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Neurohypophyseal Peptide Influences On Ethanol Tolerance and Acute Effects of Ethanol[1]

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HOFFMAN, P. L., R. F. RITZMANN AND B. TABAKOFF. *Neurohypophyseal peptide influences on ethanol tolerance and acute effects of ethanol.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 279–284, 1980.—The neurohypophyseal hormone, arginine vasopressin (AVP), was previously shown to prolong the duration of ethanol tolerance in mice. Since drug tolerance and certain memory-related processes are examples of CNS adaptation, these phenomena have been proposed to share underlying mechanisms. We investigated the effects on ethanol tolerance of two other neurohypophyseal peptides, both of which modulate memory consolidation or retrieval of information. (Des-9-glycinamide, 8-lysine) vasopressin (DGLVP), like AVP, maintained ethanol tolerance in C57Bl mice, while cyclo(Leu-Gly) (cLG), at an equimolar dose, was ineffective. Thus, various neurohypophyseal peptides may differentially influence CNS adaptive phenomena. Direct peptide effects on ethanol-induced hypothermia and "sleep time," the parameters used to evaluate ethanol tolerance, were also determined. AVP per se caused hypothermia in mice, but neither AVP nor cLG affected ethanol-induced hypothermia. Both peptides, however, increased "sleep time" after acute ethanol administration. Although these direct peptide-ethanol interactions do not account for the observed peptide effects on tolerance, the findings emphasize the importance of using several parameters to assess ethanol tolerance.

Ethanol tolerance Neurohypophyseal hormones Neurohypophyseal peptides Vasopressin
Chronic ethanol Acute ethanol Cyclo(Leu-Gly)

THE neurohypophyseal hormones, (8-arginine) vasopressin (AVP) and (8-lysine) vasopressin (LVP), can inhibit extinction of active and passive avoidance responses in rats [18,30] and can attenuate the amnesia caused by a number of agents in mice and rats [1, 16, 20, 27]. AVP has also been reported to attenuate amnesia in humans [11]. These actions are generally regarded as hormonal influences on processes of memory consolidation, or retrieval or expression of information [18, 27, 29, 30]. The effects of peptides on such processes appear to be centrally mediated [30, 31, 33].

The findings that rats genetically deficient in vasopressin show an enhanced rate of extinction of avoidance responses [32], and that intraventricular injection of vasopressin antiserum also facilitates such extinction [33], support the contention that the endogenous hormones may influence "memory," The neurohypophyseal hormones are synthesized in neuronal cell bodies in the hypothalamus [24,26]. In addition to being transported to nerve terminals in the posterior pituitary, the hormones have been localized to neuronal pathways terminating in various areas of the CNS [2, 3, 23]. Thus neurohypophyseal hormones may be released directly in the brain to modify memory and/or behavior.

Recently, both neurohypophyseal hormones and various C-terminal hormone fragments and structurally-related peptides (some of which modify amnesia, as well as extinction of avoidance responses [27,29]) have also been reported to modulate the development of morphine tolerance and dependence [19,28]. In addition, AVP was found to maintain tolerance to ethanol in mice once such tolerance had developed [6]. The results of the studies on drug tolerance are in line with the proposal that the neurohypophyseal peptides can influence the general mechanisms underlying CNS adaptive processes, since both learning and/or memory, and drug tolerance, have been regarded as adaptive responses of the CNS to perturbation by external stimuli [10].

One way to further investigate this hypothesis is to determine whether various peptides affect memory-related processes with the same relative potency with which they modulate tolerance. Structure-activity studies have been

[1]Abbreviations: AVP: arginine vasopressin, [8-arginine] vasopressin; DGLVP: [des-9-glycinamide, 8-lysine] vasopressin; cLG: cyclo(Leu-Gly).

carried out for the actions of neurohypophyseal hormones, analogs and fragments on puromycin-induced amnesia [27], inhibition of extinction of an active avoidance response [29] and, to some extent, development of morphine tolerance [28].

