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Rapid habituation of hippocampal serotonin and norepinephrine release and anxiety-related behaviors, but not plasma corticosterone levels, to repeated footshock stress in rats

É. Hajós-Korcsok[a,*], D.D. Robinson[a], J.H. Yu[a], C.S. Fitch[b], E. Walker[b], K.M. Merchant[b]

[a]*Department of Pharmacology, Pharmacia Corporation, 301 Henrietta Street, 7250-209-307, Kalamazoo, MI 49007-4940, USA*
[b]*Department of Neurobiology, Pharmacia Corporation, 301 Henrietta Street, 7250-209-307, Kalamazoo, MI 49007-4940, USA*

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Abstract

Prior stress exposure is known to alter the activation response to a subsequent stressor. In the present study, we examined neurochemical, neuroendocrinological, and behavioral correlates of short-term adaptation to homotypic stressors administered 60 min apart. An initial electric footshock significantly induced extracellular levels of both serotonin (5-HT) and norepinephrine (NE) in the rat hippocampus (650% and 200% above baseline, respectively), as measured by in vivo microdialysis. A rapid habituation in this response was evident in the inability of a second footshock to evoke similar increases. In contrast, the hypothalamic–pituitary–adrenal (HPA) response was augmented further after the second shock session: plasma corticosterone (CORT) levels were 18.1, 316.5, and 441.6 mg/ml in nonstressed, one-footshock-, or two-footshock-treated rats, respectively. In a social interaction paradigm, rats subjected to a single footshock showed several fear- and anxiety-related behaviors such as increases in freezing and decreases in rearing and active approach for social interaction. Exposure to a second footshock completely blocked the freezing response and restored rearing behavior without affecting the disruption in social interactions. Taken together, these data raise the possibility that neurochemical and neuroendocrine adaptations to short-term homotypic stressors differentially contribute to expression of different fear and anxiety-like responses in the rat.

Keywords: Stress; Adaptation; In vivo microdialysis; Monoamines; Corticosterone; Anxiety

1. Introduction

Integrated response to stressful stimuli is an essential component of adaptive processes critical for survival of the organism. Adaptation to repeated stress is associated with a complex cascade of events, ranging from neurotransmitter release (Kalen et al., 1989) to induction of gene expression (Stamp and Herbert, 2001). It is believed that maladaptations to stress may underlie affective disorders, such as major depression (Heim et al., 1997; Holsboer, 2001). A number of studies have been undertaken to examine and understand stress-induced adaptations in neural, endocrine, and behavioral responses in experimental animals. These studies demonstrate that prior stress exposure alters the response to a subsequent stressor, leading to the development of tolerance or sensitization (Natelson et al., 1988; McEwen, 2000; Stam et al., 2000). The distinct adaptive responses may also be influenced by the type, intensity, and duration of the stressors used, the time lag between stressful episodes, and the outcome measures examined.

It is noteworthy that most studies of stress-induced adaptation utilized repeated, daily stress exposures of various durations. Relatively few studies have investigated biological and behavioral responses to reexposure to the same stressor within hours after the first episode of stress (Shibasaki et al., 1995). It is well accepted that stress adaptations are critically dependent on genomic responses triggered by induction of immediate early genes, as well as transcriptional effects of nuclear hormone receptor ligands such as corticosterone (CORT). The stress-induced genomic effects are initiated within minutes of stress exposure and culminate over hours into an integrated neuroplasticity response that can produce long-term alterations in neuro-

* Corresponding author. Tel.: +1-616-833-4813; fax: +1-616-833-9763.
E-mail address: eva.hajos-korcsok@pharmacia.com (É. Hajós-Korcsok).

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endocrine, neurochemical, and behavioral effects of a subsequent stressor (Koolhaas et al., 1997). As a first step to understand the neurobiology of adaptations to repeated stress, we set out to study effects of homotypic stressors applied within a short time frame that could enable modulation of neuroplastic responses triggered by the first stressor.

