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# Modification of conditioned behavior of rats by neurohypophyseal hormones and analogues

(active avoidance response/memory/learning/structure–activity relationship/peptides)

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ABSTRACT **Vasopressin and other neurohypophyseal peptides affect various processes related to memory and/or learning. A single subcutaneous injection of vasopressin increases resistance to extinction of a pole-jumping avoidance response in rat. This test system has been applied in an attempt to relate structural aspects of neurohypophyseal peptides, analogues, and derivatives with truncated sequences to their effects on conditioned behavior. Thus far it can be concluded that there are more stringent requirements on certain residues in the 20-member covalent ring than in positions 8 and 9 of the linear peptide portion for neurohypophyseal hormones to be active. Critical are the contributions of residues in positions 2, 3, and 5; these results are reminiscent of those from conformation–activity correlations of the endocrine effects of neurohypophyseal hormones, in which the side chain of the residue in position 3 is critical for receptor binding and the side chains of residues in positions 2 and 5 are key for the activation of the receptor. Chemical modifications in position 4 yield analogues that are active and inactive in increasing the resistance to extinction of the avoidance response, depending on the particular structural substitution, similar to results from structure–activity studies of the endocrine activities of neurohypophyseal hormones. Because behavioral activities of vasopressin are more tolerant than endocrine activities to modifications of the hormone in positions 8 and 9, analogues with the most striking dissociation of potencies in learned behavior and endocrine responses are expected to be those with sequence alterations in the linear peptide portion. Peptides with linear part sequences of neurohypophyseal hormones showed little or no activity. The results obtained in this structure–activity study are compared with those of an earlier study in which the ability of various neurohypophyseal peptides to attenuate puromycin-induced amnesia in mice was evaluated.**

Learning and information storage and retrieval are thought to be influenced by pituitary hormones and by specific metabolites and synthetic derivatives of these hormones (1–6). With a pole-jumping avoidance test used as an index, the posterior pituitary hormone arginine vasopressin was found to be the most potent compound for long-term maintenance of a conditioned avoidance response (CAR) (1).

In the present investigation, arginine vasopressin and lysine vasopressin, vasotocin, and oxytocin as well as a number of analogues and COOH-terminal fragments of these hormones were assayed in intact rats by using the pole-jumping avoidance test in an attempt to relate structural aspects of the peptides to their effects on CAR.

## MATERIALS AND METHODS

[$Arg^8$]Vasopressin [AVP, 509 international units (IU)/mg rat pressor activity], [$Lys^8$]vasopressin (LVP, 300 IU/mg pressor), oxytocin (OT, 512 IU/mg avian vasodepressor), [$Leu^3$, $Ser^4$, $Arg^8$]vasopressin, and [$Mpr^1$, $Leu^4$, $Arg^8$]vasopressin were obtained from Organon International B.V., Oss, The Netherlands; [$Arg^8$]vasotocin (AVT, 234 IU/mg pressor) and [des-Gly-$NH_2{}^9$]LVP (DG-LVP) were from R. O. Studer and S. Wang, respectively, of Hoffman–La Roche & Co., Ltd.; [$Leu^4$]LVP was from V. du Vigneaud (7); [D-$Arg^8$]vasopressin (8) and its deamino analogue (DDAVP) (9) were from M. Zaoral; [tri-$Gly^1$]LVP (10) and [tri-$Gly^1$]oxytocin were from R. Vavrek, Ferring AB, Sweden. Additional peptides were available in these laboratories: [$Ala^2$]AVP (11), [$Abu^4$]AVP (12), [$Thi^3$]LVP (13), [$Abu^4$]LVP (14), and [$Ala^8$]AVP (15) and its deamino analogue (16). The linear peptides listed in Table 3 were from batches used in previous studies (17). Peptides were dissolved in saline after addition of 1 drop of 0.01 M HCl.

**Assay Procedure.** Male Wistar rats weighing 120–140 g were conditioned to jump onto a pole. The conditioned stimulus (CS) was a light presented for 5 sec. Rats that failed to jump onto the pole within 5 sec after CS onset received an unconditioned stimulus (US), electric footshock (540 V a.c.; 0.2 mA) applied through the grid floor until the animal made the response. The CS and US were terminated at the moment of the response. Ten trials were carried out each day for 3 consecutive days, with intervals between them, averaging from 40 to 80 sec, given in a predetermined random sequence. Rats that made seven or more positive avoidances (i.e., jumped onto the pole within 5 sec after presentation of the CS during the third acquisition session) were injected subcutaneously, in a volume of 0.5 ml, with the respective peptides immediately after termination of the third acquisition session. In general, 80% or more of the rats reached this criterion. At 24, 48, and 120 hr after the third acquisition session, extinction sessions of 10 trials each were run on the same schedule as that used during acquisition except that the US of shock was not applied if the rat did not make a response. The amount of peptide that induced seven or more positive avoidances (criterion) at the third extinction session (120 hr) was determined by injecting various dose levels to a maximum of 9 $\mu$g per rat. Each dose level was tested in a group of four or five rats. The activity of an unknown is expressed relative to the activity of AVP and is derived as follows: on a weight basis, the lowest dose of AVP needed to reach criterion is set at 100% and is compared to the lowest dose of unknown necessary to reach criterion.

