ELSEVIER

European Journal of Pharmacology 294 (1995) 29–39

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Vasopressin-induced sensitization: involvement of neurohypophyseal peptide receptors

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Received 16 March 1995; revised 8 August 1995; accepted 11 August 1995

Abstract

Rats pretreated with an intracerebroventricular (i.c.v.) injection of 10 pmol of vasopressin or vasopressin analogs, including deamino-D-vasopressin, [pGlu^1,Cyt^6]vasopressin, [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH$_2$, des-Gly-NH$_2^9$-vasopressin, Pro-Leu-Gly-NH$_2$, Pro-Arg-Gly-NH$_2$, became markedly hyper-responsive to the motor effects, 24 h later, to a subsequent challenge dose of vasopressin, but not vasopressin-related peptides. A vasopressin V_1 receptor antagonist, [d(CH$_2$)$_5^1$,Tyr(Me)^2]vasopressin, but not the vasopressin V_2 receptor antagonist, [d(CH$_2$)$_5^1$,Tyr(Et)^2,Val^4]vasopressin, or a more selective vasopressin V_2 receptor antagonist, [d(CH$_2$)$_5^1$,D-Ile^2,Ile^4]vasopressin, or the oxytocin receptor antagonist, [d(CH$_2$)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH$_2^9$]vasotocin ([d(CH$_2$)$_5^1$,Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT), blocked vasopressin and vasopressin analog-induced sensitization. Furthermore, both vasopressin V_2 receptor antagonists were found to sensitize the brain to a subsequent vasopressin injection. This vasopressin V_2 receptor antagonist-induced sensitization was also blocked by the vasopressin V_1 receptor antagonist. Next, we wanted to determine if this sensitization process could involve the release of endogenous vasopressin in the brain as reflected in an amplification of vasopressin mRNA expression. However pretreatment of rats with an i.c.v. vasopressin injection was not associated with an increase in vasopressin mRNA expression in the bed nucleus of the stria terminalis, medial amygdala or the paraventricular nucleus of the hypothalamus when measured 0, 1, 3, 7, 12, or 24 h after the first vasopressin injection. As many vasopressin analogs can induce sensitization, we suggest that a novel type of receptor may be involved in the sensitization process.

Keywords: Oxytocin; Structure-activity; Neuromodulation

1. Introduction

Arginine-vasopressin (vasopressin) is known to have many actions in the central nervous system (CNS) which include alteration of autonomic control of body temperature (Pittman et al., 1993) and blood pressure (McNeil, 1983) and its effects on consolidation and retrieval of learned behaviors (De Wied et al., 1984; Dantzer et al., 1988). Vasopressin is also involved in a sensitization process in which vasopressin increases the responsiveness of the brain to itself upon repeated exposure (Burnard et al., 1986; Kasting et al., 1980; Lebrun et al., 1989, 1990; Poulin and Pittman, 1993a,b; Wurpel et al., 1986). For example, pre-exposure of the rat brain to vasopressin or oxytocin, results in enhanced antipyretic (Poulin and Pittman, 1993c), cardiovascular (Lebrun et al., 1989; Poulin et al., 1994), and motor effects (Burnard et al., 1986; Kasting et al., 1980; Poulin and Pittman, 1993b; Wurpel et al., 1986) of vasopressin. Some of these effects appear as early as 6 h after the initial exposure to vasopressin.

These enhanced effects of vasopressin represent an action of the peptide at a specific V_1 vasopressin receptor (Diamant et al., 1994), in that the enhanced actions of vasopressin can be blocked by vasopressin V_1 receptor antagonists, but not by vasopressin V_2 receptor

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SSDI 0014-2999(95)00515-3

antagonists, and are not elicited by vasopressin V_2 receptor or oxytocin receptor agonists (Naylor et al., 1985; Poulin and Pittman, 1993b; Burnard et al., 1986). In keeping with this, receptor binding (Poulin et al., 1988; Jard et al., 1987; Shewey and Dorsa, 1988; Phillips et al., 1988) and in situ hybridization (Ostrowski et al., 1992; Szot et al., 1994) studies have identified vasopressin V_1 receptors in central nervous system tissue loci where vasopressin has been shown to elicit enhanced effects. Studies on receptor function have demonstrated that the phosphatidyl inositol hydrolysis response activated by vasopressin V_1 receptor occupation was enhanced in vasopressin-sensitized tissue (Poulin and Pittman, 1993a; Lebrun et al., 1990), even though receptor numbers and affinity were unaltered. Thus, a peculiarity of the vasopressin V_1 receptor-effector process in brain tissue is that it can undergo a sensitization process, such that its sensitivity to the peptide vasopressin is reversibly enhanced if it has been previously exposed to vasopressin.

Despite the unquestioned involvement of the vasopressin V_1 receptor in the manifestation of enhanced actions of vasopressin in brain, we recently discovered that the sensitization process may be more complex. We observed, for example, that pre-exposure of the rat brain to oxytocin also alters the responsiveness of the rat brain to subsequent vasopressin exposures at oxytocin-sensitizing doses even less than were required for vasopressin to act as a sensitizing agent (Poulin and Pittman, 1993b). Thus the observation that the sensitization process can be evoked by oxytocin raises the possibility that other vasopressin analogs known to have actions in brain (Croiset and De Wied, 1990; Cheng and North, 1989; Burbach et al., 1983b; De Wied et al., 1984; Kovacs et al., 1986; Szabo et al., 1991; De Wied et al., 1987; Burbach et al., 1983a; Kovacs et al., 1989) may also evoke the sensitization process. The present experiments explore the pharmacological profile of the vasopressin sensitization process, utilizing the enhanced motor actions of vasopressin as an assay system to estimate the extent of the sensitization process.

In addition, the means by which the sensitization process is brought about is yet unknown, but recent observations by Landgraf et al. (1991) demonstrate a positive feedback regulation of vasopressin upon its own release, suggesting that the sensitization process could involve an amplification of endogenous vasopressin release in the brain via an action on vasopressin neurons. To investigate this possibility, we took advantage of the coupling between activity and peptide synthesis, previously validated for vasopressin neurons (Fitzsimmons et al., 1992), to measure the activity of the vasopressin mRNA in populations of vasopressin neurons thought to provide innervation to the brain areas involved in both the enhanced motor and antipyretic actions of vasopressin in vasopressin-sensitized brain.

