The role of arginine vasopressin (AVP) dialyzed into the hippocampus or caudate nucleus as the reference structure in the acquisition and extinction of the conditioned eyelid reflex in rabbit was investigated. Phonopneumatic stimulator was used for the generation of conditioned and unconditioned stimuli, and for control of the recorder. Opto-electronic sensor transduced the behavioral responses. Microdialysis probes were chronically implanted into the brain structures. AVP was dialyzed into the brain structures during the extinction procedure. Restraining of the process of extinction was shown during AVP dialysis through the hippocampus and caudate nucleus but the effect in hippocampus was stronger and longer lasting than in caudate nucleus. The influence of AVP dialyzed through the hippocampus on the course of acquisition was biphasic. Some insignificant improvement of learning was observed at the beginning of training and then compensatory, significant restraining of learning. After AVP dialysis through the caudate nucleus only the late, insignificant tendency to improve learning was shown. The effects of AVP were dose-dependent in inversely proportional manner and long-term in nature, especially the effects in hippocampus.

Key words: conditioned eyelid reflex, hippocampus, caudate nucleus, arginine vasopressin, microdialysis of the brain structures, rabbit.

INTRODUCTION

Vasopressin (AVP) released intracerebrally may act as a neuromodulator in addition to its peripheral endocrine function. It affects several types of animal behavior directly related to learning and memory, such as active avoidance (1, 2), passive avoidance (3, 4), social recognition (5, 6) and spatial navigation — water maze procedure (7). Moreover, AVP improved memory in healthy volunteers (8, 9) and in treatment of human memory disorders (for review see 10). However, some researchers have been unable to demonstrate memory — improving actions of AVP in animals (11, 12) and in human studies (13, 14).

Various brain structures have been studied as possible sites of action of AVP on memory processes. The hippocampus seems to be the most effective region for action of the endogenous (15) and exogenous AVP (2) on memory processes.
Classical conditioning of the rabbit nictitating membrane reflex (NMR) or eyeblink reflex (ER) has been widely studied to elucidate the neurobiology of learning and memory. The neuronal circuitry that mediates the ER has been well established (16). The hippocampus plays not essential but a modulatory role in this form of learning. Even though the hippocampus is not necessary for classical eyelid conditioning in rabbit, hippocampal neurons are thought to be involved in learning this task (17).

The purpose of the present study was the evaluation whether AVP administered by microdialysis into the hippocampus during the extinction of the conditioned eyelid reflex changes the course of this process. It is believed that microdialysis causes less tissue damage than alternative in vivo push-pull technique (18).

The caudate nucleus was investigated as the reference structure for hippocampus. It is a structure, possibly important for conditional motor reflexes (19) including conditioned rabbit nictitating membrane reflex (20). Some other evidence suggests that the caudate nucleus may be related to a wider range of processes including perception, learning and memory (21).

**MATERIAL AND METHODS**

Adult male white New Zealand rabbits weighing above 3 kg were used. Rabbits were kept in separate cages and maintained on a 14-h light and 10-h dark cycle with continuous access to food and water. Animals were stereotaxically implanted with a plexiglass headpiece with guide cannulae: two for the caudate nuclei and two others for the hippocampi (22). Hippocampus was dialysed in 11 and caudate nucleus in 10 conscious animals. All experiments were carried out in accordance with the NIH guide for care and use of the laboratory animals.

*Surgery of the headpiece implantation*

Rabbits were premedicated with a subcutaneous injection of atropine sulphate (1.0 mg per animal) and anaesthetized with intravenous pentobarbital (30 mg/kg). After reaching deep surgical anaesthesia the animal was mounted on the stereotaxic frame (23). The procedure of headpiece with guide cannulae implantation and microdialysis of brain structures was described in detail by Traczyk et al. (22). Two guide cannulae leading to the caudate nuclei and two for hippocampi and cannula leading to the 3rd cerebral ventricle were implanted. The tip of the 3rd ventricle cannula was used as a reference point. Guide cannulae leading to the caudate nuclei and to the hippocampi were positioned 6 mm and 8 mm, respectively laterally on either side of the sagittal
zero plane. After the surgery each rabbit received an intramuscular injection of 100,000 IU of benzylpenicillin potassium and 0.5 g streptomycin daily during five consecutive days.