In the present study, we have evaluated the ability of two peptides which have been found to modulate memory processes, (des-9-glycinamide, 8-lysine) vasopressin (DGLVP) and cyclo(Leu-Gly), to maintain ethanol tolerance. DGLVP is an analog of vasopressin with little peripheral activity, but which is quite active behaviorally [27,31]. Cyclo(Leu-Gly), the cyclic derivative of the C-terminal dipeptide of oxytocin, is active in processes related to memory [27,29] and has also been shown to block the development of morphine tolerance [28].

The presence of ethanol tolerance following chronic exposure of mice to ethanol was determined, in the present and previous [6] work, by measuring hypothermia and duration of loss of righting reflex following a challenge dose of ethanol [21]. We, therefore, also wished to assess the direct influences of neurohypophyseal peptides on these parameters, to determine if the peptides could influence the *expression*, as well as development or maintenance of tolerance.

METHOD

(8-Arginine) vasopressin (AVP) was synthesized in the laboratory of Dr. R. Walter, and had rat pressor activity [15] of 450 U/mg. (Des-9-glycinamide, 8-lysine) vasopressin (DGLVP) was from the synthetic material used in an earlier study [27]. Cyclo(Leu-Gly) was also synthesized in the laboratory of Dr. R. Walter, and its properties have been previously described [7]. In the chronic ethanol experiments, the dose of AVP and cyclo(Leu-Gly) was 40 nmole/kg. For the acute experiments, the dose was 0.4 μmole/kg. Peptides were dissolved in saline, and AVP was freshly dissolved each day, immediately prior to use.

Male C57B1/6J mice purchased from Sprague-Dawley, Inc. (Madison, WI) were used for all experiments. Mice were housed six to a cage, under conditions of controlled temperature and lighting, for at least one week prior to being used in an experiment [21]. Two methods of chronic ethanol administration were used. For the experiments with AVP and cyclo(Leu-Gly), mice were acclimated for one day to a liquid diet containing Carnation Slender, vitamin supplement (ICN Corporation, Cleveland, OH: 3 g/l) and sucrose (96.8 g/l) (control diet) [21]. For the next seven days, they were either given diet containing 7% ethanol in place of the sucrose (Ethanol), or were pair-fed the control diet (Control). On the morning of the eighth day, all mice were again given the control diet (withdrawal) [21]. This treatment produced tolerance and physical dependence in the animals, the latter defined by the appearance of withdrawal symptomatology [21].

Following withdrawal, groups of control and ethanol-consuming animals were divided into subgroups which were to received either peptide (Control-Peptide, Ethanol-Peptide) or saline injections (Control-Saline, Ethanol-Saline). In our earlier study using AVP [6], tolerance testing was carried out at 24 hours after withdrawal, when overt withdrawal signs had disappeared, and at three-day intervals thereafter, up to 18 days after withdrawal. AVP, given once daily, starting on the day after withdrawal, maintained ethanol tolerance for as long as the AVP was administered (i.e., nine days) [6]. Ethanol-consuming animals which received saline in place of AVP showed a half-life of tolerance disappearance of about three days [6,21]. Therefore, in the present experiments with AVP and cyclo(Leu-Gly), the presence of tolerance in the ethanol-consuming animals was first evaluated at 24 hours after withdrawal. Starting on the day after withdrawal, peptides or saline were injected subcutaneously, once daily (at approximately 1600 hours) [6]. Peptide or saline administration continued for seven days, during which time the animals received lab chow and water ad lib. On the eighth day after withdrawal, the animals were again tested for tolerance.

Tolerance to ethanol was determined as previously described [21], by measuring the change in body temperature and duration of loss of righting reflex ("sleep time") after an IP injection of 3.5 g/kg of ethanol. In some experiments, animals were sacrificed at the time of regain of righting reflex on the last day of tolerance testing and brain ethanol levels were determined by a gas chromatographic method [25].

