Stress Resilience is Associated with Hippocampal Synaptoprotection in the Female Rat Learned Helplessness Paradigm

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Abstract—The synaptogenic hypothesis of major depressive disorder implies that preventing the onset of depressive-like behavior also prevents the loss of hippocampal spine synapses. By applying the psychoactive drugs, diazepam and fluoxetine, we investigated whether blocking the development of helpless behavior by promoting stress resilience in the rat learned helplessness paradigm is associated with a synaptoprotective action in the hippocampus. Adult ovariectomized and intact female Sprague-Dawley rats ($n = 297$) were treated with either diazepam, fluoxetine, or vehicle, exposed to inescapable footshocks or sham stress, and tested in an active escape task to assess helpless behavior. Escape-evoked corticosterone secretion, as well as remodeling of hippocampal spine synapses at a timepoint representing the onset of escape testing were also analyzed. In ovariectomized females, treatment with diazepam prior to stress exposure prevented helpless behavior, blocked the loss of hippocampal spine synapses, and muted the corticosterone surge evoked by escape testing. Although fluoxetine stimulated escape performance and hippocampal synaptogenesis under non-stressed conditions, almost all responses to fluoxetine were abolished following exposure to inescapable stress. Only a much higher dose of fluoxetine was capable of partly reproducing the strong protective actions of diazepam. Importantly, these protective actions were retained in the presence of ovarian hormones. Our findings indicate that stress resilience is associated with the preservation of spine synapses in the hippocampus, raising the possibility that, besides synaptogenesis, hippocampal synaptoprotection is also implicated in antidepressant therapy. © 2021 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: diazepam, fluoxetine, major depression, corticosterone, synaptic plasticity, antidepressant resistance.

INTRODUCTION

As part of the limbic system, the hippocampus is heavily implicated in the neurobiology of major depression, its dysfunction contributing to many depressive symptoms, such as cognitive decline, loss of motivation, and stress system derailment (Sapolsky, 2000a; Sousa et al., 2000; Nestler et al., 2002; Lisman and Grace, 2005; Cooper et al., 2006; Diamond et al., 2006; LeGates et al., 2018). In the context of this study, the central role that the hippocampus plays in regulating the stress system is particularly important, because stress and stress hormones appear to be key mediators both in human depression and in animal models of the disease (Sapolsky, 2000a; Sala et al., 2004; Hajszan et al., 2009; McEwen et al., 2016). Compromised hippocampal function in major depression is associated with the breakdown of hippocampal neural circuitry, especially at the synaptic level, which has prompted the synaptogenic hypothesis of major depressive disorder that postulates a causal relationship between depressive symptoms and loss of synapses in limbic brain areas (Hajszan and

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Abbreviations: aCSF, artificial cerebrospinal fluid; CA1sr, CA1 stratum radiatum; CA3sl/sr, CA3 stratum lucidum/radiatum; DGsm, dentate gyrus stratum moleculare; fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; SSRI, selective serotonin reuptake inhibitor.

MacLusky, 2006; Hajszan et al., 2010; Kang et al., 2012; Duman et al., 2016). The excitatory, asymmetric spine synapses localized within the CA1 stratum radiatum (CA1sr), the CA3 stratum lucidum/radiatum (CA3sl/sr), and the dentate gyrus stratum moleculare (DGsm) are the prime linking points of the "trisynaptic loop", the main neural circuit of the hippocampus, which is formed by sequentially organized pyramidal and granule cells (Amaral and Witter, 1995; Stepan et al., 2012, 2015). Aside from our own work (Hajszan et al., 2005, 2009, 2010; Kang et al., 2012; Baka et al., 2017), a large number of studies has investigated synaptic remodeling within the trisynaptic loop, relating it mainly to hippocampus-dependent cognitive and affective functions as summarized, e.g., in our recent review (Hajszan, 2020). As a result, the number of spine synapses provides a reliable readout of structural connectivity along the trisynaptic loop and can be considered as an anatomical correlate of hippocampal function.

