Suppression of *c-fos* Induction in Rat Brain Impairs Retention of a Brightness Discrimination Reaction

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Abstract

Recently, the induction of transcription factor-encoding immediate-early genes such as *c-fos* was observed in distinct brain regions of rats trained to acquire a footshock-motivated brightness discrimination in a Y-maze. The functional relevance of inducible transcription factors for learning and memory formation is, however, not clear. To address this question in the present study, we have used a synthetic antisense phosphorothioate oligodeoxynucleotide to suppress *in vivo* the expression of *c-fos* in rat brain. Intrahippocampal application of the oligodeoxynucleotide 10 hr and 2 hr before starting a brightness discrimination training drastically reduced the induction of *c-Fos* immunoreactivity normally observed in limbic and cortical areas after the training session. Acquisition of the discrimination reaction was not affected by this treatment. In a relearning test 24 hr after the first training, retention of the discrimination reaction was specifically impaired compared with rats pretreated with control oligodeoxynucleotide or saline. Our findings are consistent with the hypothesis that the inducible transcription factor *c-Fos* is involved in processes underlying the formation of long-term memory.

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Introduction

Long-term plastic changes in the brain, including memory formation, are assumed to depend on a sequential induction of regulatory and late structural target proteins that contribute to permanent functional alterations in neuronal cells and, in particular, in their synaptic connections (Goelet et al. 1986; Alkon and Nelson 1990; Alkon et al. 1991; Armstrong and Montminy 1993). The immediate-early gene (IEG) *c-fos* encodes a nuclear phosphoprotein, *c-Fos*, which is known to be part of the AP-1 transcription factor complex (Chiu et al. 1988). The expression of this gene is rapidly and transiently induced in the central nervous system by a variety of stimuli (for recent review, see Hughes and Dragunow 1995). Moreover, numerous reports have shown increased *c-fos* expression after learning experiments in chicken and rodents (Anokhin et al. 1991; Anokhin and Rose 1991; Campeau et al. 1991; Bialy et al. 1992; Brennan et al. 1992; Irwin et al. 1992; Nikolaev et al. 1992a,b; Ambalavanar et al. 1993; Heurteaux et al. 1993; McCabe and Horn 1993; Pezzone et al. 1993; Houpt et al. 1994; Bertaina and Destrade 1995; Hess et al. 1995). Thus, it has been suggested that *c-Fos* is involved in processes of long-term memory formation, by coupling transient extracellular stimuli to long-lasting cellular responses at the level of transcription (Goelet et al. 1986; Morgan and Curran 1989).

Previous studies on rats trained to acquire a footshock-motivated brightness discrimination reaction in a Y-maze revealed a two-stage increase in hippocampal protein synthesis. Inhibition of protein synthesis during each of these phases was am-

nesic (for review, see Matthies 1989). These findings suggested that the synthesis of proteins necessary for consolidation of the memory trace may be under the control of early induced regulatory proteins. Supporting this hypothesis, a rapid and transient induction of mRNAs encoding inducible transcription factors such as *c-Fos*, *c-Jun*, and *JunB* was observed in rat brain immediately after the discrimination training (Tischmeyer et al. 1990, 1994; Grimm and Tischmeyer 1997). However, similar results obtained in pseudotrained control rats suggested that initial prerequisites necessary for learning such as stimulus novelty led to an increased IEG expression during training, posing questions concerning the functional importance of IEG transcription factors for processes involved in acquisition and retention of the discrimination reaction.

Recent reports have demonstrated the usefulness of antisense (AS) oligodeoxynucleotides (ODNs) to study in vivo the role of specific proteins in the nervous system in a behavioral context (Chiasson et al. 1992; Dragunow et al. 1993; Heilig et al. 1993; Sommer et al. 1993; Merchant 1994; Möller et al. 1994; Suzuki et al. 1994; Hunter et al. 1995; Robertson et al. 1995; Wollnik et al. 1995; for review, see Wahlestedt 1994) and specifically during learning (Schmidt et al. 1995). Thus, we used intracerebral antisense intervention to assess the physiological significance of IEG transcription factors during acquisition and consolidation of the brightness discrimination in rats. Recent experiments revealed inhibitory influences of *c-jun* AS-ODN but not of *junB* AS-ODN on the performance of the task (Tischmeyer et al. 1994). In the present study, we demonstrate that intrahippocampally applied *c-fos* AS-ODN inhibits the induction of *c-Fos* immunoreactivity (IR) in rat brain during brightness discrimination training and impairs the retention of the task in a sequence-specific manner.

