Novel DNA dopamine aptamer pre-treatment reverses the hypoglutamatergic-induced behavioural extinction deficit in rats

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Abstract

Studies have shown that reward-based learning is under the influence of the glutamatergic and dopaminergic neurotransmitter systems: increased levels of ventral striatal dopamine as a result of cortical glutamatergic blockade impaired extinction and motor function. These effects were reduced upon intra-accumbens dopamine antagonist administration.

The present study investigates the behavioural effects of a novel dopamine (DA) antagonist (developed via DNA aptamer technology) in hypoglutamatergic rats. The effects of the DA DNA aptamer are analyzed and compared with the effects of classical D1 and D2 receptor antagonists SCH-23390 and haloperidol. 200nM aptamer pre-treatment was efficient in facilitating extinction of a previously learned behavioural task in rats that have received a subcutaneous dose a glutamatergic antagonist.

Acknowledgements

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I owe many thanks to my mother, Ana Maria Urban for supporting my studies, and Alanna I. Lawson for moral support and motivation. Last but not least, I would like to thank Etelle Bourassa for making my graduate experience at Carleton as care-free as possible.

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5-HT: 5-hydroxytryptamine (serotonin)

6-OHDA: 6-hydroxydopamine

AC: adenylate cyclase

ACA: anterior commissure

AMG: amygdala

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

BACE1: β-secretase

BH_4: (tetrahydro)biopterin

CA: catecholamine

CaMPK II: calcium- and calmodulin-stimulated protein kinase

CNS: central nervous system

COMT: catechol-O-methyltransferase

CPP: conditioned place preference

CPP: 3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (Note: different from CPP as in conditioned place preference; context specific)

CR: conditioned response

CREB: cyclic adenosine monophosphate response element bonding protein

CS: conditioned stimulus

D1-D5: dopamine receptor types and subtypes

DA: dopamine

DARPP-32: dopamine and cyclic adenosine monophosphate-regulated phosphoprotein

DAT: dopamine transporter

DNA: deoxyribonucleic acid

DOPAC: dihydroxyphenylacetic acid

EPR: extrapyramidal reaction

ERK: extracellular signal-regulated protein kinase

FDA: (US) Food and Drug Administration

FR: fixed ratio

GABA: gamma-aminobutyric acid

Glu: glutamate

HIV: human immunodeficiency virus

HPLC: high-performance liquid chromatography

IgG: immunoglobulin G

kd: dissociation constant

KMP-11: kinetoplatid membrane protein-11

LSD: lysergic acid diethylamide

LTP: long term potentiation

MAO: monoamineoxidase

MAG: myelin-associated glycoprotein

MK-801: (+)-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (dizocilpine)

NAcc: nucleus accumbens

NE: norepinephrine

NgR: Nogo-66 receptor

NMDA(R): N-methyl-D-aspartic acid (receptor)

OMgp: oligodendrocyte myelin glycoprotein

PCR: polymerase chain reaction

(m)PFC: (medial) pre-frontal cortex

PKA: protein kinase A

PP1: protein phosphatase 1

PR: progressive ratio

RNA: ribonucleic acid

SCH-23390: 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol

SELEX: systematic evolution of ligands by exponential enrichment

Ser: serine

SKF-81297: 6-chloro-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol

T: thymine

TH: tyrosine hydroxylase

U: uracil

UCR: unconditioned response

UCS: unconditioned stimulus

VMAT: vesicular monoamine transporter

VP: ventral pallidum

VTA: ventral tegmental area

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Introduction

From an industrial and academic perspective, drug research is not more than a century old, originating in chemistry then developing into its own discipline, pharmacology (Drews, 2000). This new field flourished in the early 1900's, when Chain and Florey selected penicillin (a metabolite from the penicillum mold) that could lyse staphylococci (Drews, 2000). The discovery of Penicillin by Fleming in 1929 created the beginning of a new era in pharmacology and antibiotic development. The mid 1900's were marked by the amalgamation of two previously unrelated fields: chemistry and biology, giving rise to a novel crucial new field in pharmaceutical research. The main approach biochemistry took to drug development targeted enzymes and receptors, partly based on Erlich's idea that receptors were selective binding sites for chemotherapeutic agents (Drews, 2000).

Fueled by an exponential improvement in technology and accompanied by a better understanding of molecular physiology and genetics, a new class of therapeutic agents emerged: recombinant proteins often referred to as "biotech drugs" (Drews, 2000). In the past 30 years, monoclonal antibodies (a specialized type of recombinant proteins) emerged as a new class of therapeutic agents, synthesized in three different ways. Monoclonal antibodies can be made by phage display techniques, or generated as mouse antibodies, that are subsequently "humanized". Human antibodies can also be raised on nude mice, grafted with human immune cells (Drews, 2000). These biotech drugs proved to be efficient therapeutic agents primarily due to their high binding affinity and specificity. However, they have been shown to be highly immunogenic and efficacy often differs from batch to batch (Nimjee, Rusconi, & Sullenger, 2005).

Contemporary advances in nanotechnology made it possible for single stranded nucleic acid sequences called aptamers to be synthesized, resembling (and sometimes surpassing)

monoclonal antibodies in their ability to specifically bind to their targets, making them attractive candidates as therapeutic agents, as well as strong rivals of antibodies (Nimjee, Rusconi, & Sullenger, 2005).

The present study used a DNA aptamer that binds to dopamine (and norepinephrine) molecules *in vitro* (Walsh & DeRosa, 2009). The primary goal was to investigate whether this aptamer would retain its binding properties *in vivo*, by injecting it into the nucleus accumbens (NAcc) of the rat brain. Since the aptamer works as an antagonist of the dopaminergic system by binding to dopamine molecules, thus stopping their action on the dopamine receptors, we chose to test this compound against classic dopaminergic antagonists. Due to the potential pharmacological nature of the aptamer, the clinical implications of this work apply to conditions that involve an acute or chronic surplus of dopamine within the central nervous system, such as schizophrenia, psychosis or drug addiction.

Aptamer technology

Aptamers (Latin *aptus* meaning to fit, and Greek *meros* meaning part- Ellington & Szostak, 1990), are biochemical substances, consisting of small single-stranded nucleic acids (RNA or DNA) that fold into well-defined three-dimensional structures (Proske, Blank, Buhmann, & Resch, 2005; Mayer, 2009) . Aptamers were first discovered over 20 years ago through the study of viruses, particularly HIV and adenovirus research (Sullenger & Gilboa, 2002). Due to their ability to recognize and bind to targets with great specificity and affinity, these nucleic acid ligands have been the subject of extensive bio-chemical research. Mayer's (2009) review illustrates the exponential growth and interest allocated to aptamers within the past 20 years (Figure 1).

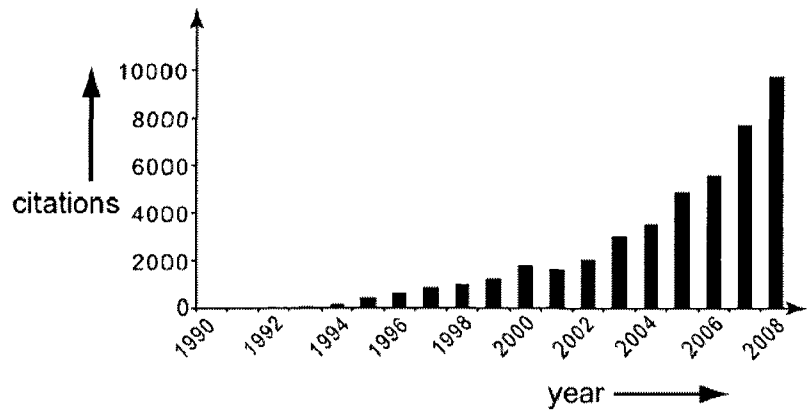


Figure 1. Citations found on the Web of Science when searching the keyword *aptamer*, prior to 21.11.2008 (Mayer, 2009).

Synthesis

Aptamers are artificially obtained via a screening technique, involving the progressive selection of highly specific ligands by repeated rounds of partition and amplification from large starting libraries of oligonucleotides (Tuerk & Gold, 1990). A simple schematic illustrating this process called the *systematic evolution of ligands by exponential enrichment* (SELEX) (Tuerk & Gold, 1990) is shown in Figure 2. Briefly, the SELEX process starts with an initial library of approximately 10^{15} single-stranded oligonucleotide templates, which are allowed to come into contact with the target molecule. The next step involves the separation of the species that bind to the target from those that did not, using specific partitioning methods. Finally, the bound clones are eluted and amplified to enrich the pool with specific sequences capable of binding to the target. Since one single round of selection is typically not sufficient in obtaining a large enough number of binding sequences, this process is repeated another 7-15 times, yielding a small number of binding clones (approximately 1-100) (Nimjee et al., 2005; Proske et al., 2005; Bouchard, Hutabarat, & Thompson, 2010).

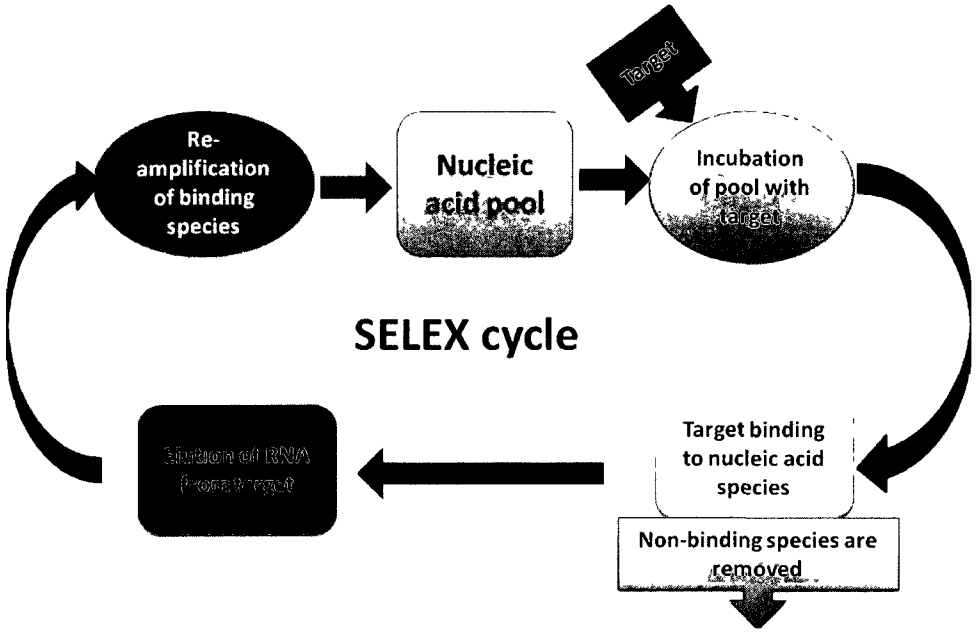


Figure 2. Simplified scheme representing the SELEX cycle of RNA aptamer synthesis.

Since its introduction in the early 1990s, the SELEX procedure has evolved, giving rise to multiple variations. Some of the patented versions include: photo-SELEX (photoreactive groups-containing aptamers binding to a target molecule), chimeric-SELEX (combination of aptamers generated using two different originating libraries), blended-SELEX (combination of aptamers with other non-oligonucleotidic groups), counter-SELEX (method generating aptamers that can discriminate between highly related molecules, such as theophyline and caffeine), tissue-SELEX (generates aptamers that bind to complex tissue targets, like Tenascin-C) and transcription-free SELEX (ligating random fragments of RNA bound to a DNA template to form the oligonucleotidic library) (Hjalmarsson, Macellaro & Norlander, 2004).

Due to their ability to fold into three-dimensional structures and bind to their targets, aptamers have emerged as a therapeutic competitor of antibodies. Compared to antibodies,

aptamers show little or no immunogenicity, uniform selectivity independent of batch synthesis, unlimited shelf-life (Nimjee, Rusconi, & Sullenger, 2005) and the ability to be neutralized through aptamer-specific antidotes (Rusconi, et al., 2004; Nimjee, Rusconi, & Sullenger, 2005), as shown in Figure 3. Aiming to control the aptamer activity independent from metabolic clearance, an active aptamer can be paired with a specific oligonucleotide antidote, altering its shape and therefore preventing it from further binding to its original target. The ability to design antidote-controlled therapeutic agents represents a promising basis for the use of aptamers in drug development (Nimjee et al., 2005).

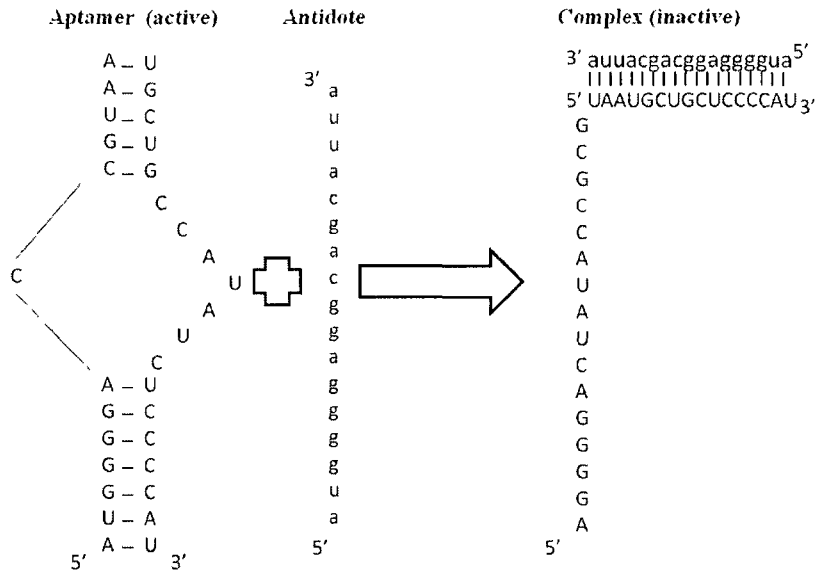


Figure 3: Inactivation of an aptamer by a complementary oligonucleotide (adapted after Nimjee et al., 2005).

Binding properties and structure

An important characteristic underlying the success of diagnostic and therapy protocols based on aptamer technology lies in the ability of an aptamer to bind to its target with high affinity and specificity (discriminating between closely-related molecules), with k_ds comparable

to those of monoclonal antibodies (Hjalmarsson, Macellaro & Norlander, 2004). Given that nucleic acids are known to be negatively charged at normal pH conditions, it had been previously assumed that aptamer-protein interactions are driven by electrostatic interactions (Hjalmarsson, Macellaro & Norlander, 2004). Additional data suggests that the binding energy in an aptamer-ligand complex also consists of hydrogen bonding, alongside other forces, like stacking of aromatic rings and Van der Waals shape complementarity (Hjalmarsson, Macellaro & Norlander, 2004). Matsugami and colleagues (2003) offered a detailed description of the bond between an RNA aptamer and the TAT protein of HIV-1.This particular aptamer has two binding sites, which fit two argininamide molecules, and bind to the aptamer's G bases through hydrogen bonds, with the binding being stabilized through stacking interactions (Matsugami et al., 2003).

Clinical potential and applications

The isolation and characterization of specific antagonists for proteins and peptides involved in disease is one of the goals of pharmacological research (Famulok & Mayer, 1999). High specificity, increased affinity, ability to be therapeutically controlled and good safety margins make aptamers optimal therapeutic and detection agent candidates (Proske, et al., 2005; Yang, Yang, Schluesener, & Zhang, 2007) . As of October 2009, seven aptamers were either on the market or in clinical trials (Bouchard, et al., 2010). Macugen^{TM} was the first FDA-approved aptamer, prescribed for age-related macular degeneration (AMD) (www.macugen.com). In a comprehensive review, Famulok and Mayer (1999) gave an overview of recent progress in oligonucleotide selections and applications of aptamers as potential tools in drug discovery, diagnostics and elucidation of cellular processes of immunological relevance (Table 1).

