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SHORT COMMUNICATION
Inhibition of c-Fos Protein Expression in Rat Spinal Cord by Antisense Oligodeoxynucleotide Superfusion

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Abstract

Peripheral noxious stimulation leads to a rapid and transient expression of c-*fos*, c-*jun* and other immediate-early genes (IEGs) in the spinal cord. However, the role of IEG encoded transcription factors in plasticity of spinal neurons remains speculative. In the present study we have shown that superfusion of rat spinal cord with antisense oligodeoxynucleotides complementary to c-*fos* mRNA suppresses heat-induced c-Fos protein expression without affecting other members of the Fos and Jun family, thus providing a technique to determine the function of IEGs *in vivo*.

Numerous studies have shown the rapid and transient induction of c-*fos* and other immediate-early genes (IEGs) in pain-processing neurons of the spinal cord as a result of noxious sensory stimulation (reviewed in Morgan and Curran, 1991). In neuronal cell culture, IEG encoded proteins act as transcription factors which regulate the expression of secondary response genes suggesting a major role in neuronal plasticity (Morgan and Curran, 1991; Naranjo *et al.*, 1991). However, in experimental animals colocalization and expression kinetics provide only indirect evidence that IEG products may activate opioid gene expression in the central nervous system (Draisci and Iadarola, 1989; Noguchi *et al.*, 1991). To show a causal relationship between induction of IEGs and subsequent alterations in secondary response gene expresson *in vivo* an experimental model is required for the inhibition of IEG expression during stimulation. Here, we present an animal model for fast and reliable examination of antisense oligodeoxynucleotides (ODNs) in a defined neuronal compartment which allows inhibition studies of IEG protein expression *in vivo*.

In cell culture assays exogenous addition of ODNs complementary to partions of c-*fos* mRNA has demonstrated efficacy in suppressing c-*fos* expression (Kindy and Verma, 1988; Colotta *et al.*, 1992; Hsieh *et al.*, 1993). However, following systemic administration in rodents, unmodified antisense ODNs were rapidly degraded by nucleases and most likely did not cross the blood-brain barrier (Agrawal *et al.*, 1991; Goodchild *et al.*, 1991). To circumvent these limitations, we used partially phosphorothioated ODNs and a local administration model. In phosphorothioate ODNs one of the non-bridging oxygen atoms in the internucleotide linkage is replaced by a sulphur atom, which renders them resistant to cleavage by nucleases (Uhlmann and Peyman, 1990). In contrast to all-phosphorothioate ODNs, which in some animals studies showed non-sequence-specific effects (Krieg, 1993), ODNs only partially modified by phosphorothioate linkages were expected to act sequence-specifically (Stein *et al.*, 1988; Katajima *et al.*, 1992). Our results indicate that superfusion of rat spinal dorsal horn with these phosphorothioate antisense ODNs sequence-specifically suppresses c-Fos protein expression induced by peripheral heat stimulation. Additionally, we describe the distribution of the radiolabelled ODNs within the spinal cord following superfusion of the cord dorsum.

End-capped phosphorothioate ODNs were synthesized on an ABI 380B DNA synthesizer using phosphoramidite chemistry (Matteucci and Caruthers, 1981) and tetraethylthiuram disulphide sulphurization (Vu and Hirschbein, 1991) for introduction of phosphorothioate linkages. The crude ODNs were purified by successive precipitation from butanol by ammonium acetate and then from ethanol by sodium chloride to give >90% full-length ODNs as judged by capillary electrophoresis in polyacrylamide gels (5% T and 5% C). The ODNs were characterized as ammonium salts by negative ion electrospray mass spectrometry and converted to sodium salts for administration to animals. Experiments were performed on pentobarbital-anaesthetized (60 mg/kg, i.p. and 10 mg/kg × h, i.v. for maintenance) male Sprague–Dawley rats (250–300 g). Following laminectomy and retraction of the dura mater, a pool was formed on the dorsum of the L3–L4 spinal cord using silicone rubber (Sandkühler *et al.*, 1991) and divided longitudinally into two chambers. The ipsilateral chamber was filled with a 20-mer end-capped phosphorothioate ODN (5′-C*G*AGAACATCATGGTCGA*A*G-3′) complementary to the translation initiation site of rodent c-*fos* mRNA (−8 to +12) since antisense ODNs are most active in inhibiting protein expression when directed against this region (Uhlmann and Peyman, 1990) (asterisks show sites of phosphorothioate modification). The contralateral chamber was filled with a random sequence ODN (5′-C*C*CTTATTTACTAC-

