seem to make less reference memory errors (although not significantly) and seem to have faster mean latency per arm (although again not significantly). Therefore, one cannot exclude the possibility that the significantly reduced latency to complete a trial between saline and amphetamine groups as a whole might be due to a collective effect on the reference memory errors and mean latency per arm. However, there is not enough evidence to conclude that this was the case. Interestingly, it was also shown that the faster mice ran the more errors they made, which could have been a result of increased impulsivity. However, separate group analysis showed that this finding was the case for the saline group and the amphetamine 5mg/kg group but not for the amphetamine 2.5mg/kg group or the amphetamine group as a whole. This is not inconsistent with the possibility outlined above, i.e. that in the saline group the faster mice ran the more errors they made, while this was not the case for amphetamine group as a whole and thus mean latency per arm and memory errors (none of which were significant but seemed to be to some extent different between groups in the graphs) could have collectively led to a significantly different latency to complete a trial (which is a measure that depends collectively on the number of errors and the mean latency per arm). Based on the present findings, however, there is not enough evidence to draw definite conclusions in this matter and thus further research is needed.

Results in chapter 4 are the first to reveal that amphetamine sensitized mice with 2.5mg/kg does, as well as the amphetamine group as a whole, have reduced social interaction

compared to saline mice in the reciprocal social interaction test. This finding possibly shows impaired ability to get pleasure from social interaction. This finding was not shown for the amphetamine group with 5mg/kg alone. The reason for this is not clear. Repetition of this study with large sample sizes is required to double-check whether an amphetamine sensitized group with 5mg/kg dose will mirror findings shown in the amphetamine 2.5mg/kg group. Overall, therefore, it seems that there is some evidence that two months withdrawal from amphetamine sensitization might induce a state that models social withdrawal and social anhedonia in schizophrenia. Another study has found depressive like symptoms between days 6 and 28 post amphetamine treatment. Such symptoms included functional impairment in nest building, an ethologically-relevant activity of daily living and reduced sucrose preference, the latter being evident on days 7-10 and 14-17 (Pathak et al, 2015). Further research is needed to test whether the social withdrawal at 2 months post drug withdrawal that we observed is accompanied by a general anhedonia problem that would model negative symptoms of schizophrenia more generally.

In contrast, results from the sociability test did not show any group differences. However, this is attributed possibly to the fact that the sociability test does not involve reciprocal social interaction between the two conspecifics. This is because one mouse is constrained and the other mouse is free. Thus, the sociability test is less anxiogenic. Results discussed in the next paragraph do not support that amphetamine sensitization increases anxiety per se. However, one cannot rule out the possibility that amphetamine sensitization might cause anxiety selectively under conditions of reciprocal social interaction with a novel conspecific, i.e. social phobia. This is because under such conditions a classic approach/avoidance conflict is generated. Thus, this test can cause conflict between the social threat and the social reward that are generated. This conflict can be anxiogenic (Kirlic et al, 2017).

More generally, however, results in chapter 4 do not support the use of amphetamine sensitization as a model of anxiety in schizophrenia and drug addiction. This might indicate, as suggested in the previous paragraph, that amphetamine sensitization causes anxiety selectively under conditions of reciprocal social interaction with a novel conspecific. Alternatively, it might indicate that results from the reciprocal social interaction test are not due to anxiety but due to anhedonia, thus an inability to get pleasure. However, there are some small potential limitations in the reciprocal social interaction test which have to do with the way the habituation phase was conducted prior to testing. In particular, only immobility was measured and not pathlength travelled. Also, only the first day of the six days of habituation was recorded. Therefore, it is unclear whether all mice had equally habituated to the arena before testing started. The levels of attention that are paid to the testing context, which are determined by the extent of habituation, might influence the levels of attention paid to the conspecific mouse.

Finally, results in chapter 4 from the recognition memory/novelty preference tests fail to provide support for the hypothesis that amphetamine sensitization can model recognition memory impairments and/or preference for novelty impairments which are characteristic of schizophrenia. While in the novel object recognition test amphetamine mice showed chance level performance there were no significant differences between groups in the preference for the novel stimulus which could have led to the conclusion of impaired preference for novelty or reduced recognition. These findings are inconclusive. Previous studies have shown mixed results with others showing no effect on novel object recognition (Che et al, 2013) and others showing compromised (Belcher et al, 2005, Reichel et al, 2011, Arai et al, 2009, Noda et al, 2010, Lu et al, 2010, Peters et al, 2016, Janetsian et al, 2015a, Kamei et al, 2006, Belcher et al, 2006, Bisagno et al, 2002, Peleg-Raibstein et al, 2009) or enhanced novel object recognition (Peleg-Raibstein et al, 2009) . However, the studies that show reduced preference

for novelty and/or recognition, have been performed with animals that had either withdrew from methamphetamine or were under the influence of methamphetamine at the time of testing. On the contrary, we used d-amphetamine. Also, different regimens (i.e. doses, duration and frequency of drug exposure) from the regimen that we used in the present study, were used in the studies that found reduced preference for novelty. The differences in drug treatment protocols between these studies and our study could have contributed to the different findings.

