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**The Effect of Intra-accumbens Injections of a Dopamine
D₂ Receptor Antisense Oligodeoxynucleotide on
Cocaine Self-administration in the Rat**

**By
Tamara L. MacIntyre**

A thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfilment of the
requirements for the degree of
Master of Science

Department of Psychology
Carleton University
Ottawa, Canada
May, 1996

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ISBN 0-612-13871-2

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ABSTRACT

The role of dopamine (DA) D₂ receptors within the nucleus accumbens (ACC) during cocaine reinforcement was examined through the use of antisense oligonucleotides (ODNs). Male Wistar rats were trained to self-administer cocaine (1.5 mg/kg/inj) under a progressive ratio (PR) schedule of reinforcement and then implanted with bilateral intracerebral guide cannulae directed toward the nucleus accumbens (ACC). Once stable self-administration baselines had been re-established, three injections of the antisense, control sequence #1 (randomization of the same base pair composition) or control sequence #2 (4 base pair exchange) in a saline vehicle (2.5 nmol/1.0 µl/inj) were administered at 12 hour intervals. The ODN sequences were phosphorothioated at all positions. The full phosphorothioated ODNs appear to produce non- sequence dependent effects on cocaine self-administration. Food reinforced responding was not affected by either D₂ antisense or control ODN injections. These data highlight the need for procedures that control for the behaviourally toxic effects of phosphorothioated ODNs.

DEDICATION

This work is dedicated to the memory of my Dad,
whose strength and encouragement carries
me through the days.

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INTRODUCTION

Antisense technology offers a new molecular biological technique for studying brain function. The inhibition of specific gene expression using synthetic oligonucleotides (ODNs) may provide an alternative to the more common irreversible genetic knockouts, receptor pharmacology and lesion studies which have been the concentration of the majority of brain research to date. The effectiveness of the strategy in the central nervous system has only begun to be characterized and until recently, has been used almost exclusively *in vitro*.

Antisense ODNs block the expression of specific proteins. Antisense ODNs are short strands of nucleotides that make up the building blocks of DNA. These compounds/drugs can be designed to recognize a unique site on a chosen gene or its corresponding mRNA transcript and are complementary to the DNA sense sequence to be targeted. Most often the experiments are performed with complementary sequences to either a part of the coding region or the untranslated but transcribed parts of the target gene. The ODNs are believed to do either of the following; 1) interfere with the reading of the sense mRNA during the translation stage of protein synthesis by inducing steric hinderance of the ribosome involved or 2) forms a double stranded cytoplasmic complex which acts as a target for the enzyme ribonuclease H to cleave the nucleic acids into small molecules thereby destroying the RNA and thus altering its function (Walder *et al.* 1988; Cohen *et al.* 1994).

***In vitro* Success**

Researchers have been able to use ODNs to block specific gene expression since the mid-1980's. Scientists first discovered that plant and animal cells sometimes employ the antisense strategy to control gene expression naturally (Cohen *et al.* 1994). This natural process occurs when an organism is challenged either by a bacterial or viral infestation or by spontaneous cell growth. It is not surprising therefore that the majority of *in vitro* success has been with antiviral and anticancer oligonucleotides (Stein *et al.* 1996; Marshall *et al.* 1993). Inhibition of neuronal gene expression has been successful *in vitro*. Antisense ODNs have been shown: (a) to inhibit the erbB2 gene which is thought to be involved in mammary carcinoma (Hunter *et al.* 1995), (b) to inhibit neuroblast migration by targeting integrin RNA (Galileo *et al.* 1992), (c) to inhibit site specific ODN repression of *c-myc* gene transcription (ultimately inhibiting the spontaneous cell growth associated with this particular gene) (Cooney *et al.* 1988) and (d) to inhibit the herpes virus (Hoke *et al.* 1991; Floresaquilar *et al.* 1994). Antisense ODNs inserted retrovirally as well as through the use of expression vectors that carry cDNAs (Hunter *et al.* 1995) have shown promise and several of these ODN antisense sequences have already gone to clinical trials to treat AIDS, herpes retinitis and leukaemia (Marshall *et al.* 1993; Stein *et al.* 1996; Cohen *et al.* 1994, Hunter *et al.* 1995, Gosztonyi *et al.* 1994).

Cell Function and Simple Animal Behaviours

The use of this technique has been successfully demonstrated: a number of studies report the successful use of ODNs in the central nervous system both *in vitro* and *in vivo*. Targets for ODNs has ranged from receptors and transporters to immediate early genes. The behavioural

consequences of the ODN induced inhibition has effected a variety of behavioural patterns (Zhou *et al.* 1994; Wahlestedt *et al.* 1993a, Wahlestedt *et al.* 1993b, Heilig *et al.* 1993, Wollnik *et al.* 1995; Tischmeyer *et al.* 1994).

An area of recent interest has been in the use of antisense ODNs to alter *in vivo* neuronal gene expression. Studies have demonstrated that antisense ODNs can be injected intracerebrally to block the synthesis of particular receptor proteins. In this manner, antisense ODNs used to inhibit production of the neuropeptide Y and N-methyl-D-aspartate receptor (Erlinge *et al.* 1993; Wahlestedt *et al.* 1993a; Wahlestedt *et al.* 1993b; Akabayashi *et al.* 1994), the oestrogen and progesterone receptors (Pollio *et al.* 1993; McCarthy *et al.* 1993; Ogawa *et al.* 1994), serotonin (5-HT₆) receptor (Bourson *et al.* 1995) and the dopamine (DA) D₂ receptor (Qin *et al.* 1995; Zhou *et al.* 1994; Zhang *et al.* 1993) have all successfully inhibited simple behaviours.