Materials and Methods

SYNTHESIS AND SEQUENCES OF ODNs

The used ODNs, 15 nucleotides in length and phosphorothioated in all positions, were of the following sequences: *c-fos* AS-ODN, 5'-GAACATCATGGTCGT-3'; control ODN, 5'-TGCTGGTAC-TACAAG-3'. The *c-fos* AS-ODN was complementary to bases -6 to +9 from the initiation codon of

the rat *c-fos* mRNA sequence. The control ODN was the reversed sequence of the *c-fos* AS-ODN. Neither ODN showed significant complementarity to any other gene sequence in the EMBL/GenBank databases. The ODNs were synthesized on an Applied Biosystems model 391 DNA synthesizer, purified by reverse-phase HPLC, detritylated with acetic acid, desalted, and extracted with diethyl ether. Lyophilized ODNs were resuspended to a concentration of 2 mM in desalted sterile water. Four independently synthesized and purified batches of each ODN were used for behavioral studies.

ANIMALS AND SURGICAL PROCEDURES

Male 7-week-old Wistar rats were deeply anesthetized with pentobarbital (60 mg/kg i.p.) and stereotactically implanted with stainless-steel microcannulae bilaterally into the dorsal hippocampus using the following coordinates: posterior -2.8 mm from bregma, lateral $\pm$ 2.2 mm, and ventral -3.2 mm from skull. According to the brain atlas from Paxinos and Watson (1986), these coordinates correspond to the CA1 region in the dorsal hippocampus. Guide cannulae were fixed using anchor screws and acrylic cement. Following surgery, rats were housed singly and given free access to standard laboratory chow and tap water on a 12-hr light/dark cycle (light on at 6 a.m.). During a 1-week recovery period before beginning the experiments, rats were handled daily to habituate to the injection procedure.

INTRAHIPPOCAMPAL INJECTIONS

For administration of saline or ODNs to freely moving rats, blunt-tipped stainless-steel injectors were placed gently into the guide cannulae such that the tips were located 0.1 mm below the cannulae. For behavioral studies, 1 μ l of 0.9% saline or of a 2 mM solution of *c-fos* AS-ODN or control ODN was injected twice into each hippocampus at a rate of $\sim$ 0.5 μ l/min. Subsequently, injectors were left in place for another minute. The first injection was given 10 hr prior to training, considering previous reports of an $\sim$ 8-hr onset latency for *c-fos* AS-ODN (Chiasson et al. 1992; Suzuki et al. 1994). To maintain efficient local concentrations of ODNs during acquisition and subsequent phases of consolidation of the discrimination reaction, a second injection

tion was given 2 hr before the beginning of training. A significant suppression of c-Fos IR within a period of 3 hr following intracerebral administration of *c-fos* AS-ODN has been demonstrated recently (Shih et al. 1996).

To immunohistochemically examine the effects of *c-fos* AS-ODN on *c-fos* expression, six rats were injected with *c-fos* AS-ODN into one hippocampus and control ODN into the opposite one using the same protocol as described above. The use of the right and left hemispheres for injecting the different ODNs was changed between animals.

