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# Peripheral injection of arginine$^8$-vasopressin increases Fos in specific brain areas

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**Abstract**

Learned behaviors and tolerance to ethanol can be maintained by peripheral injection of arginine$^8$-vasopressin (vasopressin) under conditions in which they would otherwise be lost. However, the sites of this action in the brain have not been clearly identified. Using a polyclonal antibody raised against Fos and Fos-like proteins, we have demonstrated increases in immunoreactive Fos and Fos-like proteins in the suprachiasmatic, supraoptic and paraventricular nuclei of the hypothalamus, and lesser increases in piriform cortex and amygdala, of the rat 2 h after a s.c. injection of vasopressin. Our results suggest that the exogenous vasopressin may exert its central action by activating a cellular immediate early gene in specific brain regions.

*Keywords:* Vasopressin; Fos protein; Immunoreactivity; Brain region; (Rat)

## 1. Introduction

Peripheral (s.c.) administration of vasopressin has been shown to maintain learned behaviors under conditions normally leading to extinction (Ader and De Wied, 1972; Le Moal et al., 1984). Hoffman et al. (1978) demonstrated that systemic injections of vasopressin were also able to maintain tolerance to ethanol after cessation of ethanol treatment. For these vasopressin-mediated effects to occur, integrity of the central noradrenergic (Hoffman et al., 1989) and serotonergic (Lê et al., 1982; Speisky and Kalant, 1985; Lança et al., 1993; Wu et al., 1994) systems was required. This effect of vasopressin was mediated by sites in the brain, because it was also produced by [des-glycinamide$^9$,arginine$^8$]vasopressin (des-Gly$^9$-[Arg$^8$]vasopressin) which is almost devoid of peripheral effects (Lê et al., 1982). However, the exact sites of this vasopressin action following peripheral injection were not known. By the use of [$^3$H]vasopressin as a ligand, vasopressin binding sites have been identified in the paraventricular, supraoptic and suprachiasmatic nuclei of the hypothalamus of the rat brain (Yamamura et al., 1983; Junig et al., 1985; Phillips et al., 1988) as well as the lateral septum of the mouse (Ishizawa et al., 1990) and the rat (Raggenbass et al., 1987), and the medial posterior hypothalamic nucleus of the canary brain (Voorhuis et al., 1988). The vasopressin receptors in the lateral septum have been shown to be of the vasopressin $V_1$ type (Raggenbass et al., 1987). Recent experiments have also shown that the vasopressin potentiation of acute ethanol-induced motor impairment (Wu et al., 1992) and the vasopressin maintenance of ethanol tolerance (Szabó et al., 1988) were mediated through an action on vasopressin $V_1$ receptors in the brain. Activation of brain vasopressin $V_1$ receptors leads to stimulation of inositol triphosphate ($IP_3$) formation (Stephens and Logan, 1986), and the increased $IP_3$ raises the intracellular $Ca^{2+}$ and thus activates the c-*fos* immediate early gene (Morgan and Curran, 1991). Giri et al. (1990) showed that i.c.v. injection of vasopressin in the mouse increased the amount of Fos mRNA in Northern blots of the septum and hippocampus, and sug-

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gested that the c-*fos* immediate early gene might be involved in vasopressin actions. However, this approach does not provide the necessary information to identify the precise distribution and characteristics of the neuronal population stimulated by vasopressin administration. Moreover, the i.c.v. and s.c. routes of

