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Structural Requirements for Neurohypophyseal Peptide Maintenance of Ethanol Tolerance

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HOFFMAN, P. L. *Structural requirements for neurohypophyseal peptide maintenance of ethanol tolerance*. PHARMAC. BIOCHEM. BEHAV. 17(4) 685–690, 1982.—Tolerance to the hypnotic effect of ethanol in mice is prolonged by daily subcutaneous administration of arginine vasopressin and certain analogs of this hormone. The major structural requirement for maintenance of ethanol tolerance by these peptides appears to be the N-terminal "ring" structure of vasopressin containing two amino acid residues with aromatic side chains. Peptides structurally related to the C-terminal portion of the neurohypophyseal hormones are less active in maintaining tolerance than the intact hormones. The structure-activity pattern observed for the effects of peptides on ethanol tolerance is similar to that described for neurohypophyseal peptide inhibition of extinction of an active avoidance response, an action thought to reflect peptide effects on memory consolidation. The results are in line with our hypothesis that similar CNS recognition sites may mediate neurohypophyseal peptide effects on ethanol tolerance and certain memory processes. The neurohypophyseal hormones and analogs did not affect the hypnotic or hypothermic response to an acute injection of ethanol, indicating that the determination of tolerance was not influenced by a direct peptide-ethanol interaction. The hormones themselves, however, did cause a drop in body temperature in the mice, which could be a result of either central or peripheral hormonal actions.

Alcohol Ethanol tolerance Neurohypophyseal peptides Vasopressin

ARGININE vasopressin (AVP), a mammalian antidiuretic hormone, has been demonstrated to prolong the duration of functional tolerance to ethanol beyond the time when such tolerance has dissipated in animals not treated with AVP [7,9]. As reported by us [8] and others [17], ethanol tolerance was also maintained by [des-9-glycinamide, 8-lysine]vasopressin (DGLVP), and [des-9-glycinamide, 8-arginine]vasopressin (DGAVP), vasopressin analogs which are devoid of [25], or have reduced [17], peripheral endocrinological activity. These findings suggested that vasopressin maintenance of ethanol tolerance is mediated via direct peptide action in the CNS.

Vasopressin, oxytocin (another neurohypophyseal hormone), some of their analogs and certain smaller, structurally-related peptides have also been demonstrated to have a number of other CNS effects. For example, many of these peptides inhibit the extinction of active avoidance responses and maintain passive avoidance responses, effects which have been interpreted as influences of the peptides on memory consolidation [16,28]. Some of the neurohypophyseal peptides also attenuate the amnesia caused by various agents [3, 12, 18, 24, 25], apparently by influencing retrieval or expression of information [26]. Although specific CNS binding sites have not yet been demonstrated for oxytocin or vasopressin (except for a preliminary report of such sites for the C-terminal tripeptide of oxytocin [4]), structure-activity studies of the CNS actions of these hormonal peptides have already provided an indirect characterization of putative CNS receptors which may mediate certain of the peptide effects [25,26].

In the present study, we have further examined the structural requirements for neurohypophyseal peptide maintenance of ethanol tolerance. These studies were undertaken in order to determine which characteristics of the peptides contribute to their interaction with the putative CNS receptors mediating effects on ethanol tolerance, and to compare the structural requirements for maintenance of ethanol tolerance with those for other CNS actions of the peptides.

In addition, we have investigated some of the acute physiologic effects of the neurohypophyseal peptides, as well as the influence of peptide treatment on the response of animals to acute alcohol administration.

METHOD

All peptides, with the exception of DGAVP, were synthesized in the Department of Physiology and Biophysics, University of Illinois Medical Center, in the laboratory of Dr. R. Walter, and were from the same batches used in previous studies [9, 24, 25]. AVP was bioassayed before use, and had approximately 400 U/mg of rat pressor activity [13]. DGAVP dicitrate (Organon 5459[d]) was a generous gift from Dr. J. Crabbe. The primary structure of the peptides used is shown in Fig. 1.