IMMUNOHISTOCHEMISTRY

TRAINING-INDUCED EXPRESSION OF C-FOS IR

To examine the induction of Fos protein in rat brain after brightness discrimination training, rats ($n = 4$) without surgical or pharmacological pretreatments were trained in a Y-maze as described below. Another group of rats ($n = 4$) was allowed to stay in the home cage during the time of training to serve as passive controls. Immediately after completion of one session of training, rats were deeply anesthetized with sodium pentobarbital. Following perfusion fixation first with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) and then with 4% paraformaldehyde dissolved in the same buffer, brains were removed and sectioned on a vibratome. Immunohistochemistry was performed on free-floating 50- μ m coronal sections using affinity-purified polyclonal c-Fos antibodies (Santa Cruz Biotechnology, Inc.), raised in rabbits by immunization with a peptide corresponding to human c-Fos amino acid residues 3–16. As tested by immunoprecipitation, Western blotting, and cell staining (Sambucetti and Curran 1986), these antibodies react with vertebrate *c-fos*-encoded 62-kD proteins. To reduce background staining, sections were preincubated in a mixture of 50% methanol/PBS with 0.1% hydrogen peroxide for 15 min to block endogenous peroxidases and in 5% bovine serum albumin for 1 hr to block unspecific binding. Subsequently, sections were incubated in the primary antisera (1:20,000), including 0.1% Triton X-100 for 36 hr. After thorough washing in PBS, sections were incubated with anti-rabbit-digoxigenin (1:100; Boehringer Mannheim) followed by anti-digoxigenin-peroxidase, Fab fragments (1:400; Boehringer Mannheim). Visualization of labeled

cells was performed by developing sections in 0.02% diaminobenzidine with 0.02% hydrogen peroxide.

SUPPRESSION OF C-FOS IR EXPRESSION BY *c-fos* AS-ODN

To determine the efficacy of *c-fos* AS-ODN, rats ($n = 6$) were twice injected in opposite hippocampi with *c-fos* AS-ODN and control ODN as described above and trained on a brightness discrimination reaction (see below) 2 hr after the second application of ODNs. Immediately after completion of one session of training, rats were deeply anesthetized by sodium pentobarbital and perfused transcardially with 4% paraformaldehyde. Brains were removed, postfixed in the same fixative overnight, and cryoprotected in 30% sucrose. Coronal sections (50 μ m) were prepared on a cryostat and processed for immunohistochemistry. Primary antiserum against c-Fos (Kovary and Bravo 1991) was diluted 1:12,000. Detection was performed as described previously using an avidin-biotin protocol (Herdegen et al. 1991). Briefly, sections were incubated with primary antiserum for 48–72 hr at 4°C, washed, and reincubated with secondary antibody (polyclonal anti-rabbit antiserum) for 45 min at 37°C, washed again, and processed with an ABC-Elite kit (Vectastain, Vector Laboratories, Inc.). As described previously (Herdegen et al. 1991; Kovary and Bravo 1991), the rabbit anti c-Fos antibody shows a strong reaction with the c-Fos protein, no cross-reactivity with FosB and a weak reaction with the Fos-related antigen Fra-1. For quantitative analysis, immunopositive cells in frames of 1 mm $\times$ 1 mm covering the apex of the dentate gyrus were counted separately on control ODN-treated and *c-fos* AS-ODN-treated hemispheres. Values obtained from two slices per rat were averaged for statistical evaluation.

BRIGHTNESS DISCRIMINATION EXPERIMENTS

HABITUATION

Brightness discrimination training and a relearning test 24 hr after the initial training were performed in a Y-shaped chamber. Before both the training and the relearning session, rats were allowed to habituate for 10 min to the experimental situation in the Y-maze without illumination and

footshock. To assess effects of ODNs on arousal and activity, the number of entries into each arm of the Y-maze was monitored during this period.

TRAINING

The animals were trained in the Y-maze to attain a footshock-motivated brightness discrimination, as described earlier (Tischmeyer et al. 1994). Briefly, the learning task was to escape from a mild electrical footshock (1–1.5 mA) from the grid floor in the start box by running into the illuminated alley of the Y-maze. At the beginning of each trial, the goal box was illuminated and a foot shock was delivered to the start box. When the animal escaped from the footshock into the illuminated goal box, the shock was terminated and the light remained on for another 20 sec. Entering the dark alley was punished by footshock and counted as an error. Rats stayed in the goal box for a randomly changing intertrial interval of ~1 min. For the next trial, the former goal box served as the start box, thus avoiding intertrial handling of the animals. The side of illumination was changed after three consecutive runs. A training session consisted of 40 trials and was completed within 45 min. To assess effects of ODNs on motor coordination and sensory systems, the following parameters were recorded during each run: (1) footshock intensity required to cause the rats to escape from the start box, (2) reaction time, that is, the time of escape from the start box after the onset of footshock, and (3) running time, that is, the time required to escape into the goal box.

