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Exposure to an elevated platform increases plasma corticosterone and hippocampal acetylcholine in the rat: reversal by chlordiazepoxide

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Abstract

There is evidence that the septohippocampal cholinergic system is activated in response to stressful stimuli. In addition, prior studies indicate that stimulating the hippocampal cholinergic neurotransmission increases open arm exploration in the elevated plus-maze. This raises the possibility that exposing the rat to an elevated platform, which would be similar to confining the animal to the open arms of the plus-maze, would alter hippocampal acetylcholine levels. Results from the present study suggest that an elevated platform can be used as an animal model of stress in that exposure to the platform significantly increased plasma corticosterone levels. Importantly, exposure to a platform significantly increased hippocampal acetylcholine efflux. Interestingly, the increase in plasma corticosterone and hippocampal acetylcholine levels upon exposure to an elevated platform could be prevented by chlordiazepoxide at a dose that had no effect on basal hippocampal acetylcholine or plasma corticosterone levels. However, the elevated platform-induced increase in hippocampal acetylcholine could not be blocked by prior administration of buspirone. These results provide direct evidence for the importance of the hippocampal cholinergic system in stress and provide validation for the elevated platform as a model of stress.

Keywords: Stress; Acetylcholine; Corticosterone; Benzodiazepine; Buspirone; Hippocampus; Microdialysis

1. Introduction

Emerging evidence suggests that cholinergic neurotransmission in forebrain regions plays a major role in the organisms' response to stressful stimuli (see, e.g. Imperato et al., 1991; Mark et al., 1996). The effects of stress on hippocampal acetylcholine release have been studied by subjecting animals to, for example, acute or chronic restraint stress (Imperato et al., 1991; Aloisi et al., 1997; Mark et al., 1996; Mitsushima et al., 2003; Mizuno and Kimura, 1997; Tajima et al., 1996), repeated foot shocks (Dazzi et al., 1995b), or cold (Fatranska et al., 1989). Whereas in some studies there is a significant correlation between increased plasma corticosterone and hippocampal acetylcholine levels (Mitsushima et al., 2003), other studies do not find this correlation (Imperato et al., 1991). This suggests that the increase in hippocampal acetylcholine levels may not always reflect an emotional response to stress. In addition, it is possible that the hippocampal cholinergic system may only be involved in certain stressful situations. Therefore, it is important to use a model that has been shown to involve the hippocampus when attempting to determine the importance of the hippocampal cholinergic system in modulation of stress (see below).

It is also important to note that increased cholinergic levels appear to result in decreased anxiety. For example, systemic injections of the acetylcholinesterase inhibitor physostigmine decrease novelty-induced neophobia in rats (Sienkiewicz-Jarosz et al., 2000). In addition, systemic injections of nicotinic agonists in rats or mice decrease anxiety in the elevated plus-maze, social interaction, and contextual fear conditioning tests (Brioni et al., 1993; Irvine et al., 1999; Szyndler et al., 2001). One brain structure that may mediate the effects of acetylcholine on stressful situations is the hippocampus. Increases in rats' fear reactions have been observed in a variety of animal models of anxiety following intrahippocampal infusions of both muscarinic and nicotinic receptor antagonists (File et al., 1998; Hess and Blozovski,

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1987; Smythe et al., 1998); reversely, decreases have been found following intrahippocampal infusion of physostigmine (Degroot et al., 2001; Degroot and Treit, 2002, 2003). Also, hippocampal cholinergic blockade enhances the hypothalamic–pitutitary–adrenal response to stress (Bhatnagar et al., 1997). These data suggest that an increase in hippocampal cholinergic neurotransmission may constitute an integral component of a homeostatic response to stress.

