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MEDIAL PREFRONTAL CORTICAL INJECTIONS OF C-*FOS* ANTISENSE OLIGONUCLEOTIDES TRANSIENTLY LOWER C-FOS PROTEIN AND MIMIC AMPHETAMINE WITHDRAWAL BEHAVIOURS

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Abstract—Prefrontal cerebral cortical areas display decreased expression of several transcription factor/immediate-early genes, including c-*fos*, during amphetamine withdrawal.[52] Antisense strategies can help to test possible roles for this prefrontal c-*fos* down-regulation in the behavioural correlates of amphetamine withdrawal. Medial prefrontal cortical injections delivering 1.7 nmoles of anti c-*fos* oligonucleotides revealed an approximately 3 h half-life for phosphothioate and a 15 min half-life for phosphodiester oligonucleotides. Antisense phosphothioates complementary to the c-*fos* translational start site reduced levels of c-Fos protein, while exerting modest and variable effects on c-*fos* messenger RNA levels. Neither missense phosphorothioate nor antisense phosphodiester oligonucleotides significantly reduced levels of either c-*fos* messenger RNA or protein. Animals injected with anti c-*fos* phosphothioate oligonucleotides into the medial prefrontal cortex displayed marked reductions in linear locomotor activity and repetitive movements measured in a novel environment, effects not seen when missense oligonucleotides were used or when animals were accustomed to the activity monitor prior to antisense oligonucleotide injection.

Behavioural changes produced by prefrontal cortical injections of c-*fos* antisense oligonucleotides closely mimic alterations recorded during amphetamine withdrawal. Prefrontal c-*fos* could thus conceivably play roles in the neurobiological underpinnings of psychostimulant withdrawal and of responses to stressors such as exposure to novel environments. © 1997 IBRO. Published by Elsevier Science Ltd.

Psychostimulant withdrawal syndromes are characterized by dysphoria in humans,[22,23,38,58,73] and by decreased self-stimulation, altered swim test responses and reduced locomotor activity in experimental animals exposed to novel environments.[35,36,39,47,52,59,60] Withdrawal dysphoria and drug craving contribute to relapse and maintenance of destructive addictions despite currently available treatments. Similarities between psychostimulant withdrawal and idiopathic depression also spur interest in the neurobiological underpinnings of psychostimulant withdrawal syndromes.[3,38,43,58,70,77,78]

Transcription factors could play roles in chronic amphetamine-induced neuroadaptive processes underlying tolerance, sensitization, craving and/or withdrawal by linking extracellular signals, such as those produced by psychotropic drugs, to changes in nuclear gene transcription (for review see Ref. 53). A two-week, high-dose amphetamine regimen can exert a profound impact on prefrontal immediate-early gene expression, for example.[50,52,59] Initial decreases in prefrontal c-*fos*, fos-B, jun-B, and *zif*268 mRNAs coincide with reductions in prefrontal cortical and striatal dopamine content, and with blunted repetitive exploratory behaviours in novel environments.[52,59] Behavioural alterations, however, can persist after apparent normalization of transcription factor mRNA levels and dopamine tissue levels.[52]

Delayed behavioural recovery following amphetamine withdrawal suggests the possibility that gene regulation mediated by trans-acting factors such as members of the Fos/Jun and *zif*268/NGF-I families

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Abbreviations: AP-1, activator protein-1, bp, base pairs; BSA, bovine serum albumin; EDTA, ethylenediaminetetra-acetate; ELISA, enzyme-linked immunosorbent assay; PB, phosphate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; T–PB, phosphate buffer containing 0.1% Triton X-100; T–PBS, PBS and 0.05% Tween-20.

could regulate the production of proteins whose modulation could change neuronal activities and lead to behavioural withdrawal syndromes. Administration of c-*fos* antisense oligonucleotides *in vivo* can produce behavioural alterations when they are effective in blocking c-*fos* induction.[18,28,31,44,61,79]

In this study we employed antisense technology to assess possible involvement of an immediate-early gene's products in the behavioural alterations that accompany amphetamine withdrawal. Anti-c-*fos* oligonucleotides with phosphodiester and phosphothioate backbones were delivered to rat medial prefrontal cortex, the rates of oligonucleotide delivery and degradation were monitored, and the time-courses of transcriptional and translational effects upon both baseline and psychostimulant-induced c-*fos* mRNA and protein levels were defined. These biochemical assessments of efficacy were correlated with assessments of novelty response behaviours in rats 4–26 h following oligonucleotide injections.

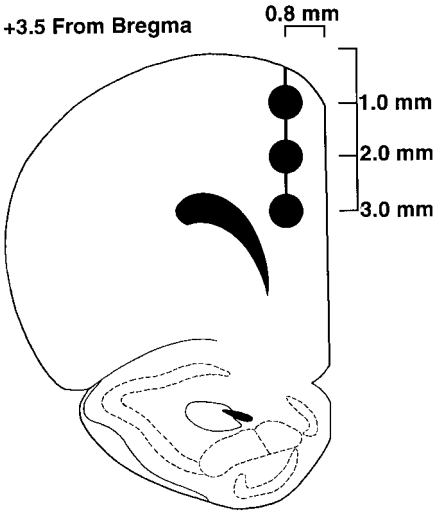


Fig. 1. Location of injection sites in the medial prefrontal cortex. Injections were made successively moving from more ventral to more dorsal sites. Coordinates are expressed in millimeters (adapted from Paxinos and Watson[48]).

EXPERIMENTAL PROCEDURES

Stereotaxic injection procedures

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g, housed three per cage, were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) and allowed free access to food and water. During study days, rat cages were kept in the animal housing facility; animals employed in all experiments were singly brought into the surgical room, rapidly anaesthetized using halothane (1.5–2.5 atm.), placed on a warm pad to maintain their temperature at 37°C, operated, and brought back to their cage in the animal facility just before coming out of the anaesthesia.

With the incisor bar adjusted to keep the bregma-lambda plane horizontal, two 28-gauge injection cannulas were inserted bilaterally into the medial prefrontal cortex 3.5 mm anterior to bregma and 0.8 mm lateral of the midline[48] (Fig. 1). The cannulae were then connected to 2 µl syringes (Hamilton, Reno, NV) placed on an infusion pump (Harvard, South Natick, MA) using P20 tubing filled with saline solution. A 1.5 µl saline volume containing 2 nmol of oligonucleotides was injected at a steady rate of 0.25 µl/min; 0.5 µl were injected at dorso/ventral locations 3.0 mm, 2.0 mm, and 1.0 mm (Fig. 1), with 2 min allowed between ejections to facilitate diffusion and 5 min before cannula withdrawal.

Oligonucleotides

Two distinct pairs of antisense and ''scrambled'' missense oligonucleotide sequences were designed for this study. The first pair includes c-*fos* antisense sequence 1 (AS1) 5′-GAACATCATGGTCGTGGT-3′, targeting c-*fos* mRNA bases 126–143[13] which span the start codon, the following two codons and nine bases of the Kozak sequence upstream,[37] and missense sequence 1 (MS1) 5′-AGTATCAGACGTTCGTGG-3′, containing the same bases present in the AS1 sequence in a different order. AS1 contains the entire sequence of antisense 15-mers [c-*fos* mRNA bases 129–143], previously shown to block amphetamine-induced Fos expression[8] and to yield behavioural effects in several distinct paradigms,[18,28,31,44,61] and spans three bases further upstream into the Kozak sequence. The second pair includes c-*fos* antisense sequence 2 (AS2) 5′-TTGGGCAAAGCTCGGCGA-3′, targeting the c-*fos* mRNA segment immediately upstream of sequence AS1 [bases 5′-108-125-3′],[13] and missense sequence 2 (MS2) 5′- GTACGTGGCCATGACAGG-3′.