**Procedure of the eyelid reflex conditioning**

A special device was designed for the acquisition and extinction of eyelid reflex and recording. The apparatus and whole procedure were described earlier (24). Briefly: the device generated periodic air puff and tones and controlled the recorder. Photoelectric transducer acting in close infrared converted the movements of rabbit’s eyelid into electric signals. When the rabbit’s eye was completely closed, maximum light emitted by an infrared light emitter was reflected from the eyelid and the maximal amplitude of the response was observed. One month after postoperative recovery (headpiece implantation) and one week before the conditioning procedure, the rabbits were accustomed to spending half an hour daily in a special box. A rabbit in the box was placed in a noise-attenuated, ventilated and illuminated camera. A holder common for the air puff nozzle and for the photoelectric transducer was mounted on the rabbit’s headpiece. Standard procedures of paired training for classical conditioning of the rabbit eyelid response was used (25). The unconditioned stimulus (US) was a 100-ms corneal air puff exerting a pressure of 0.2 kg/cm² and conditioned stimulus (CS) was a 70 dB, 450 ms, 1 kHz tone, that began 350 ms prior to the air puff onset and finished simultaneously with it. The CS and US co-terminated, resulting in a delay conditioning paradigm. The intertrial interval averaged 22 s. Each daily session of acquisition (A) consisted of 120 trials. During the extinction sessions (E) only 120 conditioned stimuli (tones) were applied. Conditioned responses were calculated as a percentage of all 120 conditioned stimuli applied during one-day session. Rabbits learned to a criterion of about 80% of conditioned responses and were overtrained to 5 days. After the acquisition training animals underwent 5 days of extinction. Such pattern of training was repeated two times – see Table 1.

**Microdialysis procedure**

The microdialysis probes CMA/Microdialysis AB (Sweden) were used (Cat.No. 8309504). The polycarbonate membrane O.D. of 0.5 mm and length of 4 mm had a molecular cut off of approximately 20,000 Daltons. The outlet tubing was cut shorter to a length of 100 mm, that is 1/3 of the inlet tubing length. After the probe implantation perfusion of the hippocampus or caudate nuclei was performed with degassed 0.9% NaCl or with AVP solution (see Experimental protocol and Table 1) at a rate of 1 µl/min using a syringe pump.
for simultaneous multiple infusions, model SP 220i, World Precision Instruments. Before the in vivo experiments passover of AVP through dialysis probe was determined in vitro using $^{125}$I AVP. The mean percent passover after reaching steady state was calculated as 10.2 ± 8.2% (mean ± SE, n = 5).

Experimental protocol

To achieve presumed level of learning preliminary conditioning training was done (see Table 1). Then the run of the acquisition and extinction was sufficiently regular and stable to the consecutive comparative studies. The microdialysis probe was implanted into the chosen structure. The next day

Table 1. The course of proceeding during microdialysis of the rabbit hippocampus. In the case of the caudate nucleus dialysis the experiments were performed in the same way except the NaCl-3 and AVP 50.0 group.