The elevated plus-maze has been validated physiologically, pharmacologically, and behaviorally as an animal model of fear and anxiety (Pellow, 1986; Pellow et al., 1985; Pellow and File, 1986). Moreover, the percentage of time spent on the open arms of the maze decreases in stressed animals (Hata et al., 2000). In addition, confinement to the open arms of the plus-maze results in significantly increased plasma corticosterone levels compared to confinement in the closed arms (Pellow et al., 1985). Evidence also suggests that hippocampal cholinergic levels can regulate anxiety levels in the elevated plus-maze. For instance, infusions of physostigmine in the dorsal or ventral hippocampus significantly increased the time spent on the open arms of the plus-maze (Degroot et al., 2001; Degroot and Treit, 2002, 2003). This would suggest that placing an animal on an elevated platform, which is similar to confining animals to the open arms of the maze, would induce stress in animals and hence increase plasma corticosterone concentrations. Also, since increased hippocampal cholinergic levels result in decreased anxiety in the elevated plus-maze, it seems plausible that placing the animal on the elevated platform would affect hippocampal acetylcholine levels. In addition, benzodiazepines are well known to reduce anxiety in the elevated plus-maze, whereas mixed results have been obtained with serotonergic compounds, such as the 5-HT_{1A} receptor agonists. Therefore, it is likely that the administration of, for example, the benzodiazepine, chlordiazepoxide, but not the 5-HT_{1A} receptor agonist, buspirone, would prevent the change in hippocampal acetylcholine levels induced by exposure to the elevated platform. These questions were addressed in the current study.

2. Materials and methods

2.1. Experiment 1

The purpose of experiment 1 was to determine if exposure to an elevated platform would affect plasma corticosterone levels.

2.1.1. Animals

All studies were performed according to the guidelines set forth by the National Institutes of Health and implemented by the Animal Care and Use Committee of Eli Lilly and Company. Male Wistar rats (250–350 g, purchased from Harlan Sprague–Dawley, Indianapolis, IN) were used. They were housed in a vivarium for at least 1 week prior to use with food and water available ad libitum. All rats were handled 3 min per day on the 4 days preceding the experiment.

2.1.2. Surgery

Twenty-four hours prior to the experiment, rats were anaesthetized with isoflurane. A cannula was implanted into the right external jugular vein of each rat for the purpose of administration of drugs and drawing of blood samples. Following surgery, the animals were individually housed in modified 18-l pails with food and water available ad libitum.

2.1.3. Elevated platform and corticosterone assay

A circular Plexiglas platform with a diameter of 28 cm was fitted 24 cm above the bottom of the individual pails. Animals were gently picked-up and placed on the platform for 60 min. A 0.3-ml blood sample was drawn 15 min prior to exposure to the platform, when the animal was placed on the platform, and 15, 60, 90, and 120 min after the animal was placed on the platform. These time intervals were based on previous studies that indicate that the biggest change in corticosterone levels are seen 60 min after stress exposure (e.g. Kinzig et al., 2003). Blood samples were stored in a tube containing 20 μl of 6% EDTA and stored at 20 °C until assayed for corticosterone using an I-125 double antibody RIA kit (ICN Pharmaceuticals, Costa Mesa, CA). In addition to exposure to the platform, animals received an intravenous (i.v.) injection of vehicle (saline) or chlordiazepoxide (1 and 3 mg/kg) 15 min prior to placing the animals on the platform. The same doses of chlordiazepoxide were also given to animals that were not placed on a platform in order to determine if there was an effect on basal plasma corticosterone concentrations.

2.1.4. Statistical analysis

Data are expressed as mean (± S.E.M.) corticosterone levels and analyzed using two-way (treatment × time) analysis of variance (ANOVA). Post-hoc comparisons were made using Bonferroni tests. A P level of 0.05 was used as the criterion for statistical significance.

2.2. Experiment 2

The purpose of experiment 2 was to determine if exposure to an elevated platform would affect hippocampal acetylcholine levels.

2.2.1. Surgery (for animals, see Section 2.1.1 above)

Seven days prior to the microdialysis experiments, the rats were anaesthetized with a mixture of chloral hydrate and pentobarbital (170 and 36 mg/kg in 30% propylene glycol and 14% ethanol, respectively), placed in a stereotaxic apparatus, and implanted with guide cannulae (BAS) in the hippocampus (coordinates AP: −5.2, ML: 5.2, DV: −3.8) according to the stereotaxic atlas of Paxinos and Watson (1998). Twenty-four hours before testing, a 4-mm concentric microdialysis probe (BAS, BR-4) was inserted

through the guide cannula. The correct location of the probes was verified histologically at the end of the experiment.

