Vasopressinergic modulation of stress responses in the central amygdala of the Roman high-avoidance and low-avoidance rat

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(Accepted 16 June 1992)

Key words: Central amygdaloid nucleus; Arginine-8-vasopressin; Oxytocin; Heart rate; Immobility; Conditioned emotional stress; Electric footshock; Roman high-avoidance rat; Roman low-avoidance rat; Genetic difference

The central nucleus of the amygdala (CEA) is selectively involved in the passive component of the behavioral (immobility) and the accompanying parasympathetic response during conditioned, stressful environmental challenges. Vasopressinergic mechanisms in the brain seem to play a role in these stress responses. The effects of the neuropeptides arginine-8-vasopressin (AVP) and oxytocin (OXT) on modulating CEA activity during conditioned stress of inescapable footshock were studied in male Roman high-avoidance (RHA/Verh) and low-avoidance (RLA/Verh) rats, psychogenetically selected on the basis of shuttle-box acquisition behavior. In RLA/Verh rats, the cardiac and behavioral responses to the conditioned emotional stressor were bradycardia and immobility, suggesting an important role for the CEA in these rats. The RHA/Verh rats, however, failed to show any change in heart rate or immobility in response to a conditioned stress situation. The low dose of AVP (20 pg) in the CEA of conscious RLA/Verh rats caused an enhancement of the stress-induced bradycardiac and immobility response. However, the high dose of AVP (2 ng) and OXT (200 pg) attenuated the bradycardiac and immobility responses in the RLA/Verh rats. Infusion of AVP and OXT in the RHA/Verh rats failed to induce any change in heart rate or immobility. Binding studies revealed that the AVP receptor selectively binds AVP with high affinity. In contrast, the OXT receptor recognizes both AVP and OXT with a similar (but lower) affinity. This suggests that the behavioral and autonomic responses of the high dose of AVP may be caused by OXT receptor stimulation. In conclusion, on the basis of the present results one may hypothesize that CEA differences in AVP and OXT innervation and/or receptor densities may contribute to the differences in coping strategy found in these animals.

INTRODUCTION

The central nucleus of the amygdala (CEA) is differentially involved in the conditioning and retention of aversively-motivated learning tasks. In contrast to a general, non-selective involvement in conditioning, the CEA seems to have a very selective role in the retention of stress-related learning tasks. In fact, the involvement of the CEA in retention is thought to be restricted to parasympathetic (bradycardia) and passive behavioral (immobility) components only, and not to sympathetic, neuroendocrine, and active behavioral stress responses. In view of these results, the role of the CEA during retention seems to be predominantly related to coping of a passive type.

A number of experiments indicate that animals may differ in their preferential coping reaction in response to a stressor. Roman high-avoidance (RHA/Verh) and low-avoidance rats (RLA/Verh) are psychogenetically selected for superior and inferior active shock avoidance acquisition, respectively, in a two-way shuttle-box. Numerous behavioral and physiological studies have indicated that this difference in avoidance acquisition is primarily due to emotional factors rather than learning ability [see, refs. 15, 16]. For example, RLA/Verh rats show greater cardiac, plasma adrenocorticotropic, corticosterone and prolactin responses, and defecation than that of RHA/Verh rats in various stressful situations. In conditioned emotional stress situations like fear of inescapable footshock or in response to novelty, the RLA/Verh rats react with a bradycardiac response and immobility, whereas the RLA/Verh rats fail to react in this way. The behav-
ioral and physiological responses of the RLA/Verh rats to environmental challenges seem to be a passive coping strategy, while the RHA/Verh rats cope actively. Therefore, one may suggest that the CEA plays an important role in the differences in coping strategy found between the RLA/Verh and RHA/Verh rats. Indeed, Henke\textsuperscript{22} showed differences in multiple unit activity in the CEA during restraint stress between both lines.