Integrative/Motivational Behaviours

The potential use of ODNs in behavioural neuroscience to study the function of a number of proteins essential to more integrative or motivational behavioural patterns, such as systems involved in reinforcement, is tempting. Several animal studies using ODNs to the inhibition of gene expression have demonstrated alterations in simple behavioural function such as locomotor activity, anxiety response, eye-blink response and phase shifts of the circadian clock (Wollnik *et al.* 1995; Wahlestedt *et al.* 1993a; Wahlestedt *et al.* 1993b; Heilig *et al.* 1993, Castroalamancos *et al.* 1994; Dragunow *et al.* 1993). It remains unclear whether the ODN technology can be used to examine the neural basis of drug reinforcement effectively

A few studies have employed antisense strategies to examine the interactions between psychostimulants, cell function and animal behaviour. Heilig et al. (1993) have used intracerebral administration (i.c.v.) of an antisense ODN to block the expression of the immediate early gene *c-fos*, and have shown that this causes an attenuation of cocaine-induced locomotor activity. Several papers have attempted to incorporate the use of ODNs targeted towards the DA receptors in the hopes of mediating or altering simple behaviours such as locomotor activity and rotational behaviour (Zhang *et al.* 1993, Weiss *et al.* 1993; Silvia *et al.* 1994). Zhang and Creese (1993) reported that intraventricular administration of antisense ODN directed against the D₂ receptor mRNA, inhibits amphetamine induced locomotor activity, but not D₁ agonist-induced grooming behaviour in mice. In addition, Weiss et al. (1993) and Qin et al. (1994), using a similar sequence in a mouse demonstrated inhibition D₂ receptor mediated behaviours following depletion of D₂ mRNA. This work, in combination with other ODN studies targeting the D₂ receptor, suggests that the sequences used to target the D₂ receptor may indeed produce similar alterations in more complex behavioural patterns. Many of these studies have demonstrated an alteration in baseline behaviour which they attribute to the ODN manipulation alone, but very little research has adequately demonstrated the DA receptor depletion as it relates to a complex behavioural paradigm.

Considerations when using Antisense ODNs

Several discussion papers have reviewed a variety of issues that must be addressed when using the antisense technology to study brain function. The mechanism of action and possible adverse effects of the ODNs are still not well understood and have not been adequately

characterized (Yee *et al.* 1994). The problematic issues of specificity, the type of ODN and its appropriate control, uptake and distribution of the ODN, the duration of ODN action and the functional recovery of the animal are important considerations.

1- Specificity / Type of ODN or control used

The number of nucleotide base pairs in an antisense sequence influences the specificity and the effectiveness of an ODN-induced disruption of gene expression. As the sequence length is increased, the specificity of the ODN improves but entry into a given cell becomes more difficult with each base pair addition and a reduction in uptake results. The ODN must have specificity for its receptor which is typically a result of hybridization interactions that depend upon hydrogen bonding. The affinity of the ODN for its target complementary mRNA is directly responsible for the specificity of the antisense oligonucleotide. To optimize the specificity for the desired interaction, a sequence of 15 to 20 nucleotides has been proposed (Stein *et al.* 1996, Hunter *et al.* 1995; Wahlestedt, 1994). The potency of the antisense ODN differs widely depending on the site of target protein selected, even for comparable analogues, therefore a number of ODNs that possess a high affinity for the target protein has been suggested.

The ODNs can be modified in a number of ways, and these modifications can affect their duration of action and specificity. Although analogues have been investigated that involve modification of several phosphodiester bonds, the replacement of all the phosphodiester (PDE) linkages of the DNA backbone with neutral achiral, nuclease resistant entities has been used for some time (S-oligonucleotide or fully phosphorothioated ODN). For application into the living brain, either analogue may be useful (Wahlestedt, 1994). The stability of the ODN is essential

to its success, since mRNA translation in the cytoplasm must be inhibited long enough to result in the depletion of the targeted protein. Some tissue damage can be expected in the case of intracerebroventricular (i c v.) injections and therefore metabolically stable ODN analogues (such as modified phosphorothioate ODNs) may be preferable. However, higher doses of the unmodified ODNs have also been used successfully in this context. Regardless of the type of ODN, effective antisense ODN site injections placed in the striatum (Chiasson *et al.* 1992), ACC (Heilig *et al.* 1993) and ventromedial hypothalamus (Pollio *et al.* 1993) have been reported. It should be emphasized that the phosphodiester ODN (unmodified) appear to have greater tissue penetration than the phosphorothioates (modified) after direct injection, although the phosphorothioates appear to be more stable than the phosphodiesters over approximately 24 hours (Hunter *et al.* 1995). Therefore, it is not surprising that sequence independent results are frequently reported (Wahlestedt, 1994; Skutella *et al.* 1994).

There is no general consensus in the literature as to an appropriate control for any given antisense ODN. Most studies have involved the use of sense or randomized sequence controls created with the presumably active ODN molecule having been taken into consideration. In many early studies researchers used a sense sequence (complementary to the antisense strand) as a control. This is no longer considered an appropriate control for an antisense ODN. It has now been shown that a more appropriate control would be an ODN containing one to four mismatched nucleotides of corresponding antisense ODN (Hunter *et al.* 1995; Wahlestedt, 1994). The "G - C" content of the ODN (either antisense or the control sequences) should be low and spread out evenly across the sequence. A high "G - C" content of the sequence is more likely to polymerize onto itself and therefore lose the sequence specificity (Hunter *et al.* 1995).

It is understood that all sequences should be run through gene databases to check their potential for non-specific cross-reactions with known sequences (accepting the limitations of these databases). Gene bank is the only option for verification of a sequence. It is not possible to be 100% sure that a nucleotide sequence is unique to the protein under investigation. Although sequences can now be compared to sequences in over 20 000 genes presently known in rodents.

