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NMDA receptors in the pontine brainstem are necessary for fear potentiation of the startle response

Markus Fendt *, Michael Koch, Hans-Ulrich Schnitzler

Tierphysiologie, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

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Abstract

The fear-potentiated startle model in rats is a valuable animal test for the investigation of the neural and neurochemical basis of fear. In this model, rats are trained to associate a neutral stimulus with an aversive stimulus, so that after conditioning the conditioned stimulus alone elicits a state of fear leading to an exaggerated acoustic startle response. The fear-potentiated startle model does not require instrumental responding for the indication of states of fear. The acoustic startle response is mediated by a simple brainstem circuit, with the caudal pontine reticular nucleus as an interface that receives input from startle-enhancing circuits. In the present study, we tested the hypothesis that *N*-methyl-D-aspartate (NMDA) receptors on neurones of the caudal pontine reticular nucleus are involved in the mediation of fear-potentiated startle. After fear-conditioning, we injected the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (AP-5), into the caudal pontine reticular nucleus of awake rats and tested the effect on the expression of fear-potentiated startle. Injections of AP-5 (0.125–0.5 nmol) into the caudal pontine reticular nucleus dose dependently attenuated fear-potentiated startle without affecting the baseline amplitude of the acoustic startle response. The results suggests that, in the caudal pontine reticular nucleus, glutamate may mediate fear-potentiated startle via NMDA receptors.

Keywords: Acoustic startle response; Anxiety; AP-5; Caudal pontine reticular formation; Fear; Glutamate receptor

1. Introduction

The investigation of the neural basis of fear and anxiety is a prerequisite for the development of strategies to treat and cure anxiety disorders. A useful model to investigate the neurobiological mechanisms underlying fear and anxiety is the fear-potentiated startle model, initially described by Brown et al. (1951). In this model, rats are trained by Pavlovian conditioning to associate a neutral stimulus, such as a light, with an aversive stimulus, such as an electric footshock. After a few pairings the light alone predicts the occurrence of the shock and acts as a conditioned aversive stimulus, which elicits a state of fear, indicated by tachycardia, blood pressure elevation, freezing, corticosteroid release and an enhancement or potentiation of the amplitude of the acoustic startle response (Davis, 1992a). A series of experiments addressed the question of which brain pathways are involved in the acquisition and expression of fear. The amygdala plays an important role for the acquisition of the conditioned stimulus (Davis, 1992a,b) and different pathways from the amygdala to the caudal pontine reticular nucleus, a part of the primary startle circuit (Davis et al., 1982; Koch et al., 1992; Lingenhöhl and Friauf, 1994), are responsible for the expression of fear-potentiated startle (Davis et al., 1993; Yeomans and Pollard, 1993; Fendt et al., 1996a).

Previous studies demonstrated that glutamate is an important transmitter which is involved in the mediation and enhancement of the acoustic startle response: microinjections of glutamate receptor antagonists into the caudal pontine reticular nucleus of awake rats reduced the acoustic startle response (Krase et al., 1993; Miserendino and Davis, 1993) corresponding to the fact that microiontophoretic application of glutamate receptor antagonists inhibited the tone-evoked activity of neurones of the caudal pontine reticular nucleus (Ebert and Koch, 1992). These studies showed that AMPA/kainate receptor antagonists are more effective than NMDA receptor antagonists. The fact that the microiontophoretic application of the NMDA receptor antagonist, AP-5, blocked the excitatory effect of amygdala stimulation on the tone-evoked activity

* Corresponding author. Tel.: (49-7071) 297-6134; Fax: (49-7071) 292-618.

of neurones of the caudal pontine reticular nucleus (Koch and Ebert, 1992) led us to the hypothesis that this subtype of glutamate receptors on neurones of the caudal pontine reticular nucleus mediates the potentiation of the acoustic startle response by fear.

The present study tested the hypothesis that NMDA receptors in the caudal pontine reticular nucleus are involved in the enhancement of the acoustic startle response by conditioned fear. After fear conditioning, we injected the competitive NMDA receptor antagonist, AP-5, into the caudal pontine reticular nucleus of awake animals and measured its effect on the expression of fear-potentiated startle. We used doses of AP-5 which had not affected the baseline amplitude of the acoustic startle response in previous studies (Krase et al., 1993; Miserendino and Davis, 1993).

2. Materials and methods

2.1. Animals

Sixteen male Wistar rats, weighing 180–230 g at the beginning of the experiments, were used. They were housed in groups of 4–6 under a continuous light/dark cycle (lights on from 7:00 to 19:00 h). The rats received 12 g rat chow/animal per day and water was freely available.