During recent years, we have reported that major depression in humans is associated with loss of synapses in the prefrontal cortex, while depressive-like behavior in rodent models is related to loss of spine synapses in the hippocampus (Hajszan et al., 2009, 2010; Kang et al., 2012; Baka et al., 2017). In addition, reproducing the loss of hippocampal spine synapses *without* exposure to stress elicits helpless behavior in the rat learned helplessness paradigm (Hajszan et al., 2009, 2010). On the other hand, our research team has also validated the theory that an antidepressant response requires the generation of new synapses in limbic brain areas, reversing the synapse loss caused by stress/depression (Hajszan et al., 2005, 2009, 2010; Li et al., 2010). Aside from synapse loss and synaptogenesis, an important aspect of the synaptogenic hypothesis remains unexplored. The synaptogenic hypothesis implies that preventing the onset of depressive-like behavior by increasing stress resilience, also prevents the loss of hippocampal spine synapses. Using a rodent model of postpartum depression, we have recently shown that chronic exposure to pregnancy levels of estradiol and progesterone indeed protects spine synapses in the hippocampus while improving stress resilience (Baka et al., 2017). Because this behavior/synapse correlation may be dependent on the special hormonal conditions of the postpartum model (Galea et al., 2001; Suda et al., 2008; Baka et al., 2017), the goal of our present study was to confirm that the protective actions we described earlier are reproducible under more generalized conditions.

To address this issue, we used a well-characterized female learned helplessness model of stress/depression (Hajszan et al., 2010) to investigate the protective actions of widely-used psychoactive drugs with and without the influence of ovarian hormones. A series of earlier learned helplessness studies demonstrates that benzodiazepines effectively prevent the development of helpless behavior when given prior to stress exposure (Drugan et al., 1984; De Pablo et al., 1991; Petty et al., 1992). Based on this confirmed preventive effect in learned helplessness, a reasonable choice for exploring the relationships between stress resilience and hippocampal synaptopro-

tection is diazepam, a GABA-A receptor benzodiazepine site agonist anxiolytic. Although a number of mechanisms may be involved, the preventive effect of benzodiazepines is most likely based on mitigating stress, because benzodiazepines are among the most potent suppressors of the stress response (File, 1982; Pohorecky et al., 1988; de Boer et al., 1990; Matar et al., 2009). Benzodiazepines are also known to reduce or even completely halt various modalities of synaptic plasticity (Evans and Viola-McCabe, 1996; Seabrook et al., 1997; Tampellini et al., 2010; Curto et al., 2016), but their influence on remodeling of hippocampal spine synapses, especially in relation to the preventive effects, is currently unknown.

Stress resilience and synaptoprotection may be highly relevant to antidepressant resistance, a major clinical problem, because antidepressant resistance and recurring depression appear to be associated with a persistent hyperactivity of the stress system (Heuser et al., 1996; Appelhof et al., 2006), while curbing the stress response seems to improve the antidepressant response (Barden et al., 1995; Jahn et al., 2004; Nemeroff and Owens, 2004; Rogoz et al., 2005). Antidepressant resistance is mostly encountered with selective serotonin reuptake inhibitor (SSRI) antidepressants (Rush et al., 2006; Trivedi et al., 2006), which is paralleled by a growing number of preclinical studies showing SSRIs being ineffective in a wide variety of paradigms (Magarinos et al., 1999; Kobayashi et al., 2008; Valentine et al., 2008; Venzala et al., 2012; Kokras et al., 2015; Workman et al., 2016). These findings suggest that the stress-mitigating effect of SSRIs may be limited, especially compared to that of benzodiazepines. A handful of reports indeed demonstrate that reduced antidepressant efficacy of SSRIs is usually observed under stressful conditions (Valentine et al., 2008; Branchi et al., 2013; Khemissi et al., 2014). Based on these data, we also selected fluoxetine, a classic SSRI, to investigate the relationships between stress resilience and hippocampal synaptoprotection under the influence of a drug highly susceptible of antidepressant resistance. Although fluoxetine is a strong initiator of hippocampal synaptogenesis under non-stressed conditions (Hajszan et al., 2005), it is currently unknown what happens to this robust synaptogenic power following stress exposure.