These four oligonucleotide sequences were chosen following a computerized analysis performed using the STEMLOOP and BLASTN programs (GCG Wisconsin Package, version 8, Genetics Computer Group Inc., 1994), to minimize stem/loop secondary structures likely to reduce c-*fos* mRNA binding efficiency, and hybridization to other mRNA sequences present in the Genbank database.[1] Oligonucleotides employed in this study were obtained from Oligo etc. Inc. (Wilsonville, OR) using phosphodiester and phosphorothioate backbone chemistries (see below).

Efficiency of oligonucleotide delivery

To assess oligonucleotide delivery, phosphodiester AS1 and MS1 oligonucleotides were initially labelled with [^{35}S]CTP using a primer extension method, as previously described,[67] with 25-base templates and 10-base primers designed to yield the AS1 (template: 5′-ACCACGA CCATGATGTTCTCGGGTT-3′; primer: 5′-GAACATCA TG-3′) and the MS1 (template: 5′-CCACGAACGTC TGATACTCCCCCCC-3′; primer: 5′-AGTATCAGAC-3′) sequences. Labelled products were then diluted to approximately 9×10^3 c.p.m./1.5 µl, which were injected bilaterally into the medial prefrontal cortex as described above, to consistently yield an overall dose of 18×10^3 c.p.m./animal. Labelled oligonucleotides flowing back out of the brain during the infusion and following withdrawal of the injectors were collected using a small cotton tip, without applying pressure on the brain surface or the injectors to avoid actively driving labelled oligonucleotide solution out of the injection track. Cotton tips were then placed in scintillation fluid and radioactivity counted. In some animals, prefrontal regions were carefully dissected out and placed in scintillation fluid to assess radiation densities.

Intracerebral oligonucleotide kinetics

Oligonucleotide half-lives were assessed by injecting radioactively-labelled AS1 and MS1 oligonucleotides into the medial prefrontal cortex, killing the animals at different time intervals following the injection, extracting DNA from the brain and assessing the sizes of radiolabelled fragments by polyacrylamide gel electrophoresis and autoradiography.

AS1 and MS1 oligonucleotides were assessed both in phosphodiester and phosphothioated form following 5′end-labelling with [^{32}P]ATP[56] to a final specific activity of 10^5 c.p.m./µl. A subset of animals was also injected with phosphodiester oligonucleotides labelled with [^{35}S]-CTP using primer extension as described above, or with chimeric [^{32}P]ATP 5′ end-labelled phosphodiester oligonucleotides with thioester bonds between T and C in positions 12–13 and 13–14 for AS1 and MS1, respectively, to closely mimic oligonucleotides obtained through primer-extension in the presence of [^{35}S]CTP.

At different time points during the 24 h following the end of oligonucleotide injections, animals were decapitated, brains were quickly dissected, prefrontal regions were homogenized using a polytron in 1.5 ml of phosphate-buffered saline (PBS) and 0.8 mM EDTA, and extracted twice with phenol:chloroform. The supernatant was then dialysed overnight in pre-washed dialysis tubing (GibcoBRL, Gaithersburg, MD), lyophilized to a final volume of 10 µl, and samples separated by electrophoresis using 8% denaturing acrylamide gels. Gels were dried and band intensities were quantified using phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Ribonuclease protection assays

Oligonucleotide effects on basal c-*fos* mRNA levels were assessed using ribonuclease protection assays. Separate sets of four animals were injected with AS1 phosphorothioated oligonucleotides in one hemisphere and with MS1 in the contralateral hemisphere. The medial prefrontal cortex of each hemisphere received AS1 oligonucleotides in two animals and MS1 in two other animals at each time point. Rats were then killed by decapitation at each of several different time points following the end of the oligonucleotide injections. Brains were quickly removed and dissected at 4°C according to a modification of the technique of Glowinski and Iversen,[24] frozen on dry ice and stored at −70°C until analysis.

Total cellular RNA was extracted from the medial prefrontal cortex, using an acid guanidinium thiocyanate–phenol–chloroform method,[9] as described.[50,52] Ribonuclease protection assays were carried out as previously described by Vandenbergh *et al.*[69] Briefly, 5 µg of total RNA were hybridized overnight in a total volume of 10 µl to radiolabelled antisense c-*fos*[65] and β-actin[27] cRNA probes, differing by approximately 150 bases in size. Unhybridized RNAs were digested with a mixture of ribonuclease A (Sigma, St Louis, MO) at 25 ng/ml and T1 (GibcoBRL, Gaithersburg, MD) at 100 U/ml, and separated using 6% denaturing polyacrylamide gel electrophoresis. Gels were dried and band intensities were quantified using phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

mRNA determinations reported here represent mean ± S.E.M. of three to four hemispheres per treatment group per time point. Each RNA sample from each animal was assessed two to three times in different assays, with c-*fos* values normalized to the value for β-actin mRNA from the same lane and averaged to yield reported values for each sample. Levels of β-actin mRNA were not affected by drug treatment (data not shown).

Effects of antisense oligonucleotides on psychostimulant-induced c-*fos* gene expression were assessed in separate groups of three to four rats receiving D-amphetamine sulphate (5 mg/kg i.p.) 2 or 4 h following medial prefrontal cortical injection of AS1 and MS1 phosphothioated oligonucleotides into opposite hemispheres. These rats were killed 1 h after D-amphetamine administration and brain tissue was processed as described above.

c-Fos immunocytochemistry

Effects of antisense and missense oligonucletide treatment on amphetamine-induced c-Fos protein levels were assessed using c-Fos immunocytochemistry. Animals were injected with AS1 or AS2 phosphorothioated oligonucleotides in one hemisphere and with MS1 or MS2, respectively, in the contralateral hemisphere. At different time intervals after the end of oligonucleotide injections, rats were treated with D-amphetamine sulphate (5.0 mg/kg i.p.). Two hours following the D-amphetamine administration, animals were deeply anaesthetized with sodium pentobarbital (>50 mg/kg), perfused with 0.1 M PBS (pH=7.4, 21°C) followed by 4% paraformaldehyde dissolved in PBS (4°C), postfixed for 12–36 h and cryoprotected in 30% sucrose dissolved in PBS.

Immunocytochemical procedures were performed on 40 µm-thick coronal sections as previously described.[46] Briefly, slices were pre-incubated 1 h at room temperature in 0.1 M phosphate buffer (PB; pH=7.4, 21°C) containing 0.1% Triton X-100 (T–PB) and 3% goat serum and were subsequently incubated for 16–48 h at 4°C with rabbit polyclonal c-Fos antibodies (Oncogene Science, Uniondale, NY) diluted 1:1000 in preincubation solution. After three 30 min washes in T–PB, slices were incubated for 1 h at room temperature with a 1:200 dilution of biotinylated goat anti-rabbit antibodies (Chemicon, Temecula, CA). After three 10 min washes in T–PB, a standard avidin/biotin detection procedure was performed using the ABC Vectastain Standard kit (Vector Laboratories, Burlingame, CA). Slices were then incubated in 0.1 M PB containing 0.05% diaminobenzidine for 10 min, followed by 0.015% H_2O_2. Slices were finally rinsed twice in PBS, mounted on gelatin-coated slides, air-dried overnight, dehydrated, cleared in xylene, and coverslipped.