2.2.2. In vivo microdialysis of hippocampal acetylcholine

Acetylcholine determination in hippocampal dialysates was performed as described (Damsma et al., 1988) with some modifications (Tzavara et al., 2003). On the day of the experiment, a modified Ringer's solution (147.0 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $Na_2HPO_4 \times 7H_2O$, 0.2 mM $NaH_2PO_4 \times H_2O$, pH = 7.25) supplemented with 0.1 μM neostigmine was perfused at a rate of 2.4 μl/min in the hippocampus. Samples were collected every 15 min and analyzed immediately on-line with high-performance liquid chromatography (HPLC) coupled to electrochemical detection, with a 150 × 3 mm acetylcholine-3 column (ESA) maintained at 35 °C. The mobile phase (100 mM di-sodium hydrogen phosphate, 2 mM 1-octanesulfonic acid, and 50 μl/l of the microbicide reagent MB, ESA; pH 8.0, adjusted with phosphoric acid) was delivered by an HPLC pump (ESA) at 0.4 ml/min. The potentiostat used for electrochemical detection (ESA Coulochem II) was connected with a solid-phase reactor for acetylcholine (ESA; acetylcholine-SPR) and with an analytical cell with platinum target (ESA 5041). Animals were given a 3-h stabilization period before baseline samples were collected (four samples). Subsequently, animals were injected with vehicle or drug (see below) and, 30 min later, were placed on the platform for 45 min (three samples). Additional five samples were collected after the animals were removed from the platform and placed back in the test cages where they were initially kept. The elevated platform consisted of a 20.5 × 20.5 cm Plexiglas platform that was elevated to a height of 36 cm. The platform was placed 68 cm below a 300-W light bulb. Prior studies indicate that high lighting conditions increase anxiety in the elevated plus-maze (e.g. Bertoglio and Carobrez, 2002; Morato and Castrechini, 1989). In the present study, the use of intense lighting allowed for a more reproducible and robust increase in hippocampal acetylcholine levels upon exposure to the elevated platform.

We wanted to determine if changes in hippocampal acetylcholine upon exposure to the elevated platform could be counteracted with administration of chlordiazepoxide or buspirone. Thus, animals received intraperitoneal (i.p.) injections of vehicle (saline), chlordiazepoxide (2.5 mg/kg), or buspirone (6 mg/kg) 30 min prior to exposing the animal to the platform. The selection of doses of these two anxiolytic drugs was based on our previous dose–response studies. These studies revealed that chlordiazepoxide (2.5, 5.0, and 10 mg/kg) dose-dependently decreases acetylcholine efflux in the hippocampus with the 2.5 mg/kg exerting only minor, nonsignificant effects (see also Fig. 3). On the other hand, buspirone (3, 6 mg/kg) transiently increased acetylcholine efflux in the hippocampus (see also Fig. 4).

It should be noted that the elevated platforms used in experiments 1 and 2 were similar but not identical (e.g. there were differences in the dimensions and height of the platform and in the use of high lighting conditions; see above). In addition, the rats used in experiments 1 and 2 were implanted with jugular catheters and intracerebral probes, respectively, and were administered compounds via a different route under a different treatment regimen (see above). These differences deemed necessary in order to accommodate for the respective experimental conditions. Importantly, once the animals were placed on the elevated platform(s), they exhibited a characteristic behavioral response that consisted of a transient (about 5 min) exploratory phase (consisting of sniffing, rearing, multidirectional movements) that was followed by a motionless phase (the animals remained still but alert in a crouching position) for the remainder of the experiment. The animals on the elevated platform also attained heightened emotionality (exhibited by urination and defecation). When the animals were returned to their testing cage, they showed high levels of exploratory activity (locomotion, rearing, sniffing) for about 10 min before they settled down for the remainder of the experiment.

2.2.3. Statistics

Data are expressed as mean (± S.E.M.) multifold changes from baseline, which is the average of the four basal values before vehicle or drug injection, and analyzed with two-way (treatment × time) ANOVA followed by the Newman–Keuls test for multiple comparisons. A P level of 0.05 was used for statistical significance.

3. Results

3.1. Experiment 1: exposure to an elevated platform significantly increased plasma corticosterone levels; reversal by chlordiazepoxide

Fig. 1 shows that exposure to an elevated platform for 60 min significantly ($P < 0.05$) raised plasma corticosterone levels at 15, 60, and 90 min post-exposure. Fig. 2 shows that prior administration of chlordiazepoxide at doses that did not affect basal corticosterone levels (1 and 3 mg/kg) prevented the raise in corticosterone levels detected 60 min after exposure to an elevated platform, in agreement with previous studies (see, e.g. File, 1982). The effects of buspirone on plasma corticosterone levels upon exposure to the elevated platform were not studied given its well-documented stimulatory actions on basal plasma corticosterone levels (e.g. Urban et al., 1986).

