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Antisense oligonucleotide to c-*fos* blocks the ability of arginine vasopressin to maintain ethanol tolerance

Gyula Szabó, Karin R. Nunley, Paula L. Hoffman *

Department of Pharmacology and Alcohol Research Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA

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Abstract

Administration of the neuropeptide, arginine vasopressin, can reduce the rate of dissipation of functional ethanol tolerance in mice that have acquired that tolerance. We previously showed that intracerebroventricular vasopressin administration can also produce an increase in septal c-*fos* mRNA levels. To evaluate the role of the increased expression of c-*fos* in the ability of vasopressin to maintain tolerance, ethanol-tolerant mice were given intracerebroventricular injections of vasopressin in the presence or absence of an antisense oligonucleotide to c-*fos*. The antisense oligonucleotide completely blocked the ability of vasopressin to maintain ethanol tolerance, while a missense oligonucleotide was without effect. The antisense oligonucleotide also attenuated the increase in septal c-*fos* mRNA levels caused by vasopressin. The results provide evidence for a role of c-*fos* expression in the maintenance of ethanol tolerance by vasopressin.

Keywords: Ethanol tolerance; Arginine vasopressin; c-*fos*; Immediate early gene

1. Introduction

The neuropeptide, arginine vasopressin, has been found to modulate neuroadaptive processes, including learning and memory (Hoffman, 1987) and functional tolerance to ethanol (Hoffman et al., 1978; Lê et al., 1982). Although acquired tolerance to ethanol usually dissipates with time if ethanol ingestion is terminated, the administration of vasopressin to mice or rats that have acquired functional tolerance to ethanol reduces the rate of dissipation of this tolerance, i.e., tolerant animals that receive vasopressin retain their tolerance to ethanol, even in the absence of further ethanol intake (Hoffman et al., 1978; Lê et al., 1982). The effect of vasopressin on ethanol tolerance is mediated through vasopressin V_1 receptors in the brain (Szabó et al., 1988). We have also shown that vasopressin, at a dose that maintains ethanol tolerance, acts through V_1 receptors in the mouse septum to produce increased levels of mRNA for the immediate early gene, c-*fos* (Giri et al., 1990). Studies using peptides related structurally to vasopressin suggested that the increase in septal c-*fos* expression was associated with the ability of various vasopressin-related peptides to maintain ethanol tolerance (Giri et al., 1990; Szabó et al., 1991). Since the Fos protein, in conjunction with the protein products of another gene family (i.e., Jun proteins), forms the AP-1 transcription factor (Rauscher et al., 1989), these observations suggested a mechanism by which the short-lived vasopressin-related peptides could have relatively long-term effects on tolerance and memory. That is, vasopressin may increase the expression of the AP-1 transcription factor that could, in turn, activate the expression of other ('late') genes that affect the maintenance or dissipation of ethanol tolerance. To assess the role of c-*fos* expression in the ability of vasopressin to maintain ethanol tolerance, we have determined the effect of an antisense oligonucleotide to c-*fos* mRNA on the behavioral and biochemical responses to vasopressin.

2. Materials and methods

2.1. Materials

C-*fos* antisense oligonucleotide (5'-GAA CAT CAT GGT CGT-3') (Chiasson et al., 1992) and c-*fos* missense oligonucleotide (5'-AGA ACT TCA GTG GCT-3') were obtained from Macromolecular Resources (Fort Collins,

* Corresponding author. University of Colorado Health Sciences Center, Department of Pharmacology, 4200 E. Ninth Ave. C-236, Denver, CO 80262, USA. Tel.: (303) 270-5690; fax: (303) 270-7097.

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CO). The missense oligonucleotide was designed by randomizing the sequence of the antisense oligonucleotide. A sequence with several mismatches was used, since 'antisense' effects have been observed using oligonucleotides with up to three mismatches (Chiasson et al., 1994). Screening of this missense oligonucleotide through the GenBank database did not reveal any matches with known sequences. Arginine vasopressin was purchased from Bachem (Torrance, CA). For Northern analyses, a 40mer oligonucleotide from exon 1 of the mouse c-*fos* sequence, and a 40mer oligonucleotide from a translated sequence corresponding to the N-terminus of mouse c-*jun*, were obtained from Oncogene Science (Uniondale, NY), and were 3'-end-labeled with [α-^{32}P]dATP (DuPont/NEN, Boston, MA) using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Piscataway, NJ).