A variety of different neuropeptide-containing pericarya and afferent axon terminals have been localized in the CEA\textsuperscript{3,9,20,21,28,44}. Evidence exists for an important role for some of these neuropeptides in function (Roozendaal et al., in preparation)\textsuperscript{23,33}. For example, arginine-8-vasopressin (AVP) and oxytocin (OXT) administered into the CEA, under stress-free resting conditions, have been shown to evoke heart rate and plasma corticosterone responses concomitant with changes in behavioral activity. Circulating catecholamine concentrations were not changed following local AVP and OXT infusion (Roozendaal et al., in preparation). The response pattern suggests that both peptides have a modulatory role on amygdaloid influences during retention rather than during acquisition.

The present experiments were designed to investigate to what extent the vasopressinergic mechanism in the CEA is involved in the active or passive coping style. Therefore, local infusions of AVP and OXT were administered to males of both the RLA/Verh and RHA/Verh lines, prior to the retention test in a conditioned emotional stress paradigm of inescapable electric footshock, and behavioral and physiological indices were determined.

MATERIALS AND METHODS

Animals

Thirty-three adult (10–15 weeks old) male RHA/Verh and 31 RLA/Verh rats (Wistar-derived), weighing 300–380 g, were used. They were obtained from a breeding colony maintained at the Behavioral Biology Laboratory (Zürich, Switzerland). The rats were housed in individual clear perspex home cages (25 × 25 × 30 cm) in a temperature-controlled environment at 21 ± 1°C under a 12-h light–dark cycle (lights on from 08.30 to 20.30 h). The experiments were carried out during the light period of the cycle (between 10.00 and 14.00 h). Food and water were available ad lib in the home cages.

Apparatus

A step-through type passive avoidance apparatus, as described by Ader et al.,\textsuperscript{1} was used to investigate the stress-related behavioral and cardiac responses. Briefly, the apparatus consisted of a dark compartment (40 × 40 × 40 cm) connected via a small opening to an elevated, well-lit platform. A sliding door could prevent access between the platform and the dark compartment. The floor of the dark compartment was made of stainless steel bars through which a scrambled electric footshock could be delivered. A waiting cage (25 × 25 × 30 cm) with a sawdust-covered floor was placed close to the passive avoidance apparatus in a sound- and light-attenuated experimental room.

Surgery

Surgery was performed under ether anaesthesia. Bilateral permanent stainless steel brain cannulae (length: 18.0 mm, o.d. 0.3 mm, and i.d. 0.15 mm) for drug infusion were stereotactically implanted into the central amygdala (coordinates: 6.7 mm rostral to interaural, lateral 4.0 mm to the midline, and ventral 6.2 mm below dura) according to Paxinos and Watson\textsuperscript{41}. The cannulae were permanently fixed to the skull of the animal by means of stainless steel screws and dental cement. Furthermore, all animals received two electrodes made of standard paper-clip to record the electrocardiogram (ECG). These electrodes were implanted transcutaneously, one between the scapulae and the other in the middle of the back\textsuperscript{5}.

Recording and analysis of the ECG

The ECG of freely moving rats was monitored telemetrically by means of a miniature FM transmitter (model SNR 102 F, Dynamic Electronics Ltd., London, UK) as described earlier\textsuperscript{6}. The transmitter was attached to a Velcro strap which was secured around the chest of the rat. The transmitter was connected to the transcutaneous electrodes. The transmitted signals were received on a commercial FM receiver, amplified with half-amplitude cut-off frequencies at 10 and 100 Hz (Grass Pr CR preamplifier) and stored on tape.

For off-line analysis, the ECG signals were processed through a cardiograph pulse generator which generated a square wave pulse at each R-wave. The time between the onset of two consecutive pulses, the interbeat interval (IBI), was measured by a personal computer (Olivetti M24). IBIs falling within the range of 100–220 ms have been selected for computing the mean IBI of each 1-min sample period. Bradycardia was considered to be an increase in mean IBI. Decrease in mean IBI indicated tachycardia. During both recording and analysis the quality of the ECG signals was continuously monitored on an oscilloscope.