3.2. Experiment 2: exposure to the elevated platform significantly increases extracellular concentrations of acetylcholine in the hippocampus; reversal by chlordiazepoxide, but not buspirone

Basal levels (mean ± S.E.M.) of acetylcholine in the hippocampus were 0.412 ± 0.08 fmol/sample ($n = 38$).

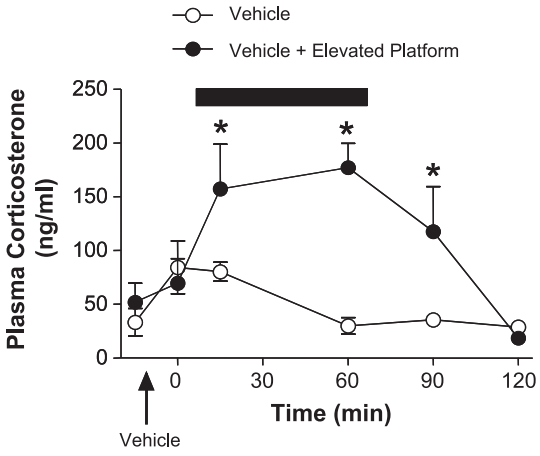

Fig. 1. Exposure to an elevated platform for 60 min (indicated by the solid bar) increases plasma corticosterone levels in the rat. Data are expressed as mean (± S.E.M.; $n = 6–8$/group) plasma corticosterone concentrations (ng/ml) 15 min before injection of vehicle (IV; indicated by the arrow), 15 min after injection of vehicle, and 15, 60, 90, and 120 min after exposure to the elevated platform (solid circles). *: $P < 0.05$ vs. the group treated with vehicle alone (open circles).

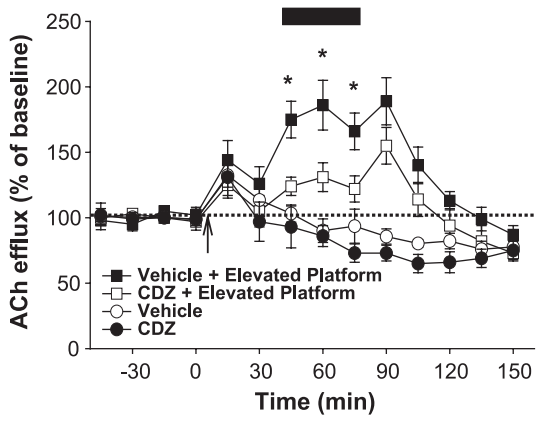

Fig. 3. Exposure to an elevated platform for 45 min (indicated by the solid bar) increases hippocampal extracellular acetylcholine levels: reversal by chlodiazepoxide (CDZ; 2.5 mg/kg, IP). Data are expressed as mean (± S.E.M.; $n = 5–7$/group) percent changes from baseline. Vehicle or CDZ was administered 30 min before the animals were placed to the elevated platform (indicated by the arrow). *: $P < 0.05$ vs. the group that received vehicle before exposure to the platform (open squares).

Fig. 3 shows that extracellular hippocampal acetylcholine levels were increased after placing the animal on the platform for 45 min. Specifically, injection of vehicle transiently increased hippocampal acetylcholine levels by about 40%. Subsequent exposure to the elevated platform produced an approximately 80% increase in extracellular hippocampal acetylcholine levels that lasted for the whole 45 min the animals were on the platform (all three samples were statistically different from the last baseline sample). Acetylcholine concentrations remained elevated during the first 15 min after the animals were placed back in their test cage, sharply reaching baseline within 45 min. Pretreatment (30 min) with chlordiazepoxide at the 2.5 mg/kg dose that by itself did not affect acetylcholine concentrations (Fig. 3) significantly ($P < 0.05$) curtailed the raise in acetylcholine levels during the three 15 min samples the animals were on the platform (Fig. 3). Interestingly, pretreatment with chlordiazepoxide did not affect the increase in acetylcholine levels observed after the animals were placed back in their test cage. Pretreatment with buspirone (30 min) at the 6 mg/kg dose that also did not significantly affect hippocampal acetylcholine levels as compared to vehicle (Fig. 4) did not significantly influence the increase in hippocampal acetylcholine concentrations found during the 45 min the animals were on the elevated platform, although there was a clear