The hypothalamic–pituitary–adrenal (HPA) axis is the central stress axis that regulates the synthesis and secretion of the neuroendocrine mediator, CORT, in rodents. It is well recognized that CORT, acting via the glucocorticoid and mineralocorticoid receptors, differentially modulates the effects of acute and chronic stress in both the CNS and peripheral systems (Stamp and Herbert, 2001). Moreover, hippocampal corticosteroid receptors are thought to provide critical neural feedback regulation of the HPA axis (Meaney et al., 1996; Reul et al., 1997, 2000). Hence, we monitored changes in plasma CORT levels, as well as hippocampal monoamine neurotransmission in rats subjected to repeated footshock stress. Among the central monoamine systems, the serotonin (5-HT) and norepinephrine (NE) systems have been implicated as important components of the central neurotransmitter network that plays a role in adaptation to stress (Stanford, 1996; Chaouloff, 2000). In vivo microdialysis studies provide evidence that acute stress induces widespread activation of the brain monoamine systems, however, repeated exposure to stress can result in either sensitization (Adell et al., 1988; Jordan et al., 1994; Jedema et al., 1999) or tolerance (Clement et al., 1998; Kirby and Lucki, 1998) to a subsequent stressful experience. Additionally, these neurotransmitters are thought to be involved in efficacy of antidepressant agents suggesting that they play a critical role in disorders associated with imbalance in the stress systems (Heninger et al., 1996).

Finally, a frequent behavioral response to stressful events is manifested as anxiety in both preclinical species and in humans. Both 5-HT and NE systems have been implicated in fear and anxiety responses. In particular, enhanced activity of the central NE system has been suggested to induce fear- and anxiety-related behaviors (Bremner et al., 1996). Similarly, facilitation of brain 5-HT neurotransmission has been proposed to increase anxiety (Wise et al., 1972; Iversen, 1984), although more recent studies are contradictory to this notion (Handley and McBlane, 1993; Hashimoto et al., 1999). Thus, in the present study we attempted to correlate adaptations in hippocampal monoamine systems to those in anxiety-related behaviors.

2. Methods

2.1. Animals

All studies were conducted with male Sprague–Dawley rats (270–300 g, Harlan, Indianapolis, IN). Animals were housed in groups of five to six, kept under conditions of controlled temperature (21 ± 1 °C) and lighting (lights on 06:00–18:00 h) and were given food pellets and water ad lib. Studies were performed between 08:00 and 16:00 h. All procedures were carried out under an approved animal use protocol and were in compliance with the Animal Welfare Act Regulations (9 CFR Parts 1, 2, and 3) and with the Guide for the Care and Use of Laboratory Animals (ILAR 1996).

2.2. Microdialysis and stress procedures

In vivo microdialysis was performed on conscious rats as described previously (Hajós-Korcsok et al., 2000). Two days before microdialysis experiments, microdialysis guide cannulae (CMA, Chelmsford, MA) were implanted into the ventral hippocampus under isoflurane anesthesia (induced at 4%, maintained at 2.5–3%). Stereotaxic coordinates for final microdialysis probe placement were AP −5.5 mm, ML −4.8 mm, DV −8.5 mm, from the bregma and dura surface (Paxinos and Watson, 1986). Following complete recovery from anesthesia, rats were returned to their home-cages.

On the day of the experiment, microdialysis probes (4 mm tip length) were inserted into the guide cannula and rats were placed into an electric footshock cage (Med Associates, Georgia, VM) to acclimatize for at least 30 min. The dialysis probe was attached to a perfusion pump via PE tubing and a liquid swivel. A microdialysis system for freely moving animals (BAS, West Lafayette, IN) was used to enable microdialysis on rats freely moving inside the shock cage. Probes were perfused continuously (2 μl/min) with artificial cerebrospinal fluid (aCSF) containing 145 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 2 mM Na_2HPO_4, and 1.2 mM $CaCl_2$ (pH 7.4). Perfusates were collected every 10 min using a refrigerated fraction collector (BAS) into glass vials containing 5 μl of 0.01 N perchloric acid and 2.5 μl of 1 mM EDTA (total volume of 27.5 μl). Samples were analyzed for 5-HT and NE by high-pressure liquid chromatography (HPLC; see below). After establishing stable basal levels of 5-HT and NE (3–4 h after commencing perfusion), a single electric footshock (ES1) was delivered (1 mA, 0.2 s duration, 1 s intershock interval, 30 s total time). Rats received a second electric footshock (ES2) 60 min after the first shock, and microdialysis samples were collected for a further 60 min.

