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Amygdaloid Noradrenaline Is Involved in the Sensitization of the Acoustic Startle Response in Rats

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FENDT, M., M. KOCH AND H.-U. SCHNITZLER. *Amygdaloid noradrenaline is involved in the sensitization of the acoustic startle response in rats.* PHARMACOL BIOCHEM BEHAV 48(2) 307-314, 1994. – The present study examined the role of noradrenaline (NA) in the central nucleus of the amygdala (cA) in the sensitization of the acoustic startle response (ASR) in rats. In the first experiment, local microinjections of 0, 0.5, 1, 2 nmol of the α_2-adrenergic antagonist yohimbine into the cA increased the magnitude of the ASR in a dose-dependent way. In the second experiment, foot shocks were applied to increase the ASR amplitude (sensitization). Local microinjections of 0, 4, 8, 16 nmol of the α_2-adrenergic agonist ST-91 into the cA dose dependently decreased the sensitizing effects of foot shocks on the amplitude of the ASR. It is conjectured that yohimbine increases and ST-91 decreases local NA release by acting at presynaptic autoreceptors. The present data suggest that the release of NA in the cA is involved in the mediation of the sensitizing effects of foot shocks on the ASR.

Acoustic startle response Amygdala Anxiety Fear Foot shocks Noradrenaline Rat
Sensitization ST-91 Yohimbine

THE acoustic startle response (ASR) is a simple behavior that can be elicited by loud acoustic stimuli with rapid onset. The ASR has a minimal response latency of 5-8 ms, as measured electromyographically in head and neck muscles (8,9), suggestive of a relatively simple circuitry. The neural substrate mediating the ASR is already fairly well understood and consists of an oligosynaptic cochleospinal pathway with the caudal pontine reticular nucleus (PnC) as an important sensorimotor interface (18,39,42,50,51,76). In addition, the PnC has been described as the recipient of modulatory input from the amygdala (33,40,63) and from the pedunculopontine tegmental nucleus (41). The ASR amplitude is regarded as a measure for fear and anxiety in mammals (21). The present study focuses on neurochemical aspects of the well documented role of the amygdala in the mediation of the sensitizing effects of foot shocks on the ASR. It has been shown that electrolytic lesions of the cA, or its efferent pathway to the brain stem, prevent the sensitization of the ASR by foot shocks (33). However, the neurochemical determinants of this effect are largely unknown as yet.

Various studies using microdialysis (67,70) or postmortem neurochemical analysis of transmitters (10,66,68) showed an enhancement of the NA release in the amygdala following stress and aversive stimuli such as foot shocks, as well as after treatment with anxiogenic drugs. Injections of NA into the amygdala facilitate aversive learning, and injections of the β-adrenergic blocker propranolol prevented this effect of NA (47) and impaired aversive learning (14,31,32). Systemic and intracerebroventricular injections of α_2-adrenergic agonists decreased the ASR amplitude and enhanced the habituation of the ASR (45), whereas injections of α_2-adrenergic antagonists increased the magnitude of the ASR in rats (17,36) and humans (56) and enhanced the effects of fear conditioning (16). Therefore, amygdaloid NA can be considered to be important for the occurrence of fear-related processes.

The objective of the present study was to investigate the role of NA in the cA in the sensitization of the ASR in rats. α_2-adrenergic antagonists have been shown to increase the NA release in different regions of the brain by blocking presynaptic α_2-adrenergic autoreceptors (44,65). Likewise, α_2-adrenergic agonists decreased the NA release by stimulating the α_2-adrenergic autoreceptors, as shown by the microdialysis technique (2,22,46,73). Because the α_2-adrenergic receptors in the cA are located presynaptically for the most part (71), it

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can be assumed that injections of the α_2-adrenergic antagonist yohimbine into the cA increase the NA release, whereas the α_2-adrenergic agonist ST-91 (a chemical analog of clonidine) should reduce, or block the amygdaloid NA release. Two experiments were carried out: first, we injected yohimbine into the cA of unrestrained rats, and measured the amplitude of the ASR. Second, we injected ST-91 into the cA immediately after administration of foot shocks to the rats, and subsequently measured the ASR amplitude.