Table 1. Summary of targeted molecules and viruses used for SELEX experiments (Famulok & Mayer, 1999)

Target molecule	**Dissociation constant (nM)**	**Therapeutic potential**
IgE	10.0	Allergic disease
L-selectin	3.0	Inflammation
CD4	N/A	Immune response
bFGF	0.35	Angiogenesis
PDGF	0.1	Tumor development
HIV-1 RT	1.0	Viral replication
PLA_2	118.0	Septic shock

Similar to small molecules such as erythromycin and Tamiflu™, aptamers have the ability to fit into clefts on the surface of their target macromolecule, allowing them to function in a similar vein as antibiotics, if selected to inhibit a bacterial protein or impede cell membrane formation (Yang, et al., 2007). Aptamers can also be used as a targeting system, carrying antibiotic agents to pathogens, or as diagnostic imaging tools. Charlton and colleagues (2007) used an aptamer as an irreversible inhibitor of neutrophil elastase for diagnostic imaging of inflammation in rats. Compared with immunoglobulin G (IgG), used clinically for diagnostic imaging of inflammatory diseases, the aptamer achieved a significantly higher target-to-background ratio in less time than IgG (Charlton, 2007).

In an effort to reduce HIV replication, Chaloin, et al. (2002) used a pseudoknot RNA aptamer selected for tight binding to HIV type 1 reverse transcriptase. T-lymphoid cells showed complete inhibition of viral replication in the presence of the aptamer post HIV-1 (low dose) infection. As the infectious dose was increased, the amount of aptamer produced by the cells was not successful in fully blocking viral replication. However, the number of particles released was

significantly lower and delayed by approximately one week compared to controls (Chalion et al., 2002).

In the early 1990's, single-stranded DNA aptamers were shown to bind to thrombin with high affinities (Nimjee, Rusconi, Harrington, & Sullenger, 2005). The 15-residue DNA aptamer ARC-183 was shown to inhibit thrombin activity by preventing the thrombin-catalyzed conversion of fibrinogen to fibrin in vitro, using either purified fibrinogen or purified plasma (Bock, Griffin, Latham, Vermaas, & Toole, 1992). DeAnda, et al. (1994) and Ingels, et al. (1994) showed that a thrombin aptamer could successfully replace heparin (anticoagulant agent) in a canine cardiopulmonary model, with no significant difference in fibrinogen consumption between the heparin and thrombin aptamer groups. The two aptamer-based anticoagulants currently in development, ARC-183 and REG1, offer unique solutions to the issues raised by classical anticoagulants: a short half-life (ARC-183) and antidote controlled compound in the case of REG1 (Nimjee, Rusconi, Harrington, et al., 2005).

Tenascin was initially shown to be an integral part of the dense mesenchyme surrounding the growing epithelium of embryonic tissues (Mackie, 1994). A closer analysis indicated that tenascin may be a marker for epithelial malignancy (Daniels, Chen, Hicke, Swiderek, & Gold, 2003). Analysis on the mammary gland tumor in humans revealed a correlation between the intensity of tenascin staining and progression of the tumor (Daniels, et al., 2003). In 2003, Daniels and colleagues used SELEX to successfully identify a DNA aptamer (GBI-10) that would bind to tenascin at 4^0C. The aptamer does not bind well at 37^0C, and is subject to nuclease activity *in vivo*, and thus cannot be used as an alternative to the anti-tenascin monoclonal antibodies that were (as of 2003) in phase II trials. However, this study adds to the growing list of aptamer-based technology within the field of therapeutics.

Recently, Meng et al (2010) used a variant of SELEX, called cell-SELEX to identify aptamers which were able to recognize leukemia cells. Since the aptamers obtained via cell-SELEX were able to differentiate between molecular signatures of healthy and disease cells, these compounds make ideal candidates to meet the challenges of cancer biomarker discovery (Meng, et al., 2010).

Moreno, et al. (2003) and Ramos, et al. (2007) have successfully isolated two aptamers that bind to *Leishmania infantum*, the parasite causing leishmaniosis in man and animals. Moreno, et al. (2003) selected a DNA aptamer binding to *L. infantum* kinetoplatid membrane protein-11 (KMP-11). Despite its unknown function, KMP-11 is a cell membrane component of all kinetoplastid parasites, suggesting its possible involvement in parasitic mobility (Moreno, et al., 2003). Similarly, Ramos, et al. (2007) generated a population of aptamers (SELH2A) that were shown to bind with high specificity to the *L.infantum* H2A antigen. Using an aptamer that blocks the effects of a protein crucial to parasitic motility indicates that this technology has the potential to be a powerful tool in the detection, diagnostics and therapy of infectious diseases.

Aptamers and the CNS

Alzheimer's disease (AD) is characterized by widespread functional and cellular disturbances within the aging human brain, with fibrilar amyloid proteins being deposited inside neurons (neurofibrilary tangles) and extracellularly as amyloid plaque cores (Kang, et al., 1987). The extracellular plaques contain aggregated β-amyloid, a peptide generated by proteolytic processing of the β-amyloid precursor protein (APP) (Rentmeister, Bill, Wahle, Walter, & Famulok, 2006). Since β-secretase (BACE1) was shown to initiate β-amyloid generation, it represents a feasible target for beta-amyloid production and possible AD treatment (Rentmeister

et al., 2006). Rentmeister, et al., (2006) developed an RNA aptamer that specifically binds the cytoplasmic domain of BACE1 *in vitro* with low dissociation constants. It is premature to suggest that these aptamers offer treatment for AD *in vivo*, but the finding that aptamers are able to bind to peptides crucial in AD etiology brings us a step closer to at least understanding the mechanisms leading to AD.

Myelin within the CNS is one of the main sources of inhibition of axonal degeneration due to injury (Wang, et al., 2010). Trauma to the CNS often results in myelin disruptions, with the products of this myelin coming in contact with the surfaces of the severed axons and inhibiting regeneration. Three myelin-derived inhibitors are known: Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp), all binding to the Nogo-66 receptor (NgR) on axonal surfaces (Wang et al., 2010). In 2010, Wang and colleagues selected a group of RNA aptamers that competitively bind to NgR, promoting axonal elongation of neurons *in vitro*, demonstrating that aptamers not only have the potential to serve as additional treatment for spinal cord and other neuronal damage, but also have the ability to act as neuromodulators (Wang et al., 2010). Other applications for aptamer technology within the CNS include the possible treatment of encephalopathies (Rhie, et al., 2003; Sayer, et al., 2004), drug addiction (Yang et al, 2007) and exploratory neurophysiology (Proske, Hofliger, Soll, Beck-Sickinger, & Famulok, 2002; Faulhammer, et al., 2004). The experiments described above emphasize the versatility of the aptamer technology and the therapeutic potential they hold across a variety of bio-medical fields. So far, these oligonucleotides have been shown to be effective in pathway elucidation, study of disease and toxicology, antibiotic production, purification, diagnosis, detection and imaging, to name a few (Hjalmarsson et al., 2004).

Considering the potential that the aptamer technology holds within the field of neuroscience, it is of no surprise that the dopamine DNA aptamer developed by Walsh and DeRosa (2009) showing improved affinity (kd = 0.7μM) for dopamine (DA) *in vitro* compared to its RNA homolog (kd = 1.6 μM) (Mannironi, Di Nardo, Fruscoloni, & Tocchini-Valentini, 1997) would be of interest in the study of hyperdopaminergic conditions. This aptamer was modified from its RNA homolog by replacing the *uracil* (U) nucleotides with *thymine* (T) nucleotides. It is worth mentioning that the DNA DA aptamer has similar affinity for norepinephrine (NE) as DA (Walsh & DeRosa, 2009). Figure 4 shows the binding specificity of the DNA aptamer with DA (center), tested against norepinephrine (NE, left) and tyramine (right) by fluorescence anisotropy experiments (Walsh & DeRosa, 2009). Maximal binding of the aptamer to both DA and NE molecules in vitro was reached at concentrations of roughly 10^{-5} M, while higher concentrations were required (10^{-3} M) for tyramine.

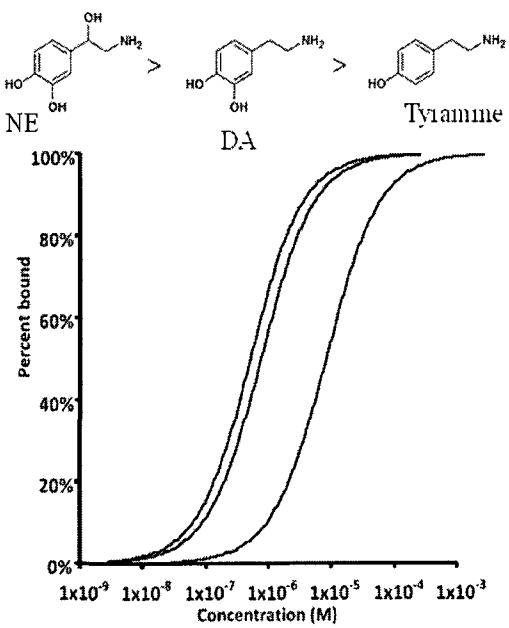


Figure 4. Comparison of the binding curves of the DNA-DA aptamer with NE (left), DA (center) and tyramine (right) (Walsh & DeRosa, 2009).

Given its high affinity for DA, the aptamer was tested using a hypoglutamatergic-mediated DA increase within the NAcc manipulation. It was hypothesized that an increase in DA levels following glutamatergic blockade would be counteracted by the DNA DA aptamer central administration. The behavioural design and rationale are described below in detail.

Adaptive learning

Behavioural psychologists have long made a distinction between *classical* (or Pavlovian) conditioning and *operant* (or instrumental) conditioning (Dayan & Balleine, 2002). In 1935, the renowned American psychologist B.F. Skinner published a theoretical paper in which he attempted to add clarity and structure to the differences between the two types of conditioning (Clark, 2004). Briefly, the two types of conditioning can be distinguished as follows: in Type I conditioning, a response (R_0) intervenes between states S_0 (lever pressing) and S, (food delivery) while in Type II, R_0 is irrelevant with respect to the presentation of S_1 (Clark, 2004). It is obvious that based on Skinner's description, Type I refers to operant conditioning and Type II represents classical conditioning.

Classical conditioning

Various environmental cues, such as specific smells, shapes or sounds are often times involuntarily associated with more or less unique experiences that elicit a hedonic or aversive response. For example, a salivatory response is automatically triggered by the ingestion of a donut. There is also a particular smell associated with the donut. On future occasions, the smell of donuts will generally result in a salivatory response. As the association of the donut (pleasurable/rewarding stimulus) with its smell is being learned upon repeated exposures, one would now exhibit a reflex response such as salivation when (s) he is subjected to the smell

alone. This adaptive type of learning, based on associations between stimuli is called classical conditioning, and was first described and more importantly investigated in detail by the Russian scientist Ivan Pavlov at the end of the 19th century. This subject has been previously studied and exemplified by Whytt in 1763: "The remembrance or idea of substances formerly applied [...] produces almost the same effect as is these substances were really present. Thus the sight, or even the recalled idea of grateful food, causes an uncommon flow of spittle into the mouth of a hungry person; and the seeing of a lemon can produce the same effect in many people" (Mackintosh, 2003).

Analyzing Pavlov's experiments, we are able to identify two stimuli (tone and food) and a response (salivation). The tone alone would initially not elicit any salivatory response, so in this context, the tone is a neutral stimulus. Placing food in a dog's mouth results in a reflexive response: salivation. Since this response is reflexive (does not need to be learned), the food is called an unconditioned stimulus (UCS), while salivation is the unconditioned response (UCR). If the tone is paired with the food, the animal will learn this association and start salivating at the sound of the tone alone. At this point, the tone will become the conditioned stimulus (CS), and the salivatory response is called the conditioned response (CR).

In the event of the CS not being immediately followed by a reinforcer or reward (food) repeatedly, this association will weaken. This process is called *extinction*. Extinction is not permanent, in a sense that this association is not forgotten, but a new, superseding association is learned (Cammarota, et al., 2004). If the same animals, after extinction, were later subjected to more pairings of tone and food, the association between the stimuli (tone-food) would be quickly re-established. This "re-learning" process is called *spontaneous recovery*.

In sum, the mechanisms underlying classical conditioning consist of *acquisition*, *extinction* and *spontaneous recovery*. This type of *reflexive* learning can be identified in higher animals, as well as insects. Similar salivatory responses have been recorded in cockroaches, where the insects successfully learned to associate a specific odor with sucrose, showing increased salivation while subjected to the odor alone (Watanabe & Mizunami, 2007). A rudimentary form of classical conditioning is the ability of the immune system to learn how to react to certain substances (endogenous or exogenous), if we consider Pavlovian conditioning to be a mechanism by which an organism learns to anticipate the onset of a biologically important event, initiating preparatory responses, such as lymphoid- and myeloid cell based responses (Schedlowski & Pacheco-Lopez, 2010).

Operant conditioning

Operant conditioning is slightly more complex than classical conditioning. Similar to classical conditioning, it relies on the pairings of environmental stimuli, but the main difference is that organisms need to produce a behavioral response to receive or remove a reinforcer from their environment (Staddon & Cerutti, 2003). In operant conditioning paradigms, animals are able to control and predict the outcomes (in this case, food delivery) of their actions (bar presses). Operant conditioning was pioneered by the American psychologist Edward Thorndike in the late 1800's and promoted by the American psychologist B.F. Skinner at the beginning of the 20th century (Passer et al., 2008).

In Thorndike's work, cats were placed in a cage that would unlock only if a lever was pressed. With food being placed outside the cage and hungry cats, they had a certain amount of motivation to escape from the cage. The animals would unsuccessfully try to escape from the

cage by digging through the floor of the cage, or scratching at the bars. By chance alone, they would hit the lever that would set them free and allow them to have access to food. When they were placed in the cage again, the animals would learn, through trial and error, that in order to have access to food, they would have to press the lever first. Thorndike called this process *instrumental learning* since the organism's behaviour is "instrumental" in eliciting desired outcomes. Thorndike also proposed the *law of effect*, which stated: "Of several responses made to the same situation, those which are accompanied or closely followed by satisfaction to the animal will, other things being equal, be more firmly connected with the situation, so that, when it recurs, they will be more likely to recur; those which are accompanied or closely followed by discomfort to the animal will, other things being equal, have their connections with that situation weakened, so that, when it recurs, they will be less likely to recur. The greater the satisfaction or discomfort, the greater the strengthening or weakening of the bond." (Thorndike, 1911, p. 244). Attention should be paid to the fact that in this context, *discomfort* and *weakening* would most likely refer to situations where an animal elicits an avoidance response to an unpleasant stimulus, which does not directly translate into the weakening of certain learned associations, but rather the forming of new associations that would elicit different responses (aversion vs. approach) by overriding the previously learned experiences.

A real life example of instrumental (or operant) learning can be seen while trying to teach a dog to perform a specific command, such as "sit". If a dog is rewarded with a treat every time it sits on command, the dog would learn (acquisition) that this behaviour is necessary in order to obtain the reward. With prolonged training, the dog will sit at the sound of the command or sight of the treat alone, having learned that this behaviour results in a treat. Just like classical conditioning, instrumental learning is acquired through numerous trials and is subject to

extinction and spontaneous recovery when the reinforcer is eliminated. In the early stages of training, if the dog is not given a treat following behaviour (sitting), the animal will eventually stop expressing this behaviour.