RELEARNING

Retention was estimated in a relearning test 24 hr after the initial training using the same test apparatus and the same behavioral procedure. Parameters were recorded as described above. The reduction in the number of errors compared with the initial training was expressed as an index of retention (% savings), calculated as

$$\% \text{ savings} = \frac{\text{training errors} - \text{relearning errors}}{\text{training errors}} \times 100\%$$

Following the behavioral experiments, the correct positions of cannulae tips were determined.

Briefly, animals were sacrificed by decapitation; brains were removed from the skull and fixed by immersion with 4% paraformaldehyde for 5 days. Localization of the cannulae was checked on 50- μ m vibratome sections. Only animals that had the cannulae inserted into the appropriate location were chosen for further analysis of the data.

STATISTICAL ANALYSIS

All values are expressed as means $\pm$ S.E.M. Owing to the small sample size, immunohistochemical data were statistically analyzed using Wilcoxon's signed rank test. Statistical evaluation of behavioral data was performed using one-way ANOVA. If significant, post-hoc comparisons were performed using Student's two-tailed *t*-test. *P* values of <0.05 were considered as statistically significant.

Results

TRAINING-INDUCED EXPRESSION OF *c-FOS* IR

Immediately after completion of a 45-min training session in the Y-maze, a distinct expression pattern of *c-Fos* IR was observed in several rat brain regions such as the hippocampal formation, the parietal, perirhinal, and piriform cortices, the amygdala, and thalamic nuclei, whereas in passive control rats, only a few scattered cells were immunopositive. In Figure 1, the hippocampal CA1 region, the dentate gyrus, and the parietal cortex (area 1) are shown as examples. Training-induced expression of *c-Fos* IR was observed in pyramidal cells of the hippocampal region CA1 (and CA3, not shown), in granule cells of the dentate gyrus, and in hilar cells. However, in the CA2 region, *c-Fos* IR remained undetectable after training (not shown). In the parietal cortex, *c-Fos* IR was predominantly induced in layers IV and VI after the discrimination training (Fig. 1). In all regions, localization of *c-Fos* IR was apparently restricted to the nuclei of labeled cells.

SUPPRESSION OF *c-FOS* IR EXPRESSION BY *c-fos* AS-ODN

To compare the effects of *c-fos* AS-ODN versus control ODN on the expression of *c-Fos* IR, a group of six rats received *c-fos* AS-ODN into one hippocampus, whereas control ODN was applied to the contralateral hippocampus of the same animal. Us-

Figure 1: Training-induced expression of c-Fos IR in rat brain. Rats ($n = 4$ per group) without surgical or pharmacological pretreatments were either trained on a brightness discrimination in a Y-maze or stayed in their home cage during the time of training to serve as passive controls. Immediately after completion of training, rats were anesthetized and perfused. Brains were removed and processed for immunohistochemistry using the digoxigenin protocol as described in Materials and Methods. Details of coronal sections at the level of the hippocampal CA1 region (CA1), the dentate gyrus (DG), and the area 1 of the parietal cortex (Ctx) of a trained rat (*right*) and a passive control (*left*) are shown as representative examples.