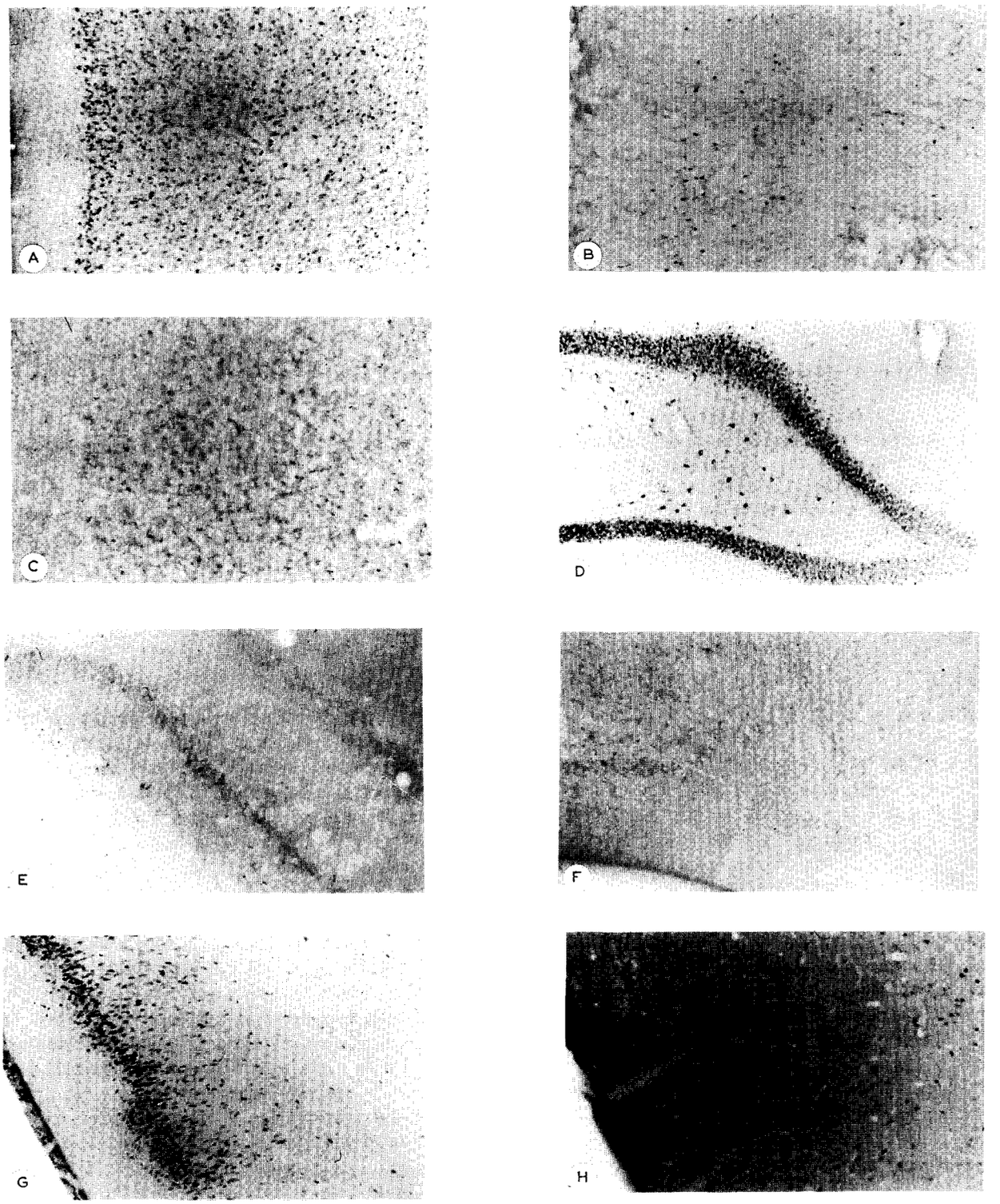


Fig. 1. Immunocytochemical demonstration of Fos protein in different regions of rat brain, after i.p. injection of pentylenetetrazole, or s.c. injection of vasopressin or saline. Bar = 150 μm. A, B and C: Anterior cingulate cortex, after pentylenetetrazole (A), vasopressin (B) or saline (C). D, E and F: Dentate gyrus, after pentylenetetrazole (D), vasopressin (E) or saline (F). G, H and I: Piriform cortex, after pentylenetetrazole (G), vasopressin (H) or saline (I).