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Abbreviations: Nomenclature is in accord with the IUPAC-IUB Rules on Biochemical Nomenclature [(1972) *Biochem. J.* **126**, 773–780, and (1967) *J. Biol. Chem.* **242**, 555–557]. All optically active amino acids are of the L configuration unless otherwise noted. CAR, conditioned avoidance response; IU, international units; CS, conditioned stimulus; US, unconditioned stimulus.

Table 1. Neurohypophyseal hormones: Effects of single injection on extinction of pole-jumping avoidance response in rats

| Hormone | Dose, μg | Extinction sessions at different times after injection* 24 hr | 48 hr | 120 hr |
|---|---|---|---|---|
| [Arg$^8$]Vasopressin (AVP) | 0.06 | 9.5 ± 0.4* | 8.0 ± 0.0 | 5.3 ± 0.5 |
| | 0.18 | 8.5 ± 0.4 | 8.8 ± 0.5 | 8.5 ± 0.4 |
| [Lys$^8$]Vasopressin (LVP) | 0.1 | 9.0 ± 0.3 | 7.2 ± 0.3 | 5.6 ± 0.4 |
| | 0.3 | 8.8 ± 0.2 | 8.8 ± 0.4 | 8.0 ± 0.4 |
| Oxytocin | 0.3 | 7.8 ± 0.2 | 7.0 ± 0.7 | 5.2 ± 1.0 |
| | 1.0 | 8.8 ± 0.5 | 7.8 ± 0.5 | 6.8 ± 0.9 |
| | 3.0 | 8.5 ± 0.6 | 8.3 ± 0.5 | 8.0 ± 0.4 |
| Vasotocin | 1.0 | 8.2 ± 0.6 | 6.8 ± 0.4 | 6.2 ± 0.4 |
| | 3.0 | 8.6 ± 0.4 | 8.8 ± 0.5 | 8.0 ± 0.3 |
| Saline | 0.5 ml | 8.6 ± 0.4 | 3.6 ± 0.8 | 0.2 ± 0.2 |
| Saline | 0.5 ml | 9.0 ± 0.3 | 6.6 ± 1.3 | 0.8 ± 0.5 |

* Results shown as mean (± SEM) avoidance responses of four or five animals.

## RESULTS AND DISCUSSION

The results of the studies with the neurohypophyseal hormones are presented in Table 1, of those with their analogues in Table 2, and of those with their linear fragments in Table 3. Several conclusions can be drawn from these studies. The linear COOH-terminal peptide portion of vasopressin (the analysis in this paper is restricted to residues 8 and 9 of the linear peptide moiety) generally tolerates considerable structural variation without drastically affecting the behavioral potency (D-[Arg$^8$]AVP is a notable exception), whereas certain substitutions in the 20-member ring are accompanied by practically a complete loss of activity (Table 2). Therefore, analogues with the most striking dissociation between effects on the expression of CAR and endocrine responses are expected to be those with sequence modifications in the linear peptide portion. A case in point is DG-LVP (Table 2). In all of the assays performed, this analogue exhibits only a minute fraction of the potency found for the endocrine activities characteristic of LVP on the maintenance of the pole-jumping avoidance response (Table 2), confirming previous findings (17–19). Similarly, the behavioral activity of AVP is relatively little influenced by the replacement of Arg$^8$ by Lys$^8$ (Table 1). Even replacement by the neutral amino acid residue Ala$^8$, giving [Ala$^8$]AVP, which has severely decreased antidiuretic and pressor activities compared to AVP (16), leads to an analogue that retains a definite potency in the pole-jumping test (Table 2). In light of the relative insensitivity to modification of the acyclic peptide portion, it is somewhat surprising that the replacement of the L-Arg in AVP by its optical isomer (D-Arg) results in a marked decrease in potency in affecting learned behavior. Studies of additional analogues with structural modifications in the linear