In a controlled laboratory setting, such behaviour can be replicated and studied using fixed and variable ratios of reinforcement. A fixed ratio of reinforcement is described by the administration of a reinforcer upon a fixed number of behavioural actions elicited by the animal (Staddon & Cerutti, 2003). Similarly, during a classical conditioning paradigm, a fixed ratio schedule would refer to the administration of a reinforcer preceded by the presentation of a cue, where the frequency of the reinforcer or cue availability remains constant throughout the testing/training period. A variable ratio schedule is based on the same concept of reinforcement delivery and cue associations as a fixed schedule, but the number of actions (i.e. bar presses) resulting in reinforcement is variable (Roane, 2008).

Summarizing classical and operant conditioning, one can state that classical conditioning is based on passively learning an association between neutral stimuli and reinforcers, while instrumental learning involves the active production of a behaviour that results in presentation or removal of a reinforcer.

The present study focused on instrumental conditioning, where food-restricted rats learned to press a lever to obtain chocolate pellets. Briefly, the operant design consisted of the following concepts: *a state space* (existence of different stimuli in the operant chamber), *a set of actions* (bar presses, programmed to result in reward delivery based on pre-set fixed or progressive schedules) and *the affectively important outcomes* (chocolate pellet delivery, which are shown to be preferred by rodents over regular diets (Dayan, Niv, Seymour, & Daw, 2006;

Hajnal, Covasa, & Bello, 2005). The outcomes, in this case have a rewarding property, since the subjects are food restricted. These affective outcomes are subject to change, as the animals become satiated over the course of a session.

Rats were exposed to five days of training, in which they learned the rules of the experimental design. Typically, by the fifth day of training, the rats have learned to successfully press the lever for food and they optimize the rate of acquisition. In previous work from this laboratory (Holahan, Clarke, & Hines, 2010), during the first two to three days of training, the rat learns that it will get food upon bar pressing, so after each press, it checks the delivery box for food (recorded via nose pokes). Despite the fact that during training, the rat is operating under a fixed ratio 2 schedule (FR2) where food is delivered only after two consecutive presses, the rat typically checks the box after each press. By the fifth day of training, the animals successfully learn that they will be rewarded after two presses, resulting in an average press/nose poke ratio of 2:1 (Holahan, Clarke, & Hines, 2010).

Dopamine systems

The main anatomical and physiological neural system behind both instrumental and classical conditioning are believed to be comprised of dopaminergic (DA) neurons within the ventral tegmental area (VTA) and their projections to the nucleus accumbens, also known as the mesolimbic DA pathway (Nishino, et al., 1986; Woodward, Chang, Janak, Azarov, & Anstrom, 1999). Dopamine is a neurotransmitter that belongs to a general class of neurosignals called cathecolamines, along with epinephrine (adrenaline) and norepinephrine (noradrenaline) (Dunkley, Bobrovskaya, Graham, von Nagy-Felsobuki, & Dickson, 2004). Dopamine, in fact, serves as a precursor in the synthetic pathways for both norepinephrine and epinephrine

(Rutledge & Jonason, 1967). The cathecolamines belong to a larger group of hormones (released peripherally) and transmitters (released centrally) called monoamines. Based on the molecular, functional and neuro-anatomical differences, the cathecolamine systems are centrally divided into dopaminergic, adrenergic and noradrenergic, respectively (Wallace, Magnuson, & Gray, 1992). DA is synthesized within the neuron terminals from the amino acid tyrosine, which is formed from the essential amino acid phenylalanine via phenylalanine 4-hydroxylase (Murdoch et al., 1970). Tyrosine depletion has been shown to result in a decrease in dopamine synthesis within the prefrontal cortex (Bongiovanni & See, 2008), while decreased levels of plasma tyrosine are associated with a decrease of systolic and diastolic arterial pressure (Moja, Lucini, Benedetti, & Lucca, 1996).

The next step in dopamine synthesis is the conversion of tyrosine into DOPA, facilitated by the rate-limiting enzyme tyrosine hydroxylase (TH, along with tetrahydrobiopterin (BH_4), O_2 and Fe^{2+} (Dunkley, et al., 2004). TH is coded for by a single gene and belongs to a family of iron-containing, biopterin-dependent amino acid hydroxylases, along with phenylalanine hydroxylase and tryptophan hydroxylase (Dunkley, et al., 2004). It is the first enzyme in DA synthesis, catalyzing the hydroxylation of L-tyrosine to DOPA. Its activity is modulated by two mechanisms: medium- and long-term regulation of gene expression (enzyme stability, transcriptional regulation, RNA stability etc) and short-time regulation of enzyme stability (feedback inhibition) (Dunkley, et al., 2004). It is critical for the maintenance of DA in tissues (post DA secretion) that TH is activated through phosphorylation, which takes place at serine residues (Ser) 8, 19, 31 and 40 by a variety of protein kinases and phosphatases (Dunkley, et al., 2004). TH activity can be inhibited by catecholamines such as DA by competing with BH_4 for the catalytic binding site, and by the substrate L-tyrosine, heparin, polyanions, phospholipids and

BH_4 bioavailability (Dunkley, et al., 2004). In a review summarizing the regulation and consequences of TH phosphorylation, Dunkley et al. (2004) described a positive correlation between the phosphorylation of TH and catecholamine (CA) synthesis *in vitro* and *in situ*. For example, incubation of striatal slices with forskolin increased TH phosphorylation at Ser40 and CA synthesis, while incubation of rat hypothalamic cells with angiotensin II increased the phosphorylation of TH at Ser19, Ser31 and Ser40, and increased CA synthesis. *In vivo* analysis indicates that phosphorylation is abolished in DA D2 null mice, and reduced by chronic haloperidol administration (for review, see Dunkley, et al., 2004). These results suggest, as mentioned earlier, that CA synthesis is highly dependent on TH phosphorylation. This is an important finding, considering that TH (phosphorylated and not) is used as a marker quantifying DA availability in tissues. Based on the research reviewed by Dunkley et al. in 2004, only phosphorylated TH should be used as a marker of CA synthesis, and since protein kinase A (PKA), calcium- and calmodulin-stimulated protein kinase (CaMKII) and extracellular signal-regulated protein kinase (ERK) are some of the few kinases shown to be responsible for the phosphorylation of TH, their activity and availability should also be analyzed and quantified in order to offer a more exact image of overall CA availability.

The final step in dopamine synthesis is the conversion of DOPA into dopamine, which is to be packaged into synaptic vesicles through the vesicular monoamine transporter (VMAT1 and VMAT2), with VMAT2 exclusively expressed within the central nervous system, while VMAT1 expression occurs mostly in peripheral endocrine tissues (Chaudhry, Edwards, & Fonnum, 2008). Blockage of VMAT results in a decreased availability of monoamines within the synaptic cleft. In 1965, Carlsson, et al., showed that inhibition of VMAT by reserpine produced depression by decreasing the availability of DA (acting on VMAT2, but was effective in treating

hypertension peripherally acting on VMAT1), giving rise to the monoamine hypothesis of affective disorders.

After its release into the synaptic cleft, dopamine will act on receptors located on the post-synaptic dendrite, as well as on transporters and autoreceptors located on the pre-synaptic terminal membrane. The dopamine transported back into the terminal is to be repackaged into vesicles for later use, while the molecules left in the cleft will be either broken down by monoamineoxidase (MAO) and catechol-O-methyltransferase (COMT), or will exert a post-synaptic effect by binding to dopamine receptors. Similar to DA's precursors, its metabolites can be quantified to measure DA activity (turn-over and clearance rates) (Zetterstrom et al., 1988; O'Connor, Drew, & Ungerstedt, 1989; Maurino, Machado, & Santiago, 2010). In a study investigating the effects of selegiline (monoamineoxidase inhibitor) on behaviour and striatal DA transmission, Themann, et al., (2002) used high-performance liquid chromatography (HPLC) to analyze the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA). DOPAC levels reflect the intraneuronal metabolism of DA, while HVA levels reflect the extraneuronal one (Maurino et al., 2010). A complete molecular map of DA transmission would then include DA activity, as well as precursor (TH) and metabolite (DOPAC, HVA) analysis.

Four dopaminergic pathways have been identified within the central nervous system, named after the location of the specific cell bodies and their projections (afferents): nigrostriatal, mesocortical, tuberoinfundibular and mesolimbic.

The nigrostriatal dopamine pathway

This pathway originates in the substantia nigra within the midbrain (mesencephalon), sending afferents to the basal ganglia. Post-synaptic blockage through antipsychotic

administration of these pathways will result in Parkinsonian-like motor deficits, also known as extrapyramidal reactions (EPRs) (Ahlenius & Hillegaart, 1986): *akathisia* (restlessness), *dystonia* (twisting movements of the face and neck), *tremor, rigidity poor balance* and *bradykinesia* (slow or lack of movement).

The mesocortical pathway

The cell bodies of the mesocortical pathway are found in the ventral tegmental area (VTA), sending their projections to the prefrontal area of the cerebral cortex. Studies suggest that this pathway, along with the mesolimbic pathway play an important role in understanding the dopaminergic hypothesis of schizophrenia (A. Carlsson, 2006).

The tuberoinfundibular pathway

The cell bodies are found in the hypothalamus and project to the pituitary gland. Dopamine receptor blockage within this pathway results in a rise in prolactin levels suggesting that the main function of this dopaminergic pathway is prolactin secretion (Andrews, Kokay, & Grattan, 2001).

The mesolimbic pathway

Similar to the mesocortical pathway, the mesolimbic pathway originates in the VTA, but sends its projections to the limbic system, including the nucleus accumbens (NAcc) and hippocampus. When overactive, this system is thought to produce delusions and hallucinations, making it the main target for antipsychotic action in the case of schizophrenia and other psychotic conditions (Yang et al., 1998). This pathway is also known as the dopamine reward circuitry, due to its contributions to the rewarding effects of drugs of abuse (S. Ikemoto, 2007).

Since the experimental manipulations in this study were performed at the NAcc level and the behavioural measurements address the reward and motor mechanisms, the mesolimbic pathway will be assessed in greater detail.

Neurons in the VTA innervate the ventromedial part of the caudate, nucleus accumbens (NAcc), olfactory tubercle, medial thalamus and hypothalamus (Nishino et al., 1986), as shown in Figure 5. Glutamatergic synapses, with cell bodies residing within the hippocampus, amygdala (AMG), pre-frontal cortex (PFC) excite post-synaptic neurons within the NAcc and the VTA, while the GABAergic cells within the NAcc inhibit the post-synaptic neurons within the ventral pallidum (VP) and the VTA (Kauer & Malenka, 2007). Dopaminergic neurons within the VTA act on post-synaptic cells within the NAcc, giving rise to the mesolimbic pathway.

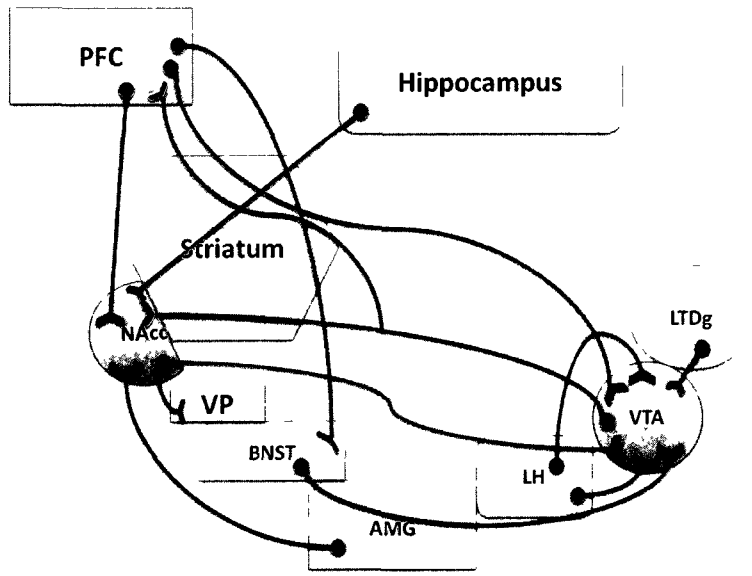


Figure 5. Mesolimbic dopamine (shown in red) system circuitry in the rat brain. Dots represent cell bodies, while the arrows depict axonal terminals. Glutamatergic projections are shown in blue, dopaminergic in red, GABAergic in orange while orexinergic projections are shown in green (adapted after Kauer & Malenka, 2007).

Dopamine receptors

A receptor for a given substance is defined as the ability of the receptor-containing tissue to recognize and respond to small quantities of the particular substance in a precise way (Kebabian & Calne, 1979). Receptors are classified according to different criteria, but most commonly, they are classified with respect to the substance that they respond to endogenously. A stricter categorization is based on the combination of pharmacology (identification of artificial agonists and antagonists), anatomy, biochemistry and physiology, giving rise to receptor subgroups (Kebabian & Calne, 1973). Based on pharmacological analysis, DA receptors were originally differentiated into two major types: D1 and D2 (Kebabian & Calne, 1973). Upon their original discovery, Kebabian predicted the existence of sub-types of DA receptors within the two major groups, which was later demonstrated and described by Baldessarini & Tarazi (1996) and Sibley, et al. (1992). Based on their similarities, D1-like receptors include the D1 and D5 receptor subtypes, while the D2-like receptor group is formed by D2, D3 and D4 receptor subtypes (Tarazi, Kula, & Baldessarini, 1997). Lesion studies indicate that these receptors are located in specific brain regions, depending on their subtype. Most D1 and D2/3 subtypes arise on postsynaptic neurons within the NAcc and striatum, while the D4 receptors seem to have a more heterogeneous distribution, with some located on postsynaptic striatal neurons and others located on corticostriatal afferents to NAcc. A detailed description of the properties of DA receptors and their subtypes is illustrated in Table 2 (Baldessarini & Tarazi, 1996).

Table 2. Configuration and binding properties of dopaminergic receptors

Types[a]	Amino acids[b]	Human chromosome[c]	Prominent tissue sites	Selective agonists	Selective antagonists[d]	Effectors
D_1 Group						
D_1 (1A)	446 (h) 446 (r)	5q 34–35	Bovine parathyroid, forebrain	Hydroxybenzazepines A-68930, CY-208-245, dihydrexidine	Halobenzazepines (SCH-23390, etc.), thioxanthenes	AC (+) (main effect), PLC (+) (?)
D_5 (1B)	477 (h) 475 (r)	4p-15.1–15.3	Hippocampus, thalamus	Hydroxybenzazepines	Halobenzazepines	AC (+)
D_2 Group						
D_{2L} (2Aα)	443 (h) 444 (r)	11q-22–23	Anterior pituitary, basal ganglia	Ergolines, (–)hydroxyaporphines, aminotetralins	Benzamides, butyrophenones, phenothiazines	AC (–), PLC (–), AA (+) K^+ channels (+), Ca^{2+} channels (–)
D_{2S} (2Aβ)	414 (h) 415 (r)	11q	Basal ganglia	Ergolines, (–)hydroxyaporphines	Benzamides, butyrophenones	AC (–), PLC (–), AA (+) K channels (+), Ca^{2+} channels (–)
D_3 (2B)	400 (h) 446 (r)	3q-13.1	Limbic system, midbrain, cerebellum	(+)7-OH-DPAT, (+)PD-128-907, pramipexole, dihydrexidines	Benzamides, (+)UH-232, S-14297	AC (–) (?), weak GTP effect
D_4 (2C)	387 (and variants) (h) 368 (r)	11p-15.5	Frontal cortex, limbic system, blood vessels	Ergolines	Clozapine, olanzapine, (+)aporphines, NGD-94-1	AC (–), AA (+)

A functionally different group of DA receptors is comprised of release-regulating autoreceptors, found predominantly within the NAcc (said to be "reuptake-dominated"), and almost absent within the medial prefrontal cortex (mPFC) (Tzschentke, 2001).