In animal models of major depression, drugs are usually given *after* stress exposure to investigate their capability of *reversing* depressive-like behavior. The goal of this study, however, is to *prevent* the development of helpless behavior via promoting stress resilience, which logically requires drug application *prior* to stress exposure. This approach obviously follows the setup of earlier learned helplessness studies that revealed the preventive effects of benzodiazepines and ovarian hormones (Drugan et al., 1984; De Pablo et al., 1991; Petty et al., 1992; Baka et al., 2017). Here we report that promoting stress resilience in learned helplessness with diazepam is associated with a synaptoprotective action in the hippocampus and with a muted stress response during escape testing. We also show that these preventive effects are more limited after fluoxetine treatment, as exposure to stress mostly destroys the

hippocampal synaptogenic power of fluoxetine. As we describe above, the influence of both diazepam and fluoxetine on various aspects of stress and stress-related behaviors has been investigated earlier. However, it is currently not known whether this influence on stress is related to synaptic remodeling in the hippocampus. As a result, our present ultrastructural investigation provides the main novel findings of this study. These results suggest that the issue of stress resilience and synaptoprotection needs further attention in future studies, as they can potentially contribute to the better understanding of antidepressant therapy and antidepressant resistance.

EXPERIMENTAL PROCEDURES

Animals

Ethical statement. Our animal protocol was approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine and by the Ethical Committee for the Protection of Animals in Scientific Research of the Biological Research Center. Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Experimental animals. Female Sprague-Dawley rats (n = 297, 200–250 g) were purchased from Charles-River Laboratories (Wilmington, Massachusetts), and only healthy, drug- and test-naive rats were allocated to experimental groups after an acclimation period of one week. We used ovariectomized and intact female rats because this study is based upon and expands the findings of our earlier experiments that also used females (Hajszan et al., 2005, 2010; Baka et al., 2017). The rationale for using ovariectomy is based on well-documented clinical observations that diminishing estradiol levels in women represent an increased risk for developing mood disorders (Freeman et al., 2006; Deecher et al., 2008; Soares and Zitek, 2008), which is in line with our own findings that withdrawal of ovarian hormones robustly contributes to depressive-like behavior in animal models (Hajszan et al., 2010; Baka et al., 2017). Ovariectomy is also essential to prevent interference from cyclic changes in the production of endogenous ovarian hormones, as the number of hippocampal synapses shows significant fluctuation during the estrus cycle (Woolley and McEwen, 1992). Animals were ovariectomized a week prior to commencing any procedures using a ketamine-based anesthetic (75 mg/kg ketamine, 3.6 mg/kg xylazine, 0.09 mg/kg acepromazine dissolved in 3 ml/kg saline, im).

Housing and husbandry. Similar to our earlier studies (Hajszan et al., 2010; Baka et al., 2017), rats were kept in a high-security-barrier animal facility and monitored periodically to be free of major pathogens. Animal rooms were maintained at 21 °C and on a 12/12-h light/dark cycle with light on at 0700 h. Rats were group-housed (n = 3 females per cage) in standard cages with tap water and rodent chow available *ad libitum*. Animal welfare was monitored daily by animal facility staff and at least weekly by a veterinarian.

Study design

Diazepam experiment. Using ovariectomized females, four experimental groups were established by treating animals with lower (5 mg/kg) or higher (10 mg/kg) doses of diazepam (ip, 30 min prior to sham stress or inescapable stress). For reference, well-documented negative and positive control groups of helpless behavior were also created by treating animals with vehicle (1 ml/kg distilled water, ip, 30 min prior to sham stress or inescapable stress) (Hajszan et al., 2010). Diazepam was obtained in the form of 5 mg/ml injectable solution from Gedeon Richter Plc (Budapest, Hungary). In earlier studies, similar diazepam regimens inhibited the behavioral effects of uncontrollable stress and instantly prevented both stress- and seizure-induced glutamate efflux in the hippocampus (De Pablo et al., 1991; Petty et al., 1992) (Bagley and Moghaddam, 1997; Khan et al., 1999).

Omitting the two groups with the lower diazepam dose, the above experiment was repeated in simulated proestrus rats to investigate whether the presence of ovarian hormones affects the actions of diazepam. Proestrus levels of estradiol and progesterone were simulated in ovariectomized females as published earlier (Scharfman et al., 2007; Baka et al., 2017), timed to achieve the required hormone concentrations by the onset of stress exposure. Briefly, a day before sham stress or inescapable stress, animals were injected with 3 μg/kg estradiol benzoate (dissolved in sesame oil, sc) at 8:30 am, followed by 4 μg/kg estradiol benzoate at 8:30 pm. Five hours prior to sham stress or inescapable stress, animals also received 2 mg/kg progesterone (dissolved in sesame oil, sc), followed by a final dose of 3 μg/kg 17β-estradiol (dissolved in sesame oil, sc) 2 h before sham stress or inescapable stress.