For the experiments in which the effects of DGLVP were compared to those of AVP, chronic ethanol exposure was carried out using an injection technique. Mice received an IP injection of 4.2 g/kg of ethanol in the morning, and were given ethanol (5 g/kg) in liquid diet by gavage in the evening. This treatment was continued for five days and, on the sixth day, mice were tested as described [21] for tolerance to the sedative and hypothermic actions of ethanol. The test dose of ethanol was 4.2 g/kg. DGLVP and AVP (40 nmol/kg) or saline were administered subcutaneously in the evening of days 7 through 13 of the experiment (no ethanol was given during this time) and the animals were again tested for tolerance on Day 14. At each time-point that ethanol tolerance was determined, the responses of ethanol-treated animals were compared to those of saline or peptide-treated animals which had not received the chronic ethanol injections.

The effect of peptides on the response to acute ethanol treatment was determined by injecting the animals subcutaneously with AVP or cyclo(Leu-Gly) two hours prior to IP injection of 3.5 g/kg of ethanol. Hypothermia and duration of loss of righting reflex were determined as described [21].

All values are reported as mean ± SEM. Significance was determined by the use of Student's *t*-test; $p<0.05$ was considered significant.

RESULTS

As previosuly shown, C57B1 mice exposed for seven days to a liquid diet containing 7% ethanol became physically dependent on, as well as tolerant to, ethanol [21]. While overt withdrawal signs had disappeared by 24 hours following withdrawal, tolerance to the hypothermic and sedative effects of ethanol disappeared with a half-life of about three days [21]. Thus, when animals received saline injections for seven days after withdrawal of the liquid diet containing ethanol, tolerance was no longer evident on the eighth day (Fig. 1). However, animals which received 40 nmole/kg of AVP daily for seven days after withdrawal, remained tolerant to ethanol when tested eight days after withdrawal. Animals treated with cyclo(Leu-Gly), on the other hand, were no longer tolerant on the eighth day; i.e., their hypothermic (Fig. 1A) and sedative (Fig. 1B) responses to 3.5 g/kg of ethanol were similar to those of ethanol-treated mice which had received daily saline injections.

