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Prevention of Learned Helplessness: In Vivo Correlation With Cortical Serotonin

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PETTY, F., G. KRAMER AND L. WILSON. *Prevention of learned helplessness: In vivo correlation with cortical serotonin.* PHARMACOL BIOCHEM BEHAV 43(2) 361-367, 1992.—Learned helplessness (LH) is prevented by pretreatment with acute benzodiazepines (BDZs), subchronic tricyclic antidepressants (TCAs), or escapable stress (ES). We have investigated the role of serotonin (5-HT) in LH prevention by these three prevention paradigms, using microdialysis to measure in vivo 5-HT release in frontal cortex (FC) after LH testing. Rats receiving pretreatment before inescapable stress with any of the three methods of prevention—BDZs, TCAs, or ES—showed escape behavior in the shuttle-box test for LH comparable to naive unstressed controls. K^+-stimulated 5-HT release in all three groups receiving pretreatment was also similar to naive unstressed controls. Rats receiving saline before inescapable stress showed significantly more LH behavior in the shuttle-box task and had significantly lower 5-HT release as well. This suggests that LH correlates with a significant decrease in intracellular releasable 5-HT in FC, and that three different techniques for LH prevention, acute BDZs, subchronic TCAs, and ES all similarly prevent this 5-HT depletion.

Frontal cortex Serotonin Diazepam Imipramine Learned helplessness
Inescapable stress Escapable stress

LEARNED helplessness (LH) is a maladaptive behavioral depression caused by exposure to inescapable stress. The neurochemical and neuroanatomic substrates for LH have been the focus of considerable research. Several neurotransmitter systems have been studied in this phenomenon, including dopamine (46), acetylcholine (25), GABA-benzodiazepine (BDZ) (10,22,27,30), glutamate (29), opiate (9,45), and norepinephrine (33,43,44). Serotonin (5-HT) has also been the focus of considerable research on the neurobiochemical mechanism of LH and the role of 5-HT in this animal model of depression seems complex, with reports of low, high, or normal 5-HT function in LH.

Support for a functional deficit of 5-HT in LH derives from our finding that microinjection of 5-HT into frontal cortex (FC) reversed helpless behavior (39). Potassium-stimulated 5-HT release was subsequently shown to be low in brain tissue slices from both FC and septum in rats subjected to inescapable stress. This functional deficit was reversed by chronic imipramine administration and the reversal correlated with the restoration of normal behavior (39). Experiments with push-pull perfusion of FC showed rats with the greatest stress-induced decrease of 5-HT became most helpless on subsequent testing (31). Additional support for a 5-HT deficit in LH derives from the demonstration that a specific 5-HT uptake blocker, alaproclate, reversed LH (32). 5-HT receptor blockade with methysergide produced LH behavior in naive rats and was reversed by the 5-HT releasing agent, *p*-chloroamphetamine (15). Decreased brain 5-HT turnover has been correlated with increased LH (41). Finally, the 5-HT_{1A} agonists ipsapirone and buspirone have been shown to reverse LH (8,14) and buspirone has also been shown to prevent LH (8).

Other work suggests that 5-HT excess occurs in LH. Intraperitoneal injection of the 5-HT precursor L-tryptophan or 5-hydroxytryptophan (5-HTP) sufficient to raise brain 5-HT levels produced LH behavior that was blocked by methysergide. Methysergide was also shown to prevent the development of stress-induced LH (4). These studies suggest that enhancement of 5-HT activity may produce LH, and blockade of 5-HT receptors with antagonists may prevent LH. Further support for elevated 5-HT in LH derives from regional levels of 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) in FC, hippocampus, and hypothalamus being higher in rats subjected to inescapable stress (16). Rats treated with the 5-HT depletor *p*-chlorophenylalanine (PCPA) did not develop LH after exposure to inescapable stress (12). Furthermore, increases in the density of the 5-HT uptake site were found to depend upon brain region in both stress-induced helpless rats and genetically helpless rats (11).