METHOD

Animals

The experimental animals were 41 male Wistar rats, weighing 200–280 g at the beginning of the experiments. They were housed in groups of six under a continuous light/dark cycle (lights on from 0700–1900 h). Food and water were freely available.

Surgery

The animals were anesthetized with chloral hydrate (420 mg/kg IP) and placed in a stereotaxic frame. Two 23 gauge stainless steel guide cannulae were implanted bilaterally into the brain aiming at the cA [bregma −2.8 mm caudal, ±4.3 mm lateral, −7.6 mm ventral, according to the coordinates of Paxinos and Watson (59)]. The guide cannulae were fixed to the skull with dental cement and three anchoring screws. After surgery and between the experiments, stylets were inserted into the guide cannulae to maintain patency. The animals were allowed to recover from surgery for 1 week. They were handled 2 days before testing began.

Apparatus

For the measurement of the amplitude of the ASR, the rat was placed in a wire mesh cage (20 × 10 × 12 cm) mounted on a digital balance (Sartorius L2200 S) inside a sound-attenuated chamber (100 × 80 × 60 cm). The rat's movements caused deflections of the balance that were digitized and fed into a computer for further analysis. The startle eliciting acoustic stimuli (10 kHz tone bursts, 100 dB SPL, 20 ms duration including 0.4 ms rise and fall times, 30 s interstimulus interval), and a continuously presented white background noise (50 dB SPL, RMS) were delivered through a loudspeaker that was mounted at a distance of 40 cm from the test cage. The whole-body startle amplitude was calculated from the peak-to-peak voltage output of the balance within a time window of 80 ms after the onset of the startle stimulus.

Foot shocks were administered through a floor grid consisting of steel bars spaced approximately 15 mm apart. The foot shocks were produced by a shock generator (custom made at the University of Tübingen) located outside the chamber. Ten foot shocks (0.6 mA, 500 ms duration) were administered at a rate of 1/s.

Testing Procedure

The rats received bilateral injection cannulae (30 gauge stainless steel tubing) aiming at the cA and were placed in the wire mesh cage. The injection cannulae were connected to two microliter syringes (1 μl, SGE) by a length of flexible PVC tubing. After 5 min time to accommodate to the startle chamber, the rats received 25 startle stimuli. These trials served to produce a stable baseline of the ASR amplitude and were not considered for further analysis. The mean ASR amplitude to the next 15 trials was taken as the pretreatment value. The first group of animals (n = 26) received bilateral injections of the α_2-adrenergic antagonist yohimbine HCl (17-hydroxy-yohimban-16-carboxylic acid methyl ester hydrochloride; RBI, MA) in concentrations of 0, 0.5, 1, and 2 nmol (dissolved in saline; pH = 7) on 4 days in a pseudorandom order. The second group (n = 15) received foot shocks and 10 s later bilateral injections of the α_2-adrenergic agonist ST-91 HCl (2,6-diethyl-N-2-imidazolidinylidene-benzenamine hydrochloride; generously supplied by Boehringer Ingelheim, Germany) in concentrations of 0, 4, 8, and 16 nmol (dissolved in saline; pH = 7) in a pseudorandom order. ST-91 is a chemical analogue of clonidine, where the chlorine atoms at the phenyl ring are substituted by ethyl groups. The injection volume was 0.5 μl and the rate of infusion was 0.1 μl/s. The injection