Animals exposed chronically to ethanol by the injection

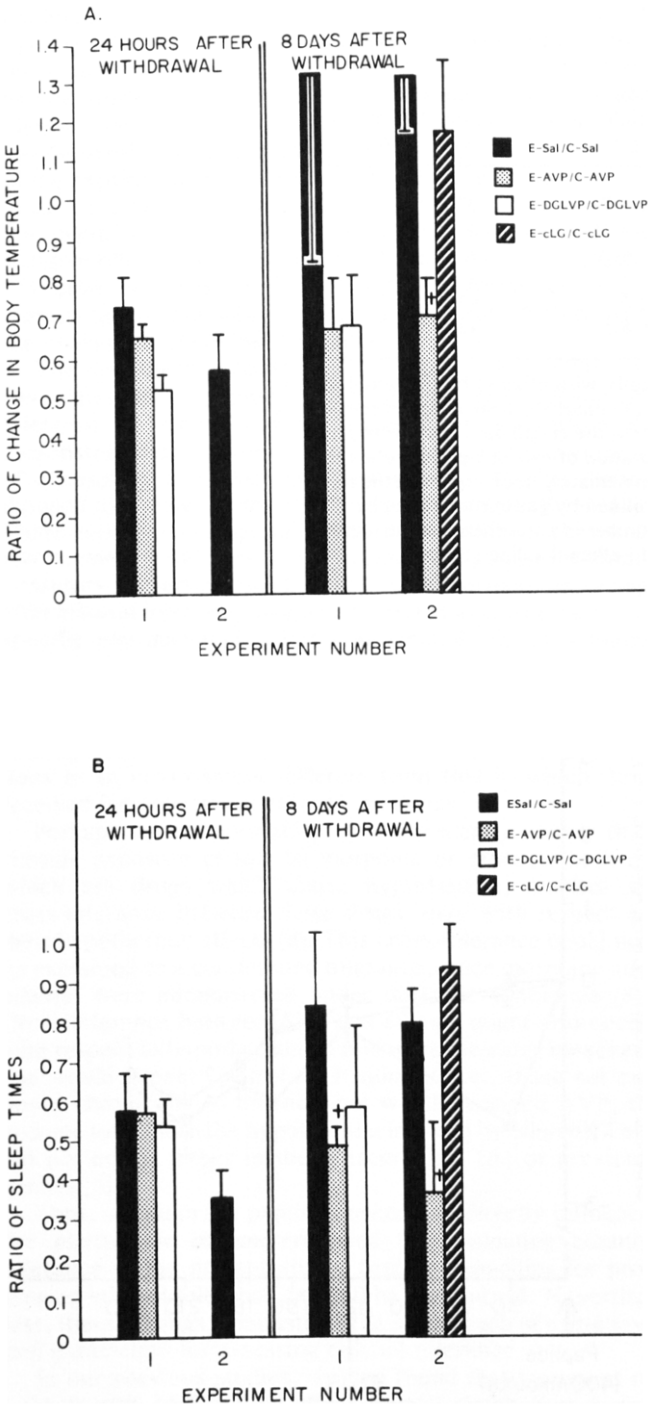

FIG. 1. Effect of AVP, DGLVP and cyclo(Leu-Cly) on maintenance of ethanol tolerance in C57B1 mice. The results of each experiment represent the means of three to five separate determinations carried out using the same method. In Experiment No. 1, animals were treated chronically with ethanol by the injection technqiue and in Experiment No. 2, the liquid diet method was used. The time after withdrawal represents either the time after withdrawal of animals from the liquid diet containing ethanol, or time after the last ethanol administration. Tolerance to ethanol was measured as described in the text, as the maximum change in body temperature (A) or duration of loss of righting reflex ("sleep time") (B) following a challenge dose of 3.5 g/kg (Experiment No. 1) or 4.2 g/kg (Experiment No. 2) of ethanol. Peptides were administered SC in saline, as described in the text; the dose was 40 nmole/kg. Values are the *ratios* of temperature drop and sleep time of ethanol-treated animals and appropriate controls. Ethanol-tolerant mice show a ratio of <1.0, and this ratio approaches 1.0 as tolerance is lost. $*p<0.01$; $\dagger p<0.05$ (Student's *t*-test; peptide-treated groups compared to saline-treated groups). n=six to 12 animals in each group. *Abbreviations:* cLG=cyclo(Leu-Gly); C-Sal=Control-Saline and C-Peptide=Control Peptide groups; E-Sal=Ethanol-Saline and E-Peptide=Ethanol Peptide groups.

technique were also tolerant to the sedative and hypothermic effects of ethanol on the day following the last ethanol injection (Fig. 1). When these animals were tested after seven more days, during which time they had received only saline or peptide injections, the saline-treated animals were no longer tolerant, while those receiving AVP or DGLVP still showed reduced responses to the challenge dose of ethanol. AVP was the more potent peptide for maintaining tolerance (Figs. 1A and B).

In certain experiments, brain ethanol levels were measured at the time of regain of the righting reflex following a challenge dose of ethanol on the eighth day after withdrawal from the liquid diet. Animals treated with AVP were found to regain the righting reflex at significantly higher brain ethanol levels, as compared to saline-treated animals (Table 1). Ethanol levels in brains of cyclo(Leu-Gly)-treated animals were slightly lower than those in controls at this time (Table 1).