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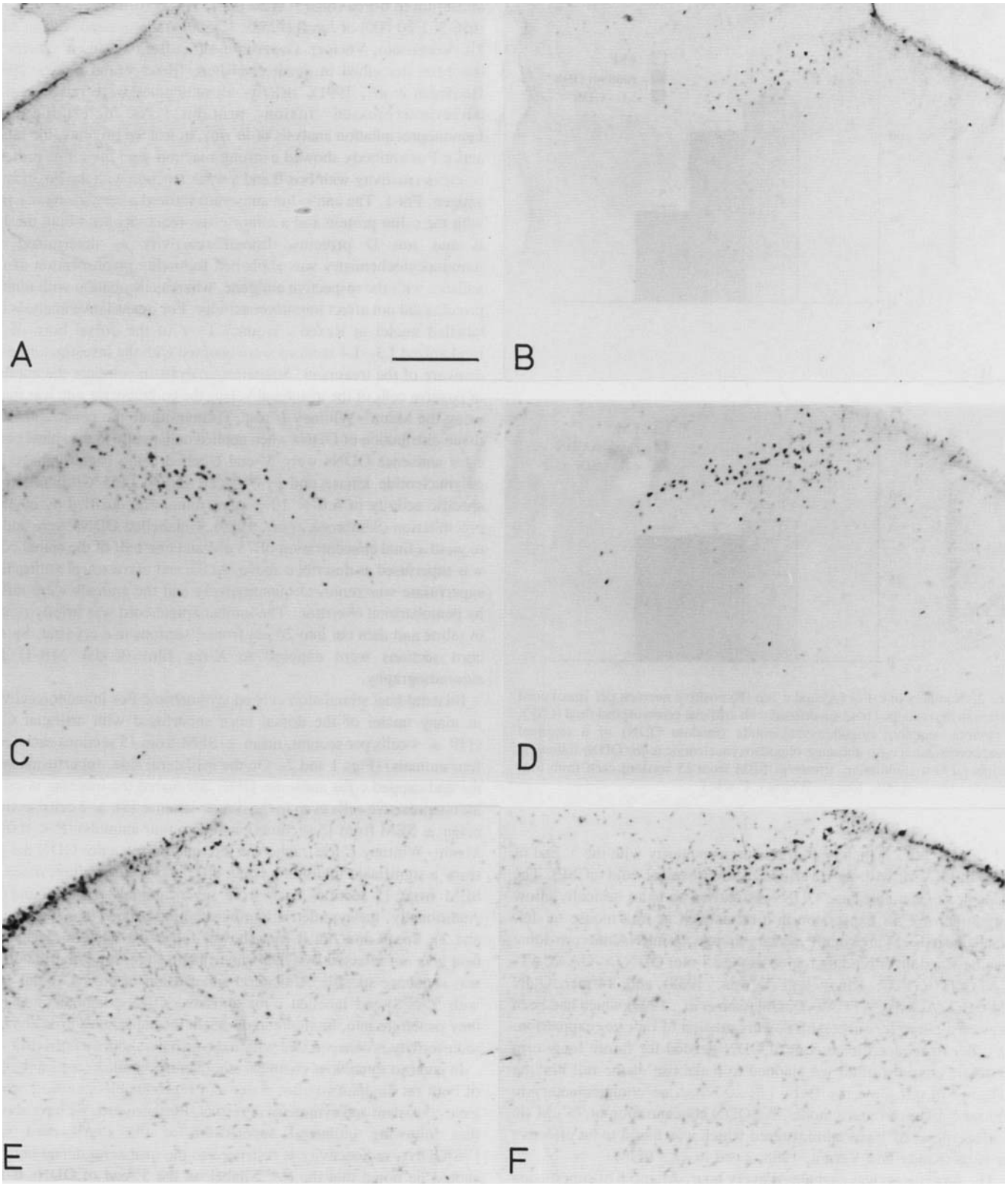

FIG. 1. Photomicrographs of 40 μm sections from rat L4 spinal cord, 1.5 h following simultaneous heat stimulation of both hindpaws. Note the smaller number of c-Fos immunoreactive nuclei in the dorsal horn superfused with (A) an end-capped phosphorothioate oligodeoxynucleotide complementary to c-*fos* mRNA compared to (B) the corresponding contralateral dorsal horn superfused with a random sequence oligodeoxynucleotide as control. A symmetric c-Fos expression pattern can be seen after unilateral superfusion with (C) the control oligodeoxynucleotide and (D) artificial cerebrospinal fluid, respectively. (E,F) Symmetric immunostaining for c-Jun following bilateral noxious stimulation and unilateral c-*fos* antisense oligodeoxynucleotide superfusion. Scale bar = 150 μm.