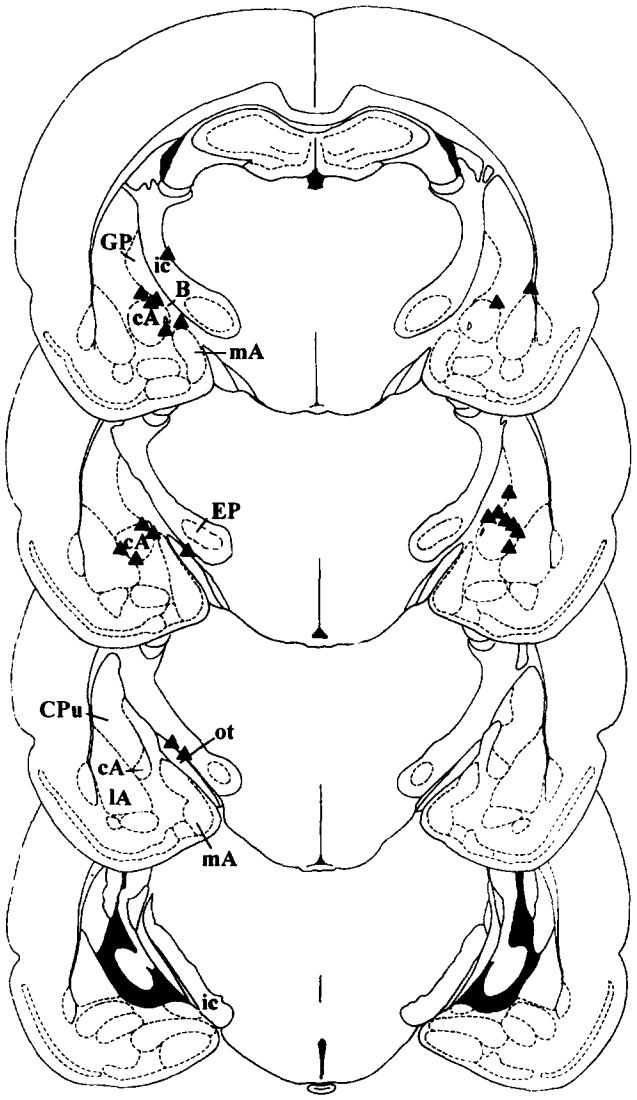

FIG. 1. Serial drawings of coronal sections of the rat brain showing the injection sites (▲) where yohimbine increased the amplitude of the ASR. Abbreviations—B: basal nucleus of Meynert; cA: central nucleus of the amygdala; CPu: caudate putamen; EP: entopeduncular nucleus; GP: globus pallidus; ic: internal capsule; IA: lateral nuclei of the amygdala; mA: medial nuclei of the amygdala.

cannulae remained in the brain during the test. The mean ASR amplitude between stimulus number 5 and 20 after the treatment was taken as the posttreatment value. The startle responses to the five stimuli after the treatment were discarded from the analysis according to Davis (19), who showed that the sensitizing effects of foot shocks are detected not until 2–4 min after presentation of the shocks. The absolute difference and the relative difference (percent change) between the pre- and the posttreatment values of the ASR amplitude were taken as the measure for the effect of the treatment. For statistical analysis of the percent difference scores and of the difference scores a one-way analysis of variance (ANOVA) was used, with post hoc Tukey's protected *t*-tests for pair-wise comparisons. For all statistical comparisons a $p < 0.05$ (two-tailed) was taken as the criterion for statistical significance.

Histology

After completion of the tests, the animals were sacrificed by an overdose of nembutal. The animals were decapitated, their brains were removed, and immersion fixed with 8% *p*-formaldehyde in phosphate-buffered saline with 20% sucrose. Coronal sections (60 μm) were taken on a freezing microtome and stained with cresyl violet. The injection sites were drawn onto plates taken from the atlas of Paxinos and Watson (59).

