Advancing rodent models of the behaviours and the neurobiological pathology associated with schizophrenia

Dan Madularu

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By: Dan Madularu

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Signed by the final examining committee:

Dr. James Grant	Chair
Dr. Adrianna Mendrek	External Examiner
Dr. Thien Thanh Dang-Vu	External to Program
Dr. Jim Pfaus	Examiner
	Examiner
Drs. Wayne Brake and Dave Mumby	Thesis Supervisor

Approved by

_____
Chair of Department or Graduate Program Director

December 15, 2014

_____
Dean of Faculty

Abstract

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Dan Madularu, Ph.D.

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Schizophrenia is a psychiatric illness affecting roughly 1% of the population worldwide. The symptomatology is generally classified in terms of the enhancement or absence of otherwise normally-functioning features, and has been partially recreated in rodents and non-human primates for research purposes. The overarching goal of this thesis is to address some of the shortcomings of rat models of the behaviours and the neurobiological pathology associated with schizophrenia. Specifically, we examined the effects of estradiol on dopamine release to address the potential role of sex differences in schizophrenia development and response to treatment. Furthermore, we explored the effects of prenatal glutamatergic blockade on cognition and locomotor activity at two different stages in rat adult development. Finally, we investigated the involvement of prefronto-cortical dopamine transmission on working memory, a cognitive function disrupted in schizophrenia. When paired with estradiol, chronic haloperidol treatment was effective in blocking the hyperlocomotor-inducing effects of amphetamine, supporting clinical findings. Although we attributed this effect, in part, to dopamine transmission within the nucleus accumbens, supplementary studies offer a complete map of possible estradiol-dopamine interactions within the central nervous system. In an effort to further investigate the developmental and glutamatergic aspect of schizophrenia, we found that prenatal glutamatergic blockade results in impairments similar to those seen in other animal models, as well as patients with schizophrenia. In investigating the role of prefronto-cortical dopamine transmission vis-a-

vis of working memory function in the rat, we found that working memory as measured by a

delay-non-match to place task, is not driven by prefronto-cortical dopamine transmission.

Acknowledgments

Table of Contents

List of figures

Chapter One

Study 1A:

Study 1B:

Chapter Two

Chapter Three

List of tables

Chapter One

General Introduction

Initially coined *dementia praecox* by Kraepelin in 1896, schizophrenia affects roughly 1% of the population worldwide and is one of the leading causes of chronic disability in Canada and the US (Bickel & Javitt, 2009; Javitt, 2010; Meyer & Feldon, 2010). Schizophrenia symptomatology is generally classified in terms of the enhancement or absence of otherwise normally-functioning features into positive, negative and cognitive symptoms (A. Becker et al., 2003; Bickel & Javitt, 2009; Nabeshima, Mouri, Murai, & Noda, 2006; Young, Powell, Risbrough, Marston, & Geyer, 2009). Positive symptoms are features that are absent in healthy individuals, but present in patients; they include, the presence of audio-visual hallucinations and delusions. Conversely, negative symptomatology describes the absence of emotions/behaviours observed in patients with schizophrenia, such as reduced emotional expression (affective flattening), as well as diminished fluency and productivity of thought and speech and delayed initiation of goal-directed behaviour. Cognitive symptoms, which often times precede the manifestation of psychosis, refer to impairment in the ability for abstract thinking, conceptual disorganization, as well as attentional and working memory deficits (Bickel & Javitt, 2009; Young et al., 2009).

Positive symptomatology is the most evident array of symptoms seen in schizophrenia, but its treatment accounts for only 5% of variance in quality of life, while the negative and cognitive symptoms are found to be the most persisting and debilitating features (Kantrowitz & Javitt, 2010). Furthermore, unlike positive symptoms, cognitive impairments are stable over time (Albus et al., 2002), continue to manifest after remission of psychosis, and are generally unresponsive to antipsychotic treatment (Keefe et al., 2007; Penn et al., 2009).

Neurobiological mechanisms implicated in schizophrenia

The focus on the neurobiological basis of schizophrenia has been primarily placed on dysfunctions of the dopaminergic and glutamatergic neurotransmitter systems. Drug-naïve schizophrenia patients elicit enhanced striatal dopaminergic activity in response to a single dose of amphetamine (AMPH), possibly due to dopamine (DA) receptor hypersensitivity (Peleg-Raibstein, Knuesel, & Feldon, 2008), a concept that will be explored in Chapter One. Similar effects have been reported in animals previously exposed to psychostimulants such as AMPH or cocaine, further implicating a sensitized dopaminergic system in schizophrenia symptomatology (Kalivas & Stewart, 1991; Vanderschuren & Kalivas, 2000). The excitatory amino acid neurotransmitter, glutamate, has also been shown to play a central role in the development of schizophrenia-like symptoms, with N-methyl-D-aspartate receptor (NMDAR) blockade in the rat resulting in cognitive deficits similar to impairments seen in schizophrenia patients, such as behavioural perseveration and working memory (WM) impairments (Adams & Moghaddam, 1998; Danysz, Wroblewski, & Costa, 1988; Holahan, Madularu, McConnell, Walsh, & DeRosa, 2011b; Reichenberg, 2010); these impairments are ameliorated by antipsychotic (8-Chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine; Clozapine) treatment (Amitai, Semenova, & Markou, 2007).

The role of dopamine dysfunction in schizophrenia

The first attempts to elucidate the neuropathology of schizophrenia implicated the dopaminergic system (Stevens, 1973, 1997; van Rossum, 1966), with blockade of DA D2 receptors (D2R) resulting in symptom alleviation (Kantrowitz & Javitt, 2010). Subsequently the DA hypothesis postulated that the development of psychotic symptoms is a result of hyperactive

mesolimbic DA transmission (Carlsson & Lindqvist, 1963). At the core of the DA hypothesis lies the seminal work of Carlsson and Lindqvist (1963), who reported that antipsychotic agents act by increasing DA metabolism and thus reducing DA transmission in the brain. Additionally, drugs that enhance DA transmission have been found to trigger psychotic episodes when administered to patients with schizophrenia (J. A. Lieberman, Kane, & Alvir, 1987). Following Carlsson and Lindquist's initial studies, a link was made between antipsychotic efficiency and their affinity to DA receptors, with inhibitory potencies of antipsychotics correlating with clinical doses used in schizophrenia (P. Seeman, Chau-Wong, Tedesco, & Wong, 1975; P. Seeman & Lee, 1975; P. Seeman, Lee, Chau-Wong, & Wong, 1976).

The original theory put forth in the 1960s suggesting that schizophrenia pathophysiology is fueled mainly by a subcortical, hyperactive dopaminergic system proved to be overly-simplistic given symptom complexity. Studies have since shown that the previously-described hyperactive state was linked to a hypoactive prefronto-cortical dopaminergic system with prefrontal DA neurons inhibiting subcortical DA activity (Carter et al., 1998; Pycock, Kerwin, & Carter, 1980). This updated approach to the DA hypothesis attempted to address both the negative and positive symptomatology of the illness, suggesting that negative symptomatology stems from prefronto-cortical hypoactivity while psychosis was linked to subcortical hyperactivity. Although this approach offered more insight into schizophrenia symptomatology, it did not address the mechanisms by which hyper-dopaminergia translates into positive symptoms and conversely, how hypo-dopaminergia translates into negative ones (for review, see Davis, Kahn, Ko, & Davidson, 1991).

In an effort to clarify the mechanism by which DA availability affects symptomatology, studies in acutely-psychotic patients showed increased pre-synaptic DA transmission using

4

positron emission tomography (McGowan, Lawrence, Sales, Quested, & Grasby, 2004). Conversely, depressive symptoms in drug-naïve patients correlated with decreased presynaptic DA transmission (Hietala et al., 1999). In order to identify the subcortical receptor type upon which the increased levels of DA were acting, *in vivo* DA occupancy of striatal D2Rs was measured in 18 untreated patients with schizophrenia (Abi-Dargham et al., 2000). This study reported increased D2R occupancy by DA in first-episode patients as well as in previously-treated patients who were experiencing a psychotic episode, supporting a direct link between DA activity mediated by D2Rs and schizophrenia. Although the updated outlook on the DA hypothesis offers a more comprehensive picture of schizophrenic pathology, the findings regarding striatal D2R density are equivocal. Meta-analyses point at a modest increase in striatal D2R density, while other studies reported reductions in D2R density (for review, see Howes & Kapur, 2009).

The evidence surrounding pre-frontal D1R transmission in WM seems to also be equivocal, especially when modelled in rodents. D1R dysfunction has been previously linked to cognitive impairment and negative symptoms, as abnormal prefrontal D1R transmission impairs WM (Mizoguchi, Shoji, Tanaka, Maruyama, & Tabira, 2009; Sawaguchi & Goldman-Rakic, 1991). It is therefore possible that hypo-dopaminergia is linked to D1R up-regulation. However, findings regarding D1R density in drug-naïve patients are conflicting, with studies finding increased (Abi-Dargham et al., 2002), decreased (Okubo et al., 1997) or no change (Karlsson, Farde, Halldin, & Sedvall, 2002) in prefrontal D1R density.

The updated DA hypothesis, based on findings reported above, appears to be as complex as the disease itself, with hypo- and hyper-dopaminergic states coexisting, and mediated by different classes of DA receptors. The most recent version of the dopamine hypothesis of schizophrenia was delineated byHowes and Kapur (2009). *The Dopamine Hypothesis of*

Schizophrenia: Version III-the final common pathway, is based on the Davis et al. (1991) approach, to which environmental and genetic factors were added. The review includes evidence supporting the involvement of social factors such as employment status, migration, isolation and childhood abuse as predictive factors in schizophrenia. Similarly, gestational complications in humans and animal models, which have been previously linked to an increased risk of illness development, have also been shown to result in long-term subcortical hyper-dopaminergia (for review, see Howes & Kapur, 2009).

The current version of the DA hypothesis offers the most comprehensive analysis vis-à-vis DA transmission in schizophrenia patients, as well as in animal models of schizophrenia. However, it omits the possible implications and/or interactions of additional neurotransmitter systems, such as glutamate, GABA, serotonin, opioid etc. Given the complexity of the illness, it may be an oversimplification to attribute its wide range of symptoms to dysfunction in only two brain regions, such as the striatum and prefrontal cortex. However, animal models of schizophrenia based on the DA hypotheses proved to be germane to the development of current therapies.

The role of glutamate dysfunction in schizophrenia

Glutamate is the major excitatory neurotransmitter throughout the central nervous system (both in the cortical and subcortical areas), accounting for roughly 60% of brain synapses (Kantrowitz & Javitt, 2010; Platt, 2007). Synthesized from glutamine and α-ketoglutarate, it exerts its effects postsynaptically on three main ion-gated ion channels (receptors): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA and kianate (Coyle, 2006; Platt, 2007). The NMDAR plays a crucial role in schizophrenia pathology, based on findings showing that NMDAR antagonists such as 1-(1-phenylcyclohexyl)piperidine) (PCP) and ketamine result in a series of

effects that are often undistinguishable from schizophrenic symptomatology (Gordon, 2010; Kantrowitz & Javitt, 2010). Such effects were first observed and documented in the 1950s when PCP, then used as an anesthetic, produced psychotic effects in humans (Neill et al., 2010). When administered to healthy individuals, PCP and ketamine results in hyperactivity, paranoia, hallucinations and cognitive impairment, while its administration to schizophrenic patients exacerbates symptoms (Neill et al., 2010). Based on earlier studies showing that NMDAR blockade induce a spectrum of behavioural and cognitive effects similar to schizophrenia (Bakker & Amini, 1961; Davies & Beech, 1960; Luby, Cohen, Rosenbaum, Gottlieb, & Kelley, 1959), the glutamate hypothesis posits that at the basis of schizophrenia pathophysiology lies a hypoactive glutamatergic system (Duncan, Sheitman, & Lieberman, 1999).

Not only do the effects of NMDAR blockade mirror the wide range of schizophrenic symptoms, it also incorporates additional neurotransmitter systems. Most importantly, the glutamate hypothesis incorporates the dopaminergic involvement in symptom alleviation, showing that antipsychotics partly block the effects of NMDAR antagonists (Duncan et al., 1999). Furthermore, striatal DA hyperactivity can be induced by glutamatergic blockade via acute NMDAR antagonist administration (Jentsch, Sanchez, Elsworth, & Roth, 2008). The over-activation of the mesolimbic DA pathway through acute glutamatergic blockade has been shown to result in motor (Tang et al., 2006) and cognitive impairments (for review, see Jentsch et al., 1998) and is reversible by DA antagonist pre-treatment (Anderson, Bari, & Pierce, 2003; Bari & Pierce, 2005).

Evidence for interaction between the glutamatergic and DA systems has been previously supported by enhanced DA release in response to treatment with an NMDAR antagonist alongside AMPH (for review, see Carlsson et al., 2001). Similar interactions have been reported, where a

non-dose dependent release of DA was observed in rats, following treatment with the NMDA antagonist, MK-801 (Miller & Abercrombie, 1996). Finally, a more recent study showed that MK-801-induced perseverative behaviour can be abated by ventral striatal infusions of a DA aptamer (Holahan et al., 2011b).

The neurodevelopmental hypothesis of schizophrenia etiology

The DA and glutamate hypotheses paint a comprehensive pathophysiological picture, accounting for positive, negative and cognitive symptomatology, however they generally omit the developmental aspect of the disease. The concept of "schizophrenic children" has been known to researchers since the late 1800s, when Kraepelin noted that roughly 3.5% of schizophrenic patients' clinical signs could be traced back to early childhood (Bender, 1953). An 11-study meta-analysis indicated that, compared to healthy controls, schizophrenic patients show minor physical abnormalities around the mouth region (Weinberg, Jenkins, Marazita, & Maher, 2007). For example, a study looking at differences in tooth size between controls and patients showed a reduction in crown size across all permanent teeth of patients suggesting that the schizophrenic profile may be pre-set sometime during the first trimester of pregnancy (Rajchgot et al., 2009). These and other similar findings led to the genesis of the neurodevelopmental hypothesis, which postulates that schizophrenia may be caused by a disruption of the neurodevelopmental processes before birth, thus preceding the post-pubertal clinical onset.

Considering the involvement of dopaminergic and glutamatergic transmission in schizophrenia, it can then be assumed that manipulating the DA and glutamate systems before, or immediately after birth would result in possible deficits similar to the ones seen in schizophrenia. Neurodevelopmental guidance cues such as netrins, are crucial in the neuronal organization of the

DA system, with pre-natal manipulation of these molecules linked to dopaminergic dysfunction later in life (Flores et al., 2005; Grant et al., 2007). In addition, mice treated with MK-801 or PCP before birth demonstrate reduced post-pubertal density of parvalbumin-immunoreactive neurons in the medial prefrontal cortex (mPFC), as well as hyperactivity in response to a PCP challenge and impaired recognition memory (Abekawa, Ito, Nakagawa, & Koyama, 2007; L. Lu et al., 2010). Other non-pharmaceutical prenatal manipulations have also been linked to schizophrenia such as gestational exposure to stress, immune activation, nutritional deficiencies and obstetric complications (for review, see Meyer & Feldon, 2010).

Animal models of behaviors and neurobiological pathology associated with schizophrenia

Most of the current understanding of schizophrenia comes from animal studies, where sets of symptoms are modelled primarily in rodents and non-human primates. Animal models, defined as experimental preparations aimed at mimicking the human condition or disease (Geyer, Olivier, Joels, & Kahn, 2012), have been used for decades, advancing disease characterization and drug development, allowing for testing hypotheses within genetically-homogeneous populations (McGonigle, 2014; McGonigle & Ruggeri, 2014). With regards to schizophrenia, animal models should not only reproduce the relevant symptomatology, but to also address the effects of antipsychotics on intended symptoms. The scientific gold-standard that needs to be met when developing animal models is primarily driven by the need of maximizing validity (McGonigle & Ruggeri, 2014; Peleg-Raibstein, Feldon, & Meyer, 2012), such that these models must abide by a set of validity criteria (Jones, Watson, & Fone, 2011; Meyer & Feldon, 2010), as shown in Figure 1. However, in order to reach this standard, a comprehensive picture of the illness has to emerge. This would allow for the classification of sets of symptoms which differentiate one condition from

another. In the case of schizophrenia, a clear clinical understanding of the symptomatology is warranted in order to further understand its neurobiological underpinnings, as described earlier.
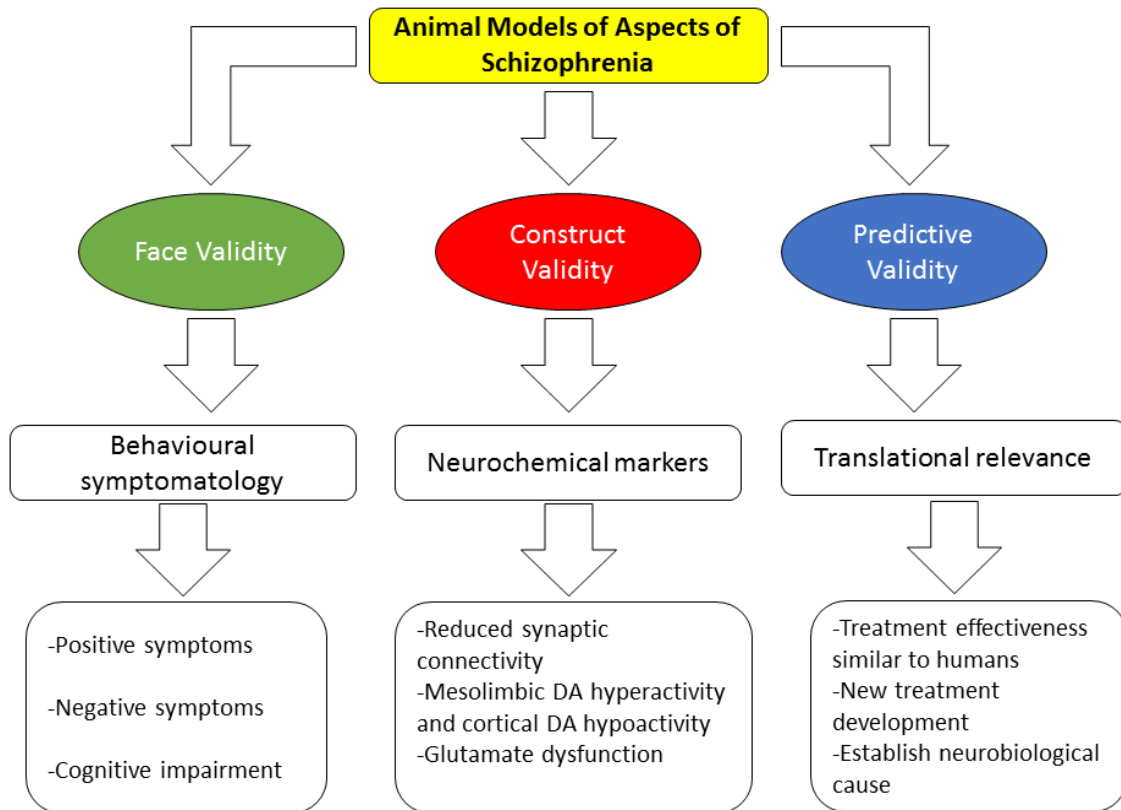
Figure 1. Schematic of validity criteria important when developing an animal model of schizophrenia (adapted from Jones et al., 2011). The ideal model should present symptoms similar to those observed in patients (face validity), resemble the underlying pathophysiology of the disease (construct validity) and the effects of antipsychotics should elicit similar responses to those seen in schizophrenia patients (predictive validity).

Several animal models of neuropathological and behavioural aspects of schizophrenia have been developed, with emphasis initially placed on the involvement of the mesocorticolimbic DA system. These models are based on the fact that repeated exposure to AMPH increases the behavioural and neurochemical responses to subsequent exposures to lower doses of the drug,

process known as behavioral sensitization (for review, see Featherstone, Kapur, & Fletcher, 2007). Although the study of positive symptomatology is not feasible through direct animal experimentation, it is possible to study behaviours that are known to share some of the underlying neurobiological mechanisms. The effects of AMPH sensitization, discussed in the following section, have been addressed in the context of cognitive deficiency in schizophrenia, focusing on attention, long-term memory and WM, as well as deficits in problem solving (Green & Nuechterlein, 2004; Kern, Green, Nuechterlein, & Deng, 2004; Nuechterlein et al., 2008; Young et al., 2009). Negative symptomatology (e.g. social withdrawal and anhedonia) has also been studied, although to a lesser extent, in animals exposed to, and withdrawing from AMPH treatment. These studies show that AMPH sensitization leads to suppression of nocturnal activity, a measure of depression and social withdrawal, immediately following AMPH withdrawal (Paulson, Camp, & Robinson, 1991; Segal & Mandell, 1974).

Although the AMPH sensitization model has been valuable in treatment development, the seemingly simplistic approach fails to address crucial aspects of the disease. As an example, this model does not take into account the developmental aspect of schizophrenia. In addition, AMPH sensitization, as studied in the context of schizophrenia, focuses mostly on dopaminergic transmission *in males*, in spite of estradiol (E2) having been previously linked to schizophrenia symptomatology and treatment. Finally, the AMPH-sensitization model does not typically address the possible involvement of the glutamatergic system in schizophrenia. It has been shown that NMDAR antagonism inhibits performance on cognitive tasks in rats, similar to impairments seen in patients with schizophrenia (for review, see Bubenikova-Valesova, Horacek, Vrajova, & Hoschl, 2008; Nabeshima et al., 2006). It is thus necessary that efforts aimed at the development

of animal models of schizophrenia should systematically address the implications of glutamatergic transmission, as well as the effects of gonadal hormones.

More recent attempts at modelling schizophrenia in animals are based on findings delineating a link between environmental factors such as obstetric complications and viral exposure, and dysfunction of neural systems (Mednick, Machon, Huttunen, & Bonett, 1988; Owen, Lewis, & Murray, 1988). As such, some of the widely-used developmental models include neonatal ventral hippocampal lesions (NVHL; Lipska, Jaskiw, & Weinberger, 1993) and prenatal immune challenge models (Meyer & Feldon, 2009; Meyer, Feldon, Schedlowski, & Yee, 2005). Developed by Lipska and colleagues in the 1990's (Lipska et al., 1993), NVHL rats show significant WM impairment tasks, similar to those seen in schizophrenia patients (for review, see Lipska, Aultman, Verma, Weinberger, & Moghaddam, 2002), while prenatal immune-challenge affected latent and prepulse inhibition, as well as WM and sensitivity to DA agonists and NMDAR antagonists (Meyer & Feldon, 2009). The following sub-sections will describe three animal model approaches of behavioural and neurochemical aspects of schizophrenia based on the DA, glutamate and developmental hypotheses of the disease. It should be noted however that this is not an exhaustive analysis, as other models (i.e. genetic, immune-based) have also been shown to mimic the behavioural aspects of the disease.

Amphetamine sensitization models

Sensitization to AMPH is a process by which repeated exposure to AMPH (induction phase) leads to an increased behavioural response to subsequent lower doses of AMPH (Featherstone et al., 2007; Schmidt & Beninger, 2006; Vezina, 1996). The process is time-dependent, such that motor effects are fully developed after a period of withdrawal (Paulson et al.,

1991; Paulson & Robinson, 1995), and not immediately after the cessation of AMPH treatment. Furthermore, the sensitizing effects of AMPH exposure last for at least one year after treatment cessation (Paulson et al., 1991), and possibly longer. The brain regions and mechanisms by which AMPH sensitization occurs are not fully elucidated. Previous research showed that D2Rs are not necessary for the induction phase, whereas D1Rs play a critical role in the development of sensitized locomotor and nucleus accumbens (NAcc) DA response to AMPH (Vezina, 1996). In a recent study, rats were treated with 2.5 mg/kg AMPH daily over 6 days, after which the extent of sensitization was assessed by means of locomotor activity and stereotypy (Scholl, Feng, Watt, Renner, & Forster, 2009). Interestingly, only sixty-two percent of rats displayed behavioural sensitization however, sensitized rats showed increased DA concentrations in the NAcc shell, as well as increased dorsal hippocampal serotonin concentrations. Furthermore, sensitization to AMPH and cocaine is sex-specific (J. B. Becker, Molenda, & Hummer, 2001), with female rats showing increased behavioural activation to acute and repeated injections of AMPH compared to males (Forgie & Stewart, 1994). In addition, E2-treated female rats show higher levels of AMPH-induced activity during induction compared to oil-treated controls (Forgie & Stewart, 1994). More recent studies showed that E2 enhances sensitization to cocaine, where sensitization occurring concomitant with E2 replacement persists even when hormone levels are lower (Hu & Becker, 2003).

AMPH-sensitized animals have been used to model the positive, negative and cognitive aspects of the disease, albeit with some limitations. Although positive and negative symptoms cannot be discarded, this thesis will focus mainly on the cognitive symptomatology, as delineated by the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS; Young et al., 2009) initiative. The MATRICS guideline identified seven cognitive domains

impaired in schizophrenia: attention/vigilance, WM, problem solving, processing speed, visual learning and memory, verbal learning and memory and social cognition (Young et al., 2009). Fletcher, Tenn, Sinyard, Rizos, and Kapur (2007) tested AMPH-sensitized rats on a set-shifting task and found that sensitized rats showed reduced accuracy and increased omissions compared to control. The effects were reversed by D1R antagonist treatment, concluding that AMPH sensitization effectively models D1R-mediated attentional deficits seen in schizophrenia. In an effort to study possible strain differences in response to AMPH sensitization in Lewis and Fischer rats, Peleg-Raibstein, Yee, Feldon, and Hauser (2009) performed a series of cognitive assessments during a 58-day AMPH withdrawal period. They assessed anxiety, fear conditioning, attention, visual learning and memory, and spatial WM. AMPH sensitization failed to show an effect on pre-pulse inhibition, a measure of attention in either strains. The Morris water maze test also yielded noteworthy results, with spatial WM being improved in Fisher rats, and impaired in Lewis rats, independent of swimming ability (Peleg-Raibstein et al., 2009). Visual memory assessment showed AMPH-treated Fischer having an enhanced preference for a novel object, whereas Lewis rats did not.

The studies described earlier support AMPH sensitization as a means of modelling some aspects of schizophrenia in animals. However, most of these data are generated from studies involving solely male animals. There are significant sex differences in patients with schizophrenia with respect to time of onset and symptom manifestation (Angermeyer & Kuhn, 1988; Guillem, Mendrek, Lavoie, Pampoulova, & Stip, 2009; Hafner, 2003; Hafner, Behrens, De Vry, & Gattaz, 1991; Jimenez, Mancini-Marie, Lakis, Rinaldi, & Mendrek, 2010; Mendrek, Mancini-Marie, Fahim, & Stip, 2007; Riecher-Rossler, Hafner, Stumbaum, Maurer, & Schmidt, 1994). In addition, women have been shown to differ in symptom severity depending on the phase of the menstrual

cycle (Hallonquist, Seeman, Lang, & Rector, 1993). Studies on medicated pre-menopausal women with schizophrenia suggest an interaction between E2 levels and their response to antipsychotic medication, which all have in common that they are D2R antagonists. For example, women receiving E2 in addition to antipsychotic treatment respond better than patients receiving antipsychotic treatment alone (Akhondzadeh et al., 2003; Kulkarni et al., 1996; Kulkarni et al., 2001). Taken together, these findings implicate ovarian steroids in schizophrenia onset, symptomatology and treatment responsiveness, a preclinical aspect of the disease that has been understudied.

Animal models involving NMDA receptor blockade

Most NMDAR antagonist-based models involve the assessment of schizophrenia-like symptomatology in response to pretreatment with PCP, ketamine or MK-801. Assessment occurs either immediately after drug treatment, or during/after a prolonged period (i.e.1-3 weeks). As mentioned earlier, positive symptomatology is rather difficult to assess in non-human mammals, thus the majority of studies focus on negative and cognitive symptoms. A suitable test for assessing negative symptomatology such as depression-like behaviour in rats and mice is the forced-swim test (Borsini & Meli, 1988; Xu et al., 2005). When placed into a cylinder containing water, animals typically try to escape by swimming around the walls of the cylinder; increased immobility in this setting is a depiction of a behavioural model of depression. PCP increases immobility on the forced-swim test in mice, but only after a prolonged treatment period (i.e. 14 days), and persists for at least three weeks after treatment cessation (Y. Noda, Yamada, Furukawa, & Nabeshima, 1995). This effect is reversed by acute treatment with atypical, but not typical antipsychotics (Mouri, Noda, Mizoguchi, & Nabeshima, 2006).