2.2. Measurement of ethanol tolerance

Male C57BL/6J mice (22–25 g) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed ten per cage under controlled environmental and lighting conditions (12 h light/dark circle) in an AAALAC-accredited facility for at least 1 week, with food and water available ad libitum, before being used in experiments. Mice were implanted with intracerebroventricular (i.c.v.) cannulas under pentobarbital anesthesia (75 mg/kg) (Szabó et al., 1988) and then housed individually. After a 3-day recovery period, the animals were fed a liquid diet for 5 days. Experimental animals received liquid diet (Bio-Serv, Frenchtown, NJ) containing ethanol U.S.P. (6.7%, v/v), and control animals were pair-fed liquid diet in which maltodextrin equicalorically replaced the ethanol. At the end of the 5-day ethanol ingestion period (on the morning of the 6th day), all animals were given the control liquid diet (withdrawal). This 5-day ethanol treatment regimen produces maximal ethanol tolerance, but only mild withdrawal signs in the ethanol-fed animals upon elimination of ethanol, indicating that physical dependence has not yet fully developed (Ritzmann and Tabakoff, 1976). At 24 h after ethanol withdrawal, mice were tested for tolerance to the hypnotic effect of ethanol by determination of the duration of loss of righting reflex after intraperitoneal (i.p.) injection of a challenge dose of 3.2 g/kg of ethanol (21% solution; 20 ml/kg) (Szabó et al., 1988). Tolerance testing was carried out between 9:00 a.m. and noon.

After the initial tolerance test, control and ethanol-fed mice were subdivided into groups that received once-daily i.c.v. injections of vasopressin (1 ng) or artificial cerebrospinal fluid (CSF) vehicle (Szabó et al., 1988). These injections were administered between 4:00 and 5:00 p.m. 45 min prior to the vasopressin or vehicle injections, designated subgroups of mice received i.c.v. injections of oligonucleotide (50 μg) or artificial CSF vehicle. Thus, there were six groups of control mice and six groups of ethanol-fed mice, each of which received one of the following treatments: vehicle-vehicle; vehicle-vasopressin; c-*fos* antisense oligonucleotide-vehicle; c-*fos* antisense oligonucleotide-vasopressin; c-*fos* missense oligonucleotide-vehicle; c-*fos* missense oligonucleotide-vasopressin. The volume of all i.c.v. injections was 2 μl. These injections were given on days 1 and 2 after withdrawal, and tolerance was again tested between 9:00 a.m. and noon on day 3 and day 6 after withdrawal.

After the last tolerance test, the placement of the cannulas was verified by injecting 10 μl of methylene blue and examining the presence of dye in the lateral ventricle (Szabó et al., 1988). Data from animals with improper cannula placement were excluded from the analysis.

2.3. Northern blots

C57BL/6J mice were implanted with i.c.v. cannulas, and were injected i.c.v. with artificial CSF vehicle or with the c-*fos* antisense or missense oligonucleotides (50 μg), followed 45 min later by vasopressin (1 ng), as described above. 15 min after vasopressin injection, the mice were killed, the septal region of the brain was dissected on ice (Giri et al., 1990) and tissue was stored at −70°C. Total RNA from 2–4 pooled septi was extracted using the Trizol reagent (Gibco BRL, Gaithersburg, MD), and 10 μg of RNA was size-fractionated on a 1.5% denaturing agarose gel (Giri et al., 1990). Ethidium bromide (83 μg/ml, Sigma Chemical Co., St. Louis, MO) was added to each sample prior to loading the sample on the gel. After the gels were photographed under UV light, the RNA was transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) by capillary elution in 10 × SSPE (SSPE = 180 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA) and cross-linked to the membrane using the Stratalinker UV crosslinker (Stratagene, La Jolla, CA). Completeness of transfer was verified by viewing the gel and the blot under UV light, and no RNA was visible on the gels after transfer.