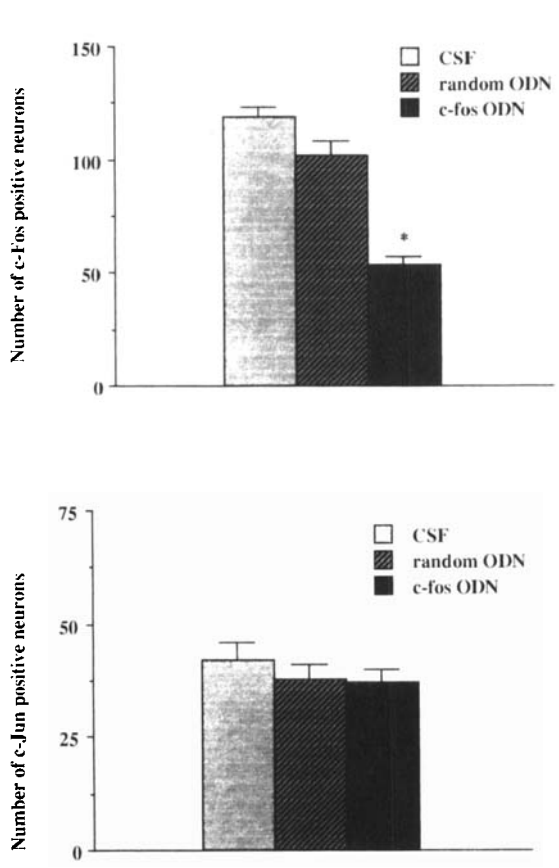

FIG. 2. Numbers of c-Fos (A) and c-Jun (B) positive neurons per spinal cord section in the rat dorsal horn superfused with artificial cerebrospinal fluid (CSF), a random sequence oligodeoxynucleotide (random ODN) or a terminal phosphorothioated c-*fos* antisense oligodeoxynucleotide (c-*fos* ODN) following peripheral heat stimulation. (mean ± SEM from 15 sections each from four animals, $*P < 0.001$, Mann−Whitney *U* test.)

TTTC*G*C-3′), which had little complementarity with the 5′ end of c-*fos* mRNA, or with sterile artificial cerebrospinal fluid (aCSF). The sequence of the antisense ODN was chosen so as to basically allow inhibition of c-*fos* expression in a rat as well as in a mouse model. Nucleotides 1−17 are complementary to rat c-*fos* mRNA thus providing a somewhat longer binding region as the 15-mer ODN (5′-GAACAT-CATGGTCGT-3′; Kindy and Verma, 1988) and 14-mer ODN (5′-CCCCAGAACATCAT-3′; Chiasson *et al.*, 1993) which had been previously used for successful downregulation of rat c-*fos* expression. In order to reduce the amount of ODN needed for future long-term systemic administration we planned to make use of the full binding capacity of our antisense ODN (all 20 bases are complementary to mouse c-*fos*) in a mouse model. An ODN concentration of 75 μM (in 5 μl sterile aCSF) was administered which was found to be effective *in vitro* (Kindy and Verma, 1988; Lord *et al.*, 1993).

The superfusate was exchanged every hour. After 6 h of superfusion both hindpaws were immersed in 52°C hot water (10 times for 20 s at 90 s intervals) (Hunt *et al.*, 1987). At 1.5 h following heat stimulation the rats were transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The lumbar spinal cord was postfixed in the same fixative overnight and cryoprotected in 30% sucrose. Frontal frozen sections of 40 μM were cut and incubated in rabbit antiserum to c-Fos (689/5; 1:20 000), Fos B (614/1; 1:2000), c-Jun (636/3; 1:20 000) or Jun B (725/5; 1:3500) and processed with an ABC kit (Vectastain; Vector). Generation of antisera and their specificity has been described in detail elsewhere (Kovary and Bravo, 1991; Herdegen *et al.*, 1991). Briefly, all antibodies were raised against bacteria-expressed fusion proteins. As determined by immunoprecipitation analysis of *in vitro* translated proteins, the rabbit anti-c-Fos antibody showed a strong reaction with the c-Fos protein, no cross-reactivity with Fos B and a weak reaction with the Fos-related antigen, Fra-1. The anti-c-Jun antiserum showed a very strong reaction with the c-Jun protein and a minor cross-reactivity with both the Jun B and Jun D proteins. Immunoreactivity as determined by immunocytochemistry was abolished following preabsorption of the antisera with the respective antigene, whereas incubation with related proteins did not affect immunoreactivity. For quantitative analysis the labelled nuclei in Rexed's laminae I−V of the dorsal horn of 15 randomized L3−L4 sections were counted with the investigator being unaware of the treatment. Statistical analysis to compare the number of positive cells of the ipsilateral versus the contralateral side was made using the Mann−Whitney *U* test. To investigate the penetration and tissue distribution of ODNs when applied unilaterally to the spinal cord, c-*fos* antisense ODNs were 5′-end labelled using bacteriophage T4 polynucleotide kinase and [γ-^{35}S]ATP (NEN 1465 Ci/mmol) to a specific activity of 6.0×10^7 c.p.m./mmol and purified by ethanol precipitation (Sambrook *et al.*, 1989). Unlabelled ODNs were added to yield a final concentration of 75 μM and one-half of the spinal cord was superfused as described above. At the end of treatment radioactive superfusate was removed quantitatively and the animals were killed by pentobarbital overdose. The lumbar spinal cord was briefly rinsed in saline and then cut into 20 μm frontal sections in a cryostat. Spinal cord sections were exposed to X-ray film (Kodak MR-1) for autoradiography.