Dopaminergic mediation of appetitive responses

DA has both immediate and long-term effects on cells (Zellner & Ranaldi, 2010). Acutely, DA serves a modulatory role, increasing post-synaptic excitability (Surmeier, Plotkin, & Shen, 2009). During longer exposure, DA plays a role in synaptic plasticity which results in a cascade of processes leading to an increased expression of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, as illustrated in Figure 6 (see Jay, 2003 for review). The D1 receptor coupled to adenylate cyclase (AC) increases AC activity, which results in the formation of cAMP that subsequently activates the

cAMP-dependent protein kinase A (PKA). Activated PKA phosphorylates both AMPA and NMDA receptors and the dopamine and cyclic adenosine monophosphate-regulated phosphoprotein (DARPP-32), which then is converted to a potent inhibitor of protein phosphatase 1 (PP1), promoting the phosphorylation of CaMKII. At the same time, activation of NMDA receptors increases the influx of Ca^{2+} into the cell, which in turn activates the protein phosphatase 2B (PP2B) calcineurin. Calcineurin dephosphorylates DARPP-32 which then promotes dephosphorylation through the disinhibition of PP1. Concurrently, PKA also phosphorylates cyclic adenosine monophosphate response element bonding protein (CREB), switching this transcription factor from an inactive to an active state. Overall, the control of PP1 via DARPP-32, a key regulator of DA transmission and NMDA receptors activity, is likely to have a significant effect on the regulation of LTP (Jay, 2003).

Based on the difference in effects with respect to exposure times, added to the fact that DA elicits various responses depending on the brain area subjected to exposure, dopamine has been shown to play crucial roles in basic reward, reinforcement and learning, effort, behavioural "switching", reward prediction error and "wanting" (for review see Zellner & Ranaldi, 2010). The present study is particularly interested in dopaminergic activity within the rat mesolimbic system and its role in reward and reinforcement.

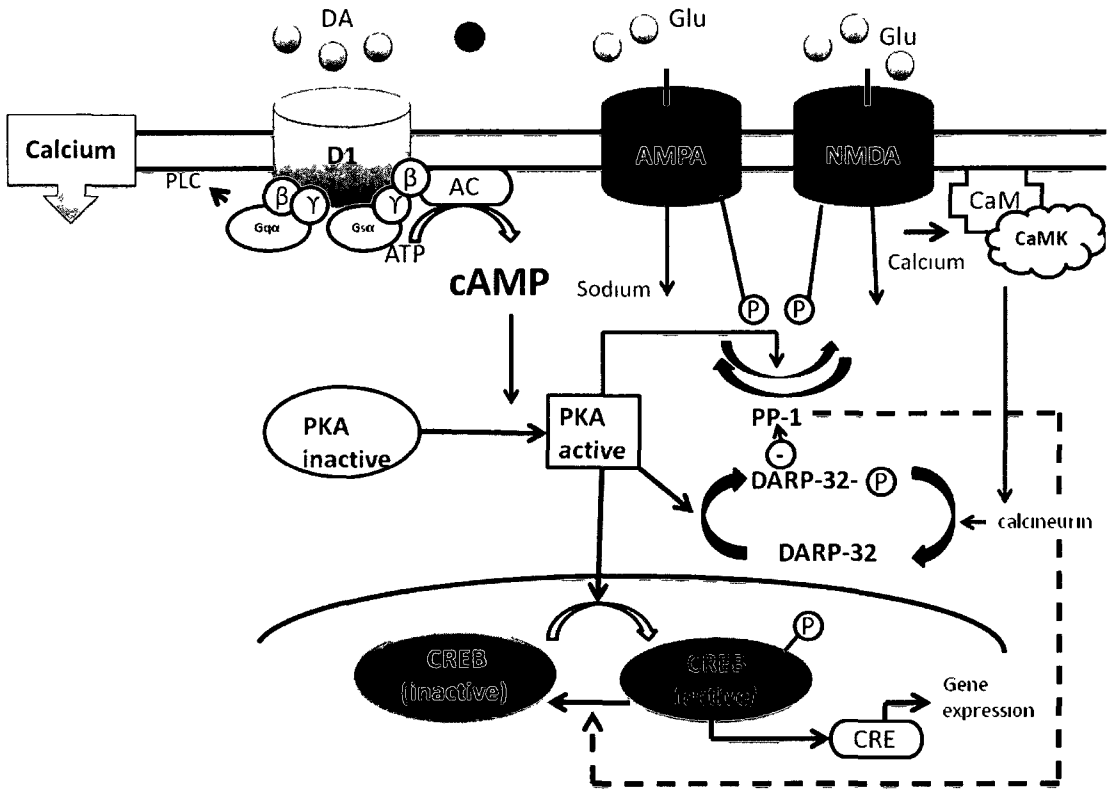


Figure 6. Schematic representation of the contribution of the D1 signaling pathway in the DA regulation of LTP (adapted after Jay, 2003).

The NAcc is a central structure connecting limbic and basal ganglia systems, receiving afferents from the prelimbic cortex, amygdala, substantia nigra and the VTA (Mogenson, Jones, & Yim, 1980). Extensive research has shown that NAcc DA has been implicated in two major roles: *invigoration of approach* and *incentive learning* (Ikemoto, 2007). DA activity in the NAcc has been shown to be related to motivational effects of rewarding stimuli, such as food or sex (Sesack, Carr, Omelchenko, & Pinto, 2003; Ahn & Phillips, 2007). In a microdialysis study, Hajnal, et al., (2004; 2005) demonstrated a sucrose concentration-dependent increase in extracellular DA levels in the NAcc. Moreover, Hajnal et al (2003) showed that intraorally applied saccharin results in an increase in extracellular levels of DA within the NAcc in naïve

rats. Similarly, Ahn and Phillips (2007) show an increased DA efflux in the NAcc during early and later training stages of an instrumental response for food in rats. These results suggest that DA activity within the NAcc is dependent on both the *behaviour* associated with reward seeking, as well as the autonomic response to *sucrose intake*.

In an exploratory study of the mesolimbic system, Woodward et al. (2006) recorded neuronal activity for long periods of time within the NAcc using arrays of microwires during freely moving reward-related behaviours. In their study, rats were trained on a FR1 schedule to press a bar for intravenous cocaine administration, hypothesizing that neural signals would be recorded in the NAcc specifically related to reward. However, phasic excitatory and inhibitory neural activity appeared prior to cocaine administration. Furthermore, small populations of neurons within the NAcc were shown to respond differently to the type of the cue presented (visual or auditory), and the type of reinforcer (cocaine vs. heroin), suggesting that the accumbens not only responds during reward administration, but is also able to code for the anticipation of reward.

Studies show that within the NAcc itself, two different anatomical and functional pathways exist: the ventral striatum (NAcc medial shell) involved in stimulus-outcome learning, and the ventro-lateral striatum (NAcc core), involved in stimulus-action association, also known as stimulus-appropriate responding (Ikemoto, 2007). For example, in a reinstatement of drug seeking paradigm, Anderson, Schmidt, & Pierce (2006) showed that following administration of the D2 antagonist sulpiride into the shell, but not core of the NAcc, rats showed an attenuated priming-induced reinstatement of cocaine seeking behaviour. Injections of the D1 receptor antagonist SCH-23390 into NAcc shell but not core dose-dependently attenuated drug seeking induced behaviour in rats (Anderson, Bari, & Pierce, 2003). This anatomical and physiological

dichotomy of the NAcc is further supported by research involving the administration of a D1 and D2 agonists (SKF-81297 and quinpirole, respectively) in the shell and core. Schmidt, Anderson, & Pierce (2006) reported that microinjections of the D1 and D2 agonists within the shell, but not the core of the NAcc reinstated cocaine-seeking behaviour in rats. Finally, Sellings & Clarke (2006) showed that 6-OHDA lesions of the medial shell, but not core of the NAcc disrupted conditioned place preference induced by intravenous administration of the psychomotor stimulant methylphenidate. In an *in vivo* electrochemical study of DA clearance within the shell and core subregions of the NAcc (David, Zahniser, Hoffer, & Gerhardt, 1998) the core and shell were shown to have different DA clearance properties as measured by extracellular DA signal amplitudes, clearance times and clearance rates. With the amount of DA controlled for, DA clearance signals recorded in the shell had greater amplitudes, but faster clearance rates compared to the core, suggesting a different activity in the DA transporter (DAT) in the shell compared to the core (David et al., 1998).

The battery of studies on dopaminergic function and organization within the mesolimbic system gave rise to a number of hypotheses, summarized by Ikemoto (2007). *The anhedonia hypothesis theory* is based on human studies, where drugs such as cocaine and amphetamines elicit subjective effects characterized as euphoric. Animal research showed that DA deficient mice starve to death, even though food and water are present *ad libitum* (Szczypka, et al., 2001). However, these mice would consume the food if directly delivered into their mouths, suggesting a DA-mediated lack in motivation based on the rewarding presentation of food.

The psychomotor stimulant reward theory addresses the fact that animals, including humans elicit forward locomotion in the presence of rewarding stimuli, such as cocaine (Ikemoto, 2007). Since DA depletion results in a lack of interest in pleasurable activities

(anhedonia), then the hyperactivity of the dopaminergic systems will result in reward-seeking behaviour, as seen in conditioned place preference studies and self-administration paradigms. Other hypotheses proposed by Ikemoto (2004) include the anergia hypothesis, the wanting vs. liking hypothesis, the incentive-salience hypothesis/incentive sensitization theory of addiction, the hedonic homeostatic dysregulation hypothesis and finally, the prediction-error hypothesis. A detailed description of each hypothesis is beyond the scope of this thesis, but these approaches needed to be at least mentioned in an effort to show the extent of research performed in this field, as well as to address the complexity of the issue.

Glutamatergic-induced DA release

Behavioral considerations

The process of learning has evolved as a basic adaptive need, responsive to the constantly changing of both the environment and personal needs. Organisms have successfully learned to exploit areas rich in primary resources, such as food, and to avoid situations that can potentially harm them. Such behaviour has been demonstrated within laboratory settings, where a food-restricted rat quickly learns to perform a behavioural task (such as pressing a bar) in order to obtain food. Similarly, in a conditioned place preference (CPP) paradigm, where rodents learn to associate certain environmental cues (such as the texture of the cage floor or the colour of the walls) with aversive (i.e. electric shock) or hedonic stimuli (i.e. cocaine administration, sexually receptive animal), the animal will learn to spend more time in the environment associated with the reinforcing stimulation and avoid settings associated with harmful experiences (Mueller & Stewart, 2000; Ferguson, Patton, Bopp, Meagher, & Grau, 2004).

The process in which a consistent behavioural response is elicited based on the repeated reinforcing nature of the stimulus is called *acquisition*. During this stage, synaptic plasticity occurs at a molecular level within the central nervous system (CNS). This phenomenon is responsible for the strengthening of pathways, as well as formation of new connections between neurons (Martin, Ledent, Parmentier, Maldonado, & Valverde, 2000). This is a form of neural adaptation in response to experiencing new environments, and is mainly under glutamatergic control, where the neurotransmitter glutamate (Glu) promotes long term potentiation (LTP) (Martin et al., 2000). In the event where the reinforcer is being removed, the animal's response (which initially resulted in rewarding stimulation) will slowly fade away. As mentioned above, this process is known as *extinction*. Extinction is a secondary type of learning: animals that previously learned that certain behaviour will result in hedonic stimulation will now learn that the behaviour that once resulted in reward is obsolete once the reinforcer is removed (or at least not associated with the previous behaviour any longer).

Since learning is the main mechanism underlying extinction, it is reasonable to consider that the key player behind this behaviour is the glutamatergic system. This hypothesis is supported by previous experiments performed in our laboratory (Holahan, et al., 2010), where rats treated with the NMDAr antagonist MK-801 prior to testing were unable to show an extinction response compared to controls. Santini, Muller, & Quirk (2001) studied a conditioned fear response where a tone was paired with a foot shock, and examined the effects of an NMDAr antagonist (CPP) on the animal's conditioned responses: freezing and suppression of bar pressing. The rats extinguished normally while under systemic CPP administration, but failed to show 24-hour memory for the experience suggesting that NMDA activation was necessary for long-term memory extinction but not short-term performance of the task. These results partly

contradict the findings of Holahan et al. (2010), where, as mentioned above, rats were not able to show short-term extinction while tested after an MK-801 injection. One reason behind this discrepancy might be due to the fact that the NMDA receptor antagonists used in these studies were different. Also, different neuronal systems or loci (for example amygdala for fear and NAcc for appetitive reward) might be responsible for extinction behaviour within these two paradigms. A third possibility is that the NMDA receptor antagonists have secondary actions on other neurotransmitter systems that are involved in the behaviors under study.

Glutamatergic-dopamine molecular interactions

DA hyperactivity within the NAcc can be induced by glutamatergic blockade via NMDAR antagonist administration. Jentsch, et al.,(2008) showed that enhanced DA transmission in the NAcc is induced by acute phencyclidine (PCP) administration (noncompetitive NMDAR antagonist). The over-activation of the mesolimbic DA pathway via acute glutamatergic blockade has been shown to result in motor (Tang, et al., 2006) and cognitive impairments (for review, see Jentsch et al., 1998) and is reversible by DA antagonist pre-treatment (Anderson, et al., 2003; Bari & Pierce, 2005). The medial prefrontal cortex (mPFC) both sends and receives glutamatergic projections to and from the VTA and NAcc directly (Tzschentke, 2002). Adding to the complexity of this network, GABAergic interneurons seem to be involved in mPFC-originating synaptic interactions (Tzschentke, 2002). Crosstalk between the glutamatergic and dopaminergic systems has been previously demonstrated by co-injecting an NMDA antagonist with amphetamine (DA agonist) resulting in the enhanced release of DA (Carlsson et al., 2001). Similar interactions have been reported, where a non-dose dependent release of DA was observed in rats, following MK-801 treatment (Miller & Abercrombie, 1996). Previous studies by Carlsson (Carlsson & Carlsson, 1990; Carlsson, 1995) revealed a synergistic relationship

between a variety of monoamines (apomorphine, SKF 38393: a mixed D1/D2 agonist, clonidine, an α2-adrenergic agonist and LSD: a 5HT2 agonist) and NMDA blockade with MK-801 on elevating dopamine release.

Previous research has shown increased locomotor activity upon both MK-801 (Tang, et al., 2006; Seillier & Giuffrida, 2009) and DA agonist administration (Swanson, et al., 2006). Based on this, a locomotor analysis was included to assess and compare possible effects of D1/D2 receptor antagonist and DA/DNA aptamer central administration. Motor activity was assessed with a cross maze measuring exploratory behaviour by the means of distance travelled, speed, arm entries and rearing behaviour.

A simplistic scheme of the interaction between the glutamatergic and dopaminergic systems, mediated by GABA is illustrated in Figure 7. Here, the cell bodies of glutamatergic neurons (red), if stimulated elicit an excitatory response on GABAergic (green) and dopaminergic neurons (brown) within the VTA. An activation of GABAergic interneurons within the VTA triggers an inhibitory response elicited on the DA VTA neurons which in turn will result in a decreased release of DA within the NAcc (blue). The cortical glutamatergic stimulation will also result in a direct stimulation of VTA dopaminergic neurons, bypassing the GABAergic interneurons. The overall amount of DA released within the NAcc following cortical glutamatergic stimulation is then mediated by the balance between the VTA neurons which are subjected to GABAergic influence and cells under direct cortical glutamatergic innervation.