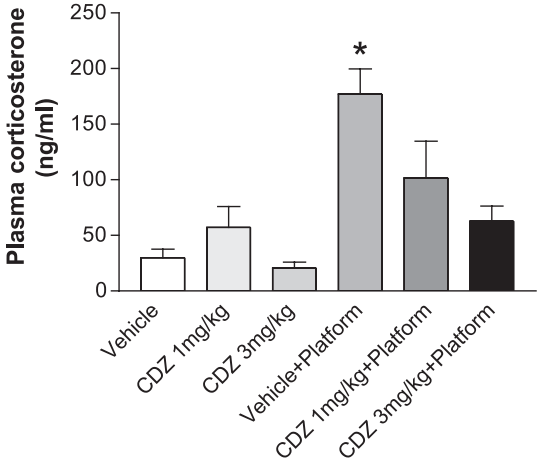

Fig. 2. Pretreatment with chlordiazepoxide (CDZ; 1 and 3 mg/kg, IV) blocks the increase in plasma corticosterone concentration in rats 60 min after exposure to an elevated platform. Data are expressed as mean (± S.E.M.; $n = 6–8$/group) plasma corticosterone concentrations (ng/ml) 60 min after the beginning of exposure to the elevated platform. Vehicle or CDZ was administered 15 min before the animals were placed to the elevated platform. *: $P < 0.05$ vs. all the other groups.

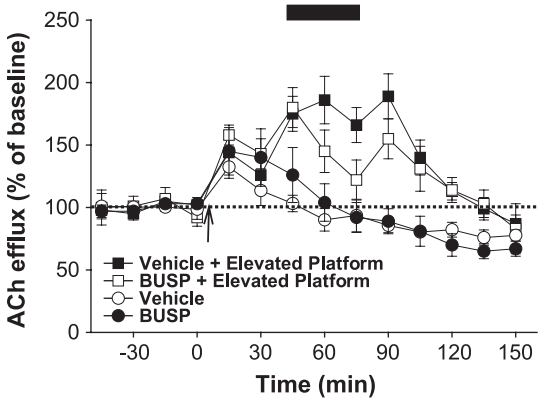

Fig. 4. Exposure to an elevated platform for 45 min (indicated by the solid bar) increases hippocampal extracellular acetylcholine levels: lack of effect of buspirone (BUSP; 6 mg/kg, IP). Data are expressed as mean (± S.E.M.; $n = 5–7$/group) percent changes from baseline. Vehicle or BUSP was administered 30 min before the animals were placed to the elevated platform (the vehicle-treated groups in Fig. 4 are the same groups depicted in Fig. 3).

tendency for a reduction during the second and third 15-min sample (Fig. 4).

It should be noted that although the behaviors of the animals were not systematically studied, it was clear that chlordiazepoxide, but not buspirone, reduced the heightened emotional responsiveness (urination/defecation) upon exposure to the elevated platform.

4. Discussion

In the present study, exposure to an elevated platform significantly increased hippocampal acetylcholine concentrations and plasma corticosterone levels in the rat. In addition, a dose of chlordiazepoxide, but not buspirone, that had no effect on basal hippocampal acetylcholine efflux, suppressed the increase in hippocampal acetylcholine levels induced by exposure to the platform. These data indicate that the elevated platform can be used to assess changes in neurochemical and physiological parameters in response to a stress-like condition in the rat. Similarly to the elevated plus-maze, the neurobiological responses in the elevated platform are sensitive to benzodiazepines, but not 5-HT_{1A} receptor agonists (for a review, see Treit et al., 2003). The results also provide further evidence for the role of the hippocampal cholinergic system in stressful situations given that upon exposure to an elevated platform, a parallel increase in plasma corticosterone concentrations and hippocampal acetylcholine levels was detected. Moreover, the well-characterized anxiolytic drug, chlordiazepoxide, was able to counteract the increase in both plasma corticosterone and hippocampal acetylcholine concentrations.