2.2. Surgery

After anaesthesia with chloral hydrate (420 mg/kg, i.p.), two 23-gauge stainless guide cannulas were implanted into the caudal pontine reticular nucleus (-9.8 mm caudal, ± 0.8 mm lateral, -9.3 mm ventral from the bregma, according to Paxinos and Watson (1986)) under stereotaxic control. The cannulas were fixed to the skull with dental cement and three anchoring screws. After surgery and between experiments, stylets were inserted into the guide cannulas to maintain patency. The animals were allowed to recover from surgery for 5 days. On the 2 days before testing they were handled daily.

2.3. Fear conditioning procedure

The rats were trained in a dark training box ($38 \times 60 \times 28$ cm^3), the floor of which was composed of steel bars spaced 15 mm apart. The conditioned stimulus was a white light produced by a 15-W bulb located at the top of the box. The unconditioned stimulus was a 0.6-mA footshock produced by a shock generator (custom-made at the University of Tübingen) located outside the training box. The rats were placed into the training box and after an acclimatisation time of 5 min, they received 10 pairings of the conditioned stimulus (light) and the unconditioned stimulus (footshock). The unconditioned stimulus was presented in the last 0.5 s of the 3.7 s conditioned stimulus at an average intertrial interval of 3 min (range 2–4 min). After an initial training period on the first day, the rats were tested for the effects of different doses of the NMDA receptor antagonist, AP-5 (2-amino-5-phosphonopentanoic acid; RBI, Natick, MA, USA; dissolved in saline, pH 7.4), in a randomised order on 4 subsequent days. To avoid extinction of fear conditioning during testing, the rats were retrained once daily 3 h before testing. Retraining and initial training procedures were identical.

2.4. Fear-potentiated startle test

To assess fear potentiation of the acoustic startle response, the rats were placed into a wire mesh cage ($20 \times 10 \times 12$ cm^3) mounted on a piezoelectric accelerometer (custom-made at the University of Tübingen), which was located inside a sound-attenuated chamber ($100 \times 80 \times 60$ cm^3). Movements of the rats resulted in changes of the voltage output of the accelerometer. These signals were amplified, digitised and fed into a computer for analysis. The presentation of the acoustic startle stimuli and the conditioned stimulus (light) was also controlled by the computer and an appropriate interface (Hortmann Universal Function Synthesiser). A loudspeaker mounted 40 cm from the wire mesh cage delivered the acoustic startle stimuli and a continuous background noise (55 dB sound pressure level, root mean square), the 15-W bulb producing the conditioned stimulus (light) was mounted 20 cm above the wire mesh cage. The whole body amplitude of the acoustic startle response was calculated from the difference between the peak-to-peak voltage output of the accelerometer within the time windows of 80 ms after and 80 ms before (spontaneous activity) the onset of the startle stimulus.

After an acclimatisation time of 5 min, 10 acoustic stimuli (100 dB SPL, 10 kHz, 20 ms duration including 0.4 ms rise and fall times, 30 s interstimulus interval) were presented to habituate baseline amplitude of the acoustic startle response. AP-5 was injected after the fifth startle stimulus bilaterally into the caudal pontine reticular nucleus through 30-gauge stainless steel injection cannulas. Each animal received 0, 0.125, 0.25 or 0.5 nmol AP-5 in a randomised order on 4 subsequent days. The injection volume was 0.5 μl and the injection rate was 0.1 μl/5 s. The injection cannulas remained in the brain during the whole test, so that no handling of the animals was necessary during the injection. After the 10 initial startle stimuli, each animal received 40 acoustic startle stimuli with half of the stimuli presented in darkness (tone-alone trials) and the other half presented 3.2 s after the onset of a 3.7-s conditioned stimulus (light-tone trials). The two trial types were presented in a randomised order.

2.5. Histology and statistical analysis

After the tests, the rats were killed, their brains were removed and immersion-fixed with 8% paraformaldehyde in phosphate-buffered saline with 20% sucrose. Coronal

sections of 60 μm were taken on a freezing microtome and stained with thionine. The injection sites were drawn onto plates taken from the atlas of Paxinos and Watson (1986).

Statistical analysis of the data was done by analysis of variance (ANOVA), post-hoc Tukey test and Student's *t*-test, using a repeated-measure design.