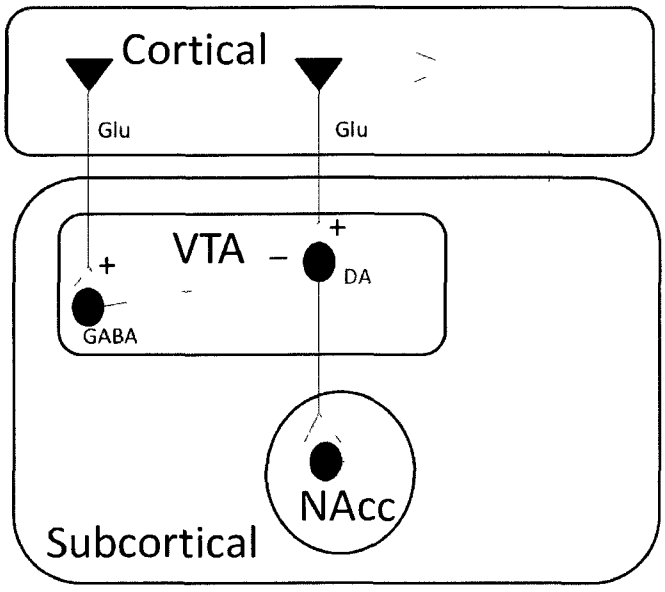


Figure 7. Hypothetical scheme showing glutamatergic/DA/GABA interactions (adapted after Carlsson et al., 2001). Dots and triangles represent cell bodies, while arrows represent axon terminals.

Rationale and study design

MK-801, administered peripherally has been shown to increase DA release within the NAcc and produce alterations in motor and cognitive behaviours. The present study explored the effects of *in vivo* administration of the novel dopamine DNA aptamer (DA antagonist) via a set of behavioural analyses on pre-treated (acute, subcutaneous injection) MK-801 male Long Evans Hooded rats.

Hypoglutamatergia was obtained through subcutaneous administration of the NMDA selective antagonist MK-801 while dopaminergic antagonism was induced via local administration (NAcc) of SCH-23390 (D1 antagonist) or haloperidol (D2 antagonist). It was hypothesized that NMDA blockade would result in disinhibition of a GABA-mediated control in

DA release within the NAcc. The GABA interneuron has an inhibitory effect between the glutamatergic and DA systems, where NMDA antagonism results in the loss of the GABAergic inhibitory effect. In turn, dopaminergic neurons (within the VTA) become hyperactive, resulting in increased release of DA within the NAcc.

The DNA DA aptamer acts by binding to dopamine (and norepinephrine) molecules *in vitro* (Walsh & DeRosa, 2009). The effects of the DA-DNA aptamer administration *in vivo* are expected to be comparable to those of typical dopamine D1 and D2 receptor antagonist administration, SCH-23390 and haloperidol as assessed *via* behavioural analysis.

In order to address the potential effects of the DNA DA aptamer on behaviour, rats were assessed on a) extinction of a *rewarded response* and b) *motor activity*.

Materials and methods

Animals

Male Long Evans Hooded rats (N = 46,Charles River, St. Constant, Quebec, Canada) were housed in groups of two in polycarbonate 48 X 26 X 20 cm^3 cages prior to surgery and individually housed post surgery, within a temperature controlled environment (21^0C). All animals were subjected to a 12-hour light/dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food (Purina rat chow) prior to surgery, and around ten days post surgery, during recovery. Additionally, rats were handled for five minutes each day for seven days, to minimize stress. Approximately ten days following the intracranial surgical procedure (described in the following section), rats were placed on a food restriction schedule for approximately eight days, until each animal reached a target of 85% of its initial weight (250-300 g). All animal procedures were approved by the Canadian Council on Animal Care (CCAC) and Carleton University Ethics Board, Ottawa, Canada.

Surgical procedure

Animals were subjected to general anesthesia, using Isoflourane (3% in pure O_2 to induce and 2-4% to maintain). The scalps were shaved, and the rats were fixed in a stereotaxic apparatus (Stoeling Instruments) using two lateral ear bars (Xylocaine was applied on the tips of the ear bars to minimize potential pain and discomfort) to assure proper placement. The exposed skin was then cleaned using 70% surgical alcohol and iodine solution. To avoid eye dryness and damage, tear gel was applied on the surface of each eye. A 1cm anterior-to-posterior incision was made, starting at the midline between the two orbital cavities, using a 10mm stainless steel scalpel. The skin was removed and fixed lateral to the incision, using four haemostats (two on

each side of the incision). The skull was exposed and cleaned with sterile gauze, and bregma was identified. Two screws were placed into the skull, approximately 4mm anterior, posterior and lateral to bregma, serving as anchors. Two stainless steel 12mm guide cannula (25 Gauge) were implanted bilaterally, targeting the nucleus accumbens (NAcc) at the following coordinates relative to bregma: antero-posterior (AP) = -1.7, latero-medial (LM) = +/-1.5 and dorso-ventral (DV) = -6 (Figure 8). Cannula were secured in place using dental cement and skull screws serving as anchors. Obturators (32 Gauge) were inserted in each cannula to avoid possible obstructions within the steel tubes. After the dental cement was allowed to dry and the cannula and obturators were fixed into the skull, antibiotic ointment (Polysporin) was applied directly on and around the incision. The incision was then closed using surgical suture and topical Lidocaine was applied around the wound. Additionally, a subcutaneous dose (0.2 ml) of the analgesic Metacam was administered. Following surgery, animals were individually placed in a clean home cage, situated on a heating pad for approximately an hour within the post-op room, under constant surveillance. Surgeries lasted approximately 60 minutes, and all surgical instruments were autoclaved prior to the first surgery and cleaned/sterilized after each consequent surgical procedure.

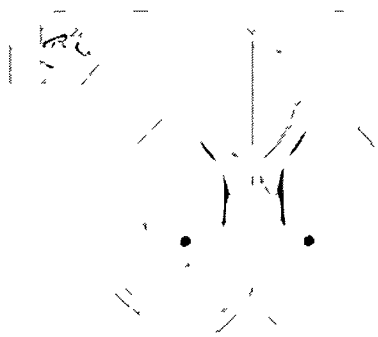


Figure 8. Estimates of cannula placements (black dots) according to stereotaxtic calculations.

Operant chambers and cross maze

Animals were trained and tested in groups of six (one per cage) using operant chambers (Habitest Operant Cage, Coulbourn Instruments; 30.5 cm W x 25.5 cm D x 30.5 cm H), housed in sound-proof Styrofoam casing. The cages were equipped with a house light, two levers, a food hopper and a set of three LED lights (red, yellow and green) located immediately above the response-generating lever, as shown in Figure 9.

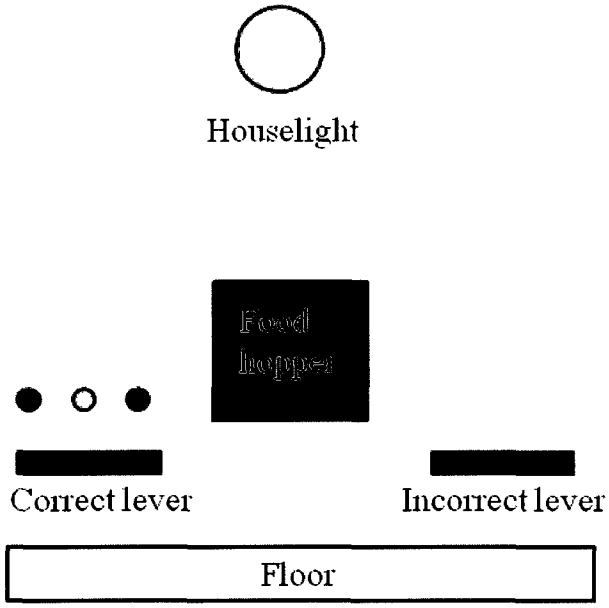


Figure 9. Illustration of the "rat's view" while facing the cued wall in the operant chamber.

The cross maze consisted of four arms (100 cm L x 6cm W) perpendicular to each other. Each arm consisted of an opaque floor and transparent Plexiglas walls (8 cm H). Motor activity was recorded using an HVS tracking system (HVS Image TM).

Drugs

MK-801 was purchased from Sigma-Aldrich and dilutions of 0.05mg/ml were prepared, using sterile 0.9% NaCl. 100nM, 200nM aptamer and random oligonucleotide doses were

obtained from the DeRosa laboratory (Chemistry Department, Carleton University, Ottawa, ON). Haloperidol (Sigma-Aldrich) was mixed into 50% ethanol at a 1 mg/ml stock solution. SCH 23390 (Sigma-Aldrich) was dissolved in 0.9% sterile saline and stored as a 1mg/ml stock solution. All solutions were stored at -20^{0}C, and thawed the day it was used.

Injections

The experiments commenced after a recovery period of at least ten days. Injections were administered a) centrally and b) peripherally.

Central administration

Obturators were removed and two stainless-steel injection cannula (13 mm, 32 Gauge) connected to two 10 µl Hamilton syringes by polyethylene tubing were inserted down the 12 mm, 25 Gauge guide cannula. Syringes were connected to an injection pump (Braintree Scientific, Inc.), programmed to deliver a 0.5 µl injection of either haloperidol, SCH 23390, DNA-DA aptamer, random oligonucleotide or Tris in the NAcc at a rate of 0.25 µl/min. The injection cannula was left in place for an additional 60 s, allowing the substance to diffuse throughout the targeted tissue. During microinjections, each animal was allowed to move freely in the home cage, in order to minimize any stress associated with prolonged restraint.

Peripheral administration

Each animal was administered a subcutaneous injection of MK-801 (0.3 ml) immediately after the microinjection protocol was completed and 15 min prior to testing.

Behavioural design

Experiment 1

The purpose of the first experiment was to determine whether DA antagonists (D1, D2 and DNA DA aptamer) influenced extinction in MK-801 injected rats, following a five-day acquisition period (training). As described above, food-deprived rats were trained to press a lever for food according to a FR2 schedule for five consecutive days. Following a two-day break, on the seventh day the animals were separated into groups as follows: Vehicle/MK-801 (n = 5), 200nM(aptamer)/MK-801 (n = 6), Random/MK-801 (n = 5), Vehicle/Saline (n = 5), 200nM(aptamer)/Saline (n = 5), D1/MK-801 (n = 7) and D2/ MK-801 (n = 7). All animals were injected according to the injection protocol described above and placed in the operant chambers for 30 min. During training (aquisition) and testing (extinction), the number of bar presses (correct and incorrect) and nose pokes were recorded every 5 min.

Experiment 2

During the second experiment, motor performance was assessed, under identical drug and dietary manipulations as described in experiment 1. Following injections, rats were placed in a cross maze and motor activity was recorded (speed, arm entries, distance travelled) over a period of 1800 s.

Histology

Approximately two days after the last experimental procedure, animals were sacrificed (decapitation) and brains were collected and stored in 4% paraformaldehyde (PFA) at 4^0C. To verify cannula placements, the tissue (both hemispheres) was sliced into 30 μm coronal sections

using a Leica CM1900 cryostat. Sections were mounted onto slides, and then stained with cresyl violet. Each slide was submerged for approximately 2 minutes in each of the following solutions: 100%, 95% and 70% ethanol. After rinsing with distilled water, slides were placed in 1% cresyl violet solution for 3 minutes. Sections were again rinsed in distilled water to wash away any excess stain, after which they were immersed in a .8% acetic acid solution for 3-5 minutes. The sections were subjected to the 100%, 95% and 70% ethanol submersion for 2 minutes each. Finally, the slides were placed in a clarene solution for 15 minutes before coverslipping (using clarion). Slides were examined (4x, 10x magnifications) and photographed using an Olympus light microscope.

Results

Experiment 1: *Operant Conditioning*

The purpose of the first experiment was to assess the effects of NAcc administration of the DA-DNA aptamer on rats that have been treated with peripheral MK-801. Rats were assigned to six groups, and each animal received a central injection of either aptamer, random oligonucleotide, tris, D1 receptor antagonist (SCH 23390) or D2 receptor antagonist (Haloperidol), followed by a subcutaneous injection of either saline or MK-801, as described in the *Methods* section above. When assigned to their respective groups, rats were matched based on correct bar pressing performance on the fifth day of training. Correct lever presses and nose pokes were recorded every 5 minutes, over a period of 30 minutes. The data were analyzed using the IBM SPSS 19 software.

Acquisition: the Aptamer cohort

In order to minimize within group variability, animals were assigned to groups based on their performance on the fifth day of training. A repeated measures ANOVA (with "Day" as the within subjects factor, and "Treatment" as the between subjects factor) was conducted to ensure that all groups were equivalent prior to beginning of testing. No effect of treatment between the Tris/MK-801 (n = 5), 200nM/MK-801 (n = 6), Random/MK-801 (n = 5), 0nM/Saline (n = 5) and 200nM/Saline (n = 5) groups was observed, $F(4,21) = 2.04$, $p = .13$, suggesting that there was no significant difference between groups during the 5 days of training (Figure 10).