2.3. HPLC measurements of dialysate 5-HT and NE

Simultaneous detection of perfusate 5-HT and NE was achieved using HPLC with electrochemical detection (EC). Samples (20 μl) were injected by a refrigerated autosampler (Model 542, ESA, Chelmsford, MA) onto a Hyperosil ODS C_{18} column (4.6 I.D. × 150 mm, 3-μm particles, 40 °C column temperature) perfused by HPLC pump (Model 582, ESA) at 0.55 ml/min rate with a mobile phase comprising 75 mM NaH_2PO_4, 1.8 mM sodium octane

sulfonate, 25 μM EDTA, and 10% (v/v) methanol (final pH 3.0). Samples were analyzed by a Coulochem II 5200A electrochemical detector with a Model 5041 high-sensitivity analytical cell and a Model 5020 guard cell (all from ESA). Electrochemical detection was performed at 220 mV with the guard cell at 350 mV.

2.4. Plasma CORT measurements

In a separate study, we established the time-course of footshock induced CORT elevations in the plasma in order to determine the time at which the second stressor can be administered. Five groups ($n = 8$ per group) of rats were used for the purpose. One group remained in the home-cage to serve as the nonstressed control whereas the other rats were subjected to ES1 (identical to that used for the microdialysis studies described above) and sacrificed at 5, 20, 60, or 180 min, respectively, after the stressor. All animals were sacrificed by decapitation between 9:00 and 11:00 a.m. to minimize alterations in plasma CORT levels due to the circadian rhythm. Trunk blood was collected and plasma separated for analysis of CORT levels by radioimmunoassay using a kit from ICN (Orangeburg, NY). For the stress adaptation studies, a separate cohort of rats was subjected to the stress procedure identical to that used in the microdialysis experiments. Animals were habituated to the footshock cage for 30 min, followed by delivery of electric footshocks (1 mA, 0.2 s duration, 1 s intershock interval, 30 s total time). Control animals were exposed to the footshock cage for 30 min but received no electric shock. A third group of rats received ES2 60 min following the first shock. During the time lag between the two footshock sessions, rats remained in the shock cage as was the case for the microdialysis study. Animals were quickly decapitated 10 min following the end of the electric footshock session, trunk blood was collected, and plasma CORT levels were determined by radioimmunoassay.

2.5. Social interaction

To evaluate the effect of single or repeated footshock on anxiety-like behaviors, a separate study was carried out. The footshock apparatus and procedure were identical to those used for the microdialysis and endocrine studies described above. Animals were divided into three separate groups ($n = 8$). The control group consisted of rats exposed to the footshock apparatus for 30 min without receiving shocks. A second group was subjected to ES1 (1 mA, 0.2 s duration, 1 s intershock interval, for 30 s) and the third group received ES2, 60 min after ES1. Social interactions were assessed in a 40×40 cm open field as follows. The experimental animal from each of the three groups described above was introduced into the open field immediately following the exposure to the footshock apparatus with or without footshock. Each experimental rat was paired with a naïve, resident rat that was unfamiliar to the experimental animal and had been habituated to the open field for at least 20 min. After introducing the experimental animal into the open field with the resident rat, behaviors initiated by the experimental rat were recorded by a person blind to the nature of the shock treatment of the experimental groups. Seven discrete behaviors were selected based on pilot studies during which the animals were videotaped and their behaviors analyzed critically at subsequent sessions. These were active approach towards the resident rat, active avoidance of the resident rat, no-contact freezing, rearing, grooming/sniffing, exploration without contact with the resident rat (no-contact exploration), and in-contact of the resident rat but without other activities (in-contact, vigilance). The dominant behavior of the experimental animal was noted every 20 s for a period of 5 min. The order of testing of each experimental group was randomized such that on a given day of testing (between 8:00 a.m. and 2:00 p.m.) each group was represented equally through the course of the day.