2- Uptake, Distribution and Localization of the Antisense ODN

Understanding the uptake and degradation properties of the targeted protein by testing the efficacy of the ODN *in vitro* is a suggested first step in the successful use of the antisense strategy. However, the mechanism of cellular uptake, distribution and the method with which an ODN is transported into their target cells has not been well characterized. Receptor mediated fluid-phase endocytosis, also known as pinocytosis, is the mechanism by which an ODN is believed to be taken up into a cell (Yakubov, 1989; Wahlestedt, 1994). Evidence has also shown that various cell types may differ greatly with respect to their ability to take up and degrade ODNs (Wahlestedt, 1994; Loke *et al.* 1989). ODNs have been shown to be endocytosed by cells other than those targeted if injected i.c.v. Several experiments have suggested that the uptake of ODNs by cells can be increased by the use of cationic lipids, considerably prolonging the ODNs effects (Hunter *et al.* 1995).

Verification of the uptake of the ODN is important, in many cases labelling the ODN to verify the localization and distribution of the injected nucleotide sequence provides immunohistochemical evidence of the antisense manipulation. Fluorescence labelled ODNs have

been observed in neurons up to 3 days after a single application (Brouwer *et al.* 1992; Schlingensiepen *et al.* 1994a; Schlingensiepen *et al.* 1994b; Hunter *et al.* 1995). It is important to note that fluorescence labelling of ODNs may influence the ODN manipulation. An under estimate of the ODN's action is possible if the label is positioned at the end of the sequence, since this has been shown to increase the susceptibility of the ODN to nucleases. The solution suggested is to label the ODN internally (Yee *et al.* 1994).

Once injected, the distribution of the ODN reportedly follows cerebrospinal fluid (CSF) flow pattern (Yee *et al.* 1994). If the brain region of interest falls along the CSF drainage pathway it may be advantageous to inject an antisense ODN i.c.v. in a CSF vehicle (Whitesell *et al.* 1993; Wahlestedt, 1994). It should be noted that the majority of literature does not clearly define the vehicle in which the ODNs are administered (Yee *et al.* 1994).

3- The Duration of Action and Functional Recovery of the Animal

Time of onset, duration and degree of effects of the antisense ODNs *in vivo* depend on several factors: the half-life of the target protein, an abundance of target mRNA and antisense ODN, and the kinetics of mRNA binding of antisense ODN (Skutella *et al.* 1994; Ramanathan *et al.* 1993). Consideration of dose and toxicity should be investigated and histochemically verified for the protein of interest.

It is imperative to consider the time course or half-life of the target protein before determining the appropriate time frame under which to inject the antisense ODN. Obviously, when studying an inducible gene product, such as *c-fos*, less time for ODN treatment is required than when attempting to affect a constitutively active gene product with a longer half-life. In the

latter case a longer ODN treatment period would be required. For example, to achieve inhibition of neurotransmitter receptor synthesis, a minimum treatment time of two to five days seems necessary (Standifer *et al.* 1994; Erlinge *et al.* 1993; Wahlestedt, 1994, Wahlestedt *et al.* 1993a, Wahlestedt *et al.* 1993b). It has been reported that Fos-like immunoreactivity and the increase in unilaterally directed rotational behaviour are both time and dose-dependent (Hooper *et al.* 1994; Heilig *et al.* 1993).

Relevant dosage of ODNs for the target protein have been reported to produce effects occurring 6 to 8 hours after administration and lasting about 10 hours; that is, the effects of antisense ODNs are relatively short lasting (Skutella *et al.* 1994). The defined interval between the effective and maximum tolerable ODN concentration must be carefully determined in any given culture *in vitro* before the ODN is used *in vivo*. Although it should be noted that the dosage and concentration of the ODN is dependant on the basal expression and turnover rate of the target protein. Some researchers have suggested that levels of behavioural change may appear earlier than the alteration of the protein levels themselves. For example, antisense to the DA D₂ receptor showed alteration in behaviour earlier than the marked effects on receptor number (Hunter *et al.* 1995). Some proteins, such as the D₁ receptor, requires a different administration regime as compared to the D₂ receptor protein in order to be maximally effective (Weiss *et al.* 1993; Hunter *et al.* 1995). This example serves as a cautionary note to researchers, successful *in vitro* laboratory results are only the first step in testing ODN efficacy in an *in vivo* model.

The virtue of the antisense ODNs lies in their ability to temporarily block the expression of existing genes. The reversibility of the manipulation differs from standard gene therapies and

traditional lesion studies and can be advantageous in the design of behavioural experiments. Functional recovery of the animals original baseline behavioural pattern serves as an estimate of the time course of the antisense manipulation. In general the recovery period post injection *in vivo* is approximately 5 - 6 days (Skutella *et al.* 1994; Zhou *et al.* 1994) and it has also been shown that the rate of functional recovery is greater in younger animals as compared to older animals (Hunter *et al.* 1995; Creese *et al.* 1994a; Creese *et al.* 1994b). Consideration of the half-life of the target protein and functional recovery of the animal is essential when attempting to interpret results on behavioural evidence alone.

Present Series of Experiments

The purpose of the present series of experiments was to establish the parameters for the use of the ODN strategy in behavioural research. The ODN was targeted toward the D₂ receptor type known to be an integral part of the reinforcing property of cocaine. To date no work has been directed at specifically interfering with mechanisms of psychostimulant reinforcement and drug-taking behaviours using an antisense ODN.

It is important to note that a correlation has been shown to exist between mRNA expression and drug addiction (Nestler *et al.* 1993). This suggests that an alteration in mRNA expression in primary reward pathways may enable researchers to dampen the expression of any given protein coded for by targeted RNA strands. It may be that by decreasing the concentration of a receptor protein, such as the DA receptor, an attenuation of the reinforcing properties of cocaine is possible. There are primarily two types of evidence to suggest that techniques which include alterations in gene expression may contribute significantly in the area of drug addiction

research. It has been shown that drug regulation of a target protein in the mesocorticolimbic system occurs at the mRNA level (Vrana *et al.* 1993, Nestler *et al.* 1993). Secondly, studies involving chronic cocaine treatment show that a regulation of mRNA expression occurs in the DA transporter of both the ventral tegmental area (VTA) and the ACC pathway (Bunzow *et al.* 1994). This connection between mRNA levels and reward properties leads us to question whether a manipulation of protein synthesis at the mRNA level would effect a complex behavioural pattern such as cocaine self-administration.