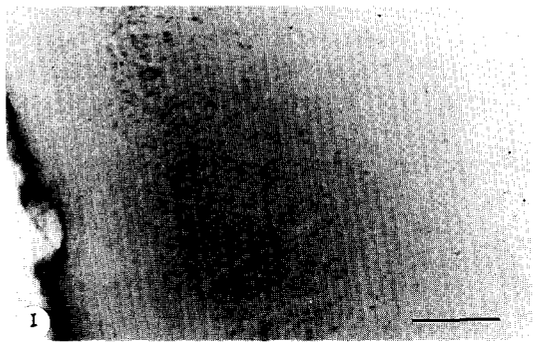

Fig. 1 (continued).

administration may result in actions at different sites in the brain, especially in the case of substances that do not readily permeate the blood-brain barrier.

In order to obtain further evidence that peripherally injected vasopressin does act in the brain, and to identify specific brain sites at which the vasopressin action may be produced, we used an immunocytochemical technique to demonstrate vasopressin-stimulated Fos and Fos-like proteins after an s.c. injection of vasopressin. Many of the brain regions examined have been implicated in learning and memory.

## 2. Materials and methods

Sheep polyclonal antibody to Fos oncoproteins was purchased from Cambridge Research Biochemicals (Wilmington, DE, USA). Biotinylated anti-sheep IgG (H + L) (antibodies directed against intact immuno gamma globulin heavy and light chains) immunoglobulin and avidin-biotin kit were obtained from Dimension Lab (Mississauga, Ontario, Canada). Glucose oxidase, 3,3′-diaminobenzidine tetrahydrochloride tablets and pentylenetetrazole were purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

Male Sprague-Dawley rats weighing 240–300 g were purchased from Charles River Laboratory (Montréal, Quebéc, Canada). The rats were housed singly in a colony complex in which the lights were on from 7:00 a.m. to 7:00 p.m., and the room temperature was maintained at 22°C. Each rat received five pellets of rat chow daily and tap water ad libitum.

For examination of c-*fos* expression, a preliminary experiment was carried out in which separate groups of rats ($n = 3$ per group) treated with saline, vasopressin or pentylenetetrazole were killed at 30 and 60 min. Staining for Fos-like immunoreactivity (see below) showed a clear increase in the pentylenetetrazole animals from 30 to 60 min, particularly in hippocampus and cortex, but no detectable staining at either time, in any brain, in the animals pretreated with saline or with vasopressin. Therefore a longer survival time (2 h) was used in the major experiment.

Rats were injected with vasopressin (10 $\mu$g/0.3 ml s.c.; $n = 6$), saline (0.3 ml s.c.; $n = 6$), saline (2 ml i.p.; $n = 3$) or pentylenetetrazole (50 mg/kg in 2 ml i.p.; $n = 3$). This dose of vasopressin is the same as that used previously in behavioral studies in which des-Gly$^9$-[Arg$^8$]vasopressin and vasopressin maintenance of ethanol tolerance was observed (Lê et al., 1982; Speisky and Kalant, 1985; Wu et al., 1994). Two hours later, each rat received an injection of pentobarbital (50 mg/kg i.p.). When the rat was fully anesthetized, intracardiac perfusion of 100 ml saline followed by 400 ml of ice-cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.25) was performed. The brain was removed and immersed in the fixative for 2 h before being stored in a 20% sucrose-phosphate buffer solution at 8°C overnight. Coronal brain sections of 48 $\mu$m thickness were cut on a freezing microtome (IEC microtome-microcryostat) and were immunostained for Fos and Fos-like proteins according to the method of Dragunow and Robertson (1987). Briefly, the sections were incubated with the primary antibody (sheep polyclonal antibody to Fos oncoproteins, 1:1500 dilution) in 1% normal rabbit serum for 48–72 h at 8°C. The sections were then washed 3 times in phosphate buffer saline (PBS) and were incubated with biotinylated anti-sheep IgG (1:500 dilution) for 1 h. After further rinsing in PBS (3 × 10 min), the sections were incubated for 1 h with the chromogen, diaminobenzidine, and visualized with the addition of glucose oxidase. In each batch, appropriate saline-treated control animals were included, as well as one rat injected with pentylenetetrazole (50 mg/kg i.p.), and the brains were processed in an identical manner. The brain sections of the pentylenetetrazole-treated rats served as positive controls for Fos and Fos-like oncoprotein immunoreactivity. The sections were dehydrated and mounted on microscopic slides in a Permount histological mounting medium (Fisher Scientific) and were observed under a Nikon Optiphot microscope. Kodak ASA-160 film was used for the photography. For the semiquantitative studies, the antibody-stained cell nuclei were counted under the microscope in a minimum of ten different fields per animal.