During these studies, it was observed that animals treated chronically with cyclo(Leu-Gly), whether or not they received chronic ethanol treatment, often displayed an increased "sleep time" in response to the challenge dose of ethanol, as compared to chronically ethanol-treated or control animals which had received saline instead of peptide (Table 2). In order to determine whether direct peptide-ethanol interactions could account for this phenomenon, and to evaluate peptide effects on hypothermia, the other parameter used to measure tolerance, peptides were administered two hours prior to an acute injection of ethanol. Table 3 shows that treatment with both AVP and cyclo(Leu-Gly) increased the "sleep time" in response to ethanol, although the increase was only statistically significant in the case of cyclo(Leu-Gly). AVP, but not cyclo(Leu-Gly), by itself caused a drop in body temperature in C57B1 mice (Fig. 2). By two hours following peptide injection, body temperature had returned to normal, and neither peptide affected the hypothermia subsequent to injection of 3.5 g/kg of ethanol (Fig. 2).

DISCUSSION

In order to demonstrate that a given treatment modulates the development or maintenance of tolerance to ethanol, or to any drug, it is necessary to use a number of measures to assess tolerance, and also to determine whether the treatment in question interferes with the parameters used to measure tolerance, rather than with the tolerance itself. In other words, the treatment may affect the animal's ability to *express* tolerance, instead of affecting the mechanisms underlying tolerance development or maintenance. Both AVP and cyclo(Leu-Gly), given prior to an acute dose of ethanol,

TABLE 1

EFFECT OF NEUROHYPOPHYSEAL PEPTIDES ON ETHANOL TOLERANCE*

Group	Duration of loss of righting reflex (min)	Brain ethanol at regain of righting reflex (mM)
Ethanol-saline (6)	30.0 ± 4.7	42.4 ± 2.5
Ethanol-AVP (6)	10.8 ± 5.6†	49.2 ± 1.2†
Ethanol-cyclo(Leu-Gly) (5)	67.0 ± 16.3†	40.7 ± 5.4
Control-saline (7)	29.7 ± 5.0	44.2 ± 2.1
Control-cyclo(Leu-Gly) (4)	44.2 ± 8.5	43.5 ± 4.7

*Male C57Bl mice were treated chronically with ethanol by the liquid diet method as described in the text. Peptides (40 nmol/kg) were administered SC daily for seven days following withdrawal. On the eighth day, mice were given a challenge dose of 3.5 g/kg of ethanol, duration of loss of righting reflex was determined, and animals were sacrificed immediately upon regaining the righting reflex. Brain ethanol levels were determined by gas chromatography [25]. Values represent mean ± SEM, and the number in parentheses is the number of animals per group. †$p<0.05$ compared to ethanol-saline group.

TABLE 2

EFFECT OF CYCLO(LEU-GLY) ON DURATION OF ETHANOL-INDUCED LOSS OF RIGHTING REFLEX IN MICE TREATED CHRONICALLY WITH ETHANOL*

Group (n)	Duration of loss of righting reflex (min)
Ethanol-saline (15)	39.8 ± 13.9
Ethanol-cyclo(Leu-Gly) (15)	57.4 ± 6.3†
Control-saline (24)	49.1 ± 3.9
Control-cyclo(Leu-Gly) (23)	63.8 ± 3.4†

*Duration of loss of righting reflex after a challenge dose of 3.5 g/kg of ethanol was determined on the eighth day after withdrawal of mice from chronic ethanol treatment by the liquid diet method (12–18 hours after the last peptide administration). See text for details. Values represent mean ± SEM of three experiments. †$p<0.05$ for cyclo(Leu-Gly)-treated animals compared to appropriate saline-treated group.

TABLE 3

EFFECT OF CYCLO(LEU-GLY) AND AVP ON DURATION OF LOSS OF RIGHTING REFLEX PRODUCED BY ACUTE ETHANOL TREATMENT*

Pre-treatment	Duration of loss of righting reflex (min)
Saline (8)	63.8 ± 5.0
AVP (10)	89.2 ± 13.7
cyclo(Leu-Gly) (9)	97.0 ± 10.0†

*Male C57Bl mice were injected SC with AVP or cyclo(Leu-Gly) (0.4 μmol/kg) two hours prior to injection of ethanol (3.5 g/kg, IP). Duration of loss of righting reflex was measured as described [21]. Values represent mean ± SEM of three experiments. The number in parentheses is the number of animals per group. †$p<0.02$ compared to saline-treated group.