Overall, research with regards to novel object recognition following amphetamine sensitization is inconclusive. In the present work, the object in place test did not work properly as saline mice did not prefer the shifted object as expected. Thus, no conclusions can be drawn with regards to any findings from this test.

Finally, there was only a trend in the preference for novelty in the social recognition test from chance level performance when using a 2-tailed t-test for the saline group. Given that this difference was not statistically significant one cannot draw any firm conclusions. However, it should be noted that if a 1-tailed t-test had been conducted on the basis of the well-known preference of mice for novelty, then there would have been significant preference for novelty in the saline group. There was a strong trend for amphetamine sensitized mice with 5mg/kg to have reduced preference for the novel conspecific compared to the saline group. However, this was not significant. Also, given that the saline group did not have significantly strong preference for novelty compared to chance levels no conclusions can be drawn.

Overall, results in the object in place and social recognition memory tests show that the performance of the saline group was not as expected and thus no definite conclusions can be drawn. Repetition of the same tests is required with larger sample sizes and using different protocols. Also, results from novel object recognition test are inconclusive.

Directions for future research

These studies should be replicated with larger sample sizes in order to verify all findings. In addition, changes to some of the experimental protocols are also recommended. For instance, in all recognition tests replication of the study is recommended with more extended habituation sessions to the experimental context. The most common protocol involves three days habituation for 3 minutes twice daily. The purpose of this habituation is to exclude the possibility of neophobia induced stress as well as the possibility of competition for attention between the experimental context and the target stimuli which could be caused by insufficient habituation at the start of the test which could interfere with exploratory behaviour for target stimuli (Leger et al, 2013). Also, in the object in place test, replication of this test with a larger and square arena is recommended, which will ensure that objects are separated by greater distances and that the distance between them is equal. In the same test, two out of four objects can exchange position instead of just one object being shifted. In addition, shorter interval times between the two phases should be tried (i.e. 5 minutes) and a longer duration for the sample phase in an attempt to improve memory performance in the control groups. Finally, one should try using triplicate objects in the test phase (i.e. different copies from those used in the sample phase) in order to avoid using the animals own scent on the objects.

As far as the social interaction test is concerned, habituation sessions should be assessed on all days and pathlength should be recorded in addition to immobility during these habituation sessions. Further screening should be conducted to test whether these animals have any general anhedonia-like symptoms such as the hedonic taste reactivity test, the sucrose consumption/preference test, the conditioned place preference test, intracranial self-stimulation and the sucrose administration test. Also, assessment of licking behaviour, through lick clusters that are used to determine palatability which indicates how much the

mice like a solution, will be informative in the overall assessment of anhedonia more than measurement of consumption alone (Lydall et al, 2010).

With regards to the T-maze test, the test should be repeated with changes in the protocol. For example, cue and spatial trials could be less intermingled, so as there is less interference. This will ensure that this potential interference has not contributed to the insufficient learning in both our study and the Ito and Canseliet (2010) study. Finally, pattern separation should be studied using other protocols that involve smaller differences between similar memory traces. This could be achieved by using arms that are more close to each other, i.e. in an 8 or 12 arm radial maze.

Interpretation of our findings within the context of the wider literature

Findings of this study with regards to amphetamine sensitization should also be seen in the context of the wider literature that compares different animal models of schizophrenia. The amphetamine sensitization model of schizophrenia is based on the direct manipulation of the dopaminergic system. On the other hand, other pharmacological models of schizophrenia including PCP, ketamine and MK-801, all of which are NMDA receptor antagonists, manipulate the glutamatergic system. These models are based on the glutamatergic hypothesis of schizophrenia, that is based primarily on the hypofunction of NMDA receptors underlying positive but mostly negative and cognitive symptoms of schizophrenia (for review Goff and Coyle, 2001, Moghaddam, 2003, Jones et al, 2011, Javitt et al, 2012, Lin et al, 2012). These symptoms are reproduced by NMDA antagonists (Krystal et al, 1994).

There are a lot of commonalities between these models and amphetamine sensitization. Like amphetamine, ketamine (Krystal et al, 1994) induces delusions and hallucinations in healthy individuals with the latter exacerbating these symptoms in schizophrenic patients (Javitt and Zukin, 1991). Also, like amphetamine, recreational use of

PCP in humans can cause long lasting and persistent psychotic symptoms (Rainey and Crowder, 1975). Overall, it is clear that both amphetamine sensitization and models that are based on NMDA receptor antagonism can mimic the positive symptoms of schizophrenia.