## 3. Results

The immunocytochemical technique used in our experiments was able to detect an increase in Fos and Fos-like proteins in many brain regions after pentylenetrazole treatment. The results were in general agreement with those reported earlier by Dragunow and Robertson (1987) (Fig. 1A, D and G). In the brain slices of pentylenetetrazole-treated animals, Fos-like

immunoreactivity was consistently robust in every experiment, especially in the hippocampus and dentate gyrus (Fig. 1D). An injection of saline (s.c.) did not result in an increase in Fos-like immunoreactivity except in the piriform cortex (Fig. 1I). In this area the number of Fos-like immunoreactive nuclei was markedly increased in the pentylenetetrazole-treated animals (Fig. 1G). Injection of vasopressin (s.c.) pro-

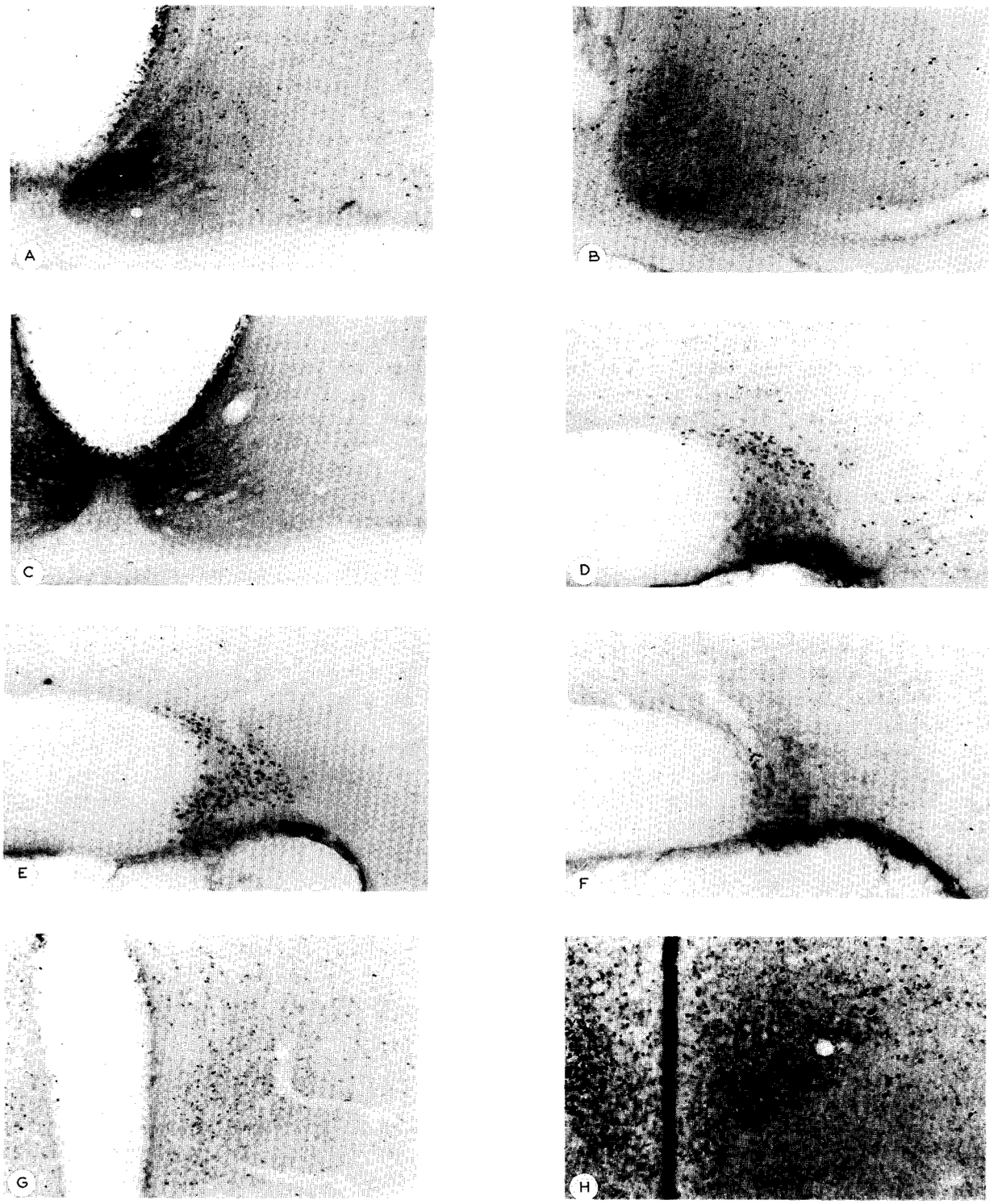


Fig. 2. Fos-like immunoreactivity in different hypothalamic nuclei of the rat; same treatments as in Fig. 1. Bar = 150 μm. A, B and C: Suprachiasmatic nucleus, after pentylenetetrazole (A), vasopressin (B) or saline (C). D, E and F: Supraoptic nucleus, after pentylenetetrazole (D), vasopressin (E) or saline (F). G, H and I: Paraventricular nucleus, after pentylenetetrazole (G), vasopressin (H) or saline (I).

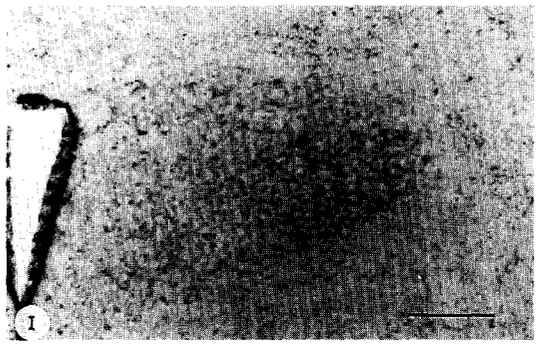

Fig. 2 (continued).

duced moderate increases in Fos-like immunoreactive nuclei in the anterior cingulate and piriform cortices (Fig. 1B and E) but not in the dentate gyrus (Fig. 1H).