The present results are in accordance with previous studies (e.g. Aloisi et al., 1997; Dazzi et al., 1995b; Fatranska et al., 1989; Mark et al., 1996), which indicate that exposure to stress increases hippocampal acetylcholine release. Although plasma corticosterone levels and hippocampal acetylcholine efflux were not measured in the same animals and under identical conditions, we clearly found parallel increases in these parameters upon exposure to an elevated platform. As mentioned, these increases in both plasma corticosterone and acetylcholine release in the hippocampus were prevented by relatively low doses of chlordiazepoxide that were ineffective by themselves. The decrease in basal hippocampal acetylcholine levels induced by higher doses of chlordiazepoxide is similar to that obtained with systemic injections of other benzodiazepines (e.g. Dazzi et al., 1995a; Ikarashi and Yuzurihara, 2002). In addition, Dazzi et al., 1995b found that the increase in hippocampal acetylcholine induced by repeated foot shocks could also be blocked by prior administration of benzodiazepines. The increase in hippocampal acetylcholine induced by exposure to an elevated platform could not be blocked by buspirone. This is not surprising given that 5-HT_{1A} receptor agonists (especially partial agonists such as buspirone) have yielded inconsistent results in the elevated plus-maze (for a review, see Treit et al., 2003). In fact, the finding that benzodiazepines, but not 5-HT_{1A} receptor agonists, have an effect in the present paradigm provides further support for the notion that this model is similar to confining animals to the open arms of the elevated plus-maze in terms of the evoked neurobiological responses. In the present study, buspirone, in contrast to its effects in the guinea pig (Wilkinson et al., 1994) and the effects of the full 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OHDPAT) in the rat (Shirazi-Southall et al., 2002), did not significantly affect acetylcholine outflow in the hippocampus, although there was a clear tendency for an increase during the first 60 min postinjection.

Thus far, the underlying mechanism and the physiological significance of the increase in hippocampal acetylcholine in a stressful situation remain unclear. Since previous studies indicate that an increase in hippocampal acetylcholine results in decreased anxiety levels (Degroot et al., 2001; Degroot and Treit, 2002, 2003), it is possible that activation of the hippocampal cholinergic system acts as a coping mechanism during stressful situations. Since an increase in hippocampal acetylcholine is also associated with enhanced cognition (e.g. Carey et al., 2001; Scali et al., 1997), it is likely that increased acetylcholine in the hippocampus facilitates cognitive processing of exteroceptive stimuli and strengthens adaptability, which might decrease anxiety. It is also possible that increased cholinergic levels in the hippocampus affect anxiety through trans-synaptic interaction with other brain structures. For instance, it has been previously proposed that stimulation of the hippocampal cholinergic system ultimately decreases anxiety by modulating the septal γ-amino-butyric-acid (GABA)ergic system (Degroot et al., 2001; Degroot and Treit, 2003). This would explain why exposure to the elevated platform fails to increase hippocampal cholinergic levels when the animal is given a prior administration of chlordiazepoxide, but not buspirone. A systemic injection of chlordiazepoxide, but not buspirone, directly stimulates GABA receptors (e.g. Mahmood and Sahajwalla, 1999; Mierlak and Farb, 1988). Since septal GABA receptors are already stimulated and anxiety is decreased, an increase in hippocampal cholinergic activity becomes redundant. The corollary of such a neurotransmitter system interaction is that the stress-evoked increase in hippocampal acetylcholine efflux is counteracted by relatively low doses of a benzodiazepine and indeed higher doses even decrease basal acetylcholine outflow (present study).

In summary, the present findings demonstrate that exposure to an elevated platform raises hippocampal acetylcholine outflow. Moreover, the data suggest that this paradigm can be effectively used as an animal model of stress since exposure to the elevated platform significantly raised plasma corticosterone levels and chlordiazepoxide blocked this effect. The fact that there was a parallel increase in plasma corticosterone and hippocampal acetylcholine levels and that these effects could be blocked by chlordiazepoxide suggest that the increased hippocampal acetylcholine efflux repre-

sents a neurobiological correlate of stress rather than of some other physiological parameter (e.g. nonspecific motoric stimulation or depression). These findings provide further support for the importance of acetylcholine in anxiety modulation and more specifically the significance of the hippocampal cholinergic system in emotional reactivity. Thus far, results from pilot data in our laboratory suggest that exposure to the elevated platform also increases acetylcholine levels in the prefrontal cortex. In addition, pilot data suggest that similar effects can be obtained in the hippocampus and the prefrontal cortex of mice. Clearly, this paradigm in rodents can be used to explore the neurobiological consequences of an aversive, stressful condition in response to pharmacological manipulations that alleviate anxiety.

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