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Mechanisms underlying executive function deficits

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Anatomy and Cell Biology

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Abstract

In our daily life, we come across situations where we meet unanticipated challenges, we must take certain decisions, pay attention, be flexible and inhibit impulsive actions to achieve goal directed behaviour. During these processes, we unknowingly use sets of interdependent cognitive processes collectively called ‘executive function’. Executive function is mainly regulated by the frontal lobe. Impaired executive function is associated with disorders such as schizophrenia, Alzheimer’s disease, autism and attention deficit hyperactivity disorder (ADHD).

In this thesis, we investigated neurotransmitters and interactions among them regulating executive function. Further, we investigated mechanisms underlying those interactions mediating executive function in rats using an operant conditioning-based set-shifting task, a common and validated test in animals to assess executive function. In our first study, we identified for the first time that systemic injections of dopamine D1 and glutamate N-methyl-D-aspartate (NMDA) receptor antagonists cause impaired set-shifting and increased the occurrence of perseverative errors only after combined administration at doses that failed to affect set-shifting following separate injections. The discovery of this novel synergistic effect of glutamate and dopamine antagonists on set-shifting prompted us to undertake our second study to determine if such synergy occurs within the medial PFC (mPFC)- an important brain area associated with executive function in rodents. Our results confirmed that mPFC is a site where seemingly mild suppression of glutamate and dopamine activities, similar to that has been reported in schizophrenia brains, may act cooperatively to manifest deficits in executive function via increasing perseverative errors. Our third study was to identify molecular mechanisms underlying such synergy. We found that protein kinase A (PKA) and extracellular signal-regulated kinase (ERK1/2) signaling cascades transduce this effect, with ERK1/2 phosphorylation in mPFC neurons as an obligatory step for set-shifting.

The present results have substantially advanced our understanding of the mechanisms underlying executive function. Our results also point to potential novel intracellular targets for therapeutic intervention in cognitive deficits.

Keywords: executive function, set-shifting, prefrontal cortex, glutamate, dopamine, synergism, PKA, ERK1/2

Co-Authorship Statement

Chapter 1 (introduction) and 5 (discussion), were written by Sagar J. Desai. Chapter 2, entitled “Combination of behaviourally sub-effective doses of glutamate NMDA and dopamine D1 receptor antagonists impairs executive function” was written by Sagar J. Desai with input from Dr. N. Rajakumar and Dr. Brian Allman. Chapter 3, entitled “Glutamate and dopamine abnormalities in the medial prefrontal cortex act synergistically to cause executive dysfunction” was written by Sagar J. Desai with input from Dr. N. Rajakumar and Dr. Brian Allman. Chapter 4 entitled “Molecular mechanisms associated with D1-NMDA receptor interaction mediated set-shifting in rats” was written by Sagar J. Desai.

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List of Abbreviations

5CERT, 5 choice serial reaction time task
ADHD, attention deficit hyperactivity disorder
ANOVA, one-way analysis of variance
cAMP, cyclic AMP
CNS, central nervous system
CREB, cAMP Response Element-Binding Protein
dlPFC, dorsolateral prefrontal cortex
DMSO, dimethylsulfoxide
DREADD, designer receptors exclusively activated by designer drugs
ERK, extracellular signal-regulated kinase
GABA, gamma-Aminobutyric acid
GAD67, glutamic acid decarboxylase-67
LTP, long-term potentiation
MAPK, mitogen-activated protein kinase
MDT, mediodorsal thalamus
MRS, magnetic resonance spectroscopy
NAc, nucleus accumbens
NMDA, N-methyl-D-aspartate
OFC, orbitofrontal cortex
PCP, phencyclidine
PET, positron emission tomography
PFC, prefrontal cortex
PKA, protein kinase A
PKC, protein kinase C
SEM, standard error of mean
SPD, schizotypal personality disorder
SPOT, self-ordered pointing task
WCST, Wisconsin card sorting task,

Chapter 1

1 Introduction

1.1 Executive functions

While driving a car, upon reaching a traffic signal that has just turned amber, the first thought that comes to our mind is about the distance of the car from the signal and how much time remains before the light turns red. Depending on the assessment, if there is sufficient time, we keep driving and get through the traffic signal safely. Alternatively, if we think that the signal will turn red before we pass through it, we apply the brakes to stop the car at the signal. In the above example, our brain works with enormous speed to calculate our distance from the traffic signal, probable time left before light turns red, are there any pedestrians looking for crossing the road, other vehicles around our car, etc. Information from our current sensory environment and relevant past experiences are considered and processed at very fast rate making it possible to drive a car safely. This is an example of ‘executive function’ where multiple interdependent cognitive processes work together synchronously to carry out a goal directed behaviour.

Executive function is a higher order cognitive ability comprising working memory, behavioural flexibility (set-shifting and reversal learning), attention, problem solving ability and inhibition (Gilbert and Burgess 2008; Diamond 2013; Miyake et al. 2000). These processes facilitate us to think, plan, get engaged, learn from the outcomes and tackle unfamiliar circumstances to carry out goal-directed behaviours in our day-to-day life (Elliott, 2003). As described by Funahashi (2001), executive function is ‘a product of the co-ordinated operation of various processes to accomplish a particular goal in a flexible manner’. It would be difficult to perform daily activities without proper executive functioning ability (Damasio, 1994). Various central nervous system (CNS) disorders including schizophrenia, Alzheimer’s disease, autism and attention deficit hyperactivity disorder (ADHD) show deficits in executive function (Millan et al., 2012). Executive function impairments may affect cognition, behaviour and personality. Behavioural component involve difficulty in pursuing goals, problem-solving ability and switching between possible strategies i.e. behavioural flexibility. These impairments affect a patient’s social life drastically and lead to immense economical loss to the individual, their family and society.

1.1.1 Executive functioning: Components

Conventionally, executive function was considered as a single construct, associated with higher order cognitive skills (Shallice, 1988). Executive function has also been conceptualized as a balanced association of multiple cognitive processes bringing about higher order cognitive function (Stuss and Alexander, 2000). As per the latter concept, there are three core components of executive functions: inhibition, working memory, and behavioural flexibility; all of which are interrelated, interdependent and work together (Miyake et al., 2000; Lehto et al., 2003).

1.1.2 Inhibition

In the example of executive functioning given in the first paragraph, when we see a traffic light turns amber from green, our brain calculates the probable time left to pass through the signal before it turns red. If that time window is too short, our brain commands to apply the brakes and stop the car at the signal. This self-inhibition is one of the components of executive functioning (Mäntylä et al., 2010). Inhibitory control involves regulating one's thoughts, attention and emotions. It helps us to focus on our aim in the presence of distractions. Inhibitory control allows us to avoid impulsive responses and to think before making a choice. Inhibition works with other functions like attention or working memory, and from our previous learnings help us to avoid making incorrect choices. Impaired inhibition can profoundly affect our daily activities causing us to react impulsively. CNS disorders including schizophrenia and ADHD, or conditions like addiction and mania display pathology of impaired inhibition (Gut-Fayand et al., 2001; Li and Sinha, 2008; Dumais et al., 2011).

The frontal lobes are thought to be associated with the regulation of inhibition. It was found that patients with frontal damage seized anything in front of them on the clinician's desk, as those objects provided affordances, and inhibition was needed to stop motor system to avoid such reactions (Lhermitte, 1983). In a lab experiment, Wallis et al, (2001) showed that monkeys with lateral PFC damage cannot control behaviour of reaching for food kept behind a transparent barrier provided, that they were trained to reach around to get the food previously.

1.1.3 Representative tasks used to assess inhibitory control

Inhibitory control can be assessed using a number of behavioural tests such as the Stroop test (Macleod, 2005), Flanker Task, and Go/No-Go tasks. In the Stroop test, a subject sees a series of colour names (black, blue, yellow, green, red). These words appear in different colours, sometimes as congruent (e.g., the word blue, written in blue), and sometimes non-congruent (e.g., the word blue, written in red). The subject is instructed to indicate, as quickly and accurately as possible, the colour in which the word is written, regardless if that matches the word itself.

Our ability to suppress inappropriate responses in a particular context can be assessed by computer-based Flanker Task (Eriksen and Eriksen, 1974). During each trial, the subject is presented with a set of arrows on screen as shown below (arranged as A, B, C, or D) and a correct response is made by choosing either a left or right button, dependent on the direction of the central arrow. The subject's ability to avoid incorrect responses is assessed in this task.

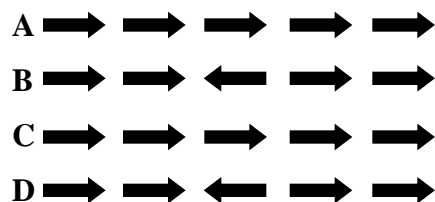


Figure 1.1 Flanker Task (Eriksen and Eriksen, 1974)

In a separate type of test, Go/no-go task, subjects are required to press a button when a stimulus appears; however, on a trial when a particular stimulus is presented, the subject must inhibit from pressing the button (Cragg and Nation, 2008).

1.1.4 Working memory

Working memory is an important component of executive function, which is characterized by the ability to hold information in the mind temporarily while processing it (Baddeley, 2012). An example of working memory would be solving a math problem without using paper and pen. Another example of working memory is finishing the

meaningful sentence while we are talking, as we need to keep track what has been said already and what we should say in order to finish the sentence. Working memory helps us to make sense of things which unfold over time as we require to hold in our mind what happened earlier and relating that to what comes later (Lett et al., 2014).

1.1.5 Representative tasks used to assess working memory

Working memory tasks are designed such that the subject must keep the information provided in mind temporarily and process it to make a correct response. Asking a subject to reorder the objects they have read, heard, smelled or seen is an excellent measure of working memory. For example, repeating the numbers flashed on screen (2, 7, 8, 1, 9) in numerical order (1, 2, 7, 8, 9) or reordering the items just heard based on their size (cat, dog, rat, elephant).

In Corsi block-tapping task, a tester taps the objects on the screen and following that, the subject must tap those objects in the same or opposite sequence in order to get the trial correct (Berch et al., 1998; Cragg and Nation, 2007). In the self-ordered pointing task (SPOT), a subject sees 12 objects on the screen (drawings or abstract designs), and is asked to click one item at a time in any order without repeating the choice until all items are clicked. After each choice, feedback is given. In addition to the tasks mentioned, researchers have used the N-Back task to assess inhibition (Owen et al., 2005; Kane et al., 2007), although it requires high levels of executive function, such as selective and sustained attention, thus not making the task selective for working memory (Miller et al., 2009).

1.1.6 Cognitive flexibility

Cognitive flexibility, which is a core feature of executive function, builds on the integration of working memory and inhibition. When we come across a problem which could not be solved by the way we know, we take a step back and try to solve it using different approaches (changing our point of view). A simple example of behavioural flexibility occurs when attempting to open a door; we elect to either pull it inside or push it outside. If pulling does not work, we change our approach and try to push the door outside.

1.1.7 Representative tasks used to assess cognitive flexibility

To investigate behavioural flexibility, researchers use set-shifting and task-switching paradigms (Monsell, 2003). The Wisconsin card sorting task (WCST) is one of the oldest tasks to assess behavioural flexibility in patients suffering from CNS disorders like schizophrenia (Braff et al., 1991; Gooding et al., 1999; Prentice et al., 2008). In WCST, reference cards with stimuli of different coloured shapes are flashed on a screen. For each trial, a new test card is offered below the reference cards. The subject must then match the test card to one of the reference cards based on the colour, shape, or number of the stimuli presented on it. Feedback is provided after each match, enabling the subject to acquire the correct rule of sorting the cards. Following a certain number of correct responses, the rule is changed without notice, and the subject must shift to a new mode of classification to get the trials correct. In set-shifting, ability of subject to switch to new strategy is assessed in presence of old strategy which is not valid anymore.

As its name implies, task switching involves two tasks and the subject's ability to switch from one task to the other. For example, subject is shown a coloured square with a number in the middle. Both sides of screen have a set of response keys: a key asking if the number flashed is odd or even, and the other is if the number is lower or higher than 10. Now based on the colour if it is black, the subject needs to answer higher or lower and if the colour is white, answer should be even or odd.

In both set-shifting or task-switching, errors are due to difficulty in inhibiting previously correct strategy, and this tendency is termed as 'attentional inertia' (Dick, 2012; Longman et al., 2014). It was found that the behavioural flexibility is developed in children by age of 7-9 (Gupta et al., 2009); during adulthood, it functions at the best and declines during old age (Kray and Lindenberger, 2000).

1.1.8 Neuroanatomical correlates of executive functioning in human

Historically executive functioning has been thought to be associated with frontal lobes. Patients with frontal cortex damage have shown impaired planning, organizing and behavioural disinhibition (Smith and Jonides, 1999; Stuss and Alexander, 2000; Roca et al., 2010; Sira and Mateer, 2014). In laboratory settings, such patients have also shown

impairments in WCST (Milner, 1963; Nelson, 1976; Stuss et al., 2000). In addition to this, patient population suffering from schizophrenia, a disorder with a prefrontal pathology, also show executive function impairments (Gooding et al., 1999; Everett et al., 2001; Prentice et al., 2008). Further, brain imaging studies in healthy individuals have shown that the dorsolateral prefrontal cortex (dlPFC) is one of the areas that is activated while performing executive function tests (Funahashi, 2001; Monchi et al., 2001). These observations over the years, led to the conclusion that executive function is strongly associated with the PFC, and hence, ‘executive function’ and ‘frontal lobe function’ became almost interchangeable terms (Ardila, 2008).

Although the association between the PFC and executive functioning has been well-documented, brain imaging studies have also identified the contribution of other brain areas in executive functioning (Monchi et al., 2001; Collette and Van Der Linden, 2002; Salmon and Collette, 2005; Alvarez and Emory, 2006; Collette et al., 2006). Neuronal system associated with executive function is complex and interrelated (Gilbert and Burgess, 2008). The PFC is highly connected with almost all brain regions including parietal, temporal, occipital lobes as well as subcortical and limbic regions, and they function interdependently (Adcock, 2000; Manoach et al., 2000; Stuss and Alexander, 2000; Cole et al., 2012; Zhang et al., 2014). Because of this, areas involved in executive functioning may be greatly expanded. Taken together, impaired executive functioning may be an outcome of PFC pathology, or it may be related to network disconnections or damage to brain regions including mediodorsal thalamus (MDT), striatum, accumbens etc.

1.1.9 Executive functioning in rodents

For decades, scientists have routinely used rodents as a main model for brain research. Numerous studies have used rats to study executive functions including working memory, attention, behavioural flexibility (set-shifting and reversal learning) and decision making (Birrell and Brown, 2000; Miller, 2000; Mirza and Bright, 2001; Floresco et al., 2009; Auger and Floresco, 2014). Studies have revealed the role of different brain nuclei including PFC, striatum, nucleus accumbens (NAc) and ventral hippocampus in executive functions (Felix and Levin, 1997; Crofts et al., 2001; Brown

and Bowman, 2002; Dalley et al., 2004; Floresco et al., 2006a, 2006b, 2009; Boulougouris et al., 2007; Brooks et al., 2012; Lindgren et al., 2013). In our experiments, we focused on set-shifting as a measure of behavioural flexibility and studied neurotransmitters and the mechanisms associated with set-shifting.

1.1.10 Behavioural flexibility in rodents

The ability to reverse a choice behaviour or the ability to shift a response rule when a previously learned strategy does not work has been widely used as a measure of behavioural flexibility. Analogous to clinical WCST used by Grant and Berg (1948), set-shifting tasks have been commonly employed for assessment of cognitive flexibility in rodents. These tasks involve training animals to follow a certain rule and once they acquire that learning (i.e. form an 'attentional set'), the rule is changed and ability of animals to figure out the new rule in presence of old one is assessed. Rats are required to: a) inhibit previously correct strategy which is no longer valid b) keep looking for a new strategy which works and c) once figured out, keep using new strategy without going back to original one (Floresco et al., 2008). The stimuli used in set-shifting are called 'dimensions' and traditionally they have been visual, related to texture, spatial or olfactory in nature. Depending on the stimuli used, there are two versions of set-shifting: a) Intra-dimensional and b) Extra-dimensional set-shifting. After forming an initial set, if the same type of stimulus used during set-shifting (i.e. after rules are changed), then it is called as intra-dimensional set-shifting, and if the different type of stimulus is used for latter phase of set-shifting, then it is called as extra-dimensional set-shifting. In accordance with clinical studies in patients with prefrontal pathology, rats with PFC lesions face more difficulties while performing extra-dimensional set-shifting than intra-dimensional set-shifting (Dias et al., 1997; Birrell and Brown, 2000). Different types of paradigms have been used to assess set-shifting in rodents. The most common examples are: a) digging task b) maze task c) operant conditioning-based task.

1.1.11 Digging task

Digging tasks have been used widely and successfully by researches to assess set-shifting in rodents (Barense et al., 2002; Young et al., 2010; Kos et al., 2011; Heisler et al., 2015). Birrell and Brown (2000) used the natural tendency of searching for food in

rats. As shown in Figure 1.2, diverse stimulus dimensions are used to distinguish between bowls: digging media filling the bowls, olfactory cues added to the medium and the texture of the bowls. Rats use tactile or olfactory cues to retrieve food rewards in this task. For example, food restricted rats are trained to dig in a bowl with filling medium smelling like mint to obtain food rewards during every trial associating mint odour of the medium with reward. This is called initial set formation. Next, during the set-shifting, rats must switch from olfactory cue to the type of texture of the bowl (sand paper) as a strategy to get rewards. So, in latter phase of the task (set-shifting), rats must dig in the bowl with sand paper texture regardless of olfactory stimulus.

Advantages of this task are that it requires simple set up of instruments, and the same rat could be tested more than once using different stimulus dimensions (Wallace et al., 2014). Although it also has certain disadvantage like more interference of researcher during the task. Furthermore, training rats takes more time as compared to the operant task (described below).

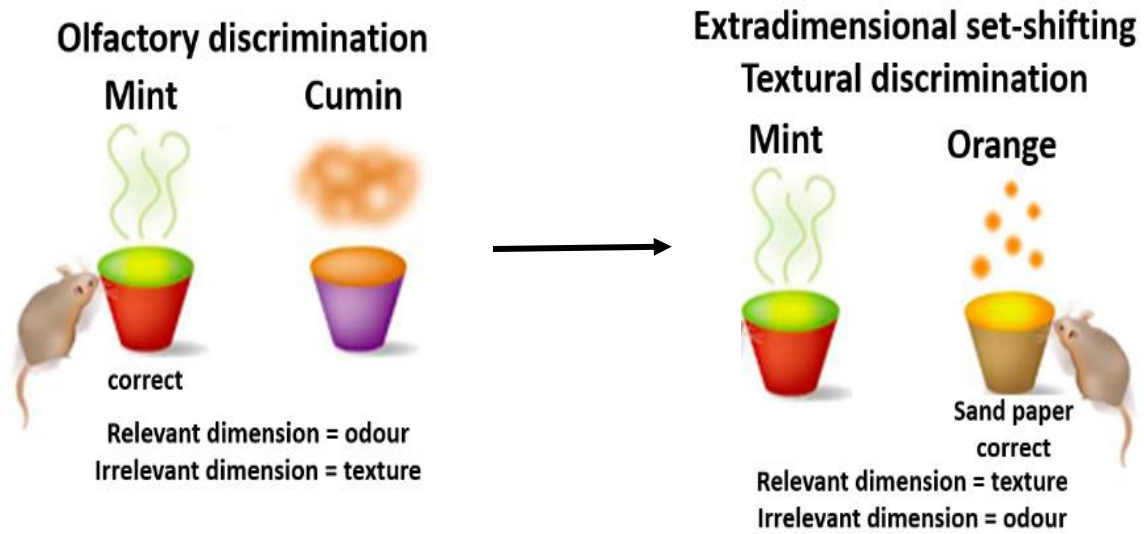


Figure 1.2 Schematic of the digging task used to assess behavioural flexibility in rodents.

In the digging task, rats are trained to dig in bowls by discriminating them based on odour, texture or the medium filled in bowls. In the given example, relevant dimension is odour and rats must dig in bowl smelling like mint to get food rewards (initial set formation). During extradimensional set-shifting, rats must dig in the bowl with sand paper like texture regardless of odour of the bowl.

1.1.12 Maze task

In the maze task, a plus maze is used where rats shift between two stimulus dimensions i.e. visual-cue and spatial-based discrimination strategies (Ragozzino et al., 2003; Stefani et al., 2003; Floresco et al., 2009). Food restricted rats are initially trained (initial set formation) to retrieve food rewards by entering in an arm with distinctive visual cue (brightened or darkened arm; Figure 1.3). For the set-shifting, rat must learn to turn in specific direction (either left or right) regardless of visual-cue in order to retrieve the food rewards. Simple experimental set up is an advantage of using maze task. Drawback of maze task would be lack of automaticity as well as an experimenter has to handle rats after every trial.

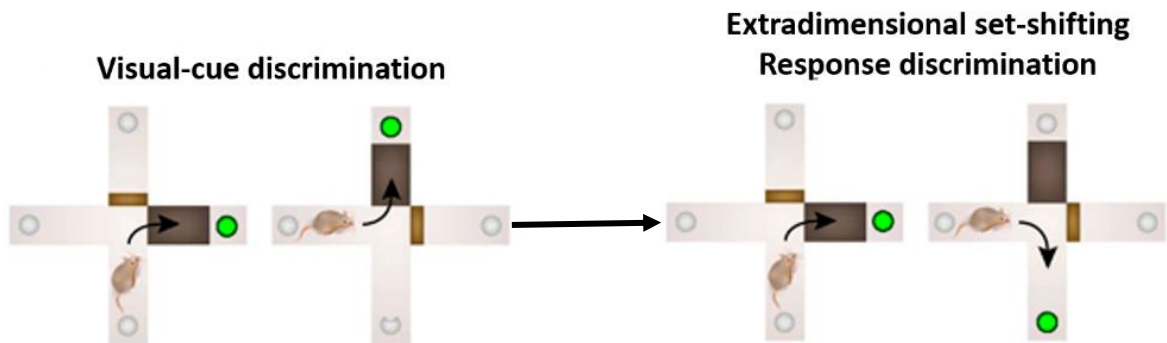


Figure 1.3 Schematic of the maze task used to assess behavioural flexibility in rodents.

For maze-based extradimensional set-shifting task, rats are trained to use a visual-cue discrimination strategy i.e., entering the arm with the visual cue by turning to right or left (initial set formation). During set-shifting, to earn food rewards, rats must use a response discrimination strategy, and make a 90° right turn to enter in an arm to get food reward regardless of position of visual-cue stimulus.

1.1.13 Operant-based task

Floresco et al. (2008) has adapted the same approach for set-shifting as that of the aforementioned plus maze. In an operant box, food restricted rats are trained to press the lever associated with an illuminated visual-cue stimulus light to earn food rewards as a part of initial training (visual-cue discrimination; Figure 1.4). Once rats achieve the criterion for visual-cue discrimination, on the next day, contingencies are altered and now rats must respond on one particular lever (response discrimination) during every trial to get food rewards regardless of location of illuminated visual-cue stimulus light.

Advantage of using this task is that operant conditioning is fully automated, thus reducing the interference of an experimenter. Furthermore, parameters like the type of errors, omissions and latency to response are precisely recorded in trial by trial manner. In addition to this, the associated training procedure in this task is short and robust as compared to maze or digging tasks. Moreover, operant conditioning does not have strong spatial or olfactory demands (Bizon et al., 2012).

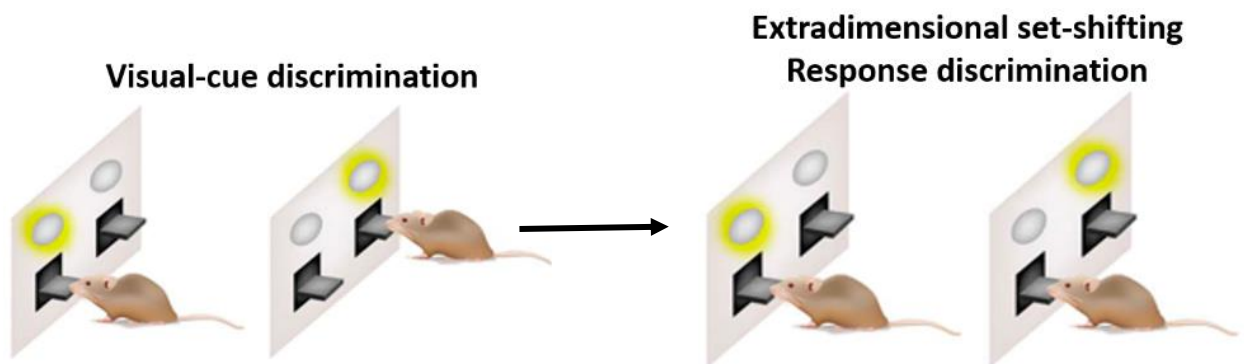


Figure 1.4 Schematic of operant-based task used to assess behavioural flexibility in rodents. In operant-based extradimensional set-shifting, rats are trained to respond on the lever with illuminated visual-cue stimulus light (i.e. rats use visual-cue discrimination strategy to discriminate between the levers). On set-shifting, rats must follow a response discrimination and press one particular lever (right or left) regardless of the position of the illuminated visual-cue stimulus light.

1.1.14 Error classification

Errors committed during set-shifting can be classified in to three categories:

Perseverative errors: Inability of rats to switch to new strategy give rise to this type of errors during set-shifting. Perseverative error is an index of how quickly animals can learn use of the correct strategy.

Regressive errors: While performing set-shifting even though rats try a novel strategy that results in a correct trial (food reward), they often go back to the original rule which is irrelevant during set-shifting making regressive errors. These regressive errors are an index of the rat's ability to maintain the newly acquired strategy.

Never-reinforced errors: While performing set-shifting, animals try to explore new response choices to figure out the novel strategies which could result in a correct response. During this process, rats try certain approaches that they never learnt during the initial acquisition, only to discover that it also does not yield a food reward during the set-shifting task. This type of error is called a never-reinforced error. Never-reinforced errors are an index of how readily rats can ignore certain strategies which do not result in a correct response.

1.1.15 Neural circuits subserving behavioural flexibility in rodents

Behavioural flexibility is not a unitary phenomenon, rather, it has been conceptualized as different functions working together. To finish set-shifting successfully, animals must meet three requirements of the task: a) Inhibit a previously correct strategy which is now irrelevant during set-shifting. b) Keep looking for a new strategy which could give food rewards. c) Once a new strategy is found, keep using it, and avoid going back to the previously correct rule. Because different brain nuclei are associated with different components mentioned, a successful set-shifting performance is thought to be the result of brain circuitry involving number of brain regions rather than one specific nucleus (Block et al., 2007; Floresco et al., 2009).

1.1.16 Prefrontal Cortex (PFC)

From the studies in human, non-human primates as well as rodents, it is well known that PFC is strongly implicated in behavioural flexibility (Brown and Tait, 2016). Inactivation or damaging medial prefrontal cortex (mPFC) in rat results in impaired set-shifting performance without affecting initial learning (initial set formation) (Ragozzino et al., 1999a; b; Floresco et al., 2008). Following inactivation/lesioning of mPFC, perseverative errors were found to be increased (rats are not able to switch to new rule, hence repeating the same incorrect response despite not receiving any food rewards). On the other hand, inactivation of orbitofrontal cortex (OFC) caused impaired reversal learning in rats (Ghods-Sharifi et al., 2008). Interestingly, inactivation of mPFC did not affect reversal learning, and lesioning OFC did not have any effect on set-shifting; findings which suggest that although set-shifting and reversal learning are components of behavioural flexibility, they are regulated by different regions of the PFC. Furthermore, increased perseverance is the abnormality shared by rats with mPFC damage and the patients with schizophrenia with frontal lobe pathology in set-shifting and WCST respectively, suggesting PFC has similar role in executive functioning across species (For review, Brown and Tait, 2016).

1.1.17 Mediodorsal Thalamus (MDT)

Mediodorsal thalamus has reciprocal connectivity with the PFC (Groenewegen, 1988). This anatomical association with PFC suggest that MDT may contribute to behavioural flexibility. The role of MDT in a simpler form of behavioural flexibility i.e., reversal learning is controversial as some studies report impairments in reversal learning following MDT lesioning (Means et al., 1975; Chudasama et al., 2001; Parnaudeau et al., 2015) while others did not see any impairments (Tigner, 1974; Beracochea et al., 1989). On the other hand, lesioning MDT produced increased perseverance in rodents in a set-shifting task which is a more complex behaviour than reversal learning (Hunt and Aggleton, 1998). Furthermore, inactivating MDT did not affect the initial learning phase in set-shifting, suggesting the MDT is associated with shifting strategy and not general learning (Block et al., 2007).

Clinical brain imaging studies have shown activation of MDT following incorrect response in WCST suggesting it may be acting as a trigger to switch the strategy (Monchi et al., 2001). Thus, across species, MDT is associated with behavioural flexibility, and abnormal functioning of MDT results in increased perseverance.

1.1.18 Nucleus Accumbens (NAc)

Floresco et al. (2006a), investigated effect of inactivation of NAc-Core and NAc-Shell on maze-based set-shifting task performance. It was found that inactivation of NAc-Core did not affect initial acquisition but impaired set-shifting. Type of errors committed during set-shifting were 'regressive' and 'never-reinforced'; error profiles different than perseverative error profile associated with mPFC or MDT lesioning. Increased regressive errors meant that rats were not able to hold on to the new strategy and more often they went back to the previously correct strategy suggesting that NAc-Core plays important role in maintenance of a novel response. Never-reinforced errors are considered as an index of how quickly rats can get rid of the strategy which would not give food pellet (correct response) (Floresco et al., 2008). An increase in never-reinforced errors suggests that NAc-Core mediates the inhibition of unsuitable response choices through learning of a novel strategy. Inactivation of NAc-Shell neither affected initial acquisition nor the set-shifting.