In contrast, vasopressin (s.c.) injection strongly stimulated Fos-like immunoreactivity in the hypothalamus and pons. In the hypothalamus, many Fos-like immunoreactive nuclei were seen in neuronal perikarya in the suprachiasmatic, supraoptic and paraventricular nuclei (Fig. 2B, E and H), whereas in the saline-injected animals only a very small number of Fos-like immunoreactive nuclei were present. Pentylenetetrazole injection led to moderate increases in Fos-like immunoreactivity in suprachiasmatic nucleus (Fig. 2A) and paraventricular nucleus (Fig. 2G) as well as in the dorsal portion of the supraoptic nucleus (Fig. 2D).

Histological examination of all the regions under study confirmed that vasopressin increased Fos-like immunoreactivity strongly in the hypothalamic nuclei and pons (ventrolateral pontine nuclei and superior and inferior olivary complex), clearly in the piriform cortex and amygdala, and more modestly in the anterior cingulate cortex, entorhinal cortex and to lesser degree, the lateral septum. The hippocampus and dentate gyrus did not show any increase in Fos-like immunoreactivity in the vasopressin-treated rats. Saline injection produced only slight increases in the piriform cortex, amygdala, olfactory tract and cortex, and agranular cortex, and no effect at all in the hypothalamus, hippocampus and pons. Detailed quantitative analysis of these regional differences is currently in progress.