The blots were pre-hybridized at 42°C for 3–4 h in a solution containing 6 × SSPE, 1.0% SDS, 10 × Denhardt's solution, 50 μg/ml yeast tRNA and 100 μg/ml herring sperm DNA. Blots were then hybridized for 18 h at 50°C in 6 × SSPE containing 1.0% sodium dodecyl sulfate (SDS) and the ^{32}P-labelled oligonucleotide probe at a concentration of 2×10^6 cpm/ml. The blots were washed (Giri et al., 1990), placed in cassettes containing intensifying screens, and apposed to X-Omat AR film (Eastman Kodak) at −70°C for 3–5 days. After removal of the c-*fos* probe by washing in a solution of 96% formamide, 10 mM Tris and 10 mM EDTA, some blots were subjected to re-hybridization with the labeled c-*jun* probe (Giri et al., 1990). The autoradiograms were quantitated by computer-assisted densitometry (Sierra Scientific CCD camera; Macintosh 2fx computer and Image 1.28 software). Total ribosomal RNA was quantitated densitometrically from nega-

tive photographs of the ethidium bromide-stained gels, and mRNA levels are expressed as a ratio to 28 S RNA.

2.4. Data analysis

The behavioral data were analyzed by a $2 \times 2 \times 3 \times 3$ repeated measures analysis of variance (ANOVA) using the multivariate approach of SAS PROC GLM. Between-subject factors were diet (two levels: control or ethanol-containing), first i.c.v. injection (three levels: vehicle, antisense oligonucleotide or missense oligonucleotide) and second i.c.v. injection (two levels: vehicle or vasopressin). Time after withdrawal (three levels: 1, 3 or 6 days after withdrawal) was a within-subjects factor. Post-hoc comparisons using Tukey's test were carried out to determine differences among treatment groups on each day of tolerance testing. Student's t test on transformed data (arcsine transformation) was used to compare ratios obtained from Northern analysis (c-*fos* mRNA/28 S RNA), which were expressed as percent of control. Values of $P < 0.05$ were considered significant. Data are presented as mean ± S.E.M.

3. Results

Treatment of control mice (mice fed the *control liquid diet*) with vasopressin, in the presence or absence of the c-*fos* oligonucleotides, did not significantly alter the response of the mice to the challenge dose of ethanol on any day tested, and data for all control groups were therefore combined (Fig. 1). Since the ANOVA showed a significant effect of time after withdrawal (i.e., dissipation of ethanol tolerance) ($F = 229.8$, df = 2, $P < 0.0001$) and a significant treatment group × time interaction ($F = 22.0$, df = 12, $P < 0.0001$), the treatment groups were compared separately at each day after withdrawal. As in previous studies (Szabó et al., 1988; Ritzmann and Tabakoff, 1976), mice that were fed the ethanol-containing liquid diet for 5 days displayed maximal tolerance to the hypnotic effect of ethanol when injected with a challenge dose of ethanol at 24 h after withdrawal (Fig. 1).

At 3 days after withdrawal, although some loss of tolerance could be observed, the duration of loss of righting reflex in all groups of ethanol-fed mice was still significantly different from that of control mice (Tukey test, $P < 0.05$). However, vasopressin delayed the loss of tolerance, since the response of the ethanol-fed, vehicle (CSF)/vasopressin-treated mice was significantly different from the response of the groups of ethanol-fed mice that did not receive vasopressin (Tukey test, $P < 0.05$) (Fig. 1). Treatment of mice with the missense oligonucleotide to c-*fos* prior to vasopressin did not alter the effect of vasopressin: the response of the mice in this group was significantly different from that of the ethanol-fed mice in all other i.c.v. injected groups except the vehicle/vasopressin