However, the necessity to establish models for negative symptoms and cognitive impairment is strong. This is because there is a negative impact of cognitive and social dysfunction on patient functionality. Also, because the pharmacotherapy that treats those symptoms is not as efficient as is the pharmacotherapy that treats the positive symptoms. Amphetamine sensitization has been traditionally considered to have poor validity as a model for cognitive impairment and negative symptoms of schizophrenia. On the contrary, models that manipulate the glutamatergic system have been considered as models not only for positive symptoms but also for cognitive impairment and negative symptoms of schizophrenia (Olney et al, 1999). The current findings that amphetamine sensitization might model social withdrawal puts into question the view that amphetamine sensitization cannot model any negative symptoms. On the other hand, the present findings support the view that amphetamine sensitization cannot accurately model cognitive impairment in schizophrenia. These findings thus change only to some extent, the way that amphetamine sensitization is considered in comparison to other animal models of schizophrenia.

The fact that both amphetamine sensitization and glutamatergic models of schizophrenia induce symptoms analogous to the positive symptoms of the disease is not the only commonality between them. Sensitization appears to happen after repeated PCP exposure, as it happens with repeated amphetamine. Also, like amphetamine, PCP sensitization is expressed in animals that had been pre-exposed to PCP by hyperlocomotion in response to a further acute PCP challenge. In both amphetamine and PCP animal models, there is a variety of different protocols that are used between different labs. Most commonly, they involve some days of drug administration followed by an abstinence period before

experimentation starts. This happens in order to ensure that any findings are the consequence of the repeated regime and not due to the immediate acute drug effects. Also, in different labs, several different species are used, as well as different drug doses. Finally, the period that the drug is administered for varies from lab to lab. These variations make more complex the comparison between studies from different labs. In this respect, it should be noted that for several experiments conducted in the current thesis, including the spatial working memory task as well as the recognition tests, the literature seems to be inconclusive.

While we found no effect of amphetamine sensitization in these tests, there are studies that support and others that do no support our findings. Variations in the protocols used in different labs could have contributed to these discrepancies in the findings. Similar problems exist in the discrepancy of findings between studies with glutamatergic models of schizophrenia which are possibly caused by the variations in the regimens administered between different labs. Overall, it seems that there are commonalities between amphetamine sensitization and glutamatergic animal models of schizophrenia. These commonalities make the comparison between our findings and findings from experiments in glutamatergic animal models both possible and meaningful.

It is well established that amphetamine sensitization is not only a model for schizophrenia but also for drug addiction. Behavioural sensitization animal models for addiction, which are based on the incentive sensitization theory for addiction (Robinson and Berridge, 1993), are also induced by other drugs including cocaine and morphine (Babbini and Davis, 1972) and ethanol (the latter only in mice but not in rats) (Didone et al, 2008, Bahi and Dreyer, 2012 a, b). These drugs can also cross-sensitize, which means that a different drug can be used to treat/sensitize animals than the drug that is later used to administer the acute challenge. There is evidence of amphetamine and cocaine cross-sensitization (Vanderschuren et al, 1999), as well as amphetamine and morphine (Vezina and Steward,

1990, Vanderschuren et al, 1997), although cross-sensitization between amphetamine and morphine is not a consistent finding (Vanderschuren et al, 1999). The incentive sensitization theory for addiction is based on the potentiation of hyperlocomotion in response to acute drug challenge following withdrawal from a repeated drug regimen. The cross-sensitization between different drugs indicates that the sensitization model reflects the underlying mechanism that mediates addiction to all drugs.

However, the face validity of behavioural sensitization models for addiction is poor. This is because modelling sensitization in humans is challenging. Also, while in animals only a few drug injections can induce sensitization, in humans longer drug exposure is required to induce addiction. Due to this reason, behavioural sensitization models do not have face validity in modelling the progressive transition phase from the initial exposure to substance abuse (Vanderschuren and Pierce, 2010). This is despite the fact that they have the advantage that they can model long-term effects of chronic drug exposure given that sensitization can last up to a year (Paulson et al, 1991).

