ELSEVIER

Behavioural Brain Research 112 (2000) 33–41

BEHAVIOURAL BRAIN RESEARCH

www.elsevier.com/locate/bbr

Research report

Impaired escape performance and enhanced conditioned fear in rats following exposure to an uncontrollable stressor are mediated by glutamate and nitric oxide in the dorsal raphe nucleus

Ruth E. Grahn [a,*], Linda R. Watkins [b], Steven F. Maier [b]

[a] *Department of Psychology, Connecticut College, Box 5275, 270 Mohegan Avenue, New London, CT 06320-4196, USA*
[b] *Department of Psychology and Behavioral Neuroscience Program, University of Colorado at Boulder, Boulder, CO, USA*

Received 27 July 1999; received in revised form 25 January 2000; accepted 30 January 2000

Abstract

Exposure to uncontrollable aversive events produces a variety of behavioral consequences that do not occur if the aversive event is controllable. Accumulating evidence suggests that exaggerated excitation of serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) is sufficient to cause these same behaviors, such as poor shuttlebox escape performance and enhanced conditioned fear that occur 24 h after exposure to inescapable tailshock (IS). The aim of the present studies was to explore the possibility that *N*-methyl-D-aspartate (NMDA) receptor activation and nitric oxide (NO) formation within the DRN might be involved in mediating the behavioral consequences of IS. To this end, either the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) or the nitric oxide synthase inhibitor Nw-nitro-L-arginine methyl ester (L-NAME), was microinjected into the DRN before IS or before testing 24 h later. Blocking NMDA receptors with APV in the DRN during IS prevented the usual impact of IS on escape responding and conditioned fear. However, injection of APV at the time of testing only reduced these effects. The DRN was shown to be the critical site mediating blockade of these behavioral changes since injection of APV lateral to the DRN did not alter the behavioral consequences of IS. Conversely, L-NAME was most effective in reversing the effects of IS when administered at the time of testing. These results suggest that there is glutamatergic input to the DRN at the time of IS that produces long-lasting changes in DRN sensitivity. This plasticity in the DRN is discussed as a possible mechanism by which IS leads to changes in escape performance and conditioned fear responding.

Keywords: Learned helplessness; Serotonin; 5-HT; Dorsal raphe nucleus; NMDA receptors; 2-Amino-5-phosphonovaleric acid; Nitric oxide synthase inhibitors; Nw-nitro-L-arginine methyl ester; NO; Conditioned fear; Shuttlebox escape performance; Inescapable tailshock; Rats

1. Introduction

The behavioral control, which an organism has over a stressor, is an important factor influencing the behavioral outcomes induced by the stressor. Behavioral sequelae of stressors that are specific to uncontrollable stressors have been called learned helplessness effects [25]. Impaired shuttlebox escape responding and enhanced conditioned fear in rats are among the best characterized behavioral effects of inescapable tailshock (IS) [19]. These behaviors do not occur if rats are exposed to the same number, duration and intensity of escapable (controllable) tailshocks (ES). These two behaviors share the same timecourse following IS and are similarly modified by pharmacological manipulations (see Ref. [20] for a review).

1.1. Role of the dorsal raphe nucleus (DRN) in learned helplessness behavior

The DRN is a neural site that has the potential to provide a common source of modulation of these and other IS-induced behaviors. The DRN projects to brain

* Corresponding author. Tel.: +1-860-4392387; fax: +1-860-4395300.
E-mail address: regra@conncoll.edu (R.E. Grahn)

0166-4328/00/$ - see front matter
PII: S0166-4328(00)00161-3

regions that modulate learned helplessness behaviors [14,32,36] and 5-HT modulates behaviors in a manner similar to IS [10,11]. Thus, a recent hypothesis suggests that IS induces a hypersensitive state in the DRN that leads to enhanced 5-HT release and mediates learned helplessness behavior [20].

Sensitization of the DRN is thought to arise from enhanced activation of the DRN during IS, relative to ES. We have demonstrated that IS is more effective than ES in activating the DRN by showing that: (1) there is more 5-HT released within the DRN during IS than during ES [26]; and (2) 5-HT neurons in the DRN are more active in IS-treated subjects, as indicated by c-Fos expression, than they are in ES-treated subjects [13]. Thus, DRN activity seems to be sensitive to the psychological dimension of controllability, and may mediate the behavioral effects of uncontrollable stressors.

Ongoing characterization of the effects of IS on DRN activity continue to support the idea that sensitization of the DRN mediates learned helplessness effects. For example, there is increased basal extracellular 5-HT in some projection regions of the DRN 24 h after IS, as well as exaggerated release of 5-HT in response to brief footshocks [4]. A series of DRN microinjection studies are also consistent with the 5-HT sensitization hypothesis of learned helplessness. Pharmacological manipulations which would be expected to increase 5-HT release mimic the effects of IS [21,34], while drugs which decrease 5-HT neurotransmission prevent the usual behavioral sequelae of IS when administered before IS [22–24]. These studies demonstrate that the DRN is more responsive than usual during the time interval in which the effects of IS are typically observed.

1.2. Possible role of long-term potentiation in learned helplessness behavior

The apparent sensitization in the DRN 5-HT neurons initiated by exposure to IS has some attributes in common with long-term potentiation (LTP). Both require intense neural activation [6], and the time course of long-term potentiation can be on the order of days [9,27], mirroring the timecourse of IS-induced behavioral consequences. Recently, there have been a number of reports demonstrating that LTP-like processes occur in brain areas outside the hippocampus, a region where LTP has been extensively characterized. For example, an LTP-like process is thought to result in sensitization of dorsal horn neurons, which convey the presence of noxious stimuli to the brain [5]. Also, an LTP-like sensitization process has been invoked to account for increased anxious behavior exhibited by cats following intense activation of the pathway from the amygdala to the periaqueductal gray [1,2]. Likewise, anxious behavior in rats that was induced by a single 5-min exposure to a cat was dependent on *N*-methyl-D-aspartate (NMDA) receptor activation [3].