RESULTS

Injections of Yohimbine

An increase of the ASR amplitude was seen following bilateral injections of yohimbine into the cA, and following unilateral injections into the cA in cases where the contralateral cannula was blocked, and in cases where the contralateral injection was located in a fiber tract (optic tract or internal capsule). These cases were pooled ($n = 14$) for statistical analysis and are shown in Fig. 1. No significant differences [ANOVA; $F(3, 52) = 0.38$, $p = 0.77$] were found in the pre-injection ASR amplitudes (Table 1). The ASR amplitude decreased by 19 ± 5% following injections of saline into the cA due to habituation. Analysis of the mean percent change of the ASR amplitude (Fig. 2) revealed a significant effect of yohimbine, $F(3, 52) = 5.29$, $p = 0.0023$. Pair-wise comparisons of the effects of different doses of yohimbine yielded statistically significant differences between the effects observed after injections of saline and 2 nmol yohimbine (Tukey test; $t = 3.1688$, $p < 0.01$), as well as a statistically significant difference between 0.5 nmol and 2 nmol (Tukey test; $t = 3.447$, $p < 0.01$). A statistically significant increase of the ASR amplitude is also observed when difference scores are used [see Table 1; ANOVA; $F(3, 52) = 4.08$, $p = 0.012$]. No significant increase of the ASR amplitude was seen following misplaced injections of yohimbine [bilaterally located in the

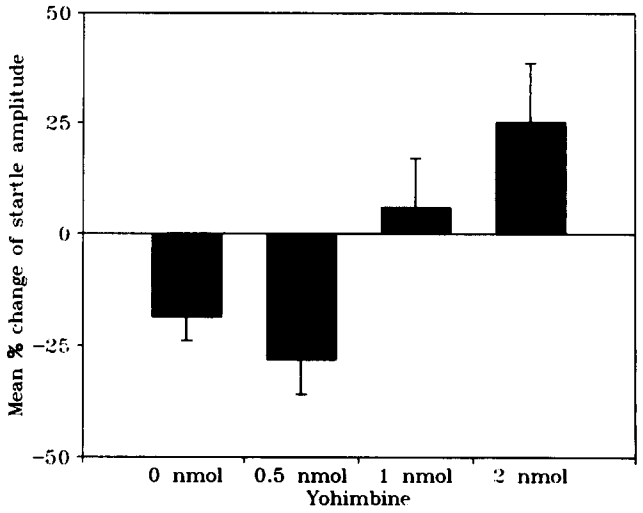

FIG. 2. Effect of yohimbine-injections into the cA on the ASR amplitude. The mean percent change (±SEM) of the startle amplitude after the yohimbine injections is shown. A percent score was computed for each rat, and these percent scores were summed to get the mean percent change for the group. A post hoc Tukey protected *t*-test reveals highly significant ($p < 0.01$) differences between injections of saline (0 nmol yohimbine) and 2 nmol yohimbine, and between injections of 0.5 nmol and 2 nmol, as well as a significant ($p < 0.05$) difference between injections of 0.5 nmol and 1 nmol yohimbine.

TABLE 1

EFFECTS OF YOHIMBINE INJECTIONS INTO THE CENTRAL NUCLEUS OF THE AMYGDALA ON THE STARTLE AMPLITUDE

Treatment (Yohimbine)	0 nmol	0.5 nmol	1 nmol	2 nmol
Mean startle amplitude (±SEM) preinjection	176 ± 28	196 ± 28	164 ± 25	159 ± 25
Mean startle amplitude (±SEM) postinjection	137 ± 23	159 ± 29	176 ± 34	179 ± 27
Difference (mean ± SEM)	−39 ± 13	−37 ± 13	12 ± 22*††	21 ± 11†‡
Percent difference (mean ± SEM)	−19 ± 5	−27 ± 7	6 ± 11††	27 ± 14†§

*$p < 0.05$ Significantly different from saline group (ANOVA followed by Tukey's *t*-test).
†$p < 0.01$ Significantly different from saline group (ANOVA followed by Tukey's *t*-test).
‡$p < 0.05$ Significantly different from 0.5 nmol yohimbine group (ANOVA followed by Tukey's *t*-test).
§$p < 0.01$ Significantly different from 0.5 nmol yohimbine group (ANOVA followed by Tukey's *t*-test).