2. Materials and methods

Adult male Sprague-Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (65 mg/kg) and a stainless steel 23-gauge guide cannula was implanted stereotaxically under aseptic conditions to allow access to a lateral cerebral ventricle. During the 5–7 days recovery, the rats were housed in groups of four per cage on a 12 h light/dark cycle and allowed food and water ad libitum. All experimental protocols were conducted according to procedures approved by the University of Calgary Animal Care Committee. Peptides were purchased as follow: arginine vasopressin (vasopressin; Bachem, CA, USA), deamino-D-vasopressin (Bachem, CA, USA), pressinoic acid (Peninsula, CA, USA), [pGlu^4,Cyt^6]vasopressin (Peninsula, CA, USA), [pGlu-Asn-Cys(Cys)] Pro-Leu-Gly-NH_2 (Peninsula, CA, USA), des-Gly-NH_2^9-vasopressin (Bachem, CA, USA), Pro-Leu-Gly-NH_2 (Bachem Bioscience, PA, USA), Pro-Arg-Gly-NH_2 (Bachem Bioscience, PA, USA), the vasopressin V_1 receptor antagonist, [d$(CH_2)_5^1$,Tyr(Me)^2]vasopressin (Bachem CA, USA), a vasopressin V_2 receptor antagonist, [d$(CH_2)_5^1$,Tyr(Et)^2,Val^4]vasopressin (Peninsula, CA, USA), a more selective vasopressin V_2 receptor antagonist, [d$(CH_2)_5^1$,D-Ile^2,Ile^4]vasopressin (Peninsula, CA, USA), and the specific oxytocin receptor antagonist, [d$(CH_2)_5^1$,Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH_2^9]vasotocin (sold as CPD 6 or [d$(CH_2)_5^1$,Tyr(Me)^2,Thr^4,Tyr-NH_2^9]OVT by Peninsula, CA, USA).

2.1. Sensitization assay: enhanced motor actions of vasopressin

A standard vasopressin sensitization protocol (Kasting et al., 1980; Burnard et al., 1986; Wurpel et al., 1986) was used in which a first intracerebroventricular (i.c.v.) injection (5 μl) of saline or a peptide solution was followed, 24 h later, by a subsequent vasopressin or peptide solution injection. All peptides were dissolved to appropriate concentration in sterile, pyrogen-free physiological saline and administered by gravitational flow through a 27-gauge injection cannula into awake, freely moving animals between 10:00 and 18:00 h. After each injection, the animals were placed in a large plexiglass chamber and observed for the development of motor disturbances for a 10-min period. Motor disturbances were defined and scored as previously described (Poulin and Pittman, 1991) as follows: 0, no effect; 1, pauses (periods of 10 s or longer of absence of activity); 2, prostration; 3, head swaying and locomotor difficulties; 4, barrel rotation as

indicated by the animal's rotation along the long axis of the body; 5, myotonus or myoclonus; 6, death. Results are presented as the highest score each animal received during the 10-min observation period. Results were statistically analysed by the Kruskal-Wallis one-way analysis of variance by ranks in conjunction with the non-parametric Wilcoxon matched-pairs signed-ranks test and the Mann-Whitney U-test. The α level of significance used was a P value of 0.05.

Vasopressin analogs and sensitization

First we tested the possibility that a pre-exposure of the rat brain to a variety of vasopressin analogs may sensitize the rat brain to the motor effects of a subsequent vasopressin exposure. In these experiments nine groups of animals were used. Animals were first given an i.c.v. injection of either saline ($n = 8$; controls) or 10 pmol of vasopressin ($n = 8$), deamino-D-vasopressin ($n = 8$), pressinoic acid ($n = 8$), [pGlu^4,Cyt^6]vasopressin ($n = 8$), [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH_2 ($n = 6$), des-Gly-NH^9_2-vasopressin ($n = 5$), Pro-Leu-Gly-NH_2 ($n = 7$), Pro-Arg-Gly-NH_2 ($n = 6$). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of vasopressin (10 pmol) and scored as described above. In another experiment, we investigated the possibility that these analogs may also induce motor disturbances when given in sensitized animals. Animals were first given as a pretreatment, an i.c.v. injection of 10 pmol of vasopressin. 24 h later, the potency of vasopressin analogs to induce motor disturbances was tested following an i.c.v. injection of either saline ($n = 6$) or 10 pmol of either vasopressin ($n = 5$), deamino-D-vasopressin ($n = 8$), pressinoic acid ($n = 4$), [pGlu^4,Cyt^6]vasopressin ($n = 8$), [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH_2 ($n = 5$), des-Gly-NH^9_2-vasopressin ($n = 4$), Pro-Leu-Gly-NH_2 ($n = 5$) or Pro-Arg-Gly-NH_2 ($n = 6$) and scored as described above.

Blockade of sensitization by specific receptor antagonists

The ability of a series of vasopressin and oxytocin receptor antagonists to inhibit the sensitization process was investigated. In these experiments, four groups of animals received a pretreatment injection of deamino-D-vasopressin (10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 7$), the vasopressin V_1 receptor antagonist (100 pmol, $n = 9$), the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,Tyr(Et)^2,Val^4]vasopressin (100 pmol, $n = 8$) or the oxytocin receptor antagonist [d(CH_2)$^1_5$,Tyr(Me)^2,Thr^4,Tyr-NH^9_2]-OVT (100 pmol, $n = 6$). Another four groups of animals received a pretreatment injection of [pGlu^4,Cyt^6]vasopressin (10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 6$), the vasopressin V_1 receptor antagonist (100 pmol, $n = 7$), the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,Tyr-(Et)^2,Val^4]vasopressin (100 pmol, $n = 7$) or the oxytocin receptor antagonist [d(CH_2)$^1_5$,Tyr(Me)^2,Thr^4,Tyr-NH^9_2]OVT (100 pmol, $n = 5$). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored as described above.

In an additional experiment, we evaluated the ability of a more selective vasopressin V_2 receptor antagonist to block deamino-D-vasopressin-induced sensitization, in which two groups of animals received a pretreatment injection of deamino-D-vasopressin (10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 6$) or the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,D-Ile^2,Ile^4]vasopressin (100 pmol, $n = 6$). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored as described above.

To test the possibility that the vasopressin receptor antagonist [d(CH_2)$^1_5$,D-Ile^2,Ile^4]vasopressin in itself may act as an agonist to induce sensitization, an additional group of animals, received a pretreatment with the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,D-Ile^2,Ile^4]vasopressin alone (10 pmol, $n = 9$) and 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored as described above.