The extensions of the established role of LTP to explain other behavioral states considered to result from intense neural activation suggests an exploration of the possible involvement of LTP-like phenomena in mediating the behavioral effects of IS. LTP can be initiated by activation of excitatory amino acid NMDA receptors. Activation of these receptors allows entry of Ca^{2+} into the cell where it acts as a second messenger, activating calmodulin. In one account of LTP, calmodulin activates the enzyme that produces nitric oxide (NO) [43]. Modulation of presynaptic processes by NO is then argued to lead to an increased amount of neurotransmitter release upon subsequent stimulation. Interestingly, there is substantial glutamatergic input to the DRN [15,16], and the enzyme that leads to the production of NO after NMDA activation (nitric oxide synthase (NOS)) is present in the DRN in very high concentrations [37,40]. We therefore began to explore the possibility that an LTP-like process in the DRN is involved in mediating learned helplessness effects by determining the effects of microinjecting the NMDA antagonist 2-amino-5-phosphonovaleric acid (APV; Experiment 1) or the NO synthase inhibitor Nw-nitro-L-arginine methyl ester (L-NAME; Experiment 2) into the DRN either before IS or before later behavioral testing for escape learning and fear conditioning. One of these drugs (APV) was also microinjected 1–2 mm lateral to the DRN to examine site specificity.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats approximately 120 days old were used as subjects. They were housed individually with free access to standard laboratory chow and water under a 12-h light/dark cycle. Experiment 1A, 1B, and 2 used 69, 32, and 57 rats, respectively. All experiments were conducted during the light part of the cycle and were approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2. Materials

The pretreatments of IS or restraint were administered in Plexiglas tubes measuring 17.5 cm in length and 7 cm in diameter. Each rat's tail was secured to a bar extending from the rear of the tube with tape. Clip electrodes were attached to the distal third of the tail of IS-treated subjects. Behavioral testing was conducted in shuttleboxes measuring $46 \times 20.7 \times 20$ cm ($L \times W \times H$). Scrambled shock was delivered through stainless

steel rods on the floor of the shuttlebox. The shuttlebox was divided by an aluminum wall with an archway cut out to allow passage from one side to the other. The inside of the shuttlebox was illuminated by a 28-V houselight. The shuttleboxes were housed in sound attenuating chambers left open to allow behavioral observations. Background noise was provided by ventilation fans.

2.3. General procedures

2.3.1. Cannula implantation

Rats were implanted with a guide cannula approximately 2 weeks prior to each experiment. In Experiments 1A and 2, rats were anesthetized with 60 mg/kg ketamine and 13 mg/kg xylazine injected intraperitoneally. Halothane, delivered by inhalation, was used as an anesthetic in Experiment 1B. In Experiments 1A and 2, a 13-mm 26 ga stainless steel cannula was positioned 1 mm above the DRN (Paxinos and Watson atlas [30] coordinates AP +0.7 mm, ML 0.0 mm, DV +4.5 mm from interaural zero), and secured with dental acrylic applied to the cannula and screws anchored in the surrounding skull. Each cannula was kept patent by a stainless steel stylet. Implantation was the same for Experiment 1B except that the medial-lateral coordinate was adjusted to 1 mm lateral to midline. Animals were monitored daily and handled twice after surgery.

2.3.2. Microinjections

Injections were made by removing the stylet and replacing it with a 14-mm microinjector made from 33 ga stainless steel tubing. The microinjector was attached to approximately 50 cm of PE-20 tubing which was attached to a 50-μl Hamilton glass syringe. Calibration indicated that fluid movement of 10.25 mm was equal to a delivered volume of 1 μl. The microinjector was left in place for 2 min to obviate drug diffusion into the cannula.

2.3.3. Inescapable tailshock

Inescapable shock or restraint began approximately 10 min after drug injections. IS consisted of 100 1.0-mA 5-s tailshocks delivered on average every 60 s, whereas the control condition was restraint in an identical Plexiglas tube without shock. Rats were returned to their home cage immediately after the 2-h session. ES groups were not included because ES does not interfere with subsequent escape or enhance later fear conditioning. Thus, ES does not produce behavioral effects relative to controls and so there are no effects to be potentially blocked by the drugs used. Controls were restrained because loose restraint does not alter escape or fear conditioning relative to home cage controls. Restraint is preferable to home cage controls because it equates IS and control subjects on all aspects other than IS itself.

2.3.4. Behavioral testing

Conditioned fear and shuttlebox escape performance were assessed in the same subject 24 h after stressor pretreatment using the procedures standard in our laboratory. Conditioned fear was measured as the occurrence of freezing, defined as the complete lack of movement except that required for respiration. Freezing is a conditioned response to the cues associated with shock in the shuttlebox [7]. Rats were placed individually in the shuttlebox and freezing observed for 10 min. The subjects were observed every 8 s by an observer unaware of group membership and scored as freezing or not freezing. Each rat then received two 0.8-mA footshocks, which could be terminated by a single crossing of the shuttlebox. Freezing behavior was then assessed for an additional 20 min as above. Interrater reliability has been assessed as 0.94. After the observations were complete, the rats received three further single-crossing escape trials (FR-1) followed by 25 trials in which a back-and-forth crossing (FR-2) was required to terminate shock. Trials occurred on average every 60 s and shock intensity continued at 0.8 mA. Escape latency was recorded automatically and each trial was terminated at 30 s if no response was made. This procedure allows measurement of fear conditioning and escape learning in the same subjects.