The effects of NMDAR blockade on cognitive symptomatology have been studied in response to PCP treatment on attention and learning. Acute PCP-treatment before a water-finding test (Ichihara, Nabeshima, & Kameyama, 1993) in water-deprived mice resulted in increased latency to find a water tube, while repeated treatment induced a long-lasting impairment on the same test (A. Noda et al., 2001). Another cognitive deficit observed in schizophrenia is behavioural perseveration, whereby patients with schizophrenia show difficulty in inhibiting behaviours on a learned task, even when it is inappropriate to do so (Waford & Lewine, 2010). When rats are tested on a behavioural perseveration test, NMDAR blockade with an acute dose of MK-801 results in pressing a previously-baited bar in an operant box, despite the lever no longer being baited during the test phase (Holahan, Clarke, & Hines, 2010; Holahan et al., 2011b). This effect is reversed by DA aptamer pretreatment, with DA DNA aptamer effectively reducing the amount of DA binding to its receptors (Holahan et al., 2011b). As previously mentioned, WM is a cognitive function impaired in schizophrenia. PCP-treated rats show impaired performance on a WM test using a T-maze (Adams & Moghaddam, 1998); this effect is accompanied by an increase NAcc DA release (approx. 3-fold). Finally, PCP and MK-801 disrupt additional domains identified by the MATRICS, such as object recognition and spatial memory in mice, with the effects reversed by the nicotinic receptor partial agonist, SSRI80711 (Pichat et al., 2007).

Neurodevelopmental models

Animal models based on the developmental hypothesis employ manipulations of environment and/or drug exposure during the sensitive pre- and peri-natal period to produce long-lasting changes in neurodevelopment. One of the most widely-used model however, is the NVHL model, developed by Lipska and colleagues in the 1990s (Lipska et al., 1993; Tseng, Chambers, & Lipska, 2009). The procedure involves lesioning the ventral hippocampus using ibotenic acid in

neonatal rats (Lipska et al., 2002). The development of this model was inspired by previous imaging studies showing ventricular enlargements and hippocampal abnormalities in patients with schizophrenia, added to research linking schizophrenia with infections and obstetrical complications during the second and third semester of pregnancy (Tseng et al., 2009; Young et al., 2009).

NVHL rats show significant impairment on WM tasks assessed in a T-maze and radial-arm maze, compared to adult-lesioned controls (Lipska et al., 2002). Interestingly, the atypical antipsychotic, clozapine, not only failed to reverse these effects, but instead exacerbated them in male, but not female rats (Levin & Christopher, 2006). In addition, NVHL rats show impairment in the 5-CSR task and hypersensitivity to PCP compared to controls and adult-lesioned controls (Le Pen, Grottick, Higgins, & Moreau, 2003). A limited number of studies assessed the effects of prenatal NMDAR blockade in mice, and found that prenatal exposure to PCP produces behavioural deficits similar to those seen in schizophrenia, such as memory and emotional dysfunction, hyperlocomotion and impaired object recognition (Abekawa et al., 2007; L. Lu et al., 2010).

Other developmental approaches to reproducing some of the hallmarks relevant to schizophrenia in rodents focused on maternal stress, malnutrition and obstetric complications (Jones et al., 2011). In addition, several genetic models have also been developed, based on the fact that a considerable amount of candidate genes have been linked to schizophrenia risk increase (for review, see Allen et al., 2008). Moreover, some of these genes code for proteins involved in glutamatergic, GABAergic and dopaminergic function, systems that have been previously identified to be disrupted in schizophrenia (Harrison & Weinberger, 2005). Specifically, the *DTNBP1* (encoding dysbindin-1) gene has been linked to schizophrenia (Schwab et al., 2003; Straub et al., 2002), with presynaptic dysbindin-1 level reduction in terminal fields in

glutamatergic connections in schizophrenia patients (Talbot et al., 2004). In addition, the *DTNBP1* gene has been associated with negative and cognitive symptoms of schizophrenia (Burdick et al., 2007; DeRosse et al., 2006).

Deletion of the *DTNBP1* gene in mice results in the manifestation of behaviours relevant to schizophrenia, such as social withdrawal and cognitive impairment (Feng et al., 2008; Jentsch et al., 2009; Papaleo et al., 2012). In addition to this behavioural profile, *DTNBP1* deletion alters dopaminergic, glutamatergic and GABAergic transmission (Bhardwaj et al., 2009; Murotani et al., 2007; Talbot, 2009) in the cerebellar cortex, hippocampus, prefrontal cortex and hypothalamus (Hattori et al., 2008; Jentsch et al., 2009; Papaleo et al., 2012). Surprisingly, some of the effects were reversed by a dopaminergic agonist, but not antagonist (Papaleo et al., 2012). It remains to be established however if these effects can be reversed by typical, as well as atypical antipsychotic treatment. Although not discussed here, other genes have been investigated as possible candidates for the development of genetic-based models, such as *disrupted-in-schizophrenia-1 (DISC1), neuregulin-1 (NRG1)* and reelin.

Given the value of animal models in studying complex diseases such as schizophrenia, it is critical that the development of these models should be focused on the inclusion of all possible factors identified as being implicated in the pathophysiological profile of the disease. Although of significant importance, the current models can be improved upon by taking into account the established implication of ovarian hormones in the course of the disease, while considering the neurodevelopmental aspect of the illness.

Rationale and Hypotheses

The overarching goal of this thesis is to develop animal models that are more representative of some aspects of schizophrenia. Specifically, the first chapter examined the effects of the ovarian hormone, E2, on DA release and in response to antipsychotics to address the potential role of sex differences in schizophrenia development and response to treatment. The second chapter explored the effects of prenatal NMDAR blockade on cognition and locomotor activity at two different stages in rat adult development. Finally, the third chapter investigated the involvement of mPFC DA transmission on WM, a cognitive function disrupted in schizophrenia. The experiments are organized and presented in three separate chapters, as detailed below.

Chapter One: Dopamine-estradiol interactions in AMPH-sensitized rats

The rationale for the experiments in Chapter One, divided into two studies (1A and 1B), is driven by two main factors. Firstly, the goal was to extend the evaluation of the AMPH sensitization model to include the female sex, as most preclinical studies focus on male rodents, despite recent evidence pointing at a sex-differentiated profile. Secondly, we aimed at further understanding the complex relationship between E2 and DA.

Behaviours elicited by AMPH sensitization are thought to reflect some of the positive and cognitive symptoms of schizophrenia (Featherstone et al., 2007; Tenn, Fletcher, & Kapur, 2003). In Study 1A, rats received either chronic low E2 alone, or chronic low plus phasic high E2 replacement to simulate two different estrogen levels during different phases of the estrous cycle in young females (Quinlan, Hussain, & Brake, 2008). Following an AMPH challenge, locomotor activity was recorded after acute and chronic HAL treatment, and NAcc DA and its metabolites were measured using *in vivo* microdialysis. Similar to previous studies investigating antipsychotic efficacy in male rats (Samaha, Seeman, Stewart, Rajabi, & Kapur, 2007), we expected a

differential response with respect to acute vs. chronic HAL. In addition, we hypothesized that the possible effects of HAL on locomotor behaviour, as well as neurochemical profile would be dependent on E2 levels.

Study 1B was aimed at investigating the interactions between the DA system and E2 through the means of functional magnetic resonance imaging (fMRI). For this study, awake AMPH-sensitized, OVX rats receiving vehicle, low E2 and phasic high E2 replacement were used. Imaging occurred after a period of AMPH withdrawal, and lasted 30 minutes, during which blood-oxygen-level dependent (BOLD) changes were recorded in response to an intraperitoneal (IP) injection of AMPH. In line with previous clinical and preclinical studies, we hypothesized a differential response to AMPH dependent on the levels of circulating E2, depicted by BOLD signal change differences in clinically-relevant brain regions of interest (ROI). Analysis was focused on, but not limited to, ROIs relevant to schizophrenia pathology, such as mesolimbic and mesocortical pathway components, as well as the habenular complex and olfactory system. Similar to Experiment 1A, it was expected that E2 would modulate AMPH-induced brain activity.

Chapter Two: Effects of prenatal NMDAR blockade on behaviour of adult offspring

NMDAR blockade has been used as a putative model of schizophrenia-like symptoms, with PCP, MK-801 and ketamine treatment resulting in schizophrenia-like effects in human participants and animals (Gordon, 2010; Kantrowitz & Javitt, 2010). On the one hand, administering PCP to healthy individuals induces hyperactivity, paranoia, hallucinations and cognitive impairment similar to that observed in schizophrenia (Javitt & Zukin, 1991). On the other hand, PCP administration to patients suffering of schizophrenia exacerbates the symptomatology (Malhotra et al., 1997). Contrary to Chapter One, where the DA hypothesis of

schizophrenia was explored, the purpose of Chapter Two was to address the glutamate and developmental hypotheses by explore the effects of prenatal NMDAR blockade on behaviour in the adult offspring. Furthermore, in order to account for possible effects throughout postnatal development, behavioural assessment was performed at two stages of rat adult development, namely postnatal day (PND) 60 and PND 210. This was done based on findings showing continuous decline in brain volume and possible subsequent decline in function in schizophrenia patients, which is likely to be accompanied by symptom exacerbation (Mathalon, Sullivan, Lim, & Pfefferbaum, 2001).

In line with previous studies (L. L. Lu et al., 2010; Lu et al., 2011), it was hypothesized that prenatal MK-801 treatment would result in impaired object recognition memory in the adult rat. Further, the offspring of the MK-801-treated dams were expected to show increased locomotor activity in response to an acute dose of MK-801. Considering that earlier findings suggested that prenatal NMDAR blockade in the rat would serve as a valid animal model for schizophrenia-like symptoms (Bubenikova-Valesova et al., 2008; Mouri, Noda, Enomoto, & Nabeshima, 2007), locomotor activity was assessed in response to a systemic AMPH injection in the adult rat. As such, increased locomotor activity in response to an AMPH challenge (Peleg-Raibstein et al., 2008) was expected in the adult offspring of the MK-801-treated dams compared to control offspring (i.e. from saline-injected dams). Finally, we hypothesized that possible differences seen between MK-801 and SAL rats would at least persist, if not be exacerbated when tested at PND 210 compared to PND 60.

Chapter Three: Rethinking the role of dopamine in working memory in the rat

Working memory is the ability to actively hold and manipulate information in order to perform various attention-based tasks (Baddeley & Della Sala, 1996), and has been identified to be affected in patients suffering from schizophrenia (Green & Nuechterlein, 2004; Nuechterlein et al., 2008; Young et al., 2009). Although the pioneering studies by Goldman-Rakic and colleagues elegantly single out the dorso-lateral prefrontal cortex (dlPFC) as crucial to WM maintenance in non-human primates, there seems to be a lack of consensus regarding WM and its biological substrates in the rat (for review, see Floresco, 2013). Given that WM is one of the functions that is often affected in patients, the aim of the experiments presented in this chapter was to address the role of rat mPFC D1R transmission in WM by assessing performance in a delayed non-match-to-place (DNMTP) task in response to systematic mPFC D1R manipulations. The translational rationale for this study is based on the fact that cognitive symptoms, such as WM deficits, are not responsive to current pharmacological treatment. Thus, it is of clinical importance to elucidate the possible mechanisms by which this function is maintained in order to develop novel ways to treat this class of symptoms.

In Experiment 1, a D1R antagonist was infused bilaterally into either the infralimbic (IL), prelimbic (PrL), or both areas of the mPFC. In Experiment 2, a D1R agonist was infused bilaterally into the IL area of the mPFC, while in Experiment 3, AMPH was infused both systemically, and bilaterally into the IL. Given the inverted U-shaped curve describing WM maintenance as a function of mPFC DA transmission via D1R receptors, three hypotheses were tested: 1) Performance on the DNMTP would decay with increasing retention delays, 2) D1R agonist and antagonist infusions into the mPFC would impair WM, thus leading to performance deficits, and 3) The impairment would be dose-dependent.

Chapter One:

Study 1A: Estrogen potentiates the behavioral and nucleus accumbens dopamine response to continuous haloperidol treatment in female rats

Dan Madularu, Waqqas M. Shams, and Wayne G. Brake

Preface

Previous studies suggest that E2 may have antipsychotic-like properties, possibly through its interaction with the dopaminergic system (Kulkarni et al., 2001). The aim of this study was to investigate this interaction in acute and chronic low-dose haloperidol-treated, AMPH-sensitized and non-sensitized female rats using behavioural and neurochemical analyses.

Abstract

Estrogen has been shown to enhance the effects of antipsychotics in humans. To investigate the mechanisms of how this may occur, the current study examined estradiol's effects on dopaminergic transmission and behavior in amphetamine-sensitized and non-sensitized female rats. Sixty-four ovariectomized female Sprague-Dawley rats were used for this study. Half of the rats were sensitized to four, once-daily injections of 1 mg/kg amphetamine, while the other half served as controls. Rats received either chronic administration of low-dose haloperidol (0.25 mg/kg/day) via osmotic minipumps implanted subcutaneously, or saline vehicle. The groups were further subdivided with respect to estradiol treatment: low chronic estrogen (subcutaneous estradiol implant, 0.36mg/pellet: 90-day release, + additional oil injection every second day) and high pulsatile estrogen (subcutaneous estradiol implant + additional 10 μg/kg estradiol injection every second day). Motor activity was assessed at day two and day twelve during haloperidol treatment, while nucleus accumbens dopamine availability was assessed via microdialysis ten days into antipsychotic treatment. Haloperidol treatment along with high, but not low, estradiol replacement was effective in reducing amphetamine-induced locomotor activity in sensitized rats. High estradiol treatment also augmented the effects of chronic haloperidol in reducing dopaminergic release in sensitized rats. These data suggest that estradiol levels affect both the behavioral and dopamine responses to chronic antipsychotic treatment.

Introduction

There are significant sex differences in patients with schizophrenia with respect to time of onset and symptom manifestation (Angermeyer & Kuhn, 1988; Hafner, 2003; Hafner et al., 1991; Riecher-Rossler et al., 1994). Women have been shown to differ in symptom severity depending on the phase of the menstrual cycle (Hallonquist et al., 1993). Studies on medicated pre-menopausal women with schizophrenia suggest an interaction between estrogen levels and their response to antipsychotic medications, which all have in common that they are dopamine (DA) D2 receptor antagonists. For example, previous research has shown that women receiving estrogen in addition to antipsychotic treatment respond better than those with antipsychotic treatment alone (Akhondzadeh et al., 2003; Kulkarni et al., 1996; Kulkarni et al., 2001). These findings implicate ovarian steroids in schizophrenia onset, symptomatology and treatment responsiveness.

The aim of the current study is to further investigate the possible interactions between antipsychotic treatment, estrogen and the dopaminergic system in a rodent model, by using female, amphetamine (AMPH)-sensitized rats. Behaviors elicited by AMPH sensitization are thought to reflect some of the positive and cognitive symptoms of schizophrenia (Featherstone et al., 2007; Tenn et al., 2003). These changes are further thought to correspond to nucleus accumbens (NAcc) DA transmission changes in both rodents and primates (Castner, Vosler, & Goldman-Rakic, 2005; Peleg-Raibstein et al., 2008; Tenn et al., 2003). In a previous study, locomotor activity was recorded in response to an acute injection of AMPH in male rats receiving chronic antipsychotic treatment over a period of twelve days (Samaha et al., 2007). Chronic, continuous antipsychotic treatment became progressively ineffective at blocking AMPH-induced locomotion, with the higher doses resulting in a potentiated response to AMPH five days after treatment cessation. In the current study, we administered the typical antipsychotic, haloperidol (HAL), at the lower

concentration of the chronic regimen used by Samaha *et al.* (2007) which is still shown to reflect efficacious doses in humans (Kapur, Zipursky, Jones, Remington, & Houle, 2000; Samaha et al., 2008; Samaha et al., 2007) to either AMPH-sensitized or non AMPH-sensitized female rats. These ovariectomized (OVX) rats received either chronic low alone, or chronic low plus phasic high 17β-estradiol (E2) replacement to simulate two different estrogen levels during different phases of the estrous cycle in young females (Quinlan et al., 2008). Following an AMPH challenge, locomotor activity was observed and NAcc DA and its metabolites were measured using in vivo microdialysis.

It has been suggested that antipsychotic administration may lead to DA receptor supersensitivity which could lead to a rebound effect when drug administration is discontinued (Antelman et al., 1986; Samaha et al., 2008) and such a rebound was observed in male rats following discontinuation of continuous HAL at a higher concentration than used here (Samaha et al., 2007). To examine this phenomenon in females, HAL administration was discontinued for one week, after which locomotor activity in response to an additional AMPH challenge was examined.

Methods

Animals

Sixty-four female, Sprague-Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing 220-250 g were pair-housed and were the original N of this study. Cages were located in a 21°C room with a 12 h reverse light/dark cycle (lights off at 9 AM), with *ad libitum* access to food and water. Bedding consisted of a 50/50 mixture of corncob and beta-chip. All testing and surgical procedures were performed during the dark phase of the diurnal cycle. All animal protocols were previously approved by Concordia University's animal research ethics

committee and were in accordance with the guidelines put forth by the Canadian Council on Animal Care as well as the European Communities Council Directive of 24 November 1986.

Drugs

HAL (0.25mg/kg/day; Sandoz Canada Inc., QC, Canada) was administered subcutaneously (SC) using Alzet osmotic minipumps (model: 2002, 14-day delivery, at a rate of 0.5µl/h; Durect, Cupertino, CA, USA). This dose, when administered chronically, has been previously shown to result in approximately 75% dopamine D2 receptor (D2R) occupancy in the striatum of rats (Samaha et al., 2008; Samaha et al., 2007) similar to D2R striatal occupancies observed following effective antipsychotic doses in humans (Kapur et al., 2000).

D-Amphetamine sulfate (AMPH: 1mg/kg, 0.5mg/kg, 0.25mg/kg; Sigma-Aldrich) was dissolved in 0.9% saline and administered intraperitoneally (IP). These doses were selected based on previous studies inducing behavioural sensitization to AMPH as well as studies examining the efficacy of antipsychotics in response to an AMPH challenge (e.g. Samaha et al., 2007).

All rats were implanted with subcutaneous E2 pellets to provide a chronic low dose of E2 (0.36mg/pellet: 90-day release; Innovative Research of America, Sarasota, FL, USA). Additionally, half of the animals received a subcutaneous injection of E2 every second day (20µg/kg dissolved in sesame seed oil) in a volume of 0.5ml/kg body weight, providing an intermittent/phasic high dose. The low E2 rats also received an injection of sesame oil vehicle every second day as a control. These doses were chosen to mimic the levels of E2 in estrous and proestrous young cycling rats (Overpeck, Colson, Hohmann, Applestine, & Reilly, 1978; Quinlan et al., 2008).

Surgery

Rats were anesthetized using Isoflurane (Inhalation Anaesthetic, Richmond Hill, ON, Canada), and two 8.3 mm stainless steel cannulae (21Ga, Plastics-One, Ronaoke, VA, USA) were stereotaxically implanted, bilaterally, toward both the left and right NAcc at the following coordinates from bregma: antero-posterior (AP) = 1.8 mm, lateral-medial (LM) = 3.0 mm and dorso-ventral (DV) = 5.4 mm, at a 10° angle. Cannulae were anchored into place with skull-screws using dental cement. Obturators (26 Ga, Plastics-One, Roanoke, VA, USA) were inserted into each cannula. Following surgery, animals were single-housed for the remainder of the experiment and were handled every day for approximately 5 minutes/day.

All surgical procedures, i.e. ovariectomies, and E2 pellet and cannula implantations, were performed at the same time in order to avoid multiple sessions of general anesthesia. All rats were ovariectomized via bilateral lumbar incisions (1cm). Post-ovariectomy, rats were implanted with E2 pellets in the nape region. They were administered the analgesic drug, Anafen (0.1 ml/rat, SC; MERIAL Canada Inc., Morgan Baie d'Urfe, QC, Canada) and the antibiotic Penicillin G (0.2 ml/rat, intramuscular; CDMV, St. Hyascinthe, QC, Canada). Antibiotic ointment (By/Par Pharmaceuticals Inc., Brampton, ON, Canada) was also applied to the incision. Rats were allowed a week to recover in their home cages following surgery.

AMPH sensitization and challenge

To avoid possible stress associated with the introduction of a novel environment, rats were habituated to the locomotor boxes two hours/day for two consecutive days. In addition, each rat received an IP injection of saline one day before the induction phase of AMPH sensitization. Half of the rats were then administered a single daily AMPH (1mg/kg IP) injection (sensitized group;

SEN) and half were administered saline (non-sensitized group; NON) for four consecutive days while locomotor activity was recorded (Robinson, 1984; Robinson & Becker, 1986).

Spontaneous locomotor behaviour was monitored in activity chambers (Truscan Activity Monitoring System/Coulbourn Instruments, Allentown, PA, USA). Each chamber (39 x 42 x 50cm) had four transparent Plexiglas walls and a removable plastic tray at the bottom. Chambers were placed in sound-attenuating boxes in a dark room. Locomotor activity was monitored for a period of 120 minutes, by recording infrared beam interruptions on two sensor rings placed around the chambers (on the outside of the Plexiglas walls), creating a 16 x16 beam matrix. The monitoring session was divided into pre-injection (30 min) and post-injection (90 min) components, during which the Truscan Software recorded total time spent moving. All rats were tested throughout the experiment in the same respective activity chamber at the same time of day.

After a one week AMPH withdrawal period, rats were administered an initial AMPH challenge (0.5 mg/kg IP) to determine if they exhibited sensitization to the locomotor stimulating effects of AMPH (see Fig.1 for experimental timeline). The doses selected for the subsequent challenge injections are based on a pilot study, where it was observed that AMPH doses higher than 0.25mg/kg resulted in stereotypy (data not shown).
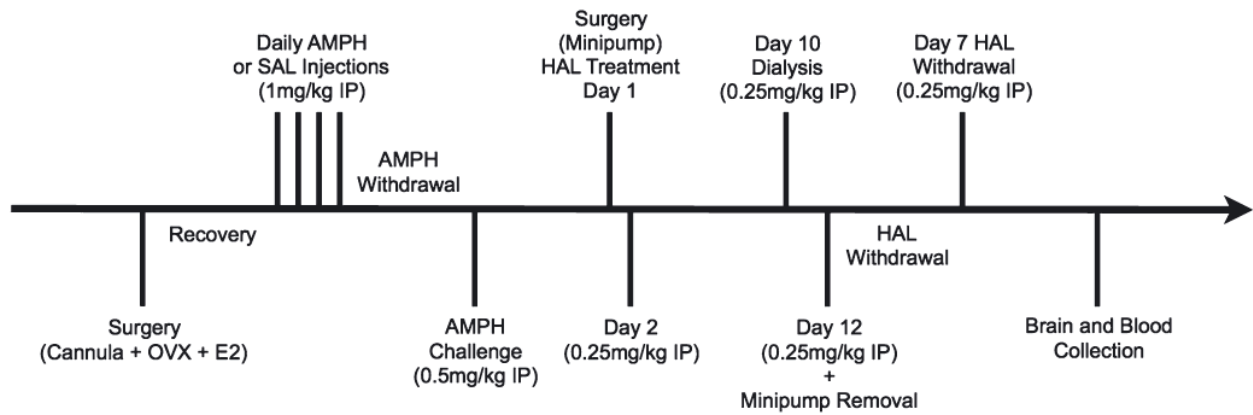
Figure 1: Schematic of the experimental timeline. After ovariectomy (OVX), cannula and estrogen replacement surgery, rats received four daily injections of either amphetamine (AMPH) or saline intraperitoneally (IP). One week after the last AMPH injection, all rats received an AMPH challenge. Following the challenge, rats were implanted subcutaneously with either haloperidol (HAL) - or saline (SAL)-containing delivery minipumps. At two and twelve days into HAL/SAL treatment, as well as one week after minipump removal, all rats were injected with AMPH and locomotor activity was measured. Dialysate collection occurred ten days into HAL/SAL treatment.

As stated previously, rats were divided into two main groups: AMPH-sensitized (SEN; n=32) and non-sensitized controls (NON; n = 32). Within each of these groups and following the initial AMPH challenge, rats were assigned to one of four E2 groups: AMPH-sensitized with low E2 replacement (low E2/SEN, n = 16); AMPH-sensitized with high E2 replacement (high E2/SEN, n = 16); Non-sensitized with low E2 replacement (low E2/NON, n = 16); And non-sensitized with high E2 replacement (high E2/NON, n = 16). These groups were each then further divided into two conditions depending upon whether they received chronic HAL or chronic saline (SAL). The final group designations were as follows: sensitized, with high E2 replacement and HAL (HE/SEN; n = 8), sensitized with high E2 replacement and SAL (SE/SEN; n = 8), sensitized with low E2 replacement and HAL (He/SEN; n = 8), low E2 replacement and SAL (Se/SEN: n = 8), non-sensitized with high E2 and HAL (HE/NON; n = 8), non-sensitized with high E2 and SAL (SE/NON; n = 8), non-sensitized with low E2 and HAL (He/NON; n = 8) and non-sensitized with low E2 and SAL (Se/NON: n = 8). Rats were administered four subsequent AMPH (0.25 mg/kg, IP) challenges on days 2, 10 and 12 of HAL/SAL treatment, and one week after discontinuation of HAL treatment. Locomotor activity was assessed on days 2 and 12 in order to compare short-versus long-term HAL treatment.

In Vivo Microdialysis

Microdialysis was performed in response to an AMPH challenge, with dialysate samples being collected from both hemispheres on day 10 of HAL treatment. Microdialysis testing occurred inside four separate operant chambers located inside foam-insulated isolation units that minimized noise and other environmental stimuli (Coulbourn Instruments; Whitehall, PA, USA). The operant chambers (28 x18 x19 cm) were equipped with a fan and a house light. The ceiling of the isolation unit had a small opening that allowed for unobstructed passage of the dialysis probe

tubing into the operant chamber. The operant chambers had grid floors with a plastic tray underneath filled with beta chip.

Probes were assembled according to previously reported methods (Sorge, Rajabi, & Stewart, 2005). They consisted of 20 µm diameter polyethylene (PE) tubing (70-75 cm long; Plastics-One) with one end connected to the stainless steel shaft of a dual-channel liquid swivel (HRS Scientific, Montreal, QC, Canada). The swivel was located on top of the isolation unit, which was connected to a variable speed electric syringe infusion pump (Harvard Apparatus, South Natick, MA, USA). Dialysate was collected from the outlet of the probe into 0.5 ml Eppendorf tubes (Sigma-Aldrich). The other end of the PE tubing was attached to a probe tip consisting of 26 Ga stainless steel tubing, 22 mm in length (Fisher Scientific, Nepean, ON, Canada) and a 2.5 mm long semi-permeable membrane (280 µm OD, 220 µm ID; with a molecular weight cut off of 13 000; Fisher Scientific). The outer end membrane was occluded with epoxy syringe glue (Henkel, Mississauga, ON, Canada) to create a closed system for the flow of dialysate. Small-diameter fused silica tubing (Polymicro Technologies; Phoenix, AZ, USA) extended into the probe 0.5 mm from the glued tip of the semi-permeable membrane. A stainless steel collar was screwed onto the cannula to secure the probe.

Ten days following minipump implantation, rats were anesthetized and microdialysis probes were lowered into each guide cannula 5 h before dialysate sampling began. When lowered, the probe extended 3.0 mm beyond the guide cannula directing the probe tip and membrane towards the center of the NAcc. Artificial cerebrospinal fluid (aCSF; 145mM Na$^+$, 2.7mM K$^+$, 1.2mM Ca^{2+}, 1.0 mM Mg^{2+}, 150mM Cl$^-$, 0.2mM ascorbate, 2mM Na$_2$HPO$_4$, pH 7.4 ± 0.1; Sigma) was perfused through the probe during a period of five hours to prevent occlusion and stabilize the baseline, at a rate of 1.0 µl/min. Following this period, 6 baseline dialysate samples were collected.

Each sample was collected for 10 min at a flow rate of 1.0 μl/min (resulting in 10 μl of dialysate/sample). Samples were immediately placed in dry ice and stored at -80 °C. After baseline, rats were administered AMPH (0.25 mg/kg IP) and another 12 samples were collected every 10 min for a period of 2 hours. Upon completion of microdialysis, the probes were removed and replaced with obturators and the animals were returned to their home cages.