Drug treatment

Animals were infused with either artificial cerebrospinal fluid (CSF), AVP (20 pg or 2 ng), or OXT (200 pg) (Organon Int., Oss, The Netherlands) to a total volume of 1 μl during an 8-min period, 30 min prior to the start of an experiment. The doses were selected on the basis of previous infusion studies, in which the physiological and behavioral responsiveness of several doses of both peptides were tested (Roozendaal et al., in preparation).

Experimental design

After transportation of the rat to the experimental room, the strap holding the transmitter was fixed around the chest of the rat. Five preshock trials were given. Each trial started with placing the rat into the waiting cage for 1 min. Only on the first trial was this followed by a 3-min adaptation to the dark compartment. Immediately afterwards, the rat was placed onto the illuminated platform and allowed to enter the dark. The sliding door was closed afterwards and the rat stayed another 3 min in the dark. On the afternoon of day 1, the morning and afternoon of day 2, and the morning of day 3, this procedure was repeated. During these trials, the rat was directly placed from the waiting cage onto the illuminated platform and, after entering, the animal was allowed to stay in the dark for 5 min. During the last trial, both the cardiac response and behavior were recorded (preshock measurement). Microinfusions into the CEA with artificial CSF or one of the drugs were given 30 min prior to testing the rats. The ECG signal was sampled three times: during 1 min in the waiting cage (Pw), and during the first (P1) and fifth (P5) min on the grid floor of the dark compartment. The duration of immobile behavior was scored during a 3-min sampling period between the 2 and 4 min in the dark compartment by direct observation. Immobility was defined as being motionless, but alert.

After a 6-day wash-out period, another trial was given in which each rat received the unavoidable aversive stimulus of a scrambled footshock (0.6 mA, AC for 3 s) immediately after entering the dark compartment. Shock thresholds in both lines of rats have been demonstrated to be identical\textsuperscript{17}. The rat was removed from the dark 60 s after termination of the footshock. One day later, the cardiac and behavioral consequences of the emotional stressor (learned fear...
of the inescapable stressor) were investigated. Once again, an infusion with one of the drugs was given 30 min before the postshock measurement. Each rat received at random CSF or a drug to avoid effects of repetitive administration of the same drug. The rat was placed into the waiting cage for 1 min and, subsequently, transferred to the grid floor of the dark compartment with a closed sliding door for 5 min (forced exposure). The ECG and behavior was sampled again using the same procedure as described for the preshock measurement. No further footshock was administered.

Histology
At the completion of cardiac and behavioral testing, the animals were deeply anaesthetized with sodium pentobarbital (90 mg/kg i.p.) and perfused with saline followed by a 4% formaldehyde solution. The brain was removed from the skull and submerged in the same fixative for at least 24 h. Subsequently, frozen sections of 40 μm were cut and the cannula placement was examined on unstained sections.

Statistics
The Mann-Whitney U test (two-tailed, corrected for ties) was used to test behavioral differences. Cardiac data of the rats were evaluated for significance using analysis of variance with repeated measures (ANOVA). The repeated measures ANOVAs were followed by Student's t-test. A probability level of $P < 0.05$ was taken as statistical significance for all tests.

RESULT

None of the animals had to be excluded from further analysis because of improper cannulae placement.

Cardiac stress responses in RLA/Verh and RHA/Verh rats
With respect to the preshock responses after central amygdaloid CSF infusion, ANOVA failed to reveal differences in IBI between the RLA/Verh and RHA/Verh rats. During the conditioned emotional test of inescapable footshock, an attenuated bradycardiac response was shown in the RHA/Verh rats compared to the RLA/Verh rats ($F_{1,22} = 7.20; P < 0.05$), with significant differences in the waiting cage (Pw; $P < 0.05$) and during the first min in the dark compartment (P1; $P < 0.01$). ANOVA showed a significant shock × time interaction in RLA/Verh rats after CSF administration ($F_{2,24} = 3.50; P < 0.05$) (Fig. 1A). Significant increases in IBI, i.e., bradycardia, were observed at Pw ($P < 0.05$) and P1 ($P < 0.01$). The conditioned stressor failed to alter heart rate in the counterpart RHA/Verh animals.