2.6. Data analysis

Microdialysis data are presented as the percentage of basal 5-HT and NE levels determined in dialysate samples. Data are corrected for sampling delay, i.e., the time to account for the outflow of the dead volume contained in the outlet tubing (from the probe to the collection vial, approximately 10 min) but not for membrane recovery. Basal levels were established using the average of the last three to four samples preceding the first footshock. The effects of footshock on 5-HT and NE were analyzed using one-way ANOVA with repeated measures followed by Fisher's PLSD test. For the behavioral analysis, the percentage of time spent in each category of behavior was computed. Effects of footshock on behavior and plasma CORT levels were analyzed by one-way ANOVA. Significant group differences were established using Fisher's PLSD test.

3. Results

3.1. Rapid adaptation of hippocampal 5-HT and NE release following repeated footshock sessions

Time-course effect of two consecutive electric footshocks (ES1 and ES2) on 5-HT release in the ventral hippocampus is shown in Fig. 1. Basal dialysate 5-HT levels in the hippocampus were 0.484 ± 0.099 fmol/μl ($n = 10$). One-way ANOVA showed a significant overall effect of footshock on 5-HT [$F(18,167) = 3.41$, $P < .00001$]. ES1 caused a marked (650%), but short-lasting increase in dialysate 5-HT levels evident only at 10 min after the stressor. Interestingly, ES2 did not alter 5-HT levels in the perfusate at any time point. Post hoc analysis (PLSD test) revealed that

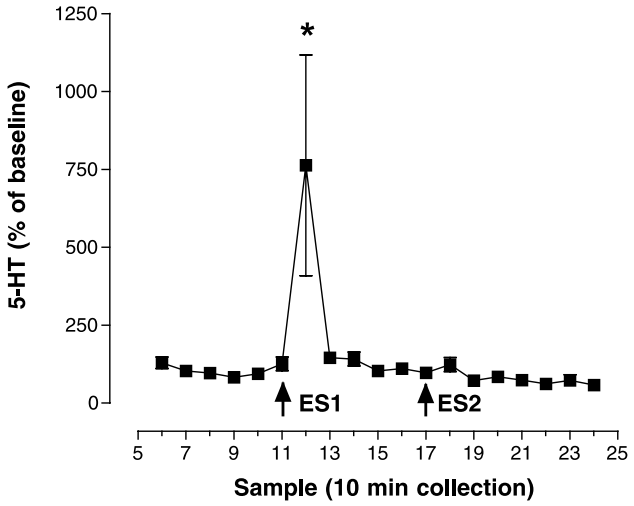

Fig. 1. Effect of two consecutive electric footshocks on dialysate 5-HT levels in the hippocampus of rats. Animals received two 0.5-s duration electric footshocks (1 mA) 60 min apart, as indicated by the arrows (ES1 and ES2). Each point is a mean ± S.E.M. ($n = 10$). * $P < .00001$ vs. preshock 5-HT levels.

dialysate 5-HT levels were significantly different between ES1 vs. preshock ($P < .0001$) and ES1 vs. ES2 values ($P < .00001$) but not ES2 vs. preshock.