globus pallidus (GP) or caudate putamen (CPu), data not shown], and following unilateral injections into the cA in cases where the contralateral cannula was located in the GP or CPu. These cases were also pooled (n = 12) and subjected to an ANOVA. A significant decrease of the ASR amplitude was observed following injections of yohimbine which missed the cA [ANOVA; $F(3, 44) = 2.83, p = 0.049$]. The decrease of the ASR amplitude observed after misplaced injections of saline (−9 ± 14%) did not differ from the decrease of the amplitude due to habituation after injections of saline into the cA (Student's t-test; $t = 1.65, p > 0.05$). Injections of 2 nmol yohimbine attenuated the ASR amplitude by 35 ± 6%. Pair-wise comparisons revealed that this decrease of the ASR amplitude was significantly different from the effects seen after injections of 0.5 nmol, which tended to increase the ASR amplitude by 6 ± 12% (Tukey test; $t = 2.2643, p < 0.05$) and of 1 nmol, which tended to increase the ASR amplitude by 14 ± 15% (Tukey test; $t = 2.706, p < 0.01$).

Injections of ST-91

The same procedure of pooling cases as described for yohimbine was used here, i.e., bilateral injections into the cA were taken as a group together with those cases where the cA was unilaterally injected, but where the contralateral injection was located within a fiber tract (n = 6). These injection sites are shown in Fig. 3. No significant differences were found in the preinjection ASR amplitudes [ANOVA; $F(3, 19) = 0.51$, $p = 0.68$; Table 2]. The effect of foot shocks and intraamygdaloid ST-91 injections are shown in Fig. 4. Foot shocks increased the ASR by 130 ± 65% (mean percent change ± SEM) if saline was injected into the cA. This effect of the foot shocks was dose dependently attenuated by ST-91 injections into the cA [ANOVA; $F(3, 19) = 3.6356, p = 0.0316$]. Pair-wise comparisons showed a significant reduction of the sensitization of the ASR by foot shocks after injections of 8 and 16 nmol ST-91 into the cA. Injections of 8 nmol ST-91 attenuated the ASR amplitude by 3 ± 11% despite of the application of foot shocks (Tukey test; $t = 2.6457, p < 0.05$; compared to saline). A total block of the sensitization by foot shocks occurred after injections of 16 nmol ST-91; in fact, the ASR amplitude decreased by 25 ± 9% (Tukey test; $t = 3.0799, p < 0.01$). This decrease of the ASR amplitude was nearly similar to the decrease of the ASR observed after a saline injection without foot shocks, which occurs as a result of short-term habituation (Student's t-test; $t = -0.9968$, $p = 0.3321$). Similar effects are observed when difference scores are used for the ANOVA, $F(3, 19) = 5.68, p = 0.006$. Injections of ST-91 into the GP or the CPu, or unilateral injections into the cA where the contralateral injection was located in the GP or the CPu, had no effect on the startle amplitude (data not shown). ANOVA, $F(3, 32) = 0.73, p = 0.5444$.