Negative or inconclusive studies of 5-HT in LH are also

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reported. Neither PCPA nor 5-HTP had an effect on LH in one study (2), while 5-HTP had a paradoxical effect in another (32). Depletion of 5-HT neurons sufficient to produce a 70% loss of tryptophan hydroxylase activity altered neither stress-induced LH nor the ability of antidepressants to reverse LH in another study (40). In pilot studies using the in vivo microdialysis technique (28), we found that in vivo levels of 5-HIAA in FC perfusate of rats receiving escapable shock were higher than those of rats receiving yoked inescapable shock or no shock, which suggests that increased 5-HT turnover in FC is associated with escapable stress and not with development of LH.

Thus, the role of 5-HT in LH is incompletely understood. We, therefore, examined in vivo 5-HT in a paradigm for LH prevention. BDZs, given acutely, prevent but do not reverse LH. Tricyclic antidepressant (TCA) drugs, given subchronically, will prevent and reverse LH (38). Also, animals preexposed to escapable stress do not develop LH when subsequently exposed to inescapable stress. Thus, the prevention of LH with three seemingly dissimilar methods—subchronic TCAs, acute BDZs, escapable training—offers a paradigm for studying the role of 5-HT mechanisms in preventing LH.

The recent development of in vivo microdialysis and its extension to behavioral studies (1,18) enables repetitive sampling in the same animal and the study of 5-HT release from discrete brain regions of conscious freely moving animals. Medial FC was chosen for study because previous research has shown this to be a key brain region in LH neurochemistry (27,39). We have, therefore, used in vivo microdialysis of FC to study 5-HT mechanisms in LH prevention.

METHOD

Behavioral

Male Wistar rats (250–300 g) were used. Animals were acclimatized to the laboratory environment for a minimum of 2 weeks prior to the experiments. There were five experimental groups, one control and four pretreatment. "Control" nonstressed rats were placed in Plexiglas tail-shock cages and had electrodes attached but received no injections or tail-shock. "Stress" rats received normal saline (0.5 cc) injection 30 min prior to tail-shock stress. "Diazepam" (DZP) rats received one injection of diazepam (10 mg/kg) 30 min prior to tail-shock stress. "Imipramine" (IMI) rats received 16 mg/kg imipramine twice daily for 4 days prior to tail-shock stress exposure with the last injection (16 mg/kg) 30 min before tail-shock exposure. "Shuttle" rats received the shuttle-box escape task described below 24 h before tail-shock stress.

All four pretreatment groups then were given 80 trials of tail-shock in Plexiglas chambers with metal electrodes (23). Each session began with an unsignaled 5-s, 1-mA shock, increased by 0.3–0.4 mA every 20 trials to a final current of 2 mA, with a 60-s variable intertrial interval. One day after tail-shock stress, all rats were tested for LH in a shuttle-box (19). Essentially, this involves a shuttle-box escape task with each trial initiated by a 5-s warning tone followed by a 1.0-mA grid shock. The first five trials require a single crossing to terminate shock (FR 1). These trials are unaffected by prior shock exposure and serve to ensure animal's having normal motor function. The subsequent 25 trials require two crossings to terminate shock (FR 2). If the escape response does not occur within 30 s, a trial is automatically terminated. There is a 60-s variable intertrial interval. Escape latencies are measured on both the FR 1 and FR 2 trials of testing. Pilot studies showed naive rats to reliably score 12 ± 4 s (mean ± SD) for the FR 2 escape latencies. A criterion was established from latencies of naive controls on pilot experiments such that prestressed rats with a mean FR 2 trial of all trials latency greater than or equal to 20 s are termed learned helpless (LH), while prestressed rats with mean latencies less than 20 s, are nonhelpless (NH). Using this procedure in our laboratory, approximately 50% of stressed animals routinely demonstrate escapes latencies in the LH range.