Immuno–polymerase chain reaction

Oligonucleotide effects upon the low basal levels of c-Fos protein were assessed using a recently-developed immuno–polymerase chain reaction (PCR) approach that provides sensitivity in the picomolar range.[57] Briefly, four animals per time point were injected with AS1 and MS1 phosphorothioated oligonucleotides into opposite hemisphere, and decapitated at different time intervals following the end of oligonucleotide injections. Brains were quickly dissected and medial prefrontal cortices were frozen on dry ice and stored at −70°C. Each well of a high-binding, flat-bottom ELISA plate (Costar, Cambridge, MA) was coated overnight at 4°C with 200 µl of a 0.1 M $NaHCO_3$ (pH 8.6) solution containing 100 µl of mouse monoclonal c-Fos antibody (Oncogene Science, Uniondale, NY). The antibody solution was then removed and replaced with 200 µl of 3% bovine serum albumin (BSA) dissolved in PBS for 2 h at 37°C. Using a polytron, tissue samples were homogenized in PBS+1% BSA and diluted to protein concentrations of 250 µg/ml. Following blocking, wells were washed five times with cold PBS and 0.05% Tween-20 (T–PBS), 100 µl of tissue protein solution were added, wells were incubated for 2 h at 37°C. Wells were then washed five times with cold T–PBS. One hundred microliters of a 1% BSA in PBS solution containing 10 µl rabbit anti-Fos polyclonal antibody/ml were added to each well and incubated for 90 min at 37°C. After five washes in cold T–PBS, 100 µl of a 1% BSA in PBS solution containing 10 µl of biotinylated donkey anti-rabbit IgG was added to each well and incubated for 45 min at 37°C. During this time, 15 µg of reporter DNA, which had been previously digested with EcoRI and labelled with biotin-14-dATP using the Klenow fragment of DNA polymerase I, was added to 100 µg avidin (Boehringer-Mannheim, Indianapolis, IN) in 4 µl 1% BSA in PBS and incubated for 30 min at room temperature. Following five more washes in cold T–PBS, wells were incubated for 30 min at room temperature with 100 µl of biotinylated reporter DNA/avidin complex. After five more washes, reporter DNA was recovered by adding 100 µl of nuclease-free water to each well, and heat-denatured for 5 min at 95°C. The eluent was collected and 5 µl used as template for a 25-cycle PCR reaction in the presence of

[^{32}P]dATP using primers specific for the reporter DNA. A 25 µl aliquot of the PCR reaction was separated by electrophoresis on a 8% polyacrylamide gel, which was then stained with ethidium bromide to visualize the 550 base pair (bp) PCR product, which was excised and quantitated by scintillation counting.

Data collected in four animals per time point, are expressed as mean ± S.E.M. change in basal c-Fos protein levels contrasted with levels recorded at the onset of surgery (zero time point), after subtraction of background. Each value was determined in duplicate for each animal.

Tubulin western blotting and Commassie staining

Western blotting to assess non-specific disruptions in tubulin protein levels was performed as described.[15] A set of animals was injected and processed, yielding gels that were stained in Commassie Brilliant Blue R250 for 30 min and destained until low background densities were achieved.

Behavioural testing

Groups of three to 10 animals/time point were tested in a ''novelty'' response paradigm, as previously described.[52,59] The locomotor exploratory activity of each animal was assessed on a single occasion in an activity monitor (Columbus Instruments, Columbus, OH), to which these rats had not previously been exposed. Distinct groups of animals were tested every 2–4 h, at times ranging from 4 to 26 h following bilateral injections of saline, AS1, or MS1 phosphorothioated oligonucleotides, with the monitor serving as a novel environment. The schedule of behavioural assessments was based on preliminary results that established 4 h as the earliest time point when rats consistently recover novelty responses within the normal range following surgery (data not shown), and to previous studies describing prominent behavioural effects of c-*fos* antisense oligonucleotides occurring within 24 h of injections.[18,28,31,44,61,79] Surgery was scheduled so that behavioural assessments would consistently be performed soon after the onset of the dark cycle, between 8.00 and 8.30 p.m., to minimize circadian variations in locomotor responses to novelty (A.M.P. and C.W.S., unpublished observation) and in transcription factor gene expression.[25]

Distinct sets of seven to 11 rats were injected bilaterally with either AS2 or MS2 phosphothioated oligonucleotides and were behaviourally tested 4, 6 or 8 h following oligonucleotide injections. Two additional sets of four rats were habituated by placing them in the behavioural testing environment twice daily for 1 h during the three days prior to surgery, injected with AS1 or MS1 oligonucleotides, and then tested for locomotion in the same apparatus under ''habituated'' conditions.

Gross locomotor activity (''distance travelled'') and time spent in repetitive motor activities, when the animal repeatedly breaks the same infrared beam (''stereotypy time'') were assessed during 60 min sessions using the auto-track system (Columbus Instruments, Columbus, OH) and are expressed as mean ± S.E.M.

Statistical analyses

Two-way ANOVAs for two treatment groups and six time points were employed to contrast normalized c-*fos* mRNA levels in antisense- vs missense-injected hemispheres. Two-way ANOVAs were also used to assess saline vs antisense vs missense oligonucleotide-induced behavioural effects recorded 4, 6 and 8 h following surgery; three treatment groups and three time points were contrasted. Significant time × treatment interactions were followed by overall comparisons among treatment groups using Fisher's least significant difference test. Student *t*-tests were used to analyse behavioural responses of habituated animals. Power analyses performed using the POWER program[19] were used to establish appropriate sample sizes for experiments on dependent variables whose dispersion in untreated or saline-treated animals could be estimated from prior work or from the literature. Whenever possible, sample sizes were set to achieve a power of 0.8, with α=0.05 and β=0.20. Data are expressed as mean ± S.E.M.