Male C57Bl/6J mice were used in all experiments. Mice were housed six to a cage under conditions of controlled

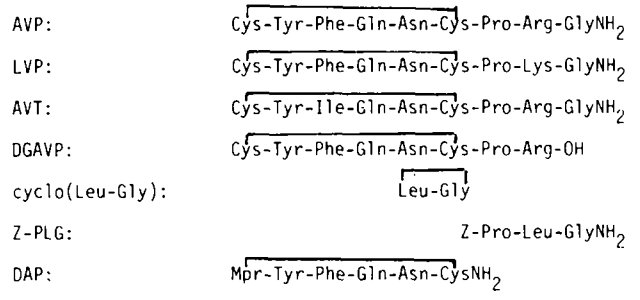

FIG. 1. Primary structure of neurohypophyseal hormones and related peptides used in the present experiments. Mpr=β-mercaptopropionic acid; Z=benzyloxycarbonyl protecting group.

temperature and lighting for at least one week prior to being used in an experiment. Chronic ethanol administration was by a liquid diet technique used previously in our laboratory [19]. Mice were acclimated for one day to a liquid diet containing Carnation Slender, vitamin supplement (3 g/l; ICN Corp., Cleveland, OH), and sucrose (96.8 g/l) (control diet). For the next seven days, control mice continued to receive the same diet, while ethanol-treated mice were given a diet in which ethanol (7%, v/v) equicalorically replaced the sucrose. The amount of diet offered to the control animals was adjusted daily to match the amount consumed by the ethanol-consuming mice. On the morning of the eighth day, all mice were again given control diet (i.e., the ethanol diet was withdrawn from ethanol-consuming mice). This treatment protocol produced functional ethanol tolerance and physical dependence (defined by the appearance of previously-described withdrawal symptomatology [19]) in the ethanol-treated mice.

Withdrawal symptoms were monitored at two-hour intervals for 12 hours following withdrawal [19]. At 24 hours after withdrawal, when overt withdrawal symptoms had dissipated, mice were tested for tolerance to the hypnotic effect of a test dose of ethanol [9,19]. Tolerance testing was carried out at 9:00 a.m., and tolerance was assessed by measuring the duration of loss of righting reflex ("sleep-time") following a dose of 3.1 g/kg of ethanol [9,19]. Following the initial test, groups of control and ethanol-withdrawn mice were subdivided into groups which received a subcutaneous injection of peptide or vehicle (saline) at 4:00–6:00 p.m. Most peptides were administered at a dose of 400 nmole/kg body weight, and in some instances, additional groups of animals were treated with a dose of 40 nmole/kg (doses calculated on the basis of free peptide). Cyclo(Leu-Gly) was administered at doses of 4, 400 or 4,000 nmole/kg. Mice continued to receive SC injections of peptide once daily (at 4:00 p.m.), and were also tested for tolerance on the mornings (9:00 a.m.) of days 3, 6 and 9 after withdrawal.

A given experiment included from two to four groups of ethanol-treated mice and corresponding groups of control mice (i.e., saline-treated and one, two or three peptide-treated groups). In general, there were six to ten ethanol-treated mice per group, and six control mice per group.

To measure the acute effect of peptides on body temperature, mice not previously exposed to ethanol were injected SC with various doses of peptide or saline. Rectal temperature was monitored by means of a Tele-Thermometer (Yellow Springs Instrument Co., Yellow Springs, OH), as described previously [19], prior to and at 30, 60, 90 and 120 minutes after peptide or saline injection. To determine the effect of the peptides on the response to acute ethanol treatment, ethanol (3.5 g/kg) was injected IP two hours after the peptide or saline injection, and body temperature was measured prior to and at 30, 60, 90 and 120 minutes after ethanol injection. In separate experiments, the effect of the peptides on the duration of loss of righting reflex following acute ethanol administration was determined, following the protocol described above. In all acute experiments, mice not previously exposed to ethanol were used.

Statistical significance was determined by analysis of variance and the Newman-Keuls test or Dunnett t-test. Values of $p<0.05$ were considered to be significant.

RESULTS

Mice which consumed the liquid diet containing ethanol for seven days became tolerant to ethanol's hypnotic effect [9,19]. Thus, at 24 hours after withdrawal, the ethanol-treated mice exhibited a significantly decreased "sleep-time," as compared to controls, in response to a challenge dose of ethanol (Figs. 2 and 3). Under our experimental conditions, tolerance to both the hypnotic and hypothermic effects of ethanol has been shown to dissipate over three to six days following withdrawal [9,19]. In the present study ethanol-exposed mice which received saline injections after withdrawal also gradually lost tolerance to the hypnotic effect of ethanol, and their responses were no longer significantly different from those of control mice within six to nine days after withdrawal (Fig. 2). However, when mice were treated daily with 400 nmole/kg of AVP, LVP or DGAVP, tolerance dissipated more slowly, and these animals were still tolerant to the hypnotic effect of ethanol on the ninth day after withdrawal (Fig. 2), at which time peptide treatment was ended. In certain experiments, the hypnotic response to ethanol of the peptide-treated mice was determined six days following cessation of peptide treatment. Tolerance had dissipated at this time.