Microdialysis

Animals received stress on day 1 and were tested in the shuttle-box about 24 h later (day 2). They were then anesthetized with phenobarbital (45 mg/kg) and secured in a stereotaxic apparatus. A loop-design microdialysis probe was inserted into the FC [3.2 mm anterior, 0.7 mm lateral, and 4.8 mm vertical from bregma (26)]. Animals recovered from surgery and were returned to individual cages, where they resumed normal feeding and grooming within 2–4 h. The day after microdialysis probe implanation (day 3), perfusion was initiated at 1.0 μl/min using a Ringer's solution (147 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$) with fluoxetine (1 μm) added to increase basal 5-HT levels. Basal levels of 5-HT obtained ranged between 5–10 pg/20 min. After obtaining a stable baseline for 5-HT in perfusate for 2 h, the perfusing solution was switched to Ringer's with 100 mM K^+ (minus the fluoxetine) for an additional 3 h. Aliquots of perfusate were collected every 20 min and immediately injected into a high-performance liquid chromatographer equipped with an electrochemical detector. At the conclusion of each experiment, rats were killed with an overdose of phenobarbital and microdialysis probe placement in FC was verified with infusion of cresyl violet dye into the probe and examination under a dissecting microscope. Animals in which the probe did not lie within the medial FC were not used for statistical analysis.

Prior to the LH experimentation, a separate series of experiments that did not involve stress or shuttle-box testing was performed to confirm that the 5-HT measured was of neuronal origin. 5-HT levels in perfusate from FC were measure with 30, 60, 90, and 120 mM K^+ in Ringer's solution to test for K^+-stimulation. FC was perfused with Ca^{++} and then Ca^{++}-free Ringer's solution to demonstrate calcium dependence of release. Perfusion was done with Ringer's containing tetrodotoxin, a sodium channel-blocking neurotoxin that stops neuronal impulse flow. Effects on 5-HT in perfusate with the specific serotonergic reuptake blocker fluoxetine, as well as imipramine and desipramine (1 mM in the perfusing medium), were examined.

RESULTS

In the experiments to determine whether the 5-HT measured in perfusate was neuronal, a dose-dependent increase in K^+-stimulated release was observed (Fig. 1A). Calcium was necessary for 5-HT release, as demonstrated by the decreased 5-HT measured in perfusate when Ca^{++}-free Ringer's solution was used (Fig. 1B). Tetrodotoxin also significantly decreased 5-HT release, suggesting release to be neuronal in origin (Fig. 1B). The specific 5-HT reuptake blocker fluoxetine significantly increased 5-HT in perfusate, as did the less specific tricyclics imipramine and desipramine to a lesser degree (Fig. 1C).

In the behavioral experiments, inescapable stress caused increased escape latencies in the FR 2 shuttle-box test but not in the FR 1 (Fig. 2A). Naive nonstressed rats had mean escape