ing the more sensitive avidin-biotin protocol, a strong expression of c-Fos IR was detected in several brain regions of the control ODN-injected hemisphere following brightness discrimination training (Fig. 2), including principal neurons of the hippocampal formation (pyramidal neurons of the *cornu ammonis*, granular cells of the dentate gyrus) and cells of the overlying cortex. In contrast, on the *c-fos* AS-ODN-injected hemisphere, strikingly less c-Fos IR was observed in large areas of the hippocampus, the dentate gyrus, and the adjacent cortex. To assess the efficacy of *c-fos* AS-ODN, immunopositive cells were counted in areas of 1 mm^2 covering the apex of the dentate gyrus. On average, the number of stained cells was reduced from 355 ± 151 on control ODN-treated hemispheres to 56 ± 13 on *c-fos* AS-ODN-treated hemispheres ($n = 6$, $P < 0.05$, Wilcoxon test), representing $>70\%$ inhibition. The action of *c-fos* AS-ODN was highly restricted to the ipsilateral hemisphere. However, a few scattered cells, for example, in the hilar region, still expressed c-Fos

IR also in this hemisphere (Fig. 2). Moreover, whereas in four out of six rats the suppression of c-Fos IR by *c-fos* AS-ODN was comparable in extent to the example shown in Figure 2, less reduction was observed in the remaining two rats (data not shown).

BRIGHTNESS DISCRIMINATION EXPERIMENTS

TRAINING

Pretraining injection of *c-fos* AS-ODN or control ODN caused no alterations in habituation compared with saline-treated rats. As shown in Table 1, the number of runs during 10 min of habituation before the initial training was comparable in saline-treated, control ODN-treated and *c-fos* AS-ODN-treated rats. Furthermore, as revealed by ANOVA, parameters recorded during the training session such as footshock intensities [$F(2,40) = 0.39$, $P > 0.10$], reaction times [$F(2,40) = 0.70$, $P > 0.10$],

Figure 2: Suppression of *c-Fos* IR expression by *c-fos* AS-ODN in hippocampal and cortical regions of rat brain. Ten hours and 2 hr before the beginning of a brightness discrimination training, the rat received *c-fos* AS-ODN to the right and control ODN to the left hemisphere of the brain. Immediately after completion of training, the animal was anesthetized and perfused. The brain was removed and processed for immunohistochemistry using the avidin–biotin protocol as described in Materials and Methods. (A) Detail of a coronal section (frontal view) showing hippocampal and cortical regions surrounding the injection sites. (B) Magnifications of the dentate granule cell layers boxed in A.

and running times [$F(2,40) = 1.27$, $P > 0.10$] did not significantly differ among groups.

Compared with saline-treated and control ODN-treated rats, performance of brightness discrimination training was not impaired in *c-fos* AS-ODN-treated animals, as documented by comparable numbers of training errors in all three groups (Table 1).

RELEARNING

During the habituation period before the relearning session, the number of runs between the arms of the Y-maze performed by saline-treated as well as control ODN-treated rats was significantly reduced compared with the exploratory behavior of these rats before the initial training (Table 1). In contrast, the number of runs performed by *c-fos* AS-ODN-treated rats during the habituation period before the relearning test was almost identical to

the pretraining habituation performance observed in this group.

As for the initial training, ANOVA revealed no significant group differences in general parameters, such as footshock intensities [$F(2,40) = 0.15$, $P > 0.10$], reaction times [$F(2,40) = 1.69$, $P > 0.10$], and running times [$F(2,40) = 3.02$, $P > 0.05$], recorded during the relearning test. However, compared with saline-treated rats, running time tended to increase in rats treated with control ODN or *c-fos* AS-ODN (Table 1). To assess interactions between running times and the brightness discrimination score, linear regression analysis was performed. It revealed no significant correlation between running times and the number of relearning errors [$r = 0.25$, $F(1,41) = 2.78$, $P > 0.10$].

Compared with the initial training, the number of errors during the relearning session was significantly reduced in saline-treated, in control ODN-treated as well as in *c-fos* AS-ODN-treated rats (Table 1), indicating an improvement of the bright-

Table 1: Data recorded during 10 min of habituation to the Y-maze, during brightness discrimination training, and during the relearning test 24 hr after the initial training