A comparison of the effects of AVP, LVP and DGAVP with the effects of other peptides on ethanol tolerance is shown in Fig. 3. In contrast to AVP, LVP and DGAVP, the other hormone analog tested, arginine vasotocin ([Ile^3, Arg^8]vasopressin; AVT), did not maintain ethanol tolerance. Similarly, ethanol-withdrawn animals treated with deamino pressinamide (DAP) (an analog of the "ring" structure of vasopressin; see Fig. 1), or N-benzyloxycarbonyl-Pro-Leu-Gly-NH_2 (Z-PLG, a derivative of the C-terminal tripeptide of oxytocin) did not exhibit tolerance to ethanol on the ninth day after withdrawal (Fig. 3). Thus, these smaller peptides, like AVT, did not prolong ethanol tolerance. Cyclo (Leu-Gly), the cyclized derivative of the C-terminal dipeptide of oxytocin, did maintain ethanol tolerance, but only at the highest dose tested (4,000 nmole/kg; Fig. 3). This peptide was inactive at lower doses ($\leq$400 nmole/kg) [8].

The peptides which maintained ethanol tolerance when given at a dose of 400 nmole/kg (i.e., AVP, LVP, DGAVP) were also active at a dose of 40 nmole/kg (data not shown). It should be noted that none of the peptides used affected the response of control animals to ethanol (Fig. 2), when ethanol was administered 17 hours after peptide treatment.

In a preliminary study, LVP was not found to maintain ethanol tolerance [10]. In the present study, a different batch of hormone was used, one that had rat pressor activity [13] of

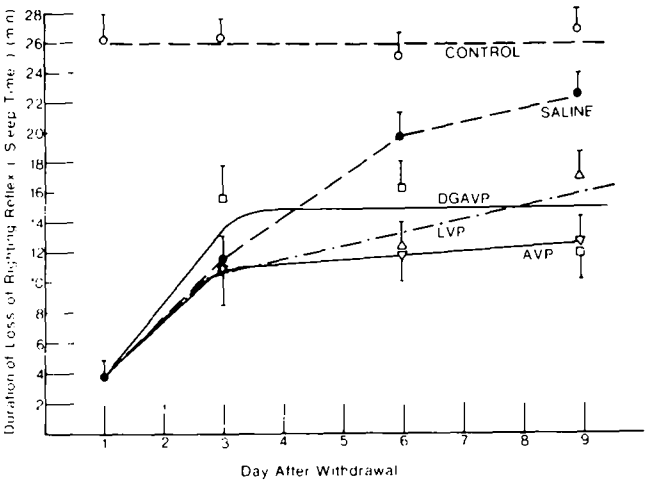

FIG. 2. Effects of AVP, LVP and DGAVP on rate of dissipation of ethanol tolerance. Mice were fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for seven days. Tolerance to ethanol was determined by measuring the duration of loss of righting reflex ("sleep-time") following a challenge dose of ethanol (3.1 g/kg). Animals received the indicated peptide or saline once daily, beginning on the day after ethanol withdrawal, as described in the text. Values represent the mean±SEM of sleep-times of control mice (○) and of ethanol-exposed mice treated with saline (●), AVP (▽), LVP (△) or DGAVP (□). The data in this figure are taken from two experiments in which the effects of all of these peptides were determined concomitantly. Sleep-times on the first day after withdrawal were obtained prior to peptide treatment. Sleep-times of saline- and peptide-treated control animals did not differ significantly at any time, and data for control animals were therefore pooled. The response of control animals did not vary significantly over days. All groups of ethanol-treated animals gradually lost tolerance (comparison of responses over days: saline-treated, $F(3,75)=33.44$; AVP-treated, $F(3,47)=7.80$; LVP-treated, $F(3,55)=21.40$; DGAVP-treated, $F(3,49)=24.78$; $p<0.01$ for all groups). However, the peptide-treated groups lost tolerance more slowly than the saline-treated group. All ethanol-treated groups were tolerant on day 3, as compared to controls ($p<0.01$, Newman-Keuls). On day 6, ethanol-exposed, saline-treated mice were less tolerant than AVP-treated and LVP-treated mice ($p<0.01$), Newman-Keuls). On day 9, ethanol-exposed, saline-treated mice were no longer tolerant (response not significantly different from that of controls; Newman-Keuls), while all groups of peptide-treated mice remained tolerant ($p<0.01$, Newman-Keuls). Abbreviations: AVP, [8-arginine]vasopressin; LVP, [8-lysine]vasopressin; DGAVP, [des-9-glycinamide, 8-arginine]vasopressin.