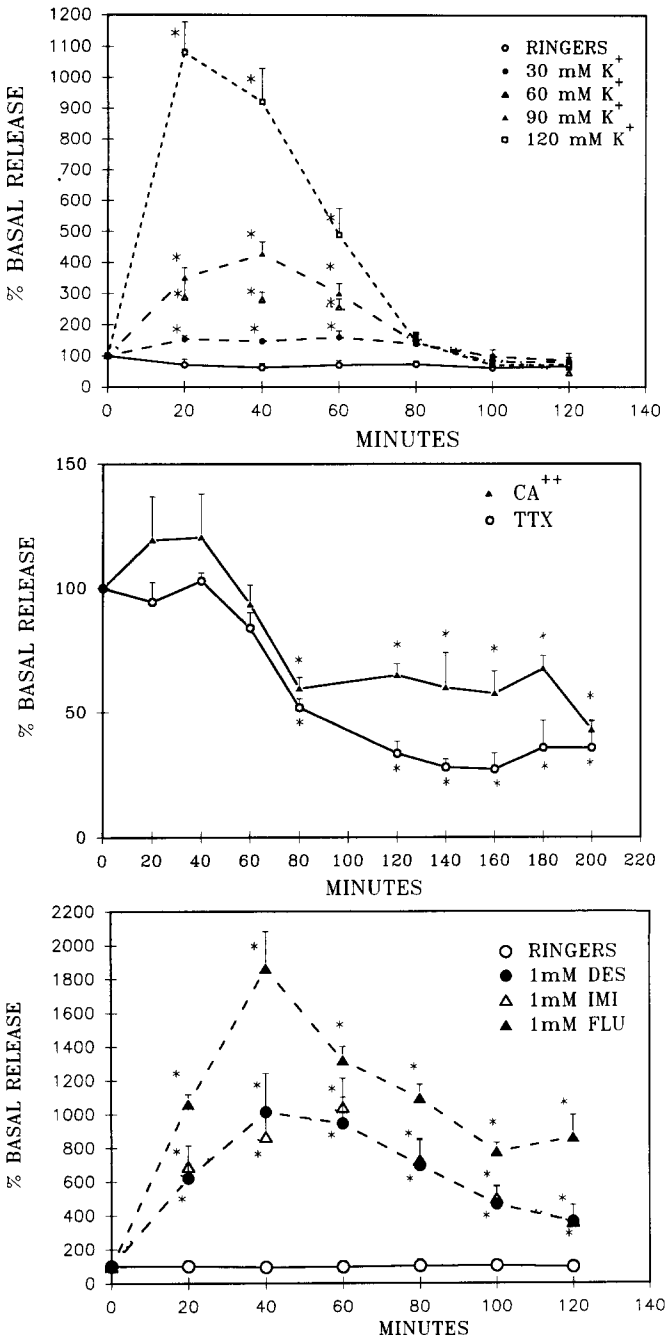

FIG. 1. Factors affecting 5-HT levels in perfusate from frontal cortex (n = 6–8 per group. (A). 5-HT levels in frontal cortex perfusate as a function of varying K^+ concentration of Ringer's solution during 60 min of perfusion after obtaining stable baseline K^+ was perfused from 0–60 min. Results expressed as percentage of basal release (mean ± SD). Indicated (*) values are significantly different from basal release $p < 0.05$, ANOVA with repeated measures). (B). 5-HT levels in frontal cortex perfusate after removing Ca^{++} from or adding tetrodotoxin (TTX) to Ringer's solution. Perfusing solution changed from Ringer's at 40 min. Results expressed as in Fig. 1A (*$p < 0.05$). Graphs represent data from two different experiments. (C). 5-HT levels in perfusate from frontal cortex. After stable baseline, perfusing solution switched to Ringer's solution with either desipramine (DES), imipramine (IMI), or fluoxetine (FLU) 1 mM for 1 h (0–60 min), then perfusion returned to Ringer's solution for another 1 or 2 h. Results expressed as in Fig. 1A (*$p < 0.05$).