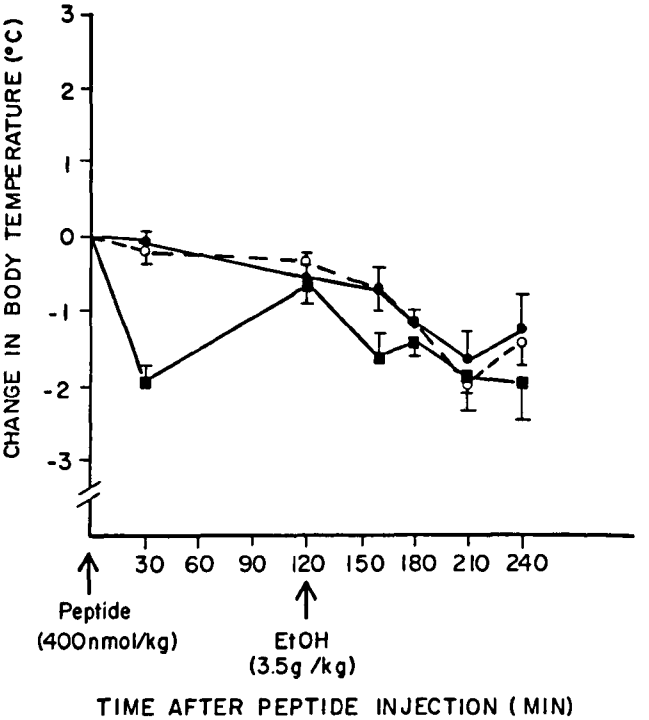

FIG. 2. Effect of neurohypophyseal peptides on the hypothermic response to acute ethanol administration. Male C57Bl mice were injected SC with AVP (0.4 μmole/kg), cyclo(Leu-Gly) (0.4 μmol/kg) or saline, two hours prior to treatment with 3.5 g/kg of ethanol. Body temperature was monitored as described [21] at the indicated times following peptide and ethanol injection.

produced an *increased* duration of ethanol-induced loss of righting reflex (Table 3). Thus, this parameter might not represent an accurate evaluation of ethanol tolerance following peptide treatment. However, since ethanol-tolerant animals display a *decreased* duration of loss of righting reflex in response to ethanol, as compared to controls, the consequence of the peptide-ethanol interaction in this case would, if anything, be a masking of the presence of ethanol tolerance in the peptide-treated animals. However, tolerance to the sedative effect of ethanol was prolonged by AVP (Fig. 1B). Therefore, one may conclude that this particular peptide influence on ethanol-induced sedation does not interfere with the evaluation of ethanol tolerance.

The hypothermia caused by AVP might be expected to have additional confounding effects on measurement of tolerance to ethanol-induced hypothermia. It has been demonstrated that animals which receive repeated injections of ethanol in a distinct environment can develop "conditioned tolerance" to the hypothermic effects of ethanol [9]. These animals will respond to placebo injections in the same environment with an *increase* in body temperature ("compensatory hyperthermia"). Such hyperthermia, occurring after ethanol injection, may be interpreted as tolerance to the specific pharmacologic effects of ethanol, but is actually non-specific conditioned tolerance to hypothermia.

It is conceivable that a similar adaptation to the hypothermic effect produced by AVP injections might occur, although our animals were given subcutaneous AVP injections in an environment different from that in which they received intraperitoneal ethanol injections.

Perhaps more importantly, it was recently shown that chronic exposure of rats to morphine or ethanol, both of which are drugs which cause hypothermia, resulted in cross-tolerance between these drugs, *only* with respect to their hypothermic effects [8]. This cross-tolerance could not be explained as a conditioned tolerance, since morphine and ethanol were administered under different conditions [8]. Cross-tolerance between AVP and ethanol might also occur with respect to hypothermia. If this were the case, however, one would expect Control-AVP animals, i.e., those not exposed chronically to ethanol, but which received AVP, to become tolerant to the hypothermia induced by ethanol. This did not occur, either in the present (Fig. 1A) or previous studies [6].