3. Results

Histological analysis revealed that 8 rats had received bilateral injections of AP-5 into the caudal pontine reticular nucleus (injection sites are shown in Fig. 1). The injection sites of 5 rats were located outside the caudal pontine reticular nucleus. Two animals were excluded from further analysis since they did not show fear-potentiated startle under control conditions. Fig. 2 shows the time course of the mean amplitudes of the acoustic startle response during tone-alone and light-tone trials of those rats which received bilateral injections of AP-5 or saline into the caudal pontine reticular nucleus. Because the effect of fear conditioning under control conditions was most prominent during the first ten tone-alone and light-tone trials, the effect of the AP-5 injections into the caudal pontine reticular nucleus was calculated from the mean amplitudes of the acoustic startle response of the first ten trials. Fig. 3 shows the mean amplitudes of the acoustic startle response in these trials after injections of saline and the different doses of AP-5 along with the corresponding difference scores. All these animals showed a significantly potentiated acoustic startle response in the presence of the conditioned stimulus after injections of saline (*t*-test: $t =$

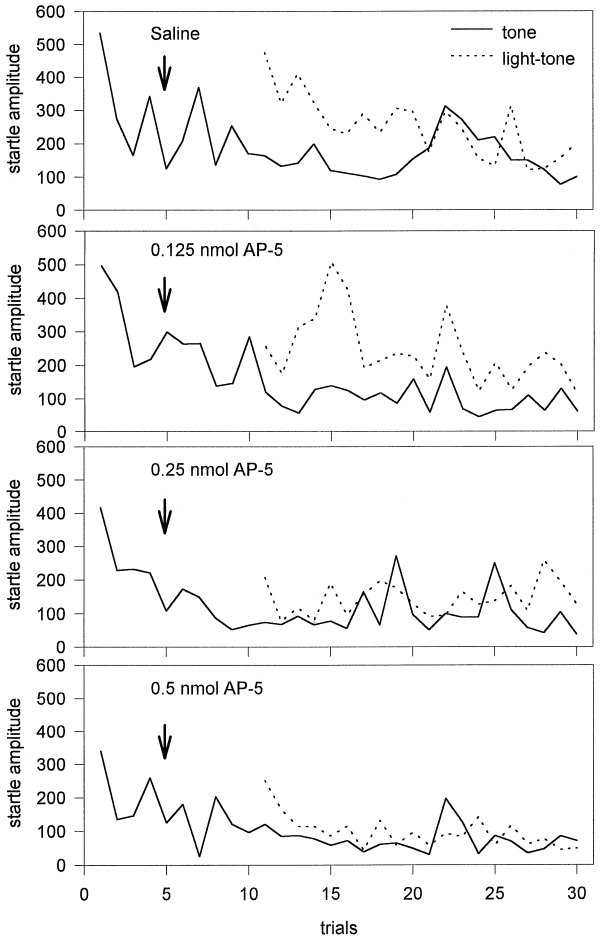

Fig. 2. Time course of the mean amplitude of the acoustic startle response ($n = 8$) on tone-alone (solid line) and light-tone (dashed line) trials shown before and after injections (arrows) of AP-5 (or saline) into the caudal pontine reticular nucleus.

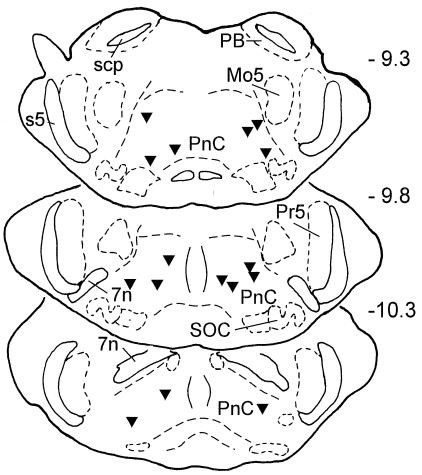

Fig. 1. Serial drawings of coronal sections through the lower brainstem depicting the injection sites of AP-5 into the caudal pontine reticular nucleus of the rats with bilateral injection ($n = 8$). Abbreviations: 7n, facial nerve; Mo5, motor trigeminal nucleus; PB, parabrachial nucleus; PnC, caudal pontine reticular nucleus; Pr5, principal sensory trigeminal nucleus; s5, sensory root of the trigeminal nerve; scp, superior cerebellar peduncle; SOC, superior olivary complex. Numbers indicate the distance from the bregma (in mm).