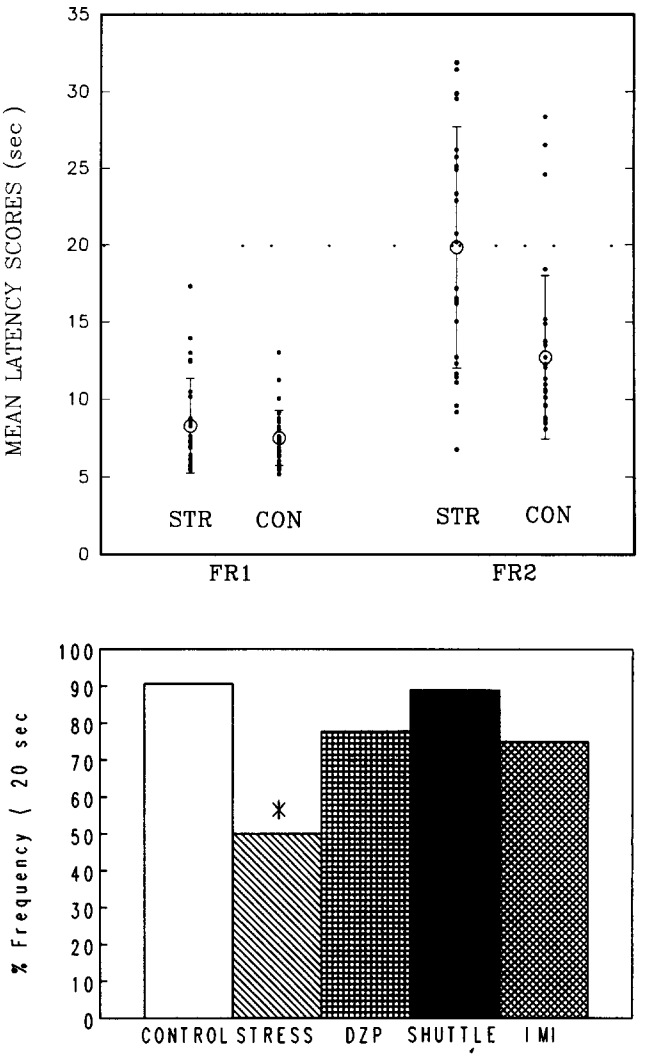

FIG. 2. (A). Shuttle-box performance for rats receiving either no stress (CON, $n = 32$) or 100 trials of inescapable tail-shock (STR, $n = 28$). Results are mean latency per trial for FR 1 and FR 2 ± SD without outliers. These data form the basis for use of a 20-s criterion, whereby rats with mean FR 2 latencies <20 s show normal behavior and >20 s are designated LH. Proportion <20 s: CON, 29/32 = 91%; STR 14/28 = 50%, $p < 0.001$ Fisher's exact test. (B). Shuttle-box performance for rats receiving either no tail-shock stress (CON, $n = 8$), or receiving tail-shock stress after pretreatment with saline (STR, $n = 24$), diazepam 10 mg/kg (DZP, $n = 12$), escapable foot-shock stress 25 trials FR 2 (SHUTTLE, $n = 12$), or imipramine 32 mg/kg for 4 days (IMI, $n = 12$). Data are presented as the percentage of rats with mean FR 2 test latencies <20 s on testing, for example, for the STR group 12 rats tested LH and 12 did not. STR group significantly lower than others (*$p < 0.05$, χ^2).

latencies of 12 ± 4 s. Therefore, a 20-s cutoff criteria was used with rats scoring mean FR 2 escape latency >20 s designated LH. About 50% of inescapably prestressed rats have scores in the LH range. All three pretreatments prevented the development of LH (Fig. 2B). Animals receiving either acute diazepam, subchronic imipramine, or escapable stress before inescapable stress all had mean shuttle-box latency scores in the normal control range. That is, for all three pretreatments the percentage of animals with mean shuttle-box latencies

<20 s was comparable to that of control. On the other hand, the group of animals receiving saline before exposure to inescapable stress had a significantly smaller proportion ($p < 0.05$, χ^2) in the normal range of latency scores.

K^+-stimulated 5-HT levels in perfusate were comparable between the three groups of animals given preventive treatment and also similar to controls (Fig. 3A). Animals receiving saline prior to inescapable stress exposure had lower levels of K^+-stimulated 5-HT release. Rats receiving saline prior to inescapable stress whose mean latencies scores were in the LH range (20s) had the lowest K^+-stimulated levels of 5-HT. Rats receiving saline prior to inescapable stress with mean latencies in the (NH) range (<20 s) had K^+-stimulated 5-HT levels intermediate between LH and preventive treatment and significantly different from both. There was a significant negative correlation between mean escape latency and K^+-stimulated 5-HT release for rats receiving saline prior to inescapable stress (Fig. 3B).

When levels of 5-HT in perfusate were compared over the course of the 3-h perfusion (Fig. 4), animals with the highest initial 5-HT levels in perfusate demonstrated a steady decrease in the second and third hour of perfusion, while animals whose initial 5-HT levels were low did not demonstrate further decrease of 5-HT in perfusate.