To test the possibility that the vasopressin V_1 receptor antagonist may also block the vasopressin V_2 receptor antagonist ([d(CH_2)$^1_5$,D-Ile^2,Ile^4]vasopressin)-induced sensitization, an additional group of animals was given a pretreatment injection of the vasopressin V_2 receptor antagonist ([d(CH_2)$^1_5$,D-Ile^2,Ile^4]vasopressin) (10 pmol, $n = 11$) preceded (2 min prior) by an injection of the vasopressin V_1 receptor antagonist (100 pmol). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored as described above.

To test whether the other vasopressin V_2 receptor antagonists may also induce the sensitization process, two groups of animals were given a pretreatment of 10 pmol of the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,Tyr(Et)^2,Val^4]vasopressin preceded (2 min prior) by an injection of either saline (5 μl, $n = 9$) or the vasopressin V_1 receptor antagonist (100 pmol, $n = 5$). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored as described above. Because the vasopressin V_2 receptor antagonist [d(CH_2)$^1_5$,Tyr(Et)^2,Val^4]vasopressin also acted as an agonist in the sensitization phenomenon, we tested the possibility that this vasopressin V_2 receptor antagonist may in itself induce motor disturbances in sensitized animals. To do this, an additional group of animals ($n = 4$) was given, on the first peptide exposure, 10

pmol vasopressin V_2 receptor antagonist, [d$(CH_2)^1_5$, Tyr(Et)^2,Val^4]vasopressin, and 24 h later, 100 pmol vasopressin V_2 receptor antagonist, [d$(CH_2)^1_5$,Tyr-(Et)^2,Val^4]vasopressin, and scored as described above.

2.2. Sensitization process and endogenous vasopressin activity

In these studies, we tested the possibility that sensitizing agents may cause release of endogenous vasopressin, as reflected in an activation of vasopressin gene expression. We tested this hypothesis by investigating the relative amount of vasopressin message expressed per cell as well as the number of cells expressing vasopressin messenger RNA (vasopressin mRNA) at several times following a pretreatment with an i.c.v. injection of vasopressin in three brain areas including the bed nucleus of the stria terminalis, the medial amygdala and the paraventricular nucleus of the hypothalamus which are known to contain vasopressin cell bodies projecting to areas though to be involved in the sensitization process (Naylor et al., 1985). The time points were chosen based on behavioural observations which have demonstrated that vasopressin sensitization becomes apparent 6 h after the first vasopressin administration and is maximal 24–48 h after the first vasopressin administration (Poulin and Pittman, 1993a). Therefore in the first series of experiments we determined the level vasopressin mRNA expression in the brain 24 h after a saline ($n = 6$, controls) or a vasopressin ($n = 6$) injection. In a second series of experiments, we analysed vasopressin mRNA expression at 0 ($n = 8$), 1 ($n = 7$), 3 ($n = 6$), 7 ($n = 4$) and 12 ($n = 6$) h after a first vasopressin injection.

To determine the level of vasopressin mRNA expression, an in situ hybridization protocol (Szot and Dorsa, 1992) was performed using a 48-base oligonucleotide complementary to bases coding for the last 16 amino acids of the vasopressin mRNA (Ivell and Richter, 1984). At the appropriate time following the injection, the animals were killed by decapitation, the brains rapidly removed and frozen at −70°C. 20 μm coronal sections were cut at −20°C and thaw-mounted onto RNase-free gelatine-coated slides. Slides were stored at −70°C until assayed. Slides were then post-fixed in 4% paraformaldehyde, washed in phosphate-buffered saline, treated with acetic anhydride (0.25% in 0.1 M triethanolamine), dehydrated, delipidated and air-dried. The probe was 3′-end-labelled with [^{35}S]-dATP (New England Nuclear, Boston, MA, USA) using terminal deoxyribonucleotidyl transferase and purified on NEN-Sorb columns (New England Nuclear). The labelled, purified probe was then diluted in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 M sodium chloride, 10 mM Tris (pH 8.0), 1 mM EDTA, 1X Denhardt's (0.2% of each bovine serum albumin, Ficoll and polyvinyl pyrrolidine), 0.5 mg/ml yeast tRNA and 10 mM dithiothreitol to a concentration of 2.5 pmol/ml (specific activity 5550–6700 Ci/mmol). 45 μl of the hybridizing solution was applied to the tissue sections and covered with a silanized coverslip. The slides were then placed in a moist chamber and incubated overnight at 37°C. Following incubation, the coverslips were removed and the sections were washed 4 times in 1 × SSC (saline sodium citrate: 150 mM NaCl/15 mM Na citrate) for 15 min at 55°C and then twice in 1 × SSC for 1 h at room temperature. Sections were dehydrated through a graded series of alcohols containing 300 mM ammonium acetate. For autoradiography, the slides were dipped in Kodak NTB2N Nuclear Track Emulsion (diluted 1:1 with 0.6 M ammonium acetate), allowed to air dry and loaded into slide boxes with desiccant capsules and exposed at 4°C for 1 day for the paraventricular nucleus of the hypothalamus and 4 weeks for the bed nucleus of the stria terminalis and medial amygdala. The slides were developed with Kodak D-19, stained with cresyl violet acetate and mounted with coverslips.

For quantification of hybridization of labelled probe to the vasopressin mRNA, two sections through the bed nucleus of the stria terminalis 0.6–0.8 mm caudal to bregma) and the medial amygdala (2.12–2.56 mm caudal to bregma) of each animal were matched anatomically with corresponding atlas sections (Paxinos and Watson, 1982). The total number of labelled cells was counted unilaterally over two sections and the amount of grains per cell was measured under dark-field illumination with a 20 × objective using an automated grain-counting analysis system, (Microcomputer Imaging Device (MCID); Imaging Research, Ontario, Canada). To estimate the number of labelled cells in the paraventricular nucleus of the hypothalamus (1.6–1.9 mm caudal to bregma) we measured the size of the area of the paraventricular nucleus of the hypothalamus covered by silver grains measured as pixels using MCID under dark-field illumination. To determine the intensity of hybridization per unit area, the relative optical density was measured under dark-field illumination. Relative brightness was obtained by subtracting the relative optical density value from 1. Data are presented as means ± S.E.M. and were analysed using analysis of variance followed by a Scheffé test for post-hoc comparisons.

3. Results

3.1. Behavioral observations

Vasopressin analogs and sensitization

In all experiments conducted in this paper, rats displayed none or minor motor disturbances (scores

less than 3) after a first peptide injection, so the motor data following a first peptide exposure are not shown.