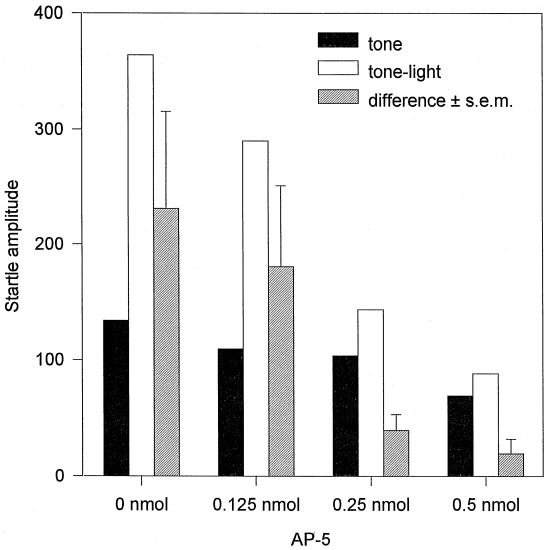

Fig. 3. Bar diagram showing the effects on fear-potentiated startle of injections of AP-5 (or saline) into the caudal pontine reticular nucleus. The mean amplitudes of the acoustic startle response (arbitrary units) of tone-alone trials (black bars) and light-tone trials (white bars), as well as the difference scores (± S.E.M., hatched bars) are plotted.

2.73; $P = 0.03$). The mean amplitude of the acoustic startle response of all 8 animals in the tone-alone trials showed no drug effect (ANOVA: $F(3,21) = 1.33$, $P = 0.29$), but a trend to a reduced amplitude of the acoustic startle response after injections of AP-5 was observed in rats with high amplitudes of the acoustic startle response, whereas animals with moderate or low amplitudes of the acoustic startle response showed no effect of AP-5 on baseline acoustic startle response. After injections of 0.125 and 0.25 nmol AP-5 into the caudal pontine reticular nucleus the potentiation of the acoustic startle response by conditioned fear was reduced, but still statistically significant (P values < 0.05, t-tests). After injections of 0.5 nmol AP-5 no significant differences between tone-alone and light-tone trials were observed (t-test: $t = 1.56$, $P = 0.16$), indicating that AP-5 in high doses blocked the fear potentiation of the acoustic startle response. An ANOVA on the difference scores showed a significant effect of various doses of AP-5 on fear potentiation ($F(3,21) = 3.98$, $P = 0.02$). Post-hoc Tukey-tests showed P values < 0.05 for the pairwise comparisons between the difference scores after injections of saline and 0.25 nmol AP-5, and between the difference scores after injections of 0.125 nmol AP-5 and 0.25 nmol AP-5. A P value < 0.01 was observed for the comparison between the difference scores after injections of saline and 0.5 nmol AP-5. Rats with injection sites outside the caudal pontine reticular nucleus (oral pontine reticular nucleus, trigeminal nuclei, superior olivary complex, tegmental area dorsal to the caudal pontine reticular nucleus) showed fear potentiation under all conditions (t-tests had $P < 0.05$), i.e., injections of AP-5 had no effect on fear potentiation of the acoustic startle response (ANOVA: $F(3,12) = 0.82$, $P = 0.49$; data not shown). The spontaneous activity of the rats during the test sessions was not affected by injections of AP-5 into the caudal pontine reticular nucleus (ANOVA: $F(3,21) = 1.24$, $P = 0.32$).

4. Discussion

The present study tested the hypothesis that glutamate receptors of the NMDA subtype in the caudal pontine reticular nucleus are involved in the expression of fear-potentiated startle in rats. Our data demonstrate a blockade of fear-potentiated startle after microinjections of the competitive NMDA receptor antagonist, AP-5, into the caudal pontine reticular nucleus without any effect on the baseline amplitude of the acoustic startle response. The time course of the tone-alone and light-tone trials during the potentiated startle test shows further that fear potentiation of the acoustic startle response was most pronounced within the first 10 presentations of tone-alone and light-tone trials, suggesting that pharmacological treatments are most effective within this time window.

The time course of the amplitude of the acoustic startle response shown in the present study demonstrates that, under control conditions, the rats showed habituation of the acoustic startle response during the 10 initial startle stimuli. Tone-alone and light-tone trials were then presented in randomised order. During the first 10 presentations of each trial type, fear potentiation of the acoustic startle response was evident, while during the last 10 presentation of each trial type this was not the case. Since we only investigated the effects of AP-5 on the first 10 presentations of each trial type, we are confident that the drug effects were measured in the phase during which fear was most strongly expressed. The reason for the relatively rapid dissipation of the fear effect on the acoustic startle response demands further experimentation. It is important to note that our experimental procedure is partly different from that of other groups. Firstly, we trained the rats in a setup different from that in which they were tested, so that we could avoid possible effects of context conditioning. Secondly, we used remote infusions so that the rats did not have to be handled for drug application.