1.1.19 Striatum

Inactivation of striatum did not affect initial acquisition in rats but impaired set-shifting. Errors committed were regressive in nature suggesting that striatum plays an important role in maintaining newly learnt strategy (Ragozzino et al., 2002b; Haluk and Floresco, 2009).

1.1.20 Neurotransmitters associated with behavioural flexibility

Various pharmacological studies in animals have provided evidence for involvement of different neurotransmitter systems regulating set-shifting. Most commonly, gamma-Aminobutyric acid (GABA), dopamine, glutamate and have been studied for their role in behavioural flexibility.

1.1.21 GABA

Involvement of GABAergic neurotransmitter in cognition has been the topic of discussion for last two decades. GABA has got the attention of researchers due to its involvement in the prefrontal pathophysiology of schizophrenia and possible role in cognitive impairments associated with schizophrenia (Coyle, 2004; Lewis et al., 2008; Vinkers et al., 2010; Lett et al., 2014). Since then several studies in human as well as in animals have investigated the role of GABA in executive functions including working memory, attention and behavioural flexibility (Michels et al., 2012; Auger and Floresco, 2014; Banuelos et al., 2014; Chen et al., 2014).

Enomoto et al. (2011) studied effect of intra-mPFC infusion of bicuculline (GABA-A receptor antagonist) on set-shifting. It was found that, bicuculline significantly impaired set-shifting in rats. Impaired set-shifting resulted from increased perseverative errors without any effect on regressive and never-reinforced errors; an error profile similar to mPFC inactivation studies (Ragozzino et al., 1999a; Floresco et al., 2008).

1.1.22 Dopamine

Historically, the role of prefrontal dopamine has been examined for its contribution in executive functioning. Evidence from human as well as animal studies have confirmed that normal dopamine receptor functioning (D1/D2) is essential for normal executive functions including working memory, attention and behavioural flexibility (Sawaguchi and Goldman-Rakic, 1994; Müller et al., 1998; Misener et al., 2004; Vijayraghavan et al., 2007; Floresco, 2013). Furthermore, decreased number of dopamine D1 receptors in dlPFC of schizophrenic patients was correlated with the poor performance on a working memory task (Okubo et al., 1997; Akil et al., 1999).

Considering the role of dopaminergic neurotransmitter in cognitive processes, researchers studied the involvement of dopamine in behavioural flexibility using pharmacological approaches in rodents. For example, intra-mPFC infusion of dopamine D1 receptor antagonist impaired set-shifting without affecting initial acquisition (Ragozzino, 2002a; Gauthier et al., 2014). Interestingly, stimulation of D1 receptors did not improve set-shifting (Floresco et al., 2006b), a finding in line with Fletcher et al. (2005), where acute intra-mPFC infusion of D1 receptor agonist treatment *per se* did not

affect set-shifting but reversed set-shifting impairments in amphetamine sensitized rats. Further, intra-mPFC infusions of dopamine D2 receptor antagonist impaired set-shifting set-shifting by increasing perseverance whereas stimulation of D2 receptors in the mPFC did not affect set-shifting. In addition to this, while D4 receptor agonist impaired set-shifting performance, stimulation of D4 receptors by an agonist improved the set-shifting above control level (Floresco et al., 2006b). These findings indicate that the “inverted-U” shaped function underlying dopamine receptor regulating working memory does not appear to hold true for set-shifting function mediated by the PFC. This finding is in accordance with

1.1.23 Glutamate

In the brain, glutamate is a major excitatory neurotransmitter. Glutamate N-methyl-D-aspartate (NMDA) receptors regulate synaptic plasticity i.e. long-term potentiation (LTP) and cognition (Villarreal et al., 2002). NMDA receptor antagonists including ketamine and phencyclidine (PCP) cause cognitive symptoms of schizophrenia in healthy volunteers or exacerbate these impairments in schizophrenic patients (Malhotra et al., 1996, 1997; Coyle and Tsai, 2004; Morgan et al., 2004; Morris et al., 2005). This led to glutamatergic hypothesis of schizophrenia where NMDA altered functioning was related to impaired cognition in schizophrenic patients (Coyle, 1996, 2006; Tsai and Coyle, 2002; Paz et al., 2008).

Due to deep association of glutamate with cognition, researchers investigated if glutamate is involved in controlling executive functions in humans as well as animals. In a clinical study (Krystal et al., 2000) found that administration of sub-anesthetic dose of ketamine in healthy volunteers impaired their performance in WCST. In animal studies, normal functioning of NMDA receptors was found to be essential for working memory as well as attention (Murphy et al., 2005; Baviera et al., 2008; Smith et al., 2011; Wang et al., 2012b; Su et al., 2014; Auger and Floresco, 2017).

In animals, behavioural flexibility was found to be regulated by NMDA receptors. Intra-mPFC infusion of NMDA receptor antagonist (MK-801) impaired set-shifting and increasing perseverative errors in animals (Stefani and Moghaddam, 2005). This finding

was replicated by acute or repeated systemic injections of either MK-801, PCP or ketamine in animals (Rodefer et al., 2005; Egerton et al., 2008; McLean et al., 2008).

1.1.24 Dopamine-glutamate interactions

Both dopamine and glutamate abnormalities in the PFC are thought to be associated with cognitive pathology of schizophrenia (Akil et al., 1999; Coyle, 2006). Role of individual neurotransmitters regulating executive functions has been studied. Although the interaction between dopamine and glutamate regulating different behaviours has been investigated, its role in executive function is yet to be explored sufficiently.

A number of studies have investigated interaction between D1 and NMDA receptors regulating different behaviours. It was found that combined infusion of sub-effective doses of D1 and NMDA receptor antagonists in the mPFC or NAc-Core impaired instrumental learning in rats (Smith-Roe and Kelley, 2000; Baldwin et al., 2002b). Similarly, co-infusion of NMDA and D1 receptor antagonists on contralateral sides of amygdala impaired glucose-conditioned learning of flavor preference in rats; co-infusion of one of the antagonists on one side and vehicle on the other side did not show any effect on behaviour suggesting that, blockade of both D1 and NMDA receptors was essential to cause impairment (Touzani et al., 2013). Furthermore, functional striatal NMDA channels are needed to control D1-stimulated locomotor behaviour (Kreipke and Walker, 2004). In addition to this, deletion of NR1 subunit of NMDA receptors in the NAc was found to attenuate apomorphine-induced decrease in acoustic startle response in rats suggesting an interaction between D1-NMDA receptors (Glass et al., 2013). Along with behaviours mentioned above, D1-NMDA interactions were found to control higher cognitive functions. Intra-medial dorsal striatum co-infusion of D1 and NMDA receptor antagonists at respective ineffective dose significantly decreased accuracy of visual discrimination in the 5 choice serial reaction time task (5CSRT) in rats (Agnoli and Carli, 2011). Moreover, Nai et al. (2010) reported that uncoupling of D1-NMDA receptor interaction by using an interfering peptide (TAT-D1-t2 peptide) eliminates D1 receptor-induced upregulation of NMDA mediated LTP in rat hippocampal cultures. In addition to this, in the same study, intra-hippocampal infusion of TAT-D1-t2 peptide impaired

working memory in mice. Intra-mPFC infusion of D1 receptor agonist rescued set-shifting in amphetamine-sensitized rats (Fletcher et al., 2005) suggesting D1-NMDA interaction controlling behaviour.

From the aforementioned literature, it seems that there are strong interactions between D1 and NMDA receptors regulating different types of behaviours in animals. These behavioural findings were supported by number of electrophysiology studies showing synergistic interaction between dopamine D1 and glutamate NMDA receptors. Kruse et al. (2009) reported that, D1 receptors and NMDA receptors are co-localized in single pyramidal neuron as well as GABA interneurons in the adult rat PFC. In the same study, activation of D1 receptors was found to potentiate NMDA receptor mediated increase in cytosolic calcium ions. Activation of D1 receptors upregulated NMDA receptor-mediated LTP in the hippocampus, PFC or striatum (Calabresi et al., 2000; Gurden et al., 2000; Kerr and Wickens, 2001; Nai et al., 2010). Furthermore, both D1 and NMDA receptors potentiated each other's expression at synaptic membrane in PFC and striatum (Jay, 2003; Hallett et al., 2006; Scott et al., 2006; Castner and Williams, 2007; Gao and Wolf, 2008).

Although interactions between D1-NMDA receptors are studied in detail, direct evidence of role of this interaction regulating set-shifting is still to be understood.

1.1.25 Mechanisms underlying D1-NMDA interactions

From the above-mentioned studies, it seems that interactions between D1-NMDA receptors are important. A number of studies investigated the mechanisms underpinning D1-NMDA receptor interactions which are complex in nature. These interactions either facilitate or inhibit responses to receptor activation (Cepeda and Levine, 2006).

1.1.26 D1-NMDA receptor interactions through protein kinase A (PKA) signaling cascade

D1-NMDA receptor interactions have been reported to be mediated by number of molecular signaling cascades in the PFC as well as the striatum (Kerr and Wickens, 2001; Cepeda and Levine, 2006; Kruse et al., 2009). The most important is of D1 activated

cAMP-PKA cascade which leads to a multitude of outcomes including phosphorylation of NR1 subunits of NMDA receptors (Snyder et al., 1998) and activation of voltage-gated Ca^{++} channels (Cepeda et al., 1998; Tseng and O'Donnell, 2004; Kruse et al., 2009). On the other hand, antagonizing D1 receptors attenuated the PKA-mediated phosphorylation of NR1 subunits (Edwards et al., 2002), and inhibiting PKA prevented D1-induced NMDA-mediated calcium signaling (Kruse et al., 2009). Thus, PKA is an important signaling molecule used by D1-NMDA receptor interactions. Table 1.1 summarizes D1-NMDA interactions mediated through PKA cascade.

1.1.27 D1-NMDA receptor interactions through extracellular signal-regulated kinase (ERK) signaling cascade

Sarantis et al. (2009) showed that combined treatment with sub-effective doses of D1 agonist and NMDA in the mPFC or hippocampus slices caused an increase in phosphorylation of NR1 and NR2B subunits of NMDA receptors. Interestingly, the signaling pathway associated with the synergism involved phosphorylation of ERK1/2. However, unlike what was described in the striatum, D1 \rightarrow ERK1/2 cascade is independent of DARPP-32 as an intermediary in the PFC. In the same study, authors stated the possibility of an involvement of PKA and protein kinase C (PKC) which would activate ERK1/2 signaling pathway as reported previously by Cahill et al. (2014). In agreement with the study of Sarantis et al. (2009), involvement of ERK1/2 signaling in D1-NMDA interaction was also reported by others in the hippocampus and NAc (Papadeas et al., 2004; Haberny and Carr, 2005; Sarantis et al., 2012).

1.1.28 Physical D1-NMDA receptor interactions

Direct physical interaction between D1 and NMDA receptors has also been reported in hippocampal neuronal cultures, PFC and the striatum (Lee et al., 2002; Pei et al., 2004; Hu et al., 2010; Groveman et al., 2012). Protein-protein interactions between C-terminals of D1 receptors and NMDA receptor subunits NR1 or NR2A allow direct cross-talk between two receptors (Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004). Interaction of D1 with NR1 increases plasma membrane insertion of D1 receptors upregulating D1 receptor function and this upregulation was found to be mediated

through Fyn kinase, a family member of Src kinase, in PFC (Pei et al., 2004; Hu et al., 2010). Table 1.1 summarizes D1-NMDA interactions mediated through direct protein-protein interaction.

Table 1.1 Summary of D1 and NMDA receptor interactions

| Receptor activated | Preparation | Region | Effect | Mechanism | References |
|---|--|----------------------|---|-------------------------------------|--|
| Physical interactions | | | | | |
| NMDA | Organotypic cultures | Striatum | D1 receptors in spines | Allosteric change, diffusion trap | Scott et al., (2006) |
| NMDA | COS7, cell cultures | Hippocampus | D1 receptors in membrane | D1-NR1 binding | Pei et al., (2004) |
| D1 | HEK293, cell cultures | Hippocampus | NMDA currents Excitotoxicity | D1-NR2A binding D1-NR1 binding | Lee et al., (2002) |
| D1 | HEK293, COS7, PSD | Striatum | Translocation of D1-NR1 D1 agonist-induced internalization | Oligomerization of D1 and NR1 | Fiorentini et al., (2003) |
| Second messenger-mediated interactions | | | | | |
| NMDA | Cell cultures | Striatum | D1 receptors in spines | Ca ⁺⁺ -dependent | Scott et al., (2002) |
| D1 | Synaptosomes from brain slices | Striatum | NR1, NR2A, NR2B in synaptosomes | Fyn protein tyrosine kinase | (Dunah and Standaert, 2001; Fiorentini et al., 2003) |
| D1 and NMDA | Brain slices, oocytes, dissociated cells | Striatum, cortex | NMDA responses | cAMP-PKA-DARPP-32, Ca ⁺⁺ | (Cepeda et al., 1992, 1998; Snyder et al., 1998; Wang and O'Donnell, 2001; Flores-Hernández et al., 2002; Seamans and Yang, 2004; Tseng and O'Donnell, 2004) |
| D1 and NMDA | Brain slices, cells | N. accumbens, cortex | NMDA responses | PKC, Ca ⁺⁺ | (Chergui and Lacey, 1999; Chen et al., 2004) |

Review Cepeda and Levine (2006)

1.2 Thesis objectives and hypotheses

Based on the overview of the literature, deficits in the behavioural flexibility and increased propensity of perseverative errors are core cognitive abnormalities in certain CNS disorders, including, schizophrenia, and are associated with abnormal functioning of interconnected brain areas, including the PFC. Post-mortem findings and previous animal studies showed marked decreases of glutamate and dopamine levels in the PFC individually and is sufficient to cause increased perseverative errors leading to impairment in behavioural flexibility. However, recent *in vivo* imaging studies have shown that, in living patients the loss of these neurotransmitters is minimal. Consequently, **our overall hypothesis was that the minimal loss of glutamate or dopamine function is sufficient to produce impaired behavioural flexibility by increased perseverative errors provided those losses co-occur** in the brain. This also suggests that there may be synergy between glutamate and dopamine neurotransmitter abnormalities. In order to test our overall hypothesis, an operant conditioning-based set-shifting paradigm was used. Following three studies were undertaken to test our overall hypothesis and to elucidate associated molecular signaling mechanisms mediating proposed synergy between deficiencies in neurotransmitter actions and how they are relevant to behavioral flexibility.

1.2.1 Study I

The role of dopamine D1 and glutamate NMDA receptors is well-recognized in the regulation of memory, cognition and executive functioning (Okubo et al., 1997; Nieoullon, 2002; Volk et al., 2015; Thomas et al., 2017; Akil et al., 2013). Contrary to the notion based on post-mortem studies, recent *in vivo* imaging studies have shown that the changes in the dopamine and glutamate levels in the PFC of living schizophrenic patients are very subtle (Laruelle et al., 2003; Théberge et al., 2007; Ohrmann et al., 2007; Galińska et al., 2009; Rowland et al., 2009; Aoyama et al., 2011; Seese et al., 2011; Szulc et al., 2011; Goto et al., 2012; Kegeles et al., 2012; Slifstein et al., 2015; Howes et al., 2015). It is possible that these subtle alterations in neurotransmitter systems ultimately result in noticeable cognitive impairments. In animal studies, the individual role of dopamine and glutamate has been investigated in regulating set-shifting behaviour

and results showed that large decreases in individual transmitters in different brain areas could impair set-shifting performance (Stefani et al., 2003; Haluk and Floresco, 2009; Tait et al., 2014; Gauthier et al., 2014). Functional neuroimaging studies have indicated that a number of cortical and subcortical areas including dlPFC, anterior cingulate cortex, striatum, hippocampus and the MDT are associated with successful performance of the WCST (Stratta et al., 1997; Rüsç et al., 2007; Wilmsmeier et al., 2010; Pedersen et al., 2012; Young et al., 2000; Kemether et al., 2003). In agreement with this, several animal studies have shown that, inactivation of number of brain areas or connections among them could lead to impaired set-shifting. Since, the hypothesized interaction between dopamine-glutamate neurotransmitters could occur in several potential areas as shown in animal studies (Block et al., 2007; Floresco et al., 2008; 2009), we decided to use systemic route of drug administration to target brain circuitry rather than specific nuclei. We tested effect of combined systemic administration of dopamine D1 and glutamate NMDA receptor antagonists on set-shifting behaviour in normal rats.

The following is the *specific aim of the study I*:

To determine the effect of systemic combined administration of “behaviourally sub-effective” doses of D1 and NMDA receptor antagonists on set-shifting in normal rats.

1.2.2 Study II

Across species, PFC is associated with executive functioning (Abbruzzese et al., 1996; Ragozzino et al., 199b; Floresco et al., 2008; Moore et al., 2009). Patients with prefrontal pathology of schizophrenia or accidental frontal lobe damage show impaired executive functions (Milner, 1963; Nelson, 1976; Smith and Jonides, 1999; Stuss and Alexander, 2000; Stuss et al., 2000; Roca et al., 2010; Sira and Mateer, 2014). In clinical studies, prefrontal cortical dopamine as well as glutamate neurotransmitters were proposed to regulate cognition and abnormal functioning of these neurotransmitters may impair cognition in schizophrenia (Krystal et al., 2000; Coyle, 2006; Akil et al., 2013). In line with the clinical studies, findings from animal studies confirm essential role of dopamine and glutamate neurotransmitters individually in the mPFC for normal

executive functioning (Ragozzino, 2002a; Stefani and Moghaddam et al., 2003; Gauthier et al 2014). In addition, several behaviours other than executive function, are regulated by interaction between D1 and NMDA receptors (Smith-Roe and Kelley, 2000; Baldwin et al., 2002; Fletcher et al., 2005; Nai et al., 2010; Agnoli and Carli, 2011; Touzani et al., 2013; Glass et al., 2013).

From our first study, it was evident that the systemic combined administration of behaviourally sub-effective doses of D1 and NMDA receptor antagonists caused set-shifting impairments and produced error profile similar to inactivation or lesioning studies in mPFC (Everett et al., 2001; Block et al., 2007; Floresco et al., 2008; Roca et al., 2010). Therefore, we hypothesized that, PFC is one of the brain sites where synergism between dopamine and glutamate abnormalities could occur. In the second study, we sought to find out if the mPFC is associated with synergism between D1-NMDA receptor antagonists. To test our hypothesis, we infused mPFC of normal rats with D1 or NMDA receptor antagonists individually to determine their dose-response profile in set-shifting. The highest but ineffective doses of these antagonists were then combined to determine whether there is a synergism between them causing increased perseverance in set-shifting.

The following is the *specific aim of study II*:

To determine the effect of intra-mPFC co-infusion of ‘behaviourally sub-effective’ doses of D1 and NMDA receptor antagonists on set-shifting in normal rats.

1.2.3 Study III

From the literature, it was evident that in different areas of the brain, interactions between D1-NMDA receptors may use mainly three molecular signaling pathways: PKA, ERK or direct protein-protein interactions between D1 and NMDA receptor complexes through Fyn kinase. Interestingly, all main three pathways (PKA, ERK and Fyn kinase mediated protein-protein interaction) have been implicated in the pathology of schizophrenia (Tardito et al., 2001; Rybakowski et al., 2007; Yuan et al., 2010; Hahn, 2011; Kunii et al., 2011; Wang et al., 2015).

In the mPFC, D1-NMDA receptors may interact via PKA signaling cascade to regulate learning in rats (Baldwin et al., 2002). Furthermore, PKA was also found to regulate synergistic effect of D1-NMDA receptors on excitability of pyramidal neuron in the mPFC (Wang and O'Donnell, 2001). Along with PKA, ERK signaling was also found to regulate D1-NMDA receptor interaction in the mPFC (Sarantis et al., 2009; Nagai et al., 2007). Moreover, physical interaction between D1-NMDA receptors seems to potentiate each other's function (Gao and Wolf, 2008; Hu et al., 2010).

From results obtained in the study II, it appears that mPFC is one of the nuclei where D1-NMDA receptors are interacting with each other to regulate set-shifting behaviour. Thus, we hypothesized that molecular mechanisms underlying D1-NMDA receptor interaction will use one or more of the following pathways in mPFC neurons to regulate set-shifting behaviour: a) PKA b) ERK1/2 c) Fyn kinase-mediated interaction between D1-NMDA receptors. To test our hypothesis, we performed a series of the following experiments: a) effects of elevating PKA levels in the mPFC in animals receiving intra mPFC infusion of a combination of D1 and NMDA antagonists that disrupted set-shifting performance in study II; b) effects of inhibiting ERK1/2 phosphorylation on set-shifting performance in naïve rats; and c) effect of inhibiting Fyn kinase in the mPFC on set-shifting behaviour.

Following *specific aims were tested in this study*:

- 1. To determine the effect of elevating PKA in the mPFC on D1-NMDA receptor antagonist combination (from study II)-induced impaired set-shifting.**
- 2. To determine the effect of inhibiting Fyn kinase and hence to verify if direct D1 → NMDA receptor cross-talk facilitates set-shifting in normal rats.**
- 3. To determine the effect of inhibiting ERK1/2-phosphorylation in the mPFC using MAPK (mitogen-activated protein kinase) inhibitors on set-shifting in normal rats.**

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Chapter 2

2 Combination of behaviourally sub-effective doses of glutamate NMDA and dopamine D1 receptor antagonists impairs executive function¹

2.1 Abstract

Impairment of executive function is a core feature of schizophrenia. Preclinical studies indicate that injections of either N-methyl D-aspartate (NMDA) or dopamine D1 receptor blockers impair executive function. Despite the prevailing notion based on post-mortem findings in schizophrenia that cortical areas have marked suppression of glutamate and dopamine, recent in vivo imaging studies suggest that abnormalities of these neurotransmitters in living patients may be quite subtle. Thus, we hypothesized that modest impairments in both glutamate and dopamine function can act synergistically to cause executive dysfunction. In the present study, we investigated the effect of combined administration of “behaviourally sub-effective” doses of NMDA and dopamine D1 receptor antagonists on executive function. An operant conditioning-based set-shifting task was used to assess behavioural flexibility in rats that were systemically injected with NMDA and dopamine D1 receptor antagonists individually or in combination prior to task performance. Separate injections of the NMDA receptor antagonist, MK-801, and the dopamine D1 receptor antagonist, SCH 23390, at low doses did not impair set-shifting; however, the combined administration of these same behaviourally sub-effective doses of the antagonists significantly impaired the performance during set-shifting without affecting learning, retrieval of the memory of the initial rule, latency of responses or the number of omissions. The combined treatment also produced an increased number of perseverative errors. Our results indicate that NMDA and D1 receptor blockade act synergistically to cause behavioural inflexibility, and as such, subtle abnormalities in glutamatergic and dopaminergic systems may act cooperatively to cause deficits in executive function.

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2.2 Introduction

Executive function is a complex phenomenon comprising attention, working memory, planning, reasoning, sequencing, inhibitory control and cognitive flexibility (Robbins et al., 1996). Severe impairment of executive function is a core feature of schizophrenia, and is an important determinant of long-term outcome and quality of life of these patients (Hutton et al., 1998; Krieger et al., 2005; Holthausen et al., 2007; Penadés et al., 2010). In addition to challenges with daily activities, patients with impaired executive function demonstrate difficulty performing standardized neuropsychological assessments of behavioural flexibility, such as the Wisconsin card sorting test (WCST) (Anderson et al., 1991; Prentice et al., 2008). Based on the findings from functional neuroimaging studies, a variety of cortical and subcortical areas have been implicated in the successful performance of the WCST, most notably the dorsolateral prefrontal cortex (dlPFC) anterior cingulate cortex, striatum, hippocampus and the mediodorsal nucleus of the thalamus (MDT) (Stratta et al., 1997; Rüsç et al., 2007; Wilmsmeier et al., 2010; Pedersen et al., 2012). Similarly, preclinical studies in rodents have confirmed that the aforementioned brain regions are necessary for behavioural flexibility during such tasks as set-shifting (Ragozzino et al., 1999; Ragozzino et al., 2002; Block et al., 2007; Floresco et al., 2008, 2009; Haluk and Floresco, 2009).

Post-mortem studies on schizophrenic brains have demonstrated a significant loss of glutamic acid decarboxylase-67 (GAD67) expression across multiple cortical areas indicating decreased activity of γ -amino butyric acid (GABA) (Woo et al., 1998; Hashimoto et al., 2003; Ishikawa et al., 2004; Duncan et al., 2010). Furthermore, a marked loss of dopaminergic fibers was described in the dlPFC of post-mortem brains of schizophrenia (Knable and Weinberger, 1997; Akil et al., 1999), and there was a severe loss of dendritic spines within the prefrontal cortex and the hippocampus, indicating possible loss of glutamatergic synapses in schizophrenia (Glantz and Lewis, 2000). Decreased levels of a number of proteins associated with glutamatergic synapses in the thalamus were also identified (Clinton and Meador-Woodruff, 2004). In addition, a significant loss of neurons has been described in the mediodorsal nucleus of the thalamus in schizophrenia brains (Galińska et al., 2009). Overall, available post-mortem studies of

schizophrenia, despite the heterogeneity of the disorder, point to a severe loss of glutamatergic, dopaminergic and GABAergic function in multiple brain areas in schizophrenia. Consistent with the findings, preclinical studies using set-shifting tasks have revealed that the separate administration of relatively high doses of either D1 dopamine, *N*-methyl *D*-aspartate (NMDA) or GABA-A receptor antagonists worsens performance (Stefani and Moghaddam, 2005; Enomoto et al., 2011; Floresco, 2013; Nikiforuk et al., 2016), suggesting that decreased activity of any one of these neurotransmitters could underlie the impaired behavioural flexibility in schizophrenia.

Although the collective results of post-mortem studies on schizophrenia brains and preclinical models suggest that severe deficits in GABA, dopamine or glutamate function may be responsible for the impairments in executive function observed in schizophrenia, the results of *in vivo* imaging studies on schizophrenic patients consistently provide a contradictory view. Studies using *in vivo* magnetic resonance spectroscopy (MRS) or positron emission tomography (PET) have identified that levels of glutamate, dopamine and GABA in the dlPFC and thalamus are not different or slightly altered in schizophrenia patients in comparison to control groups (Théberge et al., 2007; Ohrmann et al., 2007; Galińska et al., 2009; Rowland et al., 2009; Aoyama et al., 2011; Seese et al., 2011; Szulc et al., 2011; Goto et al., 2012; Kegeles et al., 2012; Frankle et al., 2015; Slifstein et al., 2015). Based on the above MRS and PET findings, it is conceivable that the extent of suppression of neurotransmitter function predicted based on post-mortem findings may not apply in patients living with schizophrenia, and if at all, the differences may be subtle, perhaps due to compensatory mechanisms. Furthermore, recent theories postulate that certain GABA abnormalities in schizophrenia might be compensatory (Gonzalez-Burgos et al., 2011; Lewis, 2014). Consequently, we hypothesized that subtle abnormalities in the glutamate and dopamine neurotransmitter systems in functionally-connected cortical and subcortical areas may cooperate synergistically to impair executive function in schizophrenia; an experimental consideration which had not been addressed in previous preclinical rodent models as they only targeted a single neurotransmitter system at a given time.

Among dopamine receptors, D1 receptor subtype is more commonly implicated in executive function (Duncan et al., 2010; Szulc et al., 2011). In addition, *in vivo* imaging studies have identified increased D1 receptor levels in the dlPFC in schizophrenia patients (Ozonoff et al., 2004). Consequently, in the present study, we focused on D1 receptor antagonism. Ultimately, we investigated the potential synergistic effect of the combined administration of “behaviourally sub-effective” doses of D1 and NMDA receptor antagonists on set-shifting in rats performing a lever-pressing task that required them to shift between visual-cue and egocentric spatial response-based discrimination strategies according to the paradigm of Enomoto et al. (2011). To that end, in separate groups of rats, we first determined the doses of the D1 antagonist, SCH 23390, and NMDA uncompetitive antagonist, MK-801, which when systemically administered alone, failed to worsen set-shifting performance compared to vehicle-treated controls. Next, these behaviourally sub-effective doses were co-administered systemically to a separate group of rats, and the results compared to controls as well as rats that received a higher (i.e., “effective”) dose of MK-801 delivered alone.

2.3 Materials and methods

2.3.1 Animals

Adult male Sprague Dawley rats (Charles River, Quebec, Canada) weighing 325-350 g at the beginning of the study were housed individually in an animal facility with temperature and humidity controlled rooms (24±2 °C, relative humidity 55±10%), 12 h light/dark cycle (lights on at 7:00 am). Animals were food restricted to ~85% of their free feeding body weight. During food restriction, rats were weighed and handled for several minutes per day to get familiarized to handling by the investigator. All procedures were approved by the Institutional Animal Care Committee and followed the Canadian and National Institute of Health Guides for the Care and Use of Laboratory Animals (NIH Publication #80-23).

2.3.2 Drugs

The glutamate NMDA receptor antagonist MK-801 [(+)-MK-801 maleate, MWt: 337; (dizocilpine); 0.075 mg/kg or 0.05 mg/kg of the salt form; Sigma-Aldrich, St.

Louise, MO], and dopamine D1 antagonist SCH 23390 [(R)-(+)-SCH 23390 hydrochloride, MWt: 324; 0.005 mg/kg of the salt form; Sigma-Aldrich] were used, with the doses chosen based on pilot studies. Despite a small difference in concentration (0.18 on a Log10 scale), 0.075 mg/kg of MK-801 consistently affected set-shifting compared to 0.05 mg/kg dose in pilot studies. Thus, in the present study, these doses of MK-801 were employed and referred to as “effective” and “behaviourally sub-effective” doses, respectively. Drugs were freshly prepared and dissolved in physiological saline. Rats received subcutaneous injections of drugs individually or in combination on the day of response discrimination (i.e., set-shifting), 25 min prior to the visual-cue retrieval trials. In pilot studies, higher doses of SCH 23390 resulted in gross motor deficits, which rendered animals incapable of performing the visual-cue and set-shifting tasks, and therefore, experiments using these higher doses of SCH 23390 were not included in the present study.