Dialysate samples were thawed and immediately analyzed for DA and its metabolites, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), using HPLC with electrochemical detection. The samples were loaded through manual injection ports (Reodyn 7125; 20 μl loop) onto C-18 reverse-phase columns (5 μm, 15 cm; Higgins Analytical). DA and its metabolites were measured on separate independent channels with dual-channel ESA coulometric detectors (Coulochem III, with a 5011 model analytical cell) for reduction and/or oxidation currents. Mobile phase was circulated through at a flow rate of 1.1ml/min by Waters 515 HPLC pumps (Waters, QC, Canada), and consisted of 20% acetonitrile 40ml, 0.076 M sodium dodecyl sulphate, 0.1 M EDTA, 0.058 M $NaPO_4$, and 0.27 M citric acid with a pH of 3.35. Known amounts of standard DA and its metabolites (concentrations: [DA] 0.384 pg/μl; [DOPAC] 90 pg/μl; [HVA] 90 pg/μl; Sigma-Aldrich) were used to calibrate the system using estimates from peak heights by comparison with standard injections. Extracellular levels of DA (elution time ~ 6.5 min) and its metabolites (DOPAC elution time ~ 2.25 min; HVA elution time ~ 3.7 min) were analyzed using the EZChrom Chromatography Software Data system (Scientific Software, San Ramon, CA, USA).

Histology and Plasma E2 Levels

Following the final AMPH challenge, rats were decapitated and brains were removed and flash frozen for later histology, while blood was collected from a subset of rats (n = 14) to determine circulating E2 levels. Blood was stored on ice and immediately centrifuged. Plasma was then collected and stored at -20^0C until assayed. E2 was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Life Technologies, Frederick, MD, USA). The assay antibodies have 100% cross-reactivity with E2 and 0.2% and 0.05% cross-reactivity with estrone and estriol, respectively. The range of the assay is between 0 and 2000 pg/ml and the reported inter-assay variation is 7–9%. Brains were sliced along the coronal plane at 40 µm using a cryostat. Sections were mounted onto glass slides and stained with cresyl violet to confirm probe placements.

Statistical analysis

Samaha et al. (2007) showed that during a 12 day chronic HAL treatment regimen, male rats respond to the locomotor activity-reducing effects of HAL in response to AMPH by day 2 but this effect disappears by day 12. To examine if this effect is similar in females and if E2 levels might influence it, here day 2 HAL treatment was compared to day 12 in both SEN and NON females with either high or low E2 replacement. Spontaneous activity was expressed as total moving time during 5-minute bins following AMPH. Data were analyzed using eight, two-way mixed analyses of variance (ANOVAs), comparing high E2 replacement paired with SAL, low E2 replacement paired with SAL, high E2 replacement paired with HAL and low E2 replacement paired with HAL, at days 2 and 12 into treatment for both sensitized and non-sensitized groups. Between factors were day (2, 12), while time following AMPH injection served as within factor.

A separate 3-way ANOVA was conducted on AMPH-induced locomotor activity following a week of HAL withdrawal, with E2 and HAL treatment as between factors and time as within factor.

For *in vivo* microdialysis, concentrations of DA and its metabolites were converted to percent baseline. That is, the three samples taken prior to drug injection were averaged as baseline and subsequent samples were converted as a percentage of this value. Four, two-way mixed ANOVAs were performed on DA levels with sensitization (SEN vs. NON) as the between factor and time as the within factor.

To determine if sensitization had occurred, an independent samples *t*-test was used, comparing average time spent moving in response to an AMPH challenge for the SEN compared to the NON group. Plasma estradiol levels were compared between the high E2 and low E2 groups using an independent samples *t*-test.

Results

Behavioural Assessment: AMPH-sensitized rats (SEN)

Three rats died during microdialysis testing (day 10) while another rat died during surgery, thus a final N of 60 was used for the locomotor analyses. Expression of sensitization was measured by administering half the dose (i.e. 0.5mg/kg) of AMPH used for induction (i.e. 1mg/kg) following a one-week withdrawal period. The locomotor response of the SEN group was significantly greater than the NON rats in response to a low dose challenge AMPH injection ($t_{34} = 2.12$, $P < 0.0001$), illustrating that sensitization to the locomotor activating effects of AMPH had occurred in the SEN group (Fig. 2).

Expression of AMPH sensitization
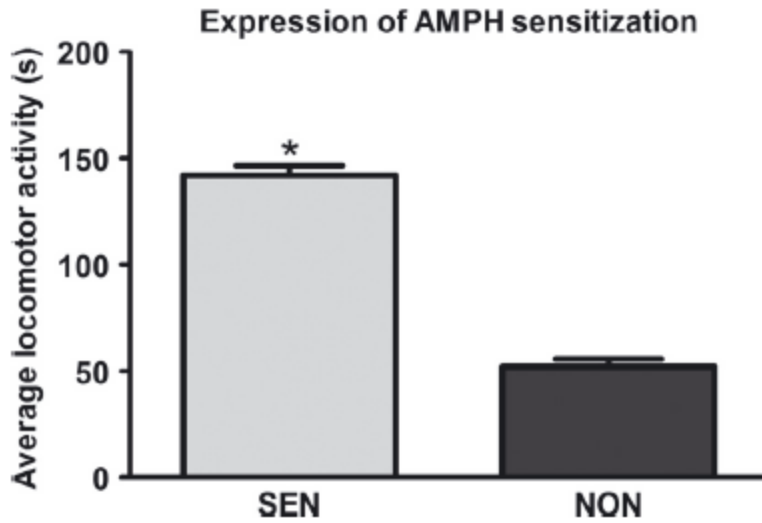
Figure 2: Mean (+SEM) locomotor activity in response to an acute amphetamine (AMPH) injection (0.5 mg/kg, IP) in rats following amphetamine withdrawal. The greater activity of the amphetamine-treated rats suggests that behavioural sensitization has occurred. Asterisk indicates AMPH-sensitized (SEN) group showing significantly greater activity than non-sensitized (NON) group in response to an AMPH challenge ($P < 0.0001$).

AMPH-sensitized, HAL-treated rats with high E2 replacement (HE group) showed a difference in AMPH-induced locomotor activity (Fig. 3a), where HAL significantly reduced AMPH-induced activity on day12 compared to day 2 of treatment ($F_{1,6}$=17.98, P = 0.005). No other comparisons were statistically significant (Fig. 3b, 3c, 3d). These findings indicate that HAL had little or no behavioural effect in female rats after 2 days of treatment but did so after 12 days – notably, only in females with high levels of E2.
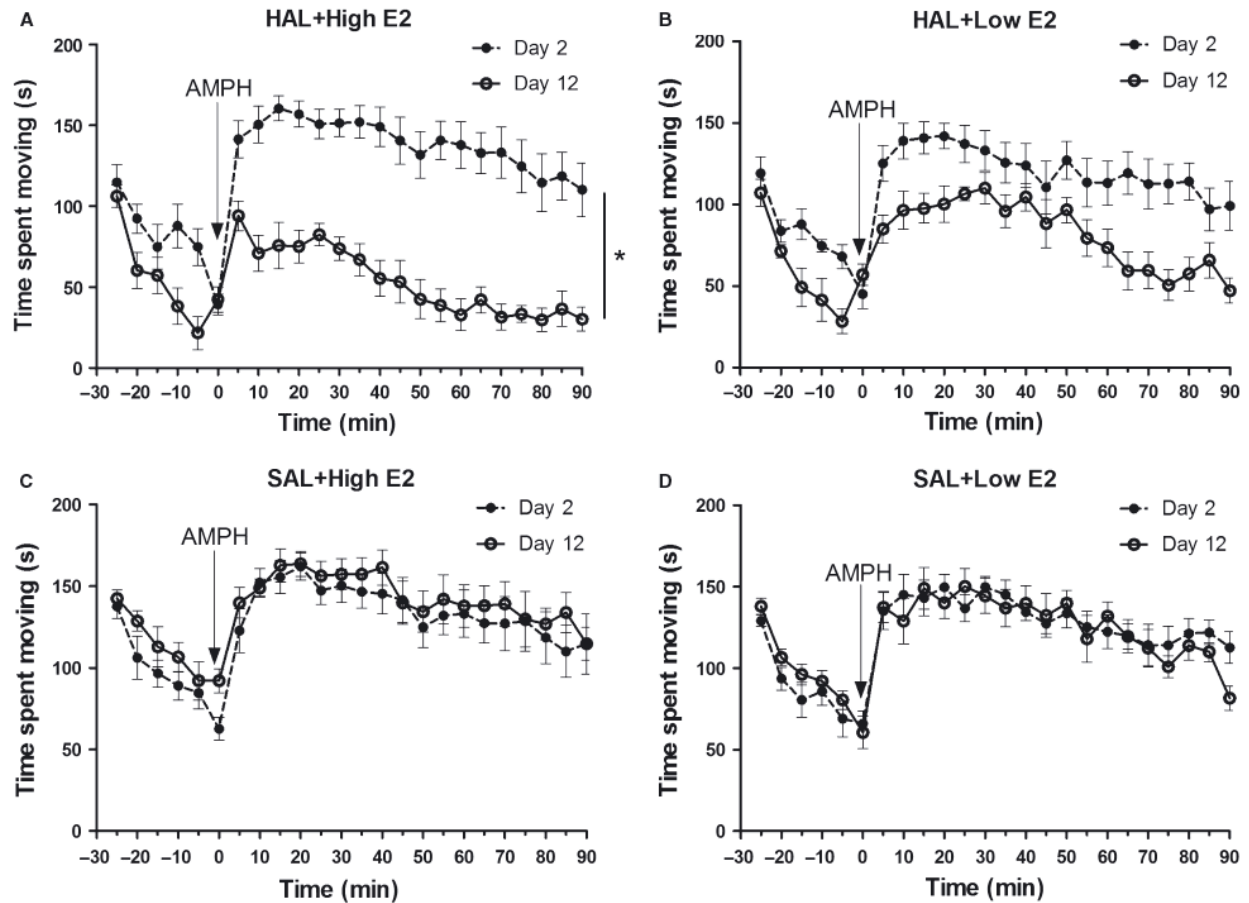
Figure 3: Mean (± SEM) locomotor activity of amphetamine-sensitized (SEN) groups (A, B, C and D) on days 2 and 12 of treatment in response to amphetamine (AMPH) challenge (0.25 mg/kg, IP). HAL: haloperidol, SAL: saline, E2: estrogen. Asterisk indicates day 12 significantly lower than day 2 ($P < 0.01$) in rats receiving high E2 replacement in conjunction with HAL treatment.

Non-sensitized rats (NON)

There was a significant difference in AMPH-induced locomotor activity between days 2 and 12 in the SAL-treated group (Fig. 4c) receiving high E2 replacement ($F_{1,6} = 13.39$, $P = 0.011$). There were no differences in activity in the other non-sensitized groups, suggesting that high E2 replacement exacerbated the effects of AMPH after 10 days of treatment (Fig. 4a, 4b, 4d).
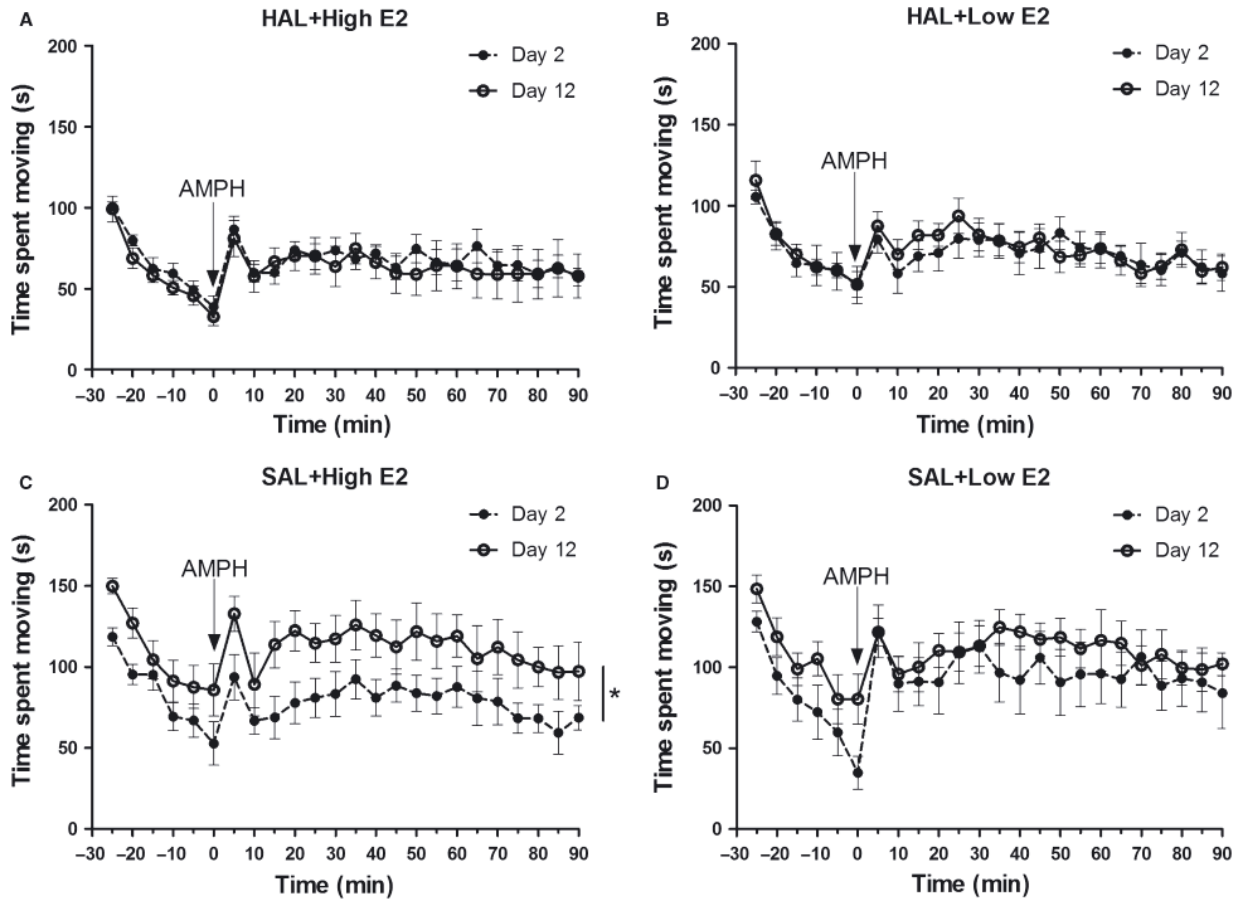
Figure 4: Mean (± SEM) locomotor activity of non-amphetamine sensitized (NON) groups (A, B, C and D) on days 2 and 12 of treatment in response to amphetamine (AMPH) challenge (0.25 mg/kg, IP). HAL: haloperidol, SAL: saline, E2: estrogen. Asterisk indicates day 12 significantly higher than day 2 ($P < 0.05$) in NON rats with high E2 replacement, in the absence of HAL treatment.

Taken together, the behavioural findings show that although in AMPH-sensitized rats, high E2 replacement enhanced the locomotor activity-reducing effects of HAL 12 days into treatment, high E2 replacement alone increased locomotor activity in non-sensitized rats after chronic administration of AMPH.

HAL withdrawal

There were no differences in locomotor activity after HAL withdrawal, regardless of sensitization protocol, antipsychotic treatment or hormone replacement (Fig. 5a, 5b).
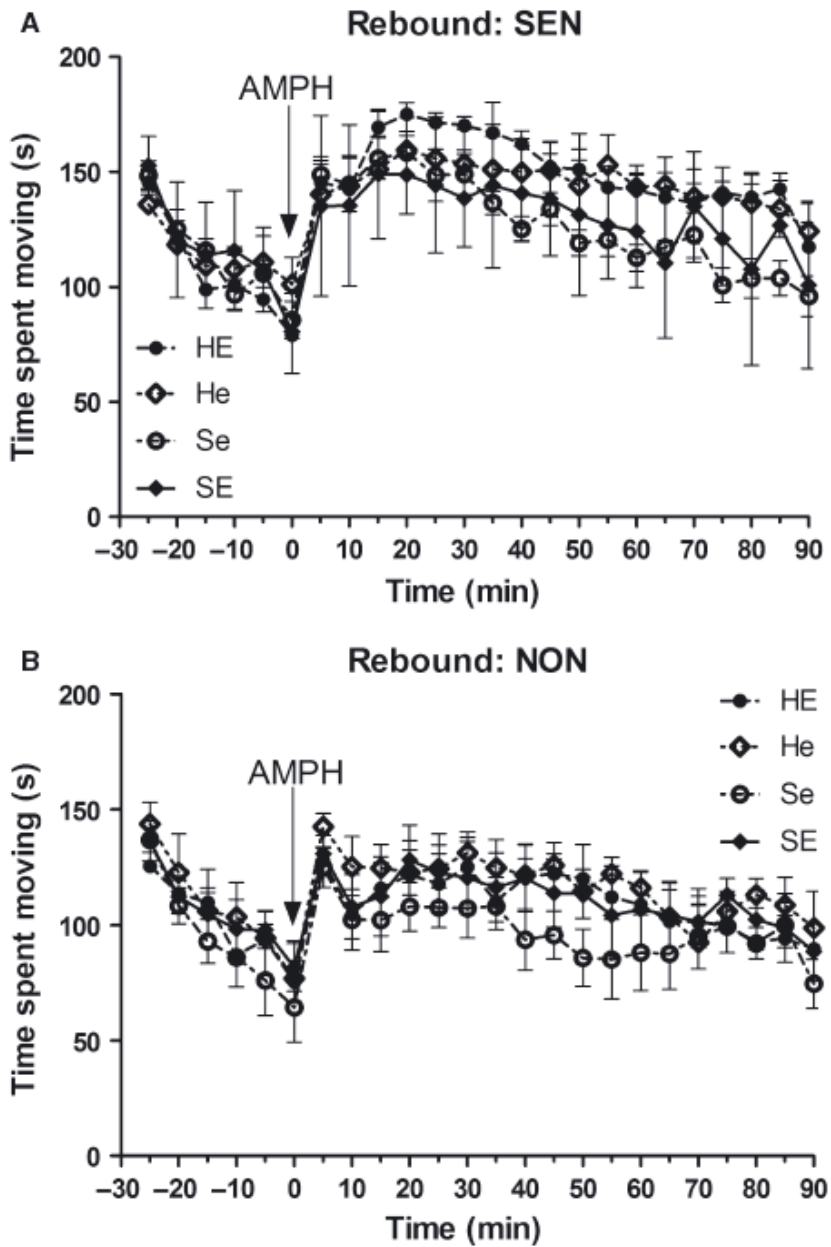
Figure 5: Mean (± SEM) locomotor activity one week after haloperidol treatment cessation in response to 0.25 mg/kg IP amphetamine (AMPH) in AMPH-sensitized (SEN; A) and non-AMPH-sensitized (NON; B) rats. HE: haloperidol with high estradiol, He: haloperidol with low estradiol, SE: saline with high estradiol and Se: saline with low estradiol.

NAcc DA Availability

During *in vivo* microdialysis, both the left and right probes of seven rats failed either because of blockage or leaking. Although there is no microdialysis data for these rats, they are included in the behavioural analyses. In 19 rats, one of the two probes failed (5 right and 14 left) during dialysate collection. In those cases, data from a single probe, viz. a single left or right NAcc collection, was used in the final analysis for that rat. In those remaining rats whose dialysate was successfully collected from both sides, an analysis on left versus right NAcc DA levels was conducted (data not shown). No differences were observed and thus data were averaged from both sides of the NAcc for each rat. Thus, a final N of 53 rats (HE/SEN = 6; HE/NON = 8; He/SEN = 6; He/NON = 6; SE/SEN = 6; SE/NON = 7; Se/SEN = 7; Se/NON = 7) were included in the analysis of NAcc DA levels.

Analysis of the DA metabolites, HVA and DOPAC, revealed that these metabolite levels changed in the same manner as previously reported in response to AMPH (data not shown). HVA and DOPAC levels decreased in tandem with DA increases as is typically observed in response to AMPH (Samaha et al., 2007). Because the dialysis probes used in this experiment were made in house, there is generally a great deal of variability between probes in absolute DA recovery. Thus, DA analysis was calculated using percent baseline values. Nonetheless, absolute DA values are shown here in Table 1.

Table 1. Absolute baseline and peak levels of nucleus accumbens dopamine

	n	Mean (pg/µL)	SEM
Baseline			
SEN			
HE	6	0.180	0.031
He	6	0.156	0.027
SE	6	0.134	0.025
Se	7	0.135	0.022
NON			
HE	8	0.116	0.027
He	6	0.116	0.027
SE	7	0.146	0.023
Se	7	0.122	0.022
Peak			
SEN			
HE	6	0.522	0.151
He	6	0.483	0.144
SE	6	0.473	0.115
Se	7	0.287	0.038
NON			
HE	8	0.274	0.040
He	6	0.232	0.024
SE	7	0.314	0.052
Se	7	0.269	0.065

Shown are baseline, absolute mean baseline dopamine levels; Peak, absolute peak dopamine levels post AMPH injection in NAcc in AMPH-sensitized (SEN) and non-AMPH-sensitized (NON) female rats. HE, haloperidol with high estradiol; He, haloperidol with low estradiol; SE, saline with high estradiol; Se, saline with low estradiol. No significant differences were observed between groups.

As can be seen in Figure 6A, in the absence of HAL, DA levels of high E2 rats were significantly ($F_{1,11}$ = 18.40, P = 0.001) greater in response to AMPH when rats were sensitized. On the other hand, following chronic HAL treatment, this effect disappeared (Fig. 6B). This suggests that chronic HAL reduces DA availability in the NAcc in response to a challenge dose of AMPH in sensitized high E2 rats. In contrast to the high E2 group, when low E2 rats were administered chronic HAL, the sensitized group had significantly ($F_{1,10}$ = 7.32, P = 0.022) greater dopamine levels than those that were not sensitized (Fig. 6D). There were no differences in NAcc DA levels between sensitized and non-sensitized rats in the groups receiving SAL paired with low E2 replacement (Fig. 6C).
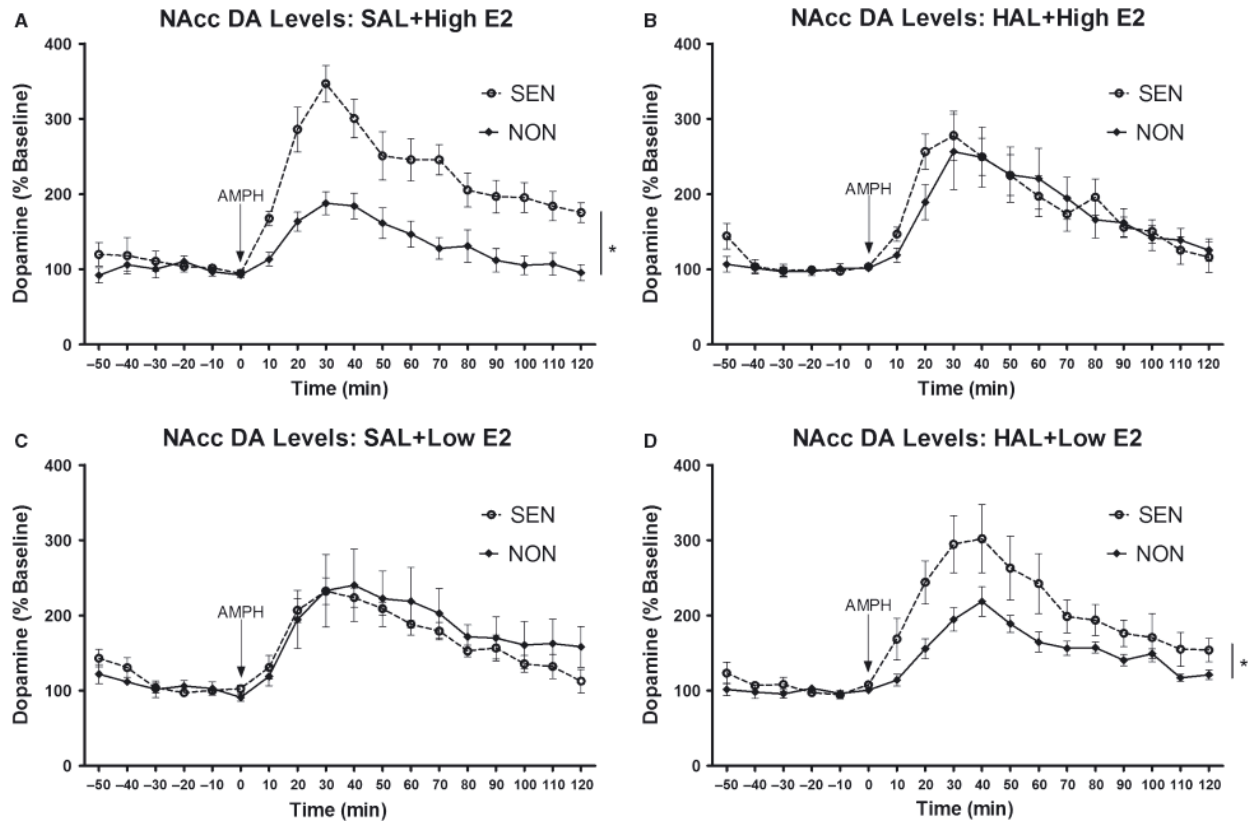
Figure 6: Mean (± SEM) dopamine levels in nucleus accumbens (NAcc) following an amphetamine (AMPH) challenge on day 10 of haloperidol (HAL) treatment in AMPH-sensitized (SEN) and non-AMPH-sensitized (NON) rats, differentiated by hormonal and haloperidol (HAL) treatment (A, B, C and D). Asterisk indicates SEN group significantly higher than NON group ($P < 0.05$) in rats receiving chronic HAL treatment in conjunction with low estrogen (E2) replacement, and in rats with high E2 replacement in the absence of HAL. NAcc: nucleus accumbens, DA: dopamine, SAL: saline.