As already suggested, the present findings show that amphetamine sensitization possibly can model social withdrawal in schizophrenia. No other negative symptoms were studied in the present thesis. In this respect it should be of interest to compare this finding with findings on negative symptoms from studies using models that manipulate glutamatergic systems. [It should be noted that NMDA antagonist ketamine is being trialled as a rapid acting anti-depressant (Berman et al, 2000, Zarate et al, 2006, 2012, Rasmussen, 2016)]. In healthy humans that are on PCP, apathy and social withdrawal have been found (Luby et al, 1959) which are analogous to negative symptoms in schizophrenia. Also, PCP produces cognitive impairment which does not persist after discontinuation of the drug (Cosgrove and Newell, 1991, Javitt and Zukin, 1991). In line with this, social withdrawal seen in schizophrenic patients is modelled in animals by PCP (Lee at al, 2005, Qiao et al, 2001,

Snigdha and Neill, 2008b, for review Snigdha and Neill, 2008a, Neill et al, 2010), ketamine (Becker & Grecksch, 2004, Georgiadou et al, 2014, Zoupa et al, 2019, Onaolapo et al, 2017, Uribe et al, 2013, Vasconcelos et al, 2015) and MK-801 (Rung et al, 2005). While all these compounds are used to model social withdrawal in schizophrenia in animals, phencyclidine (PCP) is the most commonly used compared to MK-801 and ketamine (for review see Neill et al, 2010).

Glutamatergic models of schizophrenia not only have face validity, but also predictive validity, given that atypical antipsychotic medication ameliorates schizophrenic symptoms (for review Goff and Coyle, 2001). This is supported by the following animal studies presented in this paragraph. Social withdrawal induced by MK-801 appears to be reversed by aripiprazole, an atypical antipsychotic (Faure et al, 2019). In addition, reduced social interaction induced by ketamine appears to be ameliorated by the use of positive allosteric modulators (PAMs) of Alpha 7 nicotinic acetylcholine receptors (Nikiforuk et al, 2016, Potasiewicz et al, 2017) and reversed by SB-269970 (1 mg/kg), a 5-HT7 receptor antagonist (Hołuj et al, 2015). Also, chronic Bi-acetylated l-stepholidine (l-SPD-A), a novel derivate of l-stepholidine (l-SPD), which possesses a pharmacological profile of D(1)/5-HT(1A) agonism and D(2) antagonism, improved the social interaction deficit that was induced by PCP (Guo et al, 2009). In line with this, Bruins et al (2005) provided evidence that PCP induced social withdrawal was reversed by the antipsychotics, SSR181507, a selective dopamine D2 receptor antagonist and aripiprazole, a 5-HT1A receptor agonist. Also, Takeuchi et al (2019) found that Blonanserin, an atypical antipsychotic ameliorates social deficits through dopamine-D_3 receptor antagonism. Consistently, Wang et al (2007) showed a synergistic effect of galantamine with risperidone on the impairment of social interaction in phencyclidine-treated mice.

Overall, these studies show that PCP induces social withdrawal reminiscent of

schizophrenia and that this can be reversed by atypical antipsychotics. While there is occasional evidence that social interaction is not impaired by PCP in animal models for schizophrenia (Jenkinse et al, 2008), the majority of studies support that animal models for social withdrawal in schizophrenia which manipulate the glutamatergic system have good face and predictive validity.

It is also of interest to note that while we found reduced social interaction, findings from other studies that used sensitized animals as models of drug addiction are inconsistent. It has been shown that morphine sensitized animals (Nocjar and Panksepp, 2007) have increased interest for social behaviours. This finding collectively with other findings that amphetamine sensitized animals (Fiorino and Phillips, 1999 a, b) as well as morphine sensitized animals (Nocjar and Panksepp, 2007) have facilitated sexual behaviour, might be indicative of sensitization activating reward seeking. The later study also found facilitated food seeking. These findings are not in line with the fact that drug addicts are not interested in natural and social rewards. Thus, it might be that the model that we used in this study is superior in modelling this aspect of drug addiction than the behavioural sensitization model used in those studies. Assessing sexual behaviour in the model that we used will also be of interest.

On the other hand, it should be noted that in line with our findings, there is evidence of reduced social interaction following withdrawal from repeated MDMA in adolescent rodents (Fone et al, 2002). However, no locomotor sensitization test was conducted in that study to confirm that sensitization had taken place. Also, it is of interest that while we found no anxiety in amphetamine sensitized mice, studies with cocaine models of drug addiction have shown increased levels of anxiety in animals that withdrew from repeated cocaine (Valzachi et al, 2013, Perrine et al, 2008). Increased anxiety has also been found during withdrawal from a morphine sensitizing regimen (Masukawa et al, 2020). Overall, these

findings show that contrary to our finding that amphetamine sensitization does not model anxiety, other behavioural sensitization models of drug addiction, such as cocaine and morphine, do model anxiety.

In line with our finding that amphetamine sensitized mice did not have increased levels of anxiety, evidence suggests that the social withdrawal observed in PCP animals was not due to increased levels of anxiety either (Seillier and Guiffrida, 2017). The present finding of social withdrawal induced by amphetamine sensitization challenges the view that only glutamatergic models of schizophrenia but not amphetamine sensitization can model negative symptoms of schizophrenia. However, whether the impairment observed in this study can be reversed or ameliorated by antipsychotic medication has not been tested. However, even in the event that antipsychotic medication is found effective in reversing this impairment, such a result would not indicate strong predictive validity of the model given that antipsychotic medication cannot completely treat negative symptoms. Also, there are limitations of the present study that have already been discussed. Also, it should be noted that there are a lot of different studies that support consistently that glutamatergic models of schizophrenia can model social withdrawal. On the contrary, this is the first such study showing social withdrawal with amphetamine sensitization. Thus, further studies with amphetamine sensitization are required to confirm these findings.