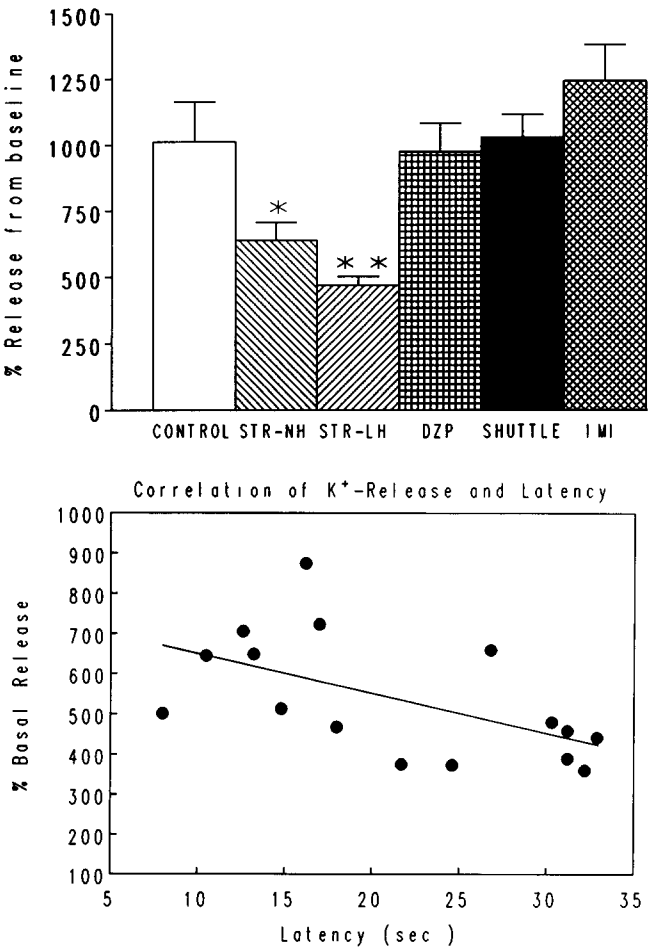

FIG. 3. (A). Potassium-stimulated release of 5-HT from frontal cortex for first hour of perfusion of rats receiving either no stress (CON, $n = 6$) or receiving inescapable tail-shock stress after treatment with saline vehicle (STR, $n = 16$), diazepam 10 mg/kg (DZP, $n = 9$), 25 FR 2 shuttle-box trials of escapable foot-shock (SHUTTLE, $n = 8$), or imipramine 32 mg/kg for 4 days (IMI, $n = 7$). Saline-treated rats were divided according to whether they scored less than 20 s mean latency on the shuttle-box test and were nonhelpless (STR-NH) or whether they scored more than 20 s mean latency and had learned helplessness (STR-LH). *$p < 0.05$ vs. CON, **$p < 0.05$ vs. STR-NH, ANOVA with Newman-Keuls posthoc test. Group size lower than in Fig. 2B due to surgical or perfusion loss. (B). Correlation of K^+ stimulated 5-HT release for first hour of perfusion with mean escape latency on FR 2 shuttle-box test for rats pretreated with saline vehicle prior to inescapable tail-shock stress (group STR in Fig. 3A, $n = 16$, $r = -0.56$, $p < 0.05$).

DISCUSSION

As shown by the nonbehavioral experiments the 5-HT release, as measured in perfusate, is K^+-stimulated, calcium dependent, blocked by tetrodotoxin, and increased by a specific serotonergic reuptake inhibitor. It is therefore likely to be of neuronal origin.

Because the purpose of the present research was to compare known LH prevention paradigms, only one dose of diazepam and imipramine was used. The dose of diazepam (10 mg/kg), while decreasing spontaneous locomotor activity, does not produce total somnolence (34). This dose does not block physiological response to stress because it is less than the 25 mg/kg used to demonstrate that diazepam did not prevent the increase in plasma corticosterone induced by placing rats on an open platform (5). Furthermore, animals in our experiment were not anesthetized by the diazepam as demonstrated by their behavior during administration of tail-shock stress, vocalizing and defecating similar to saline- or imipramine-treated animals. Thus, the dose of diazepam employed in the present experiment is a sedating anxiolytic dose that still allows a rat to experience environmentally induced and shock-induced stress. The dose of imipramine chosen for these experiments is the same dose and schedule used to completely reverse LH (40). Use of the shuttle-box for escapable stress, derived from previous work (19), showed this to be a task readily learned by naive animals in one training session.