## 4. Discussion

It has been shown that enhanced immediate early gene expression can be used to identify sites of drug action in brain (Robertson, 1993; Smeyne et al., 1992; Smeyne et al., 1993). If peripherally injected vasopressin activates vasopressin $V_1$ receptors in the brain, then the s.c. injection of vasopressin should result in the activation of c-*fos* immediate early gene in neurons possessing vasopressin $V_1$ receptors. In our experiments, the antibody was able to demonstrate increased expression of Fos or Fos-like proteins in many brain areas including the anterior cingulate cortex and hippocampus of the pentylenetetrazole-treated rats, as reported by others (Morgan et al., 1987; Dragunow and Robertson, 1987). In our hands, saline (s.c.) administration did not stimulate the production of Fos and Fos-like proteins in most brain regions except the piriform cortex and olfactory cortex as shown by Smeyne et al. (1992). The apparent increase of Fos and Fos-like immunoreactivity in these brain areas after saline injection may possibly be due to activation of c-*fos* immediate early gene in these regions by olfactory stimuli present in the test environment.

The peripheral administration of vasopressin did not markedly change Fos-like immunoreactivity over the background levels (saline-treated rat brain) in the olfactory tract, olfactory cortex and cortical layers II and III, suggesting that these four regions probably are not involved in the central actions of exogenous vasopressin. The brain regions that were most likely associated with exogenous vasopressin actions were the hypothalamic suprachiasmatic, supraoptic and paraventricular nuclei in which Fos-like immunoreactivity was markedly increased over levels seen in the saline-treated animals. Moreover, these increases after vasopressin injection were relatively much greater than those produced by pentylenetetrazole in the same area. Somewhat less marked increases were found in the piriform cortex, amygdala and thalamic paraventricular nucleus, which might also be important sites of action of exogenous vasopressin. There was a very low level of Fos-like immunoreactivity in the septum, but virtually no identifiable Fos-like immunoreactivity in the hippocampus after vasopressin treatment, despite an earlier report (Giri et al., 1990) of increased c-*fos* expression (Fos mRNA) in the septum and hippocampus after i.c.v. injection of vasopressin. Though vasopressin also produced a strong increase in the pons, it is unlikely that pontine nuclei are involved in the vasopressin effect on learned behaviors or on ethanol tolerance, because these regions are known to be mainly involved in control of peripheral and autonomic activities.

The differences between our results and those of Giri et al. (1990) may possibly be attributed to methodological factors. Firstly, different modes of vasopressin administration were used. It is possible that i.c.v. administration produces a sufficiently high concentration of vasopressin in the hippocampus to increase the expression of c-*fos* there, while the vasopressin level in the hippocampus after s.c. injection may not have been high enough to do so. Secondly, Giri et al. (1990) measured mRNA levels in blocks of dissected tissue that included the septum, but that may also have included adjacent structures such as the diagonal band

of Broca, that is also known to contain a large number of vasopressin-containing fibers (De Vries and Buijs, 1983; Sofroniew and Weindl, 1978) and vasopressinergic receptors (Baskin et al., 1983).

The present findings do not, by themselves, permit any conclusion as to whether the activation of central c-*fos* expression by peripheral administration of vasopressin is related to the peripheral actions of vasopressin on blood pressure, fluid balance, etc., or the central actions such as those on learning and tolerance. In order to help differentiate between these possibilities, further studies recently completed in our laboratory have replaced vasopressin with peripherally administered des-Gly$^9$-[Arg$^8$]vasopressin, that is virtually devoid of peripheral actions but retains the central actions of vasopressin. The most striking difference between the effects of des-Gly$^9$-[Arg$^8$]vasopressin and vasopressin observed to date is that des-Gly$^9$-[Arg$^8$]vasopressin does not stimulate c-*fos* expression in supraoptic and paraventricular nuclei, but does so strongly in the suprachiasmatic nucleus (Lança et al., manuscript in preparation). This suggests that the c-*fos* expressions in supraoptic and paraventricular nuclei are related to peripheral actions of vasopressin or to feedback control of vasopressin production, whereas the increased expression in suprachiasmatic nucleus may be related to the central actions shared by vasopressin and des-Gly$^9$-[Arg$^8$]vasopressin. This is consistent with the observation that the suprachiasmatic nucleus contains exclusively vasopressin $V_1$ receptors whereas the supraoptic nucleus and paraventricular nucleus contain predominantly vasopressin $V_2$ receptors (Johnson et al., 1993), and that the maintenance of tolerance by vasopressin is mediated by vasopressin $V_1$ receptors (Szabó et al., 1988).