Finally, there is evidence that subchronic PCP does not impair olfaction and interest for social odor in the olfactory habituation/dishabituation task (Tarland and Brosda, 2018). The same test should be conducted in amphetamine sensitized mice before excluding the possibility that the olfactory ability of sensitized mice is compromised, which could have led to compromised performance in the social interaction test. This is crucial because rodents rely on olfactory stimuli for a wide repertoire of social behaviours. Among these is recognition of conspecifics as well as sexual behaviours (Leypold et al, 2002, Arbuckle et al, 2015, Lim and

Young, 2006).

An additional reason that makes olfactory testing in amphetamine sensitized mice of interest in the interpretation of any findings relevant to social behaviour are findings in schizophrenic patients assessed with the University of Pennsylvania Smell Identification Test (UPSIT) showing deficient olfactory function (Moberg et al, 1997). It was suggested by the authors that these findings might reflect dysfunction in olfactory brain regions. In line with this finding, another study found impaired olfactory stimulus processing in schizophrenia (Turetsky et al, 2003).

It is also noteworthy that consistent with the pharmacological models of schizophrenia are findings from studies on genetic models for schizophrenia. For instance, the microtubule-associated protein (MAP)6 KO model, a microtubule-deficient model of schizophrenia that exhibits severe behavioural disorders that are associated with synaptic plasticity anomalies, seems to have reduced social investigation in the modified resident intruder test with both an awake and anaesthetized living intruder (Volle et al, 2013, Andrieux et al, 2002, Bégou et al, 2008).

Also, other studies provided evidence that neonatal lesions of the rat ventral hippocampus, another widely used model for schizophrenia, induced social interaction deficits (Sams-Dodd et al, 1997, Becker et al, 1999, Ouhaz et al, 2014) that were not normalized with clozapine (Sam Dodd et al, 1997). Also, like adult ventral hippocampal lesion models (Bannerman et al, 2002a, Bannerman et al, 2004) the neonatal lesion model reduces anxiety in the elevated plus maze (Schwabe et al, 2006). However, this is not in line with the study by Ouhaz et al (2014) mentioned above which showed increased anxiety levels, in addition to social withdrawal, in rodents with neonatal ventral hippocampus lesions. Further research is needed with amphetamine sensitization in order to ensure that it is as good a model for social withdrawal in schizophrenia as are the rest of these models. Such research

will lead to more accurate comparisons between the amphetamine sensitization animal model and other models for social withdrawal in schizophrenia.

While social withdrawal appears to be modelled by both glutamatergic models (Ellenbroek and Cools, 2000) and amphetamine sensitization, this is not the case for other behaviours that are relevant to the negative symptoms of schizophrenia. Neither PCP, ketamine nor MK-801 induce impairments in the sociability test in which one mouse is free and the other is constrained (Hanks et al, 2013). In that study both acute and subchronic regimens were tested. This finding is in line with findings from other studies on PCP (Sellier and Giuffrida, 2016) and MK-801 (Deiana et al, 2015), as well as with our study showing unaffected social approach in amphetamine sensitized mice. On the other hand, there are studies that showed impaired social approach in adolescent mice that had been given acute MK-801 (Moy et al, 2013, Kraeuter et al, 2020) and PCP (Liang et al, 2018, Spano et al, 2010). In these studies the impairment was shown after acute drug treatment, with the exception of the study of Spano et al (2010), in which the impairment was shown after both an acute and repeated regimen. In line with the study by Spano et al (2010), there is one study that found reduced sociability five days after withdrawal from a repeated ketamine regimen (Ogundele and Lee, 2018). Results from another study showed that acute but not sub-chronic administration of phencyclidine produced a reduction in measures of sociability (McKibben et al, 2014). Further studies should be conducted on sociability with both amphetamine sensitization and in glutamatergic animal models for schizophrenia before definite conclusions are drawn.

While the study by Seillier and Giuffrida (2016) showed that sociability was intact, preference for social novelty was reduced, which indicates either an impairment in social recognition or a deficit in short-term habituation for social stimuli. This finding is in line with the study with PCP by Liang et al (2018) and the study on MK-801 by Deiana et al (2015),

both described above, which showed an impairment in preference for social novelty and social recognition respectively. This is also in line with other studies (Timić Stamenić et al, 2015, Koo et al, 2020, Jeon et al, 2017, Savonenko et al, 2008) which showed that MK-801 treated mice did not show preference for social novelty, unlike the control group. On the other hand, the study by McKibben et al (2014) described above showed no effect on preference for social novelty while sub-chronic administration of phencyclidine had no effect on sociability or social novelty. As far as withdrawal from a repeated ketamine regimen is concerned, there is evidence that it induces social novelty deficits (Jeevakumar et al, 2015).