RESULTS

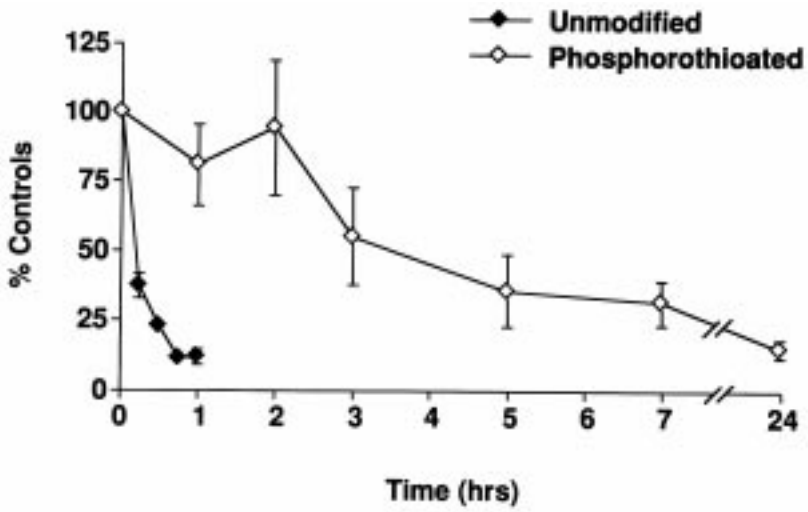
Efficiency of oligonucleotide delivery

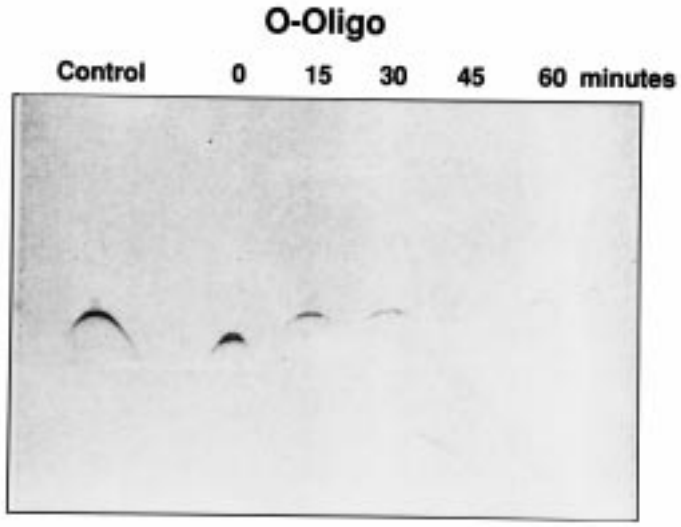
The amount of oligonucleotides actually delivered into the targetted brain region by intracerebral injection was initially assessed, to establish the dose of oligonucleotides to be employed in subsequent experiments. Ten animals were injected into the medial prefrontal cortex with AS1 and MS1 phosphodiester oligonucleotides radiolabelled using primer extension with [^{35}S]CTP to 18×10^3 c.p.m./1.5 µl; 2381 ± 385 c.p.m. could be recovered on the brain surface at the injection site during the infusion and immediately after withdrawal of the injectors. This represented a loss of 13.2 ± 2.1% (range 3.1–23.7%) of the injected dose. Medial prefrontal cortices from each of two tested animals contained ca. 85% of the injected radioactivity. Approximately 1.7–1.8 nmol of oligonucleotide are thus actually delivered into the medial prefrontal cortex when 2 nmol dissolved in 1.5 µl of saline are injected using the procedure employed here.

Intracerebral oligonucleotide kinetics

The intracerebral kinetics of unmodified vs phosphothioated oligonucleotides were defined, to choose the backbone conveying highest antisense activity *in vivo*. Phosphodiester and phosphothioated 5′ end-labelled oligonucleotides displayed dramatically different kinetics following intracerebral injection (Fig. 2). By 15 min after injection, unmodified phosphodiester oligonucleotides were degraded so that only 37.4 ± 4.5% of the injected dose remained intact. Only 12.1 ± 2.8% remained intact at 1 h (Fig. 2). Phosphorothioated oligonucleotides, on the other hand, displayed an apparent half-life of several hours; 80–95% of the injected dose could be recovered from the brain 2 h following the injection, while 15.2 ± 3.4% of the injected dose was still intact at 24 h (Fig. 2). No differences in kinetics were noted between AS1 and MS1 oligonucleotides that differed in sequence but shared phosphorothioate backbones (data not shown).

To assess whether the apparent rapid degradation rate of phosphodiester oligonucleotides could artifactually stem from phosphatase-mediated dephosphorylation of end-labelled phosphate groups, a subset of four animals received phosphodiester oligonucleotides internally labelled with [^{35}S]-CTP using primer-extension, while a second group of four animals received chimeric 5′ end-labelled oligonucleotides structurally identical to the oligonucleotides obtained using primer extension. Both displayed rapid degradation rates virtually superimposable on





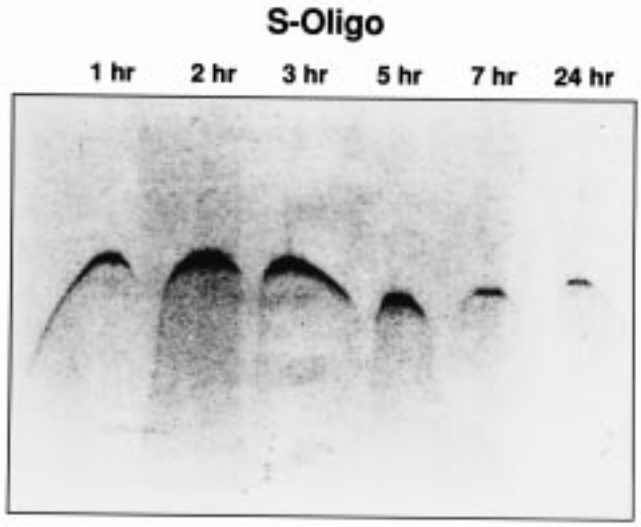


Fig. 2. Amounts of radiolabelled oligonucleotides recovered from rat prefrontal regions at different time intervals following single bilateral intracerebral injection (top panel). Data are expressed as mean ± S.E.M. (n=3–4 animals/time point). Amounts of radiolabelled phosphodiester (middle panel) and phosphorothioate (bottom panel) oligonucleotides recovered from rat prefrontal regions at different time intervals during the first hour following the injection and run on an acrylamide gel as described in Experimental Procedures.

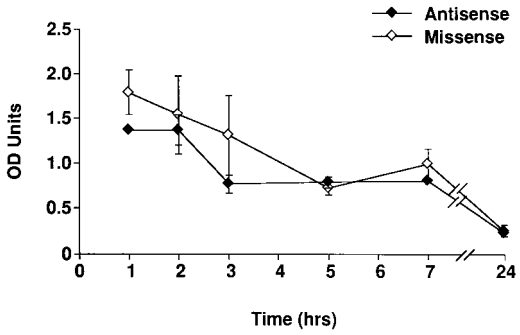


Fig. 3. Basal levels of c-*fos* mRNA detected using ribonuclease protection assays on rat medial prefrontal cortex at different time intervals following AS1 antisense or MS1 missense intracerebral injection. Each animal's RNA was assessed two to three times on different assays, with c-*fos* mRNA values normalized to β-actin mRNA values each time and finally averaged. Data are expressed as mean ± S.E.M. (n=3–4 animals/time point).

those of end-labelled phosphodiester oligonucleotides (data not shown).

Oligonucleotide effects on c-fos messenger RNA levels

Mechanisms of antisense action *in vivo* were investigated both at the transcriptional and translational levels. Phosphorothioated AS1 and MS1 oligonucleotide effects on basal c-*fos* mRNA levels, assessed using RNAse protection assays and normalized to β-actin mRNA levels, are summarized in Fig. 3. AS1-injected hemispheres display a trend toward decreased c-*fos* mRNA levels (two-way ANOVA: treatment F=3.407; d.f. 1,38; P=0.076). The impact of AS1 antisense oligonucleotides toward reducing c-*fos* mRNA levels is evident only during the first 3 h following the injection, and disappears by 5 h (Fig. 3). Missense-injected hemispheres frequently display variable stress-induced enhancements in c-*fos* gene expression 1 h after the injection, while antisense-treated hemispheres show initially blunted c-*fos* mRNA responses (Figs 3, 4). Levels of c-*fos* mRNA found in missense-treated hemispheres displayed such variability that sample sizes of 13, 110, and 25 rats would have been necessary to achieve statistically significant results at 1, 2, and 3 h, respectively, according to power calculations.

Further evidence supporting a limited impact of AS1 oligonucleotides on c-*fos* mRNA levels comes from studies of psychostimulant-induced c-*fos* gene expression assessed at 3 and 5 h. In rats killed 1 h following amphetamine injections, which were performed 2 or 4 h following AS1 and MS1 oligonucleotide injections into opposite hemispheres, c-*fos* mRNA was up-regulated in both hemispheres. Medial prefrontal cortical c-*fos* mRNA levels in AS1-treated hemispheres were 91.85 ± 1.0% of those in MS1-treated hemispheres, 3 h after oligonucleotide injections, and no differences were noted by 5 h following surgery (data not shown).

Oligonucleotide effects on Fos protein levels

The impact of antisense treatment on basal Fos protein levels was investigated using the immuno–PCR approach in prefrontal cortical tissues of hemispheres treated with phosphorothioated AS1 or MS1 oligonucleotides. These assessments revealed prominent, albeit variable effects during the first 24 h following oligonucleotide injections (Fig. 5). Antisense-treated hemispheres showed significant reductions in basal Fos levels 3 h after oligonucleotide injections, followed by rebound increases in Fos production at 5–7 h. These effects occur, however, in the context of generally elevated post-surgical Fos inductions (Fig. 5) and significant inter-individual variability (see Discussion). None of these changes are induced by control missense MS1 oligonucleotides injected in the contralateral hemispheres.