	Training			Relearning		
	saline (<i>n</i> = 12)	control ODN (<i>n</i> = 16)	<i>c-fos</i> AS-ODN (<i>n</i> = 15)	saline (<i>n</i> = 12)	control ODN (<i>n</i> = 16)	<i>c-fos</i> AS-ODN (<i>n</i> = 15)
Runs during habituation	24.7 ± 1.5	26.9 ± 1.7	21.7 ± 1.9	9.0 ± 2.8 ^a	11.6 ± 1.8 ^a	19.7 ± 3.2 ^b
Shock intensities (mA)	1.28 ± 0.01	1.30 ± 0.02	1.27 ± 0.02	1.29 ± 0.01	1.28 ± 0.03	1.30 ± 0.01
Reaction times (sec)	1.66 ± 0.26	1.74 ± 0.28	2.19 ± 0.43	1.29 ± 0.25	2.24 ± 0.36	2.19 ± 0.48
Running times (sec)	3.60 ± 0.40	4.51 ± 0.39	4.71 ± 0.63	2.25 ± 0.27 ^a	3.67 ± 0.37 ^a	3.92 ± 0.68
Number of errors	13.4 ± 1.0	14.3 ± 0.6	13.2 ± 0.7	6.5 ± 1.0 ^a	7.0 ± 0.8 ^a	9.3 ± 1.2 ^a

Rats were exposed to the Y-maze for 10 min before both the training and the relearning session to equilibrate to the experimental environment. During these habituation periods, runs between arms of the Y-maze were recorded. Footshock intensities, reaction times, running times, and the number of errors were monitored during the subsequent brightness discrimination training, and during the relearning test 24 hr later. Means ± S.E.M.. Number of animals are in parenthesis.

^a(*P* < 0.05) Significantly different from the respective values obtained on the training day of the same group of rats (paired *t*-test).

^b(*P* < 0.05) Significantly different from the respective values of saline-treated and control ODN-treated rats on the day of relearning (unpaired *t*-test).

ness discrimination score in all three groups. However, in comparison with rats pretreated with saline or control ODN, this reduction in the number of relearning errors was less pronounced in the group of *c-fos* AS-ODN-injected rats, implying a deficit in retention. Calculation of the retention index supported these findings. As shown in Figure 3, percent savings were similar in saline-treated and control ODN-treated rats, whereas in *c-fos* AS-ODN-treated rats, the retention index was significantly reduced.

Discussion

Using an antisense approach, we addressed the question of the functional relevance of inducible transcription factors for processes of learning and memory formation. As demonstrated in the present study, the expression of c-Fos IR is induced in various rat brain regions following training on a footshock-motivated brightness discrimination reaction in a Y-maze. Intracerebral injection of *c-fos* AS-ODN prior to training efficiently suppresses c-Fos expression in hippocampal and cortical areas. This has amnesic effects in a relearning test 24 hr

later, whereas the acquisition of the new behavior is not affected.

Figure 3: Retention indices expressed as percent savings. The retention indices were calculated from the number of training errors and relearning errors of saline-treated (*n* = 12), control ODN-treated (*n* = 16) and *c-fos* AS-ODN-treated (*n* = 15) rats as described in Materials and Methods. Means ± S.E.M. (*) *P* < 0.05: significantly different from the values of saline-treated and control ODN-treated rats (unpaired *t*-test).

Hybridization studies have previously revealed a differential induction of transcription factor-encoding IEGs including *c-fos* in hippocampal, cortical, and cerebellar rat brain structures following brightness discrimination training (Tischmeyer et al. 1990, 1994; Grimm and Tischmeyer 1997). These findings at the level of mRNA implied but did not prove an increased expression also of the respective proteins. Using immunohistochemical methods, we therefore examined the induction of *c-fos* gene expression at the translational level. As shown in Figure 1, c-Fos IR was induced in various rat brain regions including limbic and cortical structures immediately after completion of the Y-maze training, confirming reports on an increased expression of Fos protein in chick, mouse, rat, and rabbit brain following different behavioral paradigms (Brennan et al. 1992; Irwin et al. 1992; Ambalavanar et al. 1993; McCabe and Horn 1993; Pezzone et al. 1993; Freeman and Rose 1995). Our findings demonstrate that the induction of *c-fos* gene expression observed at the level of mRNA is continued at the translational level to increase the amount of c-Fos protein during acquisition and early phases of consolidation of the discrimination reaction, suggesting a physiological involvement of this transcription regulatory protein in the formation of the memory trace.