Bilateral heat stimulation evoked symmetric c-Fos immunoreactivity in many nuclei of the dorsal horn superfused with artificial CSF (119 ± 4 cells per section, mean ± SEM from 15 sections each from four animals) (Figs 1 and 2). On the ipsilateral side, superfusion with the end-capped c-*fos* antisense ODN attenuated the increase in c-Fos immunoreactive cells even in the deeper laminae (54 ± 3 cells/section, mean ± SEM from 15 sections each from four animals) ($P < 0.001$, Mann−Whitney *U* test), whereas the random sequence ODN did not show a significant inhibitory effect (102 ± 6 cells/section, mean ± SEM from 15 sections each from seven animals) (Figs 1 and 2). Additionally, the symmetric immunostaining pattern of c-Jun (Figs 1 and 2), Fos B and Jun B (not shown) following bilateral peripheral heat was not affected by ODN superfusion suggesting that the effect was sequence-specific. Unilateral superfusion of the rat spinal cord with 5′-[^{35}S]-end labelled c-*fos* antisense ODNs demonstrated that they penetrate into the tissue underneath the superfused area and that radioactivity is concentrated within the ipsilateral dorsal horn (Fig. 3).

In contrast to noxious chemical stimulation, simultaneous immersion of both rat hindpaws in hot water is a reproducible stimulus, which gave consistent and symmetrical results. Furthermore, we have shown that following unilateral superfusion of the spinal cord with [^{35}S]ODNs, radioactivity is restricted to the ipsilateral dorsal horn. It should be noted that the [γ-^{35}S]label on the 5′-end of ODNs is not removed by endogenous phosphatases for 48 h (Hoke *et al.*, 1991). Thus, it is possible to compare the effect of antisense and control ODN superfusion within one animal while excluding animal to animal variations. Superfusion of the rat spinal cord with 75 μM end-capped phosphorothioate c-*fos* antisense ODN (2.5 μg in 5 μl aCSF) resulted in a 50% reduction of c-Fos protein expression, whereas in a cell culture