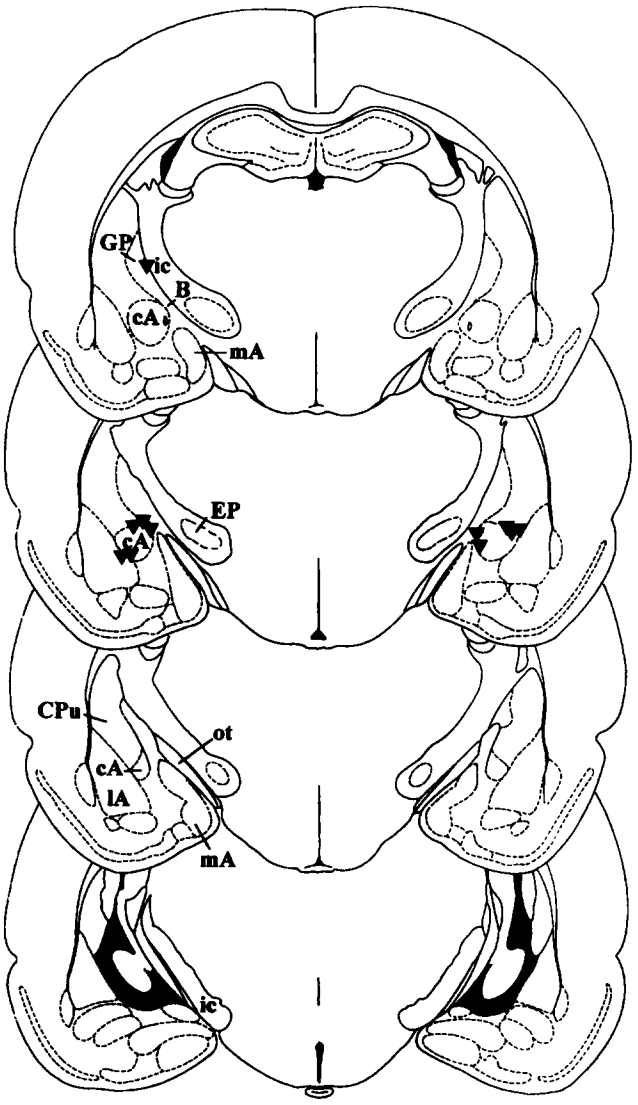

FIG. 3. Serial drawings of coronal sections of the rat brain showing the injection sites (▼) where ST-91 reduced or blocked the sensitizing effect of foot shocks. For abbreviations, see legend to Fig. 1.

DISCUSSION

In the present study, the effects of intraamygdaloid injections of the α_2-adrenergic antagonist yohimbine, and foot shocks together with injections of the α_2-adrengic agonist ST-91 into the cA on the ASR amplitude were investigated. Injections of yohimbine dose dependently increased the ASR amplitude. Injections of ST-91 dose dependently attenuated or blocked the sensitization of the ASR by foot shocks.

Studies using microdialysis in different brain areas have shown that yohimbine increases the NA release (2), and that clonidine, a structural analog of ST-91 (38,64), attenuates the NA release (2,46,73). The same effects were observed after application of other α_2-agonists and -antagonists (22,46,73). These data are supported by the effects observed after systemic application of yohimbine and clonidine (and other α_2-adrenergic agonists and -antagonists), which increase and decrease, respectively, the concentration of extraneuronal metabolites of NA in the amygdala (27). Presynaptic α_2-adrenergic receptors regulate the NA release from noradrenergic terminals by a negative feedback mechanism. It has been concluded that the effects of α_2-adrenergic drugs are exerted via blockade or activation of presynaptic autoreceptors. Activation of the α_2-adrenergic receptors leads to a reduction of the NA release; blockade of the α_2-adrenergic receptors increases the NA release (44,64,65). Therefore, it can be assumed that local injections of yohimbine and ST-91 into the

TABLE 2

EFFECTS OF ST-91 INJECTIONS INTO THE CENTRAL NUCLEUS OF THE AMYGDALA ON THE STARTLE AMPLITUDE

Treatment (ST-91)	0 nmol	4 nmol	8 nmol	16 nmol
Mean startle amplitude (±SEM) preinjection	175 ± 47	168 ± 25	202 ± 42	229 ± 41
Mean startle amplitude (±SEM) postinjection	240 ± 21	220 ± 31	184 ± 28	157 ± 12
Difference (mean ± SEM)	106 ± 33	52 ± 33	−18 ± 25*	−72 ± 36†‡
Percent difference (mean ± SEM)	130 ± 65	40 ± 26	−3 ± 11*	−25 ± 9†

*$p < 0.05$ Significantly different from saline group (ANOVA followed by Tukey's t-test).
†$p < 0.01$ Significantly different from saline group (ANOVA followed by Tukey's t-test).
‡$p < 0.05$ Significantly different from 4 nmol ST-91 group (ANOVA followed by Tukey's t-test).