In brief, evidence confirms the importance of DA in the mesocorticolimbic system, specifically the ACC and the VTA which play an intricate role in the reinforcing effect and pattern of intake of cocaine. The two classes of DA receptors, the D₁-like or the D₂-like, are distinguished by their distinct biochemical characteristics. The D₂ receptors are thought to mediate effects independent of the D₁ receptor and also to exert an opposing influence on adenylate cyclase activity (Cooper *et al.* 1991). It would therefore appear that the DA D₂ receptor is an appropriate target to further evaluate the ODN technology

We chose to examine the DA receptor, since there is overwhelming evidence that D₂ receptor subtype plays a significant role in mediating the reinforcing effects of drugs of abuse (Nakajima, 1989; Roberts *et al.* 1995; Roberts, 1992; Koob, 1992; Koob *et al.* 1987; Cooper *et al.* 1991). Pharmacological research suggests a primary role for the D₂ receptors in psychomotor stimulant effects of cocaine (Spealman *et al.* 1992; Koob, 1992). Studies have also confirmed that the terminal autoreceptors within the ACC as well as the somatodendritically located autoreceptors in the VTA are exclusively of the D₂ receptor sub-type (Jackson *et al.* 1994). DA release from axon terminals is partially regulated by D₂ receptors (Kalivas *et al.* 1990, Cubeddu

et al. 1990) It follows that alterations in autoreceptor density or sensitivity may play a mediating role in the stimulation of extracellular DA release presynaptically in the ACC. In addition, post-synaptic D₂ receptor density varies which presumably leads to an enhanced responsiveness to released DA (Kalivas *et al.* 1990). These data support the theory that the D₂ receptor is critical for cocaine's reinforcing effects in the DA mesocorticolimbic system.

By using a proven method of testing the reinforcing efficacy of cocaine to monoamine systems such as the cocaine self-administration paradigm, the merit of the antisense strategy in behavioural research can be tested. There are a host of parameters that have yet to be examined. A consideration of the basal expression and turnover rates of the D₂ receptor based on previous research (Silvia *et al.* 1994; Weiss *et al.* 1993; Zhang *et al.* 1993), toxicity of the ODN in tissue *in vivo*, the specificity of the ODN action and functional recovery of the animals behaviour post injection must be incorporated into the ODN methodology. These parameters may be investigated by targeting the role of the D₂ receptor in the ACC during cocaine self-administration, a proven paradigm for testing the motivation and reinforcing properties of this drug taking behaviour.

EXPERIMENT 1: Cocaine Self-Administration

The purpose of experiment 1 was to examine the effect of microinjections of D₂ antisense (group 1), control sequence #1 (group 2) and control sequence #2 (group 3) into the ACC on intravenous (IV) cocaine self-administration reinforced under a PR schedule.

Subjects

Male Wistar rats (Charles River, Quebec) weighing approximately 300-350 g served as subjects. Initially, rats were doubly housed in shoebox style cages and quarantined for a week. A reverse light/dark cycle was maintained throughout the experiment. Food and water were available *ad libitum*.

Food Training

Prior to entry into the self-administration experiments, animals were food deprived for 24 hours and trained to press a lever for food (a 45g Noyse food pellet) under an fixed ratio (FR) schedule of reinforcement. After completing two such training sessions with greater than 200 lever responses, an animal was accepted into the self-administration study.

Intraventricular Cannulation

Once trained to press a lever for food, rats were anesthetized with Somnotol (60 mg/kg, IP) and implanted with a chronic indwelling silastic jugular cannula (for complete description see Roberts & Goeders, 1987). Incisions were made at the mid scapular area of

the back and over the left jugular vein on the ventral side of the animal. Silastic tubing was led subcutaneously from the dorsal excision to the ventral opening. The jugular vein was exposed and punctured to allow feeding of the silastic tubing through to the heart. The vein was tied off to stabilize the tubing and the ventral incisions sutured. The dorsal incision was sutured and the cannula secured by two sutures penetrating marlex mesh that was connected to the cannula which lies subcutaneously in the dorsal portion of the animal. The tubing exiting the back was connected through a protective spring to a fluid swivel.

Operant Testing Chambers

The operant testing chambers, fashioned from plexiglass (25cm X 25cm X 25cm), were used for testing and housing of the animals. Each chamber was equipped with a water bottle, counter balance mechanism, stimulus lights and removable levers. Post-surgery the animals were placed in the testing apparatus and the fluid swivel of the cannula was affixed to a counterbalanced arm on the chamber. The swivel was further attached to a 10 ml syringe which was driven by a 5 RPM infusion pump.

Cocaine Self-Administration

Training consisted of daily five hour sessions that began with the insertion of a lever into the test chamber and a single priming injection. Rats were trained to self-administer cocaine on an FR 1 schedule. Each lever press resulted in an injection of 0.6 mg of cocaine/0.12 ml of saline over a five second period. A stimulus light was activated for a 20 seconds following the injection and any response on the operant lever during this "time-out"

had no programmed consequence and did not result in an injection. Once animals reached a stable rate of responding for three consecutive days they were then be placed into one of the experimental groups.

Intracranial Cannulation

Rats were anesthetized with Somnotol (60 mg/kg, IP) and implanted with bilateral intracranial cannulae. Animals were placed in a Kopf rat stereotaxic instrument. The dorsal surface of the skull was exposed and holes were drilled to yield an implantation site corresponding to coordinates measured from bregma (AP: + 1.0mm, DV: -5.9mm, ML: ± 1.0 mm) according to Paxinos and Watson (1986). The cannulae placement were fixed in place using dental cement and screws. Stylets which were cut flush with the end of the cannulae were inserted to maintain the patency of each cannulae. The wound was sutured closed. Upon recovery from anesthesia, animals were returned to the self-administration chamber.