Immobility stress responses in RLA/Verh and RHA/Verh rats
Both strains of animals displayed mostly exploratory behavior during the preshock measurement. However, the RLA/Verh rats displayed significantly more immobile behavior in comparison with the RHA/Verh rats ($P < 0.01$). The percentages of time spent in immobility (± S.E.M.) for the RLA/Verh and RHA/Verh rats were 10.0 ± 2.1 and 2.8 ± 0.8, respectively. Also during the postshock measurement, a significant difference was found in percentage of time spent in immobile behavior between the RLA/Verh animals and the RHA/Verh rats ($P < 0.01$). Immobility was significantly increased in the RLA/Verh animals ($P < 0.01$), but not in the RHA/Verh rats compared with the counterpart preshock values (Fig. 1B).

Cardiac responses after AVP and OXT infusion into the CEA
During the preshock measurement, in both genetically selected lines, local infusion with AVP or OXT failed to alter IBI compared to their respective CSF-treated groups (Fig. 2A). During the postshock measurement, however, ANOVA showed a significant treatment effect on heart rate in the RLA/Verh rats ($F_{3,40} = 6.36; P < 0.005$), but not in the RHA/Verh rats (Fig. 2B). The low dose of AVP (20 pg) administered into the CEA of the RLA/Verh rats significantly
increased IBI, reaching significance at P1 (P < 0.05). In contrast, the high dose of AVP (2 ng) caused an opposite effect on IBI: the conditioned bradycardiac stress response was significantly attenuated during P1 (P < 0.05). A similar effect on the conditioned heart rate response was observed after local OXT infusion (200 pg) in the RLA/Verh animals. Significantly shorter IBIs were found at Pw (P < 0.01) and P1 (P < 0.05).

Immobility responses after AVP and OXT infusion into the CEA

Infusions with either AVP or OXT failed to change the time spent in immobile behavior during the preshock measurement in both the RLA/Verh and RHA/Verh rats (Fig. 3A). However, postshock CEA manipulation with either AVP or OXT caused significant changes in conditioned stress-induced immobility responses in the RLA/Verh and RHA/Verh rats (Fig. 3B). After microinfusion with the low dose of AVP, in both lines of animals, a significant increase in time spent in immobile behavior could be observed compared to the shocked CSF-treated rats (both P < 0.05), although the total time of immobility in the RHA/Verh animals remained marginal. In the RLA/Verh rats, but not in the RHA/Verh rats, an attenuation of immobile behavior was observed after infusions with either the high dose of AVP or with OXT. The time spent in immobile behavior decreased in both drug-treated groups compared to their respective CSF-treated controls (both P < 0.01).

DISCUSSION

The two psychogenetically selected rat lines used in the present experiments showed a different pattern of autonomic and behavioral responses in relation to the conditioned emotional stress to inescapable footshock. The conditioned response of the RLA/Verh rats was characterized by an elevated parasympathetic, i.e., bradycardiac, outflow, concomitant with immobile behavior. In contrast, the RHA/Verh rats failed to show conditioned bradycardia and immobility. Significant conditioned stress responses could already be observed in the waiting cage, suggesting that the rat generalizes his fear response to the whole experimental procedure. A positive correlation between the time spent in immobile behavior and the magnitude of the bradycardiac response to a conditioned emotional stressor of fear of inescapable footshock was also found in individual
Wistar rats\(^{29,30}\). The differential response patterns found in the RLA/Verh and RHA/Verh rats fit into this individual variation pattern. The conditioned parasympathetic and passive behavioral stress responses in the Wistar rats could be disrupted by lesions in the CEA\(^{34,36}\). Accordingly, these findings suggest that a differential CEA function may contribute to the differences in expression of conditioned stress responses.