Histology and plasma E2 levels

Probe placements for all animals were confined to the NAcc, as shown in figure 7 (A, B). Probe placements were located +2.16-+1.20 mm from bregma (Paxinos & Watson, 1998). All probes were located both within the core and shell of the NAcc. ELISA results (Fig. 8) indicate an approximate 2-fold increase in E2 levels (13.31 pg/ml ± 3.55) in high E2 rats compared to low E2 rats (6.59 pg/ml ± 0.85) one day following the last high E2 injection ($t_{13} = 2.12$, $P = 0.026$).
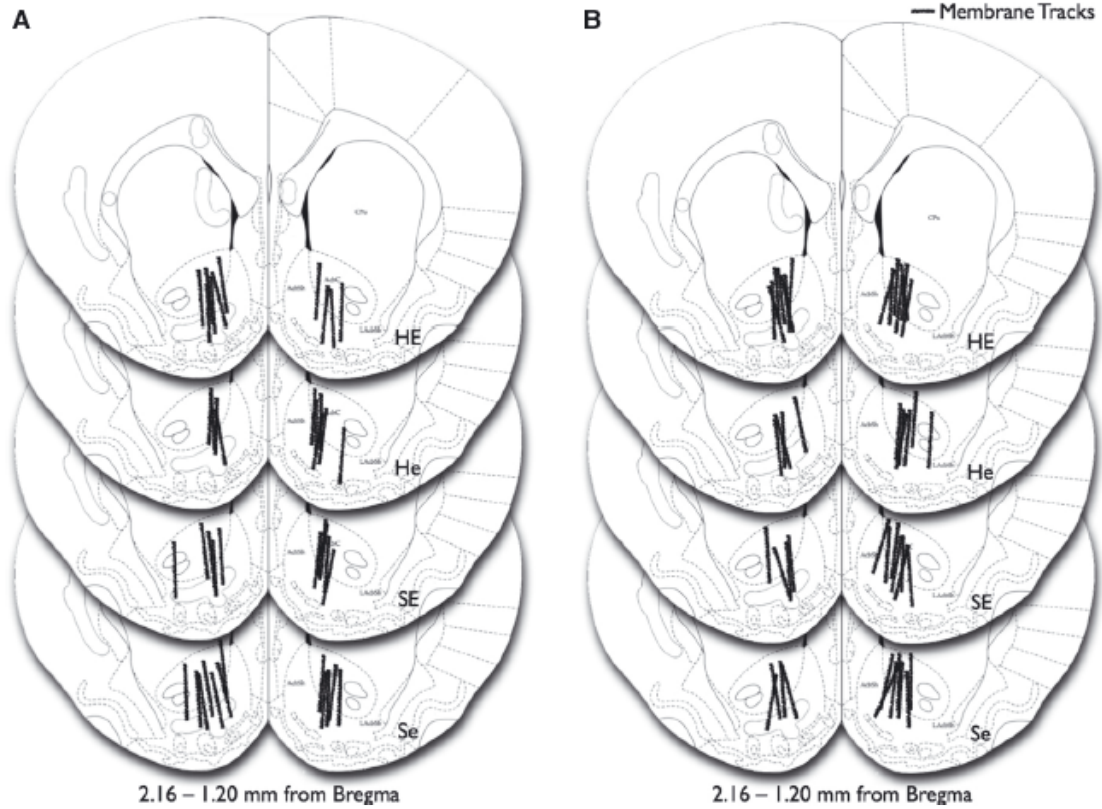
Figure 7: Schematic of rat brain showing microdialysis probe collection site placements within each group, targeting the nucleus accumbens in sensitized (A) and non-sensitized (B) rats. Lines represent the probe membrane. HE, haloperidol with high estradiol; He, haloperidol with low estradiol; SE, saline with high estradiol and Se, saline with low estradiol.
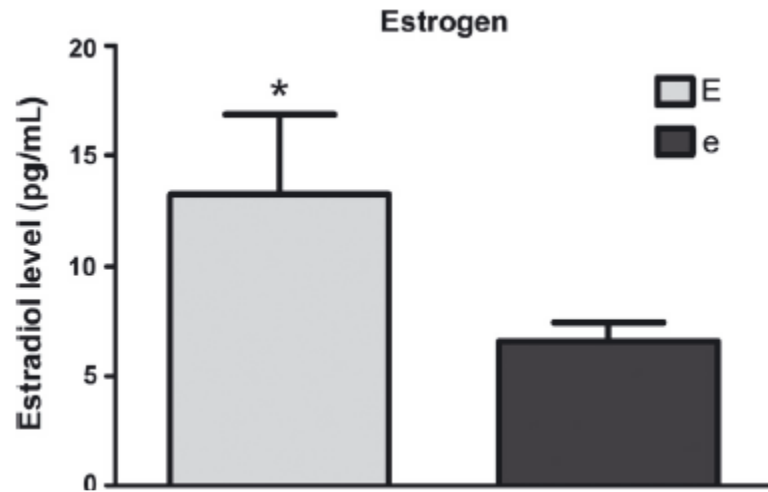
Figure 8: Mean (+SEM) estradiol (E2) levels in rats with high and low hormone replacement twenty four hours following the final E2 injection. Asterisk indicates high estrogen group showing a significant increase of circulating estrogen ($P < 0.05$). E: high estrogen replacement, e: low estrogen replacement.

Discussion

Previous studies suggest that E2 may have antipsychotic-like properties, possibly through its interaction with the dopaminergic system (Kulkarni et al., 2001). The aim of this study was to investigate this interaction in chronic low-dose haloperidol-treated, AMPH-sensitized and non-sensitized female rats using behavioural and neurochemical analyses.

A significant effect of HAL in sensitized rats was observed on day 12 compared to day 2, when AMPH-induced locomotor hyperactivity is reduced in rats receiving high E2, but not low E2, replacement. The implications of these results are twofold. Firstly, short-term (i.e. two days) antipsychotic treatment has no effect in reducing AMPH-induced locomotion at this dose in female rats, in contrast to previous findings in male rats (Samaha et al., 2007) and humans (Stern et al., 1993). Secondly, long-term (i.e. twelve days) low dose HAL treatment is efficient only in female rats receiving high E2 replacement in sensitized rats. These results partly contradict previous findings by Samaha and colleagues (2007), who observed that at day 12 neither high nor low doses of haloperidol proved to be efficient in reducing AMPH-induced locomotion in male rats. Our findings suggest that E2 has antipsychotic-like effects when paired with long-term HAL regimen in AMPH-sensitized female rats. One of the possible reasons behind the discrepancy between the current and previous findings is likely the fact that the previous study (Samaha et al., 2007) used male rats and females have been shown to require lower doses of antipsychotic drugs (Melkersson, Hulting, & Rane, 2001).

Haloperidol withdrawal had no effect on AMPH-induced locomotion, regardless of whether the rats were sensitized or not (Fig. 5). The study by Samaha *et al.* (2007) yielded similar results, where male rats treated with a low dose of HAL (0.25mg/kg) failed to show a potentiated

response to AMPH after a 5-day period of antipsychotic withdrawal, while rats treated with a higher dose did show a potentiated response to AMPH (Samaha et al., 2007). It would be interesting to see in future studies if females show a withdrawal effect at a higher dose of HAL.

Amphetamine sensitization enhanced the NAcc DA response to acute AMPH when rats received high E2 replacement (Fig. 6A). When high E2 replacement rats were administered chronic HAL, this effect went away (Fig. 6B). That is to say, HAL was effective in reducing the higher NAcc DA levels observed in sensitized rats when they were given high E2. By comparison, in rats administered low E2 replacement, HAL did not reduce NAcc DA levels in sensitized rats (Fig. 6D) to the same degree as seen in high E2 rats (Fig 6B). In other words, NAcc DA levels were significantly higher in AMPH-sensitized rats compared to non-sensitized rats when HAL was accompanied by low E2 replacement. Finally, there were no differences in DA availability between sensitized and non-sensitized low E2 rats in absence of HAL treatment (Fig. 6C). Although it has been established that AMPH sensitization and acute DA release in response to psychostimulants are at least in part mediated by estrogen, it is unclear why high levels of E2 replacement yield differential neurochemical, as well as behavioural effects compared to low E2.

The mechanisms by which E2 is effective in reducing AMPH-induced locomotion when paired with prolonged HAL treatment are unknown. The effects of E2 on striatal DA are not limited to only release, but also to DA receptor state. Dopamine D2 receptors (D2Rs) are thought to be only functional when they are in their high affinity state (D2^{High}) and are inactive when they are in their low affinity state (P. Seeman, 2006, 2010, 2011, 2013). An *in vitro* study showed that an acute physiological dose of E2 administered to OVX rat striatal tissue produces a rapid conversion of DA D2Rs from their high to low affinity state (Levesque & Dipaolo, 1988). Similarly, the affinity state of DA D2Rs fluctuates across the estrous cycle with the most DA D2Rs

in the high affinity state during diestrus when estrogen is low and most in the low affinity state during behavioural estrus and proestrus (Dipaolo, Falardeau, & Morissette, 1988). In addition, chronic replacement of E2 to OVX rats results in an increase in striatal DA D1 receptor (D1R) binding suggesting that E2 affects both the affinity state of D2Rs and the binding of D1Rs (Levesque & Dipaolo, 1989).

Previous research showed that although HAL treatment alone increased D2^{High} availability (Samaha et al., 2007; P. Seeman, 2009), when paired with AMPH, HAL reduces by 60% AMPH-elevated D2^{High} receptors (P. Seeman, 2009). One could speculate that different levels of circulating estrogen might influence the affinity state of DA D2R such that increased levels of estrogen might result in a shift in DA D2R affinity from its high state into a low one. This could potentially explain how E2 enhances the behavioural effects of HAL. Future studies should investigate the potential effects of estrogen replacement on the state of the DA D2R in the striatum of sensitized rats. On the other hand, such a post-synaptic mechanism may not explain how E2 affects the NAcc DA response to HAL. We have evidence that E2 affects D2R autoreceptor function in the dorsal striatum, such that autoreceptor function is less sensitive in high E2 rats (Hussain, Cossette, & Brake, 2013). This effect may be direct via estrogen receptors; our recent findings show that both ERα (estrogen receptor alpha) and GPER-1 (g protein-coupled estrogen receptor 1) are indeed located on DA terminals in the NAcc (manuscript in preparation). Although we have previously shown that this is not the case in the dorsal striatum (Almey, Filardo, Milner, & Brake, 2012). Thus, E2 may be acting at both pre- and post- synaptic sites in the NAcc to modulate the effects of HAL and possibly via different mechanisms.

Haloperidol became effective only in AMPH-sensitized rats receiving high E2 replacement, and only with prolonged treatment. These data mirror previous research on humans,

where estrogen, when added to antipsychotic treatment, significantly reduces schizophrenic symptoms (Akhondzadeh et al., 2003; Kulkarni et al., 1996; Kulkarni et al., 2001). In addition, the neurochemical analysis points at a direct link between NAcc DA availability and E2 levels, whereby locomotor activity in response to AMPH seems to be at least in part driven by this relationship. Although earlier studies have shown that estrogen replacement significantly increased postsynaptic striatal DA levels, as well as AMPH-induced stereotypy (Hruska, Ludmer, & Silbergeld, 1980), additional reports suggest that similar treatment reduced DA levels in the NAcc (Chavez et al., 2010). To our knowledge, this is the first study to demonstrate differences in NAcc DA availability in response to AMPH in sensitized female rats that are mediated by levels of E2. They are also the first to show that antipsychotic treatment efficacy does not decrease in female rats when administered chronically at a lower dose.

Study 1B: Integration of neural networks activated by amphetamine in females with different estrogen levels: A functional imaging study in awake rats.

Dan Madularu, Jason R. Yee, William M. Kenkel, Kelsey A. Moore, Praveen Kulkarni,

Waqqas M. Shams, Craig F. Ferris and Wayne G. Brake

Preface

The experiment outlined in Study 1A points at the nucleus accumbens as a possible site of dopamine-estradiol interaction, where it was found that the dopaminergic response to an amphetamine challenge is dependent on E2 levels, as well as antipsychotic treatment in AMPH-sensitized female rats.

It is well established that schizophrenia is not a disease of one nucleus, or a single neurochemical circuit, not is it limited by neuroanatomical delineations. Based on the findings in Study 1A, the complementary purpose of the present study is to map out the global effects of AMPH in areas and systems associated with schizophrenia. Here, the specific aim was to identify additional regions of interest vis-à-vis dopamine-estrogen interactions by using functional magnetic resonance and measuring blood-oxygen-level-dependent signal throughout the brain of AMPH-sensitized female rats.

Abstract

Previous studies demonstrate that schizophrenia symptomatology in women is dependent upon estrogen levels. Estrogen has beneficial properties when administered in conjunction with antipsychotics, and estrogen also alters dopamine neurotransmission (the target of most antipsychotic medications) in rats; suggesting a possible interaction between the two. The aim of the current study was to investigate this possible interaction using functional magnetic resonance imaging in awake, female rats. Here amphetamine-sensitized, ovariectomized rats receiving no, low, or high levels of estradiol replacement were used, and changes in blood-oxygen-level-dependent (BOLD) signal were recorded over time in response to an acute amphetamine injection. Increasing levels of estradiol increased BOLD activation in pathways previously known to be implicated in schizophrenia symptomatology, such as the mesocorticolimbic, habenular and olfactory pathways as well as more widespread areas. We propose here the first comprehensive "amphetamine activation map" integrating brain regions where dopaminergic transmission is influenced by estrogen levels.

Introduction

Schizophrenia symptoms are exacerbated in women when estrogen (E) levels are low, and women experience a change in symptoms during pregnancy when E levels fluctuate (Riecher-Rossler et al., 1994; M. V. Seeman & Lang, 1990). Women also show a second peak of onset or a worsening of symptoms after giving birth and/or experiencing menopause as E levels fluctuate and/or decline (Castle & Murray, 1993; Hafner, 2003). Thus it is suggested that E may play a role in schizophrenia onset and development in women. Women are also differentially responsive to antipsychotics. It has been consistently shown that women are more responsive to typical antipsychotics, yet women with schizophrenia require increasing doses of antipsychotics upon menopause in order to maintain remission of symptoms (M. V. Seeman & Lang, 1990). Yet, we still know surprisingly little about the neurobiological effects of E in this regard.

Sensitization to the stimulant effects of repeated amphetamine (AMPH) has been widely used as a rodent model of some of the neurochemical and behavioural aspects of schizophrenia, and there are sex differences in its emergence (for review, see Featherstone et al., 2007). For example, female rats show increased locomotor activation in response to acute and repeated injections of AMPH compared to males (Forgie & Stewart, 1994). Ovariectomized (OVX) female rats with E replacement show higher levels of AMPH-induced locomotor activity during induction compared to no-E controls (Forgie & Stewart, 1994) and E enhances sensitization to cocaine (Hu & Becker, 2003). We have shown that AMPH-sensitized, OVX rats show different behavioral and dopamine responses to a challenge injection of AMPH depending on their E replacement levels. That is, the antipsychotic, haloperidol, is effective in reducing locomotor activity in only those rats with high E replacement (Madularu, Shams, & Brake, 2014).

A recent functional magnetic resonance imaging (fMRI) study showed that the prefrontal cortex (PFC) and ventral tegmental area (VTA) of OVX rats receiving E replacement yield robust AMPH-induced blood-oxygen-level-dependent (BOLD) signal increases compared to those without E replacement (Sarvari et al., 2014). An earlier study reported that OVX rats, with and without E replacement, show increased BOLD activation in the PFC, VTA, and nucleus accumbens (NAcc) in response to cocaine, with those with E replacement showing lower positive BOLD changes compared to those without (Febo, Ferris, & Segarra, 2005). On the other hand, repeated cocaine administration increases BOLD signal changes in the NAcc, VTA and hippocampus, but only in those with 17β estradiol (E2: the most potent E during reproductive years) replacement. While much is known about the involvement of the PFC, NAcc and VTA in AMPH-sensitization models, there has been little attention paid to the integrated neural networks associated with, and independent of, the mesocorticolimbic dopamine system.

With the advent of fMRI in awake animals it is now possible to offer a global perspective of changing brain function with high temporal and spatial resolution. When combined with 3D-segmented and annotated brain atlases as well as computational analysis, it is possible to reconstruct distributed and integrated neural circuits, or "finger prints" of brain activity (Ferris et al., 2014). To this end, here we use fMRI to corroborate previous findings on E-dependent effects of AMPH. Moreover, we can report the changing pattern of activation from 172 brain areas, showing a clear delineation of neural circuits involved in learning and memory, motivation and olfaction. This experiment was carried out in awake, OVX, female rats that had been sensitized to AMPH and had either no, low constant, or low constant plus phasic high E2 replacement.

Methods

Animals

Twenty-seven OVX, Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 200-250 g were pair-housed in cages located in a 21^0C with a 12-h light-dark cycle (lights off at 19:00 h), with *ad libitum* access to food and water. Testing, injections, surgical procedures and imaging were performed during the dark phase of the diurnal cycle, in semi-dark conditions. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Northeastern University's Institutional Animal Care and Use Committee.

Drugs

AMPH (1 mg/kg, or 0.25 mg/kg; Sigma–Aldrich, UK) was dissolved in 0.9% saline and administered intraperitoneally (IP). These doses were selected based on previous studies inducing behavioural sensitization to AMPH as well as studies examining the efficacy of antipsychotics in response to an AMPH challenge (Madularu et al., 2014; Samaha et al., 2007).

Rats were divided into three groups, with respect to hormone replacement: no E2 (n = 8), constant low E2 (Low E2; n = 9) and constant low plus phasic high E2 (High E2; n = 10). The E2 (low and high) groups were implanted with sillastic capsules containing 17-β estradiol (Almey, Hafez, Hantson, & Brake, 2013). High E2-replacement rats also received a subcutaneous injection of 17-β estradiol every second day (20 μg/kg dissolved in sesame seed oil) in a volume of 0.5 mL/kg body weight, providing an intermittent phasic high dose. The no E2 and low E2 groups also received an injection of sesame oil vehicle every second day as an injection control. These doses were chosen to mimic the levels of estrogen in estrous and proestrous young cycling rats (Almey

et al., 2013; Madularu et al., 2014; Overpeck et al., 1978; Quinlan et al., 2008) and have been shown in our previous studies to affect responses to antipsychotics.

Surgery

Rats were anesthetized using isoflurane, and a 0.5 cm incision was made in the nape region. For the E2 groups, sillastic implants were placed subcutaneously, while no E2 rats received sham surgery (incision, but no implant). All rats were administered the analgesic drug, Anafen (0.1 mL/rat, SC; Merial Canada Inc., Morgan Baie d'Urfe, QC, Canada), and the antibiotic ointment (By/Par Pharmaceuticals Inc., Brampton, ON, Canada) was applied to the incision.

AMPH sensitization

AMPH sensitization was induced following E2 replacement/sham surgery. All rats were administered once- daily injections of AMPH (1 mg/kg IP) for four consecutive days, as described elsewhere (2014). This regimen has been previously demonstrated to induce sensitization to the locomotor activating effects of AMPH in our lab (Madularu et al., 2014). We showed that sensitized rats exhibit significantly greater locomotor activity (following a one-week withdrawal period) to a later, challenge injection of AMPH (0.5 mg/kg IP) compared to an AMPH challenge to saline-treated rats (Madularu et al., 2014). In the present study, imaging in response to an AMPH (0.25 mg/ml IP) challenge injection occurred after a three-week withdrawal period following the final AMPH injection during the induction phase.

Awake Animal Imaging

Acclimation. A week prior to the first imaging session, all rats were acclimated to the imaging system environment before being placed into the scanner itself. Rats were secured into a mock holding system similar to the actual coil system (Animal Imaging Research, Holden, MA) while anesthetized with 2-3% isoflurane for approximately 60 s. While under light anesthesia, the rats' heads were positioned into a flexible head-holding unit by placing the incisors onto a plastic bar. Next, the rats' head, along with the head holder, was placed into a mock coil using two lateral screws which gradually press against the flexible sides of the unit until the possibility for motion is reduced. Finally, a padded nose bar was pressed onto the rostral part of the head until the head was completely secured. Rats were habituated to this system prior to the imaging phase. The difference between the coil and mock systems is minimal. During the imaging phase, the mock coil was replaced by the actual quadrature transmit/receive volume coil, whose dimensions are identical. Following cessation of isoflurane, fully conscious rats were put into a mock scanner (a black box with a tape recording of MRI pulses) for 30 minutes at a time. Acclimation significantly reduces physiological effects of the scanner environment on the autonomic nervous system, including measures such as heart rate, respiration, corticosteroid levels, and motor movements (King et al., 2005), helping to improve contrast- to-noise and image quality.

Drug Administration. Polyethylene tubing (PE20) was inserted IP through a small incision using an 18 Ga needle as a trocar, immediately prior to the scanning procedure. Tubing was connected to an AMPH-containing syringe through a 26 Ga needle. The length of the PE 20 tubing was approximately 40 cm, of which 2-3 cm was inserted into the IP space. All injections were performed in the magnet, under awake conditions during image acquisition.

Animal Preparation with Imaging Protocol. Animals were scanned at 300 MHz using quadrature transmit/receive volume coil built into the rat head holder and restraining system for awake animal imaging (Animal Imaging Research, Holden, MA). The design of the coil provided complete coverage of the brain from olfactory bulbs to brain stem with excellent B1 field homogeneity. The design of the restraining imaging system included a padded head support obviating the need for ear bars helping to reduce animal discomfort while minimizing motion artifact as noted above. Imaging awake animals removes the obvious confound of anesthesia (Becerra, Chang, Bishop, & Borsook, 2011).

Image Acquisition. Experiments were conducted using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, Massachusetts) and a 20-G/cm magnetic field gradient insert (ID = 12 cm) capable of a 120-µs rise time (Bruker). At the beginning of each imaging session, a high-resolution anatomical data set was collected using the rapid acquisition with relaxation enhancement (RARE) pulse sequence (22 slice; 1.0 mm; field of vision [FOV] 3.0 cm; 256 × 256; repetition time (TR) 2.5 sec; echo time (TE) 12msec; NEX 2; 2 minute acquisition time. Functional images were acquired using a multi-slice Half Fourier Acquisition Single Shot Turbo Spin Echo (HASTE) pulse sequence. A single scanning session acquired 22 slices, 1.0 mm thick, every 6 s (FOV 3.0 cm, matrix size 96 x 96, NEX 1) repeated 250 times for a total time of 25 minutes. Each scanning session was continuous, starting with 50 baseline image acquisitions, then AMPH, followed by another 200 image acquisitions.

Bruker Paravision automatically finds the basic frequency, shims, power requirements for 90º and 180º pulses and sets the receiver gain. The HASTE sequence (RAREst), a spin-echo multislice pulse sequence used in these studies, corrects for field inhomogeneity, susceptibility artifact, chemical shift and other imaging distortions, and doesn't require any additional shimming

as would be the case for gradient echo pulse sequences which have been used for all previous pain imaging studies reported in the literature.

Data Analysis. Data were co-registered to a mean functional image using SPM8's co-registrational code (FIL Methods Group, UK) with the following parameters: Quality: 0.97, Smoothing: 0.35 mm, Separation: 0.5 mm. Gaussian smoothing was performed with a full width at half maximum (FWHM) of 0.8 mm. Preprocessed functional files were then exported to Medical Image Visualization and Analysis (MIVA; Boston, MA, USA) for registration and segmentation. Images were aligned and registered to a 3D rat brain atlas (Ekam Solutions, Boston, MA, USA) which is segmented and labeled with 172 discrete anatomical regions. The alignment process was facilitated by an interactive graphic user interface. The registration process involved translation, rotation and scaling independently and in all three dimensions. Matrices that transformed each subject's anatomy were used to embed each slice within the atlas. All pixel locations of anatomy that were transformed were tagged with regions of interest in the atlas, creating a fully segmented representation of each subject within the atlas. The inverse transformation matrix [Ti]-1 for each subject (i) was also calculated.

For voxel-based analysis, the BOLD % change of each independent voxel was averaged for all subjects with a baseline threshold of 2% BOLD change to account for normal fluctuation of BOLD signal in the rat brain (Brevard, Duong, King, & Ferris, 2003). A composite image of the whole brain representing the average of all subjects was constructed for each group for region of interest (ROI) analyses, allowing us to look at each ROI separately to determine the BOLD change and the number of activated voxels in each ROI. We also compared different points along the time course of the scan to assess the effects of AMPH over time. For our analyses of the AMPH neural circuit, we examined activation between 175-250 acquisitions (i.e. late scan) or 12.5 to 20

mins post-drug. We also compared activation at 100-175 acquisitions (i.e. early scan) or 5 min to 12.5 mins post-AMPH (Fig. 1). The template used to define the AMPH neural circuits was derived from previous work (Graybiel, Moratalla, & Robertson, 1990; Lin, Hou, & Jouvet, 1996; Nguyen, Kosofsky, Birnbaum, Cohen, & Hyman, 1992; Wechsler, Savaki, & Sokoloff, 1979).

Statistical Analysis. Scanning sessions consisted of 250 data acquisitions each, with a period of 6 seconds for each image, for a total time lapse of 1500 seconds or 25 minutes. Statistical *t*-tests were performed on each voxel (ca. 15,000 in number) of each subject within their original coordinate system. The average signal intensity in each voxel of the first five minutes of baseline (acquisitions 1-50) was compared to acquisitions 100-175, and acquisitions 175-250 following AMPH administration. The baseline threshold was set at 2% as noted above. The *t*-test statistics used a 95% confidence level, two-tailed distributions, and heteroscedastic variance assumptions. As a result of the multiple *t*-test analyses performed, a false-positive detection controlling mechanism was introduced (Genovese, Lazar, & Nichols, 2002). This subsequent filter guaranteed that, on average, the false-positive detection rate was below the cutoff of 0.05.

Volume of activation was compared across experimental groups using the nonparametric Kruskall-Wallis test statistic. In order to control for family-wise error rates, Bonferroni corrections were applied. The ROIs were rank ordered for their significance (Table 1). Brain areas were considered statistically different between experimental groups when comparison produced P-values less than or equal to the 0.05 cutoff.

In addition, two (early scan and late scan) separate repeated measures analyses of variance (ANOVA) were performed in order to assess the AMPH neural system time-course BOLD signal change in response to an AMPH challenge. For these analyses, the ROIs included as part of the

AMPH neural system were the substantia nigra pars compacta (SNc), substantia nigra pars reticularis (SNr), ventral pallidum (VP), ventral tegmental area (VTA) and infralimbic cortex (IL). Hormone replacement (i.e. no E2, low E2, high E2) was the between-factor while time (i.e. image acquisitions) was the within factor. For post-hoc analyses, Bonferroni corrections were performed, where appropriate.

Results

The positive BOLD changes as a percentage of the total ROI volume (i.e.$\left(\text{Normalized \# of voxels} = \frac{\text{\# activated voxels X 100}}{\text{total \# of voxels}}\right)$) for no E2, low E2 and high E2 groups in response to an AMPH challenge (0.25 mg/ml IP). The ROIs are ranked with respect to their significance as determined by Kruskal-Wallis analysis (Tables 1 & 2).

Table 1: Volume of early positive activation in response to amphetamine.

Region of Interest (ROI)	No E2			Low E2			High E2			p Value
	Med	Max	Min	Med	Max	Min	Med	Max	Min	
Dentate gyrus ventral	0	2	0	0	18	0	6.5	18	0	0.003
Habenula nucleus	0	11	0	0	3	0	7	15	0	0.004
Ventral subiculum	0	12	0	1	9	0	11.5	24	0	0.005
CA3 ventral hippocampus	0	0	0	0	25	0	5	22	0	0.005
External plexiform layer	0.5	20	0	8	61	1	28.5	102	0	0.006
Supramammillary nucleus	0	1	0	0	4	0	5.5	11	0	0.006
Granular cell layer	0	1	0	7	111	0	35	184	0	0.007
Glomerular layer	3	40	0	30	132	4	59.5	154	0	0.007
Periaqueductal gray thalamus	0	0	0	0	30	0	7.5	73	0	0.008
Medial geniculate	0	3	0	0	14	0	3	22	0	0.009
CA1 ventral hippocampus	0	1	0	0	6	0	1	6	0	0.015
Subiculum dorsal	0	7	0	0	7	0	7	40	0	0.019
Ventral tegmental area	0	0	0	0	10	0	1	12	0	0.019
Interpeduncular nucleus	0	0	0	1	18	0	3.5	14	0	0.020
Tenia tecta ctx	0	8	0	1	50	0	18	41	0	0.022
Pontine nuclei	0	6	0	10	86	0	17	35	0	0.029
Posterior hypothalamic area	0	0	0	0	9	0	0.5	11	0	0.033
Retrochiasmatic nucleus	0	3	0	0	0	0	1	3	0	0.034
Medial mammillary nucleus	1.5	11	0	0	4	0	4.5	16	0	0.037
Parafascicular thalamic nucleus	0	0	0	0	5	0	0.5	5	0	0.041
Ventral pallidum	0	0	0	0	22	0	1	15	0	0.043
Dentate gyrus dorsal	0	7	0	0	22	0	6.5	54	0	0.045
Entorhinal ctx	15	117	0	8	75	0	43	124	5	0.046
Substantia nigra reticularis	0	9	0	0	46	0	11	44	0	0.049

Ventral medial n. hypothalamus	0	7	0	0	9	0	7	16	0	0.068

Shown is a truncated list of brain areas and their median (med), maximum (max) and minimum (min) number of voxels as a percentage of the total ROI volume for No E2, Low E2 and High E2 rats following amphetamine, corresponding to the early scan. The volumes of activation for each ROI were analyzed using a nonparametric Kruskall-Wallis test and ranked with respect to their significance (cut-off at $P < 0.05$). The shaded columns highlight the median number of activated voxels for each ROI and each group.

Table 2: Volume of late positive activation in response to amphetamine.