Drug

Cocaine hydrochloride was supplied by NIDA (Bethesda, MD) and prepared in 0.9% sterile saline. D₂ antisense ODN, D₂ control sequence #1 and D₂ control sequence #2 were obtained from the DNA Synthesis Laboratory at the University of Calgary and was prepared in a sterilized 0.9% saline vehicle.

The D₂ antisense ODN was targeted at the initiation codon region of the D₂ receptor mRNA. Control sequence #1 consisted of a randomization of D₂ antisense ODN base pair

composition. Control sequence #2 consisted of a four base pair exchange from the D₂ antisense ODN. For specific sequences refer to figure 1.

GROUP 1 - D₂ Antisense ODN and Cocaine Self-Administration

After stable baseline on a PR schedule had been reestablished, animals received intra-accumbens injections of a 19-base phosphorothioated antisense oligodeoxynucleotide directed at codons 2-8 of the D₂ mRNA (5'- AGG ACA GGT TCA GTG GAT C-3'). All animals received three injections in total. The first injection was administered twelve hours prior to the subsequent self-administration session (DAY 1), the second injection was administered immediately prior to that session and the third was administered twelve hours later i.e. twelve hours prior to the subsequent self-administration session (DAY 2). Cocaine self-administration sessions continued for seven days following the first treatment (DAY 1 - DAY 7). On DAY 8 the animals were placed on a FR 1 schedule of reinforcement to ensure the patency of their IV cannulae. All rats were then be perfused through the heart with 10% formol saline or 4% paraformaldehyde and the cannulae placements verified.

GROUP 2 - D₂ Control Sequence #1 and Cocaine Self-Administration

After a stable baseline on a PR schedule had been reestablished, animals received intra-accumbens injections of a 19-base phosphorothioated randomized antisense ODN, D₂ control sequence #1 (5'- AGA ACG GCA CTT ATG GGG T- 3'). All animals received three injections in total. The first injection was administered twelve hours prior to the subsequent self-administration session (DAY 1), the second injection was administered immediately prior

to that session and the third was administered twelve hours later i.e. twelve hours prior to the subsequent self-administration session (DAY 2). Cocaine self-administration sessions continued for seven days following the first treatment (DAY 1 - DAY 7). On DAY 8 the animals were placed on a FR 1 schedule of reinforcement to ensure the patency of their IV cannulae. All rats were then be perfused through the heart with 10% formol saline or 4% paraformaldehyde and the cannulae placements verified.

GROUP 3 - D2 Control Sequence #2 and Cocaine Self-Administration

After a stable baseline on a PR schedule had been reestablished, animals received intra-accumbens injections of a 19-base phosphorothioated four base pair exchange ODN D₂ control sequence #2 (5'- AGG CCA TGT TAA GTG GAG C-3'). All animals received three injections in total. The first injection was administered twelve hours prior to the subsequent self-administration session (DAY 1), the second injection was administered immediately prior to that session and the third was administered twelve hours later i.e. twelve hours prior to the subsequent self-administration session (DAY 2). Cocaine self-administration sessions continued for seven days following the first treatment (DAY 1 - DAY 7). On DAY 8 the animals were placed on a FR 1 schedule of reinforcement to ensure the patency of their IV cannulae. All rats were then be perfused through the heart with 10% formol saline or 4% paraformaldehyde and the cannulae placements verified.

FIGURE 1. The D₂ oligodeoxynucleotide (ODN) sequences used.

Oligodeoxynucleotide (ODN) Sequences

Antisense ODN 5' - AGG ACA GGT TCA GTG GAT C - 3'
(directed at codons 2 - 8 of D₂ dopamine receptor)

Control Sequence #1 5' - AGA ACG GCA CTT ATG GGG T - 3'
(Randomization of D₂ receptor base pair composition)

Control Sequence #2 5' - AGG CCA TCT TAA GTG GAG C - 3'
(4 base pair exchanged from the antisense ODN)

EXPERIMENT 2: Food Self-Administration

The purpose of experiment 2 was to assess the effects of D_2 antisense ODN and control sequence #1 microinjections into the ACC on break point for food using a PR schedule of reinforcement.

Animals in the food reinforcement study were trained in an identical manner to the cocaine self-administration, except that 45mg Noyes pellets served as the reinforcer. Between training sessions, these animals were individually housed in holding cages. Once stable responding under the PR schedule was achieved (± 3 BP), bilateral stainless steel guide cannulae (22g) were be chronically implanted about the ACC. Intracerebral cannulae were kept patent with 28g stainless steel stylets. Responding under the PR schedule of food reinforcement was resumed until stable BP had been achieved. Rats were then tested as follows; animals received counter balanced injections of both a 19-base phosphorothioated antisense oligodeoxynucleotide directed at codons 2-8 of the D_2 mRNA (5'-AGG ACA GGT TCA GTG GAT C- 3') and a randomized sequence of the same 19 base composition. Sequences were identical to those used by Zhang and Creese (1993). All animals received six injections in total, three of which were antisense sequence injections and three were the randomized sequence. The injection procedure occurred 12 hours prior to the start of a food self-administration session (DAY 1), the second injection was administered 12 hours after the second session (DAY 2) and the third was administered twelve hours later i.e. twelve hours prior to the subsequent self-administration session (DAY 2). The animals then received the

second set of injections approximately ten days after the first treatment. Food self-administration sessions continued for seven days following both the first and second treatment (DAY 1 - DAY 7 for each treatment). All rats were then perfused through the heart with 10% formol saline and the cannulae placements verified.