AVP, locally applied at a low dose (20 pg), evoked an augmented, conditioned parasympathetic outflow, accompanied by an increase in immobility. In contrast, higher doses of AVP (2 ng), similar to OXT administration, attenuated these conditioned bradycardiac and passive behavioral stress responses in the RLA/Verh line of rats. These dose-dependent responses following AVP infusion are possibly due to a differential preference of AVP for V1a vasopressin and OXT receptors in the CEA. Receptor binding studies have shown that the CEA contains both types of receptors\(^{41,43}\). V1a receptors are predominantly found in the anterior part of the CEA, whereas OXT receptors are mostly found posteriorly. V1a receptors only bind AVP. OXT receptors, however, not only recognize OXT, but they also bind AVP with a comparably high affinity, but with a lower affinity than V1a receptors\(^{2,18,27}\). This suggests that AVP administration in the low dose leads to a selective stimulation of V1a receptors, whereas AVP infused in a high dose, or OXT, activates amygdaloid OXT receptors. This biphasic dose–response curve of AVP was also shown after infusion of this peptide under stress-free resting conditions. The effects of administration of 2 ng AVP on heart rate and behavioral activity were similar to the effects of OXT, and could effectively be blocked by a selective OXT receptor antagonist (Roozendaal et al., in preparation). The low dose of AVP (20 pg) evoked opposite effects. In contrast, administration of several doses of OXT affected CEA output in a monotonic way. Thus activation of the V1a receptor in the CEA seems to augment the activity of this nucleus, leading to a facilitation of behavioral and physiological patterns, representative of passive coping. In contrast, OXT receptor stimulation is thought to diminish CEA output.

The CEA has a scattered AVP innervation, possibly originating from cell bodies in the bed nucleus of the stria terminalis (BNST)\(^{13}\). The number of AVP-immunoreactive cell bodies in the BNST, and fibers arising from this nucleus, have been shown to be dependent on circulating levels of testosterone\(^{14,42}\). In mice genetically selected for aggression, AVP immunoreactivity in a number of brain areas has been shown to be dependent on their aggressiveness. Non-aggressive mice have a higher density of AVP-immunoreactive fibers in the lateral septum, but also in the CEA, than aggressive ones\(^{10}\). Regarding the general involvement of the physiological patterns that underlie the difference in aggression\(^{4,5}\), it seems justified to suggest that the difference in behavioral response in these mice is not restricted to aggression, but extends to other challenging situations, i.e. they differ in behavioral strategy. Additional evidence exists which shows that the RLA/Verh and RHA/Verh also differ in aggression. The RHA/Verh rats show significantly more offensive behavior towards an opponent, than do RLA/Verh rats\(^{16}\). Thus, coping in non-aggressive mice may be comparable to the RLA/Verh rat's strategy and, similarly, the aggressive mice to RHA/Verh rats. This suggests a similar difference in AVP innervation in the CEA in these rat lines, where the RLA/Verh rats should have a higher density in the CEA than the RHA/Verh rats, although immunocytochemical studies have never been performed in these lines of rats. Moreover, homozygous Brattleboro (diabetes insipidus) rats, genetically lacking in vasopressin production, have been shown to have a disrupted conditioned immobility response to inhibitory stress\(^{40}\). These studies reinforce the notion that AVP plays a crucial, general role in passive coping strategy, and the CEA may be an important site. Besides AVP innervation, the number of V1a receptors may also be essential to this phenomenon. That AVP administration was only able to modulate CEA output in the passive coping RLA/Verh rats suggests that these animals have a higher density of V1a receptors than do RHA/Verh rats. On the basis of the present results, one may hypothesize that differences in densities of AVP and OXT fibers and/or receptors in the CEA are causally related to the differences in coping strategy found in these animals.

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