Region of Interest (ROI)	No E2			Low E2			High E2			p Value
	Med	Max	Min	Med	Max	Min	Med	Max	Min	
Dentate gyrus ventral	0	3	0	2	15	0	6	20	0	0.003
Pontine nuclei	0	5	0	28	73	1	14	93	0	0.004
Granular cell layer	0.5	16	0	23	73	0	40	115	1	0.006
Reticular nucleus midbrain	0	8	0	2	40	0	9	22	1	0.006
Ventral subiculum	4.5	12	0	3	15	0	19.5	24	1	0.007
Glomerular layer	5	66	0	65	119	4	70	127	14	0.009
Supramammillary nucleus	0	2	0	0	4	0	6.5	10	0	0.010
Retrochiasmatic nucleus	0	2	0	0	0	0	1	3	0	0.012
Interpeduncular nucleus	0	1	0	2	21	0	6.5	17	0	0.015
10th cerebellar lobule	0	1	0	0	23	0	3	24	0	0.016
External plexiform layer	4	26	0	17	44	2	26.5	75	3	0.017
Ventral tegmental area	0	0	0	4	10	0	3	10	0	0.020
Frontal association ctx	2.5	14	0	17	29	1	13.5	25	0	0.022
CA1 dorsal hippocampus	0	2	0	0	17	0	5	67	0	0.025
Medial geniculate	0	4	0	2	14	0	6	21	0	0.026
Tenia tecta ctx	0	10	0	13	48	0	16	36	0	0.028
Diagonal band of Broca	0	2	0	5	12	0	1	12	0	0.032
Substantia nigra reticularis	0	16	0	8	41	0	17.5	22	0	0.034
Ventral orbital ctx	0	0	0	1	5	0	0	4	0	0.038

	No E2			Low E2			High E2			P
	med	max	min	med	max	min	med	max	min	
Substantia innominata	0	0	0	0	1	0	0	0	0	0.039
Substantia nigra compacta	0	0	0	1	12	0	0.5	6	0	0.039
Olfactory tubercles	5	41	0	41	86	0	20.5	68	0	0.040
Dentate gyrus dorsal	0	9	0	4	20	0	22.5	48	0	0.045
Lateral hypothalamus	11.5	35	0	21	94	0	42.5	79	4	0.046
Prelimbic ctx	0	0	0	1	15	0	1	10	0	0.048
Subiculum dorsal	0	9	0	2	9	0	12	35	0	0.050
Entorhinal ctx	20.5	74	0	22	67	1	67	167	14	0.050
Medial mammillary nucleus	1	13	0	3	7	0	12	15	0	0.057

Shown is a truncated list of brain areas and their median (med), maximum (max) and minimum (min) number of voxels as a percentage of the total ROI volume for No E2, Low E2 and High E2 rats following amphetamine, corresponding to the late scan. The volumes of activation for each ROI were analyzed using a nonparametric Kruskall-Wallis test and ranked with respect to their significance (cut-off at P < 0.05). The shaded columns highlight the median number of activated voxels for each ROI and each group.

Based on previous studies (Graybiel et al., 1990; Lin et al., 1996; Nguyen et al., 1992; Orzi, Dow-Edwards, Jehle, Kennedy, & Sokoloff, 1983; Uslaner et al., 2001; Wechsler et al., 1979), a 3D putative AMPH circuit was constructed using the rat brain atlas (Figure 1). Similar 3D circuits were constructed for the habenular (Figure 2) and olfactory systems (Figure 3) based on previous studies showing these systems to be affected in patients with schizophrenia (Shepard, Holcomb, & Gold, 2006; Turetsky et al., 2000). These 3D areas are collapsed into a single yellow volume the location of the average, significant change in BOLD signal. Red shows the localization of the average significant change in BOLD signal in response to AMPH with respect to hormone replacement. These same data are presented as activation maps in 2D axial sections shown in Figure 4. Post-hoc analysis for the early and late scans of the ROIs included in the AMPH system identified eight regions where estrogen potentiated the BOLD response to AMPH (Figure 1), including the substantia nigra, ventral tegmental area, prelimbic cortex and the ventral hippocampus.

Figure 5 shows time-course data depicting the change in BOLD signal intensity in five dopamine connected areas (Geffen, Jessell, Cuello, & Iversen, 1976; Gong, Neill, & Justice, 1996; Swanson, 1982; Van Eden et al., 1987) previously shown to be activated by AMPH, namely: ventral tegmental area, prelimbic cortex, substantia nigra compacta and reticularis, and ventral pallidum.

Two ANOVAs were conducted to assess the effects of AMPH on BOLD change over time. The analyses corresponded to the first half (early, 100-175 acquisitions) and the second half of the scan (late, 175-250 acquisitions). The analyses yielded a main effect of treatment (early: $F_{2,132} = 3.453, P < 0.05$; late: $F_{2,132} = 6.321, P < 0.01$), as well as time-by-treatment interaction for the early and late analyses ($P < 0.001$). Pair-wise comparisons showed a significant difference in BOLD

activation between the no E2 and high E2 groups for the early scan analysis ($P < 0.05$), and a difference between no E2 and low E2 ($P < 0.05$), as well as no E2 and high E2 for the late analysis ($P < 0.01$).
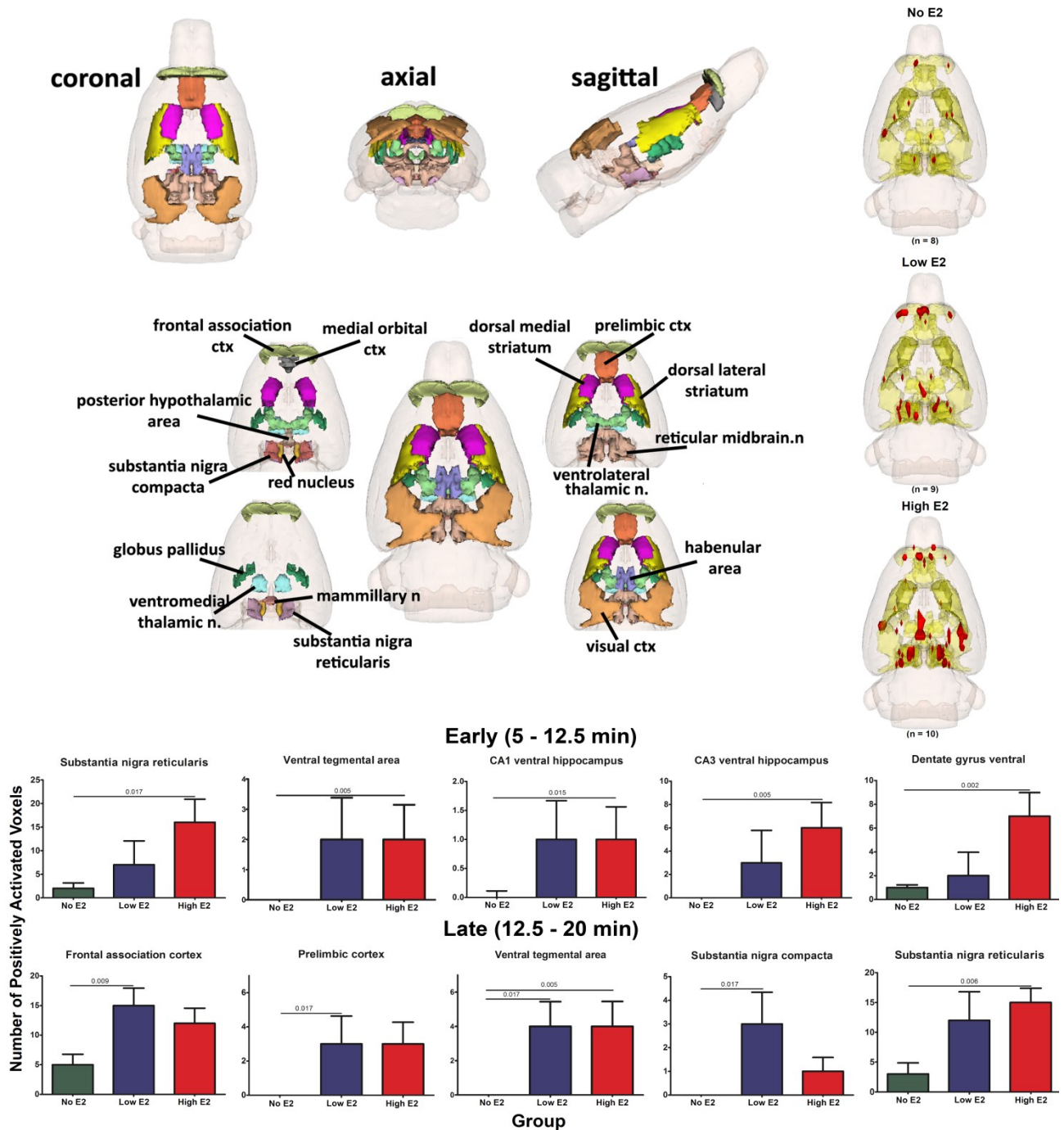
Figure 1: 17β Estradiol-dependent effect of amphetamine in the putative amphetamine neural circuit. Upper left panel depicts those brain regions included in the putative amphetamine neural

circuit. Upper right panel shows those regions (yellow) in each estrogen group. BOLD signal increases indicated in red. The lower panel represents the average number (+SEM) of positively activated voxels for the ROIs included in the AMPH neural circuit assessed early and late during the scan for No E2 (green bars), Low E2 (blue bars) and High E2 (red bars) rats. Numbers indicate P values.
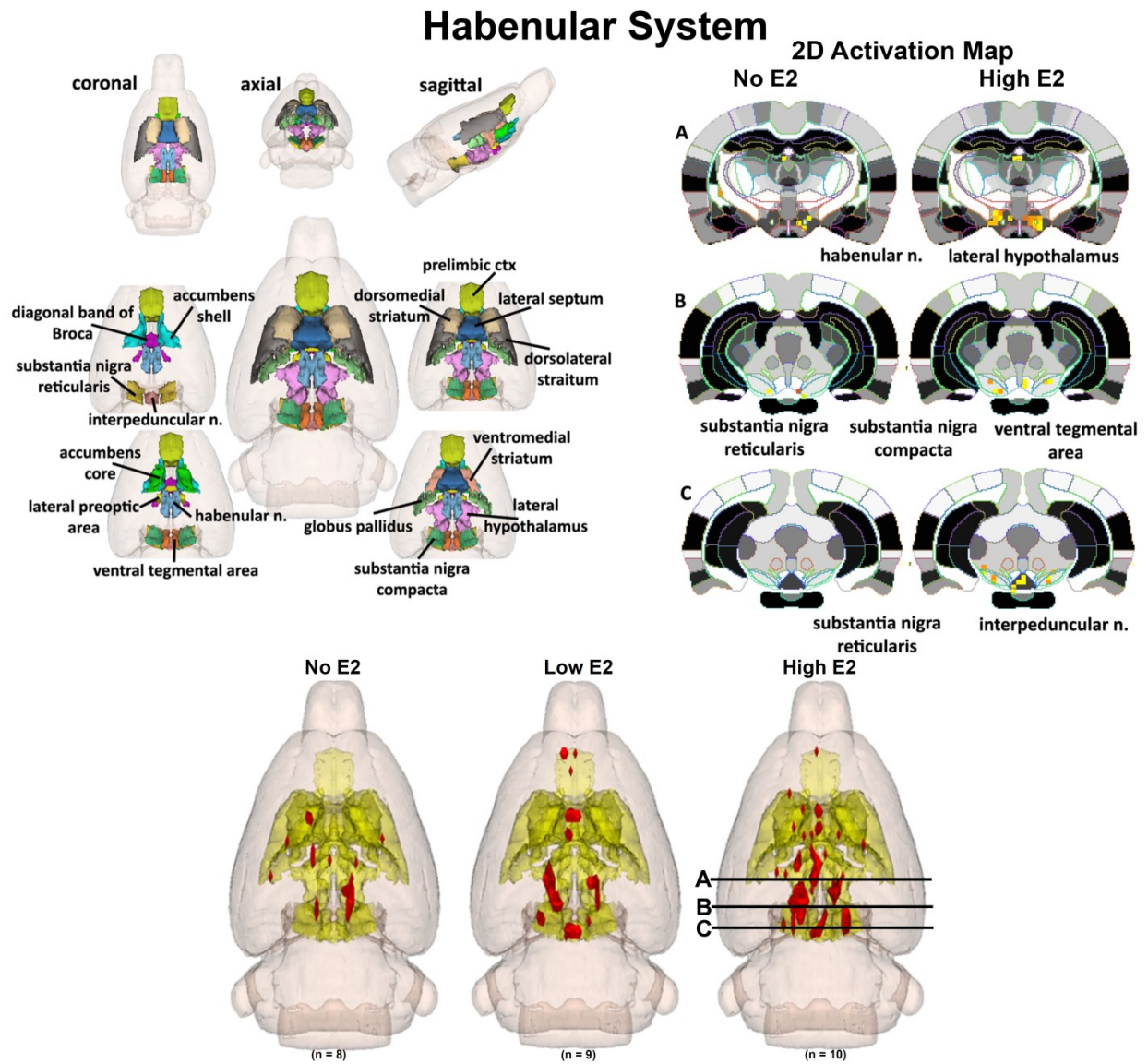
Figure 2: 17β Estradiol-dependent effects of amphetamine in the habenular system. Shown are the 3D maps of the rat habenular system (top left panel). Top right panel depicts 2D activation maps showing BOLD signal activation in red – yellow. Bottom panel shows those regions (yellow) in each estrogen group. BOLD signal increases indicated in red.
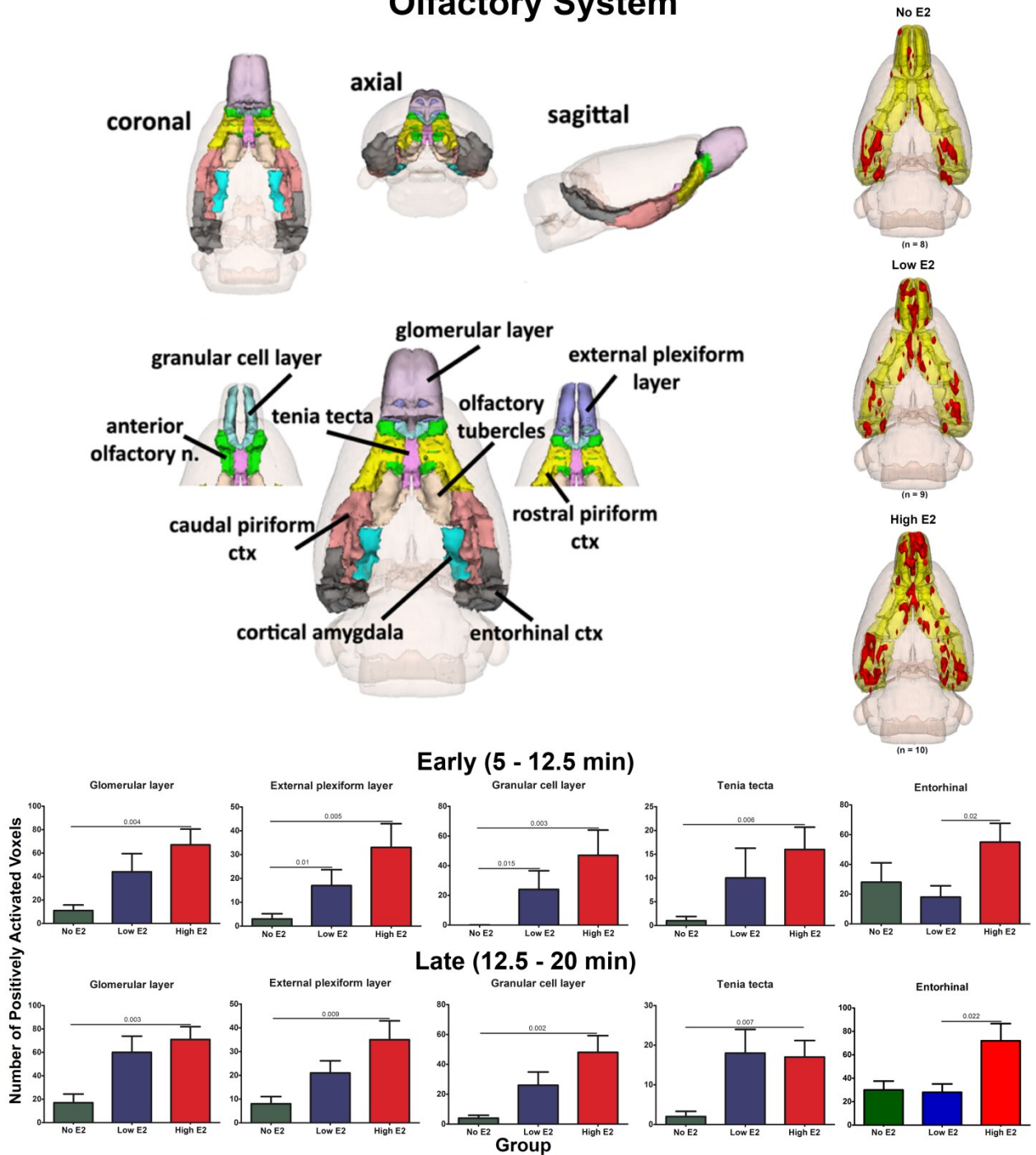
Olfactory System

Figure 3: 17β Estradiol-dependent effect of amphetamine in the olfactory system. Upper left panel depicts those brain regions included in the olfactory system. Upper right panel shows those regions

(yellow) in each estrogen group. BOLD signal increases indicated in red. The lower panel represents the average number (+SEM) of positively activated voxels for the ROIs included in the olfactory system assessed early and late during the scan for No E2 (green bars), Low E2 (blue bars) and High E2 (red bars) rats. Numbers indicate P values.

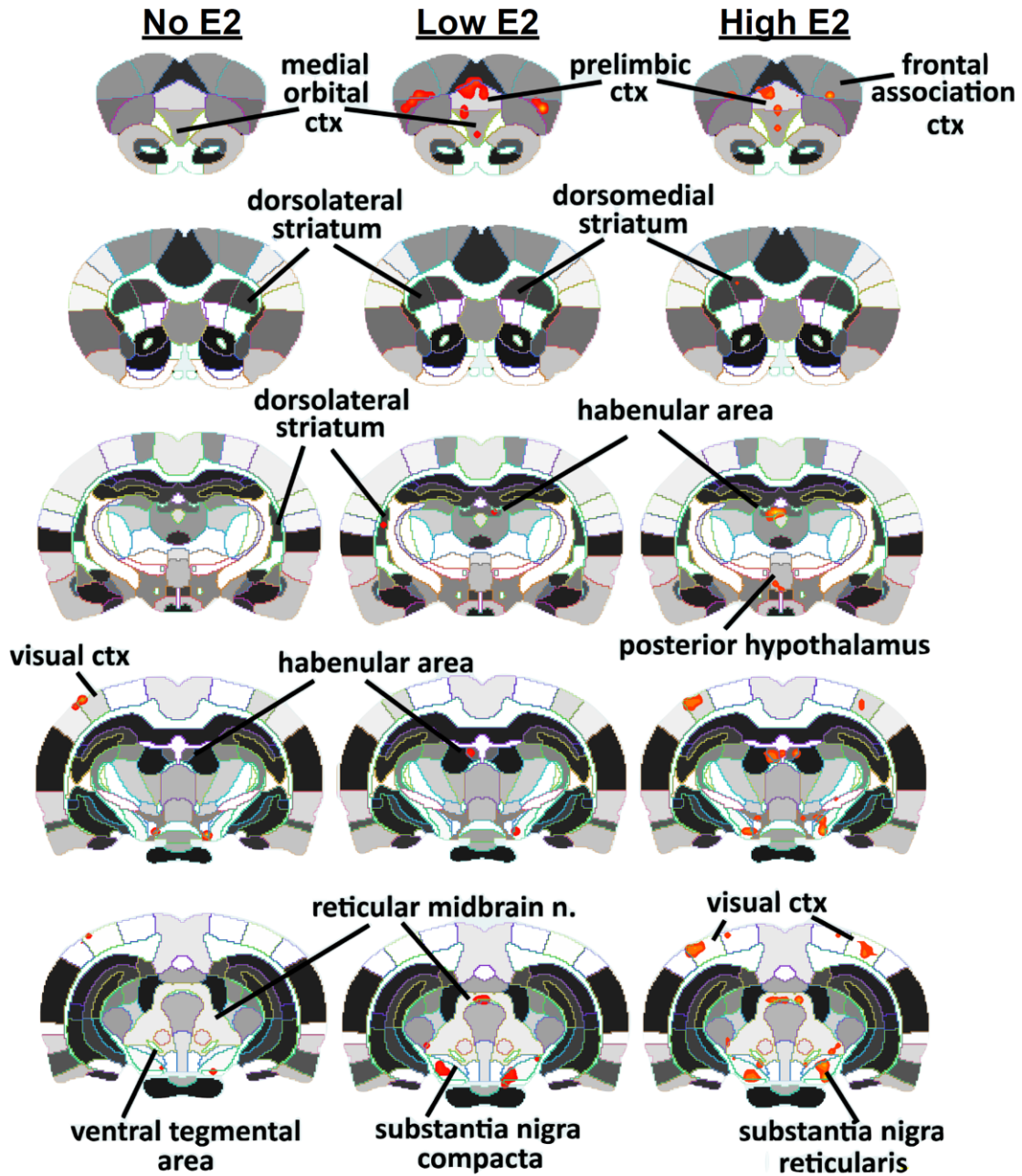# 2D Activation Maps Amphetamine Neural Circuit

Figure 4: 2D activation maps of the rat putative amphetamine neural circuit. Depicted are average significant increase in BOLD activation (red – yellow) for each of the three groups. These are the same data shown in Figure 1, but with a 2D perspective to depict signal location and strength.
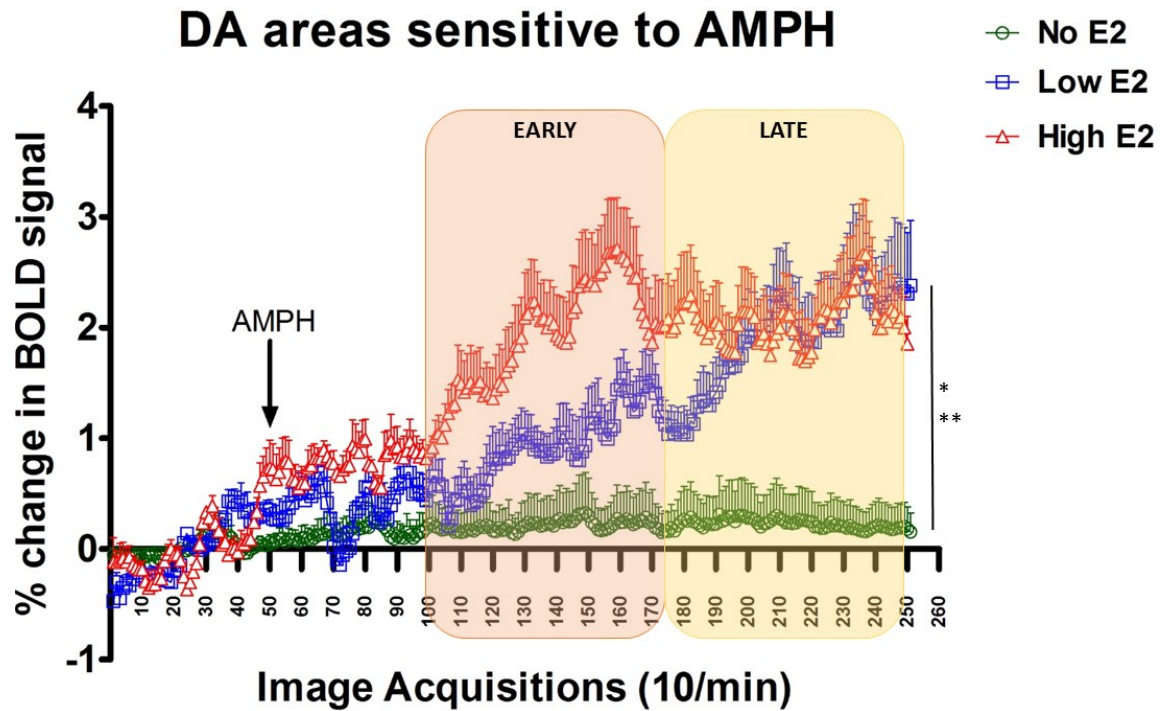
Figure 5: BOLD signal change over time in response to amphetamine. Shown are average (+SEM) changes in BOLD activity of five components of the putative amphetamine neural circuit (ventral pallidum, prelimbic cortex, ventral tegmental area and substantia nigra compacta and reticularis) for each estrogen group. The scanning session is divided into an early (red transparent box) and late period (yellow transparent box). Significant BOLD increase in High E2 rats compared to No E2 rats (*P < 0.05) during the early scan. Significant difference in BOLD activation during the late scan between No E2 and both E2 groups (**P < 0.01).

Discussion

Schizophrenia symptomatology has in part been attributed to hyperactive subcortical dopaminergic transmission, accompanied by hypoactive cortical dopaminergic transmission (Howes & Kapur, 2009). Dopamine transmission is affected by E, such that symptom severity decreases during ovulation thus suggesting that E may have protective qualities (Akhondzadeh et al., 2003; Bergemann et al., 2002; Luggin, Bernsted, Petersson, & Jacobsen, 1984; Riecher-Rossler et al., 1994). Furthermore, E exerts its effects on dopamine (but not exclusively) in areas and pathways relevant to schizophrenia (for review, see Cyr, Calon, Morissette, & Di Paolo, 2002).

In addition to further investigating the interactions between E2 and dopamine in a AMPH sensitized rats (Featherstone et al., 2007), the aim of this study was to "finger print" AMPH-induced brain activity in sensitized rats in response to E2 replacement using fMRI. We found an overall effect of E2 replacement, whereby AMPH-sensitized, OVX females receiving phasic high E showed the highest BOLD activation in response to an AMPH challenge. In addition, it appears that the effects of E2 on BOLD activation are time-dependent, as shown by the difference observed during the early scan being minimized throughout the late scan (see Figure 5). This supports earlier findings showing that AMPH activates the PFC and VTA only in OVX rats receiving E2 compared to those without E2 replacement (9). Albeit the AMPH dose was much higher in that study and the animals were not sensitized to AMPH (Sarvari et al., 2014) as they were here. Here, significant positive BOLD changes were also observed in the dentate gyrus, CA1 area of the hippocampus, enthorinal cortex, ventral medial hypothalamic nucleus and substantia nigra (Tables 1 and 2).

Dysfunction of the mesocorticolimbic pathway has been linked to schizophrenia pathophysiology (Gurevich et al., 1997; Knable & Weinberger, 1997; Koob, Stinus, & Le Moal, 1981). Peripheral AMPH has been previously shown to activate regions included within, but not exclusive to, the mesocorticolimbic pathway, such as the visual cortex, habenula, ventral thalamic nucleus, suprachiasmatic nucleus, substantia nigra, dorsal and ventral stiatum (Lin et al., 1996; Nguyen et al., 1992; Wechsler et al., 1979). Interestingly, these areas have been previously shown to be rich in estrogen receptors (for review, see Hussain, Shams, & Brake, 2014; McEwen & Alves, 1999; ER; Shughrue, Lane, & Merchenthaler, 1997). Here, we show a dose-dependent activation of the AMPH neural circuit, including the mesocorticolimbic pathway, such that high E2 rats elicit the greatest BOLD activation in response to a systemic AMPH challenge. Furthermore, the difference in BOLD activation between the chronic low E2 and chronic low plus phasic high E2 rats is accentuated during the early acquisitions. This difference is no longer present during the late segment of the scan.

Other circuits implicated in schizophrenia pathophysiology include the habenular complex (Lecourtier et al., 2006; Shepard et al., 2006), limbic system (for review, see Grace, 2000; Tamminga et al., 1992; Torrey & Peterson, 1974) and the olfactory system (Kohler et al., 2001; Moberg et al., 1999). We show that the habenular circuit also yields differential activation in response to AMPH, such that chronic low E2 and phasic high E2 rats show increased activation compared to rats with no E2 replacement. Moreover, the activation is dose-dependent, with high E2 rats showing increased activation compared to low E2. Similarly, we show dose-dependent activation of the olfactory system, driven mainly by differences in activation in the glomerular layer, granular cell layer, external plexiform layer and entorhinal cortex (Figure 3). Finally, the same dose-response pattern emerged in limbic structures, such as the ventral CA1 and CA3

hippocampal areas, as well as ventral dentate gyrus. Interestingly, rats with no E2 replacement exhibited inhibitory patterns in a smaller number of ROIs (data not shown).

The fact that we found limbic structures to be activated in response to AMPH in sensitized rats was to be expected, given that these structures have been previously linked to schizophrenia pathophysiology with regards to volume (Breier et al., 1992) and function (Tamminga et al., 1992). Interestingly, the intensity of activation reported in this study is seemingly dependent on hormonal status, such that E2-replacement groups show an overall increase in BOLD signal in response to AMPH compared to those OVX rats without E2 replacement. Given the affected structures, and considering previous findings showing E to enhance conditioned place preference to AMPH (Silverman & Koenig, 2007), one can speculate that the differential activation in response to AMPH in these limbic structures could correlate with changes in cognitive deficits, possibly similar to those seen in schizophrenia.