RESULTS

Experiment 1 was designed to assess the effects of antisense ODNs targeted at the D_2 receptor on break points derived from a PR schedule of cocaine reinforcement. Once a stable baseline was reached the subjects received intra-accumbens injections of either D_2 antisense ODN, control sequence #1 or control sequence #2. The effect of the ODN injections were assessed using a three way ANOVA on break point of cocaine self-administration over a 5 hour session. An overall analysis of break points derived from cocaine self-administration data revealed a significant main effect of ODN treatment day across all groups ($F=12.75, df(2,83)p<0.05$). A significant difference between days post-treatment as compared to the behavioural baseline levels of responding within a group. Between-group analysis of the cocaine self-administration animals revealed a non-significant main effect of Group ($F=0.67, df(2,40)p<0.05$) as well as a non-significant main effect of Group by Day interaction within subjects ($F=1.09, df(2,83)p<0.05$).

Experiment 2 was designed to assess the effects of antisense oligonucleotides targeted at the D_2 receptor on break point derived from a PR schedule of food reinforcement. Once a stable baseline was reached the subjects received intra-accumbens injections of

either D₂ antisense ODN or control sequence #1. The effect of the ODN injections were assessed using a twoway ANOVA on break point of food self-administration over a 3 hour session. An overall analysis of the food self-administration animals failed to reveal a significant overall effect of Group between subjects ($F=0.01, df(1,10)p < 0.05$). Within subject analysis revealed non-significant overall effect of both the treatment day ($F=2.38, df(3,30)p < 0.05$) as well as the Group by Treatment interaction ($F=0.66, df(3,30)p < 0.05$).

In summary, a significant overall effect of treatment day across all treatment groups for all subjects was seen in the cocaine self-administration animals. In contrast, experiment 2 revealed no significant difference between the D₂ antisense ODN group as compared to the control sequence #1 group in the food reinforced animals.

FIGURE 2. The effect of microinjections of D_2 antisense into the nucleus accumbens (ACC) on intravenous (IV) cocaine self-administration reinforced under a progressive ratio (PR) schedule. Each point represents a mean ($\pm$ SEM) break point of rats that receive antisense injections (N=24). The astericks (*) indicates the series of three injections 12 hours apart.

FIGURE 3. The effect of microinjections of D₂ control sequence #1 into the nucleus accumbens (ACC) on intravenous (IV) cocaine self-administration reinforced under a progressive ratio (PR) schedule. Each point represents a mean ($\pm$ SEM) break point of rats that receive control sequence #1 (N=12). The astericks (*) indicates the series of three injections 12 hours apart.

FIGURE 4. The effect of microinjections of D₂ control sequence #2 into the nucleus accumbens (ACC) on intravenous (IV) cocaine self-administration reinforced under a progressive ratio (PR) schedule. Each point represents a mean ($\pm$ SEM) break point of rats that receive control sequence #1 (N=7). The astericks (*) indicates the series of three injections 12 hours apart.

FIGURE 5. The effect of D₂ oligodeoxynucleotide (ODN) microinjection into the nucleus accumbens (ACC) on break point for food using a progressive ratio (PR) schedule of reinforcement in a 3 hour session. Each point represents a mean ($\pm$ SEM) break point of rats that receive antisense ODN (N=6) and control sequence #1 injections (N=6). The astericks (*) indicates the series of three injections 12 hours apart..

FIGURE 6. Schematic representation (measured from bregma AP: +1.00mm, DV: -5.9mm, ML: ± 1.0 mm) depicting cannulae placements into the nucleus accumbens (ACC) in animals exposed to cocaine self-administration or food self-administration.

GENERAL DISCUSSION

In the present series of experiments, microinjections of a DA antisense ODN were targeted at cells expressing the D₂ receptor in the nucleus accumbens. Large and long-lasting decreases in cocaine self-administration reinforced on a PR schedule were observed: however, the D₂ control sequences also caused comparable large and long-lasting decreases in cocaine self-administration. In contrast to the cocaine self-administration behaviour, food reinforced responding was minimally affected by either the antisense ODN or the control sequences. We suggest that non-specific effects of ODN injection into the ACC disrupted cocaine self-administration reinforced responding. Given that some control ODN sequences appear to be more behaviourally toxic than others, the need for procedures that control for the toxic effects of phosphorothioated ODNs must be further investigated.

Histochemical evidence for a sequence dependent effect of the D₂ antisense ODN on the expression of D₂ receptors in the ACC was collected in our lab (McGregor *et al.* 1994). Three groups of animals received a series of three injections 12 hours apart; the first group received injections of the D₂ antisense ODN, the second group received control sequence #1 (randomization of the same nucleotide base pairs as the antisense ODN) and the third group received vehicle injections. Twelve hours after the last injection the animals were perfused and alternate sections were cut with a cryostat and incubated on glass slides with a D₂ antagonist ([³H]-sulpiride). Autoradiographic data revealed that 12 hours after the third antisense treatment a 55% reduction in D₂ receptor density at the level of the injection site. This effect on DA receptor levels was confined to the ACC. D₂ binding levels within the striatum or olfactory tubercle were not effected. Additional autoradiography using a D₁

antagonist ($[^3\text{H}]$ -SCH 23390) demonstrated that the antisense treatment had no effect on D_1 receptor binding in any of the studied brain sites studied. It is important to note that the autoradiography also showed that the D_2 control sequence #1 (randomized sequence) had no effect on the D_2 receptor concentration. These data, in contrast to the self-administration data, suggest that a reduction in D_2 receptor population within the ACC was a sequence specific effect of the ODN treatment.