2.3.3 Apparatus

The operant conditioning apparatus (Med-Associates, St. Albans, VT, USA) consisted of a modular acrylic test chamber (30.5 X 24 X 21 cm), housed in a sound-attenuating box. The test chamber was equipped with grid floor, two retractable levers on either side of a central pellet receptacle, and a house light (white, 100-mA, located centrally on the top of the wall opposite to the levers). Positioned above each lever was a cue light (light emitting diode). Following a lever-press that was considered a correct response, a pellet dispenser dropped a sucrose pellet (45 mg; BioServ, Frenchtown, NJ) into the central pellet receptacle. The operation of the chamber was controlled by a customized computer software program (MED-PC IV, Med-Associates).

2.3.4 Set-shifting

Behavioural flexibility was assessed in rats using an operant conditioning-based a set-shifting task developed by Floresco et al. (2008), with minor modifications. As described in detail below, rats were exposed to a series of experimental steps which included acclimatization to the chamber, training to press the levers, determination of the rat's preference for one lever over the other (i.e., its side bias), visual-cue discrimination

ability, and finally, response discrimination ability to assess behavioural flexibility. Each rat completed this entire series of experimental steps only once.

2.3.4.1 Acclimatization

Rats were given 10 sucrose pellets in their home cage a day before their first exposure to the operant chamber. On the acclimatization day, rats were given 3 sucrose pellets in the receptacle as well as 3 crushed pellets were placed on the extended lever. Once the rat learned the relationship between lever-pressing and reinforcement, it had to achieve the criterion of 30 lever presses in 50 min. Once achieved, the second lever was inserted into the chamber, and the first lever was retracted. After achieving the performance criterion for both levers, rats were ready for the training procedure (Please see Figure 2.1 for timeline of experiments).

2.3.4.2 Training

On the training day, a trial began with illumination of the house light, and the random insertion of one of the retracted levers into the chamber. Rats were given 10 s to respond on the extended lever. If the rat pressed the extended lever, a sucrose pellet (reinforcement) was dispensed in the central receptacle, and the house light remained on for an additional 4 s (reward length). If the rat failed to respond within 10 s, the house light was turned off, the lever was retracted without pellet reinforcement, and the trial was counted as an omission. Each training trial lasted 20 s. Over a total of 90 successive trials, each of the levers was randomly inserted into the chamber, and the performance criterion set was less than 5 omissions.

2.3.4.3 Side bias determination

Once the rats achieved the criterion for the training session, their side bias was determined on the same day. A side bias trial began with the illumination of the house light and the insertion of both levers into the chamber. Rats were given 10 s to respond on either of the levers. Upon pressing one of the levers, both levers were then retracted, a pellet reinforcement was delivered, and the house light remained on for an additional 4 s before the chamber went dark. If the rat failed to respond within 10 s, the house light was

turned off, the levers were retracted without pellet reinforcement, and the trial was counted as an omission. The next trial began with the illumination of the house light and the insertion of both levers into the chamber, but this time the rat was given 10 s to respond on the lever opposite to its initial choice. If rat responded correctly, a pellet reinforcement was given. If rat pressed the wrong (i.e., initial) lever, both levers were retracted, reinforcement was not delivered, and the chamber went dark. This continued until rat responded on correct lever. In total, seven complete trials were conducted (i.e., initial choice and correct second choice). For each rat, the lever that was pressed as the initial choice more often was considered its side bias. This preference was acknowledged on the response discrimination day (set-shift day), where the lever opposite to the rat's side bias was always considered the correct lever.

2.3.4.4 Visual-cue discrimination

On the day following training and side bias determination, rats were subjected to a visual-cue discrimination task where they had to follow the cue light as a strategy to receive pellet reinforcement. A trial started with random illumination of one of the cue lights. After 3 s, the house light was turned on, and both the levers were extended into the chamber. The rat was given 10 s to respond on the lever positioned under the illuminated cue light. If the rat chose the correct lever, a pellet reinforcement was offered, both levers were retracted, the house light remained on for 4 s, and the trial was counted as a correct response. Pressing the wrong lever resulted in retraction of both levers, no pellet reinforcement being offered, and the chamber going dark. The performance criterion established for visual-cue discrimination was 10 consecutive correct responses, and the maximum number of trials performed was 100. We modified the original procedure developed by Floresco et al. (2008) by having all rats complete 100 trials instead of ending the session at the moment when a given rat achieved the performance criterion.

2.3.4.5 Response discrimination

On the day after visual-cue discrimination, rats were injected with drugs or vehicle in their home cage, and 25 min later, they were subjected to 20 visual-cue discrimination trials to determine the effect of drug treatments on retrieval of the memory

and motor function to perform the expected task. On the 21st trial, the paradigm was switched from a visual-cue discrimination to a response discrimination, in which the rats had to abandon the original rule (i.e., follow the cue light) and adopt a new rule which was previously irrelevant (i.e., lever side). During set-shifting, rats had to respond on the lever opposite to their side bias during every trial regardless of the location of the visual-cue. A trial started with random illumination of one of the visual-cue lights. After 3 s, the house light turned on, both levers were extended into the chamber, and the rat was given 10 s to respond on the lever opposite to its side bias. If the rat responded on the correct lever, a pellet reinforcement was delivered, both levers were retracted, the house light stayed for an additional 4 s, and the trial was counted as a correct response. Pressing the wrong lever was counted as an error, resulting in retraction of both levers, no pellet reinforcement being offered, and the chamber going dark. The response discrimination session ended when either the rat made 10 consecutive correct responses or if the maximum number of 120 trials was performed. Total number of trials taken and errors committed to achieve criterion were recorded.

2.3.4.6 Error analysis

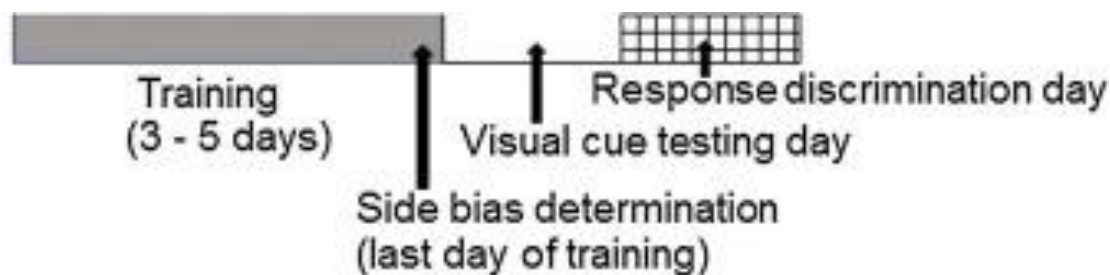
Errors committed during set-shifting (response discrimination) were divided into three different categories according to Floresco et al. (2008): perseverative error, regressive error and never-reinforced error. An error was called “perseverative” or “regressive” if during the set-shifting, the rat responded on the lever associated with visual-cue light when it was required to press the opposite lever to receive the pellet reinforcement. In a block of 16 trials, 8 of the trials required the rat to respond on the lever opposite to the visual-cue light. During those 8 trials, if the rat committed errors by pressing the lever associated with visual-cue light in 6 or more trials, all the errors committed in that block were considered “perseverative” errors. In contrast, if the rat made the same error in 5 or less number of trials, now all of these errors in the particular block were referred to as “regressive” errors. A “never-reinforced error” occurred during a trial when the visual-cue light was associated with the correct lever, yet the rat responded on opposite lever; a situation that had never been positively reinforced in either the visual-cue or response discrimination task.

2.3.4.7 Possible effect of combined drug treatment on learning

A separate experimental series was conducted on naïve rats in order to determine whether the combined treatment of MK-801 (0.05 mg/kg) and SCH 23390 (0.005 mg/kg) affected learning of a given lever-pressing rule, as opposed to actual set-shifting. One day after being trained to press the levers, these rats (n=5 per group) were administered systemically with either vehicle (saline) or the combined treatment of MK-801 (0.05 mg/kg) and SCH 23390 (0.005 mg/kg), and 25 min later, tested on their ability to learn the visual-cue discrimination task. Because this was the first time that these rats had been exposed to the visual-cue discrimination task, this experiment would determine if the combined administration of the behaviourally sub-effective doses of MK-801 and SCH 23390 caused a general inability to learn a lever-pressing rule, separate from the behavioural flexibility necessary to perform set-shifting. These rats did not go on to perform a subsequent response discrimination (i.e., set-shifting) experiment the next day.

2.3.5 Statistical analysis

All animals included in the analysis of response discrimination showed comparable performance in visual-cue discrimination on the previous day to ensure similar baseline function. A one-way analysis of variance (ANOVA) was employed to compare effect of drug treatments on visual-cue retrieval, trials and errors to criteria, and number of omissions. For type of errors and latency, a two-way mixed ANOVA was used. Results of visual-cue learning was analyzed using student's two-tailed t-test. All statistical analyses were conducted with $\alpha = 0.05$ using *GraphPad Prism 7.0* software. Following ANOVA, significant values were further analyzed using the Tukey's post hoc test. All data are presented as mean \pm standard error of mean (SEM).



On Response discrimination day:

Injection/s of drug/vehicle

↓ 25 min

Visual cue discrimination (20 trials)

↓ Immediately after

Response discrimination (120 trials max)

Figure 2.1 Timeline of set-shifting task.

Following handling and acclimatization to the chamber, the rats were subjected to training. Once the rats achieved the criterion for the training session, their side bias was determined on the final day of training. On the next day, rats were subjected to visual-cue discrimination, and the following day was the response discrimination (set-shifting) test. On the response discrimination day, the rats were injected with drugs or vehicle, and 25 min later, they were subjected to 20 visual-cue discrimination trials to determine the effect of drug treatments on retrieval of the memory and motor function. On the 21st trial, the paradigm was switched from a visual-cue discrimination to a response discrimination that lasted until either the rat made 10 consecutive correct responses or to the maximum number of 120 trials.

2.4 Results

2.4.1 Effect of MK-801, SCH 23390 or combined drug treatment on visual-cue retrieval

In order to determine whether administration of MK-801 and/or SCH 23390 produced any noticeable effect on memory retrieval or motor performance, rats were subjected to 20 trials of visual-cue discrimination just prior to the response discrimination (i.e., set-shifting) trials. Results showed that none of the drugs at the chosen doses affected the performance of visual-cue retrieval on the day of response discrimination (Figure 2.2; $P > 0.05$, One-way ANOVA).

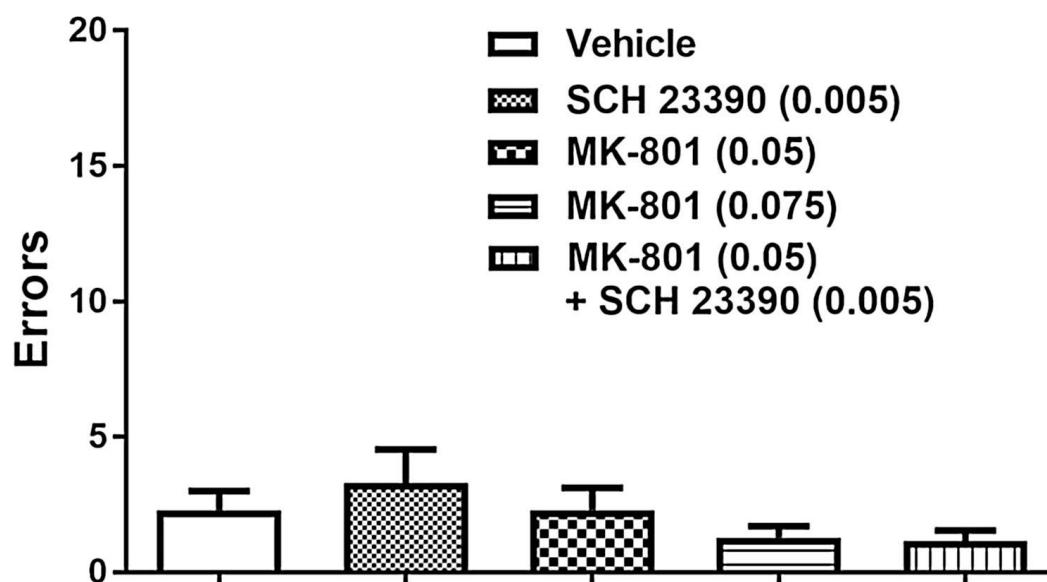


Figure 2.2 Effect of systemic administration of individual or combined antagonists on visual-cue retrieval on set-shift day.

On the set-shift day (response discrimination day), treatment with MK-801 (0.05 or 0.075 mg/kg; s.c.), SCH 23390 (0.005 mg/kg; s.c.) or the combination of MK-801 (0.05 mg/kg; s.c.) and SCH 23390 (0.005 mg/kg; s.c.) did not affect visual-cue retrieval indexed by the unchanged number of errors committed during the first 20 visual-cue trials as compared to the vehicle group ($P > 0.05$, One-way ANOVA).

2.4.2 Effect of MK-801, SCH 23390 or combined drug treatment on set-shifting

2.4.2.1 Number of trials to criterion

Rats were administered with individual antagonists ($n=7$, each group) or a combination of behaviourally sub-effective doses of antagonists ($n=7$) 25 min prior to the visual-cue retrieval trials on the day of the set-shifting test. Injections of MK-801 (0.075 mg/kg; s.c.) significantly increased the total number of trials to criterion [$F(4,30) = 15.80, P < 0.05$] compared to the vehicle-treated group (Figure 2.3; one-way ANOVA followed by the Tukey's post hoc test). Thus, the 0.075 mg/kg dose of MK-801 when administered systemically, significantly impaired set-shifting in rats. On the other hand, MK-801 at the lower dose tested (0.05 mg/kg; s.c.) did not show any change in the number of trials to criterion ($P > 0.05$), and therefore, this dose was considered to be "behaviourally sub-effective." Similarly, SCH 23390 (0.005 mg/kg) did not significantly affect trials to criterion as compared to the vehicle-treated group ($P > 0.05$), hence, this dose was deemed to be behaviourally sub-effective. In pilot studies, rats that received higher doses of SCH 23390 (0.01 or 0.02 mg/kg; s.c.) experienced noticeable motor deficits and increased response latency, which resulted in a significant number of omissions in both the visual-cue retrieval and set-shifting trials, and ultimately a failure to complete the sessions (data not shown).

Administration of a combination of the behaviourally sub-effective doses of MK-801 (0.05 mg/kg; s.c.) and SCH 23390 (0.005 mg/kg; s.c.) significantly increased the number of trials to criterion [$F(4,30) = 15.80, P < 0.05$]. Tukey's post hoc test revealed that the combined treatment increased the number of trials to criterion significantly more than vehicle treatment as well as the separate injections of the behaviourally sub-effective doses of MK-801 or SCH 23390 ($P < 0.05$). Interestingly, the effective (higher) dose MK-801 (0.075 mg/kg; s.c.) group did not differ from the group that received the combined treatment of behaviourally sub-effective doses of MK-801 and SCH 23390 in the number of trials to criterion ($P > 0.05$).

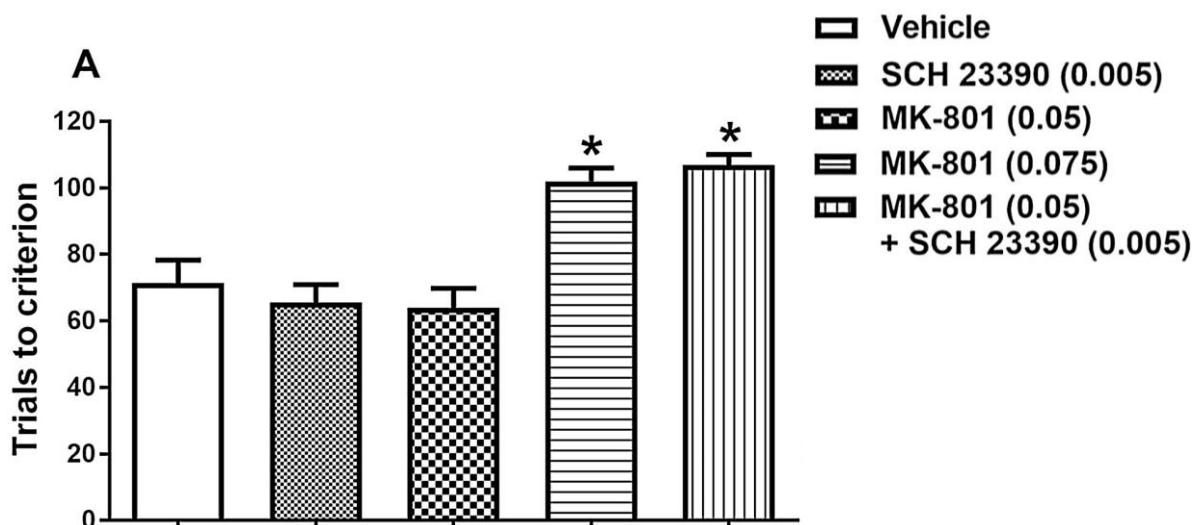


Figure 2.3 Effect of systemic administration of individual or combined antagonists on trials to criterion during set-shifting.

Low dose MK-801 (0.05 mg/kg; s.c.) or SCH 23390 (0.005 mg/kg; s.c.) did not affect the number of trials to criterion as compared to the vehicle group ($P>0.05$), and as such, these doses were considered to be “behaviourally sub-effective.” Alternatively, the higher dose MK-801 (0.075 mg/kg; s.c.) significantly increased the number of trials to criterion during set-shifting as compared to the vehicle, low dose MK-801 (0.05 mg/kg; s.c.), and SCH 23390 (0.005 mg/kg; s.c.) groups ($*P<0.05$). Consistent with our working hypothesis, the combined administration of the behaviourally sub-effective doses of MK-801 (0.05 mg/kg; s.c.) and SCH 23390 (0.005 mg/kg; s.c.) significantly increased the number of trials to criterion as compared to the vehicle group and the individual treatment groups of either low dose MK-801 (0.05 mg/kg; s.c.) or SCH 23390 (0.005 mg/kg; s.c.) ($*P<0.05$). Finally, the combined antagonist treatment group did not show significant change in the number of trials to criterion as compared to the higher dose MK-801 (0.075 mg/kg; s.c.) group ($P>0.05$). [One-way ANOVA followed by Tukey’s post hoc test].

2.4.2.1 Number of errors to criterion

Injections of MK-801 (0.075 mg/kg; s.c.) significantly increased the total number of errors committed during the set-shifting [$F(4,30) = 14.67, P < 0.05$] as compared to the vehicle-treated group (Figure 2.4; one-way ANOVA followed by the Tukey's post hoc test). On the other hand, lower dose of MK-801 (0.05 mg/kg; s.c.) did not affect errors to criterion $P > 0.05$. Likewise, SCH 23390 (0.005 mg/kg) did not significantly influence number of errors committed during the set-shifting as compared to the vehicle-treated group ($P > 0.05$), hence, this dose was considered to be "behaviourally sub-effective".

Combined administration of behaviourally sub-effective doses MK-801 (0.05 mg/kg; s.c.) + SCH 23390 (0.005 mg/kg) significantly increased number of errors committed during set-shifting [$F(4,30) = 14.67, P < 0.05$]. Tukey's post hoc analysis showed a significantly increased ($P < 0.05$) number of errors to criterion following the combined treatment in comparison to vehicle treatment or separate antagonist injections.

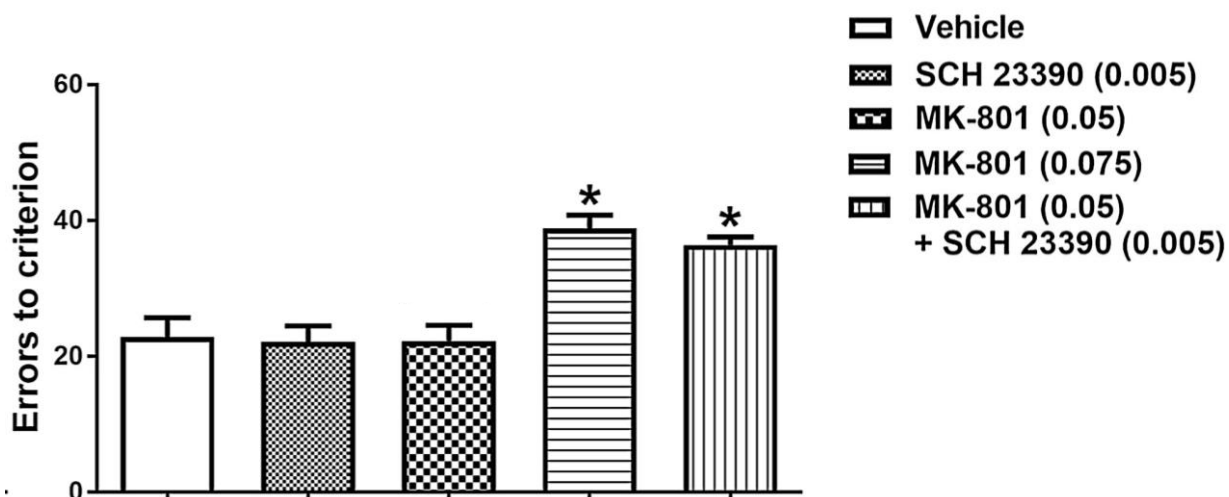


Figure 2.4 Effect of systemic administration of individual or combined antagonists on errors to criterion during set-shifting.

Similar to the trials to criterion, the number of associated errors made during the set-shifting session showed comparable treatment effects. As predicted, the number of errors to criterion was found to be increased significantly following the combined administration of MK-801 (0.05 mg/kg) and SCH 23390 (0.005 mg/kg) as compared to the vehicle group and the individual treatment groups of either MK-801 (0.05 mg/kg) or SCH 23390 (0.005 mg/kg) ($*P < 0.05$). In contrast, the combined antagonist treatment group did not show significant change in the number of errors to criterion as compared to the higher dose MK-801 (0.075 mg/kg) group ($P > 0.05$). Consistent with the combined antagonist treatment group, the higher dose MK-801 (0.075 mg/kg) treatment significantly increased the number of errors committed during the set-shifting session as compared to the vehicle, low dose MK-801 (0.05 mg/kg) or SCH 23390 (0.005 mg/kg) group ($*P < 0.05$). [One-way ANOVA followed by Tukey's post hoc test].

2.4.2.2 Type of errors

In accordance with Floresco et al. (2008), the errors committed during set-shifting trials were classified into three different types: perseverative, regressive and never-reinforced (see Methods for details). Errors were analyzed using two-way mixed ANOVA, as described previously in studies using a similar paradigm (Bornstein et al., 1990; Ishikawa et al., 2004; Szoke et al., 2008). Whenever significant interactions between the treatment conditions and the type of errors were found, the data were further analyzed using Tukey's post hoc test. Significant effects were observed for perseverative errors in the between-subject factor (treatment) [$F(4,24) = 16.94, P < 0.05$] as well as within-subject factor (type of errors) [$F(2,12) = 113.3, P < 0.05$]. Tukey's post hoc test revealed a significant difference ($P < 0.05$) between perseverative errors committed by the group treated with combination of antagonists versus the control group, low dose MK-801 (0.05 mg/kg) or SCH 23390 (0.005 mg/kg). A significant interaction between treatment X type of errors was also observed [$F(8,48) = 2.36, P < 0.05$] (Figure 2.5). The number of perseverative errors committed by the combined treatment group and effective (higher) dose MK-801 (0.075 mg/kg; s.c.) group were not significantly different ($P > 0.05$). Compared to the control group, none of the treatments affected regressive or never-reinforced errors ($P > 0.05$).

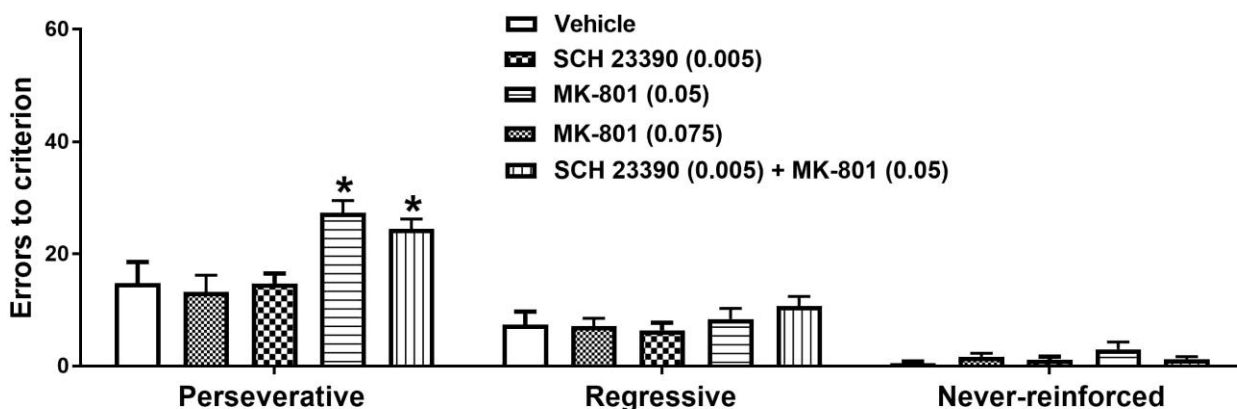


Figure 2.5 Effect of drug treatments on different type of errors committed during set-shifting.

The combined administration of the behaviorally sub-effective doses of MK-801 (0.05 mg/kg) and SCH 23390 (0.005 mg/kg) resulted in a significant increase in the number of perseverative errors as compared to the vehicle group and the individual treatment groups of either MK-801 (0.05 mg/kg) or SCH 23390 (0.005 mg/kg) ($*P < 0.05$). Similarly, the higher dose MK-801 (0.075 mg/kg) treatment significantly increased perseverative errors as compared to the vehicle, low dose MK-801 (0.05 mg/kg) or SCH 23390 (0.005 mg/kg) groups ($*P < 0.05$). Animals that received the combined antagonist treatment did not differ significantly from higher dose MK-801 (0.075 mg/kg) treated rats in any type of error ($P > 0.05$). Finally, none of the treatments significantly increased the number of regressive or never-reinforced errors beyond those made by the vehicle group. All drugs were administered s.c. [two-way mixed ANOVA followed by Tukey's post hoc test].

2.4.3 Response latencies

Because our pilot studies using higher doses of SCH 23390 (0.01 or 0.02 mg/kg; s.c.) revealed noticeable motor deficits that ultimately impaired performance, in the present study we compared the latencies of response on the extended levers for last 5 trials of the different trial types: i) correct response on non-perseverative trials, ii) correct response on trials where a perseverative error was possible, and iii) perseverative error trials. A two-way ANOVA revealed that latencies for all the groups were similar for all three trial types ($P>0.05$) (Figure 2.6). These data suggest that motor function was not affected by the various treatments.

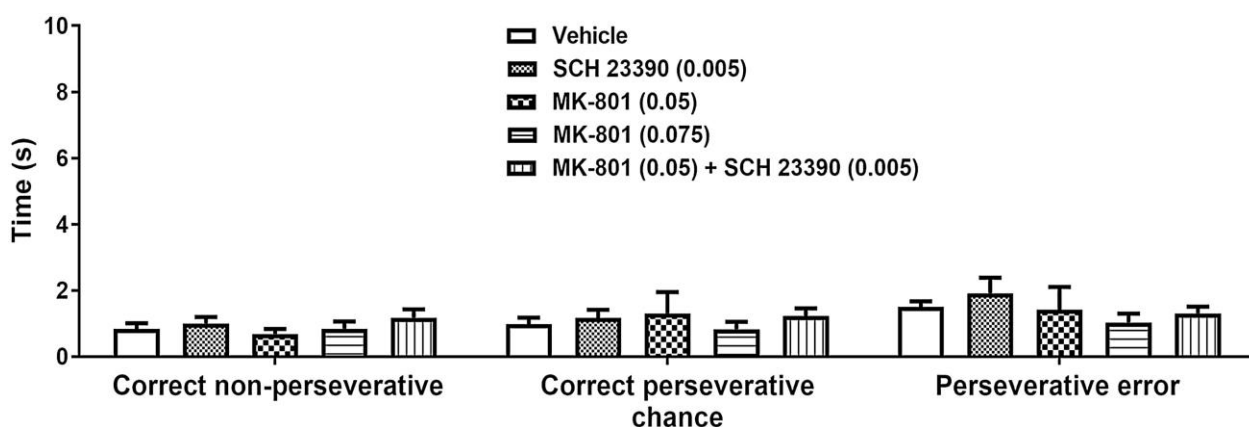


Figure 2.6 Effect of drug treatments on latencies to response during different types of trials.

During each trial, animals were given 10 s to press one of the extended levers. As compared to the vehicle group ($P>0.05$; Two-way ANOVA), none of the treatments showed significant change in latencies to response during the various trial types: i) Correct non-perseverative: A correct choice on a trial where there is no possibility of making a perseverative error; ii) Correct perseverative chance: A correct response on a trial where a perseverative error is possible; and iii) Perseverative error: An incorrect choice on a trial where a perseverative error is possible. All drugs were administered s.c. in mg/kg doses as shown.

2.4.5 Omissions

A one-way ANOVA revealed that number of omissions committed during set-shifting were comparable across all the groups ($P>0.05$) (Figure 2.7). These data suggest that motor function was not affected by the various treatments.