The habenula is associated with social recognition, predatory responses and locomotion (Dadda, Domenichini, Piffer, Argenton, & Bisazza, 2010). Dysregulation of habenular function is likely to result in disruptions in circadian rhythms (Haun, Eckenrode, & Murray, 1992), reward-based decision-making (Lecourtier & Kelly, 2005; Matsumoto & Hikosaka, 2007) and pain-stress regulation (Matsumoto & Hikosaka, 2009). Our results show that AMPH not only increased BOLD activation in the habenula and its inter-connected brain regions, but that these differences are E2-dependent in AMPH-sensitized rats. It is thus possible that E might be a stress-mediator (Lindheim et al., 1992) in schizophrenia patients (Ventura, Nuechterlein, Lukoff, & Hardesty, 1989).

In terms of the habenula and its connections in reward-based decision making, it is possible that E would have an effect on tasks that correspond to these processes, considering that patients

with schizophrenia show compromised decision-making abilities (Heerey, Bell-Warren, & Gold, 2008; Shurman, Horan, & Nuechterlein, 2005). Finally, based on our results, we would expect E-dependent effects on circadian rhythm regulation in AMPH-sensitized models. It should be noted that patients with schizophrenia show severe sleep/wake disruptions (Wulff, Dijk, Middleton, Foster, & Joyce, 2012).

Olfactory processing, which is mediated by limbic structures, is impaired in patients suffering from schizophrenia. Such impairments are specifically expressed as olfactory identification deficits, rather than deficits in olfactory acuity (Kopala, Clark, & Hurwitz, 1993). Furthermore, olfactory identification abilities have been shown to deteriorate steadily compared to other abilities that are more stable throughout the course of the illness (Moberg et al., 1997). Here, we show that, similar to the habenular and limbic systems, olfactory components are activated by AMPH in an E2 dose-dependent fashion in OVX rats.

Taken together, our results suggest that 1) E2 interacts with dopamine in ROIs previously linked with schizophrenia, and 2) aspects of schizophrenia, as modelled in the AMPH-sensitized rat, are driven by more than a few select ROIs, such as the PFC and NAcc. We show here that AMPH-induced, E2-dependent BOLD responses are rather global, involving entire pathways, as well as regulatory nuclei, such as the habenula (Hikosaka, 2010). As a result, we propose that E2 has the potential of mediating most of the cognitive and behavioural effects seen in schizophrenia, as modelled via AMPH sensitization.

Considering that E has been previously shown to have antipsychotic-like effects, further imaging studies are needed to investigate the effect of antipsychotic medication in conjunction with E2 replacement in sensitized rats. The contribution of other ovarian hormones, such as

progesterone, needs to be examined in both naturally cycling rats, as well as via replacement studies in OVX females. Finally, E has been demonstrated to have cardiovascular effects, with E2 potentiating endothelium-dependent vasodilation in healthy post-menopausal women (Gerhard et al., 1998; Gilligan, Badar, Panza, Quyyumi, & Cannon, 1994; Herrington et al., 2001; Keaney et al., 1994; E. H. Lieberman et al., 1994). One could speculate that the E2-dependent differences in BOLD signals to AMPH could be due to a nonspecific effect of E2 on the vasodilating properties of the brain's vascular system. It is unlikely that this is the case given the specificity of the BOLD signals observed here.

Chapter Two:

Behavioural consequences of prenatal exposure to dizocilpine in the adult rat

Dan Madularu, Maria Athanassiou, Cristina M. Catania, Stephanie Gallant, Loïc Welch,

Wayne G. Brake and Dave G. Mumby

Preface

The results from Chapter One support the hypothesis that estradiol interacts with dopamine, outlining its potential antipsychotic properties, when paired with haloperidol. Interestingly, these effects were only observed in rats that have been sensitized to amphetamine, a process which has been shown to mimic some of the behavioural and neurochemical aspects of schizophrenia. Furthermore, these data suggest that one possible site of interaction is the nucleus accumbens, as shown in Study 1A. Study 1B offers a global map of possible estradiol-dopamine interactions in amphetamine-sensitized female rats. The functional magnetic resonance analysis shows that estradiol enhances BOLD signal in a number of areas previously associated with schizophrenia, as well as areas known to be under dopaminergic control in healthy subjects.

Although the experiments presented in Chapter One offer further support for the DA hypothesis of schizophrenia while emphasizing the role of estradiol on dopaminergic transmission, , they do not address the implications of additional neurotransmitter systems such as glutamate in schizophrenia pathophysiology. In addition, these experiments do not take into account the developmental aspect of the disease. Thus, the study presented in this chapter is aimed at examining the possible implications of glutamate and N-methyl-D-aspartate receptors in schizophrenia. Furthermore, considering the fact that some aspects of schizophrenia are stable throughout adult development, this study is investigating the developmental aspects of the disease by manipulating the glutamatergic system before birth, while testing pups in two different stages of adult development, namely postnatal days 60 (early development) and 210 (late development).

Abstract

Blockade of glutamate N-methyl-D-aspartate receptor inhibits neuronal migration in the developing brain, and impairs cognition and emotional behaviour in adult mice. This study examined the effects of prenatal NMDAR blockade on behaviour in the adult offspring. Dams received daily injections of MK-801 or saline between gestational days 7-19. At postnatal day (PND) 60 and PND 210, male offspring received a novel-object preference (NOP) test of object-recognition memory, and their locomotor reactivity to acute MK-801 and amphetamine was assessed. Performance on the NOP test was impaired in offspring of MK-801 treated dams at PND 60, but results were inconclusive at PND 210 due to poor performance by offspring of control dams. An increase in locomotor activity seen following acute MK-801 injection was enhanced at PND 60, but not PND 210. Locomotor response to AMPH was unaffected by the prenatal treatment. The findings show that some behavioural effects of prenatal NMDAR blockade in rats persist until at least early adulthood.

Introduction

Glutamate is the major excitatory neurotransmitter throughout the central nervous system, accounting for roughly 60% of synapses (Kantrowitz & Javitt, 2010; Platt, 2007). The development of the glutamatergic system starts during embryonic stages, and is mediated by processes involving N-methyl-D-aspartate receptors (Bellinger, Wilce, Bedi, & Wilson, 2002). NMDAR antagonists such as dizocilpine ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; MK-801) or 1-(1-phenylcyclohexyl)piperidine) (PCP) have been shown to inhibit neuronal migration (Behar et al., 1999). In addition, NMDAR blockade has been used as a putative model of schizophrenia, as PCP, MK-801 and ketamine elicit schizophrenia-like effects in humans and animals (Gordon, 2010; Kantrowitz & Javitt, 2010). Administering PCP to healthy individuals induces hyperactivity, paranoia, hallucinations and cognitive impairment similar to that observed in schizophrenia, while PCP administration to schizophrenia patients exacerbates symptomatology (Neill et al., 2010).

Developmental disruptions of the glutamatergic system have been reported to result in impaired performance on cognitive and emotional behaviour tests in adult mice (Lu et al., 2011). In addition, PCP treatment in late gestation (i.e. gestational days 12-18) increase the number of [^{3}H]glutamate binding sites at postnatal day (PND) 21 in offspring (Fico & Vanderwende, 1989a). These behavioural and neurochemical effects are accompanied by subtle, but significant weight differences immediately after birth, such that offspring of either PCP or MK-801 treated dams weigh significantly less than control pups (Fico & Vanderwende, 1989b; Lu et al., 2011). In a previous study, MK-801 administration throughout gestational days (GD) 15-18 reduced the density of parvalbumin-immunoreactive γ-aminobutyric acid (GABA)-ergic cells in the rat medial prefrontal cortex; similar reports been previously described in patients with schizophrenia

(Abekawa et al., 2007; Benes, Mcsparren, Bird, Sangiovanni, & Vincent, 1991). MK-801 treatment during gestation also resulted in an enhanced locomotor response to acute PCP administration in the adult offspring.

NMDARs have been shown to be implicated in certain forms of memory, as well as playing a crucial role in long-term potentiation (Bliss & Collingridge, 1993). One type of memory ability mediated by glutamatergic transmission and impaired in patients with schizophrenia is object-recognition memory (Aleman, Hijman, de Haan, & Kahn, 1999), described as the ability to discriminate the familiarity of previously-encountered objects (Brown, Barker, Aggleton, & Warburton, 2012; Stanley, Wilson, & Fadel, 2012). Recent studies have shown that prenatal exposure to PCP results in impaired performance on a test of object-recognition memory in mice, as well as increased expression of the glial glutamate transporter, reduced extracellular glutamate levels, and reduced phosphorylation of the NR1 subunit (L. L. Lu et al., 2010; Lu et al., 2011).

Although it is clear that prenatal NMDAR blockade affects behavior in the adult offspring, the persistence of these effects throughout adult development has yet to be addressed. As such, the aim of the present study was to further explore the effects of prenatal NMDAR blockade on behaviour in the adult offspring. We assessed object-recognition memory and locomotor response to MK-801 and amphetamine (AMPH) at two stages of rat adult development, namely PND 60 and PND 210. In line with findings from earlier studies (L. L. Lu et al., 2010; Lu et al., 2011), it was hypothesized that prenatal NMDAR blockade would result in impaired object-recognition memory in the adult offspring. Furthermore, increased locomotor activity in response to a systemic injection of MK-801 was expected in the adult MK-801 offspring.

In light of previous studies suggesting that rat prenatal NMDAR blockade would serve as an animal model for schizophrenia-like symptoms (Bubenikova-Valesova et al., 2008; Mouri et al., 2007), we also assessed locomotor activity in response to a systemic injection of AMPH in the adult rat. One aspect of modelling aspects relevant to schizophrenia in the rat involves the presence of a sensitized dopaminergic system, as described in Chapter One. Thus, increased locomotor activity in response to an AMPH challenge (Peleg-Raibstein et al., 2008) in the adult offspring of the MK-801-treated dams compared to control offspring (i.e. from saline-injected dams) was expected.

Experiment 1 investigated novel-object preference using the NOP test, and locomotor activity in response to acute challenges of MK-801 and AMPH at PND 60. The NOP test is often used to assess rats' object-recognition abilities; normal performance on the NOP test is a reflection of rats' propensity to investigate novel objects more than familiar ones (Broadbent, Gaskin, Squire, & Clark, 2010; Dere, Huston, & De Souza Silva, 2007; Ennaceur, 2010; Gaskin et al., 2010). We chose to assess behaviour in early adulthood based on previous findings showing cortical NMDAR density peaks around PND 50, remaining stable throughout adulthood (for review, see McDonald & Johnston, 1990). Similar to Experiment 1, Experiment 2 investigated the effects of prenatal NMDAR blockade on behaviour at a later stage in adult development. This was done by assessing object preference and locomotor activity following MK-801 and AMPH at PND 210.

Methods

Animals

Sixteen adult female Long Evans rats (Charles River Laboratories, Montreal, QC, Canada) were used. Cages were located in a 21°C room with a 12-h reverse light/dark cycle (lights off at 9 AM). Rats had *ad libitum* access to food and water throughout the experiment. Bedding consisted of a 50/50 mixture of corncob and wood shavings. All animal protocols were previously approved by Concordia University's animal research ethics committee and were in accordance with the guidelines put forth by the Canadian Council on Animal Care.

Drugs

D-Amphetamine sulfate (AMPH: 0.5 mg/kg; Sigma-Aldrich) was dissolved in 0.9% saline and administered intraperitoneally (IP). This dose was selected based on a previous study assessing expression of AMPH sensitization in rats (Madularu et al., 2014), where four daily IP injections of 1 mg/kg AMPH resulted in a potentiated response to a subsequent challenge injection of 0.5 mg/kg AMPH after a withdrawal period.

MK-801 (0.1 mg/kg; Sigma-Aldrich) was dissolved in 0.9% saline and administered subcutaneously (SC). This dose was selected based on a previous study investigating the effects of MK-801 treatment on perseveration in rats (Holahan, Madularu, McConnell, Walsh, & DeRosa, 2011a), where subcutaneous injections 0.1 mg/kg of MK-801 resulted in prolonged behavioural perseveration in an appetitive-based task, after the reward was removed.

Apparatus

Novel Object Preference. The arena was constructed of gray PVC, measuring 60 cm X 70 cm X 70 cm (Gaskin et al., 2010; Mumby, Piterkin, Lecluse, & Lehmann, 2007). The bottom of the arena consisted of a removable stainless-steel tray, covered with wood shavings. A video camera was placed over the arena to record familiarization and test phases for subsequent analysis. Test stimuli were objects made of glass or porcelain, and varied in height and width between 6 and 10 cm. The objects were glued to the bottoms of small jars (6 cm high), which were screwed into jar lids, attached onto the steel flooring, at 27 cm from opposing corners. There were three identical copies of each object, which were used interchangeably. The objects were cleaned with water after each use.

Locomotor activity. The activity chambers measured 39 cm X 42 cm X 50 cm, and had four transparent Plexiglas walls and a removable plastic tray at the bottom. They were located in sound-attenuating boxes in a dark room. Locomotor activity was monitored for a period of 120 minutes, by recording infrared beam interruptions. The monitoring session was divided into pre-injection (30 min) and post-injection (90 min) components, during which the Truscan Software (Coulbourn Instruments, PA, USA) recorded total time spent moving. All rats were tested throughout the experiment in the same respective activity chamber at the same time of day.

Procedure

Copulation occurred in Plexiglas chambers (46 cm X 39 cm X 37 cm), divided into two compartments by a clear divider with four holes cut into the bottom (Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Ismail, Jones, Graham, Sylvester, & Pfaus, 2011; Ismail et al., 2010). The holes were large enough so that only the receptive female could pass through them,

giving her access to both compartments, whereas the larger male would be restricted to only one compartment. During mating, the male and female were placed in the two compartments, with the females being able to control the rate of copulation by entering the male's compartment at will, increasing reproductive success (Ismail et al., 2011; Martinez & Paredes, 2001). Mating sessions lasted approximately 4 hrs, and occurred during the rats' dark-cycle.

One week later, dams were placed in separate cages, and treatment began. Each dam received a daily injection of either MK-801 ($n = 9$; 0.1 mg/kg) or saline ($n = 7$; SAL) until gestational day (GD) 20. At birth (PND 0), only male pups were kept. Litter size and male:female ratio varied between dams, with a maximum of 8 male pups kept from each female, for a total of $N = 77$ pups. The MK-801-treated dams yielded a total of $n = 43$ male pups (MK), while vehicle-treated dams yielded a total of $n = 34$ male pups (SAL). Testing began on either PND 60 ($n = 49$; Experiment 1), or PND 210 ($n = 28$; Experiment 2), and thus there were four final groups: SAL/PND 60 ($n = 20$), MK/PND 60 ($n = 29$), SAL/PND 210 ($n = 14$) and MK/PND 210 ($n = 14$).

Maternal behaviour assessment commenced at PND 0 and was divided into five components (Priebe et al., 2006): 1) arched-back nursing (dam arched over nursing pups), 2) nursing prone (dam lying on its side while pups are nursing), 3) licking/grooming (dam licks/grooms pup), 4) nest building, and 5) off nest (dam not engaged in any maternal behaviour). An experimenter recorded maternal behaviour once every minute for 30 minutes during the dark cycle. The average frequency was reported for each behavioural component over two weeks (PND 0 - PND 14). Maternal assessment was conducted under dim-lit conditions, to minimize dams' stress. Pups' weight was measured at PND 8, PND 60 and PND 210. Pups were weaned at PND 21, and placed in Plexiglas cages, with *ad libitum* access to food and water. After they reached approximately 150 g, rats were housed in pairs for the remainder of the experiment.

Testing began with novel-object preference assessment, followed by locomotor assessment in response to acute systemic injections of MK-801 and AMPH (Fig. 1). NOP testing preceded locomotor assessment to avoid possible effects of systemic injections on performance. Furthermore, during the locomotor assessment segment, MK-801 treatment preceded AMPH administration by one week. The order was not counterbalanced in order to avoid possible effects related to sensitization following AMPH exposure. Thus, all rats received both challenge treatments in both experiments.
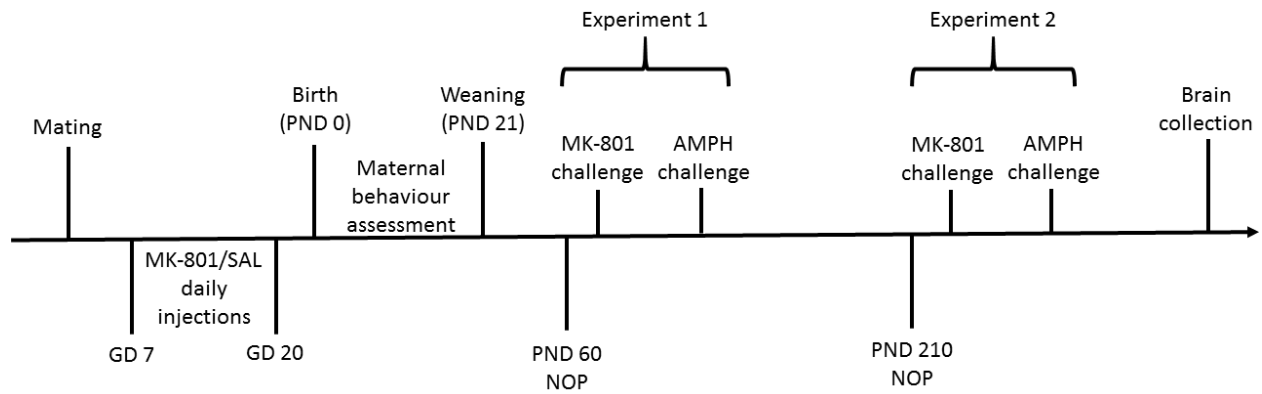
Figure 1: Schematic of the experimental timeline. After mating, females received daily injections of either MK-801 or saline, between gestational days 7 and 20. Maternal behaviour was assessed over fourteen days after birth, and pups were weaned at postnatal (PND) day 21. Novel object preference (NOP) and locomotor activity in response to MK-801 and amphetamine (AMPH) were assessed at PND 60 (Experiment 1) and PND 210 (Experiment 2).

NOP testing was comprised of three phases: habituation, familiarization (FAM) and testing (TEST). Each rat received a 15-min habituation session prior to FAM, where rats were allowed to investigate two identical objects in the same arena where subsequent testing occurred. The TEST and FAM sessions lasted 5 min each. Object preference was assessed during the first minute of the TEST phase, on two different delays (i.e. interval between FAM and TEST): 15 min and 4 hrs, in a counterbalanced fashion. Each session was video-recorded and object preference was assessed during the first minute of the 5 min session, based on an investigation ratio (IR) and compared to chance. The IR was calculated according to the following formula: $IR = \frac{Time\ spent\ investigating\ the\ novel\ object}{Time\ spent\ investigating\ both\ objects}$. A rat was considered to be investigating an object when its head was oriented within 45^0 and 1 cm from the object. Rearing with the head oriented upward was also considered, if at least one paw was placed on the object however; climbing on the object or sitting on it was not included. Different sets of objects were used between the 15 min and 4 hrs delays.

Locomotor activity was assessed approximately one week after object preference assessment. Rats were habituated to the locomotor boxes for two hours on one day prior to testing. During testing, rats were placed in activity chambers and locomotor activity was recorded as time spent moving. The monitoring session was divided into two components: 30 minutes prior to injection and 90 minutes following injection. One week following AMPH challenge, rats were decapitated, and brains were flash-frozen and stored at -80^0C.

Statistical analyses

Maternal behaviour was assessed using independent-samples t-tests, comparing average time spent engaging in each behaviour between MK-801 and SAL-treated dams. Pup weight (MK

vs. SAL) was analyzed using independent samples t-tests, at three separate time points: PND 8, 60 and 210.

Object recognition memory was assessed at PND 60 and PND 210. IR's during the first minute of the 5 min TEST session were analyzed using one-sample t-tests, and performance was compared to chance (i.e., IR of 0.5). Total time spent with both objects during the 5 min FAM sessions was recorded and analyzed using independent samples t-tests.

Spontaneous locomotor activity was recorded, and expressed as total moving time during 5-minute bins following injection. Data were analyzed using four, one-way analyses of variance (ANOVA), comparing responses to challenge injections of MK-801 and AMPH between prenatal treatment conditions (MK vs. SAL), in younger (testing starting at PND 60) and older rats (testing starting at PND 210).

Results

Maternal behaviour and weight

There were no significant differences between MK-801 treated dams and SAL controls (Fig. 2) on any of the five behaviours included in maternal assessment ($P > 0.05$). MK pups weighed significantly less than SAL pups (Fig. 3A) at PND 8 ($t_{47} = 4.46$, $P < 0.0001$). At PND 60 (Fig. 3B), the weight difference was marginal ($t_{47} = 3.37$, $P < 0.07$). There was no significant difference in weight between groups at PND 210 (Fig. 3C). Finally, there were no differences in dams' gestational weight gain between groups (data not shown).

Maternal Behaviour

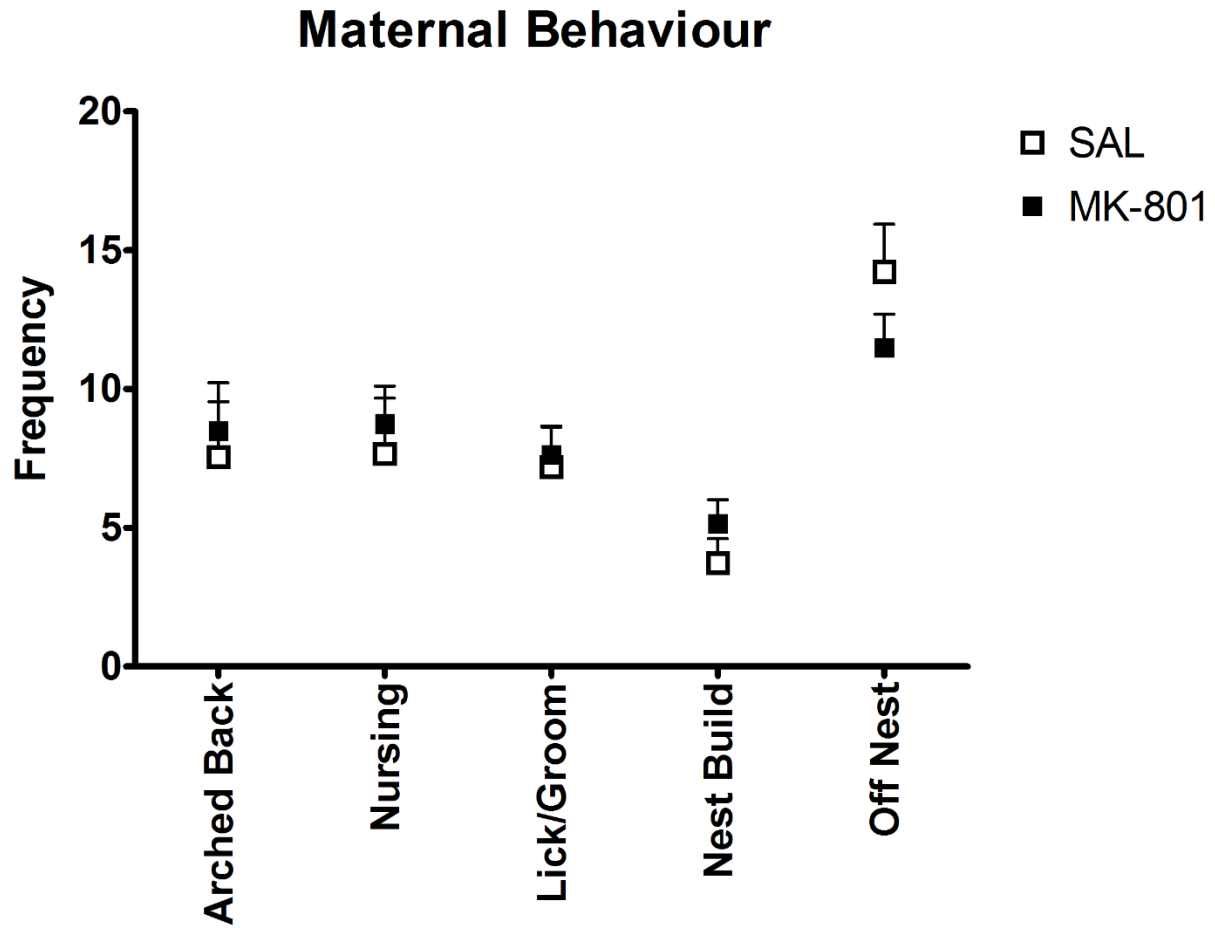**Figure 2:** Mean (+SEM) maternal behaviour assessment over 14 days on five different components, comparing MK-801-treated dams and saline (SAL) controls: arched back nursing, sideways nursing (nursing), licking and grooming, nest building and time spent off nest. No significant differences were found between SAL and MK-801-treated dams on any of the maternal behaviour components ($P > 0.05$).

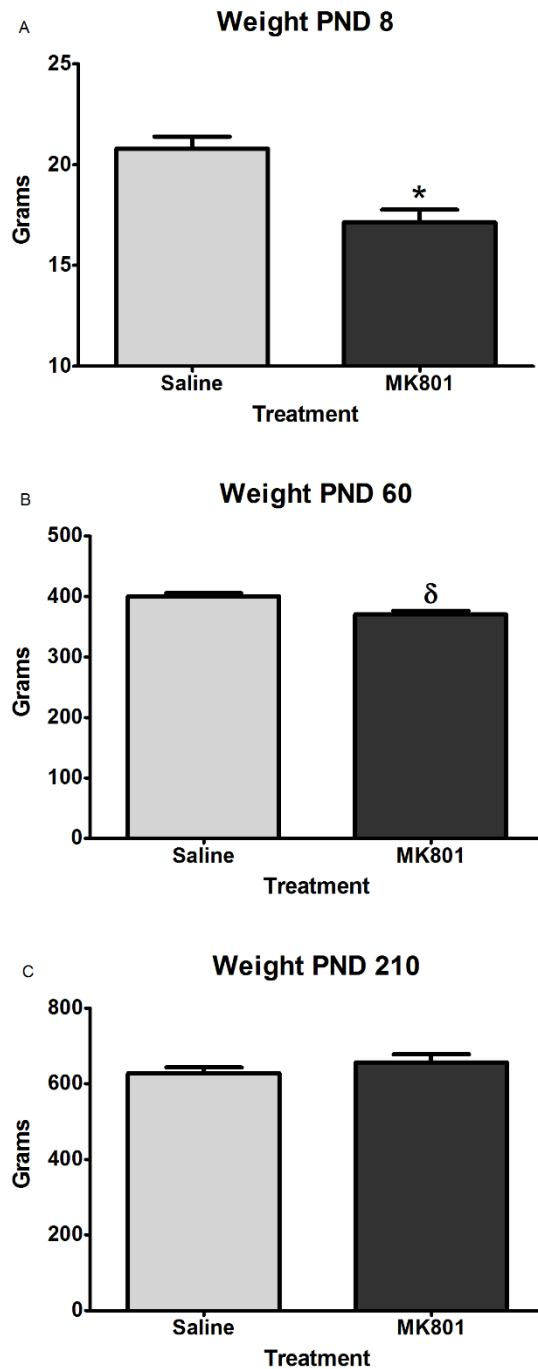**Figure 3:** Mean (+SEM) offspring weight assessment at three different times in development: postnatal (PND) day 8 (A), PND 60 (B) and PND 210 (C). At PND 8, MK-801 pups weighted significantly lower than saline controls ($P < 0.01$). At PND 60, MK-801 rats weighted marginally

less compared to saline controls ($P < 0.07$). There were no differences in weight between MK-801 rats and saline controls. Note: * $P < 0.01$; δ $P < 0.07$.