2.3.5. Cannula placement verification

The site of microinjection was determined by injecting a 1-μl volume of Evans Blue dye through the cannula while the rat was deeply anesthetized with sodium pentobarbital. After allowing the dye to be absorbed for 10 min, the rat was perfused transcardially with saline. The brain was removed and soaked in a 30% sucrose/10% formalin solution for approximately 3 days. Brains were sliced in a cryostat at −20°C. The 40-μm sections were mounted onto gelatin coated slides and allowed to dry overnight. Slides were stained with cresyl violet and examined for dye placement and tissue quality. Subjects in Experiments 1A and 2 were excluded if dye was evident in the ventricles or in brain sites other than the region of the DRN or if there was evidence of cellular damage in the DRN. Twenty-four rats from Experiment 1A and nine rats from Experiment 2 were excluded. Subjects were excluded from Experiment 1B if the cannula was within or on the border of the DRN. Two rats were excluded from Experiment 1B.

2.4. Experimental procedures

2.4.1. Experiment 1A

After recovering from surgery rats were randomly divided into six groups. Three groups received IS on

day 1, whereas the remaining three groups were restrained (R) for an equal time period. Each rat received one microinjection on each of 2 experimental days. Each rat in one IS group and one R group received 5 ng APV in 1 μl saline into the region of the DRN approximately 10 min prior to IS or restraint, and saline approximately 10 min prior to testing the next day (IS-APV(Pretreatment), R-APV(Pretreatment)). An additional IS and R group were given the opposite treatment with saline the first day and the same dose of APV on day 2 (IS-APV(Test), R-APV(Test)). The remaining IS and restraint group received saline on both days (IS-Vehicle, R-Vehicle).

2.4.2. Experiment 1B

Subjects were divided into four groups in a 2×2 factorial design. Half of the subjects were injected with APV and then were exposed to either IS or R. The remaining subjects received vehicle in combination with either IS or R.

2.4.3. Experiment 2

Surgical procedures and post-operative care were done as described in Experiment 1A. Exposure to IS and behavioral testing were also conducted exactly as described in Experiment 1A. In this experiment, 5 μg in 1 μl saline of L-NAME was injected approximately 10 min prior to IS or restraint and immediately after these treatments or it was injected approximately 10 min prior to testing the next day. Vehicle saline injections were administered when drug was not given, or before both pretreatment and testing in the control groups.

3. Results

3.1. Experiment 1A: effects of NMDA receptor blockade within DRN

The effects of APV on conditioned fear responding and escape responding are shown in Fig. 1 and Fig. 2, respectively. The data obtained from the vehicle-treated subjects is duplicated on each panel in order to make clear each effect of drug treatment. Panel A shows the effect of drug administered prior to stressor exposure while Panel B shows the effect of drug treatment prior to testing.

There was virtually no freezing in the shuttlebox before the two footshocks in any group. The effect of IS and APV on freezing following the two footshocks is shown in Fig. 1. The data are depicted in blocks of 2 min. Since the presence/absence of freezing was observed every 8 s, the maximum possible freezing score for a 2-min block is 15. Panel A contains the data for the vehicle controls and the subjects given APV before IS or R, while Panel B shows the same vehicle controls and the groups given APV before behavioral testing. The two footshocks produced substantial freezing which extinguished over the 20-min testing period. As is typical, IS potentiated the freezing occurring after the footshocks. Microinjection of APV prior to stressor pretreatment prevented enhanced conditioned fear induced by exposure to IS, while administration of APV prior to behavioral testing had only a slight effect. In addition, APV had no effect on freezing in R subjects. A repeated measures ANOVA applied to the conditioned fear data revealed that there were significant

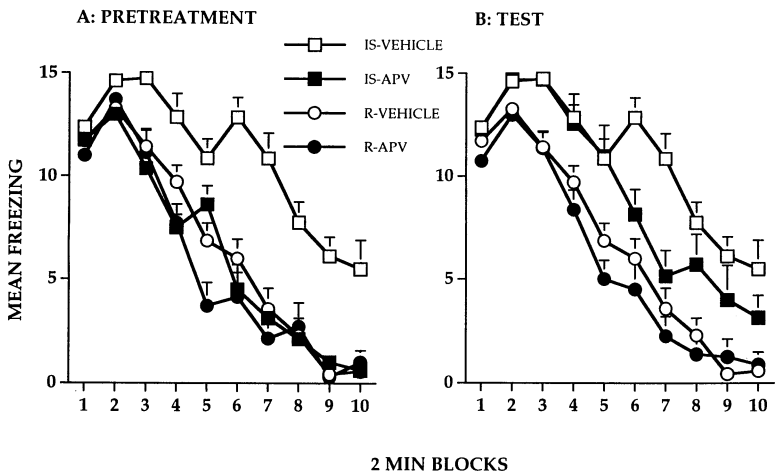

Fig. 1. Mean freezing for a 20-min period, in 2-min blocks, after presentation of two footshocks in a shuttlebox. Error bars represent standard error of the mean. Rats were exposed to pretreatment with IS or R 24 h prior to behavioral testing. Panel A includes the results obtained from vehicle-treated rats and those that received 5 ng APV just prior to IS or R. Panel B includes data from the same vehicle-treated rats and rats that received 5 ng APV prior to behavioral testing 24 h after IS or R. There were eight subjects in each of the vehicle groups, eight in the IS-APV-Pretreatment group, 6 in the R-APV-Pretreatment group, seven in the IS-APV-Test group and eight in the R-APV-Test group.