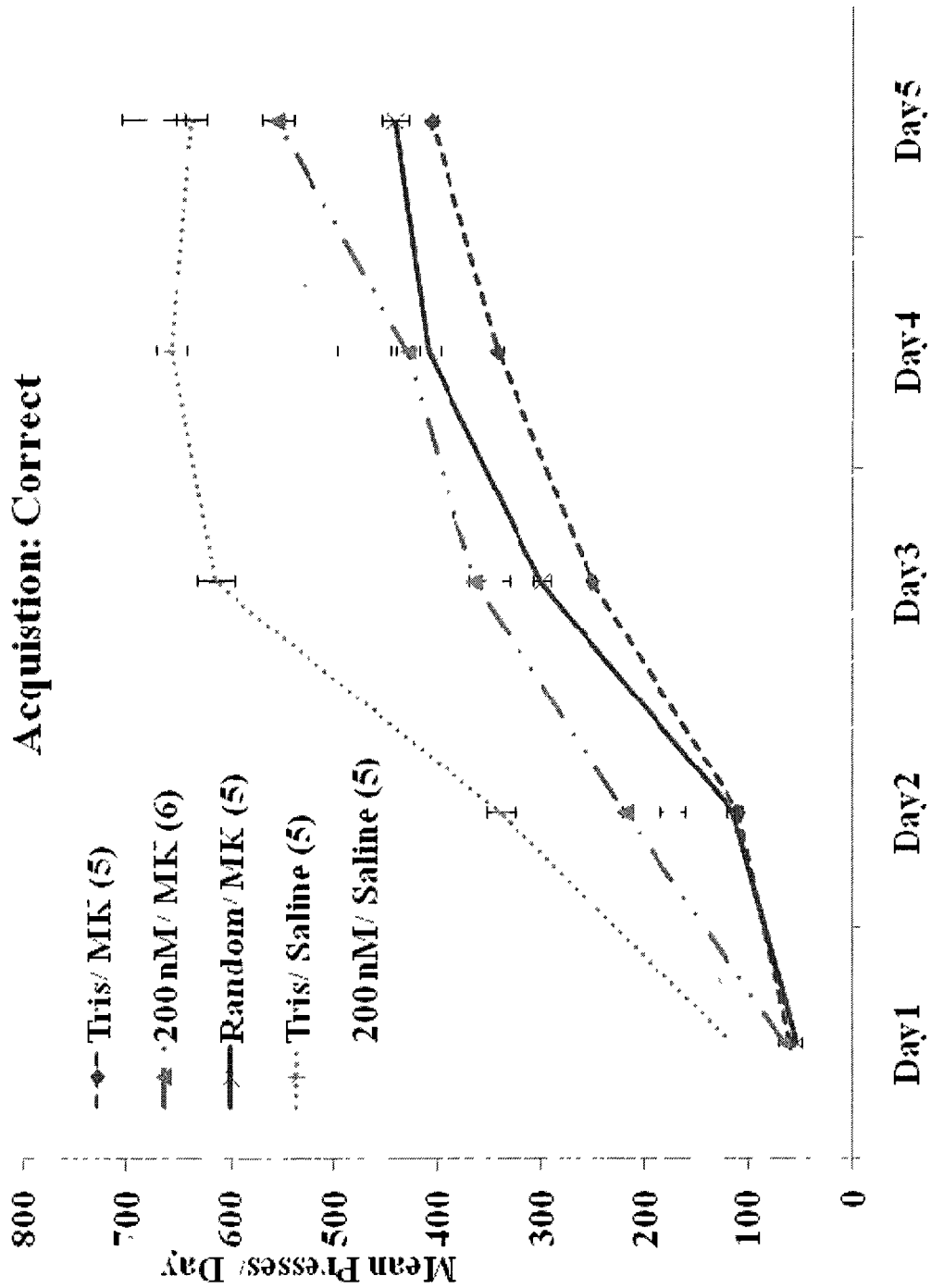
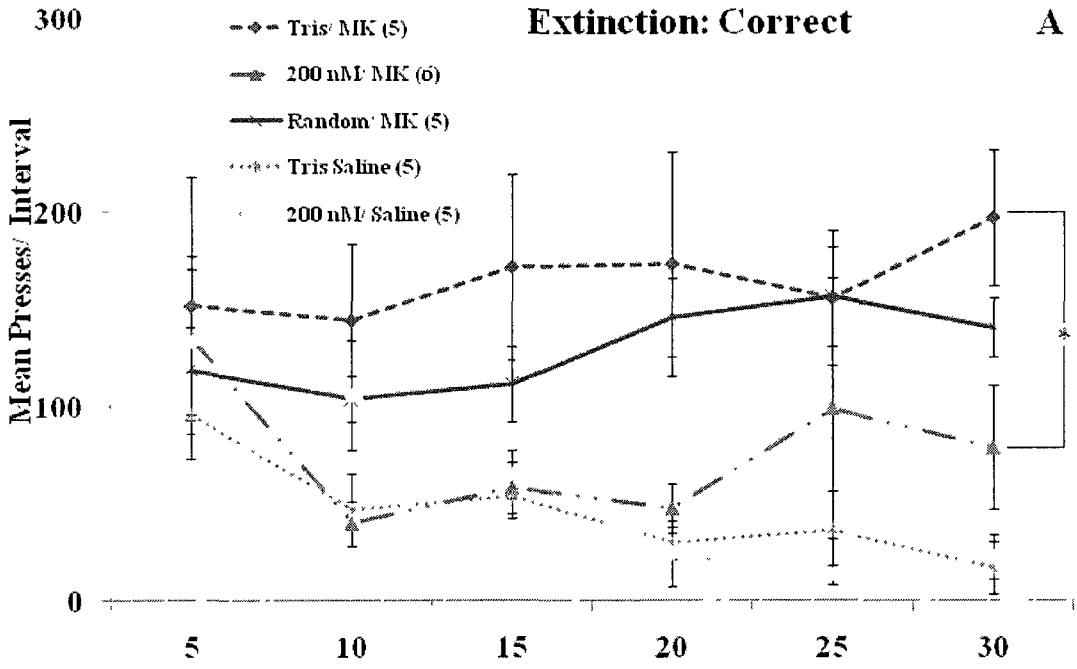

Acquistion: Correct
Tris/ MK (5)
200 nM/ MK (6)
Random/ MK (5)
Tris/ Saline (5)
200 nM/ Saline (5)
Mean Presses/ Day
800
700
600
500
400
300
200
100
0
Day1
Day2
Day3
Day4
Day5

Figure 10. Average presses across 5 days of training, prior group assessment. Following the fifth day of training, animals were divided into five treatment groups: Tris/MK-801 (n = 5), 200nM/MK-801 (n = 6), Random/MK-801 (n = 5), Tris/Saline (n = 5) and 200nM/Saline (n = 5). No significant differences were observed between assigned treatment groups at $p < .05$.

Extinction: the Aptamer cohort

During extinction, all animals were subjected to treatment, respective of the groups described above. Fifteen minutes after treatment administration, animals were placed in the operant chambers, where correct presses and nose pokes were recorded and analyzed every 5 minutes over a period of 30 minutes. In an effort to show any possible effects on correct bar presses, repeated measures ANOVA was performed, yielding a significant effect of treatment, $F(4,21) = 3.32$, $p < .05$. Post hoc analysis (LSD) showed significant differences between the Tris/MK-801 group and 200nM/MK-801 group ($p = .025$), implying that the 200nM/MK-801 group pressed significantly less than the vehicle-treated MK-801 cohort. Similarly, post hoc analysis indicates that the Vehicle/MK-801 treated animals pressed significantly higher than the Tris/Saline ($p < .05$) and 200nM/Saline ($p < .05$) groups. As expected, there was no significant difference in cumulative bar presses between MK-801 injected rats, pre-treated with Tris or random oligonucleotide mix ($p = .33$). Furthermore, there was no significant difference between rats in groups 200nM/MK-801, 0nM/Saline and 200nM/Saline (Figure 2A).

A repeated measures ANOVA was performed to assess possible effects of treatment on nose pokes over 30 minutes. A significant overall effect of treatment was observed, $F(5,24) = 3.40$, $p < .05$. LSD post hoc analysis showed that animals in the 200nM/Saline group recorded a significantly lower lumber of cumulative nose pokes compared with the Tris/MK-801 group ($p < .05$) and Random/MK-801 group ($p < .05$) (Figure 2B). The aptamer pre-treated MK-801 rats showed decreased nose pokes compared to the random/MK-801 group, but this difference was not significant at $p < .05$ ($p = .073$).


Extinction: Correct
A
Tris/ MK (5)
200 nM/ MK (6)
Random/ MK (5)
Tris Saline (5)
200 nM/ Saline (5)
Mean Presses/ Interval
300
200
100
0
5
10
15
20
25
30

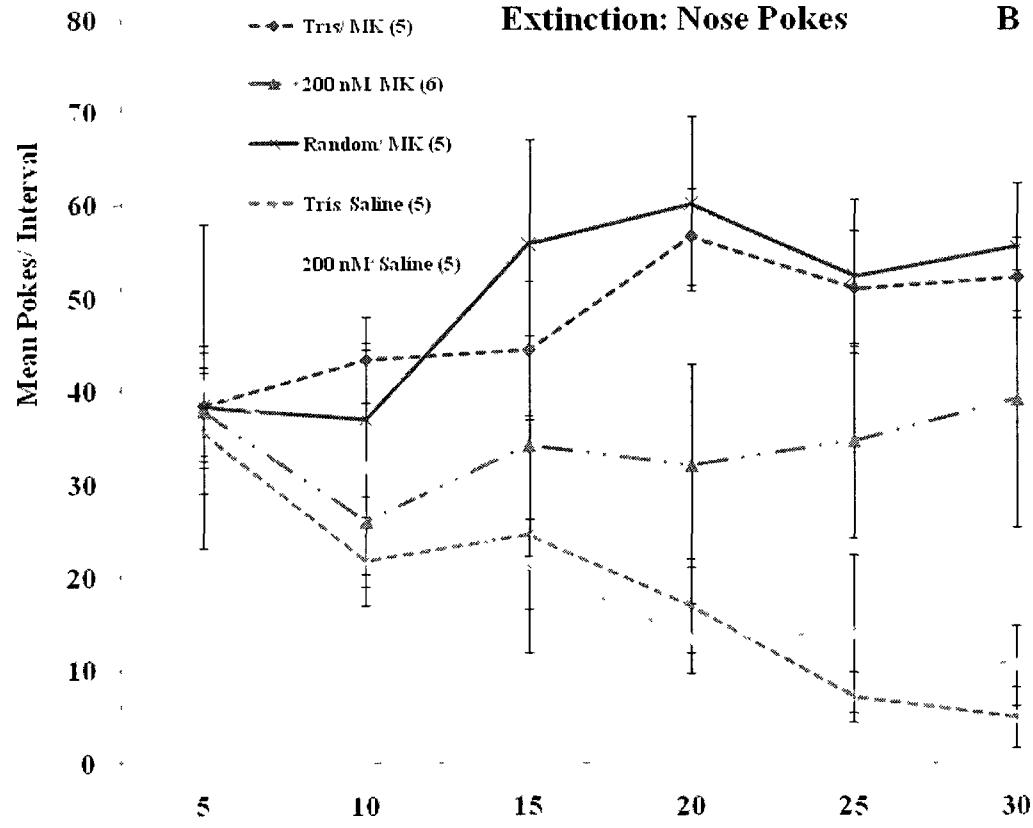

Extinction: Nose Pokes
B
Tris/ MK (5)
200 nM/ MK (6)
Random/ MK (5)
Tris Saline (5)
200 nM/ Saline (5)
Mean Pokes/ Interval
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30

Figure 11. Rat performance measured every 5 minutes over a 30 minute interval showing A) correct bar presses and B) nose pokes. MK-801 animals that received an aptamer pre-treatment pressed significantly less compared with the non-treated (random and Tris) MK-801 rats. Such difference was also observed measuring nose pokes, however, the difference failed to show significance at $p < .05$. Note: * = $p < .05$

Acquisition: the D1/D2 antagonist cohort

The training schedule, group assignment and analysis for the D1/D2 antagonist cohort followed identical steps as described for the *aptamer cohort*. A repeated measures ANOVA was performed, and no effect of treatment group was observed during the 5-day training session between the Tris/MK-801 ($n = 6$), D1/MK-801 ($n = 7$) and D2/MK801 ($n = 7$) groups, $F(2,17) = 1.15$, $p = .34$ (Figure 12).

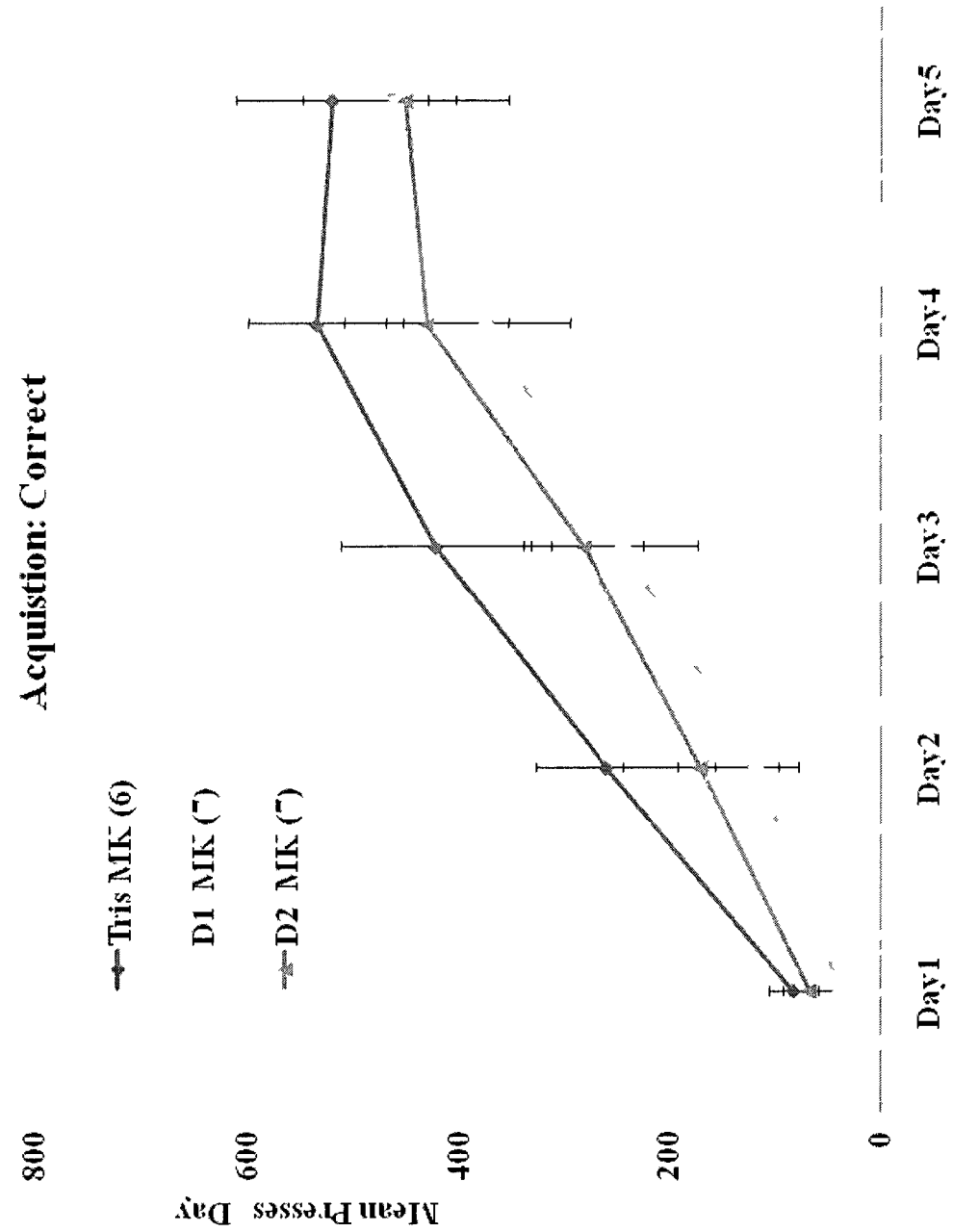
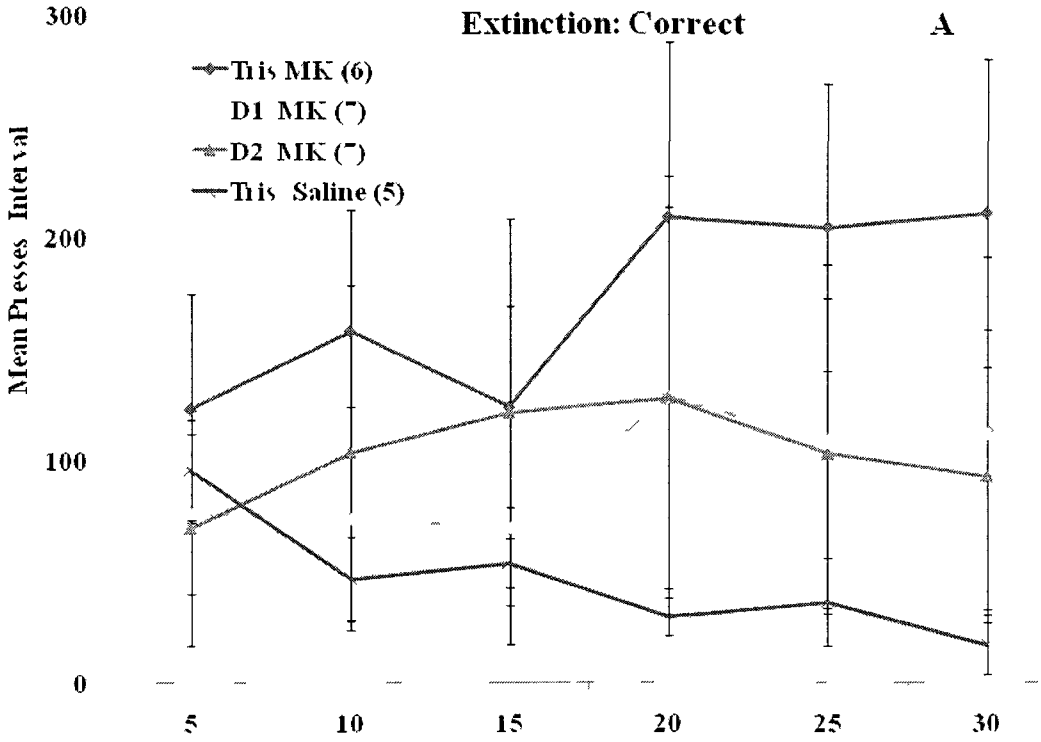

Acquistion: Correct
Tris MK (6)
D1 MK (7)
D2 MK (7)
Mean Presses Day
800
600
400
200
0
Day1
Day2
Day3
Day4
Day5

Figure 12. Average presses across 5 days of training, prior group assessment. Following the fifth day of training, animals were divided into three treatment groups: Tris/MK-801 ($n = 6$), D1/MK-801 ($n = 7$) and D2/MK-801 ($n = 7$). No significant differences were observed between assigned treatment groups at $p < .05$.