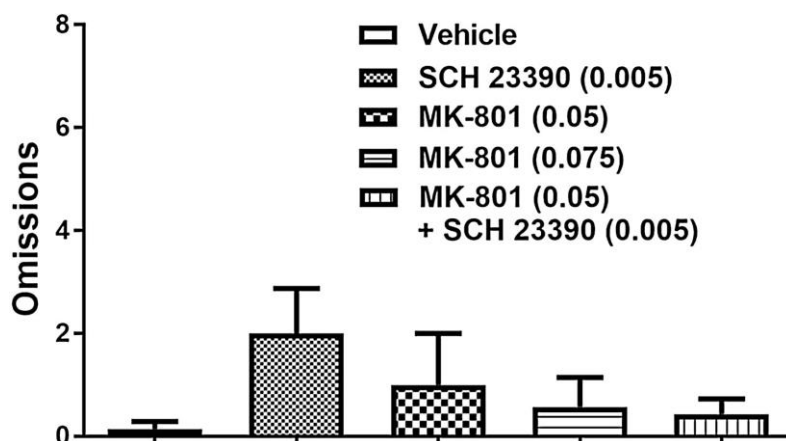


Figure 2.7 Effect of drug treatments on number of omissions committed during set-shifting.

None of the treatments significantly changed the number of omissions committed during set-shifting as compared to the vehicle group ($P>0.05$; One-way ANOVA). All drugs were administered s.c. in mg/kg doses as shown.

2.5 Discussion

The main finding of the present study was that the combined systemic administration of relatively low doses of glutamate NMDA and dopamine D1 receptor antagonists which were found to be ineffective at altering behavioural performance when injected separately caused a significant impairment in set-shifting in rats performing an operant conditioning-based task. To our knowledge, this is the first pharmacological evidence of an interaction between glutamate and dopamine systems in regulating executive function.

Our results also showed that the impairment of set-shifting ability produced by the combination of behaviourally sub-effective doses of NMDA and D1 antagonists is associated with increased perseverative errors, whereas the occurrence of regressive or never-reinforced errors was unaffected. Perseverative errors occur when participants cannot disengage from their adherence to a previously correct rule, despite prompt negative feedback that a new strategy is warranted. Previous preclinical models have shown that inactivation of the mPFC or mediodorsal thalamus results in increased perseverative errors (Block et al., 2007; Floresco et al., 2008; Parnaudeau et al., 2015). On the contrary, regressive errors occur after participants have been able to temporarily disengage from the now-incorrect rule/strategy, yet they are unable to maintain the new strategy despite receiving positive feedback. Unlike perseveration, inactivation of brain structures including the striatum and nucleus accumbens core have been shown to cause increased regressive errors (Block et al., 2007; Floresco et al., 2009). Perseverative errors may result from failure in high-order cognitive flexibility, whereas regressive errors may result from deficits in sustained attention (Sullivan and Faust, 1993; Hughes et al., 1997; Russell et al., 1999; Ozonoff et al., 2004).

Increased perseverative error is a consistent finding in schizophrenia patients performing the WCST (Bornstein et al., 1990; Szoke et al., 2008; Waford and Lewine, 2010), and it was also proposed as an endophenotype as unaffected siblings of schizophrenia patients show high degree of perseverative errors in the WCST (Saoud et al., 2000). Evidence, however, indicates that increased number of perseverative errors in the WCST is also seen in non-psychotic patients diagnosed with bipolar disorder, major

depression or autism (Rempfer et al., 2006; Griebeling et al., 2010; Waford and Lewine, 2010; Landry and Al-Taie, 2016). Our results show that a combined systemic administration of NMDA and D1 receptor blockers, at doses that do not cause behavioural effects when these antagonists were administered separately, results in increased incidence of perseverative errors. Based on the findings in the present study, it is reasonable to suggest that subtle functional decreases in glutamate and dopamine neurotransmission in circuits sub-serving successful performance of the WCST may act cooperatively to manifest perseverative errors in the above-listed neuropsychiatric disorders.

Interestingly, the extent of impairment and error profile seen following systemic administration of behaviourally sub-effective doses of NMDA and D1 antagonists are similar to that seen following administration of the effective (higher) dose of MK-801. This may suggest that the addition of the D1 antagonist may exacerbate the NMDA functional deficit in certain brain circuitries. It is also important to note that although SCH 23390 has been shown to block dopamine D5 and serotonin 5HT_{2A} receptors, the dose of SCH 23390 used in the present study may have blocked D5 receptors but not 5HT_{2A} (Suhara et al., 1992; Emmi et al., 1997). Interactions between NMDA receptors and D1 receptors has been described in several brain areas (Greengard, 2001; Chen et al., 2004; Tong and Gibb, 2008; Wigstrand et al., 2012). Acting on the same postsynaptic profile, dopamine affects the second messenger systems and protein phosphorylation through D1 receptors to facilitate NMDA receptor function (Greengard, 2001). In addition, a direct receptor-receptor interaction also has been observed between NMDA and D1 receptors in the rat (Lee et al., 2002; Martina and Bergeron, 2008). More importantly, a recent study found that SCH 23390 induced a dose-dependent decrease of [³H]MK-801 binding to membranes from rat hippocampus indicating a direct interaction between SCH 23390 and MK-801 (Wigstrand et al., 2012). Considering that increased perseverative errors have been observed following manipulation of either the mPFC, mediodorsal thalamus or hippocampus (Block et al., 2007; Enomoto et al., 2011; Shaw et al., 2012), and the present study used systemic injections of antagonists, it is possible that the interaction between NMDA and D1 receptors indicated by our results may not necessarily occur in the same neuron or even in the same brain area. Further studies are

needed to identify the precise brain regions and neuronal connections where relatively low doses of MK-801 and SCH 23390 exert their actions to cause perseverative errors. It is also possible that these antagonists might have affected different components of executive function to certain degree and these effects when combined become sufficient to affect the set-shifting performance.

Previous preclinical studies have shown that direct infusion of either dopamine D1 or NMDA receptor antagonists into the mPFC in rats resulted in disrupted set-shifting and increased perseverative errors (Stefani et al., 2003; Fletcher et al., 2005; Floresco et al., 2006). Similar to the mPFC, dopamine D1 receptor activity in the core region of the nucleus accumbens has been found to be essential for set-shifting (Floresco et al., 2009; Haluk and Floresco, 2009). Pharmacological or designer receptors exclusively activated by designer drugs (DREADD)-mediated inactivation of mediodorsal thalamic nuclei that send reciprocal glutamatergic projections to the prefrontal cortex resulted in impaired set-shifting and increased occurrence of perseverative errors (Block et al., 2007; Parnaudeau et al., 2015) indicating a potentially important role for glutamatergic activity in the mediodorsal thalamus and its prefrontal cortical connection in executive function. As mentioned, abnormalities in the prefrontal cortex, nucleus accumbens and the mediodorsal thalamus are consistently reported in post-mortem and *in vivo* imaging studies of schizophrenia. Consequently, we elected to use systemic injections to antagonize D1 and/or NMDA receptors. Despite sacrificing brain regional specificity for the site of action of these antagonists, our approach allowed us to assay several brain areas that are not only relevant to executive function but also might have abnormalities in dopamine D1 and NMDA receptor function contributing to executive functional deficits of schizophrenia. Overall, our choice to use systemic injections of the D1 and NMDA receptor antagonists in the present study is consistent with comments made in a review by Floresco et al. (2009), which stated that, "...a more complete understanding of the mechanisms underlying impaired flexibility in schizophrenia may be obtained from the elucidation of dysfunction that occurs in these cortical-subcortical circuits, rather than focusing on disruptions in functioning of the prefrontal cortex or subcortical systems alone".

2.6 Conclusions

Despite the prevailing notion that schizophrenia is associated with severe neurochemical and synaptic disturbances in several brain areas and circuitries based on post-mortem findings, recent *in vivo* neuroimaging studies provide a contradictory view in which the neurotransmitter abnormalities in the brains of patients living with schizophrenia appear to be quite subtle. Consequently, it may be more difficult than anticipated to draw sufficient parallels between these neuroimaging studies and the previous preclinical models of behavioural flexibility which induced significant pharmacological disruption of a given neurotransmitter/receptor system. In considering the potential for subtle disturbances in multiple neurotransmitter systems in schizophrenia, we investigated the consequence of combining acute systemic injections of D1 receptor and NMDA receptor antagonists on behavioural flexibility in normal adult rats as assessed with an operant conditioning based set-shifting task. Our results show that behaviourally sub-effective doses of D1 and NMDA receptor antagonists cooperate synergistically to cause disruption of set-shifting which is characterized by an increase the occurrence of perseverative errors.

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Chapter 3

3 Glutamate and dopamine abnormalities in the medial prefrontal cortex act synergistically to cause executive dysfunction

3.1 Abstract

Impairment of executive function is a core feature of schizophrenia, with patients showing perseverance in tasks requiring behavioural flexibility. Abnormalities in dopaminergic and glutamatergic neurotransmission within the prefrontal cortex have been implicated in schizophrenia, and preclinical models have confirmed the importance of these neurotransmitter systems in behavioural flexibility. The present study investigated whether intra-medial prefrontal cortex (mPFC) co-infusion of dopamine D1 and glutamate N-Methyl-D-aspartic acid (NMDA) receptor antagonists at sub-effective doses affect behavioural flexibility. Male Sprague-Dawley rats received bilateral intra-mPFC infusion of the dopamine D1 antagonist, SCH 23390, and the NMDA uncompetitive antagonist, MK-801, at various doses, either alone or in combination prior to performance of a lever-pressing set-shifting task. Task performance was sensitive to the doses infused into the mPFC; the higher doses of antagonists (0.05 µg/side SCH 23390; 0.25 µg/side MK-801) each impaired set-shifting, whereas the separate infusion of the lower doses (0.025 µg/side SCH 23390; 0.125 µg/side MK-801) was ineffective. As predicted, the co-infusion of these lower doses significantly increased the number of trials needed to complete the task and the number of perseverative errors committed, while not affecting learning or memory retrieval. The synergistic effect of SCH 23390 and MK-801 on set-shifting performance confirmed that behavioural flexibility depends on coincident activation of mPFC dopamine D1 and glutamate NMDA receptors. The collective results support the suggestion that perseverance can manifest from a subtle disruption in both dopaminergic and glutamatergic neurotransmission within the mPFC; findings which are relevant for studies attempting to model schizophrenia pathophysiology.

3.2 Introduction

Executive function, comprising cognitive processes such as working memory, attention and behavioural flexibility, enables us to solve problems, form strategies, and adapt to unexpected conditions to achieve goals (Orellana and Slachevsky, 2013; Rowland et al., 2013). Deficits in executive function are associated with a range of disorders, including Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), and schizophrenia (Elliott, 2003). For example, patients with schizophrenia show difficulties in inhibiting a previously-learned strategy (i.e., they make perseverative errors) during performance of the Wisconsin card sorting test (WCST)-a neuropsychological assessment requiring behavioural flexibility (Bornstein et al., 1990; Braff et al., 1991; Rosse et al., 1991; Abbruzzese et al., 1996; Perry and Braff, 1998; Gooding et al., 1999; Everett et al., 2001; Prentice et al., 2008; Waford and Lewine, 2010; Orellana and Slachevsky, 2013). Based on the findings from functional neuroimaging studies in healthy subjects as well as patients with schizophrenia, it is well established that the dorsolateral prefrontal cortex (dlPFC) contributes to the successful performance of the WCST (Weinberger et al., 1986; Andreasen et al., 1990; Berman et al., 1995; Nagahama et al., 1996; Fletcher et al., 1998; Callicott et al., 2000; Monchi et al., 2001; Passingham and Wise, 2014; Boschini et al., 2017). Similarly, preclinical studies in rodents have identified the role of the medial prefrontal cortex (mPFC) in executive function, as lesioning/inactivating this brain region causes impairments in working memory (Ragozzino et al., 2002b; Yoon et al., 2008; O'Neill et al., 2013; Liu et al., 2014), decision making (Sul et al., 2010; Euston et al., 2012; Lee and Seo, 2016) and set-shifting (i.e., the shifting of attention from one stimulus dimension to another) (Ragozzino et al., 1999, 2002a; Birrell and Brown, 2000; Block et al., 2007; Ragozzino, 2007; Floresco et al., 2008). Consistent with the deficits in set-shifting observed in patients with schizophrenia, altered activity in the mPFC has been shown to cause increased perseverance in rodent models (Birrell and Brown, 2000; Ragozzino, 2002a; Stefani and Moghaddam, 2005; Block et al., 2007; Floresco et al., 2008; Enomoto et al., 2011).

Preclinical models have also confirmed the importance of dopaminergic and glutamatergic neurotransmission within the mPFC in behavioural flexibility, as intra-mPFC infusion of individual D1 and NMDA receptor antagonists can impair performance during set-shifting (Ragozzino et al., 2002a; Stefani et al., 2003; Stefani and Moghaddam, 2005; Gauthier et al., 2014). In addition, we recently reported an increased perseverance in rats following the systemic co-administration of dopamine D1 and NMDA receptor antagonists at doses that were low enough to be ineffective at altering set-shifting when injected separately (Desai et al., 2017). Because these “behaviourally sub-effective” doses were injected systemically, it was not possible to conclude whether this synergistic effect of antagonists on behavioural flexibility was mediated directly through coincident deactivation of dopamine D1 and NMDA receptors within the mPFC. That said, it is reasonable to predict that set-shifting could be impaired due to local mPFC disruption of both the dopaminergic and glutamatergic neurotransmitter systems because dopamine D1 and NMDA receptors are co-localized on pyramidal neurons and interneurons within the PFC, and activation of these D1 receptors potentiates NMDA-mediated calcium responses (Kruse et al., 2009). In further support of our working hypothesis, a previous study on rats found that repetitive co-infusion of low, individually-ineffective doses of dopamine D1 and NMDA receptor antagonists into the mPFC disrupted instrumental learning over a multi-day lever-pressing task (Baldwin et al., 2002).

To determine if behavioural inflexibility requires coincident blockade of dopamine D1 and NMDA receptors within the mPFC, we exposed rats to bilateral intra-mPFC infusion of the dopamine D1 antagonist, SCH 23390, and the NMDA uncompetitive antagonist, MK-801, at various doses, either alone or in combination prior to performance of a set-shifting task. Here, we report for the first time that the combined infusion of these antagonists at doses that failed to impair performance when injected alone ultimately resulted in increased perseverance; findings which support the suggestion that a mild disruption in both dopaminergic and glutamatergic neurotransmission within the mPFC is sufficient to cause significant impairment in behavioural flexibility.

3.3 Materials and methods

3.3.1 Animals

Fifty-six adult male Sprague-Dawley adult rats were used in this study (Charles River, Quebec, Canada). All rats were kept in a facility with temperature- and humidity-controlled rooms (24 ± 2 °C, relative humidity $55\pm 10\%$), where they were maintained on a 12-h light/dark cycle (lights on at 7:00 am). In preparation for behavioural training, rats were food restricted up to 85-87% of their free feeding body weight, and they were handled for approximately five minutes per day to get familiarized to the investigator. During the last couple of days of food restriction, rats were given five sucrose pellets (45 mg; BioServ, Frenchtown, NJ, USA) in their home cage so that they could become familiar with the pellets used as a positive reinforcement in the behavioural tasks.

3.3.2 Drugs

The glutamate NMDA receptor uncompetitive antagonist (+) MK-801 (dizocilpine hydrogen maleate) and dopamine D1 antagonist (R)-(+)-SCH 23390 hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in sterile saline to achieve the following concentrations: MK-801 (0.125 and 0.25 $\mu\text{g}/0.5$ μL) and SCH 23390 (0.025 and 0.05 $\mu\text{g}/0.5$ μL). The doses of both antagonists were based upon their salt form. Fresh solutions were prepared on the day of infusion, and stored in a refrigerator. All infusions were made with solutions at room temperature.

3.3.3 Surgery

Rats were anesthetized with isoflurane (Forene®, Baxter Corporation, Mississauga, ON, Canada), mounted in a stereotaxic frame, and injected with metacam (Boehringer Ingelheim, Burlington, ON, Canada) and an antibiotic (Baytril®, Bayer Inc., Toronto, ON, Canada). For intra-mPFC drug infusion, bilateral guide cannulae (27 G, 2 mm length; RWD Life Science Inc., San Diego, CA, USA) were implanted using stereotaxic coordinates to target the prelimbic cortex (relative to bregma: AP= + 3.24, ML= ± 0.8 , DV= -1.5 mm from skull surface; Paxinos and Watson, 2007). These guide cannulae were secured to the skull with four screws and dental cement. Stainless steel

dummy cannulae were inserted in the guide cannulae to prevent clogging prior to the micro-infusion, which occurred several days later. Rats were allowed at least six days of recovery from surgery before initiation of the food restriction. Over this period, rats were handled daily during various procedures, including general holding, weighing and cleaning of dummy cannulae.

3.3.4 Micro-infusion procedure

Intra-mPFC injections were performed in awake animals using infusion cannulae that extended 2 mm beyond the length of the guide cannulae. On the experimental day, rats received bilateral infusion of either the dopamine D1 receptor antagonist SCH 23390 (0.025 µg or 0.05 µg /0.5 µL/side), the uncompetitive NMDA receptor antagonist MK-801 (0.125 µg or 0.25 µg /0.5 µL/side) or 0.9% sterile saline as vehicle (0.5 µL/side) into the prelimbic region of the mPFC 7 min before the beginning of the set-shifting session (Experiment 1) or the visual-cue learning paradigm (Experiment 2 Figure 3.1). In animals that received the combination of SCH 23390 and MK-801, appropriate doses of these antagonists were infused in a volume of 0.5 µL on each side. Both sides were infused simultaneously using a microinfusion pump and Hamilton syringes connected to the infusion cannula via Teflon tubing. Infusion were made over 5 min (0.1 µL/min), and the infusion cannulae were then left in place for an additional 2 min to allow adequate diffusion of the drug into the surrounding brain tissue.

3.3.5 Behavioural apparatus

Behavioural training and testing were performed in an operant conditioning apparatus, which included a modular acrylic test chamber (30.5 x 24 x 21 cm), housed in a sound-attenuating box. On the front wall of the test chamber were two stimulus lights, each located above a retractable lever that was positioned on either side of a central pellet receptacle. A house light was located on the back wall of the chamber. The chamber was controlled by a customized computer software program (MED-PC IV, Med-Associates). For example, when the rat pressed the lever that was considered a correct response, a dispenser was triggered to release a sucrose pellet into the central pellet receptacle.

3.3.6 Set-shifting task

Set-shifting was conducted as described in the previous chapter (see 2.3.4). One day after performing the visual-cue discrimination task, rats received an intra-mPFC bilateral infusion of one of the drug treatments (0.025 μg or 0.05 μg SCH 23390; 0.125 μg or 0.25 μg MK-801; 0.025 μg SCH 23390 + 0.125 μg MK-801; or 0.9% saline). Seven minutes later, rats were subjected to 20 visual-cue discrimination trials to determine the effect of the various drug treatments on memory retrieval of the initial strategy (i.e., follow the cue light). On the 21st trial, the paradigm was switched to a response discrimination, in which the rats had to set-shift and now respond on the lever opposite to their side bias during every trial regardless of the location of the stimulus light. Again, each trial began with the random illumination of a stimulus light, followed 3 s later by the extension of both levers. Correct lever presses within the 10 s response window resulted in delivery of a sucrose pellet. The response discrimination session ended when either the rat made 10 consecutive correct responses or if the maximum number of 120 trials was performed. Total number of trials to criterion and errors committed to achieve criterion were recorded.

In accordance with previous studies (Floresco et al., 2008; Desai et al., 2017), the errors committed during the response discrimination task (i.e., set-shifting) were subdivided into three categories as perseverative, regressive or never-reinforced errors. An error was considered “perseverative” or “regressive” if during the response discrimination task, the rat pressed the lever associated with the illuminated stimulus light when the opposite lever was correct. In a block of 16 randomized trials, 8 trials required the rat to press the lever opposite to the stimulus light (i.e., the rat was faced with a chance to make a perseverative error). If the rat committed 6 or more such errors out of the 8 chances, these errors in that block were considered “perseverative” errors, whereas 5 or fewer such errors were instead considered “regressive” errors, as the rat was following the initial visual-cue strategy in less than 75% of trials (Floresco et al., 2008). A “never-reinforced error” occurred when the stimulus light was illuminated above the correct lever, yet the rat pressed the opposite lever; a choice that had never resulted in a pellet in either the visual-cue or response discrimination task.

3.3.6.1 Visual-cue learning paradigm

To determine if the intra-mPFC co-infusion of the lower doses of SCH 23390 + MK-801 affected learning of a given lever-pressing rule as opposed to actual set-shifting, we conducted an experimental on a separate group of naïve rats (Figure 3.7). Rats were acclimatized and trained to press the levers using the same protocol as described before (2.3.4). The next day, these rats received an intra-mPFC infusion of either 0.025 µg SCH 23390 + 0.125 µg MK-801 on each side (n=7) or vehicle (saline; n=7), and 7 min later, were tested on their ability to learn the visual-cue discrimination task. These groups of rats were never subjected to the set-shifting experiment.

3.3.7 Histology

Upon completion of the response discrimination task, rats were given an overdose of sodium pentobarbital (Euthanol, 105 mg/kg, IP; MTC Pharmaceuticals, Cambridge, ON, Canada), and then perfused trans-cardially with 0.9% saline followed by a solution containing 4% formaldehyde in phosphate buffer (pH 7.4). Brains were extracted, post-fixed in 4% formaldehyde solution overnight, and then cryoprotected by storing in 30% sucrose at 4 °C for 3 days. Using a microtome, 40 µm coronal sections of mPFC were cut and collected in phosphate buffer (pH 7.4). Sections were mounted on glass slides and Nissl stained to verify cannulae placements (Figure 3.9). Every rat infused showed cannula placement within the prelimbic area bilaterally, and consequently, all are included in the analysis.

3.3.8 Statistical analysis

The effects of intra-mPFC drug infusion on visual-cue retrieval, trials and errors to criterion, and number of omissions were analyzed by separate one-way analyses of variance (ANOVA). Two-way mixed ANOVAs were used to determine the effect of treatments on the type of errors committed, as well as the latency to respond on the levers. In a separate group of rats, a two-tailed student's t-test was used to compare the performance of the visual-cue learning paradigm following infusion of the combined drug treatment (0.025 µg SCH 23390 + 0.125 µg MK-801) versus the saline control. All statistical analyses were conducted with $\alpha = 0.05$ using *GraphPad Prism 7.0* software.

When needed, Tukey's post hoc test was employed to compare the performance measures across the various drug treatment groups. All data are presented as mean \pm standard error of mean (SEM).

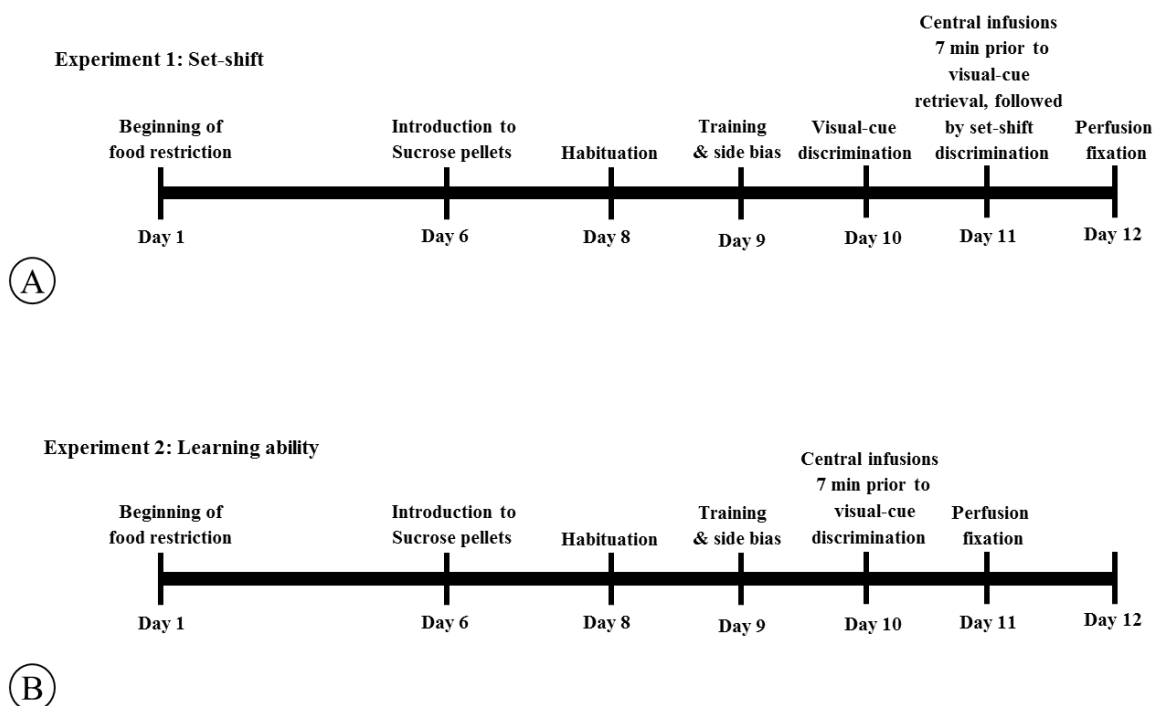


Figure 3.1 Timeline of Experiment 1 and 2.

A) In Experiment 1, following handling and acclimatization to the chamber, the rats were subjected to training. Once the rats achieved the criterion for the training session, their side bias was determined on the final day of training. On the next day, rats were subjected to visual-cue discrimination, and the following day was the response discrimination (set-shifting) test. On the response discrimination day, the rats received bilateral intra-mPFC infusion of drugs or vehicle, and 7 min later, they were subjected to 20 visual-cue discrimination trials to determine the effect of drug treatments on retrieval of the memory and motor function. On the 21st trial, the paradigm was switched from a visual-cue discrimination to a response discrimination that lasted until either the rat made 10 consecutive correct responses or to the maximum number of 120 trials. **B)** Experiment 2 was performed on a separate group of naïve rats that received the bilateral intra-mPFC infusion of drugs or vehicle prior to the initial exposure to the visual-cue discrimination task. Rats employed in Experiment 2 were never subjected to set-shifting trials.

3.4 Results

3.4.1 Effect of SCH 23390, MK-801 or combined antagonist infusion on visual-cue retrieval

On the day set-shifting task was performed, the initial 20 trials after the intra-mPFC infusion required each rat to respond on the lever associated with the stimulus light, thereby allowing for the investigation of the effect of the various drug infusion on memory retrieval of the initial visual-cue discrimination strategy. As predicted based on previous studies (Ragozzino et al., 2002a; c; Stefani et al., 2003; Stefani and Moghaddam, 2005; Gauthier et al., 2014), none of the drug infusion affected memory retrieval (one-way ANOVA, $P > 0.05$; Figure 3.2), as all rats ($n=7$ per group) showed a high level of performance.

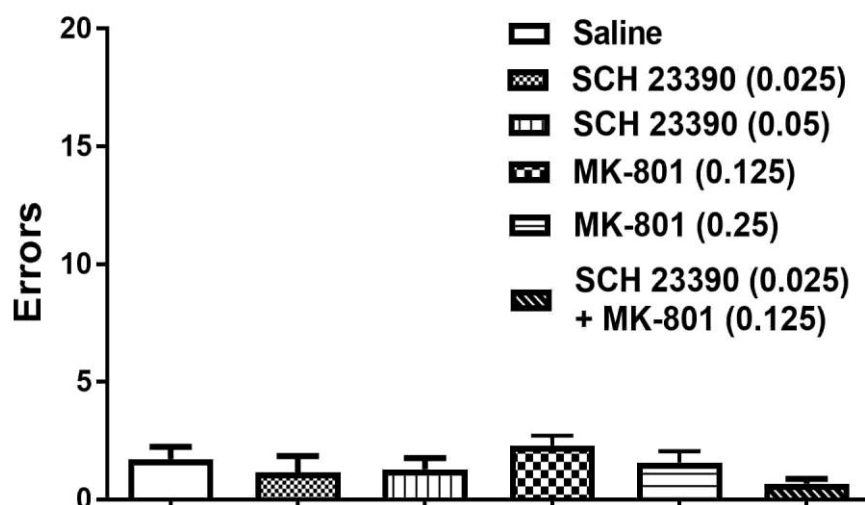


Figure 3.2 Effect of intra-mPFC infusion of SCH 23390 and MK-801 alone or in combination on visual-cue retrieval.

On the set-shift day (during the 20 trials prior to the response discrimination task), intra-mPFC infusion of SCH 23390 and MK-801 alone or in combination did not affect retrieval of the memory from the previous day (visual-cue discrimination task), as the number of errors committed during the 20 visual-cue trials was comparable across groups (one-way ANOVA, $P > 0.05$). $n=7$ per group.

3.4.2 Effect of SCH 23390, MK-801 or combined antagonist infusion on set-shifting

3.4.2.1 Number of trials to criterion

One-way ANOVA found that the various drug doses infused into the mPFC differentially affected the number of trials needed to complete the set-shifting task [$F(5,36) = 11.8, P < 0.0001$; Figure 3.3]. Tukey's post hoc testing further revealed that, compared to vehicle-treated controls ($n=7$), the groups of rats infused with the higher dose of either SCH 23390 ($0.05 \mu\text{g}/\text{side}$; $n=7$) or MK-801 ($0.25 \mu\text{g}/\text{side}$; $n=7$) showed a significant increase in the total number of trials to criterion (Figure 3.3). In contrast, the lower doses of SCH 23390 ($0.025 \mu\text{g}/\text{side}$) or MK-801 ($0.125 \mu\text{g}/\text{side}$) were ineffective at altering set-shifting performance.