Overall, therefore, the literature is inconclusive with regards to the effects of glutamatergic models on social recognition. Also, these studies cannot be compared with our findings given that in our study the saline group was only marginally significantly different from chance level performance in the preference for novelty ratio. Further studies with amphetamine sensitization as well as with glutamatergic models should be conducted in testing social recognition before any definite conclusions are reached.

Also, there is evidence that the PCP model does not mimic anhedonia in schizophrenia given that it fails to induce any significant difference in sucrose intake (Jenkins et al, 2010) which is considered, at least to some extent, relevant to reward processing. However, performance in this test cannot fully model human anhedonia. Overall, compared with all other tests that are used to study negative symptoms in schizophrenia, assessment of social withdrawal by the reciprocal social interaction test is the most straightforward compared to other negative symptoms of schizophrenia including apathy or flattened affect. Such symptoms seem to be more difficult to identify and measure in animal models. However, tests such as the hedonic taste reactivity test, the sucrose consumption/preference test, the conditioned place preference test, intracranial self-stimulation, the sucrose administration test and tests that assess palatability using lick clusters, as explained above

(Lydall et al, 2010), are useful in showing whether there is an anhedonia deficit.

As far as cognitive impairment is concerned, results from the present studies overall do not provide support that amphetamine sensitization is a good model for cognitive dysfunction. Unfortunately, the present results were inconclusive. We showed that novel object recognition was unaffected by amphetamine sensitization as shown by the lack of a difference between groups, but at the same time the amphetamine group but not saline group had chance level performance. On the other hand there is evidence that acute PCP induces a novel object recognition impairment which can be improved by acute administration of clozapine and risperidone (Grayson et al, 2007). In line with this, the subchronic PCP model induced impairment in novel object recognition which was ameliorated by subsequent administration of clozapine (Hashimoto et al, 2005). These findings show that glutamatergic models for schizophrenia might be superior to amphetamine sensitization in modeling cognitive impairment of the disease. Consistently, a study by Nagai et al (2009) showed that PCP induced impairment in object recognition was ameliorated by aripiprazole. While these findings show that antipsychotic medication improves PCP induced object recognition, one cannot fully argue that these findings show that PCP model has very high predictive validity given that antipsychotic medication has only limited efficacy in treating cognitive impairment in schizophrenia (for review Karam et al, 2010, Pratt et al, 2012, Dunlop et al, 2015).

The potency of ketamine to model novel object recognition impairments is less widely studied. Still there is some evidence that ketamine can model in animals a novel object recognition deficit (Pitsikas et al, 2008) which can be attenuated by mGluR5 positive modulators in mice (Chan et al, 2008). In line with this, MK-801 seems also to model in animals the novel object recognition deficit (King et al, 2004). This deficit seems to be reversed by pretreatment with clozapine but not haloperidol (Karasawa et al, 2008). Overall, these findings provide support that glutamatergic models for schizophrenia are good models

for novel object recognition impairment, either through causing recognition impairment in animals or through causing deficits in habituation. Further research should be conducted with amphetamine sensitization, given that its literature on novel object recognition is mixed.

Finally, with regards to spatial working memory, there is evidence that acute PCP produces spatial working memory impairments in non-human primates (Boyce et al, 1991, Rupniak et al, 1991). In line with this, Zemanova et al (2013) provide evidence that MK-801 can model a visuospatial working memory deficit. Consistently, repeated ketamine exposure appears to lead to a spatial working memory deficit (Enomoto and Floresco, 2009). This deficit appears to be reversed by acute GABA type A receptor subunits alpha2/alpha3 (A alpha2/3) agonist administration and ameliorated by D1 receptor agonists (Roberts et al, 2010, Nakako et al, 2013). Also, Castner et al (1998) provided evidence of spatial working memory deficits post-puberty in an animal model of schizophrenia involving fetal x-irradiation in monkeys. In line with this, the neonatal ventral hippocampal lesion model for schizophrenia appears to produce spatial working memory deficits (Brady et al, 2010, Chambers et al, 1996). Overall, both neonatal lesion models and glutamatergic animal models for schizophrenia seem to induce working memory deficits. Further research is required with amphetamine sensitization and working memory tasks given that the literature is more mixed in order for more accurate conclusions to be drawn.