Extinction: the D1/D2 antagonist cohort

The methodology and analysis for the *D1/D2 antagonist cohort* was performed identically as described for the *Aptamer cohort*. Two repeated measures ANOVAs were performed, to investigate the possible effects of treatment on correct bar presses and nose pokes over a period of 30 minutes. No significant difference in correct presses was found between treatment groups, $F(3,21) = .36$, $p = .78$. Similarly, there was no significant difference in nose pokes between treatment groups, $F(2,17) = .46$, $p = .64$.


Extinction: Correct
A
Tris MK (6)
D1 MK (7)
D2 MK (7)
Tris Saline (5)
Mean Presses Interval
300
200
100
0
5
10
15
20
25
30

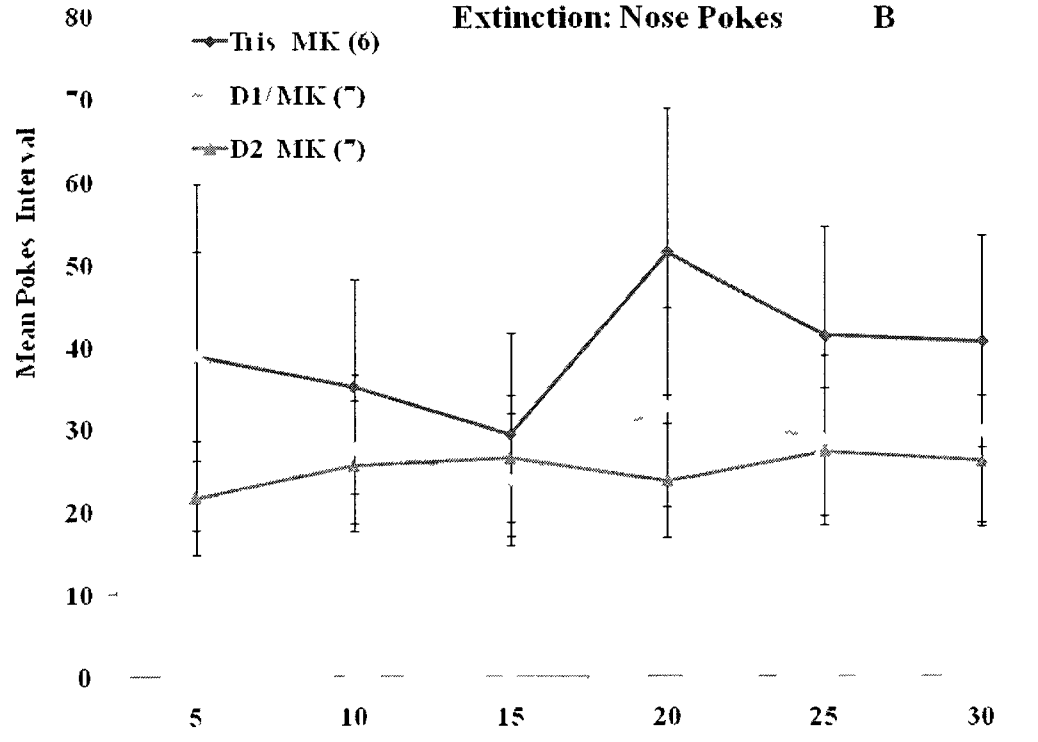

Extinction: Nose Pokes
B
Tris MK (6)
D1/MK (7)
D2 MK (7)
Mean Pokes Interval
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30

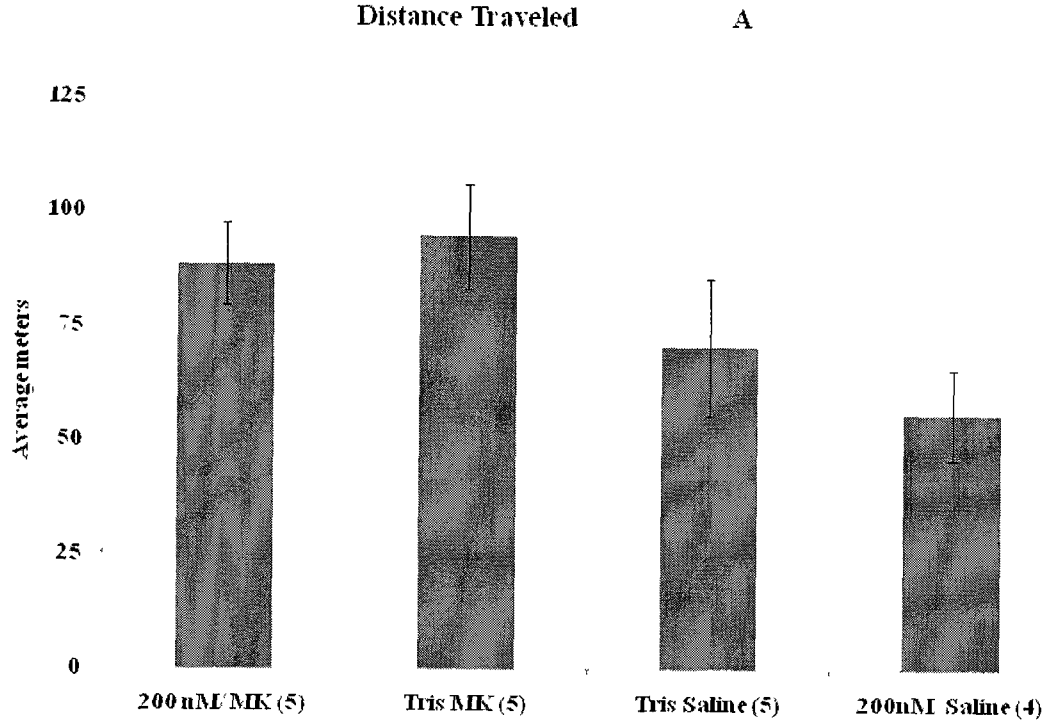
Figure 13. Rat performance measured every 5 minutes over a 30 minute interval showing A) correct bar presses and B) nose pokes. There was no significant difference in either correct bar presses or nose pokes between animals that received Tris, D1 receptor antagonist or D2 receptor antagonist prior to MK-801 subcutaneous administration.

Experiment 2: *Motor Assessment*

All animals were assessed on a motor task, described in the *Methods* section. Briefly, the purpose of the motor assessment was to investigate whether the hypoglutamatergic-induced motor hyperactivity would be affected by primarily DA-DNA pre-treatment and secondarily by D1 or D2 antagonist pre-treatment. We have also measured and analyzed the effects of Tris and random oligonucleotide pre-treatment in both MK-801 and saline injected animals. Distance traveled and arm entries over a period of 30 minutes were measured and analyzed. Group assignment remained identical to experiment 1, meaning that the animals that received a certain treatment during the first experiment will receive the same treatment during this experiment.

Aptamer cohort

Two one-way ANOVAs were performed to investigate the possible differences in distance travelled and arm entries between the four treatment groups: 200nm/MK-801, 0nM/MK-801, 0nM/Saline and 200nM/Saline. No significant difference in distance travelled between treatment groups was observed, $F(3,15) = 2.05$, $p = .15$. When assessed on arm entry performance, the analysis yielded a significant difference of treatment group, $F(3,15) = 3.97$, $p < .05$. Post hoc analysis (LSD) indicated that the 200nM/Saline animals showed significantly less arm entries compared with the 200nM/MK-801 rats ($p = .01$).


Distance Traveled
A
125
100
75
50
25
0
Average meters
200 nM MK (5)
Tris MK (5)
Tris Saline (5)
200nM Saline (4)

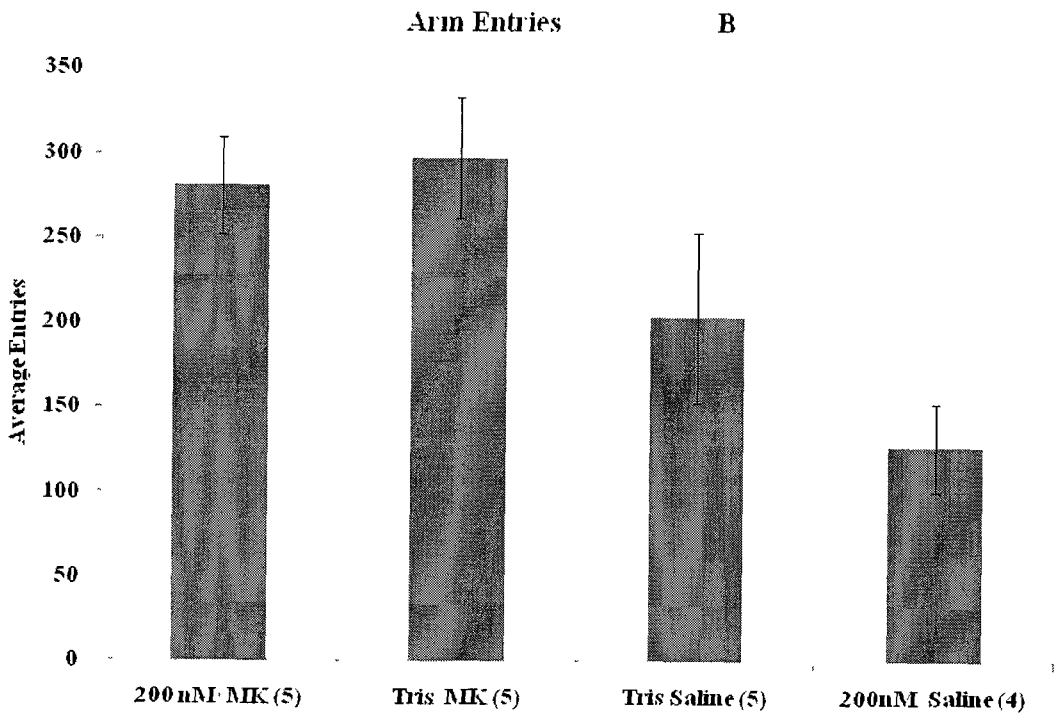
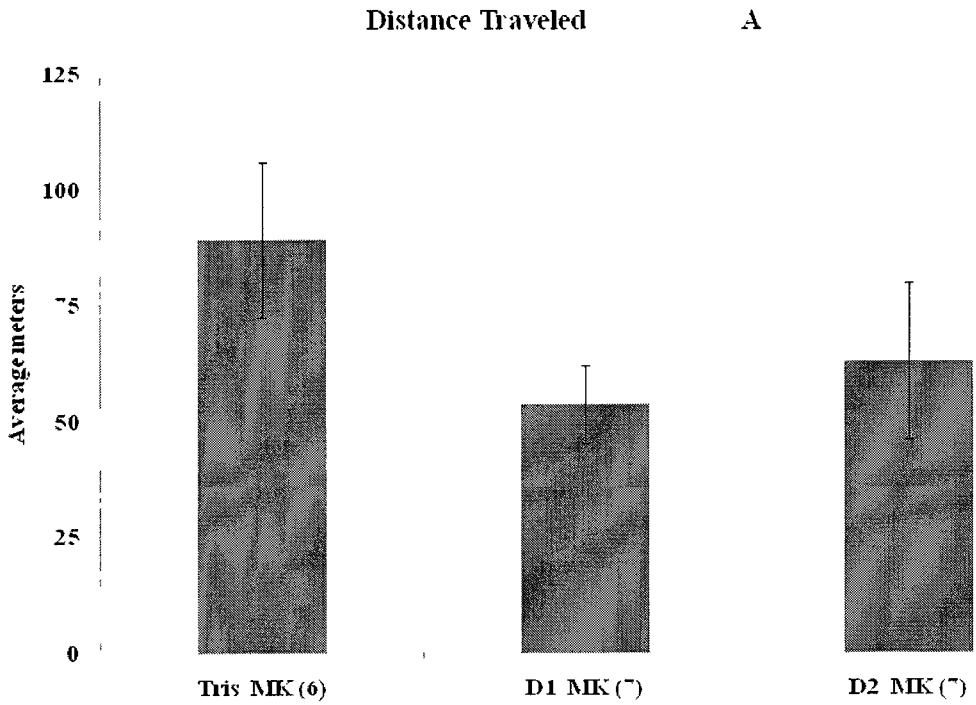

Arm Entries
B
350
300
250
200
150
100
50
0
Average Entries
200 nM MK (5)
Tris MK (5)
Tris Saline (5)
200nM Saline (4)

Figure 14. A) Distance travelled and B) arm entries measured over 30 minutes. No significant difference in distance travelled was observed between groups, however, when the number of arm entries was analyzed, we found a significant difference between the 200nM/Saline group and the 200nM/MK-801, Tris/MK-801 and Tris/Saline groups ($p < .05$).

D1/D2 antagonist cohort

The *D1/D2 antagonist cohort* testing protocol and analysis was identical with the analysis performed for the Aptamer cohort. Two one-way ANOVAs were performed, in order to analyze possible differences in arm entries or distance travelled between 0nM/MK-801, D1/MK-801 and D2/MK-801 groups. No significant difference between treatment groups in distance travelled was observed, $F(2,17) = 1.56$, $p = .24$ .Similarly, there was no significant difference between groups in total arm entries, , $F(2,17) = .95$, $p = .41$ (Figure 15).


Distance Traveled
A
125
100
75
50
25
0
Average meters
Tris MK (6)
D1 MK (7)
D2 MK (7)

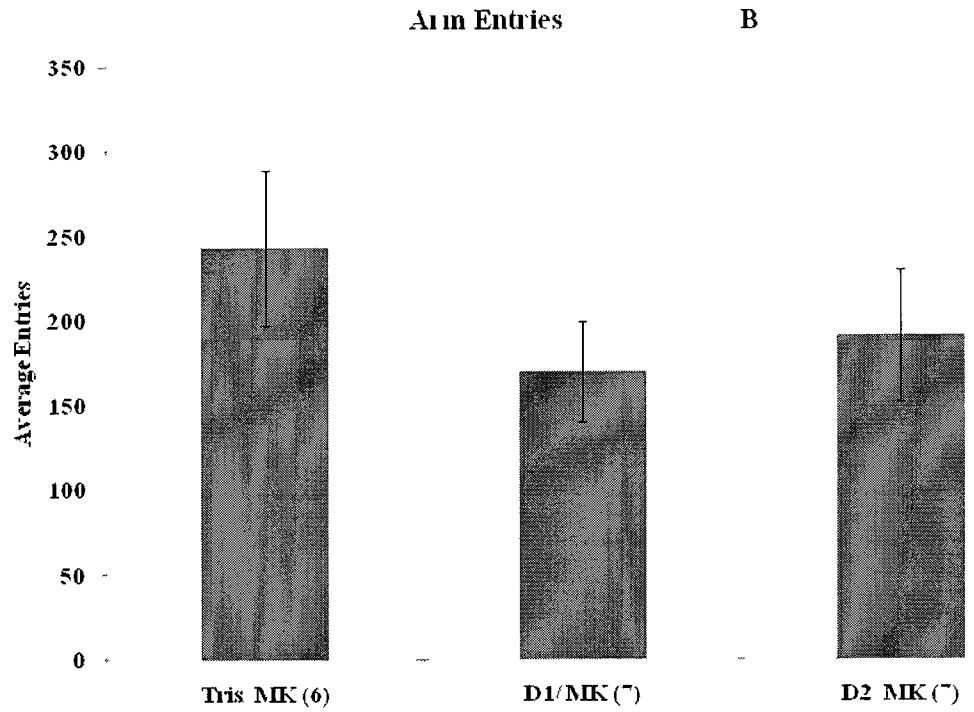

Arm Entries
B
350
300
250
200
150
100
50
0
Average Entries
Tris MK (6)
D1/MK (7)
D2 MK (7)

Figure 15. A) Distance travelled and B) arm entries measured over 30 minutes. No significant difference at $p < .05$ in distance travelled or arm entries was observed between the three groups.