We have previously proposed that the known interaction between serotonin and vasopressin in the maintenance of ethanol tolerance occurs at the level of the septum (Kalant, 1993). Studies employing combined retrograde tracing and immunocytochemistry (Lança et al., 1993) led to a modified hypothesis that such interaction could also occur at the level of vasopressin-producing cells in the hypothalamus. The present findings are consistent with this modification. Activation of c-*fos* expression in the suprachiasmatic nucleus via vasopressin $V_1$ receptors suggests the possibility of increased production of vasopressin in the cell bodies and consequently increased release of vasopressin in the projection areas of suprachiasmatic nucleus, including lateral septum (Sofroniew and Weindl, 1978).

In conclusion, our results demonstrate that peripherally injected vasopressin stimulates the expression of c-*fos* immediate early gene selectively in different brain regions, and most intensely in the hypothalamic suprachiasmatic, supraoptic and paraventricular nuclei. Such stimulation may underlie the ability of the exogenous vasopressin to maintain learned behaviors and tolerance to ethanol.

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## References

Ader, R. and D. De Wied, 1972, Effects of lysine vasopressin on passive avoidance learning, Psychonom. Sci. 29, 46.

Baskin, D.G., F. Petracca and D.M. Dorsa, 1983, Autoradiographic localization of specific binding sites for [$^3$H][Arg$^8$]vasopressin in the septum of the rat brain with tritium-sensitive film, Eur. J. Pharmacol. 90, 155.

De Vries, G.J. and R.M. Buijs, 1983, The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum, Brain Res. 273, 307.

Dragunow, M. and H.A. Robertson, 1987, Generalized seizures induce c-fos protein(s) in mammalian neurons, Neurosci. Lett. 82, 157.

Giri, P.R., J.R. Dave, B. Tabakoff and P.L. Hoffman, 1990, Arginine vasopressin induces the expression of c-*fos* in the mouse septum and hippocampus, Mol. Brain Res. 7, 131.

Hoffman, P.L., R.F. Ritzmann, R. Walter and B. Tabakoff, 1978, Arginine vasopressin maintains ethanol tolerance, Nature 276, 614.

Hoffman, P.L., H. Ishizawa, G. Szabó, J.R. Dave, L.-I. Liu and B. Tabakoff, 1989, Vasopressin and ethanol tolerance: postulated mechanism for neuroadaptation, in: Molecular Mechanisms of Alcohol, eds. G.Y. Sun, P.K. Rudeen, W. Gibson Wood, Y.-H. Wei and A.Y. Sun (Humana Press, New Jersey) p. 119.

Ishizawa, H., B. Tabakoff, I.N. Mefford and P.L. Hoffman, 1990, Reduction of arginine vasopressin binding sites in mouse lateral septum by treatment with 6-hydroxydopamine, Brain Res. 507, 189.

Johnson, A.E., S. Audigier, F. Rossi, S. Jard, E. Tribollet and C. Barberis, 1993, Localization and characterization of vasopressin binding sites in the rat brain using an iodinated linear AVP antagonist, Brain Res. 622, 9.

Junig, J.T., L.G. Abood and A.M. Skrobala, 1985, Two classes of arginine vasopressin binding sites on rat brain membranes, Neurochem. Res. 10, 1187.

Kalant, H., 1993, Problems in the search for mechanisms of tolerance, Alcohol Alcohol. Suppl. 2, 1.

Lança, A.J., W.J. Irving, P.H. Wu, H. Kalant and J.A. Saint-Cyr, 1993, Ascending projections from the dorsal (DR) and median raphe (MR) nuclei: possible functional implications with respect to ethanol tolerance, Soc. Neurosci. Abstr. 19, 1059.