cA mainly bind to α_2-adrenergic autoreceptors and regulate amygdaloid NA release. It should be noted, though, that central α_2-adrenergic receptors could also be located postsynaptically (57). However, there is evidence that the α_2-adrenergic receptors in the cA are predominantly located presynaptically. Lesions of the dorsal noradrenergic bundle resulted in a decrease of the α_2-adrenergic receptors in the cA, as measured by receptor autoradiography (71), while in other brain areas the density of the α_2-adrenergic receptors remained unchanged. In that study, it is emphasized that lesions of the dorsal noradrenergic bundle very likely only decreased α_2-adrenergic receptors in the amygdala which were located on the nerve terminals. Therefore, these findings suggest a presynaptic location of the α_2-adrenoceptors in the cA. This is further supported by the recent paper by Nicholas and coworkers (57) who showed by in situ hybridization that mRNA for different α_2-adrenergic receptors is very low in neurons of the cA. It is important to note that in situ hybridization most likely labels cell bodies only and not axons or dendrites. Because, on the other hand, several studies using autoradiography have shown a high density of α_2-adrenergic receptors in the cA (6,28,58,76), and because the presence of noradrenergic nerve terminals in the amygdala was shown by immunohistochemistry (26), these findings also suggest a predominantly presynaptic occurrence of the α_2-adrenergic receptors in the cA. An electrophysiological study has shown an inhibitory effect of clonidine on cA neurons (30).

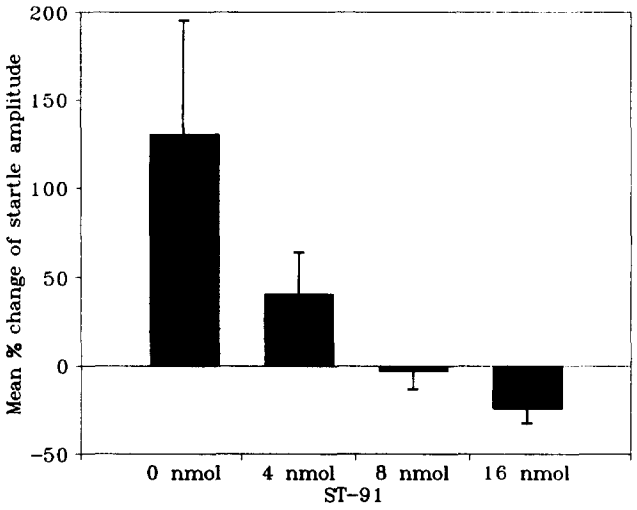

FIG. 4. Effect of ST-91-injections into the cA on the ASR amplitude after foot shocks. The mean percent change (±SEM) of the startle amplitude after the ST-91-injections is shown. A percent score was computed for each rat, and these percent scores were summed to get the mean percent change for the group. A post hoc Tukey protected t-test reveals significant ($p < 0.05$) differences between the injections of saline (0 nmol ST-91) and 8 nmol ST-91 and highly significant ($p < 0.01$) differences between the injections of 0 nmol and 16 nmol ST-91.

An increased release of NA in the amygdala has been observed after application of the anxiogenic β-carboline FG 7142 in rats, and this effect was blocked by pretreatment with clonidine. It has, therefore, been suggested that hyperactivity of NA systems is involved in the occurrence of anxiety and fear (34). Systemic injections of yohimbine have anxiogenic effects both in rats (35,60,74) and humans (56). These effects have been blocked in animal experiments by α_2-adrenergic agonists (35). In contrast, α_2-adrenergic agonists show anxiolytic effects (29). These data suggest that the anxiogenic effects of yohimbine are mediated through a blockade of α_2-adrenergic receptors, which leads to an increased NA release. The benzodiazepine-agonist diazepam did not antagonize the anxiogenic effect of yohimbine (60), while high concentrations of dopamine agonists blocked the yohimbine effect (35). Drug discrimination studies indicate that yohimbine also acts at 5-HT_{1A}-receptors if administered at a dose of 3 mg/kg (75). Because the anxiogenic effects have been observed at lower doses, in the studies cited above, it can be assumed that the anxiogenic effects of yohimbine are due to its action at α_2-adrenergic receptors, without affecting other transmitter systems. ST-91 has been reported to attenuate the nociception of rats after intrathecal injections (54,69). In contrast to many clonidine-like drugs, ST-91 has no hypotensive effects (38).