Amphetamine-induced prefrontal cortical Fos protein levels also display dramatic differences between AS1- and MS1-injected hemispheres (Fig. 6, Table 1). Phosphothioated AS1 oligonucleotides virtually block Fos induction when amphetamine is administered 1 h after oligonucleotide injection. This blockade persists at 2 h, and rapidly declines thereafter (Fig. 6, Table 1). A separate group of rats injected with phosphorothioated AS2 or MS2 oligonucleotides displayed partial AS2-induced effects only at 1 h, but revealed no differences between AS2- and MS2-injected hemispheres at later time points (Table 1).

Oligonucleotide effects on protein tissue content and tubulin levels

Generalized, non-specific effects upon translational processes due to phosphorothioate-induced toxicity were initially sought by comparing Commassie-stained proteins prepared from AS1- and MS1-injected hemispheres. No gross changes in medial prefrontal cortical protein staining patterns was evident among AS1- and MS1-injected hemispheres between 1 and 24 h following surgery (Fig. 7). Levels of tubulin immunoreactivity were also unchanged throughout the first 24 h both in AS1- and MS1-injected hemispheres (Fig. 7).

Behavioural testing

Potential links between the amphetamine withdrawal-induced decreases in prefrontal cortical immediate-early gene expression we previously described,[52] and the behavioural alterations in novelty locomotor responses we recorded during psychostimulant withdrawal[52,59] were addressed. Rats receiving bilateral medial prefrontal cortical injection of phosphothioated AS1 oligonucleotides displayed prominent decreases in novelty locomotor responses at 4 h, when contrasted with rats injected with saline or MS1 oligonucleotides (Fig. 8). Both

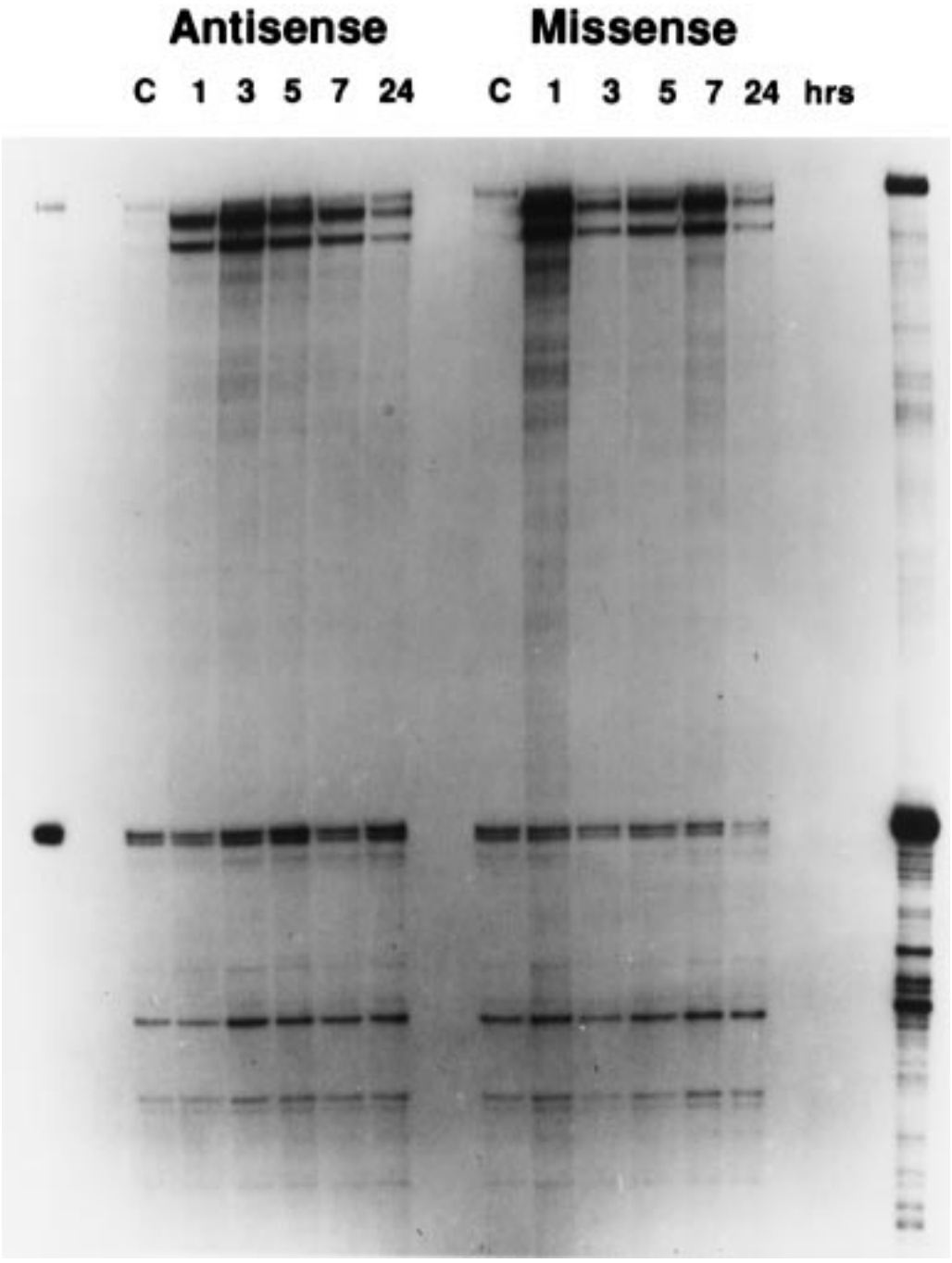


Fig. 4. Ribonuclease protection assay, displaying two upper bands produced by protected c-*fos* mRNA segments and several lower bands produced by β-actin mRNA. The first and last lanes display different amounts of labelled probe; C, uninjected control animal.

''stereotypy time'' spent in repetitive exploratory movements and assessments of linear ''distance travelled'' appeared significantly affected (Fig. 8). No difference was recorded at any other time point between 6 and 26 h (Fig. 8 and data not shown). Interestingly, no trend toward decreases in locomotor activity was recorded at 4 h in AS1-injected animals habituated to the activity monitor prior to oligonucleotide injection (distance travelled: t=0.18, 6 d.f., P=0.864; stereotypy time: t=0.47, 6 d.f., P=0.655; Table 2).

Exploratory locomotor behaviours were similar in rats injected with phosphothioated AS2 or MS2 oligonucleotides. Preliminary experiments performed on three rats injected with phosphodiester oligonucleotides also showed no differences between animals injected with AS1, MS1, or saline (data not shown).

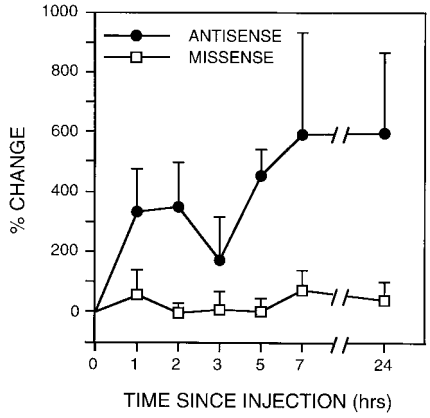


Fig. 5. Basal c-Fos protein levels recorded using immuno–PCR during the first 24 h following AS1 and MS1 injection into opposite hemispheres. Data are expressed as mean ± S.E.M. change in basal c-Fos protein levels contrasted with levels recorded at the onset of surgery (zero time point) in a distinct set of four animals, after subtraction of background (n=3–4 animals/time point). Each value was determined in duplicate for each animal and averaged.