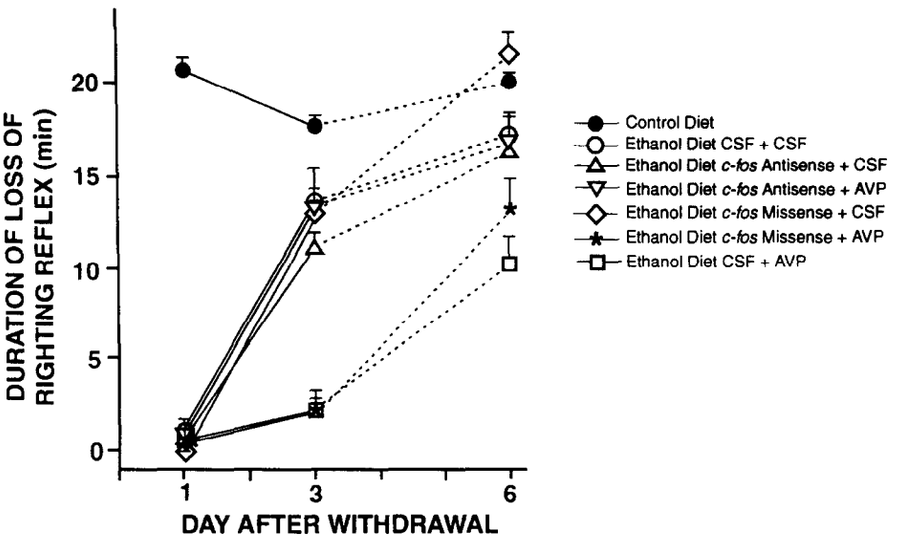

Fig. 1. Blockade of the effect of vasopressin on maintenance of ethanol tolerance by an antisense oligonucleotide to c-*fos*. C57BL/6J mice were fed ethanol in a liquid diet, or control liquid diet, for five days. At 24 h after ethanol withdrawal (between 9:00 a.m. and noon), all mice received an i.p. injection of 3.2 g/kg of ethanol and duration of loss of righting reflex was measured. Control and ethanol-fed mice were then subdivided into groups that received two i.c.v. injections (through previously implanted cannulas) of: vehicle/vehicle (artificial CSF); vehicle/vasopressin (1 ng); missense oligonucleotide to c-*fos* (50 μg)/vehicle; missense oligonucleotide/vasopressin; antisense oligonucleotide to c-*fos* (50 μg)/vehicle; antisense oligonucleotide/vasopressin. The oligonucleotide sequences are indicated in Section 2. All i.c.v. injections were given in a volume of 2 μl and the first injection was 45 min prior to the second injection. The mice received these injections on day 1 and day 2 after ethanol withdrawal (between 4:00 and 5:00 p.m.), and tolerance to the hypnotic effect of ethanol was again measured (as duration of loss of righting reflex following the challenge ethanol dose) on days 3 and 6 after withdrawal. Treatment of control mice with vasopressin and/or the oligonucleotides did not significantly alter the response of the mice to the challenge dose of ethanol, and data from all control groups have been combined. Data were analyzed by repeated measures ANOVA and post-hoc comparisons were made with the Tukey test; statistical significance of differences is discussed in the text.