Lê, A.D., H. Kalant and J.M. Khanna, 1982, Interaction between des-Gly$^9$-Arg$^8$]vasopressin and serotonin on ethanol tolerance, Eur. J. Pharmacol. 80, 337.

Le Moal, M., R. Dantzer, J. Mormede, S. Deyo, G.F. Koob and F.E. Bloom, 1984, Behavioral effects of peripheral administration of arginine vasopressin: a review of our search for a mode of action and hypothesis, Psychoneuroendocrinology 9, 319.

Morgan, J.I., D.R. Cohen, J.L. Hempstead and T. Curran, 1987, Mapping patterns of c-*fos* expression in the central nervous system after seizure, Science 237, 192.

Morgan, J.I. and T. Curran, 1991, Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*, Annu. Rev. Neurosci. 14, 421.

Phillips, P.A., J.M. Abrahams, J.M. Kelly, V. Mooser, D. Trinder and C.I. Johnston, 1988, Localization of vasopressin binding sites in rat tissues using specific $V_1$ and $V_2$ selective ligands, Endocrinology 126, 1478.

Raggenbass, M., E. Tribollet and J.J. Dreifuss, 1987, Electrophysiological and autoradiographical evidence of $V_1$ vasopressin receptors in the lateral septum of the rat brain, Proc. Natl. Acad. Sci. USA 84, 7778.

Robertson, H.A., 1993. Immediate early gene activation and long-term changes in neural function: a possible role in addiction? Activation of immediate early genes by drugs of abuse, NIDA Res. Monograph No. 125, eds. R. Grzanna and R. Brown (NIDA) p. 54.

Smeyne, R.J., K. Schilling, L. Robertson, D. Luk, J. Oberdick, T. Curran and J.I. Morgan, 1992, *Fos-lac*Z transgenic mice: mapping sites of gene induction in the central nervous system, Neuron 8, 13.

Smeyne, R.J., K. Schilling, J. Oberdick, L. Robertson, D. Luk, T. Curran and J.I. Morgan, 1993, A *fos-lac*Z transgenic mouse that can be used for neuroanatomic mapping, Adv. Neurol. 59, 285.

Sofroniew, M.V. and A. Weindl, 1978, Projections from the parvocellular vasopressin- and neurophysin-containing neurons of the suprachiasmatic nucleus, Am. J. Anat. 153, 391.

Speisky, M.B. and H. Kalant, 1985, Site of interaction of serotonin and desglycinamide-arginine-vasopressin in maintenance of ethanol tolerance, Brain Res. 326, 281.

Stephens, L.R. and S.D. Logan, 1986, Arginine vasopressin stimulates inositol phospholipid metabolism in rat hippocampus, J. Neurochem. 46, 649.

Szabó, G., B. Tabakoff and P.L. Hoffman, 1988, Receptors with $V_1$ characteristics mediate the maintenance of ethanol tolerance by vasopressin, J. Pharmacol. Exp. Ther. 247, 536.

Voorhuis, Th.A.M., E.R. De Kloet and D. De Wied, 1988, The distribution and plasticity of [$^3$H]vasopressin-labelled specific binding sites in the canary brain, Brain Res. 457, 148.

Wu, P.H., J.-F. Liu, S.J. Mihic and H. Kalant, 1992, Arginine-8-vasopressin potentiates acute ethanol intoxication, Pharmacol. Biochem. Behav. 41, 400.

Wu, P.H., J.-F. Liu, A.J. Lança and H. Kalant, 1994, Selective involvement of central 5-$HT_2$ receptors in the maintenance of tolerance to ethanol by arginine$^8$-vasopressin, J. Pharmacol. Exp. Ther. 270, 802.

Yamamura, H., K.W. Gee, R.E. Brinton, T.P. Davis, M. Hadley and J.K. Wamsley, 1983, Light microscopic autoradiographic visualization of [$^3$H]-arginine vasopressin binding sites in rat brain, Life Sci. 32, 1919.