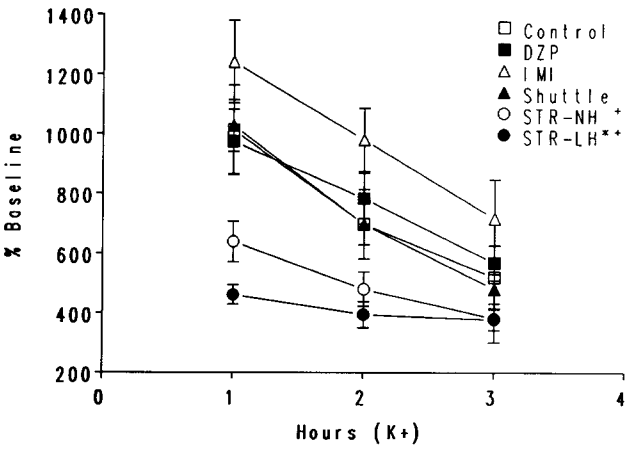

FIG. 4. Levels of 5-HT in perfusate during 3 h of perfusion with high (100 mM) K^+ Ringer's solution. Group definitions as in Fig. 3. Slopes of curves are significantly different from zero except for the STR-LH group (*$p < 0.05$ linear regression analysis), and slopes of STR-NH and STR-LH are significantly different from control ($^+p < 0.05$ multiple linear regression analysis).

The major finding of the present work was that all three treatments used to prevent the development of LH also prevented stress-induced decrease in K^+-stimulated 5-HT release such that pretreated animals had levels of K^+-stimulated release similar to unstressed controls.

A role for 5-HT in the anxiolytic effect of BDZs has been postulated for almost 20 years (48). Acute administration of BDZs leads to decreased accumulation of 5-HTP when aromatic amino acid decarboxylase is inhibited (24). This is also interpreted as a measure of decreased 5-HT turnover (36). However, regional studies suggest the presumed decrease in 5-HT synthesis by BDZs to be found in hippocampus and not cortex (37) such that the relevance of the 5-HT turnover work to the present experiments is not clear.

Diazepam increased whole brain levels of 5-HT, as well as regional levels of 5-HT in hippocampus, hypothalamus, and midbrain, but not in brainstem (34). Brain levels of 5-HT probably reflect intraneuronal 5-HT available for K^+-stimulated release and may be relevant to the present research.

In addition to these effects, diazepam enhances the electrically stimulated release of 5-HT in hippocampus and cortex (20,21). Diazepam blocks the calcium-dependent K^+-stimulated release of 5-HT from synaptosomes (3), an action interpreted as facilitation of the presynaptic inhibition of GABA on evoked release of 5-HT (35). Taken together, these findings suggest diazepam may have exerted its effect in preventing LH in the present experiments by increasing intraneuronal stores of releasable 5-HT.

TCAs, including imipramine, block neuronal 5-HT uptake. However, this effect is seen with acute treatment and may not necessarily be relevant to the effect of imipramine on LH, which requires subchronic administration. Chronic administration of a TCA increases 5-HT levels and turnover (47) and enhances the effect of 5-HT on behaviors due to postsynaptic receptor stimulation despite decreased receptor density (17). In electrophysiological studies, chronic administration of imipramine also results in enhancement of synaptic transmission of 5-HT (48), which has been proposed to be a common denominator of therapeutic antidepressant effects (6,7). We have previously shown that subchronic, but not acute, administration of imipramine increases K^+-stimulated release from slices of FC (39) and that this release was functionally related to reversal of LH. Thus, some previous research can help explain the findings of the present report, in which subchronic imipramine treatment had a protective effect on the behavioral consequences of inescapable stress.

Relatively little research has focused on neurochemical effects of escapable stress, similar to that given in the shuttle-box pretreatment in the present work. A wheel-turn tail-shock paradigm was used to show that rats receiving escapable shock had an increase in 5-HT levels in brainstem compared to both yoked inescapably stressed and nonstressed controls, but no effects on 5-HT were seen in FC (43). However, a similar experiment (16) found different results. Rats receiving escapable stress had increased 5-HT levels in FC but not brainstem compared to yoked inescapably stressed rats. The latter results (16) are compatible with escapable shock resulting in increased intraneuronal 5-HT in FC.

Thus, a rationale can be made from previous research for a possible commonality of action of the measures used to prevent LH: subchronic imipramine, acute diazepam, escapable stress in increasing intraneuronal releasable 5-HT in FC prior to the depleting effects of inescapable stress exposure. The time course of effects on 5-HT by acute BDZs (42) and subchronic imipramine (39) are compatible with their exerting a neurochemical effect during LH induction, while the persistence of effects of escapable stress is not known (16).