Table 2. Behavioral potency of various vasopressin analogues as determined in pole-jumping avoidance test

| | Peptide | Approximated potency, % |
|---|---|---|
| AVP: | H-Cys$^1$-Tyr$^2$-Phe$^3$-Gln$^4$-Asn$^5$-Cys$^6$-Pro$^7$-Arg$^8$-Gly$^9$-NH$_2$ | 100 |
| | Mpr Arg | 14 |
| | Ala Arg | 4 |
| (AVT) | Ile Arg | 7 |
| | Abu Arg | 1 |
| | Leu-Ser Arg | 1 |
| | D-Arg | 5 |
| | Mpr D-Arg | 3 |
| | Mpr Leu D-Arg | 1 |
| | Ala | 28 |
| | Mpr Ala | 4 |
| LVP: | Lys | 63 |
| | Ala Lys | 2 |
| | Thi Lys | 13 |
| | Abu Lys | 2 |
| | Leu Lys | 13 |
| | Ala Lys | 2 |
| (DG-LVP) | Lys-NH$_2$ | 67 |
| OT: | Ile Leu | 13 |

Table 3. Hormone linear fragments: Effect of single injection on the rate extinction of pole-jumping avoidance response in rat*

| Peptide | Dose, μg | Extinction sessions at different times after injection | | | Approximated potency (%) compared to AVP |
|---|---|---|---|---|---|
| | | 24 hr | 48 hr | 120 hr | |
| 1. Z-Pro-Leu-Gly-$NH_2$ | 1.0 | 7.8 ± 0.3 | 2.5 ± 1.5† | 1.3 ± 0.8† | <1 |
| 2. Pro-Leu-Gly-$NH_2$ | 3.0 | 8.2 ± 0.2 | 5.8 ± 0.3 | 3.0 ± 0.7 | 1 |
| | 9.0 | 8.0 ± 0.7 | 6.8 ± 1.8 | 5.6 ± 1.5 | |
| 3. Leu-Gly-$NH_2$·AcOH | 3.0 | 8.8 ± 0.5 | 7.8 ± 0.7 | 6.0 ± 0.3 | 2 |
| | 9.0 | 9.0 ± 0.3 | 8.6 ± 0.4 | 8.0 ± 0.0 | |
| 4. D-Leu-Gly-$NH_2$ | 3.0 | 8.4 ± 0.3 | 7.2 ± 0.4 | 5.2 ± 0.2 | 2 |
| | 9.0 | 3.8 ± 0.4 | 8.4 ± 0.5 | 7.0 ± 0.3 | |
| 5. Cyclo(Leu-Gly) | 1.0 | 9.0 ± 0.4 | 6.6 ± 0.4 | 5.4 ± 0.6 | 7 |
| | 3.0 | 8.8 ± 0.5 | 8.2 ± 0.4 | 7.4 ± 0.5 | |
| 6. Saline (control) | 0.5 ml | 8.8 ± 0.4 | 6.0 ± 0.8 | 0.4 ± 0.4 | |

* For explanation and experimental detail see legend to Table 1 and text.
† Not significantly different from control (saline).

portion of neurohypophyseal hormones are clearly warranted.

Although there seems to exist a distinct dissociation between the structural requirements in the linear segment of AVP that render it active behaviorally, as opposed to endocrinologically, it also becomes evident that the structural requirements of the 20-member covalent ring moiety of vasopressin which govern its potency in the central nervous system are similar to those necessary for high endocrinological activity. Specifically, the amino acid side chains of the two corner residues (positions 3 and 4) in the β-turn of the 20-member ring of neurohypophyseal hormones (11, 20, 21), which were found to be important for intermolecular interactions of the peptide with various receptors (22, 23), are also important for the central nervous system effects. It is suspected, although not proven by direct receptor binding studies, that the importance of an aromatic residue in position 3, found in this study, lies in its contribution to the affinity parameter, as has been shown for the endocrine effects of neurohypophyseal peptides (e.g., refs. 15 and 24). Thus, the presence of a residue with an aromatic side chain in the first corner position of the β-turn (position 3) is critical for high activity in the CAR test. As the aromaticity decreases (Phe, Thi, Ile in position 3) the activity declines; i.e., vasopressins have the highest potency, followed by [$Ala^8$]vasopressin (28%), [$Thi^3$]LVP, and [$Ile^3$]AVP (AVT) (Table 2). Moreover, the observation that AVT (with Arg in position 8) and oxytocin (with Leu in position 8) have only about 7% and 13%, respectively, of the activity of AVP speaks for the predominant importance of the aromatic residue in position 3 as compared to the nature of residue 8.