Novel Object Preference

Total investigation time during the FAM was measured for each delay. This was done to ensure that the differences in IR's during the TEST phases, when present, were not due to differences in total time investigating the objects during FAM. There were no differences between treatment groups (MK vs. SAL), at either PND 60 (Figs. 4A, B) or PND 210 (Figs. 4C, D), in total amount of time spent investigating sample objects during the FAM phases ($P > 0.05$).
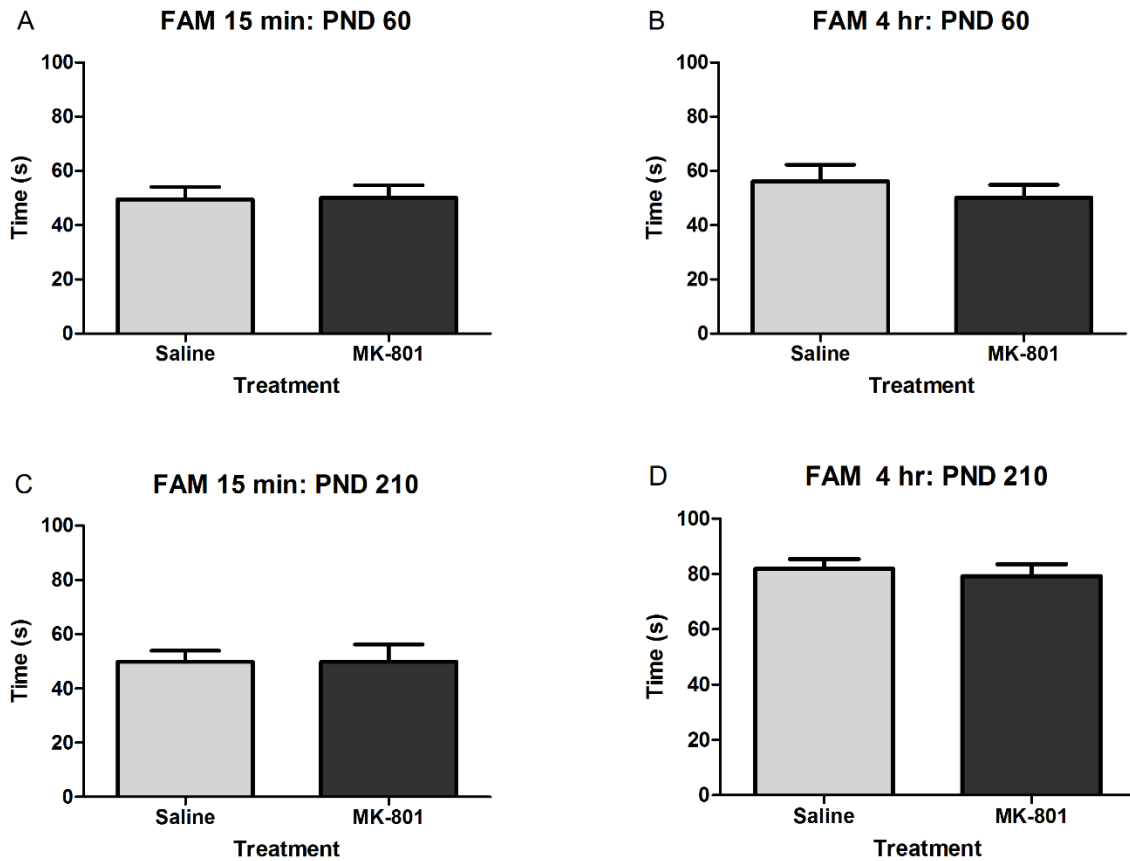
Figure 4: Mean (+SEM) time spent investigating both objects during the 5 min familiarization (FAM) phase of the novel-object preference test. There were no differences in time spent investigating the objects between MK-801 and saline rats, independent of delay or developmental stage.

At PND 60, both MK and SAL groups showed IR's significantly different from chance after a 15 min delay (Fig. 5A) between the FAM and TEST phases (SAL: $t_{19} = 3.37$, $P < 0.01$; MK: $t_{27} = 2.63$, $P < 0.05$). At 4 hrs delay (Fig. 5B), SAL rats investigated the novel object significantly more than chance ($t_{17} = 5.317$, $P < 0.01$), whereas MK rats did not ($P > 0.05$). At PND 210, neither SAL nor MK rats investigated the novel object significantly more than chance (Figs. 5C, D), regardless of the delay ($P > 0.05$).
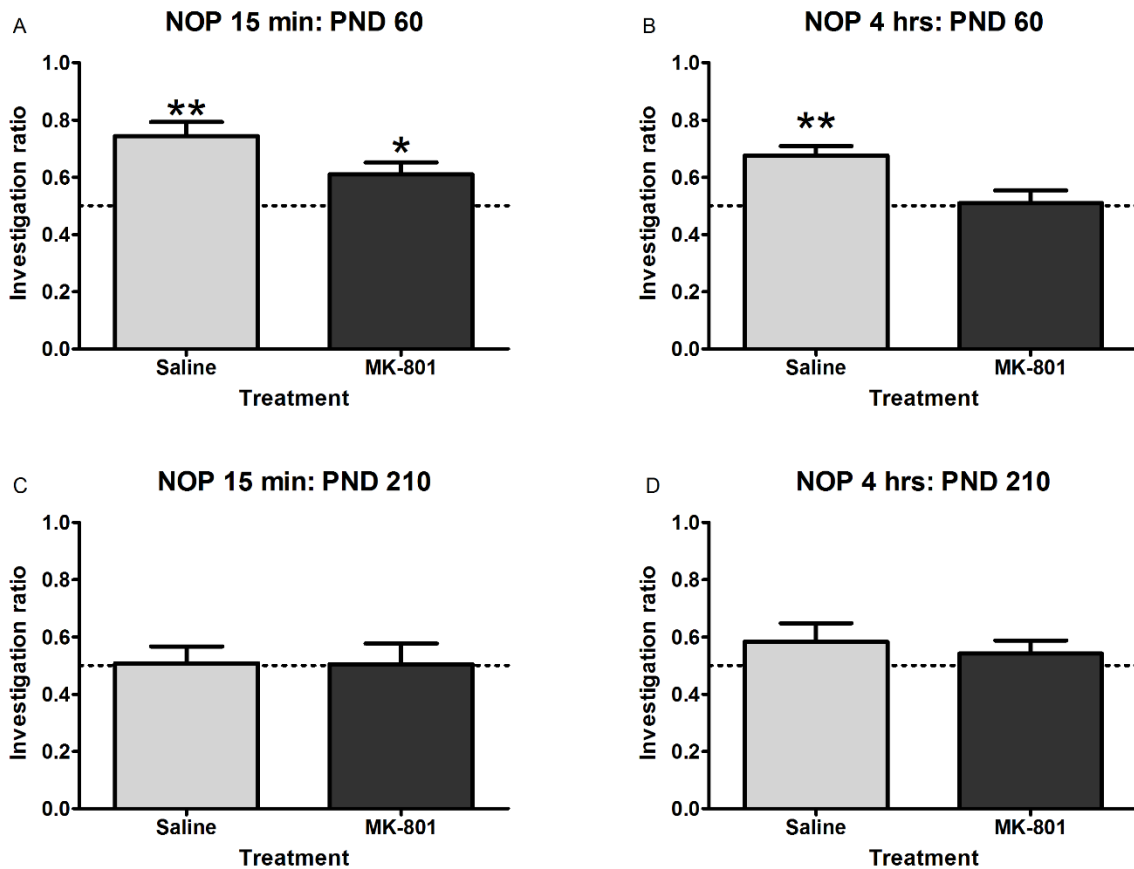
Figure 5: Mean (+SEM) investigation ratios during the first minute of the test phase of the novel-object preference test at two delays and two different stages in adult development. At postnatal day (PND) 60 (A), both saline and MK-801-treated rats investigated the novel object significantly more than chance (SAL: $P < 0.01$; MK: $P < 0.05$). At the 4 hr delay (B), saline rats investigated the novel object significantly more than chance ($P < 0.01$), whereas MK-801 rats did not ($P > 0.05$). Note: ** $P < 0.01$; * $P < 0.05$. Dotted line represents chance performance as defined by an investigation ratio of 0.5.

Locomotor activity

MK rats showed a significant increase in locomotor activity in response to an MK-801 challenge ($F_{1,47} = 6.947$, $P < 0.05$) at PND 60 (Fig. 6A), whereas no significant differences in MK-801-induced activity were observed at PND 210 (Fig. 6B). There were no differences in AMPH-induced locomotor activity between MK and SAL rats at PND 60 and 210 (Figs. 7A, B).
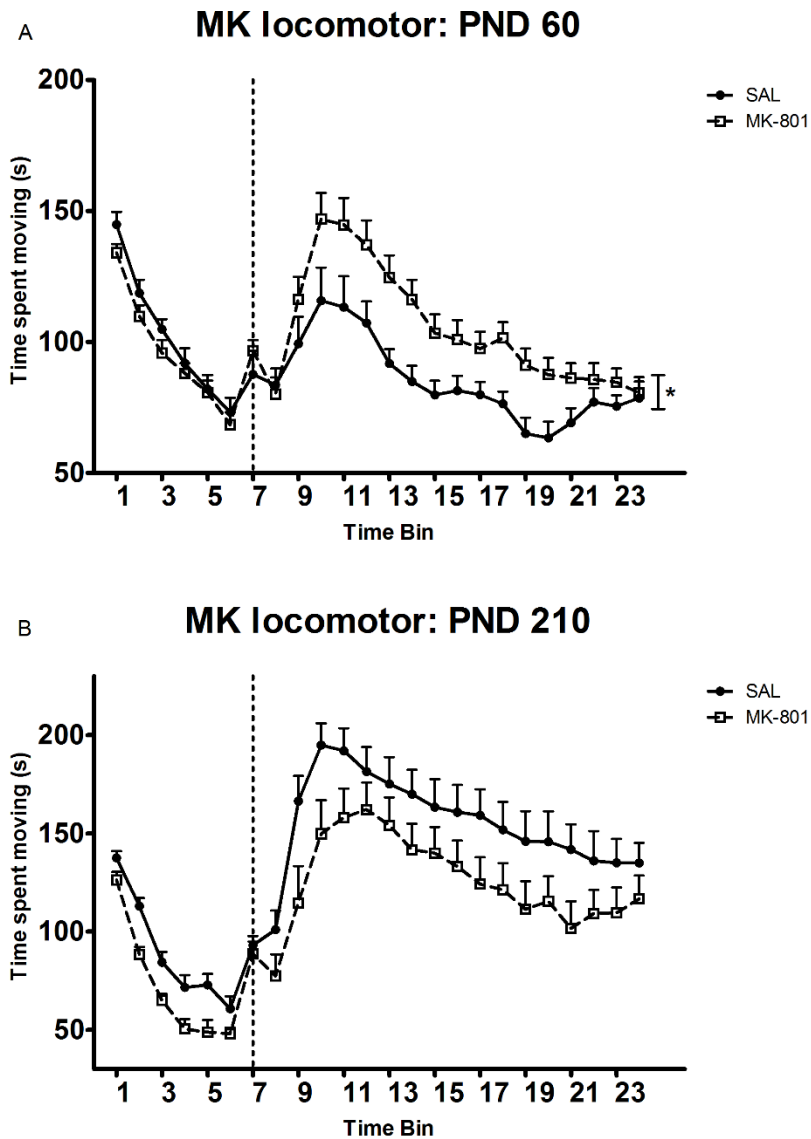
Figure 6: Mean locomotor activity (+SEM) measured in 5-minute bins, of MK-801-treated rats compared to saline (SAL) controls in response to a challenge subcutaneous injection of 0.1 mg/kg MK-801 on postnatal (PND) 60 (A) and PND 210 (B). At PND 60, MK-801 rats showed greater activity in response to an MK-801 challenge compared to SAL controls. Note: * P < 0.05. Dotted line represents time of challenge, 30 minutes into locomotor activity assessment.
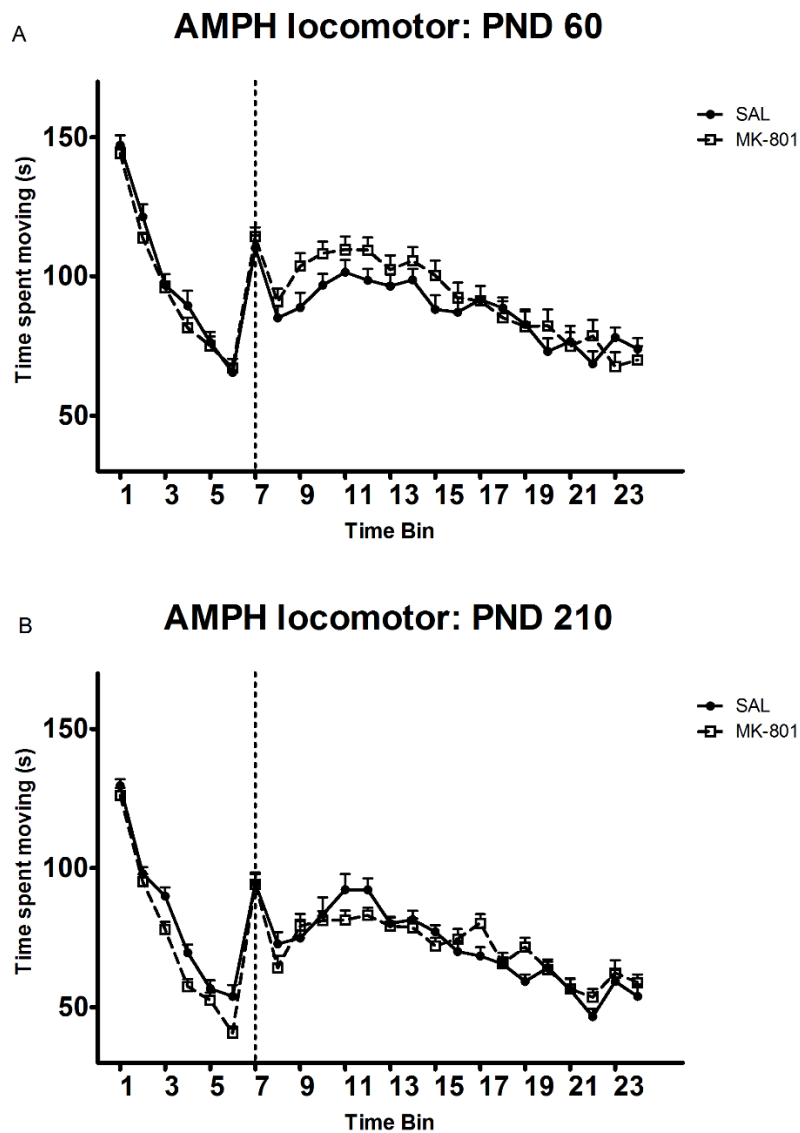
Figure 7: Mean locomotor activity (+SEM) measured in 5-minute bins, of MK-801-treated rats compared to saline (SAL) controls in response to a challenge intraperitoneal injection of 0.5 mg/kg amphetamine (AMPH) on postnatal (PND) 60 (A) and PND 210 (B). There were no significant differences in AMPH-induced locomotor activity between the groups, regardless of developmental stage. Note: Dotted line represents time of challenge, 30 minutes into locomotor activity assessment.

Discussion

The aim of this study was to investigate the effects of prenatal NMDAR blockade on object recognition memory and locomotor activity in response to MK-801 and AMPH at two different stages into adult development in the adult male rat. Maternal care was assessed during the first two weeks after birth, and rats were tested at two stages into adult development: PND 60 and PND 210. At PND 60, both SAL and MK rats showed preference for the novel object after a short delay, however this effects was abolished at a longer delay.

Object memory has been previously shown to be dependent on glutamatergic function, such that NMDAR blockade via systemic MK-801 injections significantly impairs object recognition memory at short and long retention delays (for review, see Winters, Saksida, & Bussey, 2008). Specifically, it has been demonstrated that NMDAR blockade within the perirhinal cortex (Winters & Bussey, 2005) or dorsal hippocampus (Baker & Kim, 2002) affects object recognition memory after a 3 hrs delay, whereas there was no effect after a 5 min delay. In a subsequent study, perirhinal cortical NMDAR blockade resulted in impaired recognition at a longer (24 hrs), but not shorter (20 min) delay, only when both NR2A- and NR2B-subunit antagonists were infused together (Barker et al., 2006). Our findings support previous results in demonstrating that NMDAR function is critical in object recognition memory. Although it remains to be empirically established, it is possible that the mechanisms by which recognition memory impairment occurs are similar when NMDAR blockade occurs prenatally or in adulthood. The lack of novelty preference in either groups at PND 210 may be attributed to the fact that object preference has been previously shown to be age-dependent, such that older rats would not discriminate between familiar or novel objects (Scali et al., 1997). Although previous studies (Scali et al., 1997) assessed object preference at a much older age (i.e. > 2 yrs), it is not clear whether

function was impaired around this age, and not earlier. The PND 210 data would indicate that in this case, object recognition is affected before the rat reaches an age of 2 years, resulting in an overall floor effect.

Locomotor activity in response to systemic challenges of MK-801 and AMPH was assessed at two different stages of adult development: PND 60 and PND 210. At PND 60, MK rats showed increased locomotor activity in response to a subcutaneous injection of MK-801 compared to SAL rats. There was no difference in MK-801-induced locomotor activity at PND 210 between MK and SAL rats. The discrepancy between the results obtained at the different stages in adult development could be due to a possible delay in normal age-dependent NMDAR up-regulation throughout postnatal development (for review, see Scheetz & Constantine-Paton, 1994). In the rat visual associated structures, [^{3}H]glutamate binding increases by 30-50% between PND 10 and 15, whereas in other cortical areas, [^{3}H]glutamate binding rises between PND 5 and PND 50 (for review, see McDonald & Johnston, 1990). Perinatal rats treated with MK-801 show increased [3H]glutamate binding at 2 and 24 hrs post-treatment (McDonald, Silverstein, & Johnston, 1990), while NMDAR density increased in cultured cortical neurons after exposure to NMDA antagonist D-AP-5 (K. Williams, Dichter, & Molinoff, 1992). A subsequent study showed that chronic D-AP-5 treatment up-regulates the NMDAR mRNAs and polypeptides in cortical neurons (Follesa & Ticku, 1996). Based on these previous findings, our findings suggest that chronic prenatal NMDAR blockade may result in prolonged NMDAR up-regulation, shown by increased locomotor activity in response to an MK-801 challenge.

Taken together, the data presented in our study suggest that NMDAR blockade during rat prenatal development affects cognition, possibly due to changes in receptor density and/or sensitivity, and that these effects are limited to early adulthood (PND 60). Furthermore, the

110

locomotor responses seem to be specifically NMDAR-driven, without compromising dopaminergic sensitivity, as shown by a lack of difference between groups at either PND 60 or 210. The lack of a difference in locomotor activity in response to an AMPH challenge may also be attributed to the relatively low dose used (i.e. 0.5 mg/ml). It is thus possible that higher doses might yield significant differences between groups, and this should be further investigated.

Chapter Three:

Evidence that medial prefrontal cortical dopamine is not critical for working memory in the rat

Dan Madularu, Arne Hantson, Amy L. Bilodeau, Melissa A. Berman-Rosa, Ramzi Houdeib, Devon J. Merza, Andrea DiCarlo, Stephanie M. Rosenbaum, Nigel J. Hessing, Joshua Oliel, Waqqas M. Shams, Dave G. Mumby and Wayne G. Brake

Preface

The studies performed in Chapters One and Two investigated different approaches to modelling some behavioural and neurochemical aspects of schizophrenia in the rat, and found that both the dopamine and glutamate neurotransmitter systems play a crucial role in schizophrenia-like symptoms as modelled in the rat.

Although in the previous chapters we did investigate some cognitive aspects of schizophrenia, the purpose of the studies performed in Chapter Three was set to investigate a key cognitive element which was previously reported to be deficient in schizophrenia, namely, working memory. The reason for investigating this cognitive aspect of schizophrenia is based on findings linking working memory integrity to overall functional outcome in schizophrenia. In addition, current treatment is virtually ineffective in addressing cognitive impairments in schizophrenia and thus, it is critical that a mechanism by which working memory impairment is delineated for the purpose of effective treatment development.

Working memory integrity has been previously linked to prefronto-cortical dopaminergic transmission in humans and non-human primates, however results yielded by rodent research have been equivocal. Thus, the aim of the experiments presented in this chapter is to assess working memory performance following dopamine receptor agonist and antagonist infusions in the rat medial prefrontal cortex.

Abstract

Working memory (WM) has been conceptualized as the active holding of information for the performance of attentional tasks. In non-human primates, WM is modulated by dopamine in the prefrontal cortex, primarily through dopamine D1 receptors. The relationship between dopamine in the prefrontal cortex and WM has been described as an inverted U-shaped curve of D1R transmission, where either blockade or augmentation of DA is linked to impairments in tasks that require WM. This study aimed to determine whether a similar relationship exists in rats. Male rats received infusions of either a dopamine D1 receptor agonist or antagonist into the medial prefrontal cortex during training on a delayed non-match to place task in a T-maze. Rats also received central or peripheral infusions of amphetamine prior to T-maze testing. Accuracy decreased as the retention delay increased (0, 30, 60, 120, 180 sec) but there was no interaction with any of the drugs. The data suggest that substantial changes in medial prefrontal cortical dopamine transmission is inconsequential for performance on a benchmark test of WM in rats.

Introduction

First described by Baddeley and Hitch in 1974, the concept of working memory (WM) is the ability to actively hold and manipulate information in order to perform various attention-based tasks (Baddeley & Della Sala, 1996). Studies using non-human primates have shown that WM, as measured primarily by delay-dependent tasks, requires intact function of the dorso-lateral prefrontal cortex (dlPFC; Brozoski, Brown, Rosvold, & Goldman, 1979; Fuster, 1973; Goldman-Rakic, Muly, & Williams, 2000). Depletion of dlPFC dopamine (DA) impairs performance on a spatial alternation task, which is reversed by the DA precursor, L-Dopa, as well as the DA agonist, apomorphine (Brozoski et al., 1979). Later research showed that WM function is, at least in part, mediated by DA transmission via dopamine D1 receptors (D1Rs), such that D1R antagonists induce errors on a WM task (Sawaguchi & Goldman-Rakic, 1991). Conversely, D1R agonists enhance WM performance in older rhesus monkeys that have impaired D1R transmission (Castner & Goldman-Rakic, 2004). Thus, the relationship between dopamine in the dlPFC and WM maintenance has been described as an inverted U-shaped curve of D1R transmission, where either blockade or augmentation of DA is linked to impairments in tasks measuring WM (G. V. Williams & Castner, 2006).

It is difficult to identify any one area of the rodent PFC as having the exact same function with the non-human primate dlPFC. It has been suggested that the rodent medial prefrontal cortex (mPFC) is homologous to the primate dlPFC based upon its anatomy and function (Uylings, Groenewegen, & Kolb, 2003; Vertes, 2004). The dorsal component of the rodent mPFC is formed by the frontal cortex area 2, dorsal anterior cingulate area, and dorsal prelimbic area (PrL), while the ventral component is comprised of the ventral PrL, infralimbic (IL), and medial orbital areas (Heidbreder & Groenewegen, 2003; Kolb, 1984; Kolb, Nonneman, & Singh, 1974; Uylings et al.,

2003). Notably, the ventro-medial PFC (i.e. the IL/PrL mPFC) has been implicated in performance on cognitive tasks (for review, see Floresco & Magyar, 2006) and is considered the homologous rodent structure where WM is concerned.

Whereas primate studies clearly implicate DA transmission in the dlPFC in WM maintenance as assessed *via* delay-dependent tasks, similar experiments involving DA in the mPFC of the rat yield equivocal results. Infusions of the D1R full agonist, SKF 81297, into the PrL area of the rat mPFC resulted in a dose-dependent impairment of delayed-alternation performance, which is reversed by systemic D1R antagonist pretreatment (Zahrt, Taylor, Mathew, & Arnsten, 1997). A subsequent study showed similar results, where the D1R antagonist, SCH 23390 (SCH), when infused into the PrL area, impaired performance on a delayed spatial win-shift task (Seamans, Floresco, & Phillips, 1998). Although several studies have observed an effect of mPFC DA on WM (Dent & Neill, 2012; Floresco & Phillips, 2001; Rios Valentim, Gontijo, Peres, Rodrigues, & Nakamura-Palacios, 2009), others (for review, see Floresco, 2013; Romanides, Duffy, & Kalivas, 1999) found no effect. Finally, intravenous infusions of SCH 23390 and SKF 38393 did not affect WM performance (Bushnell & Levin, 1993). Thus there seems to be a lack of consensus regarding the biological substrates for WM in the rat.

The aim of the present study was to clarify the role of rat mPFC D1R transmission in WM by assessing performance in a delayed non-match-to-place (DNMTP) task in response to systematic D1R manipulations of the mPFC. The DNMTP task is an example of a delayed-alternation task, and it is among the classic methods of assessing WM in rats. In the learning phase of a trial, the rat finds a food reward in one of the two goal arms in a T-maze. Following a retention interval, the rat is returned to the maze and allowed to choose between the two goal arms. It receives a food reward only if it chooses the arm opposite to the one in which it found food in the

learning phase. There are several trials per session, and the same two choices recur on every trial of a test session; after a few trials, the rat has found food multiple times in each arm of the T-maze, and it will be able to remember both rewarded locations. Only the most recently visited arm is relevant during the choice phase on any particular trial, however. Thus, in order to make accurate choices on a consistent basis, on each trial the rat must remember specifically which of the two arms contained a reward during the learning phase. This procedure fits the operational definition of a working-memory task for rats (Dudchenko, 2004; Frick, Baxter, Markowska, Olton, & Price, 1995; Olton, Becker, & Handelmann, 1980), as accurate performance depends on the rat's ability to keep certain transitory information "active" until it can use the information to guide a choice response.

In light of the possibility that D1R treatment effects depend on the difficulty of WM task, performance was assessed over a range of retention intervals. Given the inverted U-shaped curve describing WM maintenance as a function of mPFC DA transmission via D1R, increasing doses of either D1R agonists or antagonists should also increasingly impair performance on the DNMTP task. Thus, we had three hypotheses: 1) Performance on the DNMTP would decay with increasing retention delays. 2) D1R agonist and antagonist infusions into the mPFC would impair WM, thus leading to performance deficits. 3) The impairment would be dose-dependent. Three experiments were carried out to test these hypotheses. In Experiment 1, the D1R antagonist, SCH, was infused bilaterally into either the IL, PrL, or both areas of the prefrontal cortex. In Experiment 2, the D1R agonist, SKF 38393 (SKF), was infused bilaterally into the IL area of the mPFC. In Experiment 3, *D*-amphetamine sulfate (AMPH) was infused either systemically, or bilaterally into the IL, to determine the role of mPFC dopamine on performance on the DNMTP task.

Methods

Animals

Ninety-three male Sprague-Dawley rats (Charles River, St-Constant, Quebec, Canada), weighing 250-300 grams were housed in pairs in polyurethane cages and maintained on a reverse 12h:12h light/dark cycle with lights off from 09:00- 21:00h. Thirty-eight rats were used for Experiment 1: saline infused into the IL (SAL; n=7), SCH infused into the IL (SCH-IL; n=17), SCH infused into the PrL (SCH-PrL; n=7) or SCH infused into both the IL and PrL (SCH-PrL/IL; n=7). SCH-IL rats were further randomly assigned to one of two doses: 0.1 µg/µl (n=9) or 0.25 µg/µl SCH (n=8). For Experiment 2, twenty-six rats were used. SKF-treated rats were randomly assigned to one of two doses: 2 µg/µl (n=11) or 6 µg/µl SKF (n=8). Thirty-seven rats were included in Experiment 3, divided into two main groups: those with infusions into the infralimbic area (AMPH-IL; n=20) or those with systemic, intraperitoneal injections (AMPH-IP; n=17). AMPH-IL rats were randomly assigned to either SAL (n=11) or AMPH (10 µg/µl; n=9) infusions. Similarly, AMPH-IP rats were randomly assigned to either SAL (n=8) or AMPH (0.5 mg/kg; n=9) injections. Note that the vehicle control (SAL) groups were the same animals for Experiments 1 and 2. All tests were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Drugs

The DA D1R antagonist, SCH, and D1R agonist, SKF (both Sigma-Aldrich, UK) were dissolved in 0.9% saline and infused into the mPFC (1μl/side over 2 min). The ranges of doses were chosen as the either the upper or lower/moderate effective doses from previous studies where they were shown to affect behavior (Beninger, Musgrave, & Dickson, 1990; Ramos, Goni-Allo, & Aguirre, 2005). AMPH (Sigma-Aldrich, UK) was dissolved in 0.9% saline and administered IP or centrally into the IL area of the mPFC (1μl/side over 2 min). Doses of AMPH were selected based on previous studies where it was administered either peripherally or centrally (Ben-Shahar & Ettenberg, 1998; Lacroix, Broersen, Feldon, & Weiner, 2000).