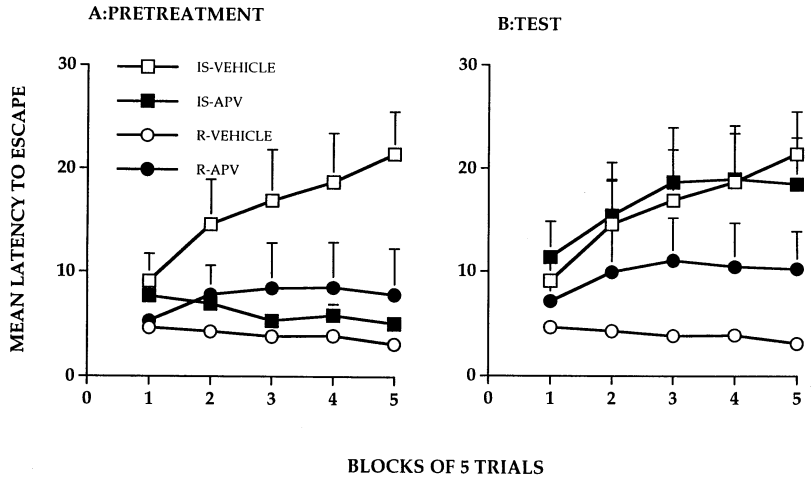

Fig. 2. Mean latencies to escape footshock trials in a shuttlebox in blocks of five trials, measured 24 h after IS or R. Error bars represent standard error of the mean for blocks of five trials. Panel A includes the results obtained from vehicle-treated rats and subjects that received 5 ng APV just prior to IS or R. Panel B includes results from the same vehicle-treated rats and rats that received 5 ng APV prior to behavioral testing 24 h after IS or R. The number of subjects in each group was as described in Fig. 1.

effects of shock, $F(1, 39) = 34.427$, $P < 0.0001$; drug condition, $F(2, 39) = 11.785$, $P < 0.0001$; the shock × drug interaction, $F(2, 39) = 6.076$, $P < 0.01$; blocks of time $F(9, 531) = 218.290$, $P < 0.0001$; the shock × blocks of time interaction, $F(9, 351) = 4.826$, $P < 0.0001$; the drug × blocks of time interaction, $F(18, 351) = 2.779$, $P < 0.001$; and the three-way interaction, $F(18, 351) = 2.230$, $P < 0.01$. Newman–Keuls post-hoc analysis revealed that both IS-APV (Test) and IS-Vehicle groups were each different from all other groups, which did not differ among themselves. IS-APV (Test) and IS-Vehicle groups also differed reliably.

Neither IS nor APV had an effect on escape latencies on the FR-1 trials that preceded freezing measurement ($F < 1.0$), and so the enhancement of fear conditioning produced by IS and the reduction produced by APV cannot be explained as occurring because the treatments altered shock durations. It is typical in our laboratory that IS has no effect on FR-1 escape latencies. Shuttlebox escape latencies on FR-2 trials are shown in Fig. 2. Exposure to IS strongly interfered with FR-2 escape responding. Fig. 2 also indicates that APV microinjected prior to stressor pretreatment prevented the effects of IS on impaired shuttlebox escape performance. APV did not have an effect on the performance of IS-treated subjects when microinjected into the DRN prior to behavioral testing. However, APV impaired escape performance in restraint subjects. A repeated measures analysis of variance conducted on FR-2 trials revealed a significant effect of shock, $F(1, 39) = 5.937$, $P < 0.05$; a significant effect of blocks of trials, $F(4, 156) = 5.3$, $P < 0.001$, and a significant three-way interaction between the shock condition, the drug condition and blocks of trials, $F(4, 156) = 2.763$, $P < 0.01$. A Newman–Keuls post-hoc analysis ($P = 0.05$) revealed that the IS-Vehicle group differed from all other groups except the IS-APV(Test) group. Also of interest was that the R-APV(Test) group differed from both the IS-Vehicle and R-Vehicle groups.

3.2. Experiment 1B: effects of NMDA receptor blockade outside of DRN

As noted above, two subjects were excluded because of cannula placements within or on the border of the DRN. Acceptable cannula placements were typically 1–2 mm lateral to the DRN, either in the ventrolateral periaqueductal gray area or in the neighboring intercollicular nucleus. A few placements were in the dorsolateral periaqueductal gray.

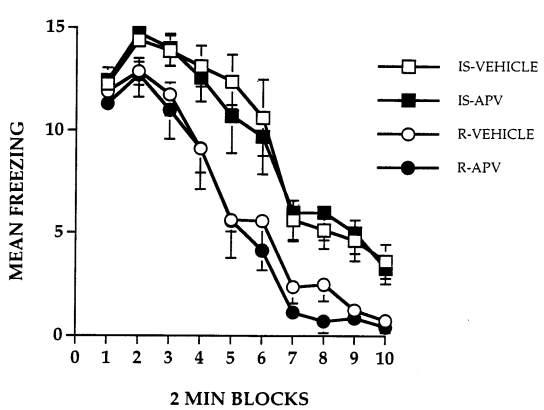

Fig. 3. Mean freezing for a 20-min period, in 2-min blocks, after presentation of two footshocks in a shuttlebox. Error bars represent standard error of the mean. Rats were administered 5 ng APV or vehicle lateral to the DRN before IS or R. Behavioral testing occurred 24 h later. There were eight subjects in each of the vehicle groups and seven subjects in each of the APV groups.