<table>
<thead>
<tr>
<th>day of the experiment</th>
<th>procedure (group of variables)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1– 5</td>
<td>acquisition</td>
</tr>
<tr>
<td>6–10</td>
<td>extinction</td>
</tr>
<tr>
<td>11-15</td>
<td>acquisition</td>
</tr>
<tr>
<td>16–20</td>
<td>extinction</td>
</tr>
<tr>
<td>21</td>
<td>implantation of the microdialysis probe</td>
</tr>
<tr>
<td>22–26</td>
<td>acquisition and dialysis of the hippocampus with 0.9% NaCl</td>
</tr>
<tr>
<td>27–31</td>
<td>extinction and dialysis of the hippocampus with 0.9% NaCl (control E), Fig. 3</td>
</tr>
<tr>
<td>32–36</td>
<td>acquisition and dialysis of the hippocampus with 0.9% NaCl (control A), Fig. 2</td>
</tr>
<tr>
<td>37–41</td>
<td>extinction and dialysis of the hippocampus with AVP in concentration of 0.05 µg/ml (AVP 0.05), Fig. 3</td>
</tr>
<tr>
<td>42–46</td>
<td>acquisition and dialysis of the hippocampus with 0.9% NaCl (NaCl-1), Fig. 2</td>
</tr>
<tr>
<td>47-51</td>
<td>extinction and dialysis of the hippocampus with AVP in concentration of 0.5 µg/ml(AVP 0.5), Fig. 3</td>
</tr>
<tr>
<td>52–56</td>
<td>acquisition and dialysis of the hippocampus with 0.9% NaCl (NaCl-2), Fig. 2</td>
</tr>
<tr>
<td>57–61</td>
<td>extinction and dialysis of the hippocampus with AVP in concentration of 5.0 µg/ml(AVP 5.0), Fig. 3</td>
</tr>
<tr>
<td>62–66</td>
<td>acquisition and dialysis of the hippocampus with 0.9% NaCl (NaCl-3), Fig. 2</td>
</tr>
<tr>
<td>67-71</td>
<td>extinction and dialysis of the hippocampus with AVP in concentration of 50.0 µg/ml (AVP 50.0), Fig. 3</td>
</tr>
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</table>
microdialysis of the structure with 0.9% NaCl solution was started simultaneously with the acquisition training and then simultaneously with the extinction (control A and control E respectively). After this control microdialysis, AVP solutions were dialysed through the hippocampus or caudate nucleus during the extinction training. Four concentrations of AVP were used in case of the hippocampus microdialysis (0.05; 0.5; 5.0 and 50.0 μg/ml – AVP 0.05, AVP 0.5, AVP 5.0 and AVP 50.0 groups of variables, respectively). AVP was dissolved in 0.9% NaCl solution and infused through the Hamilton syringe with the rate of 1 μl/min during all time of extinction procedure, that is, about 45 minutes. The amounts of AVP delivered via dialysis probes were estimated at about 0.2; 2.0; 20.0; 200.0 ng. After each extinction training in the presence of successive concentrations of AVP, the acquisition procedure was performed with 0.9% NaCl solution dialyzed through the brain structure (NaCl-1, NaCl-2 and NaCl-3 groups of variables in case of the hippocampus). Table 1 summarizes the course of proceeding in the

Fig. 1. Frontal sections of rabbit brain in P8 (on the right — hippocampus) and A1 (on the left — caudate nucleus) and the headpiece with position of microdialysis probe. Arrows indicate outflow and inflow of the fluid perfusing the microdialysis probe. Stilettes in the guide cannulae are shown contralaterally to the probes. Plane modified from the Sawyer et al. atlas.
case of hippocampus dialysis. In the case of caudate nucleus dialysis experiments were performed in the same way except the NaCl-3 and AVP 50.0 groups.

Following the experiments the probe position in the brain structure was marked by 10% Ianus Green microdialysis and rabbit’s brain was fixed with 10% formalin solution, frozen with solid CO₂ and cut parallel to the frontal plane. Correct positioning of the microdialysis probe and cannula in the 3rd cerebral ventricle was checked in sections under the stereomicroscope. Dialyzed places in the hippocampus and caudate nucleus are shown in Fig. 1.

Statistics

Analysis of variance (ANOVA) involving the factors of day and group with repeated measures on day factor followed by the test of least significant differences (LSD) was used to compare percentage of responses in all groups of variables. Percentage of responses was subjected to a 2x arcsine square-root transformation according to the formula: $2 \arcsin \sqrt{p}$. Differences were considered significant if $P < 0.05$.

RESULTS

Influence of AVP dialyzed into the hippocampus on the acquisition and extinction

Analysis of acquisition data yielded a significant effect of day ($F_{4,120} = 4.19$), $P = 0.003$. Both effect of group and day by group interaction were not significant. Mean percentage of conditioned responses observed during acquisition after AVP dialysis into the hippocampus had smaller values than in control data during all five days of training and after all applied doses of AVP except the value on the 1st day in NaCl-1 and NaCl-2 group and on the 5th day in NaCl-3 group of variables (Fig. 2). But only the value reached on the 4th day in NaCl-2 group (80.9 ± 3.9%) was significantly smaller than adequate value in control (88.9 ± 2.1%), $P = 0.026$. It is suggested that the least (0.05 μg/ml) and the middle (0.5 μg/ml) doses of AVP dialyzed into the hippocampus during the 5 consecutive days preceding acquisition had a slight improving action on learning on the 1st day and a slight inhibitory, as if compensatory, effect on the progress of learning on consecutive days. The course of acquisition after the higher dose (5 μg/ml) of AVP infusion was similar to the control course.