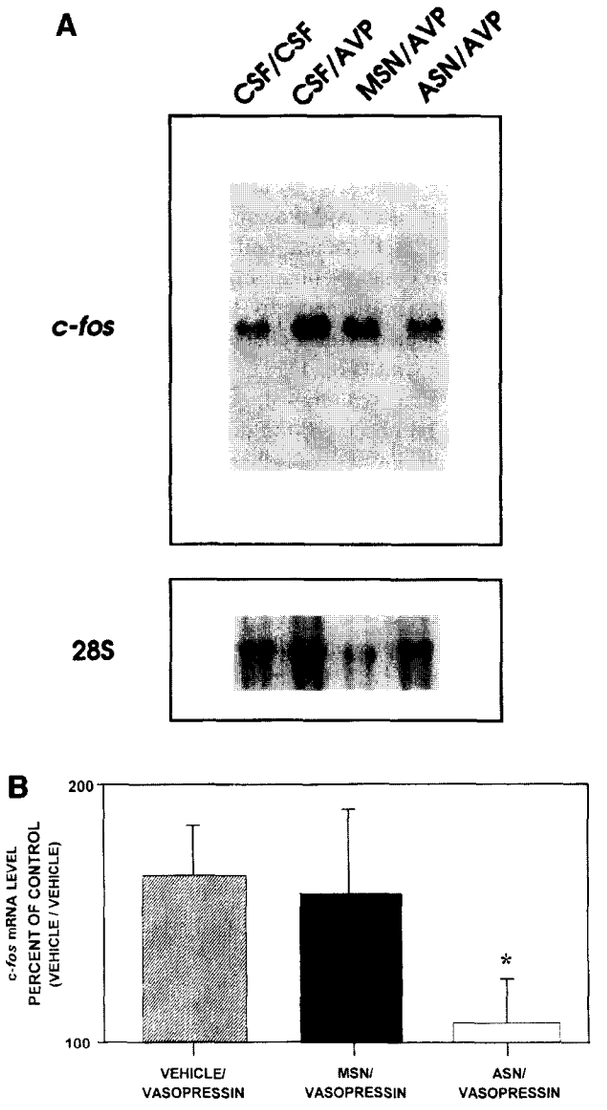

Fig. 2. A: Autoradiogram of Northern blot of c-*fos* mRNA levels in mouse septum. C57BL/6J mice were injected i.c.v. with vasopressin and artificial CSF vehicle or the missense or antisense oligonucleotides to c-*fos*, as described in Section 2 and the legend to Fig. 1. All i.c.v. injections were made via previously implanted cannulas and were in a volume of 2 μl. Vehicle or the oligonucleotides were injected 45 min prior to vasopressin, and mice were killed 15 min later. Total RNA was extracted from two pooled septi and Northern analysis was performed as described in Section 2. Lane 1: vehicle (CSF)/vehicle; lane 2: vehicle/vasopressin (AVP); lane 3: missense oligonucleotide (MSN)/vasopressin; lane 4: antisense oligonucleotide (ASN)/vasopressin. B: Effect of c-*fos* oligonucleotides on vasopressin-induced increase in mouse septal c-*fos* mRNA. C57BL/6J mice were injected i.c.v. with vasopressin (1 ng) in the presence or absence of the missense (MSN) or antisense (ASN) oligonucleotides (50 μg) to c-*fos* as described above. Autoradiograms of Northern blots were quantitated by computer-assisted densitometry. Data are presented as percent of control (i.e., mice injected with vehicle (CSF)/vehicle only) septal c-*fos* mRNA levels. Values represent mean ± S.E.M. from three separate experiments (septal tissue from 2–4 mice was pooled in each experimental group). * $P < 0.05$, compared to vehicle/vasopressin group (Student's *t* test on transformed data).

group (Tukey test, $P < 0.05$) (Fig. 1). In contrast, treatment with the antisense oligonucleotide to c-*fos* blocked the ability of vasopressin to delay the loss of tolerance. The response of the ethanol-fed mice treated with the antisense oligonucleotide and vasopressin was significantly different from the responses of the ethanol-fed mice that received vasopressin or the missense oligonucleotide and vasopressin (Tukey test, $P < 0.05$), but was not significantly different from the responses of the groups of ethanol-fed mice that did not receive vasopressin (Fig. 1).

By the 6th day after withdrawal, 4 days after cessation of i.c.v. injections, all groups of ethanol-fed mice, except those that received vasopressin or the missense oligonucleotide plus vasopressin, had lost tolerance (i.e., their responses were not different from those of controls, Tukey test). The rate of loss of tolerance is consistent with the rate of loss of tolerance that we have observed earlier (Hoffman et al., 1978; Szabó et al., 1988). We have also previously found that vasopressin-treated mice lose tolerance within 6 days after cessation of vasopressin treatment (e.g., Szabó et al., 1988), and the current data are also consistent with that finding, in that tolerance was clearly decreased in the vehicle/vasopressin and missense oligonucleotide/vasopressin groups at 4 days after cessation of vasopressin treatments, compared to 1 day after cessation of treatments (Fig. 1).