First we tested the possibility that a pre-exposure of the rat brain to a variety of vasopressin analogs may sensitize the rat brain to the motor effects of a subsequent vasopressin exposure. Shown in Fig. 1A are the behavioral scores of rats receiving an injection of 10 pmol vasopressin 24 h following a pretreatment with either saline, as control, or a variety of vasopressin analogs. When compared with saline pretreatment controls, all animals pretreated with vasopressin analogs, with the exception of pressinoic acid pretreatment, exhibited significantly enhanced ($P > 0.05$) motor disturbances to a subsequent challenge to a standardized dose of vasopressin; thus many of the vasopressin analogs sensitize the rat brain to vasopressin-induced motor disturbances.

The finding that many of the vasopressin analogs sensitized the rat brain to vasopressin-induced motor disturbances led us to further investigate the possibility that these analogs may also induce motor disturbances when given in sensitized animals. As can be seen in Fig. 1B, when vasopressin-pretreated animals were administered, 24 h later, either saline or vasopressin as controls, or a vasopressin analog, the behavioral responses of the animals receiving vasopressin analogs, were not significantly different from those of saline-pretreated controls. Only when vasopressin-pretreated animals received vasopressin were the motor responses of the animals significantly enhanced ($P > 0.05$).

Blockade of sensitization by specific receptor antagonists

To further investigate the pharmacological characteristics of the receptor(s) involved in the sensitization process, we tested the ability of a series of vasopressin and oxytocin receptor antagonists to inhibit the sensitization process initiated either by the vasopressin V_2 receptor agonist deamino-D-vasopressin (Fig. 2A) or by the vasopressin metabolite [pGlu^4,Cyt^6]vasopressin (Fig. 2B), using the vasopressin V_1 receptor antagonist, [d(CH$_2$)$^1_5$,Tyr(Me)^2]vasopressin, a vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Et)^2,Val^4]vasopressin

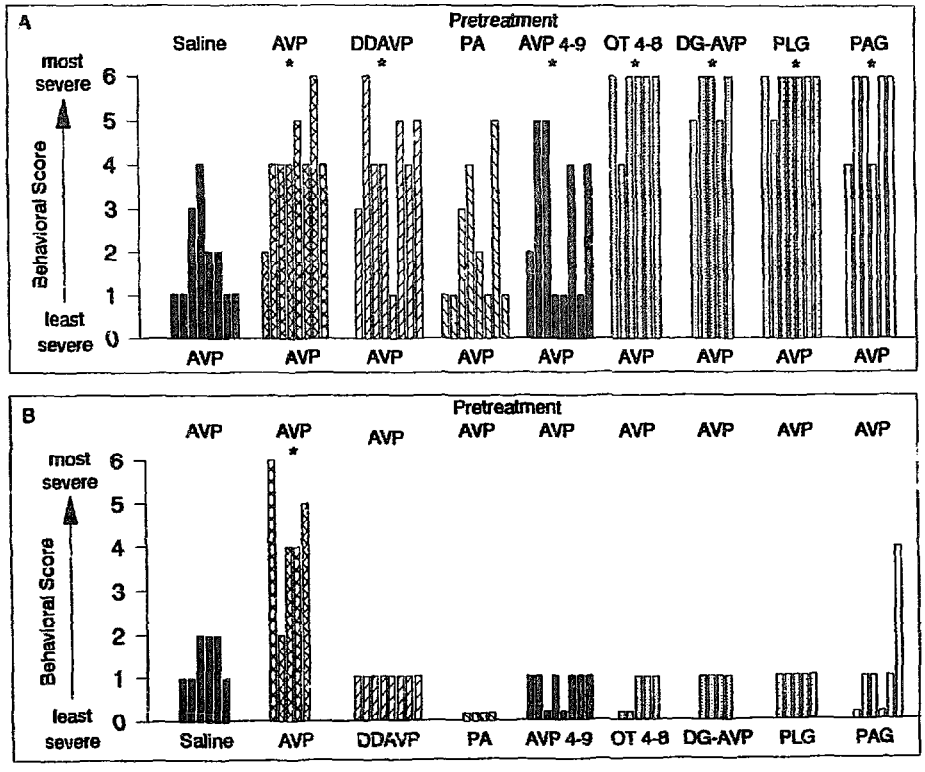

Fig. 1. Vasopressin analogs and sensitization. In these experiments, 18 groups of male Sprague Dawley rats were first given a peptide pretreatment as an i.c.v. injection of either (panel A): 5 μl saline ($n = 8$; controls) or 5 μl of saline containing 10 pmol of vasopressin (AVP; $n = 8$), deamino-D-vasopressin (DDAVP; $n = 8$), pressinoic acid (PA; $n = 8$), [pGlu^4,Cyt^6]vasopressin (AVP 4–9; $n = 8$), [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH$_2$ (OT 4–8; $n = 6$), des-Gly-NH$^9_2$-vasopressin (DG-AVP; $n = 5$), Pro-Leu-Gly-NH$_2$ (PLG; $n = 7$), Pro-Arg-Gly-NH$_2$ (PAG; $n = 6$) or (panel B): 10 pmol of vasopressin (AVP). 24 h later, the potency of vasopressin (AVP) (panel A) or the potency of saline ($n = 6$, controls), or 10 pmol of either vasopressin (AVP; $n = 5$), deamino-D-vasopressin (DDAVP; $n = 8$), pressinoic acid (PA; $n = 4$), [pGlu^4,Cyt^6]-vasopressin (AVP 4–9; $n = 8$), [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH$_2$ (OT 4–8; $n = 5$), des-Gly-NH$^9_2$-vasopressin (DG-AVP; $n = 4$), Pro-Leu-Gly-NH$_2$ (PLG; $n = 5$) or Pro-Arg-Gly-NH$_2$ (PAG; $n = 6$) (panel B) to induce motor disturbances was tested and scored as follows: 0, no effects; 1, pauses; 2, prostration; 3, head swaying and locomotor difficulties; 4, barrel rotation; 5, myotonus/myoclonus; 6, death. Each bar represents the most severe behavioral score of an individual animal in response to the second peptide treatment. * Significant ($P < 0.05$, Mann-Whitney U-test) difference in motor responses between the control and treatment groups.

and the specific oxytocin receptor antagonist, [d(CH$_2$)$^1_5$,Tyr(Me)^2,Thr^4,Tyr-NH$^9_2$]OVT. Fig. 2A demonstrates that the vasopressin V_1 receptor antagonist, but not the vasopressin V_2 receptor antagonist or the oxytocin receptor antagonists, significantly reduce the vasopressin V_2 receptor agonist deamino-D-vasopressin-induced sensitization. Similarly, Fig. 2B demonstrates that vasopressin V_1 receptor antagonist, but not the vasopressin V_2 receptor or the oxytocin receptor antagonist, could significantly reduce the vasopressin metabolite [pGlu^4,Cyt^6]vasopressin induced sensitization.