Figure 3 shows the course of extinction in control and AVP groups of variables. There was a significant effect of day factor ($F_{4,176} = 61.35$), $P = 0.000$. Effects of group as well as day by group interaction were not significant. AVP
Fig. 2. Influence of AVP pre-dialyzed into the hippocampus on the course of acquisition of the conditioned eyelid reflex in rabbit during 5 days of training. Means ± SE of 6–11 variables in 11 animals in each group. Asterisk (*) indicates significant difference as compared to the untreated control in this day.

Fig. 3. Influence of AVP dialyzed into the hippocampus on the course of extinction of the conditioned eyelid reflex in rabbit during 5 days of training. Means ± SE of 8–11 variables in 11 animals in each group. Asterisk (*) indicates significant difference as compared to untreated control in each day.
dialyzed into the hippocampus changed the course of extinction and the values of percentage responses but in a different manner depending on AVP concentration. In AVP 0.05 group AVP considerably stopped the effect of extinction, i.e. progressive forgetting of the learned task, beginning from the 1st day of extinction. Differences between percentage of responses on the successive days of extinction (1 v. 2 and 2 v. 3) were not significant in this group of variables in contrast with control group. Beginning from the 3rd day values of percentage responses were significantly higher than corresponding values of controls, because AVP treated rabbits remembered the learned task better than control ones. In AVP 0.5 group restraining of forgetting was observed between the 2nd and the 3rd day of extinction. Values of percentage responses from the 3rd to the 5th day were significantly higher than corresponding values in control. The courses of extinction in the next two groups of variables: AVP 5.0 and AVP 50.0 were very similar as in the control. It seems that AVP dialyzed into the hippocampus during the course of extinction prevented forgetting of the learned task – eyelid conditioned reflex. Two concentrations of AVP – 0.05 µg/ml and 0.5 µg/ml were the most effective. The effect diminished with increasing concentration of AVP. It seems that the effect of AVP was inversely proportional to the applied peptide dose.

Influence of AVP dialyzed into the caudate nucleus on the acquisition and extinction

Analysis of variance showed a significant effect of day factor during the acquisition training ($F_{4,104} = 2.58$), $P = 0.042$. Effects of group factor as well as day by group interaction were not significant. Some progress in learning appears in the NaCl-2 group from the 3rd day of acquisition (Fig. 4). In this group of variables AVP applied into the caudate nucleus slightly, not significantly, increased the learning abilities in rabbits.

Fig. 5 presents the course of extinction in control and AVP groups of variables. The effect of day factor was statistically significant in this group of variables, $P = 0.000$. However, the effects of group as well as day by group interaction were not significant. AVP dialyzed into the caudate nucleus prevented the great decrease of percentage responses on the 3rd day in AVP 0.05 and AVP 0.5 groups of variables because there were no significant differences between the values achieved on the 2nd and on the 3rd day of training in these groups. Moreover, mean percentage of responses observed during the whole 5 day training had greater values in AVP 0.05 and AVP 0.5 groups than appropriate values in control group, but only the values obtained on the 3rd day of extinction in these groups were significantly greater than the value observed on the 3rd day in control group. It seems that AVP infused into
Fig. 4. Influence of AVP pre-dialyzed into the caudate nucleus on the course of acquisition of the conditioned eyelid reflex in rabbit during 5 days of training. Means ± SE of 9—10 variables in 10 animals in each group.

Fig. 5. Influence of AVP dialyzed into the caudate nucleus on the course of extinction of the conditioned eyelid reflex in rabbit during 5 days of training. Means ± SE of 9—10 variables in 10 animals in each group. Asterisk (*) indicates significant difference as compared to untreated control in this day.
the caudate nucleus in concentration of 0.05 \( \mu g/ml \) and 0.5 \( \mu g/ml \) somehow restrained the process of forgetting of the learned task, but only temporarily. This restrained process of forgetting renewed after the four days of extinction. AVP in concentration of 5.0 \( \mu g/ml \) dialyzed into the caudate nucleus not significantly accelerated forgetting of the learned task.