The schedule and sample sizes of the diazepam experiment are detailed in Table 1.

Fluoxetine experiment. Using ovariectomized females, four experimental groups were established by treating animals with lower (5 mg/kg) or higher (20 mg/kg) doses of fluoxetine (ip, once daily for 15 days prior to sham stress or inescapable stress). For reference, well-documented negative and positive control groups of helpless behavior were also created by treating animals with vehicle (1 ml/kg distilled water, ip, once daily for 15 days prior to sham stress or inescapable stress) (Hajszan et al., 2010). Fluoxetine was obtained in the form of 4 mg/ml aqueous solution from Eli Lilly and Company (Indianapolis, Indiana). In earlier studies, we have demonstrated that lower doses of fluoxetine elicit antidepressant responses in a chronic unpredictable stress paradigm and induce strong proliferation of hippocampal spine synapses, while higher doses reverse escape

Table 1. Schedule of the diazepam experiment.

Group	Day-1	Day-7	Day-8	Day-9
Diazepam experiment in ovariectomized females				
veh/ns	ovariectomy	—	veh + ns	EM ($n = 4$) LTP ($n = 6$) AE + CS ($n = 10$)
veh/is	ovariectomy	—	veh + is	EM ($n = 4$) LTP ($n = 5$) AE + CS ($n = 10$)
dz5/ns	ovariectomy	—	dz5 + ns	EM ($n = 4$) AE + CS ($n = 10$)
dz5/is	ovariectomy	—	dz5 + is	EM ($n = 4$) AE + CS ($n = 10$)
dz10/ns	ovariectomy	—	dz10 + ns	EM ($n = 4$) AE + CS ($n = 10$)
dz10/is	ovariectomy	—	dz10 + is	EM ($n = 4$) LTP ($n = 6$) AE + CS ($n = 10$)
Diazepam experiment in simulated proestrus rats				
veh/ns	ovariectomy	eb3 + eb4	pe + veh + ns	EM ($n = 4$) AE + CS ($n = 10$)
veh/is	ovariectomy	eb3 + eb4	pe + veh + is	EM ($n = 4$) AE + CS ($n = 10$)
dz10/ns	ovariectomy	eb3 + eb4	pe + dz10 + ns	EM ($n = 4$) AE + CS ($n = 10$)
dz10/is	ovariectomy	eb3 + eb4	pe + dz10 + is	EM ($n = 4$) AE + CS ($n = 10$)

Treatments: dz5, low-dose diazepam (5 mg/kg, ip, 30 min prior to ns or is); **dz10**, high-dose diazepam (10 mg/kg, ip, 30 min prior to ns or is); **eb3**, estradiol-benzoate (3 µg/kg, sc, at 8:30 am); **eb4**, estradiol-benzoate (4 µg/kg, sc, at 8:30 pm); **is**, inescapable stress; **ns**, sham stress; **pe**, progesterone (2 mg/kg, sc, 5 h prior to ns or is) and 17β-estradiol (3 µg/kg, sc, 2 h prior to ns or is); **veh**, vehicle (1 ml/kg distilled water, ip, 30 min prior to ns or is). **Measurements: AE**, active escape testing; **CS**, blood sampling for corticosterone immunoassay immediately after the completion of escape testing; **EM**, sampling for electron microscopic stereology immediately before the onset of escape testing; **LTP**, recording long-term potentiation at a timepoint that represents the onset of escape testing.

deficits and block the downregulation of hippocampal neurogenesis in the rat learned helplessness paradigm (Malberg and Duman, 2003; Hajszan et al., 2005; Valentine et al., 2008). Omitting the two groups with the lower fluoxetine dose, the above experiment was repeated in intact rats to investigate whether the presence of fluctuating ovarian hormones modifies the actions of fluoxetine.

The schedule and sample sizes of the fluoxetine experiment are detailed in Table 2.

Sample size. We have shown in earlier studies that a sample size of $n = 10$ rats/group provides sufficient statistical power to differentiate between helpless and control animals based on escape performance and corticosterone levels (Hajszan et al., 2009, 2010; Baka et al., 2017). Considering our previous spine synapse analyses, a sample size of $n = 4$ rats/group is sufficient for ANOVA to detect at least 25% change in synapse numbers with the desired 80% power at $\alpha = 0.05$ (Hajszan et al., 2005, 2009, 2010; Baka et al., 2017). In the case of long-term potentiation (LTP) measurements, we also followed our earlier experiments that suggest a sample size of $n = 5$ rats/group (Marosi et al., 2009; Kocsis et al., 2014).