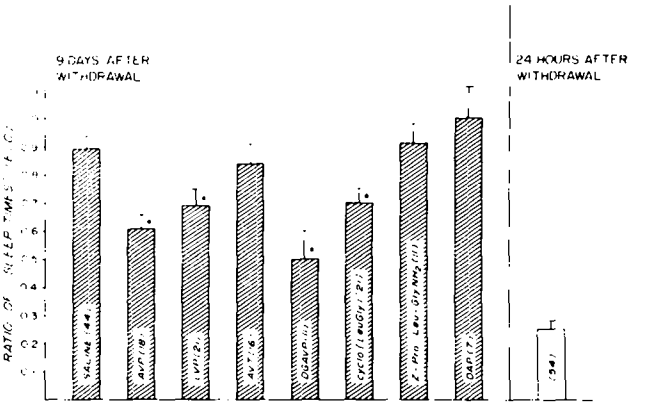

FIG. 3. Effect of neurohypophyseal hormones, analogs and related peptides on maintenance of ethanol tolerance in C57Bl mice. Treatment of mice with ethanol-containing diet (E) and control diet (C), and peptide administration, are described in the text and legend to Fig. 2. The dose of all peptides except cyclo(Leu-Gly) was 400 nmole/kg. The dose of cyclo(Leu-Gly) was 4,000 nmole/kg. Values are the mean±SEM of the ratios of "sleep-time" in ethanol-treated mice and their matched controls. When the ratio for ethanol-fed mice is less than 1.0, these animals are exhibiting tolerance to ethanol. This ratio approaches 1.0 as tolerance is lost. Number in parentheses is number of ethanol-fed animals tested. $*p<0.05$, peptide-treated compared to saline-treated groups (analysis of variance: $F(7,147)=4.98$ and Dunnett t-test). Abbreviations: AVP, [8-arginine]vasopressin; LVP, [8-lysine]vasopressin; AVT, [8-arginine] vasotocin, [Ile^3,Arg^8]vasopressin; DGAVP, [des-9-glycinamide, 8-arginine]vasopressin; DAP, deamino pressinamide; Z, benzyloxycarbonyl protecting group.

240 U/mg. Differences in activity were also noted between different batches of DGAVP. These results emphasize the fact that hormones and peptides used in behavioral studies must be thoroughly characterized and, when possible, bioassayed.

The acute response to the neurohypophyseal peptides, and peptide effects on responses to acute ethanol administration, are shown in Table 1. In each instance, the response to the peptide is compared to the response to saline administered in the same experiment. Subcutaneous injections of AVT or LVP resulted in dose-dependent drops in body temperature, which were not seen following injection of the other peptides. A similar hypothermic response to AVP had previously been reported [8]. This drop in body temperature was maximal at 30 minutes after peptide treatment, and body temperature had returned to control values by 120 minutes after peptide treatment. When ethanol was administered two hours after peptide treatment, none of the peptides significantly affected the hypnotic or hypothermic responses to the acute ethanol injection.

DISCUSSION

As is the case for other CNS effects of neurohypophyseal peptides related to vasopressin [25,26], distinct structural requirements exist for peptide maintenance of tolerance to the hypnotic effects of ethanol. The most important structural characteristic for this activity appears to be the presence of two amino acid residues with aromatic side chains within the N-terminal ("ring") portion of the peptides. This N-terminal structure is characteristic of vasopressins (see Fig. 1). Thus, AVT, which contains an arginine residue in its linear C-terminal portion, but has the oxytocin "ring" structure as its N-terminal portion, was inactive in maintaining tolerance. Similarly, in previous studies, oxytocin was found to be ineffective in maintaining alcohol tolerance [7,9]. In general, our results resemble those found both for peptide inhibition of extinction of an active avoidance response and for peptide attenuation of puromycin-induced amnesia [25,26]. The major difference between the structural requirements for these two peptide effects on memory was that small peptides, structurally related to the C-terminal portions of the neurohypophyseal hormones, were active in at-