The "Success" of Antisense ODNs targeted towards the D_2 Receptor

To date, little work has been directed at specifically interfering with mechanisms of psychostimulant reinforcement and drug-taking behaviour using an antisense ODN. The use of the antisense technique has been successfully demonstrated in tissue culture and a number of studies have reported the successful use of antisense ODNs targeted toward the D_2 receptor. Zhang and Creese (1993) reported a depletion of rat striatal D_2 receptors by 48% using antisense ODNs. Autoradiography indicated a homogeneous down-regulation of the D_2 receptor by approximately 50% throughout the striatum and over 70% in the nucleus accumbens. The control sequence used by Zhang and Creese (1993) (a randomization of the same base pair composition as the antisense ODN) failed to affect either the striatal D_2 or D_1 receptor density. Behaviourally, the antisense treatment inhibited a D_2 receptor agonist (quinpirole) induced locomotor activity without altering a D_1 receptor agonist (SKF38390) induced grooming behaviour.

Silvia et al. (1994) demonstrated a role for nigrostriatal D_2 autoreceptors in the motor actions of cocaine using a D_2 receptor antisense ODN targeting the same initiation codon

region as Zhang and Creese (1993). Weiss et al. (1993) and Zhou et al. (1994) both reported that antisense ODNs targeted toward the D₂ receptor successfully inhibited D₂ receptor mediated behaviours and produced a depletion in the D₂ mRNA concentration. More recently, Valerio et al. (1994) showed inhibition of prolactin release from rat pituitary cells. Qin et al. (1995) demonstrated the inhibition of the synthesis of an entire functional pool of D₂ receptors in the mouse striatum; both studies incorporated an ODN targeted at the initiation codon region of the D₂ receptor suggested by Zhang and Creese (1993).

These studies suggest that an investigation into the use of an antisense sequence for the D₂ receptor mRNA to examine the role of the D₂ receptor in cocaine self-administration (a complex behavioural paradigm) in the rat might be viable. The mechanisms involved in cocaine self-administration are known to be intrinsically linked to DAergic function and a role for both the D₁ and D₂ receptor subtypes in psychostimulant reinforcement mechanisms has been widely documented (Roberts, 1992; Caine *et al.* 1994; Nakajima, 1989; Koob *et al.* 1987; Koob, 1992). Assuming that sequence dependent effects of an antisense ODN and not control ODNs have led to a reduction in D₂ receptor population in the ACC, the present experiments set out to investigate the viability of using an antisense sequence targeted at the D₂ receptor to examine the role of the D₂ receptor in cocaine self-administration in rat.

Determining Antisense and Control ODN Sequences

When using the antisense strategy, researchers cannot predict which nucleotide sequences will have a high affinity for the complementary mRNA of a specific sequence. It is common among researchers to target the initiation codon region of the mRNA based on the

assumption that the region is easily assessable to the antisense ODN (Uhlmann *et al.* 1990; Wagner, 1994; Hunter *et al.* 1995; Skutella *et al.* 1994). Zhang and Creese (1993) and others, chose to target codons within the initiation codon region of the DA D₂ receptor mRNA sequence with the fully phosphorothioated D₂ antisense ODN (S-oligo).

ODNs are susceptible to degradation and specific hybridization and therefore researchers must address the time course of the treatment injections or the duration and stability of the ODN action (Skutella *et al.* 1994). The most common modification used to increase the stability of the ODN *in vitro* has been alterations in the phosphodiester backbone, although many other forms of modification are under investigation. Some of these modifications target the ribose and phosphodiester backbone and completely replace it with a phosphoramidate linkage. A second modification is targeted at the replacing the sugar of the backbone with methoxyethoxy linkage. Many of these modifications decrease the affinity of RNase H to cleave the ODN when bound to mRNA sequence. In doing so, the bound mRNA is not as rapidly cleaved by the RNase H and the specificity and selectivity of ODN is eliminated. Many of these modifications are still under investigation *in vitro* and therefore are not viable alternatives to the S-oligos and native ODNs currently being used with some success *in vivo* (Wagner, 1995).

There is currently no consensus of appropriate choice of control sequence and researchers are still investigating a variety of options (Wagner, 1994; Brysch *et al.* 1994; Wahlestedt, 1994). Recent literature suggests that the best control sequences are those sequences that differ only slightly from the antisense sequences (Wagner, 1995; Hunter *et al.* 1995). For this reason, sense sequences as well as randomized sequences are no longer

advised. We chose to use the same randomized sequence outlined by Zhang and Creese (1993) as well as a mismatch control (the exchange of 4 base pairs) from the original D₂ antisense ODN.

Dose and Injection Procedure

Weiss et al. (1993) determined that significant inhibition due to a D₂ receptor antisense ODN could be obtained with a series of three injections given 12 hours apart. A dose of 2.5 nmol/2 μ l/injection i.c.v. (lateral cerebral ventricle) was administered unilaterally. The recovery of the animals from the antisense ODN inhibition reportedly occurred approximately two days after cessation of the antisense treatment. Similarly Zhou et al. (1994), Silvia et al. (1994) and Qin et al. (1995) used a comparable dose and i.c.v. injection procedure. In contrast, Silvia et al. (1994) used a five injection regime with each injection separated by a 12 hour interval and injected unilaterally above the substantia nigra pars compacta. Following the protocol of Weiss, we chose to cannulate the animals bilaterally just above the ACC and inject a series of three injections 12 hours apart at a dose of 2.5nmol/1 μ l/injection.

More recently osmotic minipumps have been used to deliver low doses of unmodified antisense ODNs over a longer treatment period. By using an unmodified antisense ODN targeted at the M₃ muscarinic receptor in the ventral tegmental area, Yeomans et al. (1995) successfully demonstrated inhibition of self-stimulation behaviour. This alternative delivery method may prove to be a more suitable, and less toxic, protocol for behavioural neuroscientists.