**DISCUSSION**

Summarizing the effects of AVP dialyzed through the hippocampus and caudate nucleus on the course of acquisition and extinction of the eyelid reflex it may be stated that the influence of AVP dialyzed through the hippocampus is more evident than its influence in the caudate nucleus. Moreover, the course of extinction is more changed than the course of acquisition. The direction of changes observed during the extinction sessions were the same in the hippocampal and the caudate nucleus groups of variables, that is restraining of the process of extinction was observed when AVP in concentrations of 0.05 and 0.5 \( \mu g/ml \) was applied. A similar effect of AVP on the course of extinction was observed earlier in mice and in rats (26, 27, 2). Peripherally injected AVP increased the resistance of conditioned passive avoidance reaction to a sharp extinction in mice (27), and lysine vasopressin microinjected into the ventral hippocampus stopped the extinction of active avoidance conditioned reflex in rats (2), like AVP i.c.v injected after the last learning session (26). An increased resistance to extinction in the present experiments occurred because AVP somehow strengthened the associative bond between the tone and the air puff shock, maybe because of increased excitability of hippocampal neurons treated with AVP (28, 29).

The influence of AVP on the course of acquisition appeared to be a little complicated. Some improvement of learning was observed at the beginning of training and following compensatory restraining after AVP dialysis through the hippocampus. Thus, a biphasic effect of AVP was observed. It was earlier stated that animal experience prior to AVP treatment and the level of proficiency in doing the learned task modify the effects of the treatment (30, 31). Whatever time AVP was intrahippocampally injected in mice — pre-session or post-session, little improving, deleterious or no effect was observed in the learning of a visual discrimination task (31). In the present experiment AVP was dialyzed through the hippocampus always before the acquisition training. When AVP was administered pre-session in Paban et al. experiments, deleterious learning was noted just in the further sessions not in the 1st session of learning (31). The present findings support Paban’s et al. notion. Moreover, AVP is supposed to affect memory processes by its metabolites (32). Different phases of information processing can be selectively
affected by putative brain metabolites of administered AVP, sometimes more or less active than mother molecule of AVP, during 5 day of acquisition training in the present studies. After AVP dialysis through the caudate nucleus only the late, not significant tendency to improve learning was shown.

Histological verification of the probe tips showed the great spatial distribution of the dye dialyzed through the brain structure. The spatial distribution of radiolabeled AVP was determined in the rat brain (33). It was found within an area of approximately 6 mm (frontal-caudal), 3 mm (sagittal) and 3.5 mm (dorsal-ventral) after 30 min of dialysis. Probably in the present experiments AVP diffused into both dorsal and ventral parts of the hippocampus and to more remote locations. Some comparative studies indicated the greater involvement of the ventral than dorsal hippocampus in the behavioral expression of AVP (32, 34, 35). However, the study of AVP influence on the locomotor activity revealed that when AVP was injected into the dorsal hippocampus the peptide failed to alter the locomotor activity in mice. When the treatment was performed into the ventral hippocampus a reduction of locomotor activity was recorded (35). A hypothesis exists that motor changes might contribute to any observed behavioral effects of treatment as the non-specific factor. This wide distribution of peptides applied to distinctive brain areas is the generally existing disadvantage and must be taken into consideration during discussion of the results.

The effects of AVP were dose-dependent in an inversely proportional manner and long-term in nature, especially the effects in hippocampus.

Behavioral effects of AVP are mediated mainly by AVP receptors. At least three receptor subtypes for AVP (V_{1a}, V_{1b} and V_{2}) have been identified (36). A chronic exposure to agonist results in a down-regulation of the target receptor system. This may be the reason for the decreased responsiveness of rabbit brain structures on rising amounts of AVP.

Caudate nucleus up to now has not been considered to be the site of AVP action in memory processes. The idea that caudate nucleus may be the site of AVP action may be supported by the fact that AVP was found in caudate nucleus of the rat brain, in amounts about twice as high as in hippocampus (37). Although V_{1a} receptors are absent in the caudate — putamen complex in the rat brain (38), oxytocin binding sites were revealed (36). But cerebral oxytocin (OXT) receptors discriminate rather poorly between AVP, OXT and vasotocin. Results of the study with receptors’ antagonists suggest the existence of a separate neurohypophyseal hormone receptor complex in the brain affecting memory processes that differs from the peripheral V_{1}, V_{2} and OXT receptor (4).

In summary, the hippocampus appears to be related to learning and memory processes in rabbits by AVP.
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