TABLE 1

EFFECTS OF NEUROHYPOPHYSEAL PEPTIDES ON THE ACUTE RESPONSE TO ETHANOL

	Peptide Alone	Peptide and Ethanol	
	Δt 30' (°C)	"Sleep-Time" (min)	Max Δt (°C)
Saline	+0.01 ± 0.1	129 ± 2	−1.9 ± 0.2
LVP (400 nmole/kg)	−2.7 ± 0.2*	132 ± 19	−2.4 ± 0.2
LVP (40 nmole/kg)	−1.9 ± 0.2*	118 ± 20	−2.2 ± 0.2
LVP (4 nmole/kg)	−0.8 ± 0.2*	172 ± 27	−2.3 ± 0.2
Saline	+0.2 ± 0.1	112 ± 16	−1.7 ± 0.2
AVT (400 nmole/kg)	−2.5 ± 0.2*	98 ± 13	−2.2 ± 0.2
AVT (40 nmole/kg)	−1.3 ± 0.1*	100 ± 22	−2.2 ± 0.2
AVT (4 nmole/kg)	0.3 ± 0.1†	96 ± 5	−2.2 ± 0.2
Saline	−0.1 ± 0.1	92 ± 6	−1.9 ± 0.3
DGAVP (400 nmole/kg)	−0.2 ± 0.1	82 ± 7	−1.7 ± 0.2
DGAVP (40 nmole/kg)	−0.2 ± 0.1	97 ± 11	−2.5 ± 0.3
DGAVP (4 nmole/kg)	+0.3 ± 0.1	88 ± 9	−2.2 ± 0.2
Saline	−0.3 ± 0.1	98 ± 11	−1.9 ± 0.6
Z-PLG (400 nmole/kg)	+0.03 ± 0.1	103 ± 10	−2.0 ± 0.2
Z-PLG (40 nmole/kg)	+0.1 ± 0.1	118 ± 5	−2.1 ± 0.2
Z-PLG (4 nmole/kg)	−0.2 ± 0.1	100 ± 8	−2.1 ± 0.2
Saline	+0.2 ± 0.1	123 ± 4	−1.9 ± 0.2
DAP (400 nmole/kg)	+0.1 ± 0.1	124 ± 7	−2.4 ± 0.2
DAP (40 nmole/kg)	−0.04 ± 0.1	129 ± 15	−2.5 ± 0.3
DAP (4 nmole/kg)	+0.2 ± 0.1	123 ± 7	−2.2 ± 0.2

Peptides or saline were administered two hours before ethanol (3.5 g/kg). Duration of loss of righting reflex and rectal temperature were measured as described previously [19]. The maximum change in body temperature after peptide treatment occurred at 30 minutes following injection. The response to the peptides was compared to the response to saline which was obtained during the same experiment. There were 10–20 animals per group. Values represent mean ± SEM.

*$p<0.01$; †$p<0.05$ peptide-treated animals compared to saline-treated animals within each group (Analysis of variance and Newman-Keuls test; for LVP, $F(3,66)=36.9$; for AVT, $F(3,83)=56.38$.)

tenuating amnesia, but were less active or inactive in inhibiting extinction of avoidance behavior [25,26]. In this respect, the structure-activity profile for peptide maintenance of ethanol tolerance resembles that for inhibition of extinction of an active avoidance response. Thus, Z-Pro-Leu-Gly-NH_2, a peptide which is structurally similar to the C-terminal tripeptide of oxytocin, had no detectable effect on ethanol tolerance. In contrast, Z-Pro-Leu-Gly-NH_2 was even more active than AVP in attenuating puromycin-induced amnesia [6,24]. Cyclo(Leu-Gly) was found to maintain ethanol tolerance only at the highest dose administered. Similarly, this peptide was much less potent (about 15-fold) than AVP in inhibiting extinction of active avoidance behavior, but did show activity [26]. Cyclo(Leu-Gly) was relatively more potent in attenuating puromycin-induced amnesia [6,24].