DISCUSSION

Previous studies have shown that repeated amphetamine administration can produce withdrawal-induced behavioural alterations, changes in monoamine turnover rates, and prefrontal decreases in mRNAs encoding members of the FOS/JUN and Zif transcription factor families, including c-*fos*.[52,59] This work describes behavioural effects produced by phosphothioated c-*fos* antisense oligonucleotide injections into the medial prefrontal cortex of adult rats, defines temporal correlations with Fos protein reductions, and provides support for roles of medial prefrontal cortical gene regulation in psychostimulant withdrawal-related behavioural alterations. Data from distinct converging approaches employed here support the efficiency and region-specificity of oligonucleotide deliveries, and provide specific assessments of the kinetics, translational, transcriptional, and behavioural effects of intracerebral oligonucleotide administration.

Several features of the currently available *in vivo* antisense approaches were selected to enhance reliability (for reviews see Refs 12, 29, 45, 64, 71, 72). Rats were consistently handled, since inconsistent handling can provide interindividual differences in stress responses, prefrontal Fos levels[2,5] and behavioural responses to novel environments. Halothane was used since it allows animals to recover normal behavioural responses by 4 h following anaesthesia and because it exerts minimal influences on c-*fos* mRNA levels.[42] Single intracerebral injections were performed, since preliminary experiments revealed substantial injection-related behavioural disruption following two injections into each hemisphere (data not shown). Intracerebral stereotaxic injections used here documented that approximately 85–90% of oligonucleotide was successfully delivered, allowing local tissue concentrations in the 10–30 µM range that have proven effective in translational arrest and shortening mRNA half-lives *in vitro*.[21,68] Behavioural measurements in both the novelty and habituated paradigms were performed according to procedures previously standardized in rodent psychostimulant withdrawal-related studies.[50,52,59] These and other experimental features should provide the maximal opportunity to observe behavioural effects likely to represent specific results of c-*fos* inhibition.

c-fos *antisense-induced blunted novelty responses, amphetamine withdrawal behaviours, and stress*

Rats injected with AS1, and not MS1, phosphothioated oligonucleotides displayed decreased locomotor responses when exposed to a novel environment, but not when placed in a familiar environment. Repetitive components of exploratory movements were more profoundly affected than linear components. Both of these behavioural response patterns were strikingly similar to alterations previously recorded during withdrawal from amphetamine.[52,59,60] These parallels strengthen suggestions of potential relationships between the altered novelty responses recorded during amphetamine withdrawal and those observed following c-*fos* antisense treatments. The failure to find alterations in the locomotor activities of animals habituated to the activity monitor prior to antisense treatment underscores the specificity of medial prefrontal cortical roles in exploratory behaviour, as distinct from gross locomotor activity *per se*. Prominent involvement of medial prefrontal cortical brain regions in motivation, drug self-administration and locomotor responses to novelty has been supported by numerous studies (for review see Refs 16, 20, 40, 54).

Failure of c-*fos* knockout mice ''to respond in a typical fashion to stress (in the presence of) normal motor skills''[34,49] suggests that c-Fos involvement in stress responses may be at least as great as its role in learning, memory, and motor behaviours. Acute stressors enhance prefrontal dopamine turnover.[16] Repeated handling and saline-injection stress also produce profound alterations in brain transcription factor gene expression, cerebral monoamine levels and locomotor responses in novel environments.[50] Stress-induced decreases in transcription factor mRNA levels are, however, evident throughout the brain and not limited to prefrontal cortical regions.[52] Furthermore, frontal cortical hypoactivation also appears to link amphetamine withdrawal and idiopathic major depression,[3,10,43,70] syndromes displaying striking similarities in humans.[38,58,73,77,78] Our results may nonetheless be compatible with prefrontal mechanisms playing a more general role in stress responses, rather than being specifically implicated in psychostimulant withdrawal.

Blunted locomotor responses in novel environments were consistently recorded in animals tested

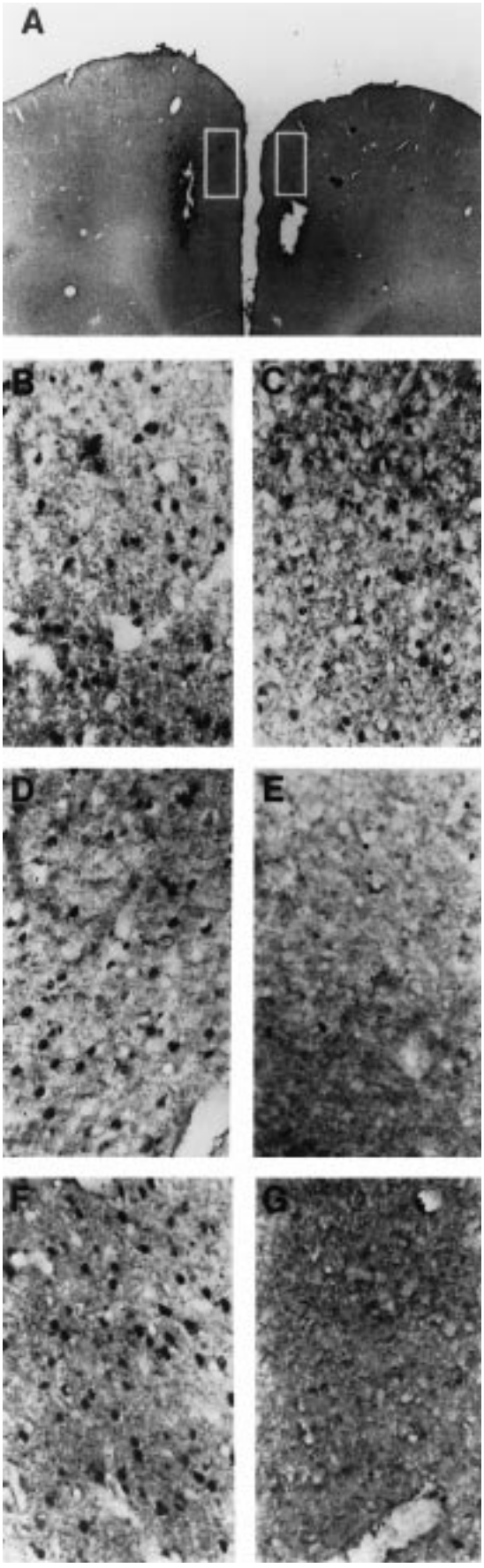


Table 1. Intensity of Fos-like immunostaining in the medial prefrontal cortex of rats injected with phosphothioated oligonucleotides, followed by systemic administration of D-amphetamine sulphate (5 mg/kg i.p.) at different time intervals, and killed exactly 2 h after psychostimulant administration

Time interval between oligonucleotide injection and amphetamine administration	AS1	MS1	AS2	MS2
1 h	0	+++	+	+++
2 h	0	+++	++++	+++
3 h	+	+++	+++	+++
4 h	++	++	++	+++
5 h	++	++	++	+++
6 h	++	++	++	+++
8 h	++	++	++	+++

4 h after injection of phosphothioated AS1 oligonucleotides. Considering the 2 h half-life of Fos protein, this time course provides plausible temporal coincidences between behavioural alterations, effective c-*fos* mRNA translational blockade, and significant decreases in basal Fos protein levels. Behavioural recovery following psychostimulant withdrawal does not simply mirror normalization of neurotransmitter turnover; conceivably, it may require longer-term adaptations that could include return to baseline levels of proteins relevant to neuronal function.[51,52] The temporal coincidence between induced and possibly basal Fos decreases with the behavioural alterations noted in the current study is in line with convergent results obtained in other studies employing c-*fos* antisense technology *in vivo*. Altered behavioural responses coincident with peak antisense-mediated effects on intracellular Fos levels have been recorded following unilateral striatal injections of c-*fos* antisense oligonucleotides that enhance susceptibility to apomorphine- and amphetamine-induced ipsilateral rotation.[18,31,61] Injections of c-*fos* antisense oligonucleotides in the nucleus accumbens dramatically blunt cocaine-induced rat locomotor activation concomitant with c-*fos* translational blockade.[28] Amygdaloid injections of antisense oligonucleotides block conflict-induced c-*fos* expression and produce benzodiazepine-like ''anxiolytic'' behavioural effects with similar time-courses.[44] Anti-c-*fos* oligonucleotides block light-induced phase shifts in the suprachiasmatic nucleus.[79] Intrastriatal injections of c-*fos* antisense, but not sense, oligonucleotides produce dramatic pathway-specific decreases in GABA

Fig. 6. Fos-like immunoreactivity in medial prefrontal cortical regions (panel A) of rats injected with D-amphetamine sulphate (5 mg/kg i.p.), either 0 h (panels B and C), 1 h (panels D and E), or 2 h (panels F and G) after intracerebral injections of AS1 antisense (panels C,E,G) or MS1 missense (panels B,D,F) oligonucleotides into opposite hemispheres. Panels B–G correspond to anatomical areas highlighted in panel A.