Experimental outcomes. The following endpoints were assessed in both the diazepam and the fluoxetine experiments: (a) performance in an active escape task to detect helpless behavior; (b) serum corticosterone concentrations at the completion of active escape testing to measure the magnitude of stress response evoked by the active escape task; (c) number of spine synapses in the CA1sr, CA3sl/sr, and DGsm areas to estimate the status of structural connectivity along the hippocampal trisynaptic loop at the onset of active escape testing. In addition, (d) LTP of the Schaffer collateral-CA1 synaptic response was evaluated at the onset of active escape testing in a subset of groups from the diazepam experiment with the sole purpose of verifying the effectivity of stress exposure in diazepam-pretreated animals.

Experimental procedures

Learned helplessness. Our laboratories routinely use a modified learned helplessness model as published previously (Valentine et al., 2008; Hajszan et al., 2009, 2010; Baka et al., 2017). Even the standard learned help-lessness paradigm is highly-rated as a rodent model of stress/depression based on its excellent face, predictive, and construct validities (Seligman, 1968; Thiebot et al., 1992; Cryan et al., 2002). Our modified model surpasses the standard paradigm by providing an improved rate of helplessness and an ability to differentiate between antidepressant classes by reproducing the clinical obser-

Table 2. Schedule of the fluoxetine experiment

Group	Day-1	Days 8–21	Day-22	Day-23
Fluoxetine experiment in ovariectomized females				
veh/ns	ovariectomy	veh	veh + ns	EM (*n* = 4) AE + CS (*n* = 10)
veh/is	ovariectomy	veh	veh + is	EM (*n* = 4) AE + CS (*n* = 10)
fx5/ns	ovariectomy	fx5	fx5 + ns	EM (*n* = 4) AE + CS (*n* = 10)
fx5/is	ovariectomy	fx5	fx5 + is	EM (*n* = 4) AE + CS (*n* = 10)
fx20/ns	ovariectomy	fx20	fx20 + ns	EM (*n* = 4) AE + CS (*n* = 10)
fx20/is	ovariectomy	fx20	fx20 + is	EM (*n* = 4) AE + CS (*n* = 10)
Fluoxetine experiment in intact rats				
veh/ns	—	veh	veh + ns	EM (*n* = 4) AE + CS (*n* = 10)
veh/is	—	veh	veh + is	EM (*n* = 4) AE + CS (*n* = 10)
fx20/ns	—	fx20	fx20 + ns	EM (*n* = 4) AE + CS (*n* = 10)
fx20/is	—	fx20	fx20 + is	EM (*n* = 4) AE + CS (*n* = 10)

Treatments: fx5, low-dose fluoxetine (5 mg/kg, ip, once daily on Days 8–21 and 30 min prior to ns or is on Day-22); **fx20**, high-dose fluoxetine (20 mg/kg, ip, once daily on Days 8–21 and 30 min prior to ns or is on Day-22); **is**, inescapable stress; **ns**, sham stress; **veh**, vehicle (1 ml/kg distilled water, ip, once daily on Days 8–21 and 30 min prior to ns or is on Day-22). **Measurements: AE**, active escape testing; **CS**, blood sampling for corticosterone immunoassay immediately after the completion of escape testing; **EM**, sampling for electron microscopic stereology immediately before the onset of escape testing.

vations of reduced responses to serotonergic drugs (Trivedi et al., 2006; Valentine et al., 2008). As published earlier, we utilized inescapable stress to induce helplessness, followed by an active escape task to assess the behavioral deficit. Briefly, the testing apparatus consisted of a commercially available and fully automated shuttle avoidance system (Med Associates, St. Albans, Vermont). Animals subjected to inescapable stress received randomized exposure to 60 scrambled footshocks with 0.85 mA intensity, 15 s average shock duration, and 45 s average intershock interval. Footshocks were administered via the wire grid flooring in a closed shuttle box compartment with no opportunity to escape. Sham-stressed controls underwent the same protocol, but the shock generator was switched off during the entire procedure.