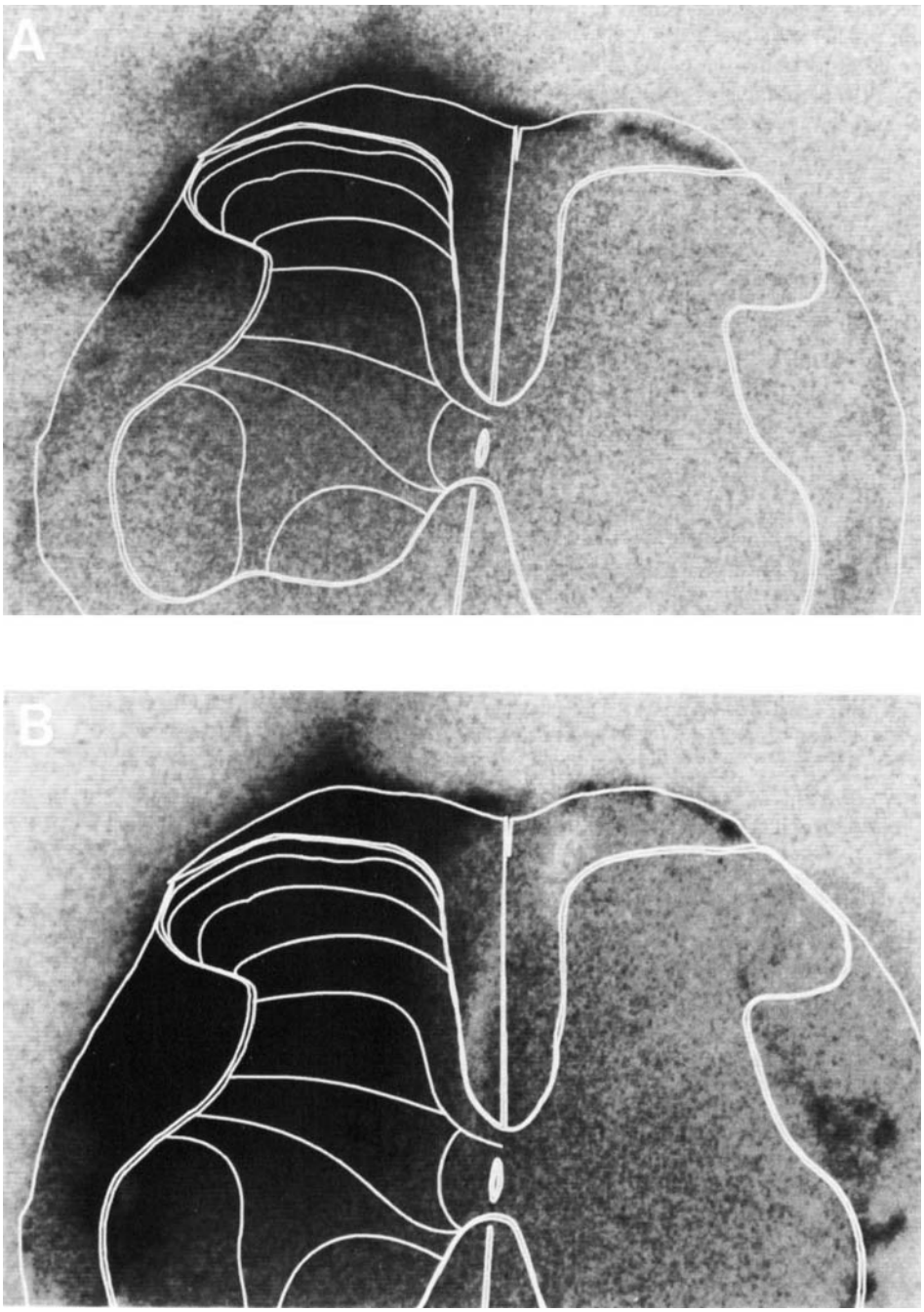

FIG. 3. Autoradiograms of 20 μm frontal sections of rat lumbar spinal cord following unilateral superfusion with 5′-[^{35}S]oligodeoxynucleotides for 1 h (A) and 6 h (B).

assay preincubation with only 15 μM c-*fos* antisense ODN had the same effect (Kindy and Verma, 1988). This discrepancy may be due to different assay systems (*in vivo* versus *in vitro*), to the selection of slightly different target sequences or to different modification of ODNs (end-capped-phosphorothioates versus all-methylphosphonates). In recent *in vivo* studies local delivery of similar concentrations of a fully-phosphorothioated c-*myb* antisense ODN (1 μg/μl solution of pluronic gel) inhibited c-*myb* expression in rat carotid artery (Simons *et al.*, 1992) and direct injections of a c-*fos* phosphorothioate antisense ODN (2 μl of 1 mM) into the brain substance strongly reduced amphetamine-induced Fos-like immunoreactivity (Chiasson *et al.*, 1993).

Phosphorothioate end-capped-antisense ODNs have been chosen for this study since they (i) are highly resistant to exonucleases, (ii) hybridize with RNA more efficiently than all-phosphorothioate and methylphosphonate analogues, (iii) retain good aqueous solubility, and (iv) are synthesized conveniently by automated phosphoroamidite ODN synthesis. Different mechanisms may contribute to the inhibition of c-Fos protein expression by our antisense ODN, including a translational block, enhanced c-*fos* mRNA degradation by RNase H, or specific blockage of nuclear mRNA transport (Neckers *et al.*, 1992). The findings that suppression was observed in protein levels of c-Fos but not related and co-expressed IEGs and that the random sequence

control ODN was ineffective, strongly suggest that the inhibitory activity is sequence-specific and not due to non-specific interaction with proteins. In summary, we have shown that our model provides a tool to test the *in vivo* efficacy of antisense ODNs and may be useful in defining the role of IEG encoded proteins in stimulus-transcription coupling and neuroplasticity *in vivo*.

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Abbreviations

aCSF artificial cerebrospinal fluid
IEG immediate-early gene
ODN oligodeoxynucleotide

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