An alternative possibility is that the pretreatment caused an increased intraneuronal 5-HT setpoint that persisted for the 48–72 h between treatment and testing. To our knowledge, there are no reports examining 5-HT release several days after treatment with subchronic imipramine, acute BDZs, or escapable stress to examine this possibility. Future research should clarify the time course of these effects.

Other theoretical explanations of the data should also be considered. The three pretreatments may have lowered the "psychological" stress reaction during tail-shock LH induction such that the stressor was sensed but not perceived as stressful at some higher CNS level of integration. Because the neurochemical measures were taken 24 h after LH testing, the findings with 5-HT may only reflect a post test index of the LH state, and it would be presumptive to interpret the data as demonstrating a causal relationship.

It is possible that the decreased K^+-stimulated 5-HT release seen in the LH rats was merely due to the extended shock exposure in the shuttle-box during testing and not to the LH phenomenon per se. However, we do not think this explanation can account for all the data. In the present work, rats treated with saline before inescapable tail-shock stress had significantly lower levels of K^+-stimulated 5-HT release than controls and rats receiving active pretreatments, whether the saline-treated rats were helpless on shuttle-box testing or not. In other words, if shock exposure during testing were the only factor involved in decreasing K^+-stimulated 5-HT release saline-treated NH rats should have had similar K^+-stimulated 5-HT release as rats receiving active treatment and testing NH because the level of shock exposure during testing was similar for all NH groups. This was not found. Of the saline-treated rats, the NH rats had K^+-stimulated release significantly higher than similarly treated rats that became LH, suggesting increased shock exposure during testing may have contributed to decreasing the K^+-stimulated 5-HT release. One possible explanation is that there is a threshold of releasable 5-HT that must be preserved to mediate normal behavior. According to this explanation, rats becoming LH depleted their frontal cortical 5-HT stores below this threshold and sustained this depletion for 24 h. Additional future experiments examining doses of drugs, trials of escapable shock, and time course of neurochemical effects should help clarify this possibility.

Perfusion with the high K^+ Ringer's solution for 3 h depleted 5-HT, except in the LH group. These rats seemed to have already expended all releasable 5-HT and showed no further decrease with K^+ over time. This supports a low 5-HT hypothesis of LH in FC. However, the present study measured neurotransmitter 24 h after the shuttle-box test. Therefore, the possibility remains that the measurements reported reflect a compensatory rebound or shift in 5-HT values compared to what would have been observed immediately after shuttle-box testing. Future research should clarify this, as microdialysis technology continues to be improved.

Two previous LH studies that reported pretreatment of rats with serotonergic agents merit comment. One showed that depletion of 5-HT with PCPA prevented LH (12). This finding is in apparent conflict with our present research because our results would predict that 5-HT depleted rats should become spontaneously helpless. Another study using a similar experimental paradigm produced a 70% decrease in serotonergic neurons with infusion of the neurotoxin 5,7-DHT, which did not affect the ability of inescapable shock to induce LH (40). These findings appear contradictory and there is no sim-

ple explanation for this. The fact that the interaction of 5-HT with LH is complex is further demonstrated by the recent findings that some specific inhibitors of 5-HT reuptake do not reverse LH at high doses (13) and reverse LH at intermediate doses only when administered *after* the shuttle-box testing session, unlike imipramine, which is effective in reversing LH when administered before testing sessions.

The involvement of other neurotransmitters in LH, including acetylcholine, dopamine, GABA, and norepinephrine, is well documented and probably plays a role in the findings here reported. Also, the involvement of other brain regions, particularly hippocampus and hypothalamus, is important in mediating frontal cortical 5-HT. Future research should address how multitransmitter, multiregional data can be integrated into a unified model of stress-induced behavioral depression in the rat.

In summary, this research suggests that prevention of LH may occur in part by maintenance of intraneuronal stores of 5-HT in FC at a level below which rats become helpless. Alternative explanations are also possible. For example, the present data are also compatible with the notion that LH prevention pretreatments increased the intraneuronal levels of 5-HT in FC irrespective of stress so as to overcompensate for stress-induced reductions. The data presented are correlative and not causative, and any conclusions reached are therefore tentative particularly because the contributions of other neurotransmitter systems have not yet been studied with the present protocol.

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