Overall, our findings tentatively support the face validity of amphetamine sensitization to model social withdrawal in schizophrenia. This finding is of interest given that negative symptoms of schizophrenia are not adequately treated by second generation antipsychotic medication and given that the development of new drugs has been inadequate too (Hanson et al, 2010). However, further research should be conducted to verify this finding. Our findings do not yet support the use of amphetamine sensitization as a model for cognitive impairment in schizophrenia. However, some of our findings cannot be interpreted

as evidence that amphetamine sensitization cannot model cognitive impairment given that in some of them the performance of the saline group was inadequate. Given that the literature is also inconclusive with regards to the potential of amphetamine sensitization to model cognitive impairments further research should be conducted. Overall, glutamatergic models and neonatal lesion models seem to be better models for cognitive impairment in schizophrenia.

Conclusions

Our results may support that amphetamine sensitization can model social withdrawal in schizophrenia but only under conditions in which reciprocal social interaction is allowed. Further testing is required to verify this finding. Neither reduced novelty preference nor reduced recognition memory performance was found. Nonetheless, replication of these studies is required under conditions which yield better memory performance levels in the controls. Results do not support that amphetamine sensitization can induce anxiety. Also, results do not support that amphetamine sensitization can produce spatial working memory deficits, a spatial pattern separation impairment, or a reduction in cue learning when it takes place in parallel with spatial learning.

Also, amphetamine sensitization does not appear to model facilitated formation of long-term associations between contextual cues and rewarding stimuli that are seen in schizophrenia and drug addiction. These results overall do not support that amphetamine sensitization can model aspects of cognitive impairment in schizophrenia. This is in line with many relevant previous studies. The fact that amphetamine sensitization can possibly model social withdrawal in schizophrenia is of interest given that current pharmacotherapy is more effective in treating positive symptoms compared to negative symptoms and cognitive impairment (Nuechterlein et al, 2004, Keefe et al, 2007) and thus the amphetamine

sensitization model might be a useful model in further pharmacological testing. Also, the above finding is important because most rodent models of schizophrenia model selectively the alterations in the mesolimbic dopaminergic function, which underlie the positive symptoms of the disease and not model negative symptoms and cognitive impairment. Alterations reminiscent of negative and cognitive symptoms may be as a result of changes in the frontal cortical-limbic circuit. They are modelled in some animal models including the neonatal ventral hippocampus lesion model, chronic PCP, isolation rearing from weaning, and pre-natal methylazoxymethanol (MAM) models.

Overall, it is important to note that animal models of schizophrenia and drug addiction have certain limitations. These limitations are despite their important advantages. Among their advantages is the fact that they offer a straightforward way to monitor the progression of the disease and invasively monitor molecular and structural alterations. Also, they are used to test novel treatments that cannot be tested in humans. On the other hand, one of the main limitations of animal models is that some of the symptoms of schizophrenia like thought, verbal learning and memory are specific to humans and do not constitute animal traits. Also, most behavioural symptoms of schizophrenia can only be indexed and not directly measured. Thus, tasks that monitor them have only translational relevance and only help to make inferences about the disease. Such limitations exist in the amphetamine sensitization animal model that we studied. Another limitation is that there is not a medication treating all the symptoms which can be used in preclinical studies as a definite positive control. Also, schizophrenia is a complex and heterogeneous disorder. It is being caused by unclear interactions between genes and environmental factors. Thus, there can be no single animal model that can mirror all the complex aspects of the disease. Most animal models do not focus on the prevention of the progression of the underlying causal structural changes which could be assessed in neurodevelopmental animal models. Instead they focus on the reversal of

symptoms. In addition, most animal models focus on the acute effects of antipsychotics rather than on chronic administration. Thus, any observations are of limited translational significance. Finally, the unclear cause of the disease limits the creation of better animal models.

It should be added that similar limitations exist with the animal models for drug addiction, given that it is a complex psychiatric disorder in which progression is also dependent on factors such as social and economic factors. There is no single animal model for all aspects of drug addiction. Instead different animal models mimic different behavioural features of drug addiction such as escalation of drug taking, compulsive drug use, withdrawal and relapse. Also, different molecules and brain circuits regulate drug seeking and drug taking. Finally, for these reasons, reverse translational animal models that mimic treatments that are successful have been proposed to be of higher translational value for investigating the substrate and treatment of drug addiction (for review see Venniro et al, 2020).