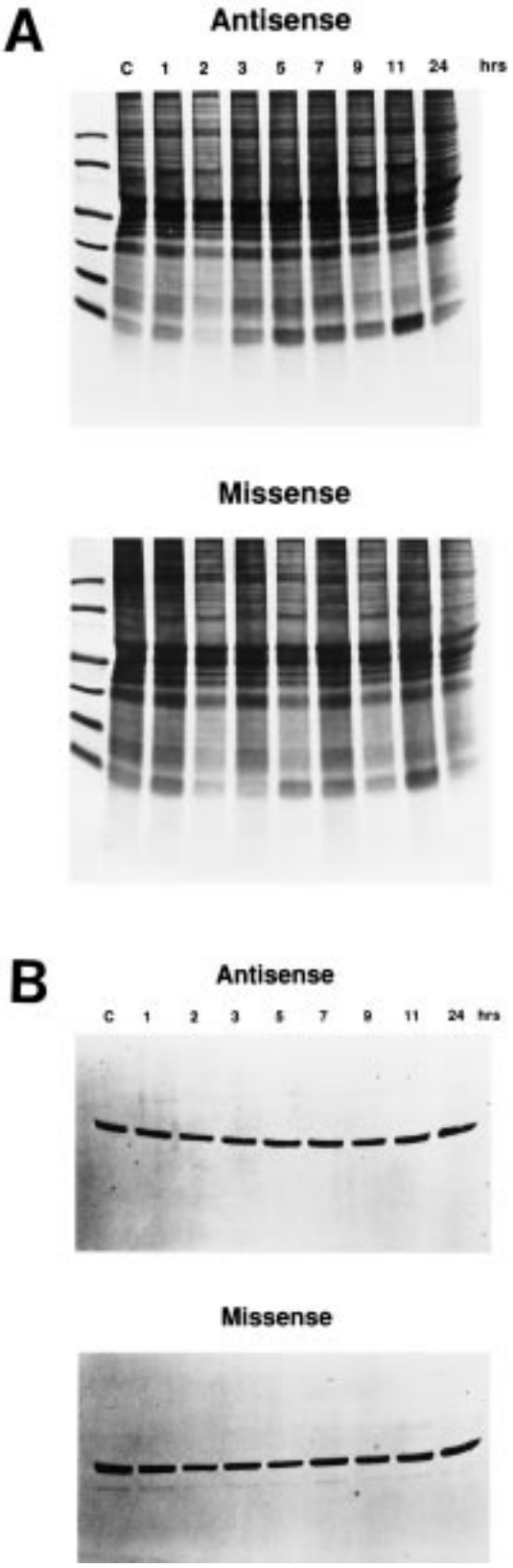


Fig. 7. Commassie staining of medial prefrontal cortical proteins (panel A) and prefrontal tubulin protein levels detected performing western blotting (panel B) following intracerebral AS1 and MS1 oligonucleotide injections into opposite hemispheres.

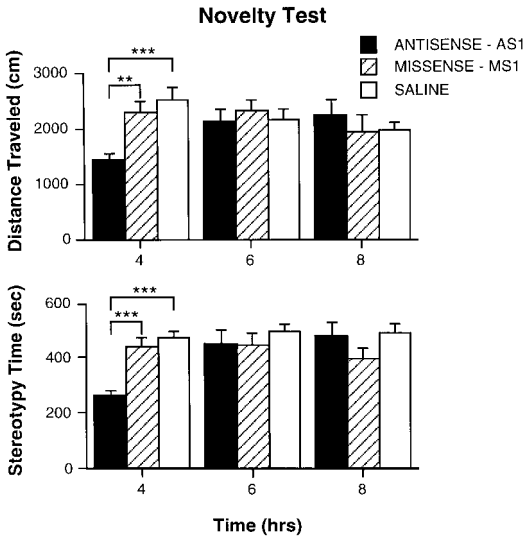


Fig. 8. Locomotor responses to novelty, recorded 4, 6, and 8 h after saline, AS1 antisense, or MS1 missense oligonucleotide bilateral injection into the medial prefrontal cortex of nine to 11 rats per sample (results recorded between 10 and 26 h are not shown). The linear and repetitive components of exploratory behaviour were measured in centimeters as ''Distance Travelled'' and in seconds as ''Stereotypy Time'', respectively. Data are expressed as mean ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$.

Table 2. Locomotor responses recorded 4 h after AS1 antisense, or MS1 missense oligonucleotide bilateral injection into the medial prefrontal cortex of groups of four rats habituated to the activity monitor prior to oligonucleotide injection

	Distance Travelled (cm)	Stereotypy Time (s)
AS1 Antisense oligos	1289.3 ± 280.0	358.1 ± 5.0
MS1 Missense oligos	1382.9 ± 443.3	323.8 ± 72.9

Linear and repetitive components of locomotor activity were measured in centimeters as ''Distance Travelled'' and in seconds as ''Stereotypy Time''. Data are expressed as mean ± S.E.M.

release in the substantia nigra and not in the globus pallidus; this effect is apparent by 60 min after the injection, peaks at 2 h and is attenuated at 3 h.[55] The rapid time-courses demonstrated in the current study and in these other investigations suggest that an activator protein 1 (AP1)-mediated transcriptional regulatory effect would need to be prompt and likely to alter levels of a protein with a very short half-life to adequately explain the behavioural changes observed. Alternatively, neuronal responses might be modulated by members of the Fos/Jun family acting through mechanisms other than direct alterations of transcription rates by AP1 complexes. Members of the Fos/Jun family can heterodimerize with members of other transcription factor families through leucine zipper motifs.[4,26] Protein–protein interactions can also use Fos protein regions distinct from the zipper

motif. Fos homodimers or Fos:Jun heterodimers can stably complex the glucocorticoid receptor and inhibit its transactivating properties, for example.[17,41,66] Similar direct protein–protein interactions between Fos/Jun factors and other transcription factors have been described also outside the central nervous system.[32] Finally, leucine zipper-like motifs have been identified in membrane proteins as distant from Fos/Jun family members as the recently-cloned thyroid iodide transporter, where leucines may be involved in oligomerization.[14] Conceivably, Fos interactions with these or other cellular mechanisms could contribute to its rapid actions. C-*fos* antisense oligonucleotides might acutely alter medial prefrontal cortical output, resulting in enhanced mesolimbic dopamine turnover and increased sensitivity to stressors, effects similar to those produced in rodents by excitotoxic prefrontal lesions.[33,40]