As predicted, Tukey's post hoc testing confirmed that the groups of rats that received the co-infusion of the lower, "behaviourally sub-effective" doses of SCH 23390 ($0.025 \mu\text{g}/\text{side}$) and MK-801 ($0.125 \mu\text{g}/\text{side}$) showed an increased number of trials to complete the set-shifting task compared to controls ($P < 0.0001$) as well as the individual, lower dose groups ($0.025 \mu\text{g}/\text{side}$ SCH 23390 alone, $P < 0.01$; $0.125 \mu\text{g}/\text{side}$ MK-801 alone, $P < 0.001$).

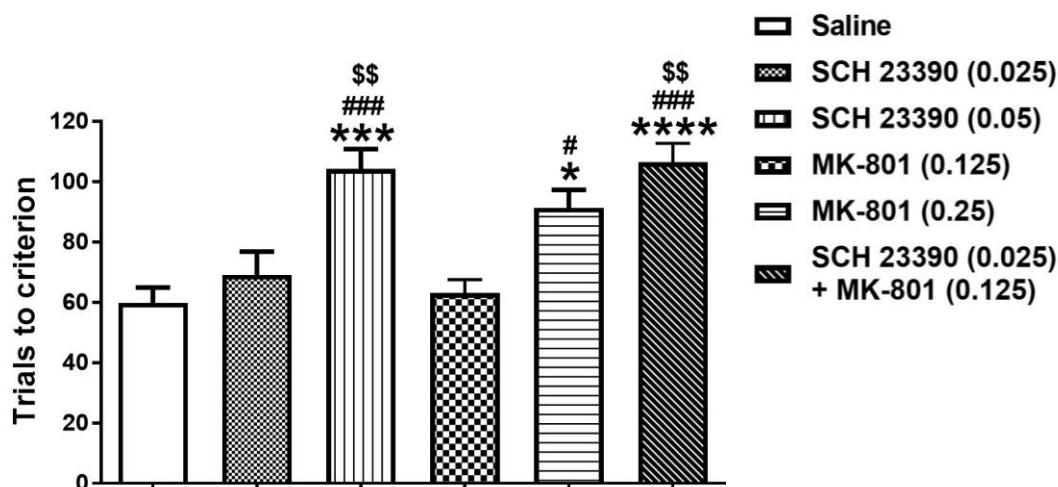


Figure 3.3 Effect of intra-mPFC infusion of SCH 23390 and MK-801 alone or in combination on trials to criterion during set-shifting.

Intra-mPFC infusion of the individual lower doses of SCH 23390 (0.025 $\mu\text{g}/\text{side}$) or MK-801 (0.125 $\mu\text{g}/\text{side}$) did not affect number of trials to criterion ($P>0.05$) as compared to control group. In contrast, the higher dose of SCH 23390 (0.05 $\mu\text{g}/\text{side}$) significantly increased the number of trials to criterion as compared to the control (*** $P<0.001$), lower dose SCH 23390 ($^{\$}P<0.01$) and lower dose MK-801 groups (### $P<0.001$). Similarly, infusion of the higher dose of MK-801 (0.25 $\mu\text{g}/\text{side}$) significantly increased the trials to criterion as compared to the control (* $P<0.05$) and lower dose MK-801 groups (# $P<0.05$). As predicted, the co-infusion of the lower doses of SCH 23390 and MK-801 significantly increased the trials to criterion as compared to the control (**** $P<0.0001$), lower dose SCH 23390 ($^{\$}P<0.01$) or lower dose MK-801 groups (### $P<0.001$). Finally, the number of trials to criterion following the individual higher dose infusion was comparable to the co-infusion group ($P>0.05$). One-way ANOVA followed by Tukey's post hoc tests. $n=7$ per group.

3.4.2.2 Number of errors to criterion

One-way ANOVA revealed effects of different drug treatments on number of errors committed during set-shifting task [$F(5,36) = 14.82, P < 0.0001$; Figure 3.4]. Post hoc analysis revealed that, compared to control group ($n=7$), the groups infused with the higher dose of either SCH 23390 ($0.05 \mu\text{g}/\text{side}; n=7$) or MK-801 ($0.25 \mu\text{g}/\text{side}; n=7$) showed a significant increase in errors to criterion.

Co-infusion of lower doses of SCH 23390 and MK-801 significantly increased number of errors committed as compared to the controls ($P < 0.001$) or separate lower dose infusion of SCH 23390 ($P < 0.001$) or MK-801 ($P < 0.0001$).

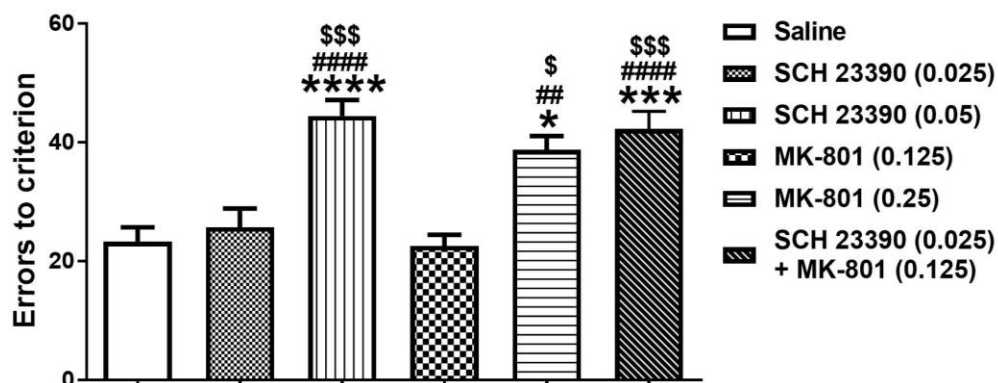


Figure 3.4 Effect of intra-mPFC infusion of SCH 23390 and MK-801 alone or in combination on errors to criterion during set-shifting.

The number of errors committed by either of the groups receiving the lower doses of SCH 23390 (0.025 $\mu\text{g}/\text{side}$) or MK-801 (0.125 $\mu\text{g}/\text{side}$) were comparable to those committed by the control group ($P > 0.05$). Following infusion of the higher dose of SCH 23390 (0.05 $\mu\text{g}/\text{side}$), rats committed significantly more errors as compared to the control (**** $P < 0.0001$), lower dose SCH 23390 (*** $P < 0.001$) and lower dose MK-801 groups (#### $P < 0.0001$). In agreement, intra-mPFC infusion of the higher dose of MK-801 (0.25 $\mu\text{g}/\text{side}$) significantly increased the number of errors committed versus the control (* $P < 0.05$), lower dose SCH 23390 ($P < 0.05$) and lower dose MK-801 groups (## $P < 0.01$). Co-infusion of the lower, individually-ineffective doses of SCH 23390 and MK-801 significantly increased the number of errors made compared to the control (*** $P < 0.001$), lower dose SCH 23390 (*** $P < 0.001$) and lower dose MK-801 groups (#### $P < 0.0001$). Lastly, the number of errors committed by either of the higher dose groups was comparable to those of the co-infusion group. One-way ANOVA followed by Tukey's post hoc tests. $n = 7$ per group.

3.4.2.3 Type of errors

Errors committed during the set-shifting task were divided in three subgroups (perseverative, regressive and never-reinforced errors) and the effect of the different drug infusion on these error types was analyzed using a two-way, mixed ANOVA with “treatment” as the between-subject factor and “error type” as the within-subject factor, as described previously (Darrah et al., 2008; Floresco et al., 2008; Snyder et al., 2015; Desai et al., 2017). In addition to reporting significant main effects for both treatment [$F(5,30) = 15.94, P < 0.0001$] as well as error type [$F(2,12) = 967.7, P < 0.0001$], the two-way, mixed ANOVA found an interaction between the factors [$F(10,60) = 11.19, P < 0.0001$]. Ultimately, Tukey’s post hoc testing revealed a significant increase in perseverative errors committed by the co-infusion group (0.025 $\mu\text{g}/\text{side}$ SCH 23390 + 0.125 $\mu\text{g}/\text{side}$ MK-801) compared to the saline ($P < 0.0001$), lower dose SCH 23390 ($P < 0.0001$) or lower dose MK-801 groups ($P < 0.0001$) (Figure 3.5). The number of regressive and never-reinforced errors did not differ between groups ($P > 0.05$).

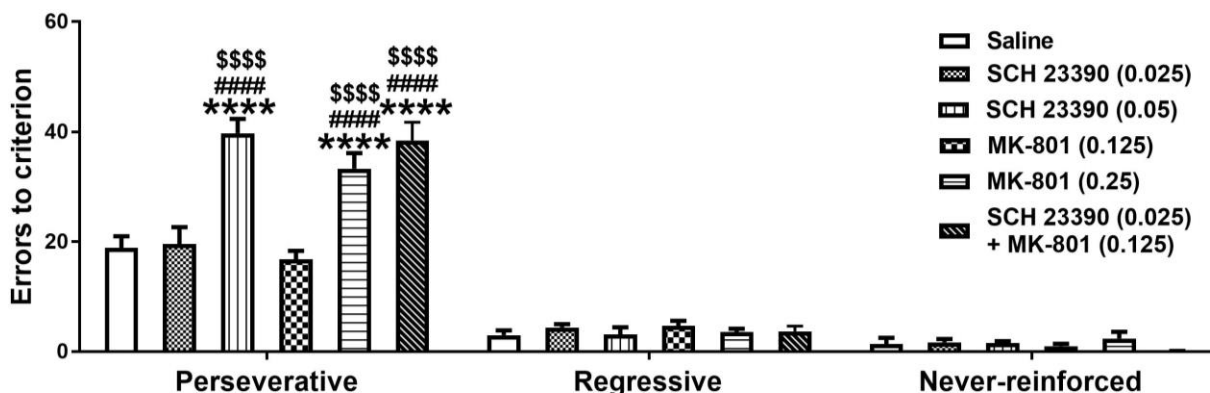


Figure 3.5 Intra-mPFC infusion of SCH 23390 and MK-801 alone or in combination differentially affected the error profile during set-shifting.

Both groups that received the individual higher doses of the antagonists (SCH 23390 0.05 $\mu\text{g}/\text{side}$; MK-801 0.25 $\mu\text{g}/\text{side}$) showed a significant increase in the number of perseverative errors made compared to the control (**** $P < 0.0001$), lower dose SCH 23390 (0.025 μg) (\$\$\$\$ $P < 0.0001$) and lower dose MK-801 groups (0.125 μg) (#### $P < 0.0001$). Similar results were observed for the group co-infused with the lower doses of the SCH 23390 + MK-801. The number of regressive and never-reinforced errors was not changed across groups ($P > 0.05$). Mixed two-way ANOVA followed by Tukey’s post hoc tests. $n = 7$ per group.

3.4.3 Response latencies and omissions

For rats in each of the drug infusion groups, the latency to respond on the extended levers was compared for last five trials of the following trial types: i) correct response on non-perseverative trials, ii) correct response on trials where a perseverative error was possible, and iii) perseverative error trials. Response latencies did not differ between groups or during the various trial types (two-way mixed ANOVA, $P>0.05$; Figure 3.6). These data suggest that motor function was not affected by the various drug infusion.

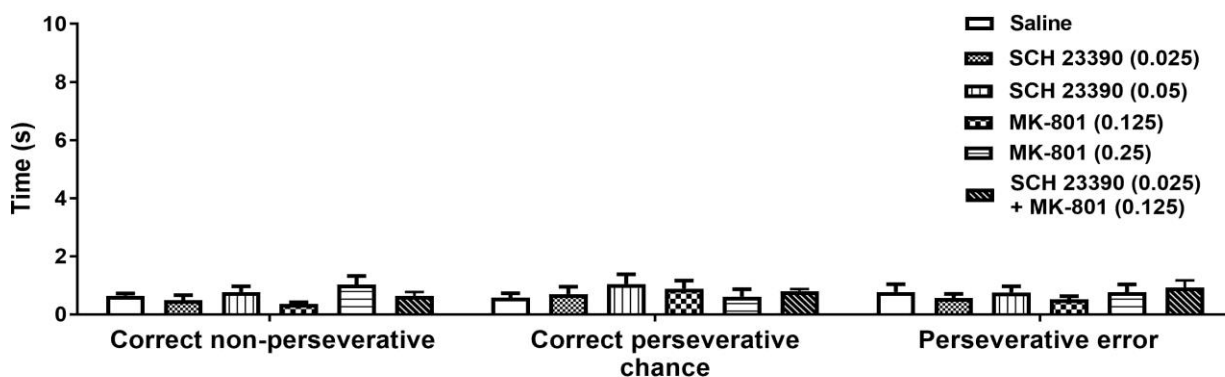


Figure 3.6 Effect of intra-mPFC infusion of antagonists alone or in combination on latencies to respond during different types of trial.

Latencies to respond on the extended lever during the different types of trials were not affected by the treatment groups (two-way ANOVA, $P>0.05$). Different types of trial included: i) Correct/non-perseverative: A trial where the rat chose the correct lever when the illuminated visual-cue light was associated with the correct lever; ii) Correct perseverative chance: A correct response on a trial where a perseverative error was possible; and iii) Perseverative error: An incorrect choice where the rat responded on the lever with the visual-cue light illuminated above it when the opposite lever was correct. $n=7$ per group.

3.4.4 Omissions

The number of omissions made during the set-shifting task was comparable across all the groups (one-way ANOVA, $P > 0.05$; Figure 3.7).

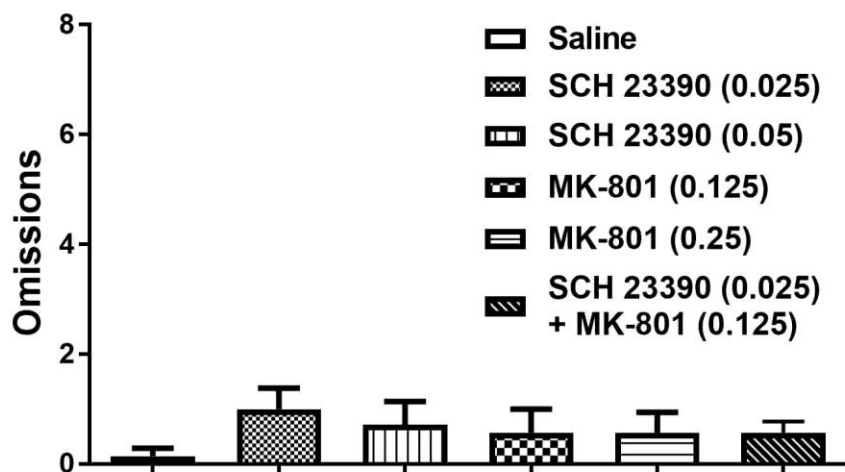


Figure 3.7 Effect of intra-mPFC infusion of antagonists alone or in combination on the number of omissions committed during set-shifting.

None of the treatments significantly changed the number of omissions committed during set-shifting as compared to the vehicle group (One-way ANOVA, $P > 0.05$, $n = 7$ per group).

3.4.5 Effect of co-infusion of receptor antagonists on visual-cue learning

In Experiment 2, the separate group of naïve rats ($n=7$) that received the bilateral intra-mPFC saline infusion prior to the visual-cue learning paradigm were able to complete the task in ~ 30 trials (Figure 3.8). Compared to these controls, the rats ($n=7$) that received the co-infusion of the lower doses of SCH 23390 + MK-801 ($0.025 \mu\text{g}/\text{side}$ SCH 23390 + $0.125 \mu\text{g}/\text{side}$ MK-801) did not differ in the number of trials to criterion [two-tailed student's t -test, $t(12)=0.775$, $P>0.05$]. Thus, the intra-mPFC co-infusion of these “behaviourally sub-effective” doses of antagonists did not affect the rats' ability to learn a new set of rules.

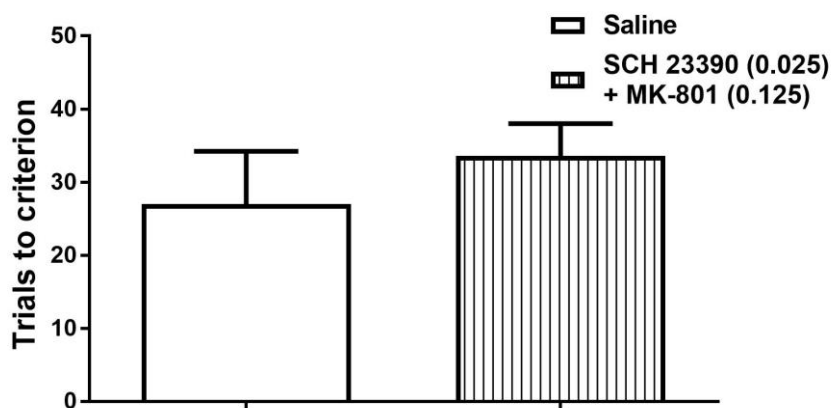


Figure 3.8 Intra-mPFC co-infusion of the lower, individually-ineffective doses of antagonists did not impair learning.

In Experiment 2, in a separate group of rats ($n=7$), prior to performance of the initial visual-cue discrimination task, the lower doses of SCH 23390 ($0.025 \mu\text{g}/\text{side}$) and MK-801 ($0.125 \mu\text{g}/\text{side}$) were co-infused into the mPFC. This combined treatment did not affect visual-cue learning as compared to the control group ($n=7$) that received saline infusion prior to performing the task (student's t -test, $P>0.05$).

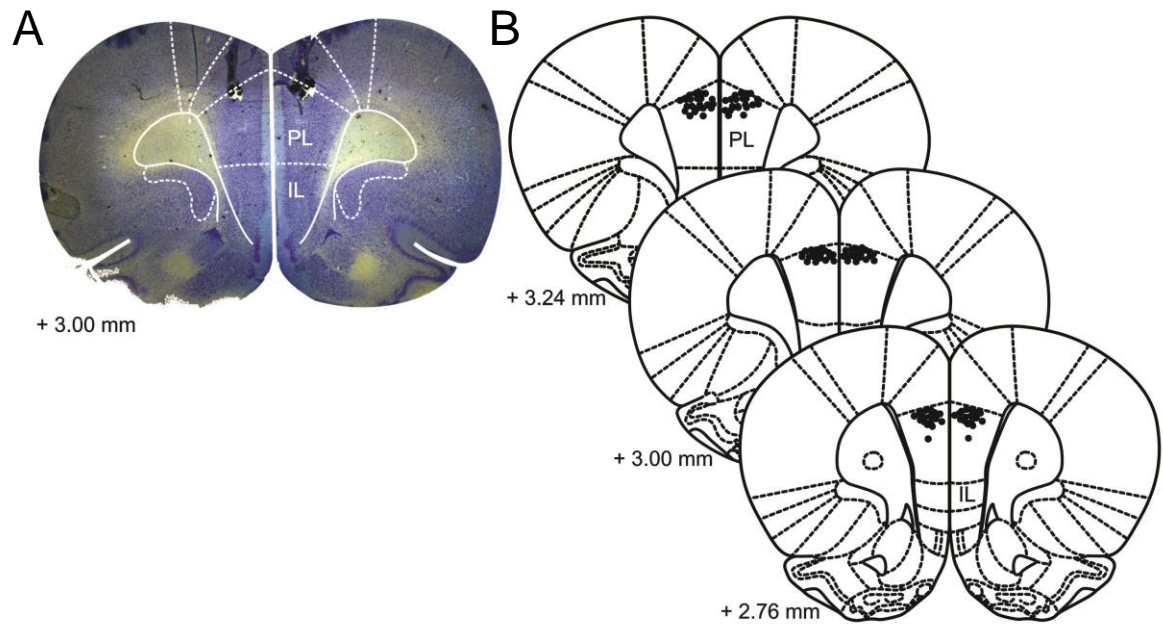


Figure 3.9 Representative histology and reconstruction of infusion cannulae location in mPFC.

A) A photomicrograph showing that the bilateral infusion cannulae targeted the prelimbic (PL) region of the mPFC in a representative animal. **B)** Schematic images of successive coronal sections (adapted from Paxinos and Watson 2007) showing the location of the ends of the infusion cannulae in all animals used in the present study (n=56).

3.5 Discussion

In the present study, we used an operant conditioning-based task in rats to investigate if the combined, intra-mPFC infusion of low, individually-ineffective doses of dopamine D1 and NMDA receptor antagonists would be sufficient to impair set-shifting. Ultimately, in agreement with our working hypothesis, we report for the first time that coincident blockade of dopamine D1 and NMDA receptors in the mPFC impairs behavioural flexibility. Moreover, our collective findings support the suggestion that perseverance can manifest from a seemingly mild but coincidental disruption in both dopaminergic and glutamatergic neurotransmission within the mPFC.

Over the past two decades, a variety of preclinical rodent models have been developed to investigate the brain regions and neurotransmitter systems underlying deficits in set-shifting, with many of these models being based on digging behaviour, maze navigation or conditioned lever-pressing in operant chambers (Birrell and Brown, 2000; Stefani et al., 2003; Ghods-Sharifi et al., 2008; Enomoto et al., 2011; Tait et al., 2014). Largely consistent among these set-shifting tasks, rodents must suppress their actions associated with a previously-learned response, rule or strategy, successfully sample novel strategies and eliminate those that are disadvantageous, and finally, adhere to the newly-effective strategy (Block et al., 2007; Floresco et al., 2008, 2009). Importantly, these overlapping stages of set-shifting appear to have a differential susceptibility to disturbed activity in certain brain regions, as evidenced with distinct error profiles. For example, local damage/inactivation of the mPFC results in an inability to disengage from the initial strategy (i.e., increased perseverative errors) (Ragozzino et al., 1999; Ghods-Sharifi et al., 2008; Enomoto et al., 2011), whereas as the number of never-reinforced or regressive errors were increased following inactivation of the nucleus accumbens (Floresco et al., 2006a) or dorsal striatum (Ragozzino et al., 2002c), respectively.

In addition to lesioning/inactivating the mPFC, it is well established that the number of perseverative errors also increases following local blockade of the dopaminergic (Crofts et al., 2001; Ragozzino, 2002a; Floresco et al., 2006b) or glutamatergic neurotransmitter systems (Stefani et al., 2003; Stefani and Moghaddam,

2005). In agreement with these previous studies, we found that intra-mPFC infusion of the higher doses of either the dopamine D1 receptor antagonist, SCH 23390, or the uncompetitive NMDA receptor antagonist, MK-801, caused impaired set-shifting via increased perseverance. In considering the effect of the separate disturbance of dopaminergic or glutamatergic neurotransmission within the mPFC, it is worth noting that the individual doses used to impair set-shifting in the present study (SCH 23390: 0.05 µg/side; MK-801: 0.25 µg/side) were previously found to be ineffective at altering performance during various working memory tasks (Seamans et al., 1998; Romanides et al., 1999; Rios Valentim et al., 2009; Rodrigues et al., 2011; Auger and Floresco, 2017). Taken together, these findings highlight the seemingly heightened susceptibility of behavioural flexibility to altered neurotransmission within the mPFC.

To investigate our working hypothesis that intra-mPFC synergism between dopamine and glutamate abnormalities cause impaired set-shifting, a group of rats were infused intra-mPFC with a combination of the doses of SCH 23390 and MK-801 that were low enough to be ineffective at impairing set-shifting when infused separately. As predicted, the intra-mPFC co-infusion caused a significant increase in the number of trials and errors to criterion as well as perseverative errors committed. To our knowledge, these results provide the first evidence that behavioural flexibility is sensitive to a mild degree of co-disruption of dopamine D1 and NMDA receptors within the mPFC. In fact, set-shifting appears to be particularly sensitive to disruption in both dopaminergic and glutamatergic neurotransmission as the doses of antagonists used to cause perseverance failed to affect either the acquisition of the visual-cue strategy or memory retrieval. However, in contrast to these null effects, Baldwin et al, (2002) reported that daily co-infusion of low, individually-ineffective doses of dopamine D1 and NMDA receptor antagonists into the mPFC impaired the acquisition of a lever-pressing behaviour over a multi-day instrumental learning task. It is reasonable to suggest that these disparate results on learning could be due to the actual doses used (e.g., SCH 23390: 0.025 µg/side in the present study vs. 0.05 µg/side in Baldwin et al, (2002) as well as differences in the task demands (e.g., unlike in the present study, the first exposure their rats had to lever-pressing occurred following co-infusion of the antagonists). Irrespective of the

experimental differences between studies, the collective findings identify the importance of coincident activation of dopamine D1 and NMDA receptors in the mPFC.

At present, it remains unknown, which cellular mechanisms underlie the impaired set-shifting observed following co-infusion of the dopamine D1 and NMDA receptor antagonists. That said, considerable research has investigated the interactions between D1 and NMDA receptors. There is evidence of co-localization of dopamine D1 receptors and NMDA receptors in the same pyramidal neuron or interneuron in the PFC (Kruse et al., 2009), and numerous in vitro studies have shown a synergism at the receptor level between the D1 and NMDA receptors. For example, NMDA receptors were shown to modulate D1 receptor-mediated functions, as blocking NMDA receptors leads to an attenuation of the ability of the D1 receptor to affect neuronal activity (Huang et al., 1998; Zheng et al., 1999). Reciprocally, activation of D1 receptors is known to upregulate the activity of NMDA receptors (Gurden et al., 2000; Wang and O'Donnell, 2001; Chen and Yang, 2002; Flores-Hernández et al., 2002; Chen et al., 2004; Hallett et al., 2006; Kruse et al., 2009; Sarantis et al., 2009; Varela et al., 2009; Li et al., 2010; Nai et al., 2010). Moreover, uncoupling of the D1-NMDA interaction was found to prevent the D1 receptor-induced upregulation of long-term potentiation (LTP) (Nai et al., 2010).

In addition to the aforementioned D1-NMDA interactions, it has been shown that D1 receptors can modulate NMDA currents through a PKA-dependent intracellular signaling pathway (Flores-Hernández et al., 2002; Cepeda and Levine, 2006). At the level of animal behaviour, Kelley and colleagues reported that the repeated infusion of a selective PKA inhibitor into either the mPFC (Baldwin et al., 2002) or nucleus accumbens (Smith-Roe and Kelley, 2000) of rats attenuated instrumental learning during a multi-day lever-pressing task; findings that were consistent to those following co-infusion of individually-ineffective doses of dopamine D1 and NMDA receptor antagonists. Given that we found an impairment in set-shifting following similar co-infusion of receptor antagonists into the mPFC, it will be important for future studies to determine if the selective inhibition of the PKA-pathway influences behavioural flexibility.

The increased perseverance observed in the present study following the intra-mPFC co-infusion of individually-ineffective doses of dopamine D1 and NMDA antagonists is consistent with the error profile of schizophrenia patients performing the WCST, as patients demonstrate difficulties in inhibiting a previously-learned strategy (Braff et al., 1991; Rosse et al., 1991; Abbruzzese et al., 1996; Perry and Braff, 1998; Gooding et al., 1999; Everett et al., 2001). Based on post-mortem studies of schizophrenia brains, a considerable loss of γ -amino butyric acid (GABA)-ergic, dopaminergic and glutamatergic activity in multiple cortical and subcortical areas has been proposed (Akil et al., 1999; Clinton and Meador-Woodruff, 2004; Lewis et al., 2005; Lewis, 2014; Hu et al., 2015; Poels et al., 2015; Weinstein et al., 2017). For example, within the dlPFC of post-mortem schizophrenia brains, there is a 30% loss of dopaminergic fibers, as well as a severe loss of dendritic spines (Glantz and Lewis, 2000), which suggests a corresponding loss of glutamatergic synapses. On the contrary, a recent study using positron emission tomography (PET) described a significant but small decrease in dopamine levels in the dlPFC in schizophrenia patients compared to controls, and proposed that the loss of dopaminergic activity could be minimal (Slifstein et al., 2015). Ultimately, the results of the present study along with those of Baldwin et al. (2002) offer an important consideration for the future modeling of schizophrenia pathophysiology, as minimal but simultaneous blockade of dopamine D1 and NMDA receptors in the mPFC were found to be sufficient to significantly impair cognitive function.

3.6 References

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Chapter 4

4 Molecular mechanisms associated with D1-NMDA receptor interaction mediated set-shifting in rats

4.1 Abstract

Prefrontal cortical interactions between dopamine and glutamate receptors are known to control cognitive functions. In our previous study, synergistic interaction between dopamine D1 and glutamate N-Methyl-D-aspartate (NMDA) receptor antagonists in the medial prefrontal cortex (mPFC) caused impaired set-shifting in rats. To find out molecular mechanisms underlying D1-NMDA receptor interaction in mPFC, we investigated i) involvement of protein kinase A (PKA) pathway in D1-NMDA receptor interaction mediated set-shifting behaviour ii) role of cortical extracellular regulated kinase (ERK) transduction signaling in set-shifting and iii) role of D1-NMDA receptor protein-protein interaction in set-shifting behaviour. We observed that, elevating mPFC levels of PKA by rolipram ameliorated set-shifting deficits caused by intra-mPFC co-infusion of behaviourally sub-effective doses of D1-NMDA receptor antagonists. Inhibiting ERK phosphorylation by intra-mPFC infusion of mitogen-activated protein kinase (MAPK) inhibitor PD98059 resulted in deficits in set-shifting behaviour. PD98059-induced set-shifting impairments were not ameliorated by rolipram. Blocking protein-protein interaction between D1 and NMDA by Fyn kinase inhibitor- PP2 did not have any effect on set-shifting behaviour. In conclusion, in the mPFC, a strong synergistic interaction between D1 and NMDA receptors exist, which regulates set-shifting behaviour through PKA pathway. Further, for normal set-shifting, activation of ERK cascade is essential in the mPFC; and ERK signaling is downstream to PKA regulating set-shifting. Lastly, protein-protein interaction between D1-NMDA receptors through Fyn kinase does not have any role in set-shifting behaviour.