The present study was based on results of previous studies of the role of glutamate receptors in the mediation and modulation of the acoustic startle response. Glutamate has an excitatory effect on the tone-evoked activity of neurones of the caudal pontine reticular nucleus (Ebert and Koch, 1992), while the AMPA/kainate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) potentially inhibits the tone-evoked activity. In contrast, the NMDA receptor antagonist, AP-5, has a weaker inhibitory effect on the activity of the caudal pontine reticular nucleus. This finding is complemented by results of behavioural studies (Krase et al., 1993; Miserendino and Davis, 1993) showing that microinjections of low doses of AMPA/kainate receptor antagonists into the caudal pontine reticular nucleus of awake rats decrease the amplitude of the acoustic startle response, whereas injections of NMDA receptor antagonists are less effective. Furthermore, the excitatory effect of amygdala stimulation on the tone-evoked activity of neurones of the caudal pontine reticular nucleus is reduced by local applications of the NMDA receptor antagonist AP-5 (Koch and Ebert, 1992). The reduction of fear-potentiated startle by the NMDA receptor antagonist, AP-5, demonstrates that glutamate mediates the effects of fear on the acoustic startle response via the NMDA receptor. Because the projections from the amygdala to the caudal pontine reticular nucleus are known to be necessary for the expression of fear-potentiated startle (Davis, 1992b; Davis et al., 1993; Hitchcock and Davis, 1991; Fendt et al., 1996b) and since glutamate may be a possible transmitter of this projection (Price et al., 1987), we speculate that a glutamatergic projection from the amygdala and/or the central gray to the caudal pontine reticular nucleus might be important for the expression of fear-potentiated startle, but further anatomical and electrophysiological studies are necessary to address this possibility. The idea that NMDA receptors mediate the enhancement of the acoustic startle response is supported by results of electrophysiological

studies (Collingridge and Lester, 1989) showing that the AMPA/kainate receptor is fast-acting, whereas the NMDA receptor is more tonic, probably suggesting that NMDA currents play a modulatory, rather than a mediating role in the transmission of acoustic information at the level of the caudal pontine reticular nucleus (Krase et al., 1993; Miserendino and Davis, 1993). The suggestion that glutamate is involved in the mediation of fear-potentiated startle is supported by the fact that injections of the somatostatin receptor agonist, sandostatin, into the caudal pontine reticular nucleus blocked the expression of fear-potentiated startle *and* blocked the excitatory effect of glutamate on the tone-evoked activity of neurones of the caudal pontine reticular nucleus (Fendt et al., 1996c). In the present study, AP-5 up to 0.5 nmol did not affect the baseline acoustic startle response. We used the same doses as in our previous work (Krase et al., 1994), but it should be noted that Miserendino and Davis (1993) observed an effect on the baseline acoustic startle response after injections of 0.39 nmol AP-5 into the caudal pontine reticular nucleus. However, methodological differences are likely to account for this discrepancy.

We suggest the following neural circuitry to mediate the effects of conditioned fear on the acoustic startle response: the amygdala is the locus of acquisition of conditioned fear. The expression of fear-potentiated startle is mediated by several parallel pathways from the amygdala to the caudal pontine reticular nucleus (Hitchcock and Davis, 1991; Fendt et al., 1996a). One direct projection from the amygdala excites the caudal pontine reticular nucleus (Hitchcock and Davis, 1991; Rosen et al., 1991; Koch and Ebert, 1993), other pathways involve indirect projections from the amygdala via the central gray (Fendt et al., 1994, 1996a,c), the rostrolateral midbrain (Yeomans and Pollard, 1993; Frankland and Yeomans, 1995) and the laterodorsal tegmental nucleus (Krase et al., 1994; Kungel et al., 1994) to the caudal pontine reticular nucleus or perhaps brain sites downstream from the caudal pontine reticular nucleus. The transmitters within these pathways could be corticotropin-releasing factor as an excitatory transmitter of the direct pathway from the central amygdala to the caudal pontine reticular nucleus (Fendt et al., 1996b), somatostatin as an inhibitory transmitter of the indirect pathway via the central gray (Fendt et al., 1996c) and substance P as an excitatory transmitter of the indirect pathway via the laterodorsal tegmental nucleus (Krase et al., 1994; Kungel et al., 1994). The present study adds glutamate to this list of transmitters, although the origin of the glutamatergic projection is still unclear. A further transmitter which could be involved in the expression of fear-potentiated startle is the neuropeptide cholecystokinin, since we showed that cholecystokinin in the caudal pontine reticular nucleus potentiates the acoustic startle response amplitude (Fendt et al., 1995).

We assume that for the expression of fear-potentiated startle all these different pathways with a number of different transmitters must be active, that is to say that fear-potentiated startle is the result of an interactive network of different nuclei and destruction of only one part of this network impairs the function of the entire system.

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