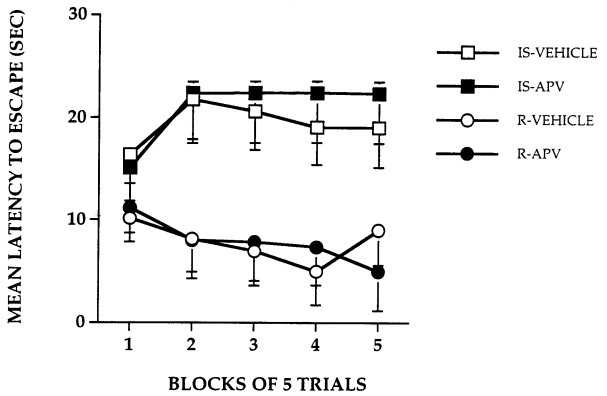

Fig. 4. Mean latencies to escape footshock trials in a shuttlebox in blocks of five trials, measured 24 h after IS or R. Error bars represent standard error of the mean for blocks of five trials. Rats were administered 5 ng APV or vehicle lateral to the DRN before IS or R. Behavioral testing occurred 24 h later. The number of subjects in each group is as indicated in Fig. 3.

There was no freezing in the shuttlebox prior to two footshocks. Fig. 3 shows freezing following the two footshocks. The footshocks led to substantial freezing, which was again potentiated by prior IS. However, here APV given before IS did not attenuate the effect of IS. ANOVA yielded reliable effects of IS, $F(1, 26) = 23.891$, $P < 0.0001$, trials, $F(9, 234) = 137.333$, $P < 0.0001$, and the interaction between trials and IS, $F(9,234) = 4.09$, $P < 0.0001$. Newman–Keuls post hoc analysis indicated that the IS-Vehicle and IS-APV groups differed from the R-Vehicle and R-APV groups, but did not differ from each other.

There were no significant differences between groups in FR-1 shuttlebox escape latencies. FR-2 latencies are shown in Fig. 4. IS interfered with escape performance, and this interference was not reduced by APV. ANOVA yielded reliable effects of IS, $F(1, 26) = 13.153$, $P < 0.002$, trials, $F(4,104) = 55.615$, $P < 0.006$, and the interaction between trials and IS, $F(4, 104) = 3.231$, $P < 0.02$. Newman–Keuls post hoc analysis indicated that the IS-Vehicle and IS-APV groups differed from the R-Vehicle and R-APV groups, but did not differ from each other.

3.3. Experiment 2: effects of inhibiting NO formation

There was very little freezing in the shuttlebox before the presentation of footshocks. As shown in Fig. 5, IS produced the expected enhancement of fear conditioning in the shuttlebox after two footshocks. L-NAME injected at the time of IS attenuated the effects of IS and had no effect on responding in R subjects. Injection of L-NAME prior to behavioral testing completely eliminated the effect of IS on fear conditioning after the two footshocks.

A repeated measures ANOVA on conditioned fear responding indicated that there were significant effects of shock, $F(1, 42) = 12.88$, $P < 0.01$; drug, $F(2, 42) = 6.17$, $P < 0.05$; the shock × drug interaction, $F(2, 42) = 14.72$, $P < 0.01$; blocks of trials, $F(9, 369) = 113.78$, $P < 0.0001$; the blocks of trials × shock interaction, $F(9, 369) = 3.04$, $P < 0.05$, and the three-way interaction, $F(9, 369) = 4.12$, $P < 0.01$. Newman–Keuls post-hoc analysis revealed that the IS-Vehicle group differed from all other groups. The remaining groups did not differ among themselves.

Escape latencies did not differ among the groups during FR-1 trials. Fig. 6 shows that IS produced clear interference with FR-2 escape performance. L-NAME

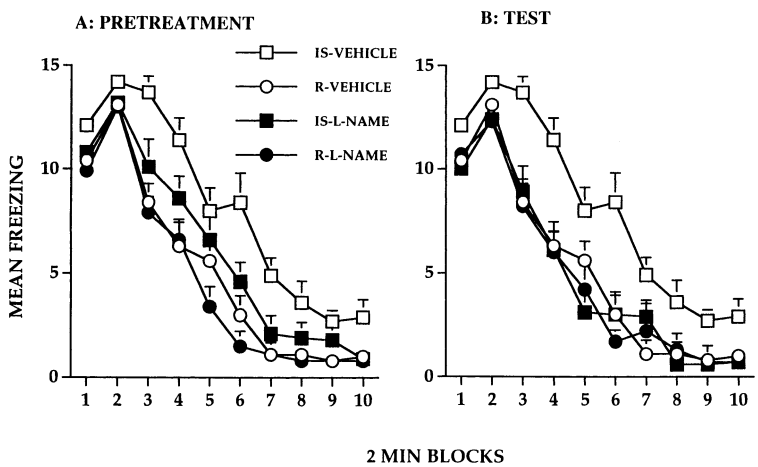

Fig. 5. Mean freezing for a 20-min period, in 2-min blocks, after presentation of two footshocks in a shuttlebox. Error bars represent standard error of the mean. Rats were exposed to pretreatment with IS or R 24 h prior to behavioral testing. Panel A includes the results obtained from vehicle-treated rats and those that received 5 μg L-NAME prior to and after IS or R. Panel B includes the results from these vehicle-treated rats and rats that received 5 μg L-NAME prior to testing 24 h after IS or R. Each vehicle group included nine subjects. There were nine subjects in the IS-L-NAME-Pretreatment group, eight in the R-L-NAME-Pretreatment group, seven in the IS-L-NAME-Test group, and six in the R-L-NAME-Test group.