4.2 Introduction

Executive functions are higher-order cognitive processes comprising behavioural flexibility (set-shifting and reversal learning), working memory and selective attention; and they are necessary for normal daily life activities including planning, problem-solving, learning from the outcomes, changing responses and carrying out goal-directed behaviours (Hughes and Graham, 2002). A number of psychiatric disorders including schizophrenia, Alzheimer's disease, autism and attention deficit hyperactivity disorder (ADHD) show impaired executive functions (Bornstein et al., 1990; Braff et al., 1991; Ozonoff, 1995; Collette et al., 1999; Perry and Hodges, 1999; Duke and Kaszniak, 2000; Elliott, 2003; Hill, 2004; Orellana and Slachevsky, 2013; Reddy et al., 2016). Across species studied, the prefrontal cortex (PFC) plays an important role in mediating executive function (Ragozzino et al., 1999; Monchi et al., 2001; Floresco et al., 2008; Moore et al., 2009; Tsuchida and Fellows, 2013; Déziel et al., 2015; Dalton et al., 2016).

Infusion of dopamine D1, glutamate *N*-Methyl-*D*-aspartate (NMDA) or gamma-Aminobutyric acid (GABA)-A receptor antagonists separately into the medial PFC (mPFC) cause impairment in working memory, set-shifting as well as attention in rodents (Seamans et al., 1998; Granon et al., 2000; Smith-Roe and Kelley, 2000; Ragozzino, 2002; Stefani et al., 2003; Moghaddam and Jackson, 2003; Stefani and Moghaddam, 2005; Paine and Carlezon, 2009; Enomoto et al., 2011; Su et al., 2014; Gauthier et al., 2014; Paine et al., 2015; Auger and Floresco, 2017). We have recently reported impaired set-shifting in normal adult rats following systemic or intra-mPFC administration of D1 receptor antagonist, SCH 23390, and a non-competitive NMDA receptor antagonist, MK-801, in combination at doses that are ineffective when administered separately (Desai et al., 2017; see results, 3.4.2). This suggest a synergistic role for D1 and NMDA receptors in the mPFC in regulating set-shifting. Extensive literature has reported functional interactions between D1 and NMDA receptors playing critical role in reward, attention, locomotor activity, positive reinforcement, latent learning and working memory (Pulvirenti et al., 1991; Smith-Roe and Kelley, 2000; Kreipike and Walker, 2004; Missale et al., 2006; Castner and Williams, 2007; Mouri et al., 2007; Agnoli and Carli, 2011; Bishop et al., 2011; Wang et al., 2012a, 2012b). It is possible that D1 and NMDA

receptors from separate neurons of the mPFC to mediate such interaction via synaptic connections. Nevertheless, D1 receptors and NMDA receptors have been found to be present in same glutamatergic and GABAergic neurons of the mPFC and possibly localized in close proximity to each other (Kruse et al., 2009), and therefore, an intraneuronal D1-NMDA interaction cannot be overlooked.

Synergistic interactions between D1 and NMDA receptors in the PFC and the nucleus accumbens in regulating cognitive processes such as working memory and instrumental learning have been investigated previously (Smith-Roe and Kelley, 2000; Baldwin et al., 2002b; Mouri et al., 2007; Rios Valentim et al., 2009). Baldwin et al. (2002b) reported that combined infusion of sub-effective doses of NMDA and D1 receptor antagonists into the mPFC impaired instrumental learning in rats. In the same study, authors found that inhibiting protein kinase A (PKA) in the mPFC replicated effect of combined blocker treatment suggesting that cyclic AMP (cAMP)-dependent PKA cascade is involved in D1-NMDA interaction associated with the instrumental learning. Furthermore, in mice infusion of a D1 receptor agonist into the PFC attenuated the latent learning impairment caused by phencyclidine (PCP; a NMDA receptor antagonist), as well as decreased levels of learning associated phosphorylation of NR1 subunit of NMDA receptors in the mPFC suggesting a functional link between dopaminergic D1 and glutamatergic NMDA receptor signaling through PKA (Mouri et al., 2007). In addition, NMDA-mediated cytosolic Ca^{++} elevation was enhanced by a D1 receptor agonist and this effect was blocked by PKA inhibitor (Kruse et al., 2009) (Figure 4.1). Moreover, in hippocampal-PFC synapses, NMDA-induced long-term potentiation (LTP) and excitability of pyramidal neurons were increased by D1 agonist or PKA activator while D1 antagonist or PKA blocker attenuated this effect (Gurden et al., 2000; Wang and O'Donnell, 2001). Although from aforementioned studies, it seems that PKA cascade plays a facilitatory role in D1-NMDA interaction and regulates cognitive behaviours and LTP, deleterious effect of PKA activation on working memory has also been reported (Taylor et al., 1999).

In addition to PKA, signaling mediated by the extracellular signal-regulated kinases (ERKs) also appears to play an important role in the synergistic interaction

between D1 and NMDA receptors. Co-infusion of small concentrations of D1 agonist and NMDA receptor agonists increased the levels of phosphorylation of NMDA and AMPA receptor subunits as well as ERK1/2, levels and this effect was blocked by inhibitors of ERK phosphorylation (Sarantis et al., 2009) suggesting that ERKs are downstream molecules involved in D1-NMDA interaction, and in turn ERK may facilitate excitatory neurotransmission by phosphorylating NMDA and AMPA receptor subunits (Figure 4.1). A separate mechanism underlying D1-NMDA receptor interaction has been proposed by Nai et al. (2010) who reported NMDA-D1 direct protein-protein interaction, which facilitated LTP and working memory in rats.

Evidence indicate that interactions between D1 and NMDA receptors also occur through Fyn kinase pathway allowing functional cross-talk and potentiation of both types of receptors independent of PKA mechanism (Lee et al., 2002; Scott et al., 2002, 2006; Fiorentini et al., 2003; Cepeda and Levine, 2006; Li et al., 2010; Nai et al., 2010) (Figure 4.1). Fyn kinase, a Src kinase family member (non-receptor tyrosine kinases), is activated following D1 receptors stimulation via β , γ subunits of Gs protein and in turn phosphorylates NR2B subunit of NMDA receptors at Tyr1472, rendering NMDA receptors to be more effective in Ca^{++} conductance (Nakazawa et al., 2001). Moreover, a recent study identified strong evidence for reduced Src-family kinase activity in the PFC of post-mortem brains of patients with schizophrenia, a condition characterized by impaired executive function, and it has been proposed that Src-family kinases in the PFC might play a central role in cognitive deficits of schizophrenia (Hahn, 2011; Banerjee et al., 2015).

Based on the above evidences, it appears that there is at least three main signaling pathways potentially mediate intraneuronal interaction between D1 and NMDA receptors in the mPFC. These are, i) PKA signaling; ii) ERK phosphorylation cascade and iii) Fyn kinase-mediated augmentation of NMDA function (Figure 4.1). In current study, we sought to investigate the role of PKA, ERK and Fyn kinase in regulating set-shifting.

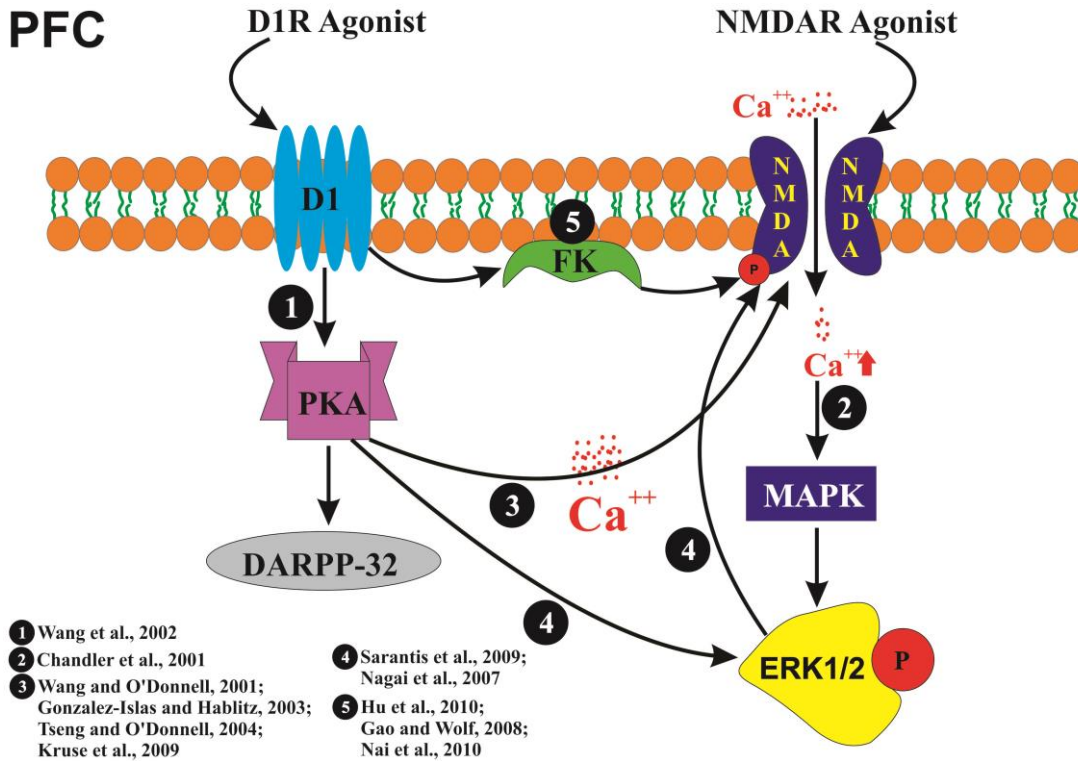


Figure 4.1 Prefrontal cortical D1-NMDA receptor interactions and molecular signalling cascades associated with them.

Stimulation of D1 receptors trigger adenylyl cyclase (AC)/cAMP/PKA cascade (Wang et al., 2002). Although DARPP-32 is present in PFC, it may not be playing a crucial role in D1 stimulation-activated protein kinase A (PKA) signalling cascade in the PFC (Sarantis et al., 2009). NMDA receptor stimulation results in activation of signalling mechanism which ultimately increase phosphorylation of extracellular regulated kinase 1/2 (ERK1/2) through mitogen-activated protein kinase (MAPK) pathway (Chandler et al., 2001). D1-induced increase in PKA levels were found to potentiate NMDA mediated excitability as well as Ca⁺⁺ influx of cortical pyramidal neurons (Wang and O'Donnell, 2001; Gonzalez-Islas and Hablitz, 2003; Tseng and O'Donnell, 2004; Kruse et al., 2009). Further, coincidental activation of D1 and NMDA receptors or stimulation of D1 receptors found to cause increased phosphorylation of NMDA receptor subunits through ERK1/2 pathway (Nagai et al., 2007; Sarantis et al., 2009). Along with PKA and ERK pathways, D1-NMDA receptors interact with each other by direct protein-protein interaction through Fyn kinase-dependent mechanism; and a Fyn kinase inhibitor can disrupt this interaction (Gao and Wolf, 2008; Hu et al., 2010; Nai et al., 2010).

4.3 Materials and methods

4.3.1 Animals

Adult male Sprague-Dawley rats were used in this study (Charles River, Quebec, Canada). All rats were kept in facility with temperature- and humidity-controlled rooms (24 ± 2 °C, relative humidity $55\pm 10\%$). A 12 h light/dark cycle (lights on at 7:00 am) was maintained. In preparation for behavioural training, rats were food restricted up to 85-87% of their free feeding body weight, and they were handled for approximately five minutes per day to get familiarized to the investigator. During the food restriction, rats were given five sucrose pellets for the last five days prior to the start of training (45 mg; BioServ, Frenchtown, NJ) to make them to be familiar with the pellets that are used as a positive reinforcement in the behavioural tasks. All measures were taken to minimize the pain and suffering to animals at all time. Animals showing set-shifting deficit following drug treatments were reused one more time in this study. From our data, in naïve untreated animals, a 5-day washout period following the first run of set-shifting did not alter their performance on second run as the number of trials and errors to criterion as well as different types of errors committed during the first and second set-shifting were comparable (see results, 4.4.1). Our data is in accordance with Wallace et al. (2014). After washout period (5 days), animals to be reused were taken through exact same steps as their first exposure of set-shifting (n=14). All procedures were approved by the Institutional Animal Care Committee and are following the Canadian and National Institute of Health Guides for the Care and Use of Laboratory Animals (NIH Publication #80-23).

4.3.2 Drugs

The glutamate NMDA receptor uncompetitive antagonist (+) MK-801 (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine hydrogen maleate; dizocilpine hydrogen maleate) and selective dopamine D1 receptor antagonist (R)-(+)-SCH 23390 hydrochloride (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) were dissolved in sterile saline to get desired concentrations. Fyn kinase inhibitor, PP2 (4-amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine; a selective inhibitor of Src family kinases with

very high affinity to Fyn kinase), mitogen-activated protein kinase (MAPK) inhibitor, PD98059 (2'-Amino-3'-methoxyflavone; a selective inhibitor of MAP kinase kinase – MEK), and a selective inhibitor of phosphodiesterase 4 (PDE4), rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone) were dissolved in 3 % dimethylsulfoxide (DMSO) to achieve desired concentrations. All the drugs were procured from Sigma-Aldrich (St. Louis, MO, USA). Fresh solutions were prepared on the day of injection, and stored in a refrigerator between injections in successive animals. All infusions were made with solutions at room temperature. Control group of animals received infusion of sterile saline or 3% DMSO in saline as appropriate.

4.3.3 Surgery

Rats were anesthetized with isoflurane (Forene®, Baxter Corporation, Mississauga, ON Canada), mounted in a stereotaxic frame, and injected with Metacam (Boehringer Ingelheim, Burlington, ON, Canada) and an antibiotic (Baytril®, Bayer Inc., Toronto, ON, Canada). For intra-mPFC drug infusion, bilateral guide cannulae (27 G, 2 mm length; RWD Life Science Inc., San Diego, CA, USA) were implanted using stereotaxic coordinates to target the prelimbic cortex (relative to bregma: AP= + 3.24, ML= ± 0.8, DV= -1.5 mm from skull surface; Paxinos and Watson, 2007). These guide cannulae were secured to the skull with four jeweller's screws and dental cement. Stainless steel dummy cannulae were inserted into the guide cannulae to prevent clogging in between the micro-infusion. During surgical recovery period, rats were handled daily at various procedures, including general care, weighing and cleaning of dummy cannulae.

4.3.4 Micro-infusion procedure

Intra-mPFC injections were performed in awake animals using infusion cannulae that extended 2 mm beyond the length of the guide cannulae. On the experimental day, rats received one of the following bilateral infusion into the mPFC: i) co-infusion of rolipram (2.5 µg) with SCH 23390 (0.025 µg) + MK-801 (0.125 µg); ii) rolipram alone (2.5 µg); iii) PD98059 alone (2.67 µg); iv) co-infusion of rolipram (2.5 µg) + PD98059 (2.67 µg); v) PP2 alone (3.0 ng); vi) vehicle (3% DMSO or saline). For co-infusion, drugs solutions were diluted to obtain the appropriate concentrations of individual drugs in 0.5 µL volume per site. Doses of PD98059 and PP2 we chose were commonly used in

in vivo studies where they showed impaired cognitive functions and expected changes in the levels phosphoproteins (Grosshans and Browning, 2001; Bevilaqua et al., 2003; Gerdjikov et al., 2004; Nagai et al., 2007; Liu et al., 2014). In addition, in pilot studies, we have verified efficacy and possible motor effects of rolipram, PD98059 and PP2 that could interfere non-specifically with behavioural testing (2-3 doses per drug; 2-3 rats per group). Both sides were infused simultaneously using a microinfusion pump and Hamilton syringes connected to the infusion cannula via Teflon tubing. Infusions were made over 5 min (0.1 μ L/min), and the infusion cannulae were left in place for an additional 2 min to allow diffusion of the drug into the surrounding brain tissue and to minimize solutions escaping via the cannula tract.

4.3.5 Behavioural apparatus

Behavioural training and testing was performed in an operant conditioning apparatus, which included a modular acrylic test chamber (30.5 x 24 x 21 cm). Front wall of the test chamber had two stimulus lights, each located above a retractable lever that was positioned on either side of a central pellet receptacle. A house light was located on the back wall of the chamber. The chamber was operated through a customized computer software program (MED-PC IV, Med-Associates). Trial by trial data was recorded during all the procedures performed in the box.

4.3.6 Set-shifting task

Behavioural flexibility was assessed using a set-shifting task adapted from Floresco et al. (2008). This task has been described in detail in our recent study (Desai et al., 2017 see 2.3.4). Following series of steps were involved in testing set-shifting: acclimatization and training, side-bias determination, initial set formation (visual-cue discrimination) and set-shifting (response discrimination).

4.3.7 Histology

Following set-shifting, rats were given an overdose of sodium pentobarbital (Euthanol, 105 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, ON, Canada), and intracardially perfused with 0.9% saline followed by a solution containing freshly prepared 4% formaldehyde in phosphate buffer (pH 7.4). Brains were extracted, post-

fixed in the same formaldehyde solution overnight, and then cryoprotected by storing in 30% sucrose in phosphate buffer at 4 °C for 3-4 days. Using a microtome, 40 µm coronal sections through the mPFC were cut and collected in phosphate buffer. Sections were mounted on glass slides and Nissl stained to verify cannulae placements (Figure 4.12).

4.3.8 Statistical analysis

Effect of co-infusion of rolipram with dual combination on visual-cue retrieval, trials and errors to criterion, and number of omissions committed during set-shifting was analyzed by one-way analysis of variance (ANOVA). A two-way mixed ANOVA was used to determine the effect of treatments on the type of errors committed, as well as the latency to respond on the levers. Following ANOVAs, if needed, Tukey's or Bonferroni's post hoc test was employed to compare the performance measures across the groups. A two-tailed student's test was used to assess effect of re-exposure of set-shifting, effect of PD98059 and PP2 on visual-cue retrieval, trials and errors to criterion during set-shifting and number of omissions committed during set-shifting in comparison with respective control groups. Further, student's t-test was also used to assess effect of PD98059 on visual-cue learning. All statistical analyses were conducted with $\alpha = 0.05$ using *GraphPad Prism 7.0* software. All data are presented as mean \pm standard error of mean (SEM).

4.4 Results

4.4.1 Effect of re-testing rats on behavioural pattern during set-shifting.

4.4.1.1 Trials and errors to criterion

Following first set-shifting rats were given washout period of 5 days. Re-exposure to training and set-shifting did not affect number of trials [$t(10)=0.4593$, $P>0.05$] ($n=6$) and errors to criterion [$t(10)=0.3339$, $P>0.05$] ($n=6$; Figure 4.2 A,B; student's t-test).

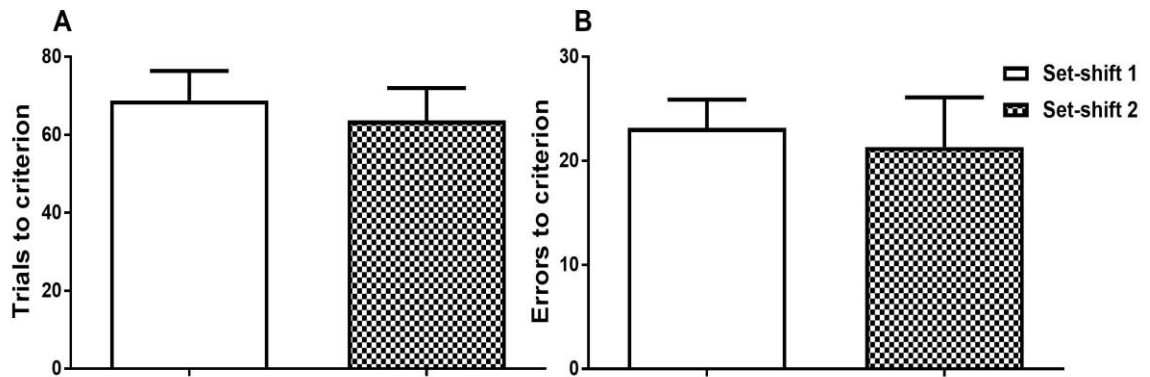


Figure 4.2 Effect of re-exposure to set-shifting on performance.

A) Number of trials to criterion by normal untreated naïve rats during first and the second exposure to set-shifting were comparable ($P>0.05$). Student's t-test; $n=6$ per group. **B)** Number of errors committed by rats performing set-shifting for second time were comparable to the number of errors committed during their performance on the first time ($P>0.05$). Student's t-test; $n=6$ per group.

4.4.1.2 Type of errors

During re-exposure to set-shifting, number of perseverative, regressive and never-reinforced errors were comparable between two runs of set-shifting [$F(1,5) = 0.1018, P=0.7626$] (Figure 4.3; two-way ANOVA).

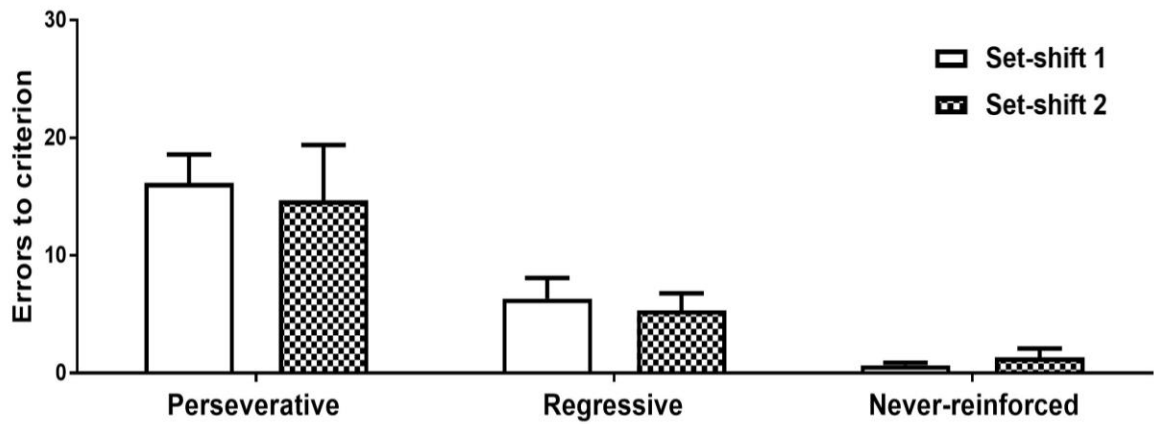


Figure 4.3 Effect of re-exposure to set-shifting on type of errors.

Number of perseverative, regressive and never-reinforced errors committed during both set-shifting sessions were comparable ($P=0.7626$) two-way ANOVA; $n=6$ per group.

4.4.2 Reduced Fyn kinase activity in the mPFC does not affect set-shifting

4.4.2.1 Trials and errors to criterion

Intra-mPFC infusion of PP2 (3 ng/side) in naïve rats did not affect set-shifting. Student's t-test did not show any significant change in the number of trials to criterion [$t(12)=0.2269$, $P>0.05$; $n=7$ each] (Figure 4.4 A) or the number of errors to criterion [$t(12)=0.1572$, $P>0.05$; $n=7$ each] in rats infused with PP2 in comparison to vehicle-infused animals (Figure 4.4 B).

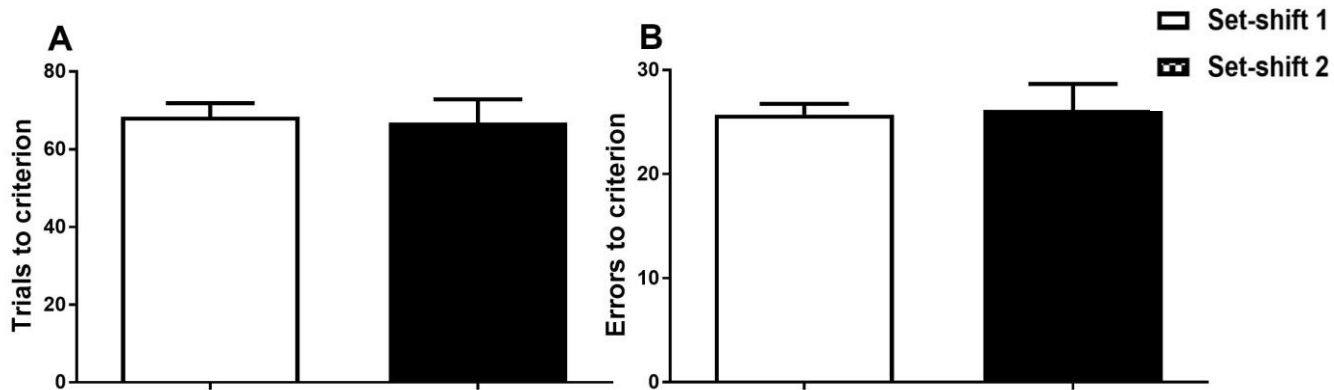


Figure 4.4 Effect of intra-mPFC infusion of PP2 on number of trials and errors to criterion during set-shifting.

A) As compared to vehicle group, intra-mPFC infusion of Fyn kinase inhibitor PP2 (3 ng/side) did not affect trials to criterion during set-shifting ($P>0.05$). Student's t-test; $n=7$ per group. **B)** Intra-mPFC PP2 treatment did not affect errors to criterion as compared to vehicle group ($P>0.05$). Student's t-test; $n=7$ per group.

4.4.2.2 Type of errors

A two-way, mixed ANOVA with “treatment” as the between-subject factor and “error type” as the within-subject factor, was used in the analysis of error profile as described in the literature (Darrach et al., 2008; Floresco et al., 2008; Jones et al., 2014; Snyder et al., 2015; Desai et al., 2017). Results showed that in naïve rats PP2 (3 ng/side) treatment did not show any significant difference compared to control group in committing perseverative errors while performing set-shifting task [$F(1,6) = 0.017, P=0.9003$]. Furthermore, regressive and never-reinforced errors were comparable between PP2 and vehicle groups ($P>0.05$) (Figure 4.5).

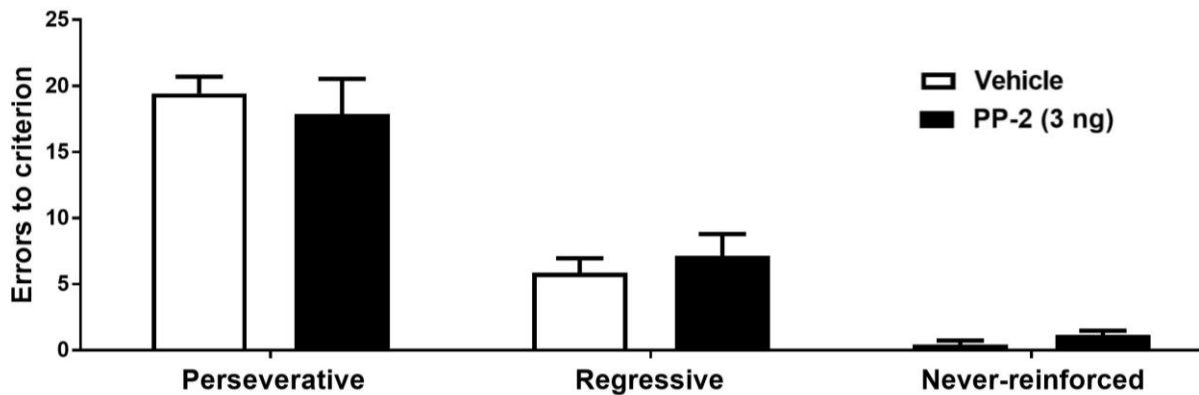


Figure 4.5 Effect of intra-mPFC infusion of PP2 on type of errors during set-shifting.

Number of perseverative, regressive as well as never-reinforced errors was comparable between PP2 and vehicle groups ($P>0.05$). Two-way ANOVA; $n=7$ per group.

4.4.3 Increasing PKA activity in the mPFC rescues co-infusion of D1 and NMDA blockers-induced impairment in set-shifting

4.4.3.1 Trials and errors to criterion

From our previous study, (see results 3.4.2), co-infusion of behaviourally sub-effective doses of D1 receptor and NMDA receptor blockers (0.025 µg/side SCH 23390 + 0.125 µg/side MK-801; referred to as dual combination treatment) bilaterally into the mPFC caused impairment in set-shifting in rats by increasing trials to criterion [$F(3,24) = 13.75, P < 0.0001$] as well as errors to criterion [$F(3,24) = 10.7, P < 0.0001$] (one-way ANOVA followed by Tukey's post hoc test; $n=7$), whereas separate infusion of the same low doses of SCH 23390 or MK-801 into the mPFC bilaterally did not affect set-shifting ($P > 0.05$).

In order to identify the potential signaling cascade downstream to D1 receptors in mediating set-shifting, we augmented the activity of the most prominent D1 receptor signaling pathway, the PKA signaling by co-infusing animals with dual combination treatment and rolipram (2.5 µg/side). The number of trials required to achieve the criterion [$F(3, 24) = 13.75, P < 0.0001$] and the number of errors committed before reaching the criterion [$F(3, 24) = 10.7, P < 0.0001$] were significantly decreased compared to those following the dual combination treatment (Figure 4.6 A,B; one-way ANOVA followed by Tukey's post hoc test). As hypothesized, co-infusion of rolipram with the dual combination treatment significantly ameliorated the deficits. The group that received co-infusion of rolipram and the dual combination treatment showed comparable number of trials as well as number of errors to criterion with vehicle- or rolipram alone-treated groups ($P > 0.05$). Infusion of rolipram alone, however, did not affect set-shifting and the observed trials and errors to criterion were similar to that of vehicle treatment ($P > 0.05$), indicating a lack of pro-cognitive effect of rolipram *per se* in naïve rats at this dose when infused into the mPFC.