Because the N-terminal portion of vasopressin was necessary for the maintenance of ethanol tolerance, an analog of the N-terminal "ring" of vasopressin was also tested. Deamino pressinamide (DAP; see Fig. 1), was inactive in maintaining ethanol tolerance (Fig. 3). It is of interest that pressinamide, although inactive when administered SC, did inhibit extinction of an active avoidance response when it was administered intraventricularly [27]. Similarly, the C-terminal tripeptide of arginine vasopressin (Pro-Arg-Gly-NH_2) was active when administered centrally, and inactive when given subcutaneously [27]. It is possible that these peptides are rapidly metabolized in the periphery, or do not easily enter brain following peripheral administration. Deamino pressinamide, as well as the derivatives of the C-terminal portion of oxytocin that were tested in our studies, might maintain ethanol tolerance in a paradigm in which they could be administered centrally.

On the other hand, there is strong evidence that the route of administration is not the major determinant of peptide effects on ethanol tolerance or on memory-related phenomena. Cyclo(Leu-Gly), for example, has been detected in its intact state in mouse brain following subcutaneous injection, and the concentration of this peptide in the synaptosomal fraction correlated positively with its potency in attenuating puromycin-induced amnesia [14]. Furthermore, peptides related to the C-terminal structure of oxytocin and vasopressin were very active in blocking

puromycin-induced amnesia in mice following peripheral injection [6, 24, 25]. Since this activity also appears to be mediated centrally [25], it may be concluded that these peptides do, in fact, reach the brain in high enough concentrations to exert behavioral effects following subcutaneous injection.

Therefore, differences in response to these neurohypophyseal hormones and analogs cannot be attributed purely to differences in uptake and/or metabolism following systemic administration, although these factors may play a role. Instead, it seems likely that the entire vasopressin molecule is important for maintenance of ethanol tolerance, as well as for inhibition of extinction of an active avoidance response. In this respect, it should be noted that, for this latter activity, both pressinamide and Pro-Arg-Gly-NH_2 were less active than AVP, even when they were administered centrally [27]. The total vasopressin structure is also important for attenuation of puromycin-induced amnesia [25]. However, CNS pathways which are also sensitive to the C-terminal peptides or their derivatives appear to influence this response.

In general, our results are in line with the hypothesis that ethanol tolerance shares certain underlying mechanisms with memory-related processes, as assessed by the response to the neurohypophyseal peptides. In particular, the maintenance of tolerance and the maintenance of a conditioned avoidance response may be mediated by certain common pathways, and neurohypophyseal peptide effects on these processes may occur, in part, through the same or similar recognition sites. However, it is likely that all of the behavioral effects being considered are influenced in a complex manner by many neuronal pathways, each of which may be differentially sensitive to various neurohypophyseal peptides.

The influence of vasopressin and related peptides on the acute response to ethanol was investigated in order to determine whether direct peptide-ethanol interactions might contribute to the observed effects on tolerance. We have examined peptide effects on the hypothermic response to acute ethanol administration, as well as on ethanol-induced hypnosis. We previously showed that AVP and cyclo(Leu-Gly), at a dose of 400 nmole/kg, potentiated the hypnotic effect of an acute dose of ethanol administered two hours after peptide treatment [8]. Since tolerance to ethanol involves a reduced "sleep-time" response, it was concluded that such direct interaction did not mask the effects of AVP on tolerance [8]. None of the other peptides tested in the present study significantly affected the hypnotic or hypothermic response to an acute dose of ethanol administered two hours after the peptides. It should also be reiterated that, during chronic experiments, ethanol was administered 17 hours after peptide treatment. Thus, these peptides had little influence on the tested acute responses to ethanol, and the interactions that did occur did not interfere with the evaluation of peptide effects on tolerance.

The neurohypophyseal hormones LVP and AVT, in addition to AVP [8], did cause a short-lived drop in body temperature in mice. This drop was dose-dependent (AVP data not shown). The fact that DGAVP did not cause a significant drop in body temperature suggests that the change in body temperature may involve a peripheral response to the hormones. In rats, however, AVP was previously shown to cause hypothermia following intraventricular, as well as peripheral, injection [5,11].

In summary, our results suggest that the maintenance of functional tolerance to ethanol involves modulation of a CNS adaptive response by the tested peptides, and that this peptide action may be mediated by neuronal recognition sites which are relatively specific for vasopressin. Vasopressin has been shown to have both electrophysiological [1,2] and biochemical effects in the CNS [15,23]. In addition, recent studies indicate the widespread distribution of this peptide in brain areas other than the hypothalamus [20–22]. Thus, vasopressin may well be found to be an endogenous regulator or modulator of a number of CNS adaptive processes.

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