Basal dialysate NE levels in the hippocampus were 0.475 ± 0.190 fmol/μl ($n = 10$). One-way ANOVA showed a significant overall effect of footshock on NE [$F(18,165) = 2.53$, $P < .01$]. Similar to changes in 5-HT, ES1 robustly increased (200%) dialysate NE levels while ES2, administered 60 min later, failed to induce such an effect (Fig. 2). Within group comparisons (PLSD test) revealed that dialysate NE levels were significantly different from preshock levels at both 10 ($P < .02$) and 20 min

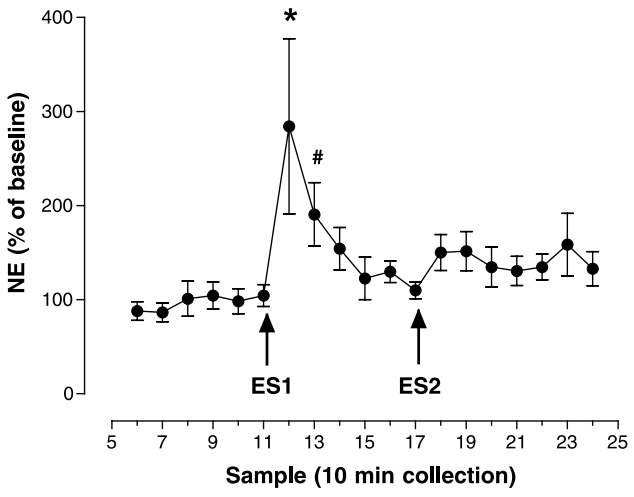

Fig. 2. Effect of two consecutive electric footshocks on dialysate NE levels in the hippocampus of rats. Animals received two 0.5-s duration electric footshocks (1 mA) 60 min apart, as indicated by the arrows (ES1 and ES2). Each point is a mean ± S.E.M. ($n = 10$). * $P < .00001$ and $^{\#}P < .05$ vs. preshock NE levels.

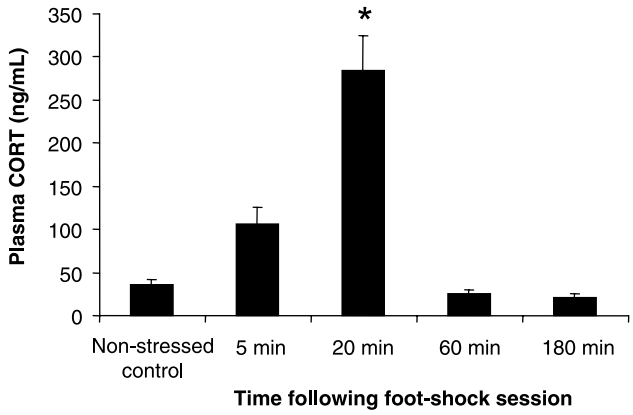

Fig. 3. Time course of changes in plasma CORT levels in rats subjected to ES1. Four groups of rats ($n = 8$ per group) were subjected to ES1 and sacrificed at 5, 20, 60, or 180 min after the footshock. A separate group of rats was sacrificed without being subjected to footshock to serve as the control. Each bar represents a mean ± S.E.M. * $P < .001$ vs. nonstressed control.

($P < .04$) after ES1. The effect of ES2 was not significantly different from basal levels.

3.2. Sensitization of plasma CORT response to repeated footshock

Initially, we examined the time course of changes in plasma CORT following a single footshock session. These data indicate that plasma CORT levels return to baseline within 60 min after subjecting rats to ES1 (Fig. 3). Next, we used the same stress protocol employed for the microdialysis and plasma CORT studies. One-way ANOVA revealed a significant effect of stress session on CORT [$F(2,20) = 43.39$, $P < .0001$]. ES1 induced a marked, 18-fold increase in plasma CORT levels at 10 min after the stressor, when compared to nonstressed rats (Fig. 4). There