Specificity of oligonucleotide effects in vivo

Oligonucleotide injections could potentially produce non-specific effects through mechanisms that might include (a) non-sequence-specific, non-antisense inhibition; (b) sequence-specific, non-antisense (aptameric) inhibition; or (c) sequence-specific antisense inhibition of additional mRNA species distinct from the target mRNA.[6] Evidence from control experiments in the current study argues against a generalized disruption in translational processes due to phosphothioate toxicity, a common mechanism for non-sequence-specific/non-antisense inhibition. AS1 and MS1 oligonucleotide injections do not alter either Commassie-staining patterns for medial prefrontal cortical proteins or levels of tubulin immunoreactivity. Non-sequence-specific interactions between phosphothioated oligonucleotides and albumin,[63] acetaminophen,[11] or α-adrenergic receptors (Iversen P.L., personal communication) have been described and attributed to the thioated backbone. The failure of phosphothioated AS2, MS1 and MS2 oligonucleotides to mimic antisense AS1 effects argues against non-specific phosphorothioate backbone effects contributing substantially to the behavioural results observed in the current studies. Contributions of sequence non-specific, non-antisense, aptameric effects on mRNAs other than c-*fos* to the current results cannot be entirely excluded. The likelihood of non-sequence-specific aptameric interactions has been minimized, since no oligonucleotide contains the dG quartets or C/G-rich stretches that typically exert such nonspecific effects in other studies.[6,30,81] Sequence-specific antisense inhibition of mRNA species other than c-*fos* is also conceivable.[80] However, the Genbank database contains no significant identity (i.e. <10 bases) between AS1-targeted c-*fos* sequences and those of other mRNAs encoding proteins expressed in the brain. It seems unlikely that an unidentified protein target of these antisense treatments would have both behavioural effects and a much shorter half-life than that displayed by most receptor proteins, for example.[72,74]

In contrast to the minimal evidence for non-specificity of the antisense treatments used in the current work, strong evidence is produced for substantial effects on c-*fos*. Improved evidence for specificity could also come from studies replicating the effects noted here using antisense oligonucleotides recognizing different c-*fos* mRNA sequences. Unfortunately, attempts by several laboratories to identify alternate c-*fos* sequences whose recognition by different antisense oligonucleotides would reduce expression and allow parallel behavioural assessments have been ineffective. Neither Hooper *et al.*,[31] who selected sequences flanking the start site in the 3′ direction, nor the present work, examining sequences 5′ of the start site and Kozak sequence, have been successful at blunting c-*fos* expression with a second antisense oligonucleotide, lending some caution to interpretation of results. Furthermore, the c-*fos* antisense 15-mer employed in previous studies, which displays sequence overlapping the AS1 18-mer employed in the current studies, can block not only c-*fos* but also Jun-B expression.[18] AS1 could thus also impact jun-B expression, an indirect effect not mediated by direct oligonucleotide–mRNA interactions, as Jun-B mRNA does not contain sequences complementary to those of AS1-like c-*fos* antisense oligonucleotides. Decreases in jun-B expression could also conceivably be potentiated by the halothane anaesthetic used in these studies.[42] Transcription factors interact with one another in a complex network where alterations of a single factor may conceivably affect several other factors, making it difficult to discern those directly responsible for behavioural or neurochemical effects under scrutiny.

Mechanisms of action of c-fos *antisense oligonucleotides* in vivo

Phosphorothioated AS1 oligonucleotides displayed modest effects on basal and amphetamine-induced c-*fos* mRNA levels that did not reach statistical significance, possibly due to interindividual differences in surgical stress effects in missense-injected hemispheres (Fig. 3). Striking antisense effects on both basal and amphetamine-induced Fos protein levels were instead recorded as early as 1 h following AS1 injection. These data fit with AS1 oligonucleotide recognition of c-*fos* mRNAs translational start regions, where antisense binding could be anticipated to block translation, rather than to significantly increase mRNA degradation rates.[7,29,62,72] Ribonuclease H-mediated double-stranded mRNA degradation may take place, however, as revealed by apparent blunting of the mRNA production in antisense-injected hemispheres elicited during the first 3 h following surgical stress (Fig. 3).

The current results reveal for the first time that transient translational blockade by phosphoro-

thioated AS1 antisense oligonucleotides may affect not only Fos induction but also basal Fos protein synthesis, albeit in a complex fashion. Undetectable basal levels of Fos protein, well below levels found in missense-injected animals, were observed in two of the four AS1-treated animals 3 h following oligonucleotide injection. These animals also displayed dramatic rebound increases in basal Fos protein levels at 5 h, with a time-course consistent with predictions based on rates of oligonucleotide intracellular uptake, Fos protein half-life, and Fos transcriptional auto-inhibition. Basal Fos levels in the remaining two animals, however, displayed only minimal effects, yielding the intermediate mean values shown in Fig. 5. This interindividual variability could partly stem from the prompt induceability of Fos protein by a wide array of environmental stimuli. Despite methodological limitations suggesting caution in interpreting these results, evidence that antisense oligonucleotides might at least transiently reproduce the neurochemical ''hallmark'' of rat amphetamine withdrawal syndromes that we previously described,[52] namely transient prefrontal cortical decreases of AP1 transcription factor-encoding mRNAs such as c-*fos*, spurred further interest in assessing its potential relationship with behavioural alterations.

Intracerebral oligonucleotide kinetics

Assessments of intracerebral oligonucleotide delivery and kinetics revealed similar results whether oligonucleotides were labelled at their 5′-end or internally,[75] leading to the use of 5′ end-labelling for subsequent experiments with phosphorothioated oligonucleotides. The parallel time-courses for persistence of intracerebral phosphorothioated oligonucleotide kinetic and the antisense transcriptional, translational and behavioural effects, each support the estimated half-life of approximately 3 h for phosphorothioated 18-mers injected into the medial prefrontal cortex. The absence of prominent partial degradation products (cf. Fig. 2) could reflect rapid oligonucleotide degradation once DNase digestion has begun[75] and/or greater losses of lower molecular weight degradation products during the dialysis steps used for oligonucleotide extraction from brain tissue.

The rapid degradation of phosphodiester AS1 and MS1 oligonucleotides is consistent with their lack of effects on c-Fos protein levels and behaviour (data not shown), and with the modest efficacy often displayed by phosphodiester oligonucleotides in cell culture or *in vivo* paradigms involving exposure to serum or cerebrospinal fluid.[30,75,76] The half-life of phosphothioated oligonucleotides injected into the medial prefrontal cortex, while significantly longer than that of phosphodiester oligonucleotides, is also somewhat shorter than those reported for other oligonucleotides injected into other brain regions, confirming previously described site- or sequence-dependent variability in oligonucleotide degradation rates.[29] Translational and/or behavioural effects of intraparenchymal injections of phosphorothioated 15-mers displaying sequences identical to the 5′-most 15 bases of our AS1 oligonucleotide can persist for longer periods after administration to the striatum,[8,18,31,61] nucleus accumbens,[28] or amygdala.[44] Oligonucleotide half-life could differ in distinct brain regions due to factors including the non-homogeneous distributions of α-exonucleases in different central nervous system regions and, possibly, in different subcellular compartments. The potential existence of distinct brain region-, cell type- and subcellular distributions of degrading enzymes is further supported by the recovery of undegraded phosphodiester oligonucleotide, ca. 15% of the injected dose, 24 h after the injection (Fig. 2). Specific central nervous system cell types or intracellular districts with low exonuclease activity and/or high concentations of protectors from exonucleases may thus allow enhanced persistence of intact oligonucleotides.

CONCLUSIONS

The current results, taken together with evidence from other reports employing c-*fos* antisense oligonucleotides, suggest that members of the Fos/Jun family, exerting well-explored roles in transcriptional regulation or possibly other extranuclear modulatory roles, remain strong candidates to contribute to behavioural features of psychostimulant withdrawal syndromes.

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