From the limited information available it may be suggested that the effects on resistance to extinction of the CAR that are observed upon modification of the residue in position 4 (second corner position in the β-turn), a residue that has been proposed to play a role in the active center of neurohypophyseal hormones (22), will depend on the kind of substitution made. This again is reminiscent of results obtained when comparing the endocrine activities of vasopressin and its analogues (e.g., refs. 22–27). The minimal activity of [$Ala^5$]LVP (Table 2) in the CAR assay reveals that the Asn residue in position 5 is key for this activity of neurohypophyseal hormones, as also is the case with respect to the full spectrum of endocrine activities of these hormones (22, 23, 25). The very low behavioral activities of [$Ala^2$]AVP and [$Ala^2$]LVP calls for further exploration of the possibility that the hydroxy group of $Tyr^2$ is an "active element" as has been proposed for oxytocin when bound to the uterine receptor (25).

The replacement of the cysteine residue in position 1 by a 2-mercaptopropionic acid (deamino analogues) has a negative effect on the activity of the resultant analogue in the pole-jumping avoidance test. Although not shown in Table 2, the analogues extended at position 1 ($N^{\alpha}$-triglycyl derivatives of LVP and oxytocin) exhibited 14% and 11%, respectively, of the activity of AVP. Thus, the attachment of the triglycyl moiety to the $NH_2$-terminal amino group decreases the behavioral effect of LVP but, unexpectedly, not of oxytocin. Enzymatic release of the respective hormone *in vivo* from these "hormonogens," as has been suggested for several endocrine responses with these "synthetic" precursors (24), apparently does not affect the behavioral profile of these peptides.

Fragments and derivatives of the linear COOH-terminal sequence of oxytocin were also tested. At a dose of 100 μg in mice, these compounds had been found previously to possess protective effects against the amnestic action of puromycin (17); most potent were Z-Pro-Leu-Gly-$NH_2$, cyclo(Leu-Gly), and the L and D isomers of Leu-Gly-$NH_2$. Flexner *et al.* (28) recently confirmed and extended these results by obtaining dose-response plots. Furthermore, Pro-Leu-Gly-$NH_2$ and cyclo(Leu-Gly) were found to be as potent as oxytocin in facilitating morphine dependence in rats (29), an adaptive response that may be considered analogous to learning or memory (29, 30). Although some of these peptides were found in this study to be effective in increasing resistance to extinction of the avoidance response, their potency by the criteria set for the extinction sessions was low. Noteworthy is the lack of any detectable activity with Z-Pro-Leu-Gly-$NH_2$ (Table 3); this compound was highly active in protecting against puromycin-induced amnesia (17, 28).

On comparison of the effects of identical neurohypophyseal hormone analogues in their ability to delay extinction of the pole-jumping avoidance response in rats with their ability to attenuate puromycin-induced amnesia in mice (17, 28), striking

analogies in the structure–activity relationship can be detected. Modifications of residues in the 20-member ring of neurohypophyseal peptides bring about similar changes in the potencies of the resultant analogues in both test systems. The combination of a cyclic moiety containing $Tyr^2$ and $Phe^3$ is important for significant activity in both test systems, position 4 shows selective sensitivity to modifications, and the Asn residue in position 5 is essential for maintenance of activity.

However, marked differences are found as well. For example, the linear portion of the vasopressin molecule is highly active in attenuating puromycin-induced amnesia and in facilitating morphine dependence, whereas the cyclic moiety (referred to as pressinoic acid) is inactive in both assays (17, 29). In contrast, pressinoic acid is active in increasing resistance to extinction of pole-jumping avoidance behavior, particularly after intraventricular administration (31), whereas Pro-Arg-Gly-$NH_2$ is much less active. These discrepancies might be explained by assuming that amnesia and resistance to extinction of active avoidance behavior measure different processes. The influence of neurohypophyseal hormones in reducing amnesia and on resistance to extinction may reflect peptide effects on distinct memory (information storage, retrieval) processes. Other explanations for the discrepancies may be offered. In the amnesia test, much more material is administered than in the avoidance test. Large amounts of peptide fragments, for example, may affect the release of neurohypophyseal hormones; in fact, cyclo(Leu-Gly) was reported (32) to increase radioimmunoassayable AVP in rat plasma after the subcutaneous injection of 20 $\mu$g but not after 1 $\mu$g.

Accordingly, conclusions regarding the structure–activity relationship of peptides of neurohypophyseal origin should be drawn with care as long as the interrelationship of release and metabolism of hypothalamic and pituitary hormones is not known. Nevertheless, a defined structure–activity relationship can be expected to emerge with respect to neurohypophyseal hormone-induced CAR.

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