AS-ODNs are commonly designed to inhibit the synthesis of specific proteins by hybridization to the respective mRNA. One of the major problems for in vivo use of these compounds is their rapid degradation by nucleolytic enzymes. More stable ODNs can be generated by replacing a non-bridging oxygen of the phosphodiester backbone by sulfur at each base. The resulting phosphorothioated ODNs are known to be effectively taken up by brain cells and to be stable there for >24 hr after topical application in vivo (Whitesell et al. 1993; Wahlestedt 1994). Using intrahippocampal injection in rats, we have demonstrated earlier that labeled phosphorothioated ODNs were detectable in hippocampal pyramidal cells for at least 26 hr and, as shown for *c-jun* AS-ODN, behavioral effects were also still evident at this time (Tischmeyer et al. 1994). On the other hand, unmodified *c-fos* AS-ODN intrahippocampally applied at the same dosage as the phosphorothioated one was ineffective in the present study (data not shown), confirming previous observations in rat brain after intrastriatal injections (Hooper et al. 1994). Thus, to examine the role of Fos protein in rat brain during learning

and memory formation, we used phosphorothioated ODNs.

The *c-fos* AS-ODN used in our experiments has been shown previously to successfully inhibit c-Fos expression in rat brain and spinal cord following topical application (Chiasson et al. 1992; Dragunow et al. 1993; Heilig et al. 1993; Sommer et al. 1993; Hooper et al. 1994; Merchant 1994; Möller et al. 1994; Hunter et al. 1995; Quercia and Chang 1996; Shih et al. 1996). Similarly, using an intrahippocampal application protocol in the present study, we observed an efficient suppression of c-Fos IR in hippocampal and cortical regions ipsilateral to the injection site of *c-fos* AS-ODN compared with the contralateral, control ODN-injected hemisphere of the same rat after brightness discrimination training (Fig. 2). However, single scattered cells of the ipsilateral hemisphere, for example, in the hilar region, still expressed c-Fos IR, probably owing to cell type-specific differences in the uptake of the ODN (Sommer et al. 1993). Furthermore, inhibition of *c-fos* induction by *c-fos* AS-ODN is not always complete. Thus, in four out of six rats a strong suppression of c-Fos IR was evident, whereas in two rats the inhibition was clearly weaker. The reasons for this latter finding are not known.

Intracerebrally applied phosphorothioated ODNs may show toxic effects, including tissue damage (Chiasson et al. 1994). However, neither behavioral effects nor signs of structural damage were observed when amounts of *c-fos* AS-ODN comparable to those used in the present study were applied to the amygdala several days (i.e., with an interval that allows degradation of ODNs) before training or shortly before testing of rats in a conditioned taste aversion paradigm (Lamprecht and Dudai 1996). Similarly, in the present study no tissue damage additional to injuries caused by the insertion of cannulae was observed in ODN-treated versus saline-treated rats during determination of cannulae positions 5 days following intrahippocampal injections (data not shown). Nevertheless, to control for sequence-independent effects of *c-fos* AS-ODN, behavioral experiments were performed in comparison with saline-treated as well as control ODN-treated rats. The control ODN used was a reversed AS-ODN; that is, the nucleotide sequence was assembled in inverted orientation to prevent hybridization to *c-fos* mRNA and gene. Compared with commonly used control ODNs, such as sense, mismatched, or scrambled ODNs, reversed AS-ODNs advantageously have identical

nucleotide compositions and structural features as the corresponding AS-ODNs. Furthermore, physiological effects occasionally observed with sense (Cameron and Jennings 1991; Scherzinger et al. 1992) and mismatched (Woolf et al. 1992; Chiasson et al. 1994; Tischmeyer et al. 1994) sequences should not occur with reversed AS-ODNs.