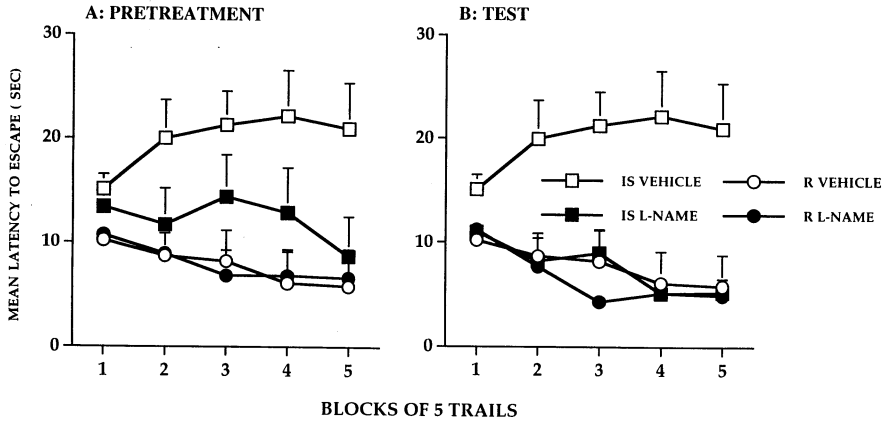

Fig. 6. Mean latencies to escape footshock trials in a shuttlebox in blocks of five trials, measured 24 h after IS or R. Error bars represent standard error of the mean for blocks of five trials. Panel A includes the results obtained from vehicle-treated rats and those that received 5 μg L-NAME prior to and after IS or R. Panel B includes the same vehicle-treated subjects and groups that received 5 μg L-NAME prior to testing. The number of subjects in each group is as indicated in Fig. 5.

administered before IS attenuated the escape deficit, while administration of L-NAME at the time of behavioral testing completely eliminated the effect of IS. L-NAME did not interfere with responding in R subjects.

A repeated measures ANOVA conducted on FR-2 response latencies revealed significant effects of shock, $F(1, 42) = 29.16$, $P < 0.0001$; drug, $F(2, 42) = 7.18$, $P < 0.01$; the shock × drug interaction, $F(2, 42) = 10.13$, $P < 0.0001$; blocks of trials, $F(4, 163) = 2.97$, $P < 0.05$ and the three-way interaction, $F(8, 163) = 3.55$, $P < 0.01$. Newman–Keuls post-hoc analysis revealed that the IS Vehicle group differed from all other groups except the IS L-NAME (Pretreatment) group. There were no differences among the remaining groups.

4. Discussion

NMDA receptor blockade before IS completely prevented both subsequent impairment of shuttlebox escape responding and enhanced conditioned fear. NMDA receptor blockade before behavioral testing had a much more ambiguous effect. It did attenuate the IS-induced enhancement of freezing (groups IS-Vehicle and IS-APV (Test) differed reliably), but the IS-Vehicle and IS-APV (Test) groups did not differ with regard to shuttle latencies. However, APV given before testing interfered with shuttle performance in R subjects, and so the lack of a difference between IS-Vehicle and IS-APV (Test) escape latencies could therefore be interpreted as a small attenuation in IS-induced escape failure produced by APV. Unfortunately, the fact that APV did not interfere with performance in the IS subjects could also reflect a ceiling effect. Inhibition of nitric oxide synthase before IS somewhat attenuated the effects of IS, while the same treatment before testing completely reversed the effects of IS. Thus, the conservative conclusion is that NMDA receptor activation is primarily involved in the processes initiated at the time of IS that produce escape deficits and enhancement of fear conditioning, while NO production is primarily involved in the processes that occur at the time of behavioral testing.

These processes would appear to be in the region of the DRN, since APV microinjected just outside the DRN was without effect. We are confident that the effects of APV were DRN-specific since diffusion of drug into the ventricle would have been just as likely from the control injections as from the DRN injections and the control injections did not prevent the effects of IS. The pattern of results showing that APV prevented IS-induced effects and that L-NAME reversed those effects suggests that the L-NAME effects were dependent on NMDA receptor activation at the time of IS. Glutamate receptor blockade was effective only if microinjected at the DRN site, indicating that the involvement of NO processes are also likely to be specific to the DRN. Thus, L-NAME was not injected lateral to the DRN as a control procedure.

4.1. Glutamatergic mediation of learned helplessness behavior

Glutamate projections to the DRN have been characterized as originating primarily from the lateral habenula, the lateral hypothalamus, and the PAG [15]. The best characterized of these is the projection from the lateral habenula [8,16,17,29,33,41]. However, the exact effect of lateral habenula stimulation on DRN activity has been a source of debate. Initial studies using unit recording in the DRN suggested that lateral habenula stimulation causes inhibition of DRN neurons [33]. These results were consistent with the original report by

Wang and Aghajanian [38] proposing that the lateral habenula projection to the DRN is GABAergic. Indeed, the inhibitory effect of habenular stimulation on DRN neurons was blocked by a GABA antagonist. Further investigation suggested that lateral habenula input was not GABAergic, but that its influence acted on GABAergic interneurons in the DRN. Kalen et al. [15] convincingly demonstrated that glutamate was the neurotransmitter released in the DRN upon lateral habenula stimulation.

One attempt to reconcile the GABA and glutamate accounts of lateral habenula input to the DRN yielded evidence that the influence of lateral habenula stimulation on DRN serotonin neurons was inhibitory and in some cases monosynaptic [29]. In direct contrast, more recent investigations indicate that the influence of lateral habenula stimulation on serotonin neurons is NMDA receptor-dependent and is excitatory [41]. A GABA-dependent inhibitory influence of lateral habenula stimulation was shown to occur when stimulation parameters were intense [8]. Although a glutamate anatomical connection to the DRN is established, the function of activity in this neural pathway during IS has yet to be established.