Thus, although the peptides tested can directly influence the physiologic parameters used for evaluating ethanol tolerance in the present study, peptide capacities for prolonging ethanol tolerance can still be determined. Nevertheless, these findings demonstrate the importance of using several parameters for assessing ethanol tolerance.

In our previous studies, we had found that treatment of animals with AVP did not affect ethanol metabolism during the drinking period, during withdrawal or during tolerance testing [5,6]. We therefore suggested that AVP maintained functional (CNS) tolerance to ethanol, rather than influencing metabolic or dispositional tolerance. The present results confirm this conclusion, since, on the last day of tolerance testing, animals treated with AVP regained the righting reflex at higher brain ethanol levels than controls (Table 1). Thus, AVP maintained an adaptive change in the CNS which permitted the tolerant animals to function nearly normally in the presence of a level of ethanol which is hypnotic in a control animal.

The maintenance of ethanol tolerance by DGLVP is in line with the idea that neurohypophyseal peptides affect tolerance via central mechanisms, since DGLVP is nearly devoid of the peripheral activities of the neurohypophyseal hormones [27,31]. The finding that both AVP and DGLVP, but not cyclo(Leu-Gly), can maintain ethanol tolerance, however, contrasts somewhat with results obtained in other behavioral studies [27,29]. Both AVP and DGLVP were about equally potent in inhibiting extinction of an active avoidance response, while cyclo(Leu-Gly), although less potent, did show definite activity [29]. AVP and DGLVP were also about equally potent in attenuating the amnesia caused by puromycin in mice [27]. Cyclo(Leu-Gly) also attenuated this amnesia, but a higher dose than that necessary for the vasopressins was needed to achieve a maximal response [4]. It is possible that higher doses of cyclo(Leu-Gly), which in the present study was administered at a dose equimolar to that of vasopressin, would also prolong the duration of ethanol tolerance, and dose-response studies are in progress. On the other hand, since cyclo(Leu-Gly) is not readily metabolized [7], and has been shown to remain in mouse brain for up to 96 hours after subcutaneous injection [17], the amount of the peptide present in the ethanol-treated mice at the time of tolerance testing would presumably be higher than that in the mice tested for retention of memory, which had received only a single peptide injection [4,27].

A comparison of neurohypophyseal peptide effects on ethanol tolerance with those on morphine tolerance is more difficult. In one study, both AVP and oxytocin were found to facilitate the development of morphine tolerance [19], while in another study, these peptides did not influence morphine tolerance [22]. Cyclo(Leu-Gly), on the other hand, has been found to be highly active in *blocking* the development of morphine tolerance [28].

These differences in the ability of various neurohypophyseal peptides to influence memory-related CNS processes and the development and maintenance of drug tolerance would be expected if the neurohypophyseal peptides can influence particular CNS adaptive phenomena via distinct receptors or neuronal pathways. In fact, it is likely that each type of CNS adaptation results from perturbations of several underlying neuronal systems, each of which may be differently influenced by particular neurohypophyseal peptides.

The question of the possible role of endogenous neurohypophyseal hormones in the development or maintenance of ethanol tolerance remains open. The findings that DGLVP maintains tolerance, and that AVP maintains a functional change in the CNS following ethanol exposure, are in line with the idea that neurophypophyseal homones released directly into brain could influence the development or maintenance of ethanol tolerance under physiological conditions. To date, studies of the effects of ethanol on AVP release have examined peripheral hormone levels [13,14] or excretion of the hormone [12]. One may speculate that the more difficult task of measuring neurohypophyseal hormone levels or turnover in the hypothalamus or other brain areas (e.g., [2, 23, 24, 26]) following ethanol exposure, might begin to provide the information necessary for examining the influence of these peptide hormones on CNS adaptive phenomena.

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