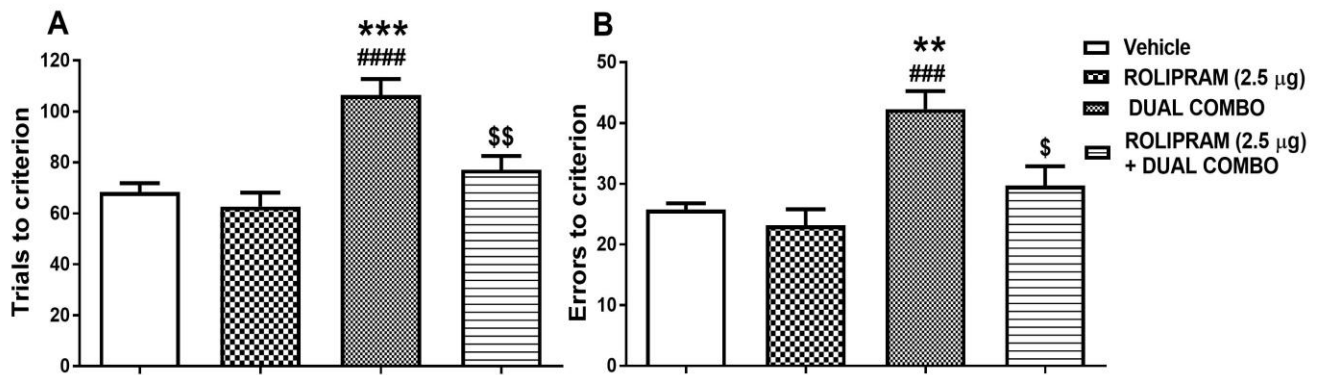


Figure 4.6 Effect of rolipram on dual combination-induced set-shifting impairments.

A) Intra-mPFC infusion of dual combination (0.025 µg/side SCH 23390 + 0.125 µg/side MK-801) significantly increased trials to criterion during set-shifting as compared to vehicle ($***P=0.0002$) and rolipram alone groups ($####P<0.0001$). This effect was attenuated significantly by co-infusion of rolipram (2.5 µg/side) ($$$P<0.01$). Number of trials taken by co-infused group and vehicle group were comparable ($P>0.05$). One-way ANOVA followed by Tukey's post hoc test; $n=7$ per group, for rolipram alone group. **B)** Dual combination treatment (0.025 µg/side SCH 23390 + 0.125 µg/side MK-801) significantly increased number of errors committed during set-shifting as compared to vehicle ($**P<0.01$) and rolipram *per se* ($###P<0.001$) groups. Combining rolipram with dual combination significantly ameliorated this effect ($$P<0.05$). Further, co-infused group and vehicle group committed comparable number of errors during set-shifting ($P>0.05$). One-way ANOVA followed by Tukey's post hoc test; $n=7$ per group.

4.4.3.2 Type of errors

A significant main effects of treatment [$F(3,18) = 7.862, P < 0.001$] as well as error type [$F(2,12) = 1376, P < 0.0001$], and a significant interaction between the factors [$F(6,36) = 10.42, P < 0.0001$] were seen. Post hoc testing revealed a significant increase in perseverative errors committed following the dual combination group as compared to the vehicle and rolipram alone ($P < 0.00001$) (Figure 4.7). Co-infusion of rolipram (2.5 $\mu\text{g}/\text{side}$) with the dual combination treatment significantly decreased perseverative errors compared to dual combination group ($P < 0.00001$). The number of regressive and never-reinforced errors did not change across all groups tested ($P > 0.05$).

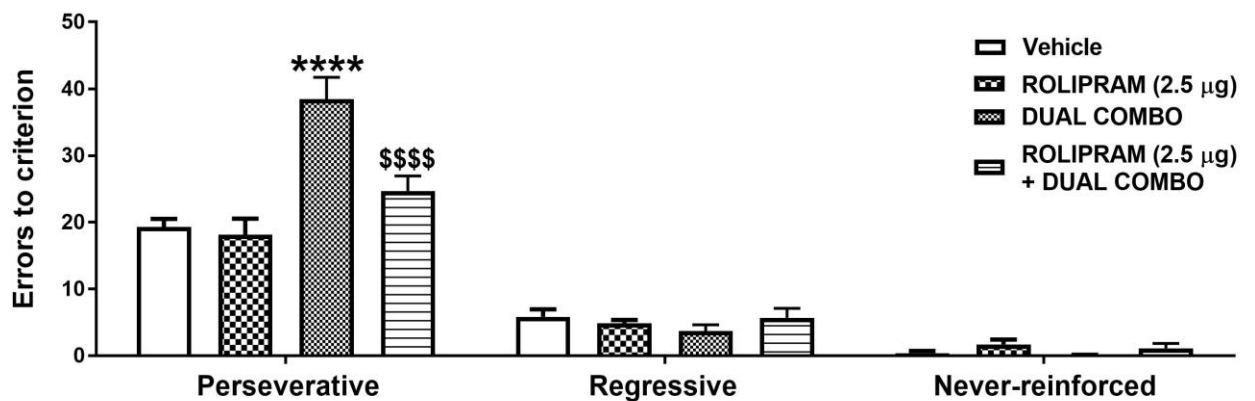


Figure 4.7 Effect of rolipram on dual combination-induced increased perseverance.

Intra-mPFC treatment of dual combination (0.025 $\mu\text{g}/\text{side}$ SCH 23390 + 0.125 $\mu\text{g}/\text{side}$ MK-801) significantly increased number of perseverative errors committed during set-shifting as compared to vehicle and rolipram alone groups (**** $P < 0.0001$). This effect was ameliorated by rolipram co-infusion with dual combination (\$\$\$\$ $P < 0.0001$). Perseverative errors committed by co-infusion group and the vehicle group were comparable ($P > 0.05$). Further, regressive and never-reinforced errors committed by all the groups were comparable ($P > 0.05$). Two-way ANOVA followed by Tukey's post hoc test; $n = 7$ per group.

4.4.4 ERK phosphorylation in mPFC neurons is essential for set-shifting

In order to identify the potential signaling cascade downstream of NMDA receptors in mediating set-shifting, we attempted to inhibit phosphorylation of ERKs, a common downstream signaling event of NMDA-mediated Ca^{++} influx, and an important intermediary transducing NMDA signaling to the nucleus. To this end, groups of naïve animals received intra-mPFC infusion of a MEK inhibitor PD98059 prior to testing.

4.4.4.1 Visual-cue learning and visual-cue retrieval before set-shifting

In different group of naïve animals, intra-mPFC infusion of MEK inhibitor; PD98059 (2.5 $\mu\text{g}/\text{side}$) did not affect visual-cue learning on visual-cue day (initial set formation) as compared to vehicle group [$t(12)=0.3234$, $P>0.05$] ($n=7$; Figure 4.8 A; student's t-test). Further, visual-cue retrieval on set-shift day was not affected by PD98059 treatment [$t(6)=0.6882$, $P>0.05$] ($n=7$; Figure 4.8 B; student's t-test).

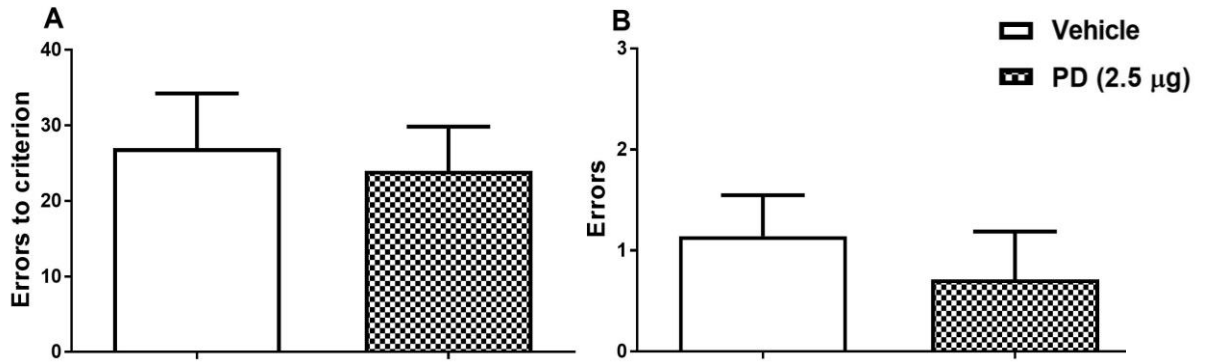


Figure 4.8 Effect of PD98059 treatment on visual-cue learning and visual-cue retrieval before set-shifting.

A) On the visual-cue discrimination day, prior intra-mPFC infusion of PD98059 (2.5 µg/side) did not affect learning of visual-cue discrimination strategy. The number of errors committed during the visual-cue learning was comparable between PD98059 and vehicle groups ($P > 0.05$). Student's t-test; $n = 7$ per group. **B)** On the set-shift day (during the 20 trials prior to the response discrimination task), intra-mPFC infusion of PD98059 (2.5 µg/side) did not affect retrieval of the memory from the previous day (visual-cue discrimination task), as the number of errors committed during the 20 visual-cue trials was comparable between the groups ($P > 0.05$). Student's t-test; $n = 7$ per group.

4.4.4.2 Trials and errors to criterion during set-shifting

Results indicate that bilateral infusion of PD98059 into the mPFC impair set-shifting in naïve rats. PD98059 at dose 2.5 μg per side, significantly impaired animal's ability to shift from one strategy to the other. The number of trials to criterion [$t(12)=6.963$, $P<0.0001$] as well as number of errors to criterion [$t(12)=8.169$, $P<0.0001$] were significantly increased following PD98059 infusion compared or vehicle group ($n=7$, each; student's t-test; figure 4.9 A,B).

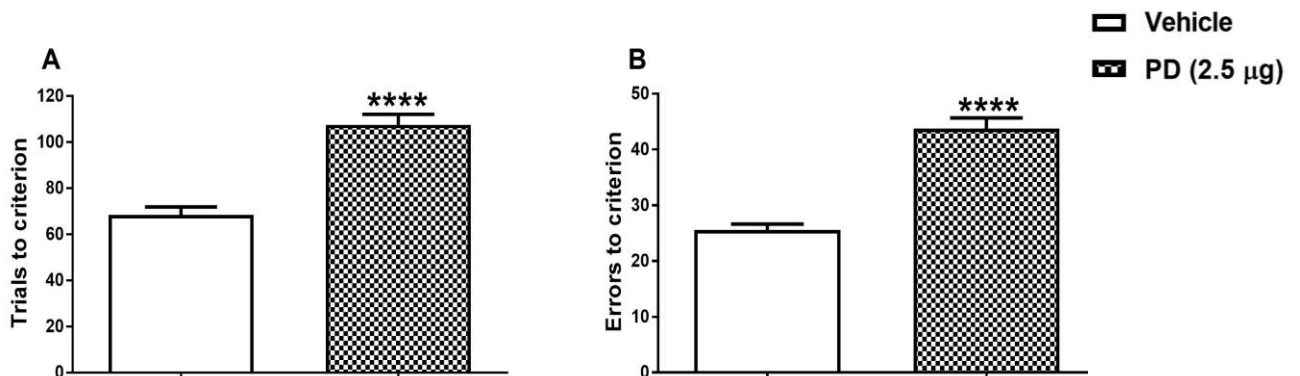


Figure 4.9 Effect of intra-mPFC infusion of PD98059 on trials and errors to criterion during set-shifting.

A) Intra-mPFC infusion of the PD98059 (2.5 $\mu\text{g}/\text{side}$) significantly increased number of trials to criterion (**** $P<0.0001$) as compared to vehicle group. Student's t-test; $n=7$ per group. **B)** Following infusion of PD98059 (2.5 $\mu\text{g}/\text{side}$), rats committed significantly more number of errors as compared to the vehicle group (**** $P<0.0001$). Student's t-test; $n=7$ per group.

4.4.4.3 Type of errors

Two-way ANOVA revealed a significant main effect of treatment [$F(1,6) = 64.95, P=0.0002$] as well as type of errors [$F(2,12) = 58.57, P<0.0001$] following 2.5 μg per side of PD98059. Bonferroni's multiple comparisons test showed significant increase in perseverative errors following PD98059 infusion as compared to vehicle-treated group ($P<0.05$) (Figure 4.10). PD98059 treatment did not affect regressive or never-reinforced errors compared to vehicle group ($P>0.05$).

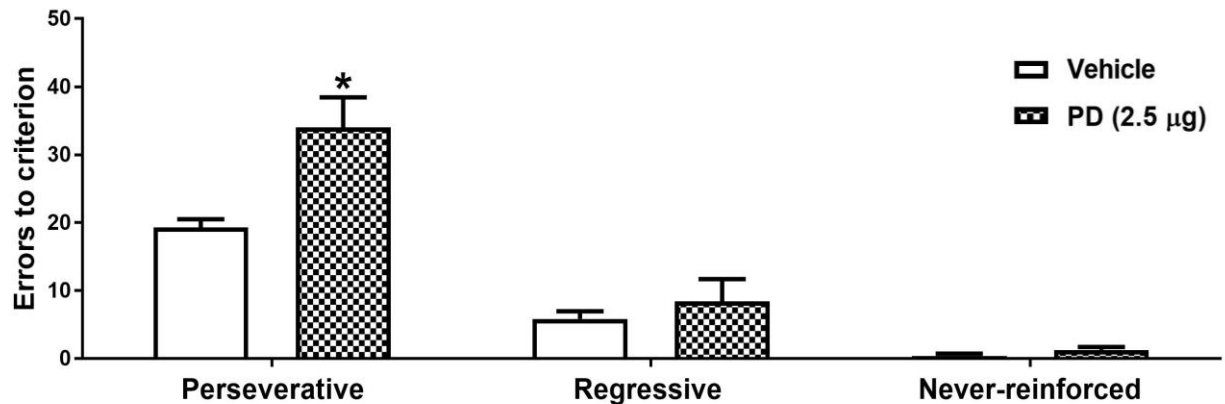


Figure 4.10 Effect of PD98059 treatment on type of errors during set-shifting.

Intra-mPFC infusion of PD98059 (2.5 $\mu\text{g}/\text{side}$) prior to set-shifting increased number of perseverative errors committed significantly as compared to vehicle group ($*P<0.05$). Regressive and never-reinforced errors were remained unaffected by PD98059 ($P>0.05$). Two-way ANOVA followed by Bonferroni's multiple comparison test; $n=7$ per group.

4.4.4.4 Omissions and latency to response

Number of omissions committed by PD98059 treated group was comparable to vehicle group [$t(12)=0.9487$, $P>0.05$] ($n=7$; Figure 4.11 A; student's t-test). Latency to response for different type of trials was comparable between PD98059 and vehicle groups [$F(1,6) = 0.000244$, $P=0.9881$] ($n=7$ Figure 4.11 B; two-way ANOVA).

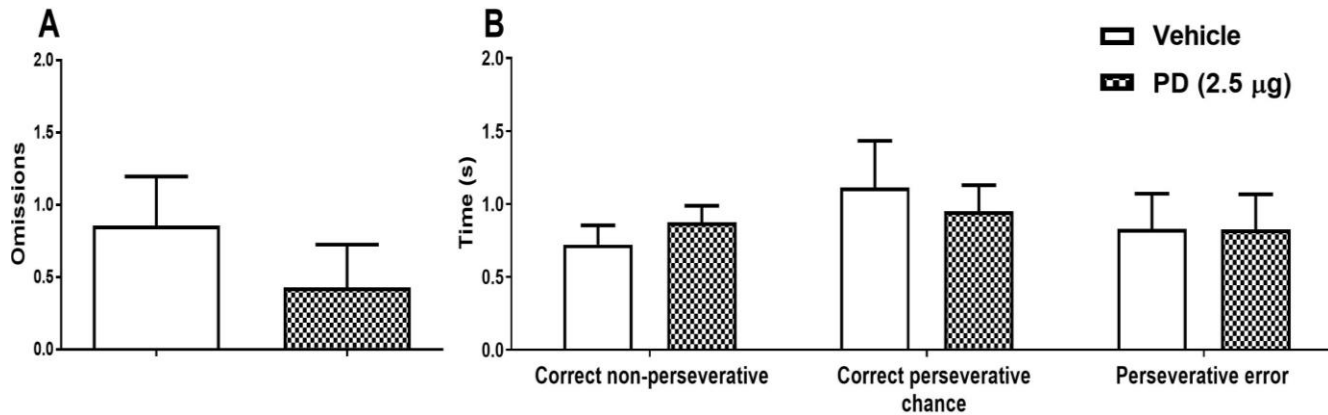


Figure 4.11 Effect of PD98059 treatment on number of omissions and latency to response during set-shifting.

A) Number of omissions committed by PD98059 treated group were comparable to vehicle group ($P>0.05$). Student's t-test; $n=7$ per group. **B)** Latency to response on extended levers during different type of trials was not altered in both PD98059 and vehicle groups ($P>0.05$). Two-way ANOVA; $n=7$ per group.

4.4.5 Augmenting PKA activity does not affect the set-shifting deficits caused by the inhibition of ERK phosphorylation

Our results so far showed that augmenting PKA activity ameliorates set-shifting deficits caused by simultaneous inhibition of D1 and NMDA receptors (i.e., dual combination treatment) and inhibiting ERK phosphorylation impairs set-shifting. PKA activity may affect NMDA receptor-mediated ERK phosphorylation by targeting multiple members along the pathway. The main proposed effect of phosphorylated ERK is on transcriptional regulation. Interestingly, PKA translocate to the nucleus and capable of affecting transcription including phosphorylating nuclear (cAMP response element-binding protein) CREB independent of ERK phosphorylation. To verify whether augmented PKA activity facilitates set-shifting via ERK phosphorylation or by other cascades, we sought to augment PKA activity while inhibiting ERK phosphorylation in animals performing set-shifting task. Rats were infused into the mPFC bilaterally with a combination of rolipram and PD98059.

4.4.5.1 Trials and errors to criterion

Co-infusion of rolipram (2.5 µg/side) and PD98059 (2.5 µg/side) did not cause any improvement in PD98059-induced set-shifting deficit. Rolipram did not attenuate PD98059-induced increased number of trials to criterion [$F(2,18) = 24.08, P < 0.0001$] and number of errors to criterion [$F(2,18) = 22.38, P < 0.0001$] (one-way ANOVA followed by Bonferroni's test). Post hoc analysis showed that compared to the vehicle group, co-infusion of rolipram and PD98059 resulted in significant increase in number of trials to criterion ($P < 0.001$) and number of errors to criterion ($P < 0.001$). Importantly, group that received co-infusion did not show any difference in number of trials or errors to criterion as compared to the group infused with PD98059 alone ($P > 0.05$) (Figure 4.12 A, B; one-way ANOVA).

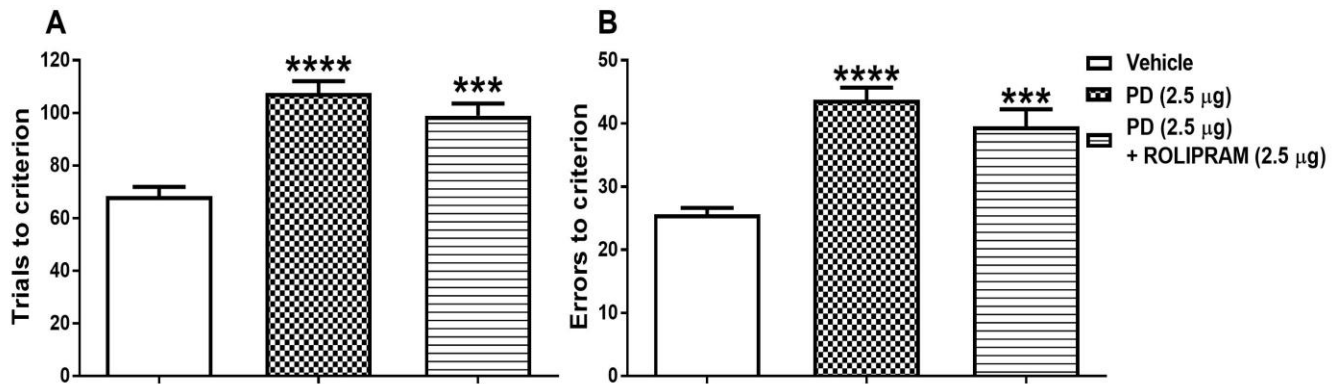


Figure 4.12 Effect of rolipram on PD98059-induced set-shifting impairments.

A) Intra-mPFC infusion of PD98059 (2.5 µg/side) significantly increased number of trials to criterion during set-shifting as compared to vehicle group (**** $P < 0.0001$). Co-infusing PD98059 with rolipram did not alter number of trials to criterion as compared to PD98059 *per se* group ($P > 0.05$). Further, co-infusion group showed significant increase in trials to criterion as compared to vehicle group (*** $P < 0.001$). One-way ANOVA followed by Bonferroni's test; $n = 7$ per group. **B)** Prior treatment with PD98059, animals showed a significant increase in number of errors committed during set-shifting as compared to vehicle group (**** $P < 0.0001$). Combined treatment of rolipram with PD98059 did not decrease number of errors to criterion as compared to PD98059 alone group ($P > 0.05$). As compared to vehicle, combined infusion group showed a significant increase in errors to criterion (*** $P < 0.001$). One-way ANOVA followed by Bonferroni's test; $n = 7$ per group.

4.4.5.2 Type of errors

Co-infusion of rolipram (2.5 µg/side) and PD98059 (2.5 µg/side) did not attenuate PD98059-induced increased perseverative errors. Combined treatment with rolipram and PD98059 showed increased number of perseverative errors [$F(1,6) = 21.89, P=0.0034$] (two-way ANOVA; Figure 4.13). Bonferroni's post hoc analysis showed significant increased perseverative errors following the combined treatment as compared to vehicle group ($P<0.01$). Further, the number of perseverative errors seen following co-infusion of rolipram and PD98059 did not differ from PD98059 alone treatment group ($P>0.05$). There was no change in regressive and never-reinforced errors across groups ($P>0.05$).

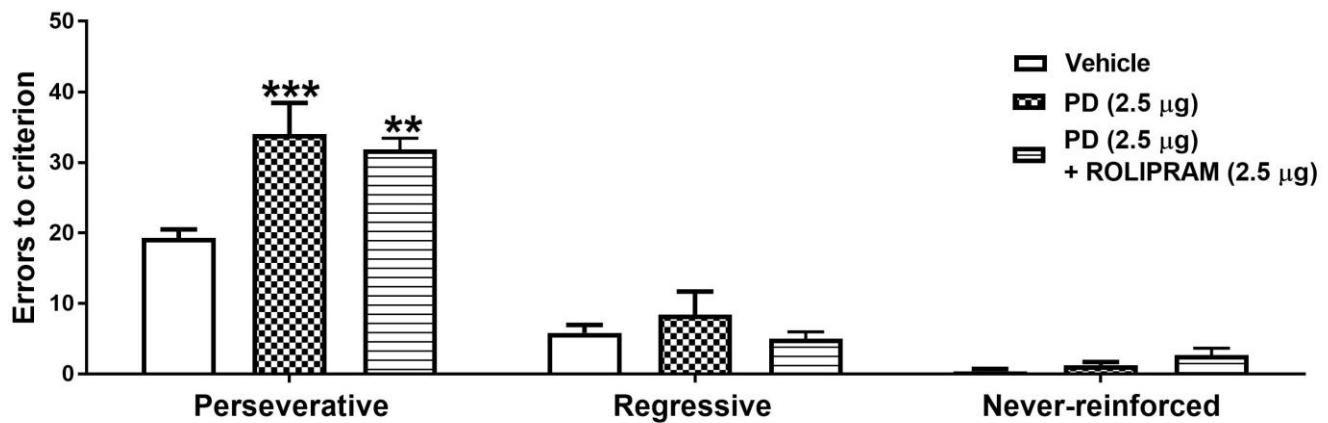


Figure 4.13 Effect of rolipram on PD98059-induced increased perseverance.

PD98059 infusion in mPFC significantly increased number of perseverative errors as compared to vehicle group ($***P<0.001$). Co-infusion of rolipram with PD98059 did not decrease number of perseverative errors committed during set-shifting as compared to PD98059 group ($P>0.05$). In comparison with vehicle group, animals receiving combined infusion showed a significant increase in number of perseverative errors committed during set-shifting ($**P<0.01$). Two-way ANOVA followed by Bonferroni's test; $n=7$ per group.

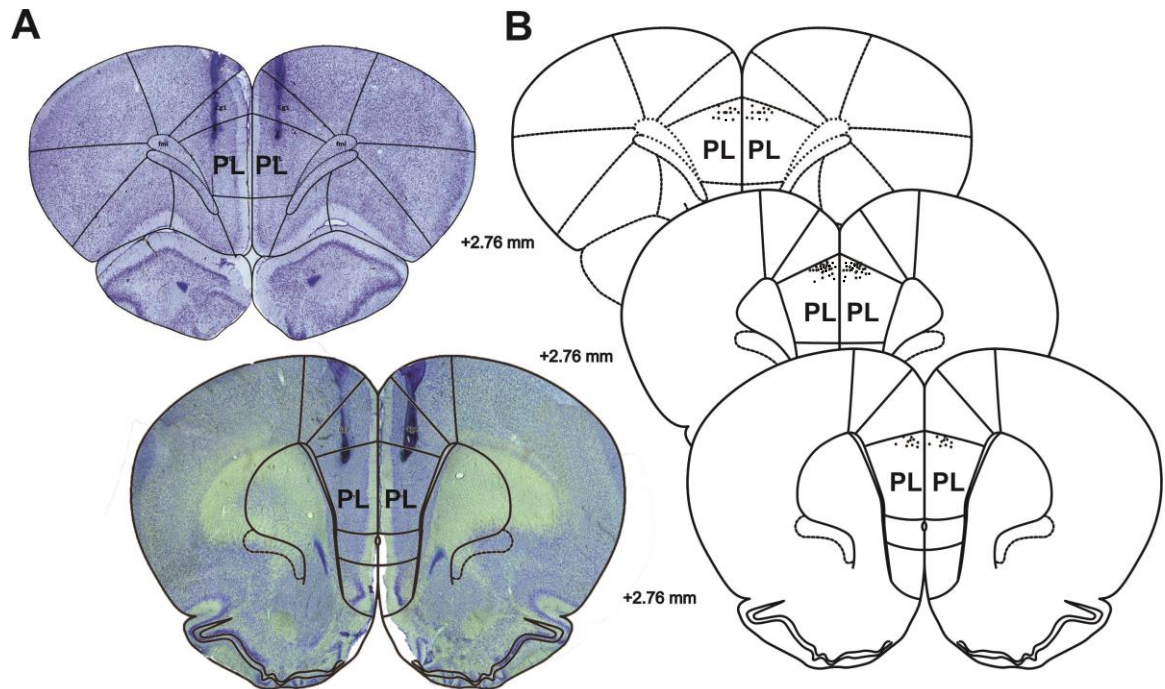


Figure 4.14 Representative histology and reconstruction of infusion cannulae tracks in mPFC.

A) Photomicrographs showing that the bilateral infusion cannulae targeted the prelimbic (PL) region of the mPFC in a representative animal. **B)** Schematic images of successive coronal sections (adapted from Paxinos and Watson, 2007) showing the location of the ends of the infusion cannulae in all animals used in the present study (n=48).

4.5 Discussion

We found that, set-shifting impairments caused by intra-mPFC infusion of dual combination were reversed co-infusion of PDE4 inhibitor- rolipram. Further, inhibiting ERKs phosphorylation by MAPK inhibitor (PD98059) impaired set-shifting, while co-infusion of rolipram failed to ameliorate this impairment. Surprisingly, disrupting D1-NMDA receptor cross-talk by inhibition of Fyn kinase in the mPFC did not affect set-shifting performance.

4.5.1 Effect of PKA elevation on dual combination-induced set-shifting deficits

We have shown that co-infusing D1 and NMDA receptor antagonists into the mPFC impaired set-shifting at doses that when infused separately failed to affect set-shifting performance (see 3.4.2). We found that increasing PKA levels in mPFC by intra-mPFC infusion of PDE4 inhibitor, rolipram, attenuated this dual combination treatment-induced set-sifting impairments by decreasing the number of trials and errors to criterion as well as the number of perseverative errors committed during set-shifting. Several behavioural and electrophysiological studies have shown that PKA signaling cascade may underlie the D1-NMDA receptor interaction in the PFC (Snyder et al., 1998; Aujla and Beninger, 2001; Wang and O'Donnell, 2001; Baldwin et al., 2002a; Flores-Hernández et al., 2002; Tseng and O'Donnell, 2004; Kruse et al., 2009; Li et al., 2010; Cahill et al., 2014). Increasing levels of PKA should, therefore, override the deleterious effects of combined D1 and NMDA receptor antagonism, and our results support this notion.

Rolipram was chosen based on its proven effectiveness for the PFC where the most predominant PDE is of type 4 (Bolger et al., 1994; Suvarna and O'Donnell, 2002; Richter et al., 2013). Our results are in accordance with Rodefer et al. (2012) who showed that rolipram reversed set-shifting impairment caused by sub-chronic phencyclidine (PCP, NMDA receptor antagonist) treatment in rats. In addition, number of behavioural studies have reported effectiveness of rolipram in ameliorating cognitive deficits due to NMDA antagonism (Zhang et al., 2000, 2004, 2005; Davis and Gould, 2005; Rodefer et al., 2005).

We found that intra-mPFC infusion of rolipram alone in naïve rats did not improve their set-shifting performance. In accordance with our results, Rodefer et al. (2005) failed to see improvement in the performance in set-shifting using PDE10 inhibitors *per se*. However, Nikiforuk et al. (2016) reported improvement in set-shifting performance in rats following infusion of a PDE10 inhibitor into the striatum. Our study investigated the role of PFC and used an operant conditioning-based set-shifting task where as Nikiforuk et al. (2016) studies striatal role and used a digging task-based set-shifting, and strategies used by animals may differ between these different type of tasks.