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Appendix

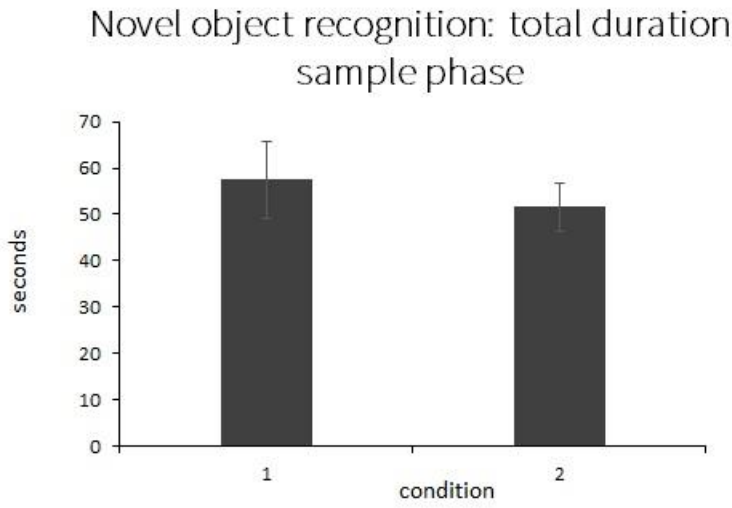


Figure 1. Mean (±SEM) total duration of investigation (seconds) of both objects during sample phase. Condition 1: pair of wooden rectangular boxes (n=11), condition 2: pair of plastic bottles (n=10).

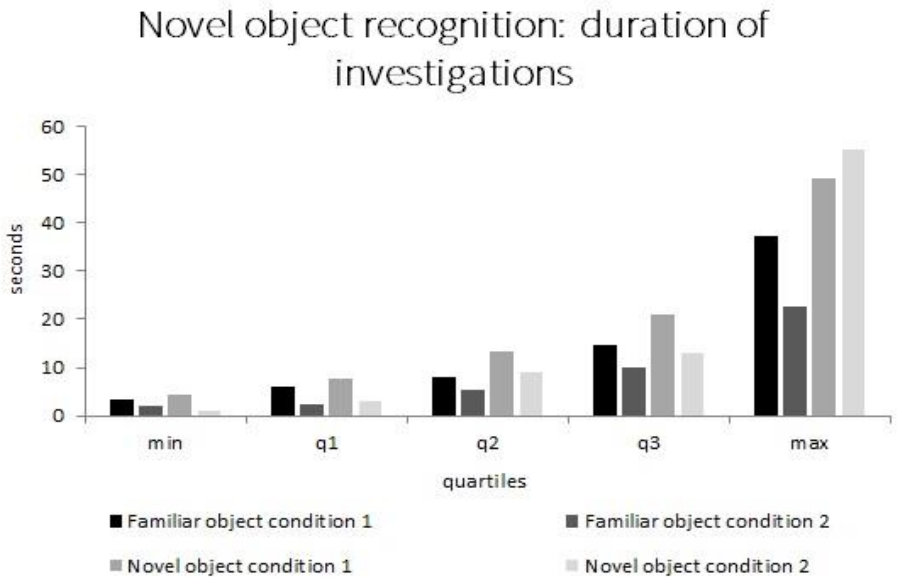


Figure 2. Quartiles for duration of investigation (seconds) of novel and familiar objects during choice phase for condition 1: mice pre-exposed to pair of rectangular boxes during sample phase (n=11), condition 2: mice pre-exposed to pair of plastic bottles (n=10). Quartiles are presented as data were not normally distributed.

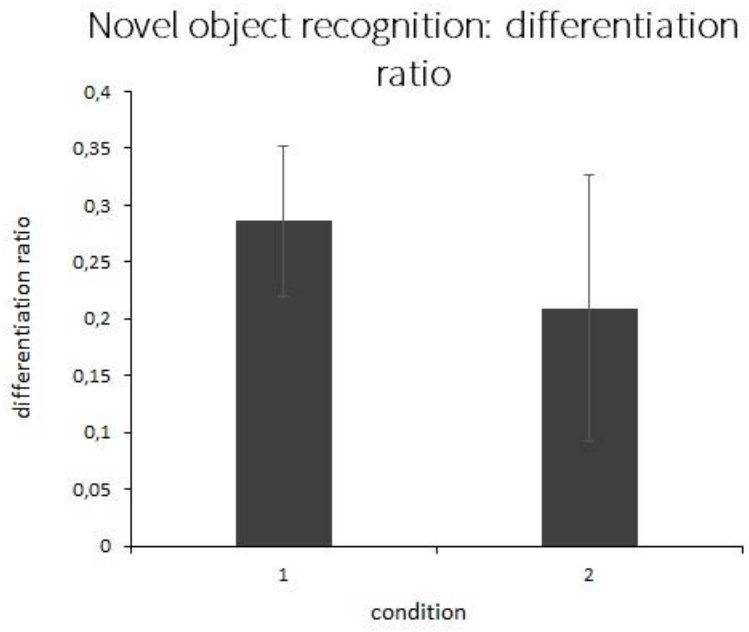


Figure 3. Mean (±SEM) discrimination ratio for duration of exploration during choice phase for condition 1: mice pre-exposed to pair of wooden rectangular boxes during sample phase (n=11), condition 2: mice pre-exposed to pair of plastic bottles (n=10).