Surgery

Rats were anesthetized with isoflurane (Inhalation Anaesthetic, Richmond Hill, ON, Canada), and two 8 mm stainless steel cannulae (23Ga, Plastics-One, Ronaoke, VA, USA) were stereotaxically implanted bilaterally, directed at the IL area of the mPFC (Fig. 1A). The coordinates were relative to bregma: antero-posterior (AP) = + 2.7 mm, latero-medial (LM) = ± 1.5 mm and dorso-ventral (DV) = -5 mm, at a 15° angle. A group was implanted targeting the PrL area of the mPFC at similar coordinates except for DV, which was - 3.1 mm (Fig. 1B). An additional group, PrL/IL rats, was implanted in a similar fashion however, the DV coordinate was - 2.8 mm, placing the tip of the cannula immediately dorsal to the PrL (Fig. 1C). Obturators (26 Ga, Plastics-One, Roanoke, VA, USA) were inserted into each cannula, extending 1 mm from the tip of the cannulae for the IL and PrL rats, and 3 mm for the PrL/IL rats Thereafter, rats were single-housed and were handled every day for approximately 5 minutes/day. One week post-

surgery, all rats were placed on a food-restricted diet, where the weight was reduced to 90% of the individual free-feeding weight for the duration of the experiment.

Behavioural assessment

Behavioral training for all animals was carried out in a T-maze placed on a table, one meter above the floor in a dim-lighted room. The dimensions are described in detail elsewhere (Quinlan et al., 2013; Quinlan et al., 2008). The T-maze version of the delayed alternation task is the most commonly used DNMTP task and is noted to be particularly sensitive for evaluating WM (Mizoguchi, Shoji, Tanaka, & Tabira, 2011). For three consecutive days, rats were allowed 15 min of exploratory behavior within the maze. Following the habituation sessions, acquisition training began. Ten sessions were carried out daily for each rat. Within each session, there were two phases: a forced-trial and a choice-trial. During the forced-trial, one arm of the maze was occluded, the other left open and baited with 1/4 of a Kellogg's FrootLoop®. Both arms contained FrootLoop crumbs under the mesh floor to diminish the possibility of solving the task by using odor cues. The rat was placed in the start arm and allowed to enter the open arm and consume the reward. Immediately after, the rat was removed from the maze and placed in a cage on a chair facing away from the maze for approximately 5 sec, to minimize possible confounds related to spatial cues and postural bias (Castner, Goldman-Rakic, & Williams, 2004). During the choice-trial, the opposite arm was baited and both arms were available for entry. Alternation of the baited arm between trials takes advantage of rats' tendency to spontaneously alternate between arms of the T-maze and explore the novel arm not previously reinforced (Hughes, 2004). Therefore, the rat has to remember the arm visited during the forced-trial in order to correctly alternate during the choice-trial and obtain the food reward. Once the rat performed 9/10 correct trials for three consecutive days it was considered to have met the performance criterion.

In order to rule out the possibility of rats using food-odor cues to solve the task, a subset of rats (n = 10) was tested on match-to-place (MTP) probe trials randomly interspersed within the regular DNMTP trials. On these probe trials, the reward during one of the ten trials was placed in the same arm during the choice phase as the forced phase, and entries into the arm were recorded. If rats were using food-odor cues to find the reward, rats should have then entered the same arm during the choice trial as they did in the forced trial. Each rat received one probe trial per session, for a total of ten probe trials. All ten rats entered the opposite arm during the random probe trials, suggesting that they were not solving the task by detecting the odor of the food reward.

During the test phase, five delays (0, 30, 60, 120 or 180 sec) were introduced between the forced and choice phases of each of the ten daily trials. A different delay was introduced each day in a counterbalanced order (Latin square design). In the case of intra-mPFC infusions drugs were infused 15 minutes prior to the first trial. Drug was delivered over 2 min at a constant rate of 0.5 μl/min/side. For the PrL/IL group, injectors extended 3 mm from the tip of the cannula, targeting the IL area. After the first minute of the infusion, the injectors were moved approximately 1.5 mm dorsally, to allow infusion into the PrL area of the mPFC. In order to ensure diffusion within the tissue, the injectors were left in place for another minute after delivery ceased. The procedure for Experiment 3 was similar to the one described in Experiments 1 and 2 above however, in addition to the central infusion segment, rats received either AMPH or vehicle intraperitoneally (IP) 15 minutes prior to the testing phase. Prior to euthanasia, methylene blue was infused into the cannulae for later histological confirmation of cannula placements (Fig. 1).

Statistical analyses

For Experiments 1 and 2, four mixed analyses of variance (ANOVAs) were performed, with delay as the within factor and dose as the between factor. Similarly, for Experiment 3, two mixed ANOVAs were performed, with delay as the within factor and drug as between.

Results

For Experiment 1, there was a main effect of delay for SCH infusions into the IL ($F_{4,84} = 12.27$, $P = 0.001$), PrL ($F_{4,48} = 6.92$, $P = 0.001$) and PrL/IL ($F_{4,48} = 5.00$, $P = 0.002$), with response accuracy decreasing as delay increased (Figs. 2A, C, D). There were no interactions (IL: $F_{8,84} = 1.14$, $P = 0.340$; PrL: $F_{4,48} = 0.77$, $P = 0.548$; PrL/IL: $F_{4,48}= 0.57$, $P = 0.683$), nor were there main effects of drug for the IL ($F_{2,21} = 3.02$, $P = 0.070$), PrL ($F_{1,12} = 1.06$, $P = 0.322$) and PrL/IL ($F_{1,12} = 0.13$, $P = 0.716$) groups. These data show that there is a significant decrease in performance as delays increase, however these effects are not treatment-dependent.

Similarly, there was an overall effect of delay ($F_{4,92} = 10.59$, $P = 0.0001$) when SKF was infused into the IL (Fig.2B). There were no main effects of drug ($F_{3,22} = 2.02$, $P = 0.155$), nor a significant drug by delay interaction ($F_{12,88} = 1.29$, $P = 0.254$), indicating that although performance decreased as delays increased, it was not treatment-dependent.

Analyses yielded main effects of delay for the AMPH-IP (Fig. 2E) group ($F_{4,60} = 5.90$, $P = 0.001$), as well as for the AMPH-IL (Fig. 2F) group ($F_{4,60} = 3.22$, $P = 0.018$), with performance on the DNMPT decreasing as delays increase. There were no main effects of treatment in the centrally-infused ($F_{1,15} = 0.008$, $P = 0.932$), nor in the peripherally-injected rats ($F_{1,15} = 1.58$, $P = 0.227$). There was no drug by delay interaction in the centrally-infused groups ($F_{4,60}= 0.73$, $P = 0.569$) nor in the peripherally-injected groups ($F_{4,44} = 1.29$, $P = 0.283$). Similar to Experiments 1 and 2, these data point at a delay-dependent decrease in performance, regardless of treatment.

122
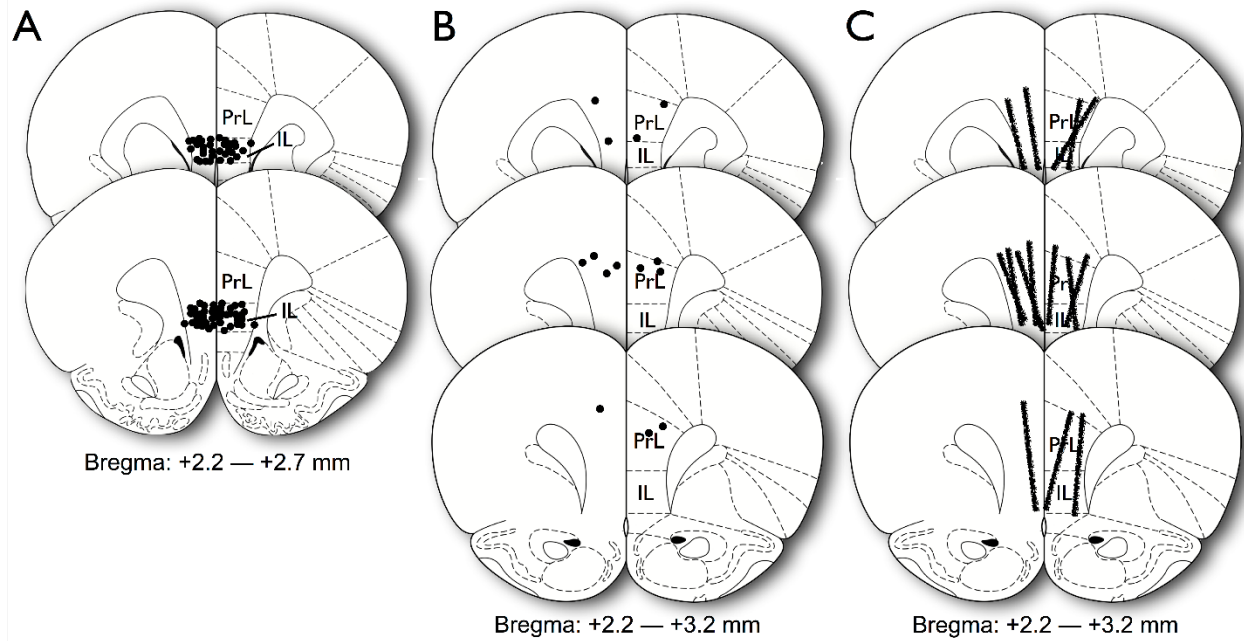
Figure 1: Schematic of rat brain with dots indicating deepest point of injector penetration, and lines representing injector-made tracts. Depicted are the infralimbic (A), prelimbic (B) and prelimbic/infralimbic (C) areas of the rat medial prefrontal cortex (mPFC). IL: infralimbic; PrL: prelimbic.
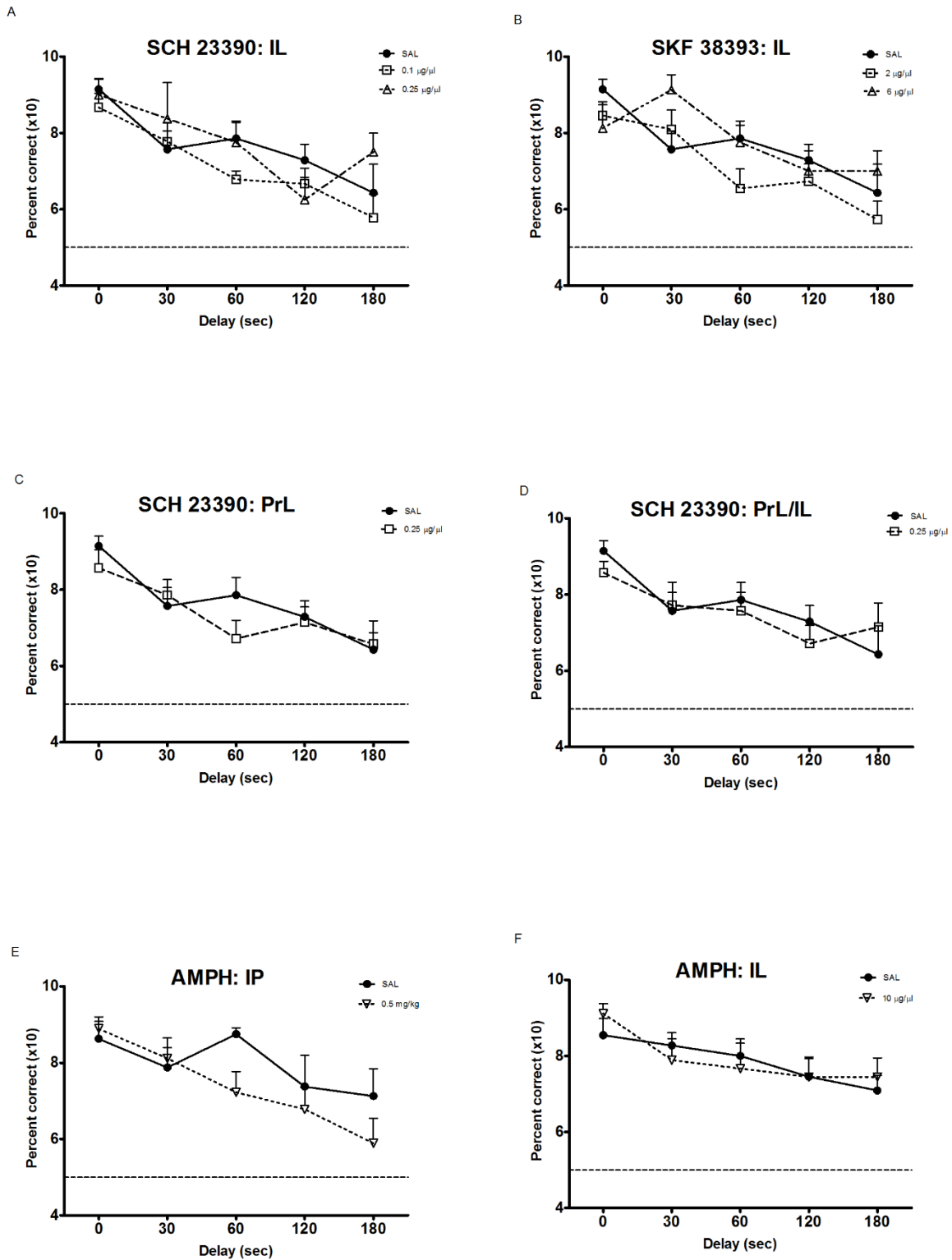
Figure 2: Mean (+SEM) performance in the delayed non-match to place task in a t-maze in response to medial prefrontal cortical (mPFC) infusions of the dopamine D1 receptor antagonist,

SCH 23390 (A: infralimbic; C: prelimbic; D: prelimbic and infralimbic), D1 receptor agonist SKF 38393 (B: infralimbic) and AMPH (E: intraperitoneal; F: infralimbic). Results yielded significant effects of delay in each case, however there were no main effects of drug, nor significant drug by delay interactions. Dotted line represents chance performance (i.e. 50%). IL: infralimbic; PrL: prelimbic; IP: intraperitoneal; AMPH: amphetamine.

Discussion

The aim of this study was to assess the role of DA in WM in the rat, by assessing performance on a DNMTP task in response to centrally-administered D1R agonist and antagonist. Performance was also assessed in response to AMPH, administered both centrally, as well as systemically. The lack of a dose by delay interaction in Experiment 1 shows that D1R blockade does not impair performance on the DNMTP task, regardless of infusion site. Furthermore, results from Experiments 2 and 3 did not show a significant dose by delay or drug by delay interaction. Neither the D1R receptor agonist, SKF, nor AMPH impaired performance compared to SAL in the DNMTP task. The lack of an effect of AMPH contrasts with a previous report of improved WM performance at some delays using the same dose (Shoblock, Maisonneuve, & Glick, 2003).

Despite the fact that studies in non-human primates have consistently shown that frontal cortical DA is important for WM function, rat studies have been equivocal (Romanides et al., 1999). A possible caveat might stem from the functional discrepancy between the IL and PrL areas of the rat mPFC. Although some studies (Delatour & Gisquet-Verrier, 1999; Gisquet-Verrier & Delatour, 2006; Joel, Weiner, & Feldon, 1997; Ragozzino, Adams, & Kesner, 1998; for review, see Uylings et al., 2003) do not differentiate between these areas of the mPFC, other studies (for review, see Dalley, Cardinal, & Robbins, 2004) point to discrete anatomical and, most importantly, functional differences between the two (Gutman et al., 2012; Mendez et al., 2008), with the PrL implicated in cognitive function (Vertes, 2004). Accordingly, we added groups that were infused into the PrL exclusively, as well as both PrL and IL combined, which all provided the same negative results. Future studies should also examine the cingulate cortex in WM function as some effects have been shown in more dorsal injection site by others. Finally, in this study only a partial D1R agonist was used here. Although the partial agonist, SKF 38393, has been previously reported

to significantly impair choice accuracy in the radial-arm maze (Levin & Eisner, 1994), future studies should examine if a full D1R agonist may affect WM performance.

Several studies suggest that mPFC function may not be essential for normal WM abilities (Gisquet-Verrier & Delatour, 2006; Joel, Tarrasch, Feldon, & Weiner, 1997; Joel, Weiner, et al., 1997), although an effect of lesion was recorded following the introduction of interference. Despite the fact that the lesion studies do not directly address the involvement of mPFC dopaminergic transmission on WM, they do raise serious questions about whether mPFC functions are critical for normal performance on tests that require working-memory in rodents. Further lesions studies are needed to assess these questions directly, but if it is the case that mPFC makes only noncritical contributions to WM in rats, this might explain why performance on the DNMTP task was not sensitive to our D1R manipulations.

In summary, the present data suggest that mPFC D1R transmission is not critically involved in the maintenance of WM in the rat, as there were no significant effects on DNMTP performance of infusing either a D1R agonist or antagonist into the IL and/or PrL subregions, nor was there a drug x delay interaction. It is possible that differences exist in behavioural strategies employed between tasks, some of which could be more taxing on mPFC dopaminergic transmission than others. Perhaps this is why no effect was observed here using a DNMTP task whereas others did see an effect of mPFC dopamine manipulations using delayed-alternation (Zahrt et al., 1997) and delayed win-shift (Seamans et al., 1998) tasks. Although the current study does not support the theory that WM is D1R-dependent in the rat using the T-maze, future studies should examine mPFC D1R manipulations in other WM tasks, such as those utilizing radial-arm mazes and water-mazes, as well as operant-based tasks. Nonetheless, it is not possible to reconcile

the present data with the notion that D1R transmission in rat mPFC and WM performance in this task have an inverted U-shaped relationship.

General Discussion

The goal of this thesis was to address some of the shortcomings of pharmacologically-based rat models of schizophrenia-like symptoms. Accordingly, the effects of E2 on HAL in AMPH-sensitized rats as well as E2-dependent BOLD response to AMPH were investigated in Chapter One. The effects of prenatal NMDAR blockade in adulthood were assessed in Chapter Two, and the involvement of mPFC DA in WM was addressed in Chapter Three.

When paired with E2, chronic HAL treatment was effective in blocking the hyperlocomotor-inducing effects of AMPH, supporting clinical findings. Although in Study 1A we attributed this effect, in part, to DA transmission within the NAcc, Study 1B offers a more complete map of possible E2-DA interactions within the central nervous system. In an effort to further investigate the developmental and glutamatergic aspect of schizophrenia, in Chapter Two it was demonstrated that prenatal NMDAR blockade results in impairments similar to those seen in other animal models, as well as patients with schizophrenia. Chapter Three investigated the role of mPFC DA transmission relating to WM function in the rat, considering that WM deficits are challenging to treat with traditional antipsychotics in the clinic. It was established that that WM, a cognitive function affected in patients with schizophrenia, is not driven by mPFC D1R transmission when rats are assessed on a DNMTP task.

Animal models have added to the understanding of disease throughout the past century, by offering a more accessible venue to monitor disease progression (Jones et al., 2011). Although one of the main and final aims of animal research lies in the translatability of the findings to human research, only a third of studies translate at the level of clinical research (Hackam & Redelmeier, 2006), due to the studies' inadequate external and internal validity (van der Worp et al., 2010). Briefly, internal validity refers to the differences observed between groups being attributed to different treatments, whereas external validity is concerned with the generalization of results.

Generally, issues surrounding internal validity are present in every study whether animal-based or clinical, whereas the external validity of an animal model is driven by disease-specific factors (van der Worp et al., 2010). In conclusion, the main effort driving the development of animal models of disease should be placed in maximizing their internal and external validity.

Although the primary goal of the studies presented in Chapter One was not to increase the external validity of AMPH sensitization models per se, the experiments conducted in this study add to the better understanding of the interaction between DA and E2 in this rat model of some aspects of schizophrenia. Study 1A shows that E2 can enhance the effects of HAL in sensitized rats, while Study 1B offers a comprehensive map of DA-E2 interaction in AMPH-sensitized, OVX rats. Consequently, these results, in consideration with data yielded from clinical studies point at the possible involvement of E2 in the development of treatment of schizophrenia. Furthermore, the data support previous findings showing that treatment efficacy is different in females compared to males (Samaha et al., 2007) and thus, suggesting that schizophrenia is a sex-specific, rather than uniform illness. Study 1B is unique as it suggests novel, as well as previously-implicated, pathways affected in schizophrenia, such as the mesocorticolimbic, habenular and olfactory systems. Moreover, activation of these systems is E2-dependent in the sensitized female rat, further supporting the involvement of E2 in schizophrenia related behaviour, as modeled by AMPH sensitization.

The aim of the studies conducted in Chapter Two was to address the behavioural consequences of NMDA blockade during prenatal development. This neurodevelopmental approach to modelling schizophrenia-like symptomatology enhances the external validity of rat models, considering the increasing amount of evidence supporting the neurodevelopmental aspect of the disease. As previously mentioned, schizophrenia is a developmental disease and as such, in

132

an effort to increase external validity, the animal models mimicking symptomatology should be developmental in nature. Here, we assessed the effects of glutamatergic blockade during prenatal development followed by juvenile and adult behavior analyses, thereby offering a more comprehensive timeline of symptoms. It was found that disruption of normal development of the glutamatergic system before birth negatively affects cognition and enhances the sensitivity of the glutamatergic system to MK-801 when assessed early in adult development (i.e. PND 60). Interestingly, these effects were, at least in part, abated when measured at a later stage in development, viz. PND 210. Although these data partly support the hypothesis that glutamatergic transmission is involved in schizophrenia, further studies are needed in order to establish it as a neurodevelopmental model. These studies should both expand behavioural and cognitive assessment to include all domains delineated by MATRICS, and attempt to reverse the effects via antipsychotic treatment.

The studies comprising Chapter Three took a different approach. Instead of assessing and expanding on an already-existing animal model, it addressed whether WM, one of the cognitive functions impaired in schizophrenia, is mediated by mPFC DA in the rat. This focus is crucial given that, as opposed to human and non-human primate studies, the evidence supporting the implication of mPFC DA in WM in rodent is equivocal. Seminal studies conducted on non-human primates suggested that WM is a function of prefronto-cortical DA transmission, whereby excessive or insufficient DA binding at dlPFC D1Rs results in WM impairment. We, as well as others (for review, see Floresco, 2013; Romanides et al., 1999), have not been able to replicate these findings in the rat.

The implications of these findings are two-fold. With respect to this thesis, the data suggest that WM, as assessed using the T-maze, is not a suitable measure for the assessment of WM

function in rat models of schizophrenia. On a greater scale, however, our findings point at the possible involvement of other DA receptors, or other neurotransmitter systems and brain regions in WM maintenance. There is evidence that WM can be affected by systems such as the cholinergic (Levin, Kaplan, & Boardman, 1997), GABAergic (DeSousa, Beninger, Jhamandas, & Boegman, 1994; Givens & Olton, 1990), opioid (Kalivas, Jackson, Romanidies, Wyndham, & Duffy, 2001), and/or glutamatergic systems (Danysz et al., 1988). Although it cannot be excluded that the effects of other neurotransmitters on WM may occur in concert with dopaminergic transmission, it can be stated that, based on these data, WM is not solely mediated by D1Rs in the rat mPFC.

There have been attempts at modelling schizophrenia in rodents, and although not all experiments led to clinical trials, most proved to be pieces of a larger, more complex puzzle. The effort placed on mimicking classes of symptoms of schizophrenia led to the development of a comprehensive and integrative picture, one that includes a variety of symptoms driven by more than one neurotransmitter system (for review, see Benes & Berretta, 2001; Tandon, 1999; Watson, Akil, Berger, & Barchas, 1979). The current pharmacology-based models are often based on acute manipulations of the DA and glutamate neurotransmitter systems. Rats pre-treated with AMPH, cocaine, PCP or MK-801 are assessed on various behavioural tasks which have been developed to parallel some of the tests administered to patients suffering from schizophrenia. Finally, in an effort to increase construct, face and predictive validity of animal models, the behavioural and neurochemical effects should to be reversed, at least partially, by antipsychotic (pre)treatment.

As described earlier, the experiments outlined in this thesis addressed some of the shortcomings of pharmacological and neurodevelopmental animal models by addressing the involvement of E2 and its effects on AMPH-induced locomotion in an AMPH-sensitized model. In addition, the neurodevelopmental and glutamatergic hypotheses were combined in an effort to

develop a novel approach to studying schizophrenia-like symptoms in the rat. Finally, cognitive deficits in schizophrenia were addressed by revisiting the hypothesis stipulating that WM is an mPFC, DA-driven construct. As such, the overall results are three-fold. First, E2 plays a mediating role in dopaminergic transmission in AMPH-sensitized rats. Second, prenatal glutamatergic disruption results in schizophrenia-like effects in early, but not late adulthood, and third, WM, a cognitive function affected in schizophrenia and unresponsive to treatment is not solely driven by D1R transmission, as previously suggested. From a therapeutic point of view, our data add support for the implication of ovarian hormones as adjuvants in treating some aspects of schizophrenic symptomatology. Furthermore, we provide indirect evidence that systems other than dopaminergic, and areas outside the mPFC are involved in rat WM function.

Limitations and future directions

Future studies should address the nature of the E2-DA interaction in animal models of schizophrenia, in an effort to develop E2-based therapies (Akhondzadeh et al., 2003; Kulkarni et al., 1996; Louza et al., 2004). Although Study 1B, offers a comprehensive E2-dependent activation map in response to an acute injection of AMPH in AMPH-sensitized rats, additional studies are required to determine whether activation was mainly driven by DA or E2 receptors (or both). In addition to its interaction with the DA system, E2 has been reported to affect central and peripheral blood flow (Hurn, Littleton-Kearney, Kirsch, Dharmarajan, & Traystman, 1995; Magness, Phernetton, & Zheng, 1998). Given that the results yielded in Study 1B are measures of regional blood flow, it is possible that BOLD activation differences seen with respect to hormonal treatment could be attributed to a facilitating effect of E2 on blood flow. Although possible, this is less likely since the activation patterns are based on ROIs previously reported to be rich in DA and E2 receptors. Furthermore, the experiments conducted as part of Chapter One do not address the

possible involvement of additional neurotransmitter systems, and their interaction with E2. Lastly, throughout Chapter One, the focus was mainly on E2. As such, additional work is required to investigate the effects of additional ovarian hormones such as progesterone in animal models of schizophrenia.

The experiments described in Chapter Two show that prenatal NMDAR blockade results in cognitive and locomotor impairments in the adult rat. As only male rats were used for this study, future experiments should address the involvement of ovarian hormones in this possible model of schizophrenia, given the findings from Chapter One. In addition, the effect of typical and atypical antipsychotics on locomotor and cognitive impairment should be assessed. Finally, additional doses of MK-801 and AMPH should be tested. Although prenatal MK-801 treatment resulted in locomotor hyperactivity in the adult offspring, we did not find such an effect in response to an AMPH challenge. It is possible that the AMPH dose was too low (i.e. 0.5 mg/kg) compared to other studies (Robinson, Jurson, Bennett, & Bentgen, 1988; Steinpreis, Sokolowski, Papanikolaou, & Salamone, 1994; Weiner, Lubow, & Feldon, 1988) where higher doses have been used (i.e. 1-5 mg/kg).

Although it was concluded that DA transmission via mPFC D1Rs is not crucial for WM maintenance in the rat, further studies should address the possible involvement of other DA receptors or neurotransmitter systems, as well other ROIs. As previously mentioned, the primate dlPFC is believed to be the anatomical and functional homolog of the rat mPFC (Uylings et al., 2003). However, it is likely that the functional concordance between these areas is not linear nor complete, which may result in discrepancies between the primate and rodent findings.

The studies described in this thesis indicate that, although AMPH sensitization has been a process widely used in schizophrenia research, the community still far from developing a comprehensive animal model. In order to maximize the validity of such models and ultimately their translatability, it is critical that the involvement of neurotransmitter systems other than DA and glutamate should be addressed systematically. Given the evidence presented in this thesis, the ideal animal model of schizophrenia would have to include a developmental component, while including multiple neurotransmitter systems. Most importantly, it would explore the dynamic interaction between these systems and be responsive to antipsychotic treatment. Finally, considering the growing evidence supporting the endocrine involvement in schizophrenia, animal models should mimic groups of symptoms in the presence of ovarian hormones.

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