The findings that the vasopressin V_2 receptor antagonist did not block the specific vasopressin V_2 receptor agonist (deamino-D-vasopressin)-induced sensitization led us to further evaluate the ability of a more selective vasopressin V_2 receptor antagonist ([d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin) to block deamino-D-vasopressin-induced sensitization. Fig. 3 (A1) clearly demonstrates that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin also failed to block deamino-D-vasopressin-induced sensitization. The finding that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin did not block deamino-D-vasopressin-induced sensitization and the observation that the motor disturbances of the animals which received the vasopressin V_2 receptor antagonist appeared even more severe than those which received a pretreatment with deamino-D-vasopressin preceded by saline led us to investigate the possibility that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin itself may act as an agonist to induce sensitization. Fig. 3 (A2) demonstrates that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin itself can sensitize the animals to vasopressin-induced motor disturbances. Here again, the unexpected finding that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin could act as an agonist in the sensitization paradigm lead us to further test the possibility that the vasopressin V_1 receptor antagonist may also block the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin-induced sensitization. Fig. 3 (A3) demonstrates that the vasopressin V_1 receptor antagonist is effective in blocking the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin-induced sensitization.

The findings that the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin could act as an agonist in the sensitization of vasopressin-induced motor disturbances and could also be blocked by the

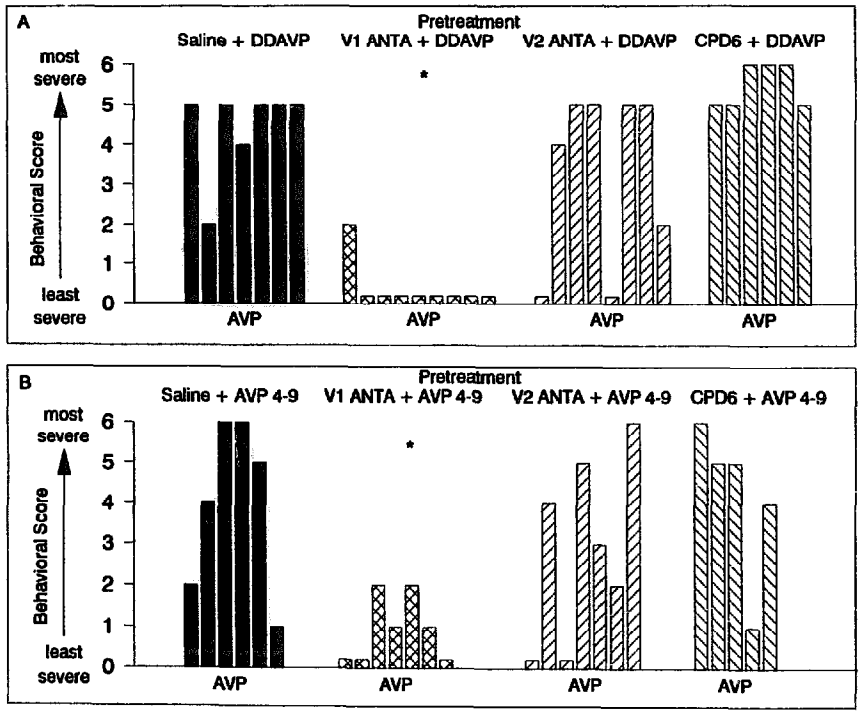

Fig. 2. Blockade of sensitization by specific receptor antagonists. In these experiments, four groups of animals received a pretreatment injection of deamino-D-vasopressin (DDAVP; 10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 7$, controls), the vasopressin V_1 receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Me)^2]vasopressin (V_1 ANTA; 100 pmol, $n = 9$), the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Et)^2,Val^4]vasopressin (V_2 ANTA; 100 pmol, $n = 8$) or the oxytocin receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Me)^2,Thr^4,Tyr-NH$^9_2$]OVT (CPD 6; 100 pmol, $n = 6$) (Fig. 2A). Another four groups of animals received a pretreatment injection of [pGlu^4,Cyt^6]vasopressin (AVP 4–9; 10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 6$, controls), the vasopressin V_1 receptor antagonist (V_1 ANTA; 100 pmol, $n = 7$), the vasopressin V_2 receptor antagonist (V_2 ANTA; 100 pmol, $n = 7$) or the oxytocin receptor antagonist (CPD 6; 100 pmol, $n = 5$) (Fig. 2B). 24 h later, the potency of vasopressin to induce motor disturbances was tested and scored. Refer to Fig. 1 for details. * Significant ($P < 0.05$, Mann-Whitney U-test) difference in motor responses between the control and treatment groups.

vasopressin V_1 receptor antagonist led us to test whether the other vasopressin V_2 receptor antagonists may also induce the sensitization process. Fig. 3 (B1) shows that the other vasopressin V_2 receptor antagonist can also sensitize the brain, and that this effect can be blocked with the vasopressin V_1 receptor antagonist. Because the vasopressin V_2 receptor antagonist acted as an agonist in the sensitization phenomenon, we also tested the possibility that the vasopressin V_2 receptor antagonist may in itself induce motor disturbances in sensitized animals. Fig. 3 (B2) demonstrates that in sensitized animals, when given on the second peptide exposure, the vasopressin V_2 receptor antagonist did not cause significantly enhanced motor disturbances.