We also wished to determine whether the antisense oligonucleotide treatment, that blocked the ability of vasopressin to maintain ethanol tolerance, blocked the increase in c-*fos* mRNA levels produced by vasopressin injection. As shown in Fig. 2, i.c.v. injection of vasopressin resulted in an increased level of c-*fos* mRNA in the mouse septum, consistent with our earlier findings (Giri et al., 1990) and those of others (Andreae and Herbert, 1993). When mice were treated with the antisense oligonucleotide to c-*fos* prior to vasopressin, the increase in c-*fos* mRNA was significantly attenuated. The effect of vasopressin on c-*fos* mRNA, however, was not significantly affected by prior treatment with the missense oligonucleotide to c-*fos* (Fig. 2). When the Northern blots were re-probed with the c-*jun* probe, we found that the expression of this immediate early gene was not significantly affected by treatment of the mice with the c-*fos* antisense oligonucleotide (Fig. 3).

4. Discussion

The use of antisense oligonucleotides that can, theoretically, specifically inhibit the transcription or translation of specific genes provides a potentially powerful method for determining the role of a particular molecule in a behavioral or biochemical response of an organism (Brysch and Schlingensiepen, 1994). The immediate early genes, such as c-*fos*, are particularly appealing targets for the use of antisense technology, since they are not expressed constitutively, or are expressed at very low levels, and their

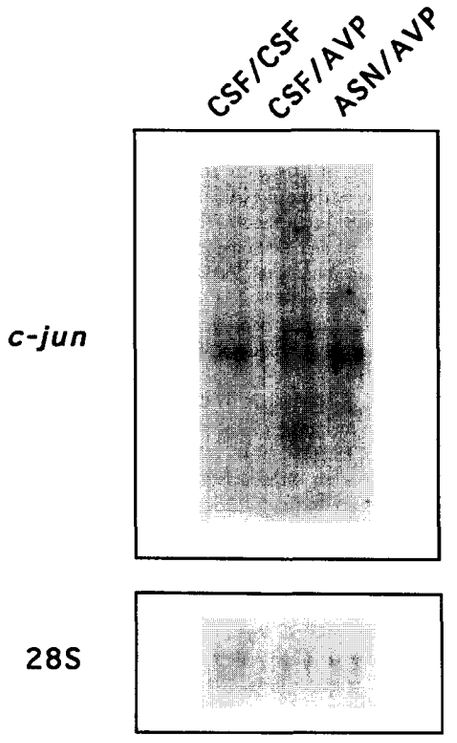

Fig. 3. Autoradiogram of Northern blot of c-*jun* mRNA levels in mouse septum. The blots used to measure c-*fos* mRNA levels in the experiments described in the text and legend to Fig. 2 were re-probed with the ^{32}P-labeled c-*jun* probe. Lane 1: vehicle (CSF)/vehicle; lane 2: vehicle/vasopressin (AVP); lane 3: antisense oligonucleotide (ASN)/vasopressin.