4.2. NO mediation of learned helplessness behavior

The NMDA receptor-dependent and NO-dependent nature of impaired escape responding and enhanced conditioned fear following IS is consistent with the DRN sensitization hypothesis of learned helplessness. The DRN contains nitric oxide synthase, thereby making local NO production possible [18,37,40]. One of the best characterized mechanisms by which NO is produced is through the activation of NMDA receptors [43]. In the present experiments blocking NMDA receptors at the time of IS prevented poor escape responding and enhanced conditioned fear, but was not very effective in reversing these effects of IS. Conversely, inhibiting the synthesis of NO was most effective in reversing IS effects, not in preventing them. This pattern is consistent with a scenario in which NMDA receptor activation during IS initiates a cascade of events leading to NO synthesis and consequent sensitization of the DRN to subsequent input during behavioral testing.

Several effects of NO, in addition to its potential role in DRN neural sensitization via a LTP-like mechanism, could account for its role in mediating IS effects. It has been demonstrated that NO enhances the effects of β-endorphin [35]. For example, inhibition of NOS activity attenuates the reduced pain sensitivity that occurs after intracerebral administration of β-endorphin. Since the release of opioid peptides in the DRN is involved in mediating IS effects [12], the enhancing effects of NO on opioid activity could contribute to the heightened responsiveness of the DRN to activation during testing procedures.

In addition, NO interferes with $GABA_A$ receptor function. The formation of NO reduces $Cl-$ accumulation in cortical and cerebellar granule cells [31]. NO has been shown to increase levels of cGMP in amacrine cells by activation of guanylate cyclase [42]. Reduced $GABA_A$ receptor-mediated $Cl-$ currents occur when the receptor is phosphorylated by cGMP-dependent protein kinase [39]. We have demonstrated that interfering with $GABA_A$ receptor mediated inhibition in the DRN is sufficient to produce learned helplessness behavior [21,34]. Thus, NO interference with $GABA_A$ receptor function provides yet another mechanism by which increased NO production in the DRN could result in learned helplessness behavior. NO production could also alter 5-HT transmission by increasing re-uptake of 5-HT. Formation of NO and the consequent increase in cGMP levels phosphorylates the serotonin transporter as shown in rat basophilic leukemia cells [28]. If NO had this effect in the DRN, the reduced amount of 5-HT acting at inhibitory somatodendritic autoreceptors could lead to increased 5-HT transmission in projection regions. It is possible that some or all of these mechanisms contribute to the behavioral consequences of IS.

Acknowledgements

We would like to thank Reza Ghaboosi for his assistance conducting Experiment 1A, Carolyn Robbins and Boyd Schwartz for their help with Experiment 1B, and Matthew McQueen for assistance with Experiment 2. This research was supported by grants NIMH MH50479 and RSA MH00314 to S.F. Maier.

References

[1] Adamec R. Transmitter systems involved in neural plasticity underlying increased anxiety and defense-implications for understanding anxiety following traumatic stress. Neurosci Biobehav Rev 1997;21:755–65.

[2] Adamec R. Evidence that limbic neural plasticity in the right hemisphere mediates partial kindling induced lasting increases in anxiety-like behavior: effects of low frequency stimulation (quenching?) on long term potentiation of amygdala efferents and behavior following kindling. Brain Res 1999;839:133–52.

[3] Adamec R, Burton P, Shallow T, Budgell J. NMDA receptors mediate lasting increases in anxiety-like behavior produced by the stress of predator exposure-implications for anxiety associated with posttraumatic stress disorder. Physiol Behav 1999;65:723–37.

[4] Amat J, Matus-Amat P, Watkins L, Maier S. Escapable and inescapable stress differentially alter extracellular levels of 5-HT in the basolateral amygdala of the rat. Brain Res 1998;812:113–20.

[5] Baranauskas G, Nistri A. Sensitization of pain pathways in the spinal cord: cellular mechanisms. Prog Neurobiol 1998;54:349–65.