Several studies have investigated the effects of noradrenergic drugs on the ASR. Systemic application of α_2-adrenergic antagonists, e.g., yohimbine, increased the ASR amplitude in rats (36) and humans (56) and facilitated fear conditioning

by foot shocks (16). On the other hand, α_2-adrenergic agonists, e.g., clonidine, reduced the ASR amplitude (15), facilitated habituation (45), attenuated fear conditioning (16), and showed no effect on sensitization to background noise (15). Intracerebroventricular injections of ST-91, but not intrathecal administration, decreased the ASR magnitude (20,37) so that an action of ST-91 in the brain, and not in the spinal cord, can be assumed. Neurotoxic lesions with 6-hydroxydopamine of the locus coeruleus, which contains the greatest number of noradrenergic neurons in the brain, decreased the ASR amplitude (3). The findings of the present study support the hypothesis that an important site of action for NA for the modulation of the ASR is the cA. Moreover, the present data suggest that the sensitization of the ASR by foot shocks might be mediated by the release of NA in the cA. Because associative processes during sensitization of the ASR cannot be ruled out (5), it would be of interest to investigate the effects of intraamygdaloid NA on fear conditioning. The effects of NA in the cA on the acquisition or expression of conditioned fear has not been investigated in the fear-potentiated startle paradigm so far. Microinjections of propranolol, a β-adrenergic blocker, into the lateral nucleus of the amygdala showed no effect on the acquisition of conditioned fear (53), indicating that NA in the lateral amygdala is not important for fear conditioning. Moreover, fear conditioning, but not sensitization of the ASR, can be blocked by injections of the G-protein inhibitor pertussis-toxin into the basolateral nucleus of the amygdala (52). It has been suggested that fear conditioning occurs in the basolateral nucleus of the amygdala, possibly through long-term potentiation (13). This kind of enhanced neurotransmission is then transferred to the cA by intraamygdaloid pathways. Therefore, it can be concluded that fear conditioning is mediated by the lateral amygdala, while, sensitization of the ASR is mediated by the cA.

An important source of noradrenergic afferents of the cA is the locus coeruleus (4,25,26,43,55,61,62) and, therefore, it might be possible that the locus coeruleus is also involved in the effects reported in the present paper. The firing rate of the locus coeruleus neurons is increased by foot shocks (1,11), and the nucleus paragigantocellularis in the rostroventral medulla has been identified as the source of somatosensory input into the locus coeruleus (12). The data presented thus far suggest that foot shocks increase the activity of the nucleus paragigantocellularis via a spinoreticular pathway. The nucleus paragigantocellularis activates the locus coeruleus, which, in turn, would increase NA release in the cA.

The idea that the sensitization of the ASR by foot shocks is regulated by the release of NA in the cA, is further supported by two recent papers by Liang and co-workers (48,49). Intracerebroventricular injections of corticotropin releasing factor (CRF) resulted in long-lasting facilitation of the ASR. This effect could be blocked by intracerebroventricular injections of propranolol, or lesions of the cA. Local microinjections of CRF into the cA, however, did not facilitate the ASR. The findings by Liang and co-workers can be interpreted as an effect of CRF on the noradrenergic system. Several studies support the conception that intracerebroventricular CRF increased the ASR via an increase of NA release from the locus coeruleus: local injection of CRF into the locus coeruleus has anxiogenic effects (7) and increases the electrical activity (72) and NA release (23) from locus coeruleus neurons. Intracerebroventricular injections of CRF increases the NA level in several brain regions, including the amygdala (24).

In summary, the present data, together with several findings from the literature, strongly suggest that the release of NA in the cA plays a decisive role for the sensitization of the ASR by foot shocks.

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