Histology

Histological analysis (cresyl violet) was performed on each brain post mortem, to verify confirm the placements of the cannula. The cannula and injectors reached the previously calculated stereotaxic coordinates corresponding to the nucleus accumbens (Figure 13).

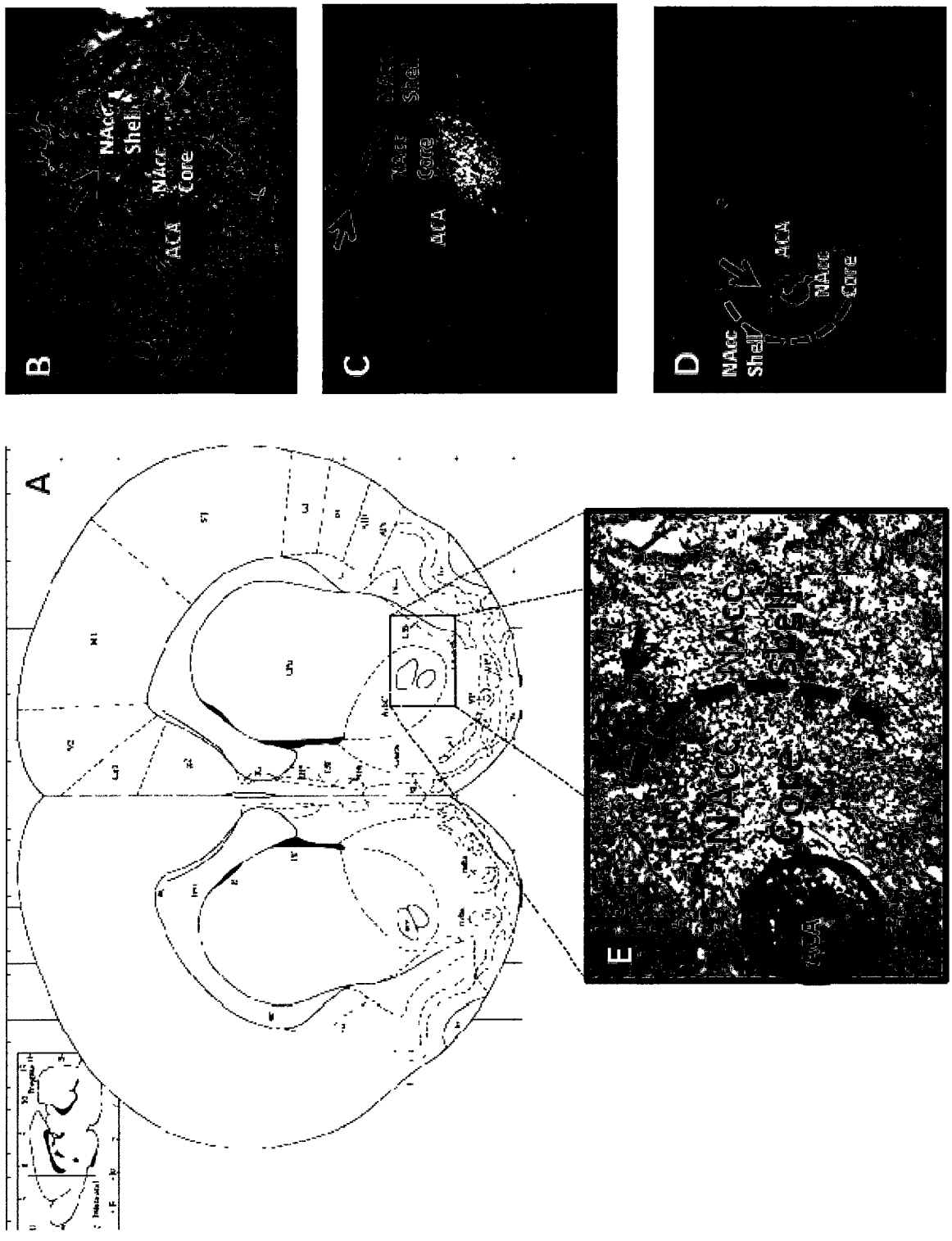

B
NAcc Shell
NAcc Core
ACA
C
NAcc Shell
NAcc Core
ACA
D
NAcc Shell
ACA
NAcc Core
A
E

Figure 16. A) Location of the nucleus accumbens (NAcc) (Paxinos & Watson). B, C and D) Cresyl violet stain of brain coronal sections (4x) of aptamer pre-treated three rats, showing the placements of the cannula and injector (orange arrows). E) Cresyl violet stain of a brain brain coronal section, shoving the placement of the injector (orange arrows) (10x). ACA represents the anterior commisure, the green dotted line shows the histological delimitation between the nucleus accumbens shell and core.

Discussion

Throughout this experiment, rats were tested on both cognitive (acquisition and extinction) and motor abilities, following central (dopaminergic) and systemic (glutamatergic) manipulations. The main objective was to investigate the possible effects that the novel DNA dopamine aptamer might have on rats that have been systemically injected with the glutamatergic antagonist MK-801. Since *in vitro*, the aptamer works by binding to dopamine molecules with increased affinity, we suggested that its effects *in vivo* would resemble those of classical D1 and D2 receptor antagonists. Previous research suggests that glutamatergic blockade achieved through systemic administration results in an increase in accumbal dopaminergic output. Furthermore, hypoglutamatergia has been shown to impair the rats ability to extinguish a previously acquired behaviour, an effect that was successfully reversed by systemic D1/D2 antagonist administration (Holahan, et al., 2010) .

Although the rats were tested following identical chemical and behavioural paradigms, the training/testing protocols were divided with respect to the dopaminergic manipulations, yielding two major cohorts. The first cohort consisted of the animals that received the aptamer central administration, while the second squad received central D1 and D2 antagonist pre-treatment. Each of these two groups had their own, separate control sub-groups.

Acquisition

Food-restricted rats were trained to press a bar twice in order to have access to one chocolate pellet. The training carried on for five days. Throughout days 1-4, animals showed different learning capabilities; however on day 5, no significant differences in bar presses

between animals were recorded. On day 5, the animals were assigned to treatment groups, as described above.

Extinction

DNA dopamine aptamer pre-treatment facilitates extinction in hypoglutamatergic rats

Extinction was quantified via bar-presses and nose pokes for a period of 30 minutes. Bar presses were not accompanied by food delivery during this time. Analysis showed that MK-801 injected rats pre-treated with a 200nM dose of the DNA dopamine aptamer, pressed significantly less ($p < .05$) than the MK-801 animals who received vehicle (Tris) pre-treatment. Furthermore, there was no significant difference in bar presses between rats who received central 0nM and 200nM aptamer pre-treatment and saline peripheral administration, suggesting that aptamer pre-treatment was successful in reversing, or at least minimizing the effects of glutamatergic blockade achieved by peripheral MK-801 administration. Additionally, there was no significant difference between animals receiving peripheral MK-801 administration and 0nM or random oligonucleotide central pre-treatment. The current finding that hypoglutamatergic animals fail to extinguish a pre-learned behaviour is consistent with previous research performed in out lab (Holahan, et al., 2010).

Measuring the number of nose-pokes every five minutes for 30 minutes, we recorded similar patterns, where MK-801 rats pre-treated with random or Tris showed increased nose poke activity (numbers and pattern) compared to the other groups (Figure 2B). The 200nM pre-treated rats that received subcutaneous MK-801 injections did show a lower nose poke activity compared to the other MK-801 groups, but failed to reach significance at $p < .05$.

These results indicate that 200nM aptamer pre-treatment was successful in instating extinction in hypoglutamatergic/hyperdopaminergic animals. If the failure of achieving extinction in MK-801 treated animals is partly caused by an overactivation of the mesolimbic dopaminergic system, we can then conclude that the aptamer was successful in binding to dopamine molecules within the nucleus accumbens, resulting in a "normal" extinction process, by showing no significant difference in bar-pressing pattern and numbers measured every five minutes over a period of 30 minutes (Figure 11A).

D1 or D2 receptor antagonist pre-treated failed to show extinction in hypoglutamatergic animals

Animals belonging to the *D1/D2 antagonist cohort* were tested on an identical extinction task as the *Aptamer cohort* rats. There was no significant difference in either bar presses and nose pokes between MK-801 rats pre-treated with either Tris, D1 or D2 receptor antagonist (Figure 13A and B), contradicting previous results reported by (Anderson, et al., 2003), (Bari & Pierce, 2005), as well as data yielded from our own lab (Holahan et al., 2010). These divergences may be attributed to the fact that compared to previous studies (Holahan et al., 2010), our animals received D1/D2 pre-treatment centrally, as opposed to systemic injections. While injecting directly into the accumbens, we did not dissociate between the functionally and anatomically different components of this formation: the core and the shell (David, Zahniser, Hoffer, & Gerhardt, 1998; Anderson, Bari, & Pierce, 2003; Anderson, Schmidt, & Pierce, 2006; Sellings & Clarke, 2006; Ikemoto, 2007). Furthermore, these discrepancies might be attributed to the fact that for the present experiment, the increased dopaminergic output within the nucleus accumbens was achieved indirectly, through peripherally-induced glutamatergic blockade, rather than via direct, central manipulation.

Motor analysis

Aptamer and D1or D2 antagonist pre-treatment has no motor effect in MK-801-injected animals.

Holahan et al. (2010) showed that peripheral administration of MK-801 increased motor activity. Previous studies showed that MK-801-induced motor hyperactivity was abolished or reduced in animals pre-treated with dopaminergic antagonists, administered peripherally (Verma & Kulkarni, 1992; Yan, Reith, Jobe, & Dailey, 1997). During our experiment, motor activity was assessed by measuring the animal's distance travelled and arm entries in a plus maze for 30 minutes, 15 minutes post treatment. The aptamer pre-treated MK-801 animals travelled less compared to the Tris pre-treated mk-801 rats. However this difference failed to reach significance at $p < .05$. When analyzing motor performance measured via arm entries, here was a significant difference between the aptamer pre-treated/MK-801 animals and the Tris/saline groups, with the Tris pre-treated animals showing significantly less arm entries compared to the 200nM/MK-801 group. This result, combined with the finding that there was no significant difference between the groups mentioned above, may be explained by the various stereotypical motor effects previously reported in MK-801 injected rodents. Verma & Kulkarni (1992) showed that a 0.1 mg/kg dose of IP administrated MK-801 produced intense stereotyped sniffing, rearing, lateral head-weaving, body rolling, falling, ataxia and jumping. Although the behaviours mentioned above were not quantified during the present study, it is possible that an increased number of arm entries might be just another motor effect fitting the stereotypical motor battery caused by MK-801 administration. To our knowledge, motor activity quantified via arm entries in a plus maze has not been reported elsewhere and thus, we cannot clearly confirm whether or not this effect is entirely attributed to MK-801 treatment. The motor analysis performed on the

D1/D2 antagonist cohort indicated that D1 and D2 pre-treatment had an effect on the animals' motor activity expressed by suppressing number of arm entries and decreased distance travelled (not significant at $p < .05$).

Histology

Histological analysis (Figure 13) showed that the cannula and injectors were placed according to the previously calculated stereotaxic coordinates corresponding to the nucleus accumbens. No functional or histological differences within the nucleus accumbens (core *vs.* shell) were accounted for during this experiment. We acknowledge the fact that the insertion of 25 Gauge cannula into the brain alone implies physical damage as well as risk of infection. However, the placements did not seem to have any effects on the animals' cognitive and motor performance, with rats performing at similar levels compared to non-implanted rats (Holahan et al., 2010).

Overall, these results suggest a functional dissociation within the effects of the DNA dopamine aptamer, showing higher selectivity towards reward-related tasks rather than motor effects. Not only dopamine, but also serotonin and norepinephrine showed dose-dependent elevated levels within the nucleus accumbens following focal or systemic administration of MK-801 (Hatip-Al-Khatib, Mishima, Iwasaki, & Fujiwara, 2001; Yan, et al., 1997). Aptamer pre-treatment seemed to be overall more efficient in counteracting the MK-801 induced increase in accumbal DA levels compared with D1 or D2 antagonist pre-treatment. An explanation may arise from the fact that the DNA aptamer synthesized by Walsh & DeRosa (2009) shows similar binding properties to norepinephrine as well, and thus D1 or D2 antagonist pre-treatment alone might not be sufficient in abolishing the effects of MK-801 injections. Further studies are needed

in order to fully mimic the effects of this aptamer using classical dopaminergic antagonists, where additional to individual D1 and D2 receptor antagonist pre-treatment, animals will also receive pre-treatment consisting of a combination of the antagonists mentioned above, as well as noradrenergic antagonist pre-treatment.

The cognitive performance of the aptamer-treated MK-801 animals was not different than the control groups, suggesting that these animals were successful at extinguishing a previously learned behaviour. Contrarily, dopamine antagonist pre-treated rats failed to show extinction. Further research is needed to investigate the pharmacokinetics of this aptamer *in vivo*. This can be achieved by measuring renal filtration, elimination of non-bound oligonucleotides and rapid bio-distribution from the plasma into the tissues (Bouchard, et al., 2010). However, aptamer blood-brain-barrier (BBB) bypass is necessary in order to investigate the compounds' pharmacokinetical properties and consider it a feasible therapeutic agent. Once these crucial requirements are met, aptamers must be modified to increase metabolic stability, slow and steady distribution from the central compartment and slow filtration (Bouchard et al., 2010). Unmodified DNA therapeutics showed limited applications due to their relatively short duration of action. Chemical modifications are available and routinely utilized to increase nuclease resistance, and the most frequently used for aptamers consist of changing the 2'-OH group of ribose to 2'-amino (NH_2) or 2'-fluoro (F) groups on pyrimidines (see Hjalmarsson, 2004 for detailed review). Other methods summarized by Hjalmarsson (2004) include the enlargement of the molecule through extended sequence (not adequate if the goal is BBB passage), conjugation of aptamers with larger macromolecules like polyethylenglycol (PEGilation) and embedding into liposomes. Another technology useful in increasing aptamer stability is called the Spiegelmer technology (Faulhammer, et al., 2004).

In conclusion, we can state that the 200nM dose DNA dopamine aptamer used for this study was successful in binding to dopamine molecules *in vivo*, demonstrated via cognitive and motor testing. The therapeutic potential of the DNA dopamine aptamer revolves around clinical conditions and diseases that have a hyperdopaminergyc system as one of the main or underlying causes. Such cases include schizophrenia, psychosis and drug abuse.

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