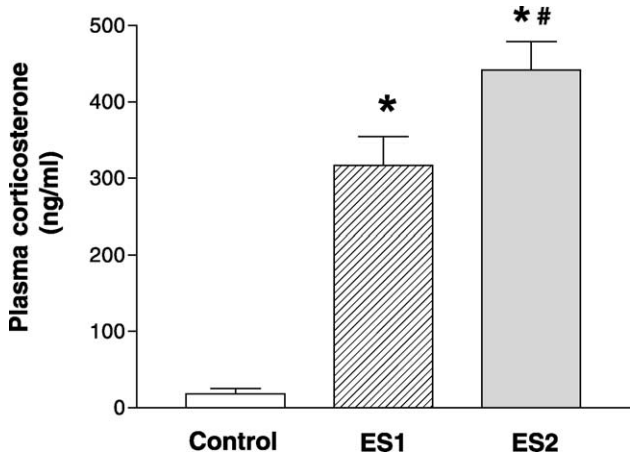

Fig. 4. Plasma CORT response to single and repeated electric footshocks. Animals received ES1 or ES2 60 min apart. Plasma levels were determined 10 min following stress. Control animals were exposed to the shock cage for 10 min but received no shock. Each bar represents a mean ± S.E.M. ($n = 7–8$ per group). * $P < .0001$ vs. control, $^{\#}P < .01$ vs. ES1.

Table 1
Effects of repeated footshocks on behaviors during the social interaction test

	% Time spent in each activity (mean ± S.E.M.)				
	Active approach	Active avoidance	No-contact exploration	Grooming/sniffing	In-contact vigilance
Nonstressed control	55.83 ± 2.16	0.83 ± 0.83	15.00 ± 1.67	3.33 ± 1.26	1.67 ± 1.67
ES1	14.17 ± 4.62***	4.17 ± 1.22	16.67 ± 3.09	6.67 ± 1.78	31.67 ± 8.33**
ES2	14.17 ± 4.95***	6.17 ± 2.50*	10.83 ± 2.8	10.00 ± 2.18*	35.00 ± 8.43**

Data are expressed as mean percentage of time spent in each activity (± S.E.M.) for the group. Note that in addition to the categories shown here, two other behaviors (freezing and rearing) were monitored and are reported in Fig. 5.

* $P < .02$ when compared to respective nonstressed control.

** $P < .005$ when compared to respective nonstressed control.

*** $P < .0001$ when compared to respective nonstressed control.

was further augmentation in this response upon exposure to ES2. The ES2 group was significantly different from both control ($P < .0001$) and ES1 animals ($P < .01$).

3.3. Rapid adaptation in some behavioral responses to repeated footshock stress

Rats subjected to ES1 showed several behaviors consistent with heightened anxiety. Thus, compared to the nonshocked animals, ES1-exposed rats showed increases in no-contact freezing, decreases in active approach of the resident rat, decreases in rearing, and an increase time spent in contact with the resident rat without any activity (in-contact vigilance) (Table 1 and Fig. 5). Rats subjected to ES2 displayed negligible no-contact freezing and engaged in rearing behavior to the same extent as nonstressed rats (Fig. 5). Statistical analysis (one-way ANOVA) showed a significant effect of stress on freezing [$F(2,21) = 17.48$, $P < .001$] and rearing [$F(2,21) = 5.22$, $P < .01$] with the ES2 group being significantly different from the ES1 animals ($P < .0001$ and $P < .02$, respectively). On the other hand, measures of social interaction (active approach and active avoidance of the resident rat) and in-contact vigilance did not differ between rats subjected to ES1 and ES2 (Table 1). Finally, exploratory activity (no-contact exploration) was the one behavior that was not affected significantly by either ES1 or ES2.

4. Discussion

In the present study, we have demonstrated that a short-lasting (0.5 min), acute electric footshock session induces a marked increase in both 5-HT and NE synaptic levels in the hippocampus. Interestingly, in the same animals, the magnitude and time-course of the dialysate 5-HT and NE changes to footshock appeared distinct, the NE response being more modest but longer lasting than 5-HT. Direct comparison of the magnitude and time-course of stress-induced monoamine responses in the present study with those reported in the literature is hampered by the use of different stress protocols and sampling times. However, in agreement with the present findings, numerous microdialysis studies have shown that exposure to an acute stress increases extracellular levels of 5-HT and NE in the rat hippocampus (Abercrombie et al., 1988; Kalen et al., 1989; Kirby et al., 1997; Vahabzadeh and Fillenz, 1994), although no change (Kirby et al., 1997) or reduction (Adell et al., 1997) in monoamine release is also reported. This