3.2. Sensitization process and endogenous vasopressin activity

In these studies, we tested the possibility that the sensitization phenomenon may be reflected in an activation of vasopressin gene expression. We tested this hypothesis by determining the relative amount of vasopressin message expressed per cell as well as the number of cells expressing vasopressin messenger RNA (vasopressin mRNA) at several times following a pretreatment with an i.c.v. injection of vasopressin in three brain areas containing vasopressin cell bodies, including the bed nucleus of the stria terminalis, the medial amygdala and the paraventricular nucleus of the hypothalamus. These vasopressinergic nuclei are known to project to areas thought to be involved in the sensitization process (Naylor et al., 1985). Fig. 4A shows that although there is a tendency for the number of vasopressin-expressing cells in the bed nucleus of the stria terminalis to increase, 24 h following an vasopressin injection, no significant differences were found either in the number of cells or in the relative amount of grains/cells in the bed nucleus of the stria terminalis or the medial amygdala. There are also no significant differences in the total area covered by silver grains or in the intensity of hybridization per unit area (relative brightness) in the paraventricular nucleus of the hypothalamus. To further investigate the possibility that vasopressin mRNA alterations may be more apparent

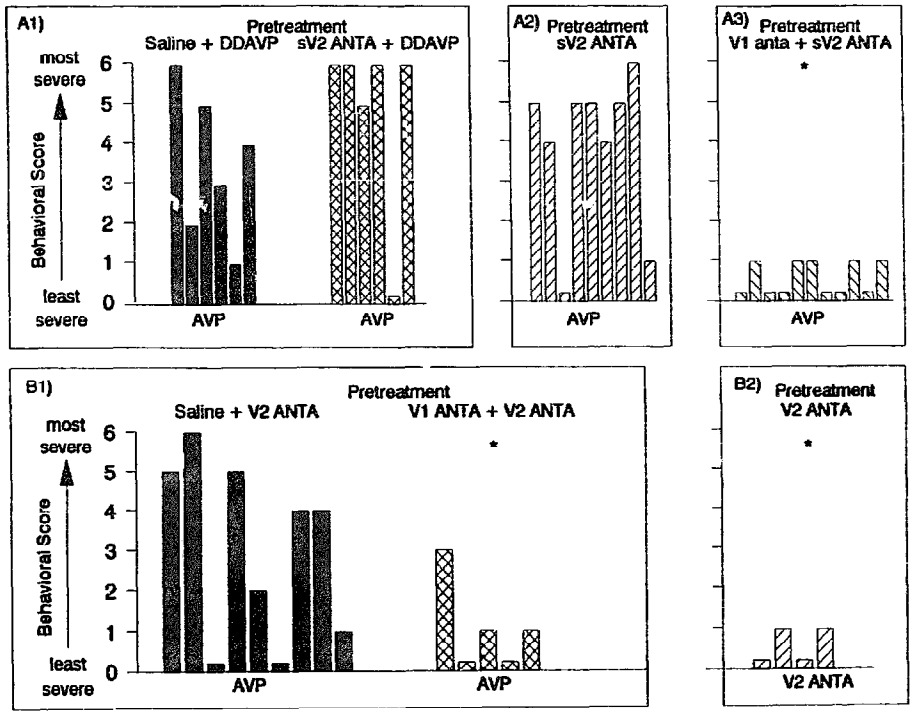

Fig. 3. Vasopressin V_2 receptor antagonist and sensitization. Panel A1: in these experiments male Sprague Dawley rats received a pretreatment injection of deamino-D-vasopressin (DDAVP; 10 pmol) preceded (2 min prior) by an injection of either saline (5 μl, $n = 6$) or the selective vasopressin V_2 receptor antagonist ([d(CH$_2$)$_5^1$,D-Ile^2,Ile^4]vasopressin) (sV$_2$ ANTA; 100 pmol, $n = 6$); panel A2: an additional group of animals received a pretreatment with the selective vasopressin V_2 receptor antagonist ([d(CH$_2$)$_5^1$,D-Ile^2,Ile^4]vasopressin alone (sV$_2$ ANTA; 10 pmol, $n = 9$); panel A3: another group of animals was given a pretreatment injection of the selective vasopressin V_2 receptor antagonist [d(CH$_2$)$_5^1$,D-Ile^2,Ile^4]vasopressin (sV$_2$ ANTA; 10 pmol, $n = 11$) preceded (2 min prior) by an injection of the vasopressin V_1 receptor antagonist (V_1 ANTA; 100 pmol). 24 h later, the potency of vasopressin (AVP) to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin and scored (see Fig. 1 for details). Panel B1: in these experiments rats received a pretreatment of 10 pmol of the vasopressin V_2 receptor antagonist, [d(CH$_2$)$_5^1$,Tyr(Et)^2,Val^4]vasopressin (V_2 ANTA) preceded (2 min prior) by an injection of either saline (5 μl, $n = 9$) or the vasopressin V_1 receptor antagonist (V_1 ANTA; 100 pmol, $n = 5$). 24 h later, the potency of vasopressin to induce motor disturbances was tested following an i.c.v. injection of 10 pmol vasopressin (AVP). Panel B2: an additional group of animals ($n = 4$) was given, on the both the first and the second (24 h later) peptide exposures, 10 pmol of the vasopressin V_2 receptor antagonist (V_2 ANTA) to determine if it could induce motor disturbances as well as sensitize. * Significant ($P < 0.05$, Mann-Whitney U-test) difference in motor responses between the control and treatment groups.