From available preclinical data as well as our findings, it seems that PKA activators (phosphodiesterase inhibitors) improve cognition in animals. Executive functions including working memory, attention and set-shifting were found to be improved (Nikiforuk et al. 2016; Zhang et al., 2000, 2004, 2005; Davis and Gould, 2005; Rodefer et al., 2005). Potential use of rolipram like compounds as cognitive enhancers is being tested in clinical trials by different pharmaceutical companies against cognitive symptoms associated with CNS disorders including schizophrenia and Alzheimer's disease (Houslay et al., 2005; Kanos et al., 2007; García-Osta et al., 2012; Maurice et al., 2014). Effect of inhibiting ERK cascade on set-shifting.

Our results for the first time indicate that inhibiting ERK phosphorylation in the mPFC impaired set-shifting without affecting new learning or retrieval of memory. Intra-mPFC infusion of a MAPK inhibitor, (PD98059; 2.5 µg/side), increased number of trials and errors to criterion while performing set-shifting task and significantly increased perseverative errors.

Stimulation of NMDA receptor induces activation of MAPK signaling cascade leading to phosphorylation of ERKs and this plays an important role in learning (Chandler et al., 2001; Krapivinsky et al., 2003; Shiflett et al., 2010; Shiflett and Balleine, 2011). Although involvement of NMDA receptors in set-shifting is well documented (Stefani et al., 2003; Stefani and Moghaddam, 2005), signaling mechanisms downstream to receptors regulating set-shifting are not clear. Sarantis et al. (2009) has reported that synergism between D1 and NMDA receptors in the PFC and hippocampus is ERK-dependent. Recognizing a potential role of D1 and NMDA receptor synergism in regulating set-shifting, we propose that the ERK signaling was disrupted following co-

infusion of D1 and NMDA receptor antagonists in the mPFC causing set-shifting deficits in rats (Figure 4.15). Effect of PKA activation on set-shifting deficit caused by inhibition of ERK signaling

Our results showed that augmenting PKA levels in the mPFC ameliorates set-shifting deficits caused by the combined blockade of D1 and NMDA receptors, suggesting an important role for PKA in D1-NMDA receptor synergism in mediating set-shifting. We also found that ERK phosphorylation, a downstream effect of NMDA stimulation in the PFC, is critical for set-shifting. The most widely studied effect of ERK phosphorylation is to affect transcriptional regulation. Interestingly, several transcription factors, such as CREB, can be activated by phosphorylated ERKs as well as by activated PKA trafficking into the nucleus. Consequently, we tested whether augmenting PKA would compensate for impaired ERK phosphorylation in relevance to set-shifting. To this end, we investigated effect of mPFC infusion of rolipram (2.5 $\mu\text{g}/\text{side}$), the regimen ameliorated the deficits following dual combination treatment, in presence of PD98059 (2.5 $\mu\text{g}/\text{side}$) on set-shifting. Surprisingly, we did not see any effect of rolipram on PD98059-induced set-shifting impairment. Therefore, it is likely that increased PKA cannot compensate for the impaired ERK function, and ERK is acting downstream of PKA.

Zhang et al. (2004) reported that PKA elevation mediated by PDE4 inhibitors reverse the reference memory impairment caused by MAPK inhibitors in rats. In that study, intra-hippocampus infusion of MAPK inhibitor U0126 impaired working and reference memory in a radial arm maze task. Co-infusion of rolipram into the hippocampus reversed the reference memory deficits with no effect on working memory. Mechanism subserving reference memory might be different from that underlying set-shifting. In fact, our results show that doses of blockers and receptor antagonists that impair set-shifting do not affect memory formation or retrieval. Further, in the same study, it was shown that, rolipram dose which reversed U0126-induced reference memory deficit, did not ameliorate U0126-induced decreased ERK-phosphorylation. This suggests that rolipram-mediated increased PKA activity might have reversed the reference memory impairment via a different signaling cascade (Yan et al., 2016).

Interestingly, activation of D1 receptor in mPFC, presumably activating PKA signaling, also increased phosphorylation of NMDA receptor subunits, and this effect was blocked by inhibitors of ERK phosphorylation (Nagai et al., 2007; Sarantis et al., 2009) suggesting a cascade involving $D1 \rightarrow PKA \rightarrow ERK \rightarrow NMDA$, and our findings are in support of this possibility (Figure 4.15). Thus, based on mentioned studies, we speculate that phosphorylated ERKs may be regulating set-shifting behaviour by targeting cytoplasmic substrates. It may be possible that phosphorylated ERKs are responsible to phosphorylate NMDA receptors and increasing their functioning which ultimately benefits set-shifting behaviour as proposed by Sarantis et al. (2009). Further, increased activation of NMDA receptors found to increase activation of PKA (Roberson and Sweatt 1996; Jay et al. 1998; Nayak et al. 1998). As our results suggest that PKA restores abnormal set-shifting behaviour, overall effect of increased ERKs phosphorylation should promote set-shifting behaviour.

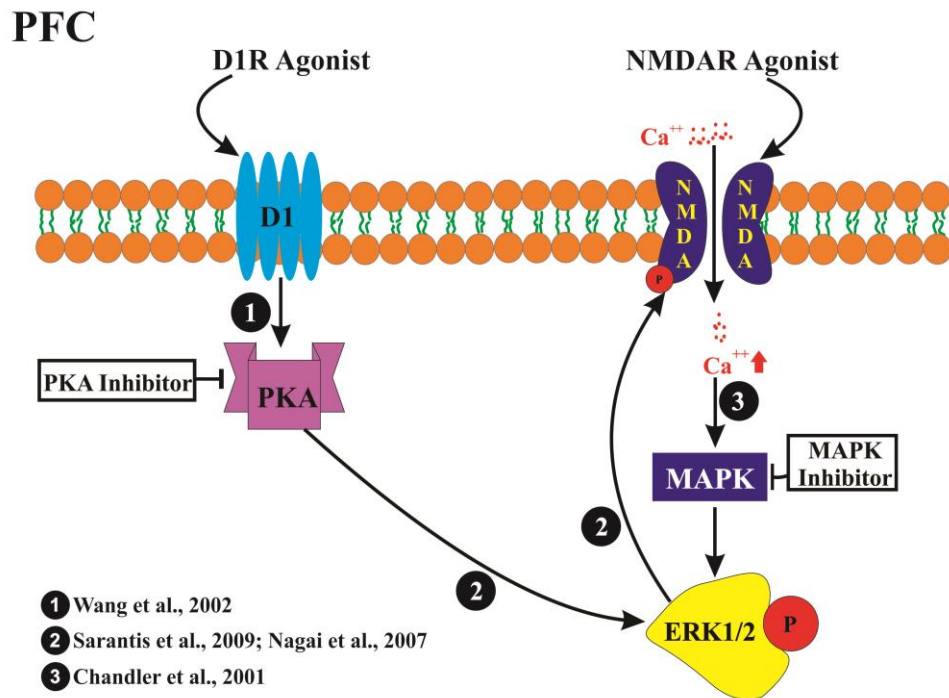


Figure 4.15 Possible mechanisms underlying D1-NMDA receptor interaction mediated set-shifting.

We observed that, co-infusing D1 and NMDA receptor antagonists in the mPFC at sub-effective doses (dual combination) impaired set-shifting in rats. This impairment was reversed by rolipram infusion in the mPFC. Further, inhibiting ERKs phosphorylation in mPFC also caused set-shifting deficits and co-infusion of rolipram failed to reverse these impairments. Based on available literature, it is possible that, D1 and NMDA receptor antagonists at sub-effective doses act synergistically at the level of molecular signalling affecting behaviour (Baldwin et al., 2002b). It is known that, D1 and NMDA receptors stimulation triggers PKA and ERK pathways respectively (Chandler et al., 2001; Wang et al., 2002). Further, PKA-ERK signalling cascades interact with each other where, D1 receptor stimulation or combining sub-effective doses of D1 and NMDA receptor agonists resulted in increased phosphorylation of ERKs as well as NMDA and AMPA receptor subunits; and this effect was blocked by MAPK inhibitor (Sarantis et al., 2012). Based on our findings and the literature, we propose that, set-shifting is regulated by PKA as well as ERK signalling and ERK is downstream to PKA as rolipram did not reverse the set-shifting deficits caused by inhibition of ERK phosphorylation. Thus, it seems that, set-shifting is regulated by $D1 \rightarrow PKA \rightarrow ERK \rightarrow NMDA$ pathway.

4.5.2 Effect of inhibiting Src kinase on set-shifting

Besides PKA and ERK signaling, evidence indicates that D1-NMDA receptor interaction may involve direct physical coupling and functional cross-talk (Lee et al., 2002; Fiorentini et al., 2003; Cepeda and Levine, 2006; Scott et al., 2006). This protein-protein interaction is associated with phosphorylation and trafficking of NMDA receptor subunits from cytoplasm to the cell membrane (Hallett et al., 2006; Gao and Wolf, 2008; Hu et al., 2010). Fyn kinase (a member of Src family kinase) mediates D1-induced increased surface expression of NMDA receptors in the PFC (Dunah et al., 2004; Hu et al., 2010). Consequently, we predicted that Fyn kinase activity might mediate part of the D1-NMDA interaction associated with set-shifting. Intra-mPFC infusion of PP2 (3 ng/side) did not affect set-shifting in rats. The dose of PP2 was chosen from previous studies where PP2 impaired cognitive functions (Bevilaqua et al., 2003) and suppression of NR2B phosphorylation (Grosshans and Browning, 2001; Lu et al., 2015). Unlike our finding, Bevilaqua et al. (2003) reported that, infusing the same dose of PP2 into the hippocampus impaired memory formation and retrieval. In addition, although mice with Fyn mutation showed impaired LTP and spatial learning in water maze task (Grant et al., 1992), they did not show spatial learning deficit in radial arm maze (Miyakawa et al., 1996); neither they had deficits in conditioned taste aversion (Schafe et al., 1996). A recent post-mortem study demonstrated evidence for reduced Src-family kinase activity in the PFC of patients with schizophrenia (Hahn, 2011; Banerjee et al., 2015). Among other cognitive symptoms, impaired behavioural flexibility and poor performance in WCST are consistent findings in these patients (Rosse et al., 1991; Abbruzzese et al., 1996; Haut et al., 1996; Waford and Lewine, 2010). Based on these evidence it was proposed that impaired Src kinase function in the PFC might serve as a hub for the cognitive dysfunction of schizophrenia (Banerjee et al., 2015). Although relatively less is known of signaling mechanisms of Src kinase in the PFC, our results indicate that Src kinase activity is unlikely playing a major role in set-shifting ability.

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Chapter 5

5 General discussion

Our studies investigated executive function (set-shifting), interaction between dopamine and glutamate neurotransmitters regulating set-shifting and the signaling mechanisms underlying the interaction. We found that, dopamine D1 and glutamate NMDA receptors synergistically regulate set-shifting behaviour. We also found that D1-NMDA interaction in the mPFC regulates set-shifting behaviour through PKA pathway. Further, we found obligatory role of ERK signaling cascade in the mPFC mediating set-shifting behaviour. For the first time, our studies explored role of molecular mechanisms associated with set-shifting behaviour in rats. Our results propose possibility of targeting molecular signaling pathways including PKA and ERKs for new drug discovery and development.

In our first study, we examined the role dopamine and glutamate in regulating set-shifting particularly when their activities are decreased only slightly. We hypothesized that a minimal loss of glutamate and dopamine activity in certain brain areas as long as they occur simultaneously would impair set-shifting and promote perseverative errors. We employed an operant conditioning-based set-shifting task (Floresco et al., 2008) in rats to determine behaviourally sub-effective doses of D1 or NMDA receptor antagonists using dose-response studies. We then injected the behaviourally sub-effective doses of D1 and NMDA antagonists together in naïve adult rats systemically to test the hypothesis. Results showed that the combined systemic administration of behaviourally sub-effective doses of NMDA and D1 receptor antagonists resulted in significant impairment in set-shifting and increased perseverative errors. To our knowledge, this is the first experimental evidence of an interaction between glutamate and dopamine systems in regulating set-shifting behaviour in rats.

Based on post-mortem studies of schizophrenia brains consistently showing evidence of considerable loss of GABA, dopamine or glutamate activity in the PFC, it was widely believed that suppression of any one of these neurotransmitters may facilitate impairment in executive function seen in schizophrenia (Okubo et al., 1997; Akil et al., 1999; Goldman-Rakic et al., 2004; Gonzalez-Burgos et al., 2011; Hu et al., 2015). However, recent studies using *in vivo* MRS and PET have identified that levels of

glutamate, dopamine and GABA in the dlPFC and thalamus are normal or slightly altered in schizophrenia patients in comparison to control groups (Théberge et al., 2007; Ohrmann et al., 2007; Galińska et al., 2009; Rowland et al., 2009; Aoyama et al., 2011; Seese et al., 2011; Szulc et al., 2011; Goto et al., 2012; Kegeles et al., 2012; Frankle et al., 2015; Slifstein et al., 2015). Above MRS and PET findings suggest that the extent of decrease of neurotransmitter function foreseen based on findings from post-mortem studies, may not be applicable in living patients of schizophrenia, and if at all, the differences may be subtle, perhaps due to compensatory mechanisms. Furthermore, recent theories postulate that certain GABA abnormalities in schizophrenia might be compensatory (Gonzalez-Burgos et al., 2011; Lewis, 2014). In addition D1 and NMDA receptors are found to regulate cognitive functions in animal models (Baldwin et al., 2002; Yang and Chen, 2005; Kruse et al., 2009; Wang et al., 2012a). Consequently, we hypothesized that subtle abnormalities in the glutamate and dopamine neurotransmitter systems in functionally-connected cortical and subcortical areas may cooperate synergistically to impair executive function in schizophrenia, an experimental consideration never addressed in previous preclinical rodent models as they only targeted single neurotransmitter system at a time.

In our first study, we chose systemic route of drug administration to achieve blockade of receptor function in multiple brain areas to be in agreement with the proposal that executive function is regulated by neuronal circuits rather than single brain nucleus (Floresco et al., 2009). As predicted results showed a significant increase in perseverative errors following combined systemic administration of behaviourally sub-effective doses of D1 and NMDA receptor antagonists. Interestingly, animal studies have shown that inactivation of mPFC or MDT results in increased perseverative errors (Block et al., 2007; Floresco et al., 2009). Our finding is also in line with the clinical literature where schizophrenic patients performing WCST show increased perseverance (Crider, 1997; Gooding et al., 1999; Prentice et al., 2008; Orellana and Slachevsky, 2013), points to abnormal glutamate and dopamine function in multiple brain areas.

Based on preclinical studies Floresco (2013) proposed that inactivation of mPFC or MDT or disconnecting these two nuclei results in increased perseverative errors while

no change in regressive or never-reinforced errors. In our first study, systemic co-administration of NMDA and D1 antagonists produced a pattern of error profile with increased perseverative errors that is similar to that proposed following damage to mPFC and MDT or their connection (Block et al., 2007; Floresco et al., 2009). The results pointed to mPFC and MDT as the most likely sites responsible for sub-effective doses of D1 and NMDA receptor antagonists to act to produce deficits in set-shifting and increased perseverative errors. Considering parallels with schizophrenia, we propose that systemic administration of D1 and NMDA antagonists in naïve adult rats may model certain aspects of cognitive deficits of schizophrenia and may form a potential high throughput screening tool for testing drug candidates against cognitive symptoms of schizophrenia.

As reasoned above, systemically-administered D1 and NMDA antagonist combination has likely acted in the mPFC and MDT to impair set-shifting and to increase perseverative errors. Since MDT receive very sparse dopamine innervation (Block et al., 2007; Floresco et al., 2008, 2009) and dopamine abnormalities have never described in the MDT of patients with schizophrenia, a disease characterized by executive functional deficits and increased perseverative errors, we considered the mPFC as the most likely candidate site. Consequently, in the second study, effects of combined blockade of D1 and NMDA receptors were investigated in the mPFC. However, it is still possible that D1 blockers might be acting in the PFC while NMDA blockers simultaneously acting in MDT (or even the hippocampus) to cause their combined effects, as these are structurally and functionally interconnected centers (Vertes, 2006; Floresco et al., 2009). In the second study, following direct microinfusion of various doses of D1 and NMDA antagonists separately into the mPFC bilaterally, we determined the behaviourally sub-effective doses for each of these blockers. When these seemingly ineffective doses were combined, increased number of trials and errors to criterion were evident while performing set-shifting task, suggesting that the mPFC is one of the sites where synergism between D1 and NMDA receptor antagonism might be occurring. The impaired set-shifting was accompanied by significant increase in perseverative errors following intra-mPFC co-infusions. This finding is in accordance with clinical studies in schizophrenic patients performing WCST (Gooding et al., 1999; Prentice et al., 2008;

Desai et al., 2017). It appears that PFC may play an important role in executive function and its abnormality may manifest perseverative errors across species.

From our results, it seems that normal functioning of dopamine and glutamate neurotransmitters is essential for executive functioning and abnormalities among them lead to deficits. This notion opens doors for different strategies that could be used to ameliorate executive dysfunction due to abnormal functioning of PFC. For example, using drugs such as PDE4 inhibitors (rolipram) to increase the levels of cAMP, a downstream effector of D1 stimulation, and obligatory activator of PKA cascade. Based on our finding that co-infusion of D1 and NMDA blockade would cause impairment of set-shifting while only one of these drugs at the same dose is unable cause this effect suggesting that for normal set-shifting, co-incident activation of both D1-NMDA receptors is necessary. Thus, we postulated use of multi-drug therapy strategy (i.e., combining antipsychotic drugs and cognitive enhancers) could be more beneficial rather than conventional mono-therapy approach used so far in schizophrenia. Nevertheless, more comprehensive understanding of the signaling cascade mediating set-shifting and preventing perseverative errors is essential for future strategies to investigate potential novel pharmacological targets. It is important to note that virtually nothing is known of signaling beyond neurotransmitter receptors when it comes to set-shifting or perseverance. Consequently, in the third study, we sought to investigate signaling mechanisms underlying synergism between D1 and NMDA receptor antagonism in the mPFC and those cascades important for set-shifting and preventing perseverative errors.

In the third study, we found that synergistic interaction between dopamine D1 and glutamate NMDA receptor antagonists cause impairment of set-shifting and this effect was reversed by intra-mPFC co-infusion of PDE4 inhibitor (rolipram). This is an example of using adult naïve rats with combined infusion of D1 and NMDA antagonists into the mPFC as a putative model of executive function deficits of schizophrenia to investigate a potential effect of cognitive enhance, a proposal we made above. Findings from this study suggested that D1-NMDA receptor interaction might use PKA signaling pathway and elevating PKA levels using rolipram rescues set-shifting from effect of dual combination of antagonists. Our findings are in accordance with previously published

studies where PDE inhibitors successfully rescued animals from certain types of cognitive deficits (Rodefer et al., 2005, 2012; Nikiforuk et al., 2016). In addition to this, use of PDE inhibitors in number of CNS disorders has been initiated (Fore review, Maurice et al., 2014). Post-mortem studies as well as imaging studies in schizophrenic patients have showed abnormal dopamine neurotransmission in the PFC (Okubo et al., 1997; Akil et al., 1999; Harrison, 2000; Abi-Dargham, 2003; Howes et al., 2015). This lead to notion that, increasing D1 receptor activity in PFC would be beneficial for cognition. In line with the hypothesis, Rosell et al. (2015) found that, D1 receptor agonist treatment was helpful to improve cognition in patients with schizotypal personality disorder (SPD). Although peripheral side-effects of the drugs tested prevented further human studies (Salmi et al., 2004).

Another novel finding of our study is that, ERK signaling pathway is essential for normal set-shifting and inhibition of ERK1/2 phosphorylation using a MAPK inhibitor PD98059 resulted in increased perseverative errors. Interestingly, PD98059-induced set-shifting impairments were not rescued by co-infusion of rolipram suggesting that ERK pathway is downstream to PKA regulating set-shifting. Association of ERK signaling in cognition has been studied extensively (Kelly et al., 2003; Duvarci et al., 2005; Giovannini, 2006; Peng et al., 2010). In addition, evidence suggesting attenuated ERK signaling in the PFC of schizophrenic patients has been reported in post-mortem studies (Yuan et al., 2010; Hirayama-Kurogi et al., 2017). From our results and the literature published on ERK signaling and its role in cognition, it seems that ERK could be a potential target for development of drugs against cognitive deficits. In this regard, use of ERK pathway modulators as therapeutic agents has been proposed in literature (Pearson et al., 2001; Peng et al., 2010; Eishingdrelo, 2013). Researchers have investigated possible use of modulators of ERK signaling in animal models of cognition, fragile X syndrome, autism and reward (David Sweatt, 2001; Gerdjikov et al., 2004; Beninger and Gerdjikov, 2005; Weng et al., 2008; Peng et al., 2010; Shiflett and Balleine, 2011; Wang et al., 2012b, 2015; Papale et al., 2016; Sun et al., 2016). Further understanding of signaling mechanisms is necessary for this line of effort to improve cognition.

A Number of studies have explored ERK signaling and its association with chronic or sub-chronic conditions including addiction, stress or memory consolidation. It is generally considered that phosphorylated ERK1/2 would translocate to the nucleus and interact with cAMP Response Element-Binding Protein (CREB) and other nuclear targets (Roberson et al., 1999; Dudman et al., 2003; Waltereit and Weller, 2003). In our studies, rats were subjected to set-shifting following acute intra-mPFC treatment with MAPK inhibitor (PD98059). This suggests that, even acute blocking of ERK signaling impairs set-shifting. Preliminary results from our laboratory suggest that in the context of set-shifting, phosphorylated ERK1/2 might be acting mainly in the cytoplasm in mPFC neurons, a proposal suggested previously by Sarantis et al. (2009). Although ERK interactions with its nuclear targets like CREB cannot be ruled out.

In our study, inhibition of ERK phosphorylation with MAPK inhibitor (PD98059) impaired set-shifting; and rolipram failed to attenuate this impairment. In addition to this, disrupting cross-talk between D1 and NMDA receptors by inhibiting Fyn kinase did not affect set-shifting. To our knowledge, this is the first study investigating signaling mechanisms underpinning D1-NMDA receptor synergistic interaction regulating set-shifting behaviour in rats.

5.1 Future directions

Taken together, present series of studies represent a big step forward towards understanding executive function and underlying molecular mechanisms associated with executive function deficits. We revealed molecular signaling pathways regulating set-shifting behaviour and facilitating perseverative errors. This information will likely pave future research endeavours in direction of developing potential drug candidates to improve executive functions by targeting unconventional molecular pathways. With the novel information, there are a number of new questions rise. From our studies, it seems that multiple neurotransmitters act simultaneously to mediate successful executive function. Future studies in animals as well as in human may use this approach to find out potential therapeutic combinations of drugs to improve cognitive symptoms.

In the final study, our results suggest critical and obligatory involvement of ERK signaling mediating set-shifting. Further investigations are needed to find out what targets in cytoplasm/nucleus ERK interact with to mediate set-shifting. Future research should also address if NMDA/D1 independent activation of ERK pathway in mPFC would ameliorate set-shifting deficits in putative models of schizophrenia.

Importance of dopamine-glutamate interactions in implications for development of neuropsychiatric treatments has attracted researchers (Castner and Williams, 2007; Wang et al., 2012a). For example, conventional medications used to suppress positive symptoms of schizophrenia mainly act by blocking dopamine D2 receptors. On the other hand, cognitive and negative symptoms are associated with D1 receptors in the PFC (Seamans et al., 1998; Akil et al., 1999; Goldman-Rakic et al., 2004). Similarly, glutamate NMDA receptors have become an important target as preclinical studies have proven that, blocking NMDA receptor function induces positive, negative and cognitive symptoms of schizophrenia whereas enhancing NMDA receptor functions potentially reverse these impairments (Fletcher et al., 2005; Nabeshima et al., 2006; Mouri et al., 2007; Hashimoto et al., 2008; Seillier and Giuffrida, 2009; Castañé et al., 2015). Although the success of drug candidates modulating D1 or NMDA receptors seems very promising in preclinical studies, recent clinical trials using these modulators have met with limited success (Chaves et al., 2009; Tandon et al., 2010; Miyamoto et al., 2012; Menniti et al., 2013; Dimitrelis and Shankar, 2016). The main reason behind failure of compounds to pass clinical trials is that simply blocking or enhancing receptor functions would result in unintended side-effects. Further, number of conventional preclinical models cause impairments in animal behaviours following use of high dose of antagonists which not the case in actual physiology. In this context, our results have indicated that future studies should model cognitive deficits using minimal but combined suppression of receptor/molecular function to mimic those changes might be happening in living patients with tremendous compensatory mechanisms trying to normalize the defect. Finally, targeting receptor interactions and molecular pathways might be a useful avenue in correcting pathological imbalance in signaling mechanisms of neurotransmitters rather than using molecules that bind to cell surface receptors.

5.2 Limitations:

In pilot testing for our first study, we tested individual dose-dependent effects of glutamate NMDA receptor antagonist (MK-801), dopamine D1 receptor antagonist SCH 23390 and GABA A receptor antagonist bicuculline on set-shifting behaviour following subcutaneous injections. We obtained behaviourally sub-effective doses of each antagonists. To study any interactions, we injected all three sub-effective doses of antagonists simultaneously and rats were subjected to set-shifting task. Animals treated with combined three antagonists showed noticeable abnormalities in behaviour including reduced latency to response on the levers as well as they did not eat food pellets following correct responses for 20-25 trials. On the other hand, rats treated with combined behaviourally sub-effective doses of D1 and NMDA receptors showed only behavioural flexibility deficits not affecting latency or omissions committed during set-shifting. For the same reason, we used D1 and NMDA receptor antagonists in our first study.

Further, in our first study, while testing dose-dependent effects of dopamine D1 receptor antagonist SCH 23390 on set-shifting behaviour, we observed that subcutaneous injections of higher dose of SCH 23390 (0.01 or 0.02 mg/kg) caused noticeable motor deficits and increased response latency, which resulted in a significant number of omissions in both the visual-cue retrieval and set-shifting trials, and ultimately a failure to complete the sessions (data not shown). For the same reason, we could not find effective dose of SCH 23390 in our study and used lower dose of the antagonist which did not affect locomotor activity and set-shifting in rats. In our last study (Chapter-IV), we examined role of prefrontal cortical Fyn kinase in set-shifting behaviour in normal rats. We found that used dose of Fyn kinase inhibitor-PP2 in the mPFC did not affect set-shifting behaviour. Although, the dose of PP2 was chosen from previous studies where PP2 impaired cognitive functions (Bevilaqua et al., 2003) and suppression of NR2B phosphorylation (Grosshans and Browning, 2001; Lu et al., 2015), running western blot studies to see if there is actual decreased NMDA receptor subunit phosphorylation would confirm the previous findings.

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Appendices

Appendix A Formal License for material used with permission for Study I


6/25/2017

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Appendix B: Research Ethics and Approval Number for Study I

2013-030::1:

AUP Number: 2013-030

AUP Title: Molecular mechanisms of spatial memory: Role of calcineurin signalling.

Yearly Renewal Date: 09/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2013-030 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
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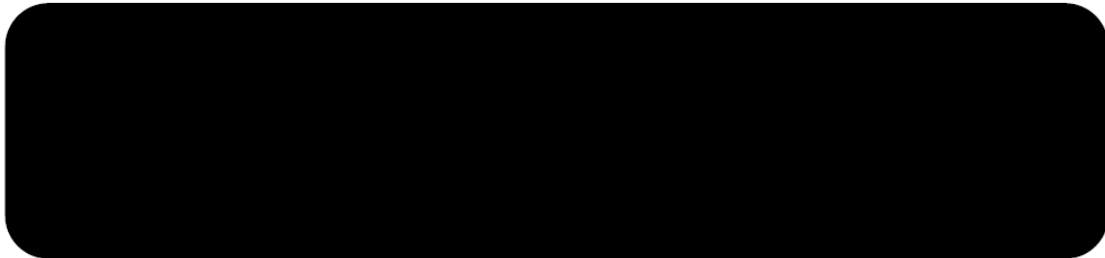
REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D

on behalf of the Animal Use Subcommittee



Appendix C: Research Ethics and Approval Number for Study II and III

2013-030::1:

AUP Number: 2013-030

AUP Title: Molecular mechanisms of spatial memory: Role of calcineurin signalling.

Yearly Renewal Date: 09/01/2016

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2013-030 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
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Submitted by: Kinchlea, Will D

on behalf of the Animal Use Subcommittee



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2014-2015

Peer-Reviewed Publications:

Desai SJ, Allman BL, Rajakumar N (2017) Combination of behaviourally sub-effective doses of glutamate NMDA and dopamine D1 receptor antagonists impairs executive function. *Behav Brain Res* 323:24-31.

Desai SJ, Allman BL, Rajakumar N. Partial ablation of frontal cortical subplate leads to deficits in behavioural flexibility: A novel neurodevelopmental model of schizophrenia (Under revision, *Schizophr Bull*).

Levit A, Regis AM, Garabon JR, **Desai SJ**, Rajakumar N, Hachinski V, Agca Y, Agca C, Whitehead SN, Allman BL. Novel analyses of behavioural inflexibility in a comorbid rat model of striatal ischemic injury and mutant hAPP overexpression (Manuscript accepted, *Behav Brain Res*).

Desai SJ, Borkar CD, Nakhate KT, Subhedar NK, Kokare DM (2014) Neuropeptide Y attenuates anxiety- and depression-like effects of cholecystokinin-4 in mice. *Neuroscience* 277:818-30.

Desai SJ, Bharne AP, Upadhya MA, Somalwar AR, Subhedar NK, Kokare DM (2014) A simple and economical method of electrode fabrication for brain self-stimulation in rats. *J Pharmacol Toxicol Methods* 69:141-9.

Desai SJ, Upadhya MA, Subhedar NK, Kokare DM (2013) NPY mediates reward activity of morphine, via NPY Y1 receptors, in the nucleus accumbens shell. *Behav Brain Res* 247:79-91.