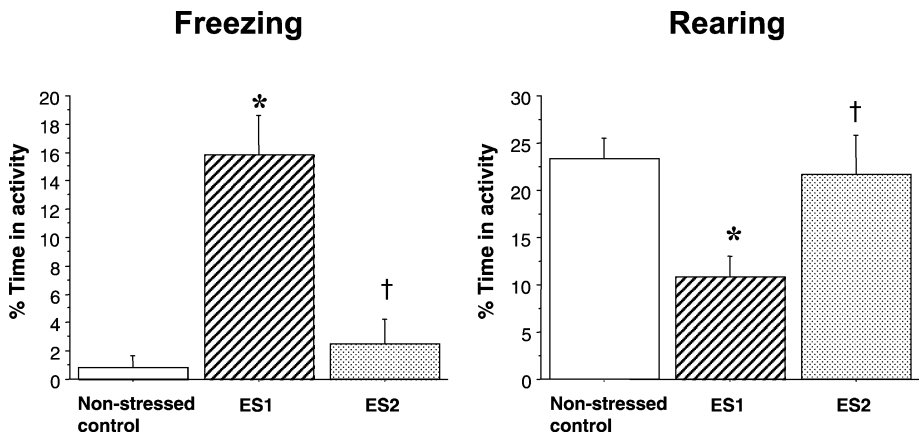

Fig. 5. Behavioral responses following repeated footshocks in the rat. Animals received ES1 or ES2 60 min apart. Control animals were exposed to the shock cage but received no shock. Test animals were monitored for anxiety related behaviors in an open field while being paired with a naïve, habituated rat. This figure shows the percentage of time spent in two of seven distinct behavioral categories, freezing and rearing. Each bar represents a %mean ± S.E.M. ($n = 8$ per group). * $P < .0001$ vs. control, † $P < .01$ vs. ES1.

heterogenous response to stress is likely to be related to differences in the quality of stressors used by the various studies.

An interesting finding of the present study is the apparent adaptation to footshock stress that led to the inability of ES2 to induce 5-HT and NE response when administered 60 min following the first session. The lack of monoamine response to the second stress is not likely to be due to depletion of the neurotransmitters during microdialysis since studies using depolarizing pulses of electrical or chemical (high K^+) stimulation showed that monoamine neurons are capable of releasing similar amount of neurotransmitter following two subsequent stimulations administered 60 min apart (McQuade and Sharp, 1995; Hughes and Stanford, 1998; Gartside et al., 2000; Hajós-Korcsok and Sharp, 2002). There seem to be conflicting reports in the literature with regards to habituation or sensitization of 5-HT and NE release after repeated stress (see Introduction). While the reason for these inconsistencies is not clear, different experimental conditions are likely to play a role. Present findings are in agreement with several studies demonstrating habituation in 5-HT and NE release in various brain areas following daily exposure to homotypic stressors (Clement et al., 1998; Kirby and Lucki, 1998; Pacak et al., 1993). In addition, similarly to our findings, a rapid habituation in NE release is reported when restraint stress, but not tail pinch, is administered repeatedly at 2-h intervals (Shibasaki et al., 1995).