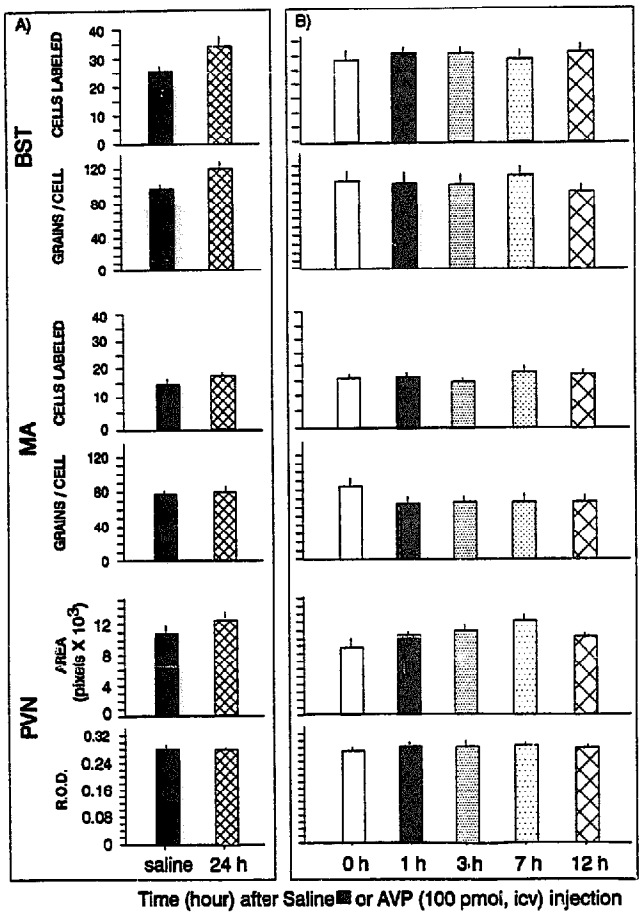

Fig. 4. Sensitization process and endogenous vasopressin activity. In a first series of experiments (Fig. 4, panel A) we investigated vasopressin mRNA gene expression in the brain 24 h after a saline ($n = 6$, controls) or an vasopressin ($n = 6$) injection. In a second series of experiments (panel B), we investigated the time course development of vasopressin sensitization by analyzing vasopressin mRNA gene expression at 0 ($n = 8$), 1 ($n = 7$), 3 ($n = 6$), 7 ($n = 4$) and 12 ($n = 6$) h after a first vasopressin injection. Each bar graph represents the mean ± S.E.M. of six to eight animals for the number of cells and grains/cell in the and medial amygdala and the Area and relative brightness (1 – relative optical density) in the paraventricular nucleus of the hypothalamus from emulsion-coated slides. Cells were labeled by the ^{35}S-labeled oligonucleotide complementary bases coding for the last 16 amino acids of the vasopressin mRNA. For each animal two sections were quantified bilaterally using a digitized image analysis system.

at an earlier time point, a second series of experiments was performed. Vasopressin mRNA levels were measured at 0 ($n = 8$), 1 ($n = 7$), 3 ($n = 6$), 7 ($n = 4$) and 12 ($n = 6$) h after a first vasopressin injection. Fig. 4B shows no significant differences in the number of cells or in the relative amount of grains/cells in the bed nucleus of the stria terminalis and the medial amygdala at any of the time points analysed. Similarly, there are no significant differences in the total area covered by silver grains or in the intensity of hybridization per unit area (relative brightness) in the paraventricular nucleus of the hypothalamus at any of the time points studied.

Taken together, these results suggest that, under the present conditions, an i.c.v. injection of vasopressin does not appear to result in a significant alteration in the expression of vasopressin mRNA in vasopressin-containing cells of the bed nucleus of the stria terminalis, medial amygdala or paraventricular nucleus of the hypothalamus up to 24 h after an injection.

4. Discussion

Sensitization of a response can be defined as the processes which facilitate an organism's response to the repeated presentation of a stimulus. Vasopressin is involved in a sensitization process in which vasopressin increases the responsiveness of the brain to itself upon repeated exposure. The availability of an array of ligands related structurally to vasopressin provides a diverse set of pharmacological tools for the study of the pharmacological characteristics of vasopressin-induced sensitization. In this study, we demonstrated that animals pretreated with vasopressin or vasopressin analogs, including deamino-D-vasopressin, [pGlu^4, Cyt^6]vasopressin, [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH_2, des-Gly-NH^9_2-vasopressin, Pro-Leu-Gly-NH_2 and Pro-Arg-Gly-NH_2 became markedly hyper-responsive to the motor effects of a subsequent challenge dose of only vasopressin, but not vasopressin-related peptides. Using a series of available neurohypophysial antagonists, we demonstrated that the V_1 antagonist [d(CH$_2$)$^1_5$,Tyr(Me)^2]vasopressin but not the vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Et)^2,Val^4]-vasopressin, or the more selective vasopressin V_2 receptor antagonist [d(CH$_2$)$^1_5$,D-Ile^2,Ile^4]vasopressin or the oxytocin receptor antagonist [d(CH$_2$)$^1_5$,Tyr(Me)^2, Thr^4,Tyr-NH^9_2]OVT blocked vasopressin analog-induced sensitizations, including those induced by deamino-D-vasopressin and [pGlu^4,Cyt^6]vasopressin. Furthermore, while both vasopressin V_2 receptor antagonists were ineffective in blocking the induction of sensitization, both acted as agonists in the sensitization phenomenon when given on the first peptide exposure. Both vasopressin V_2 receptor antagonist-induced sensitizations were also blocked by the vasopressin V_1 receptor antagonist. Thus these data clearly demonstrate that, while many vasopressin analogs sensitize the rat brain to vasopressin-induced motor disturbances, only vasopressin can induce the enhanced motor responses. These findings indicate that the sensitization phenomenon induced by vasopressin and vasopressin-related peptides and the sensitized response induced by vasopressin appear to be controlled by different determinants.

The mechanism underlying the sensitization phe-

nomenon is not known but our investigation of vasopressin mRNA expression in sensitized animals indicate that the sensitization phenomenon does not appear to involve an increase in the expression of the mRNA. As there is now good evidence that increased mRNA expression reflects activation of neurons (Fitzsimmons et al., 1992), our data would suggest that the sensitization agents were not acting simply to release endogenous stores of vasopressin. While we cannot discount the possibility that a possible presynaptic release of vasopressin (as has been described at a vasopressin V_2 receptor in septum; (Landgraf et al., 1991) may not be a sufficiently strong stimulus to activate the gene expression in the cell body, these results suggest that it is unlikely that vasopressin and vasopressin-related peptides induced sensitization via enhanced synthesis and release of endogenous vasopressin. Whatever the type of receptor which may be involved in the sensitization process, the actual cellular locus of this receptor appears unlikely to be on the endogenous vasopressin neurons.

While the pharmacological characteristics of the vasopressin sensitization process appears somewhat strange at first glance, it has similarities to that of a number of other central actions of vasopressin and vasopressin analogs. For example, vasopressin, [pGlu^4,Cyt^6]vasopressin, oxytocin, a vasopressin V_2 receptor agonist, deamino-D-vasopressin, and the vasopressin V_2 receptor antagonist, [d-(CH$_2$)$_5$[d-Ile^2,Ala^4]-vasopressin), have all been demonstrated to act as agonists in inducing intracellular accumulation of cAMP in cultured hippocampal neurons (Brinton and Brownson, 1993). Furthermore, the pharmacological characteristics of the development of physical dependence induced by vasopressin-related peptides to morphine are also similar to the pharmacological characteristics of vasopressin analog-induced sensitization in that the physical dependence to morphine is facilitated in rats by des-Gly-NH$_2^9$-vasopressin, vasopressin, oxytocin and the *C*-terminal fragments of these peptides Pro-Arg-Gly-NH$_2$ and Pro-Leu-Gly-NH$_2$, but not by pressinoic acid (Van Ree and De Wied, 1976). In yet another example, vasopressin analogs, including oxytocin and oxytocin fragments (Kovacs et al., 1979), have been shown to have vasopressin-like effects in facilitation of passive avoidance behavior, results which are similar to our findings that oxytocin (Poulin and Pittman, 1993b) and oxytocin analogs (this study) have vasopressin-like effects in sensitizing the motor actions of vasopressin. Therefore, while the pharmacological characteristics of the vasopressin sensitization phenomenon appear at variance with the specific activations of the enhanced motor disturbances via a V_1 receptor, they are similar to other neuromodulatory actions of vasopressin and vasopressin analogs in the brain.