[6] Chen C, Tonegawa S. Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. Annu Rev Neurosci 1997;20:157–84.
[7] Fanselow M, Lester L. A functional behavioristic approach to aversively motivated behavior: predatory imminence as a determinant of the topography of defensive behavior. In: R.C. Bolles, M.D. Beecher, editors. Evolution and Learning. Hillsdale, NJ: Erlbaum, 1988.
[8] Ferraro G, Montalbano M, Sardo P, La Grutta V. Lateral habenular influence on dorsal raphe neurons. Brain Res Bull 1996;41:47–52.
[9] Frey U, Morris R. Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. Trends Neurosci 1998;21:181–8.
[10] Gonzalez L, Andrews N, File S. 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res 1996;732:145–53.
[11] Graeff F, Brandao M, Audi E, Schutz M. Modulation of the brain aversive system by GABAergic and serotonergic mechanisms. Behav Brain Res 1986;21:65–72.
[12] Grahn R, Maswood S, McQueen M, Watkins L, Maier S. Opioid-dependent effects of inescapable shock on escape behavior and conditioned fear responding are mediated by the dorsal raphe nucleus. Behav Brain Res 1999;99:153–67.
[13] Grahn R, Will M, Hammack S, Maswood S, McQueen M, Watkins L, Maier S. Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. Brain Res 1999;826:35–43.
[14] Imai H, Steindler D, Kitai S. The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. J Comp Neurol 1986;243:363–80.
[15] Kalen P, Karlson M, Wiklund L. Possible excitatory amino acid afferents to nucleus raphe dorsalis of the rat investigated with retrograde wheat germ agglutinin and D-[^{3}H]aspartate tracing. Brain Res 1985;360:285–97.
[16] Kalen P, Pritzel M, Nieoullon A, Wiklund L. Further evidence for excitatory amino acid transmission in the lateral habenular projection to the rostral raphe nuclui: lesion-induced decrease of high affinity glutamate uptake. Neurosci Lett 1986;68:35–40.
[17] Kalen P, Strecker R, Rosengren E, Bjorklund A. Regulation of striatal serotonin release by the lateral habenula-dorsal raphe pathway in the rat as demonstrated by in vivo microdialysis: role of excitatory amino acids and GABA. Brain Res 1989;492:187–202.
[18] Krukoff T, Khalili P. Stress-induced activation of nitric oxide-producing neurons in the rat brain. J Comp Neurol 1997;377:509–19.
[19] Maier S. Role of fear in mediating shuttle escape learning deficit produced by inescapable shock. J Exp Psychol An Beh Proc 1990;16:137–49.
[20] Maier S. Learned helplessness, fear and anxiety. In: S.C. Stanford, P. Solomon, editors. Stress: From Synapse to Syndrome. London: Academic Press, 1993:207–248.
[21] Maier S, Busch C, Maswood S, Grahn R, Watkins L. The dorsal raphe nucleus is a site of action mediating the behavioral effects of the benzodiazepine receptor inverse agonist DMCM. Behav Neurosci 1995;109:759–66.
[22] Maier S, Grahn R, Maswood S, Watkins L. The benzodiazepine receptor antagonists flumazenil and CGS-8216 block the enhancement of fear conditioning and interference with escape behavior produced by inescapable shock. Psychopharmacology 1995;121:250–8.
[23] Maier S, Grahn R, Watkins L. 8-OH-DPAT microinjected in the region of the dorsal raphe nucleus blocks and reverses the enhancement of fear conditioning and interference with escape produced by exposure to inescapable shock. Behav Neurosci 1995;109:404–12.
[24] Maier S, Kalman B, Grahn R. Chlordiazepoxide microinjected into the region of the dorsal raphe nucleus eliminates the interference with escape responding produced by inescapable shock whether administered before inescapable shock or escape testing. Behav Neurosci 1994;108:121–30.
[25] Maier S, Seligman M. Learned helplessness: theory and evidence. J Exp Psychol 1976;105:3–45.
[26] Maswood S, Barter J, Watkins L, Maier S. Exposure to inescapable but not escapable shock increases extracellular levels of 5-HT in the dorsal raphe nucleus of the rat. Brain Res 1998;783:115–20.
[27] Matthies H. Neurobiological aspects of learning and memory. Ann Rev Psychol 1989;40:381–404.
[28] Miller K, Hoffman B. Adenosine A3 receptors regulate serotonin transport via nitric oxide and cGMP. J Biol Chem 1994;269:27351–8.
[29] Park M. Monosynaptic inhibitory postsynaptic potentials from lateral habenula recorded in dorsal raphe neurons. Brain Res Bull 1987;19:581–6.
[30] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press, 1986.
[31] Robello M, Amico C, Bucossi G, Cupello A, Rapallino M, Thellung S. Nitric oxide and GABAA receptor function in the rat cerebral cortex and cerebellar granule cells. Neuroscience 1996;74:99–105.
[32] Steinbusch H. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience 1981;6:557–618.
[33] Stern W, Johnson A, Bronzino J, Morgane P. Effects of electrical stimulation of the lateral habenula on single-unit activity of raphe neurons. Expl Neurol 1979;65:326–42.
[34] Sutton L, Grahn R, Wiertelak E, Watkins L, Maier S. Inescapable shock-induced potentiation of morphine analgesia: involvement of opioid, GABAergic, and serotonergic mechanisms in the dorsal raphe nucleus. Behav Neurosci 1997;111:816–24.
[35] Tseng L, Xu J, Pieper G. Increase of nitric oxide production by L-arginine potentiates i.c.v. administered beta-endorphin-induced antinociception in the mouse. Eur J Pharmacol 1992;212:301–3.
[36] Vertes R. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. J Comp Neurol 1991;313:643–68.
[37] Wang Q, Guan J, Nakai Y. Distribution and synaptic relations of NOS neurons in the dorsal raphe nucleus: a comparison to 5-HT neurons. Brain Res Bull 1995;37:177–87.
[38] Wang R, Aghajanian G. Physiological evidence for habenla as a major link between forebrain and midbrain raphe. Science 1977;197:89–91.
[39] Wexler E, Stanton P, Nawy S. Nitric oxide depresses GABAA receptor function via coactivation of cGMP-dependent kinase and phosphodiesterase. J Neurosci 1998;18:2342–9.
[40] Wotherspoon G, Albert M, Rattray M, Priestley J. Serotonin and NADPH-diaphorase in the dorsal raphe nucleus of the adult rat. Neurosci Lett 1994;173:31–6.
[41] Zagami M, Montalbano M, Ferraro G, Sardo P, Caravaglios G, La Grutta V. Electrophysiological and iontophoretic aspects of the habenular influence on hippocampal neurones. Arch Physiol Biochem 1995;103:59–63.
[42] Zarri I, Bucossi G, Cupello A, Rapallino M, Robello M. Modulation by nitric oxide of rat brain $GABA_A$ receptors. Neurosci Lett 1994;180:239–42.
[43] Zhang J, Snyder S. Nitric oxide in the nervous system. Annu Rev Pharmacol Toxicol 1995;35:213–33.