The HPA axis serves as a major effector system to maintain homeostasis during stress exposure. The stress hormone, CORT in rodents, is a critical endogenous stress signal as well as a mediator of stress-induced plasticity (reviewed by Bohus et al., 1996). We assessed the activity of the HPA axis in the acute, repeated stress protocol by measuring plasma levels of CORT. Using identical stress procedures as in the microdialysis experiments, we found that rats subjected to ES1 displayed significantly elevated levels of CORT compared to nonstressed rats. Interestingly, in rats subjected to ES2, CORT levels were increased even further. Our time-course experiment indicates that plasma CORT levels return to baseline by 60 min post-ES1, the time when the ES2 was administered. Taken together, these data indicate that the augmented CORT response to the ES2 is not a result of additive effects of ES1 and ES2. Thus, in contrast to the monoamine changes, the HPA response was sensitized after the ES1. A similar divergence in 5-HT release, and CORT response is reported following exposure to two sessions, 24 h apart, of forced swim stress in rats (Kirby and Lucki, 1998). However, unlike the present data, Kirby and Lucki (1998) found no evidence of sensitization in CORT response, even though forced swim-induced 5-HT release was blunted during the second session. The sensitized HPA response to a homotypic stressor demonstrated here is in contrast to reports of either no change in plasma CORT response after 5-day immobilization stress (Clement et al., 1998) or a tolerance in plasma CORT release following repeated restraint stress (Chen and Herbert, 1995; Stamp and Herbert, 1999, 2001; our own unpublished data). The differences in adaptive responses to the homotypic stressors may be due to differences in the stress modality, duration, or time lag between stress episodes.

The functional significance of these divergent neuroendocrine and neurochemical adaptations were evaluated further in a behavioral paradigm aimed at examining fear- and anxiety-related behaviors in an open field. Rats subjected to ES1 showed several behaviors consistent with heightened anxiety such as freezing, decreases in rearing, deficits in social interactions, and increases in in-contact vigilance. Interestingly, two of these behaviors, freezing and decreased rearing, were not observed in rats subjected to ES2. These results are consistent with a published report from Thorsell et al. (1999), who reported that restraint-induced anxiety assessed using the elevated plus maze was no longer evident following 9–10 days of repeated restraint sessions in rats. In contrast, Zafar et al. (1997) found no habituation in restraint stress-induced inhibition of behaviors in an open field following eight consecutive, daily restraint sessions in Wistar–Kyoto rats. Several factors, including the strain, type, and duration of stress could account for the apparent differences between findings reported here and those of Zafar et al. (1997). It is noteworthy, that only those behaviors that may be classified strictly as innate, fear-related responses (freezing and reduced rearing) showed adaptations in the ES2 animals. This may be attributable to the homotypic nature of the two successive stressors used in the present study. It will be interesting to determine whether differing stress modalities when applied during a short time can induce tolerance to fear responses. On the other hand, social interactions represent complex behaviors that can be modulated not only by fear and anxiety but also motivational states appear to be more resistant to manipulations by short-term, homotypic stressors. One implication for these data could be that some simple fear responses may be more amenable to modulation and this could underlie the relatively high success rate of exposure therapy of certain phobias (Fava et al., 2001).

Although the neurochemical, endocrine, and behavioral measures were undertaken in different groups of rats, one could draw cautious conclusions from the apparent correlations between different measures. Thus, it appears that increases in certain fear-like responses induced by ES1 may be related to augmented 5-HT and NE synaptic levels in the hippocampus. On the other hand, the anxiety-induced disruption in complex social interactions appears to be independent of the acute increases in synaptic 5-HT and NE levels in the hippocampus but may be related to sustained activity of the HPA–limbic systems. Clearly, further studies examining effects of a variety of homo- and heterotypic stressors and direct application of 5-HT and NE ligands in the hippocampus are needed to clarify the association between the neurotransmitter systems and stress adaptations shown here.

In summary, findings of the present study provide evidence of rapid adaptation in neurochemical, endocrine, and behavioral responses to a homotypic stressor, mild-intensity footshocks delivered over a short duration of 30 s. The habituation in hippocampal synaptic monoamine release correlates with some innate fear-related behaviors but not plasma CORT levels. These data raise the possibility that acute augmentation in synaptic 5-HT and/or NE levels in the hippocampus may be associated with certain types of clinical anxiety disorders. On the other hand, sustained or sensitized activity of HPA system may contribute to other anxiety-related behaviors.

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