The observation that a wide variety of vasopressin analogs induced the sensitization phenomenon could indicate that the sensitization phenomenon is a non-receptor-mediated event. However, neither pressinoic acid nor saline were effective sensitizing agents. Furthermore, the observation that the vasopressin V_1 receptor antagonist, [d(CH$_2$)$_5^1$,Tyr(Me)^2]vasopressin, was able to block sensitization phenomenon induced by vasopressin as well as that induced by [pGlu^4,Cyt^6]-vasopressin and deamino-D-vasopressin suggests that the sensitization phenomenon is a receptor-mediated event. Although this could be interpreted as meaning that the blockade of vasopressin sensitization by the V_1 antagonist is non-specific in nature, there are other studies (Thornhill and Pittman, 1990) that indicate that actions of non-neurohypophysial-related compounds are not blocked by this vasopressin V_1 receptor antagonist and that it is without activity in itself; these observations argue against possible non-specific, neurodepressant actions of [d(CH$_2$)$_5^1$,Tyr(Me)^2]vasopressin.

The observation that the vasopressin V_1 receptor antagonist can block a wide variety of vasopressin analog-induced sensitizations could be interpreted as meaning that the vasopressin V_1 receptor antagonist binds to some yet unknown vasopressin-like receptors, in brain tissue. Indeed, as more work is done with this antagonist, it has become clear that it can bind more than the classical V_1 vasopressin receptor (Di Scala-Guenot and Strosser, 1992). In further support of this, it was recently demonstrated (Brinton et al., 1986) that the vasopressin V_1 receptor antagonist [d(CH$_2$)$_5^1$,Tyr-(Me)^2]vasopressin blocked vasopressin, [pGlu^4,Cyt^6]-vasopressin and oxytocin induced 3H-IP1 accumulation in cultured hippocampal neurons. Similarly, De Wied et al. (De Wied et al., 1991) observed that the vasopressin V_1 receptor antagonist could block the influence of neurophyophysial hormones, including vasopressin, vasopressin 4–8, oxytocin and [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH$_2$, on avoidance behavior.

Furthermore, evidence is available to support the hypothesis that several vasopressin analog receptors exist in brain tissue. For example, [pGlu^4,Cyt^6]-vasopressin binding sites have been reported in brain (Brinton et al., 1985, 1986; De Kloet et al., 1985; Jurzak et al., 1993). The oxytocin fragment Pro-Leu-Gly-NH$_2$ has also been found to have specific binding sites in the brain (Zadina et al., 1982). Szot et al. (1989) have also reported a unique pharmacological profile for vasopressin receptors in the cingulate gyrus of rat pups during development. A novel type of oxytocin receptor with equal binding ability for oxytocin, the vasopressin V_1 receptor antagonist and the vasopressin V_2 receptor agonist has been described on astroglial cells (Di Scala-Guenot and Strosser, 1992). Additional possibilities that other, not yet characterized, vasopressin receptors exist in brain tissue is further sug-

gested by the work using deamino-D-vasopressin, the classical vasopressin V_2 receptor agonist. For example, despite the physiological and pharmacological evidence in support of vasopressin V_2 receptor existence in the brain, there has been no biochemical or molecular (Ostrowski et al., 1992) evidence of vasopressin V_2 receptor in the brain. Furthermore, the demonstration that deamino-D-vasopressin can behave in vitro as a vasopressin V_1 receptor antagonist makes it difficult to reconcile with the concept that there are only two types of vasopressin receptors (Vallotton, 1991). Electrophysiological studies also indicate the presence of a novel receptor responsive to arginine vasotocin in the rat brain (Ingram and Tolchard, 1994). Thus, the observations that several vasopressin-related peptide receptors are present in brain tissue, that vasopressin-related peptides can induce the sensitization phenomenon, and that the vasopressin V_1 receptor antagonist is able to block the sensitization phenomenon induced by vasopressin and vasopressin-related peptides leads us to suggest that a novel type of vasopressin receptor may be involved in the sensitization process.

While the present data concern a possible pathological expression of vasopressin action (i.e. motor disturbances), the sensitization has also been reported for a number of other transmitter actions of vasopressin, including those on fever (Poulin and Pittman, 1993c) and cardiovascular (Poulin et al., 1994; Lebrun et al., 1989) regulation. Thus the sensitization phenomenon reported herein may have relevance to experience-and time-dependent alteration in fever (i.e. tolerance). Similarly, sensitization of the vasopressin receptor involved in the central cardiovascular control may implicate vasopressin in such diseases as hypertension.

Vasopressin and its analogs have been shown to act as neuromodulators of neuroadaptive phenomena such as learning and memory (De Wied et al., 1991), and maintenance of functional tolerance to ethanol (Szabo et al., 1991). It is interesting to note here that the sensitization phenomenon described here and the neuromodulator actions of vasopressin (Szabo et al., 1991; De Wied et al., 1991) have similar pharmacology. Thus, the sensitization effect may be seen as a neuromodulatory action of vasopressin and vasopressin-related peptides on the neurotransmitter-like action of vasopressin in brain. It is interesting to note that the neuromodulatory actions of vasopressin appear to be dependent on interaction with CNS receptors which recognize deamino-D-vasopressin, [pGlu^4,Cyt^6]vasopressin, Pro-Leu-Gly-NH_2, Pro-Arg-Gly-NH_2, oxytocin, [pGlu-Asn-Cys(Cys)]Pro-Leu-Gly-NH_2, the vasopressin V_2 receptor antagonist as agonists and the vasopressin V_1 receptor antagonist as antagonist. Such a receptor has not yet been identified in the rat brain.

Acknowledgements

Supported by MRC (Canada) and NIH Grant 20311. P.P. was an MRC Fellow and Q.J.P. an Alberta Heritage Foundation for Medical Research Scientist and a MRC Senior Scientist.

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