Recently, severe behavioral deficits have been described in mutant mice lacking *c-fos* expression (Paylor et al. 1994). However, these mice showed impaired sensitivities of sensory systems and had heterogeneous genetic backgrounds, making interpretation of learning deficits rather difficult. Hence, to look for side effects of the administered ODNs per se and of the suppression of *c-fos* expression on mechanisms that may interfere with the learning performance, we have analyzed whether ODN-induced alterations occur during adaptation of the rats to the test apparatus. Runs between the arms of the Y-maze were counted during the habituation period before both the training and the relearning test and assessed as an index of arousal and activity. On the day of training, habituation performances of control ODN-treated rats as well as of *c-fos* AS-ODN-treated animals did not differ from exploratory behavior of the saline-treated control group (Table 1), indicating that neither control ODN nor *c-fos* AS-ODN caused alterations in the state of arousal and activity. These findings are in accordance with previous results demonstrating that intrahippocampally applied phosphorothioated ODNs did not alter the activity of rats in an open field test (Tischmeyer et al. 1994). On the relearning day, again saline-treated and control ODN-treated rats showed almost identical habituation performances, but the number of runs performed by these rats was reduced significantly compared with their pretraining exploratory activity, probably reflecting some kind of familiarity with the training chamber and the experimental situation. In contrast, exploratory behavior of *c-fos* AS-ODN-treated rats during the habituation phase on the relearning day did not significantly differ from that on the day of initial training when the test apparatus was novel to these rats, suggesting that they did not remember the experimental situation well and therefore behaved like naive animals.

During training and relearning of the brightness discrimination reaction, general parameters such as footshock intensities as well as reaction times and running times required to escape from

the start box and to arrive in the goal box, respectively, were recorded. These parameters reflect the sensitivity of sensory and motor systems. They did not vary significantly among the differently pretreated groups of rats. A tendency to increased running times was noticeable both in control ODN-treated and *c-fos* AS-ODN-treated rats during relearning. This trend, however, did not correlate with the brightness discrimination score. Thus, neither the ODNs per se nor the suppression of *c-fos* expression in *c-fos* AS-ODN-treated rats caused deficits in sensory transduction that could account for learning deficits.

In agreement with the observed normal motor and sensory behavior, the number of training errors did not differ between groups (Table 1). This shows that acquisition of the brightness discrimination reaction was not affected by the two ODNs. In contrast, after 24 hr, the degree of retention of the task was clearly diminished in *c-fos* AS-ODN-treated rats, as reflected in a significantly reduced retention index compared with the level of both the saline-treated and the control ODN-treated groups (Fig. 3). However, pretraining application of *c-fos* AS-ODN did not completely prevent brightness discrimination learning, probably reflecting an incomplete inhibition of *c-fos* expression or interindividual variations in the application, penetration, or efficiency of *c-fos* AS-ODN as could be expected from the immunohistochemical data. Alternatively, related transcription factors might compensate to a certain degree for c-Fos.

Recently, we have shown that suppression of c-Jun expression by intrahippocampally applied AS-ODN affected mainly the acquisition of brightness discrimination in rats (Tischmeyer et al. 1994), implying that this transcription regulatory protein, expressed at a relatively high basal level in rodent brain, is necessary for normal neuronal function. In contrast, suppression of another member of the Jun family, JunB, was without effects on the discrimination performance. Our present results demonstrate a sequence-specific impairment of retention of the brightness discrimination reaction in rats by intrahippocampally applied *c-fos* AS-ODN. The parameters monitored during the behavioral experiments indicate that this retention deficit does not result from influences on sensory or motor systems that may interfere with the acquisition of the discrimination task, suggesting that c-Fos, certainly in combination with other transcription factors, is involved in mechanisms of consoli-

dation and/or retrieval of the discrimination reaction.

Furthermore, as published during the reviewing of this manuscript, transient expression of *c-Fos* in rat amygdala is required for encoding conditioned taste aversion memory (Lamprecht and Dudai 1996). Thus, results yielded by use of different learning paradigms support the hypothesis that *c-Fos* is a link in the chain of metabolic events induced by neuronal signals during learning and leading finally to the formation of a long-term memory trace.

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