Verification of mRNA and/or Protein concentration

It is essential to show proof of the antisense manipulation by verifying either the change in mRNA levels post antisense injection or the change in the concentration of the targeted protein. Although the antisense manipulations have been primarily verified through autoradiographical evidence, it has recently been suggested that alterations in mRNA levels are better assessed by Northern Blot Analysis or *in situ* hybridization with or without polymerase chain reaction (PCR). While autoradiography allows determination of receptor density or population in the targeted brain region and is most commonly used to verify the antisense manipulation, it is possible to verify indirectly the ODNs effects on the mRNA by examining protein expression through immunohistochemistry techniques (Hunter *et al.* 1995; Wahlestedt, 1994). It should be noted that mRNA levels are measured directly while protein concentration is measured indirectly. Nevertheless, the exact time course of the antisense ODN action can be isolated using both of these techniques.

There are no behavioural papers to date that have incorporated this type of rigorous demonstration of specific inhibition by verifying both decreased mRNA levels and protein density. Our results highlight the necessity for this type of verification of specificity and affinity of the antisense ODN for the complementary mRNA target. It is clear from our results that specificity cannot be solely inferred from the behavioural effects of antisense as compared to the control ODNs without measuring the protein concentration and/or the levels of targeted mRNA (Wagner, 1994).

Stability versus Toxicity of the Antisense ODN¹

The effectiveness of the antisense ODN seems to be determined by a variety of factors including its structure, stability, *in vivo* concentration and ODN access to the target mRNA (Skutella *et al.* 1994). However, a variety of biological effects that have been reported that cannot be attributed to the antisense mechanisms alone. Similar to other drugs, antisense ODNs can cause non-specific and potentially toxic side effects. Modification of an ODN increases the half-life of the sequence substantially thereby increasing the stability of the ODN. Unfortunately an increase in stability might also produce an increase in the toxicity of the sequences. These modified sequences (S-oligonucleotides) have been repeatedly linked to toxic side effects and sequence-independent results (Brysch *et al.* 1994).

A number of potential impurities are produced during the biosynthesis of the ODN that may not be removed before shipment from the supplier (Wagner, 1995; Wagner, 1994; Wahlestedt, 1994). Often the only purification step taken is a trityl-on reverse phase HPLC aimed at separating large, full-length fragments from shorter fragments. Many of the toxic by-products such as high salt and sulphur or ammonium ions are not adequately removed by this treatment alone. Toxicity is no doubt also caused by ODN fragments due to the degradation of the ODN nucleotides. The abundance of intra- and extra-cellular nucleotides can affect cell proliferation and cell differentiation (Wagner, 1994). The high concentrations and excess quantity of antisense ODNs injected could also account for the observed side effects reported in animals. Although substantially lower doses of ODNs are typically used for site injections as compared to i.c.v. injections, similar toxic behavioural responses are reported

(Weiss *et al.* 1993; Wahlestedt *et al.* 1993a; Wahlestedt *et al.* 1993b; Simons *et al.* 1992; Chiasson *et al.* 1992).

Inhibition of behaviour or toxicity response? Is food an appropriate control?

The question of toxicity and non-specific effects due to the modification of the ODN backbone is one that requires further investigation. A balance must be maintained between ODN stability *in vivo* due to modification and the toxic effects the modification may cause. Our results suggest that disruption of self-administration behaviour is not sequence specific due to the fact that all animals across all groups reacted similarly to the antisense ODN and control treatments.

Of particular concern was our observation that in some animals, the phosphorothioated ODN (both antisense and control sequences) produced toxic-like responses including piloerection, decreased body temperature and reduced food and water intake. Although a similar malaise was observed in some animals in the food reinforcement group, behavioural disruption was not observed. Furthermore, although slight decreases in responding were seen, any decreases in responding were not as large or long lasting as that observed in the cocaine self-administration group. For this reason it should be questioned whether the observed side effects were due to the toxic by-products of the ODNs or to the dose and its toxicity for the animal. It should be noted that the return to baseline levels of responding seen in many animals is not a reassurance of the lack of toxicity in these animals (Wahlestedt *et al.* 1993a; Wahlestedt *et al.* 1993b; Wahlestedt, 1994). The failure to find a significant behavioural effect

of the antisense ODN with the food animals suggests that food reinforced responding is more robust to the potential toxic effects of the ODN treatment.

The possibility exists that there is a biochemical difference between the brain of a chronic cocaine self-administration animal and that of a cocaine naive animal (as in the food reinforced responding animals) with respect to metabolism and brain chemistry. It should be questioned whether chronic exposure to a drug that effects the DA system like that of cocaine could potentially effect either the toxicity or the specificity of the ODN, and whether or not the antisense ODN functions similarly in a cocaine reinforced animal as compared to a food reinforced (drug naive) animal. It follows that the changes in brain chemistry of the chronic cocaine animals may interact differently following ODN treatment and two very different types of brain receptor changes would be in evidence.

Summary

There have been some encouraging reports on the use of the ODN approach. However, the mechanism of antisense ODN action remains speculative and may include specific genetic as well as toxic non-specific mechanisms. Clearly demonstrating that specific gene inhibition has occurred and that it is due to an antisense ODN requires meticulous isolation of the results from any non-specific effects the ODN may have had. Although the successes of the antisense strategy both *in vitro* and in clinical trials (Marshall *et al.* 1993; Stein *et al.* 1996; Cohen *et al.* 1994; Hunter *et al.* 1995; Gosztanyi *et al.* 1994) are encouraging, the use of ODNs to inhibit specific proteins as a basic research tool in behavioural paradigms is premature. Toxicity of an ODN and ensured stability of that

particular ODN in the functional time frame of complete protein turnover must be understood before *in vivo* manipulation can be properly interpreted. Sequence dependance of the antisense ODN is the ultimate goal of the antisense strategy and the approach of first testing the ODN efficacy *in vitro* is advisable. Our results suggests that a demonstration of sequence dependance does not allow the ruling out of toxicity due to the injection alone. Until distinction between specificity and toxicity is made, the antisense strategy is not yet a valuable tool for investigating complex and motivational behaviours.

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