expression increases substantially but transiently after a stimulus (Curran et al., 1990). The sequence of the antisense oligonucleotide to c-*fos* mRNA that was used in the present study is identical to the sequence of the oligonucleotide that was previously shown to alter D-amphetamine-induced rotational behavior (Hooper et al., 1994) and to block the locomotor stimulant action of cocaine (Heilig et al., 1993) in rats. In those studies, the oligonucleotide was infused into a particular brain area (striatum, nucleus accumbens) 8–10 h before the rats were treated with amphetamine or cocaine and tested behaviorally (Hooper et al., 1994; Heilig et al., 1993). With this oligonucleotide treatment regimen, the increase in c-*fos* immunoreactivity that occurred 2 h after amphetamine injection was attenuated (Chiasson et al., 1992; Hooper et al., 1994). The oligonucleotides used in these previous studies were fully phosphorothioated, a modification that reduces oligonucleotide breakdown and permits the use of a lower dose of oligonucleotide (Brysch and Schlingensiepen, 1994). However, in our initial studies, we found that i.c.v. injection of chromatographically purified phosphorothioated oligonucleotides resulted in seizures in our mice. We therefore used a higher dose of unmodified oligonucleotides in the present study, and did not observe seizures or any other overt behavioral signs of toxicity. It has been demonstrated that both unmodified and phosphorothioated oligonucleotides are taken up by cultured neural cells (Meeker et al., 1995). The concentration of unmodified oligonucleotides reached a peak at 30 min after administration, and declined to a low steady-state level by 2–4 h. In contrast, phosphorothioated oligonucleotide concentration rose more slowly to a peak at 6 h after administration, and declined over the next 18 h (Meeker et al., 1995). Based on this time course, and the relatively short half-life reported for unmodified oligonucleotides (Brysch and Schlingensiepen, 1994), we administered the oligonucleotides 1 h prior to the expected maximal increase in c-*fos* mRNA produced by vasopressin (i.e., c-*fos* mRNA levels were previously shown to peak in the septum at 15 min after vasopressin injection (Giri et al., 1990)).

In the earlier studies with the c-*fos* antisense oligonucleotide, a sense oligonucleotide was used as a control for specificity (Hooper et al., 1994; Heilig et al., 1993). Because of possible effects due to sense oligonucleotide binding to double-stranded DNA (Brysch and Schlingensiepen, 1994), however, a missense oligonucleotide was chosen for the current experiments. The chosen oligonucleotides and treatment regimen resulted in a block of the ability of vasopressin to maintain functional ethanol tolerance by the antisense oligonucleotide to c-*fos*, with no effect of the missense oligonucleotide on this neuroadaptive phenomenon.

Several mechanisms have been proposed to explain the action of antisense oligonucleotides. Antisense oligonucleotides that are targeted toward the start codon and surrounding bases of the mRNA are designed to interfere with translation (Brysch and Schlingensiepen, 1994). However, another postulated mode of action is the degradation of the DNA-RNA duplex by RNase H (Brysch and Schlingensiepen, 1994). Binding of antisense oligonucleotides can also destabilize mRNA, possibly by disrupting secondary or tertiary structures (Brysch and Schlingensiepen, 1994). These mechanisms may be important for the action of the antisense oligonucleotide to c-*fos*, since we found that this oligonucleotide interfered with the ability of vasopressin to increase c-*fos* mRNA. This effect was specific to the antisense oligonucleotide, since the increase in c-*fos* mRNA levels produced by vasopressin was not significantly affected by treatment of mice with the missense oligonucleotide (Fig. 2). In addition, the effect of the c-*fos* antisense oligonucleotide appeared to be specific for c-*fos* mRNA, since the oligonucleotide produced no detectable change in septal c-*jun* mRNA levels (Fig. 3).

Basal levels of c-*fos* mRNA and Fos protein in the brain are low to nonexistent (Curran et al., 1990), and vasopressin administration increases not only c-*fos* mRNA but also Fos protein in the septum (Andreae and Herbert, 1993). The fact that c-*fos* mRNA was not increased over baseline in mice that were treated with the c-*fos* antisense oligonucleotide and vasopressin indicates that Fos protein would also not be increased under these conditions. The

ability of the c-*fos* antisense oligonucleotide to attenuate vasopressin-induced stimulation of septal c-*fos* mRNA, and also to block the ability of vasopressin to maintain ethanol tolerance, provides strong correlative evidence that activation of septal c-*fos* expression by vasopressin plays a role in the effect of this neuropeptide on tolerance. These findings lend support to a hypothesis that vasopressin may produce its effects on neuroadaptive processes by generating the AP-1 transcription factor, a Fos-Jun heterodimer (Rauscher et al., 1989), and thus leading to altered expression of 'late genes', the identity of which is currently not known. As mentioned in the introduction, such a mechanism could account for the relatively long-term effects of vasopressin on processes involving central nervous system plasticity, including memory and functional ethanol tolerance.

Acknowledgements

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