Twenty-four h after exposure to inescapable stress or sham stress, 30 randomized trials of *escapable* footshocks were administered with 0.65 mA intensity, 35 s maximum trial/footshock duration, and 60 s average intertrial interval. During each footshock, animals were allowed to escape by passing between shuttle box compartments. The initial five trials required one shuttle crossing, while the following 25 trials required two shuttle crossings to terminate footshocks. Escape latencies and escape failures were registered, representing the time to escape footshocks and the number of trials during which escape requirements were not met, respectively. Maximum escape latency (35 s) was registered for each escape failure. Behavioral testing was conducted in a dimly-lit room between 1000 h and 1600 h.

Serum corticosterone assay. Trunk blood samples were collected under ketamine-based anesthesia (see above) from each behaviorally tested animal immediately after the conclusion of active escape testing as described earlier (Hajszan et al., 2009; Baka et al., 2017). Serum was separated by centrifugation and stored frozen until assayed. Serum total concentration of corticosterone was determined using a commercially available enzyme immunoassay kit by following the manufacturer-recommended protocol (Assay Designs, Ann Arbor, Michigan). Samples from the diazepam and the fluoxetine experiments were analyzed in separate sessions, but all samples from the same experiment were analyzed in a single session in duplicates. In the concentration ranges pertinent to the present study, the intraassay coefficient of variation was 8.4%.

Electron microscopic stereology. The number of asymmetric spine synapses was calculated in three hippocampal sampling areas, the CA1sr, the CA3sl/sr, and the DGsm, as published earlier (Hajszan et al., 2009; Baka et al., 2017). Four animals were randomly selected and sacrificed from each experimental group to assess synapse numbers at a timepoint representing the onset of active escape testing. Selected rats were perfused transcardially under ketamine-based anesthesia (see above) using phosphate-buffered saline followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed overnight in the same fixative without glutaraldehyde. It needs to be mentioned that ketamine is well known to rapidly induce antidepres-

sant and synaptogenic responses (Li et al., 2010), which may introduce a bias into our synapse measurements. However, such responses occur at subanesthetic doses of ketamine (5–15 mg/kg) (Maeng et al., 2008; Li et al., 2010; Ardalan et al., 2017), and we are not aware of any studies reporting antidepressant and/or synaptogenic effects at higher doses of ketamine. Moreover, we have specifically confirmed that the rapid signaling mechanisms that mediate antidepressant and synaptogenic responses to ketamine are dose-dependent, appearing only at sub-anesthetic doses (5–10 mg/kg) and not seen at the high anesthetic dose (75 mg/kg) we are using in this study (Li et al., 2010). Each perfused hippocampus was cut into 100-μm thick serial sections in the coronal plane using a vibratome. Every tenth section was then selected in a systematic random manner and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania). Using the embedded sections, the volume of each sampling area (V_{SA}) was estimated utilizing the Cavalieri Estimator software module of the Stereo Investigator computerized stereology system (MicroBrightField, Villiston, Vermont).

To apply the disector technique (Sterio, 1984) at the electron microscopic level, 20 counting sites were localized in each sampling area along the entire septotemporal axis of the hippocampus using a systematic random approach as modified from MacLusky and colleagues (MacLusky et al., 2006). Notable differences in function and connectivity patterns suggest that the hippocampus is divided into dorsal and ventral domains involved in cognitive operations and emotional processing, respectively (Moser and Moser, 1998), although this dorsal/ventral distinction appears to be preferential rather than absolute (Bannerman et al., 2004). Based on the following clinical and preclinical findings, however, we do not differentiate between these two domains in our studies. (1) Considering the co-occurrence of cognitive and emotional dysfunctions (Nestler et al., 2002; Belmaker and Agam, 2008), the symptomatology of major depression does not suggest differing involvement of dorsal vs. ventral hippocampal regions. (2) Morphometric imaging studies in depressed patients show a typical hippocampal atrophy that affects the *entire* organ (Sheline et al., 1999), which is reproduced in animal models of stress and depression (Donohue et al., 2006; Chen et al., 2010; Kassem et al., 2013; Ardalan et al., 2017). (3) Despite differences in its input/output connectivity patterns, the intrinsic circuitry, i.e., the trisynaptic loop, basically repeats itself along the septo-temporal axis of the hippocampus (Andersen et al., 1971). We are not aware of any systematic *ultrastructural* studies reporting discrepancies in intrinsic synaptic architecture between dorsal vs. ventral hippocampal regions under any conditions, e.g., (Santuy et al., 2020). Actually, the rostrocaudal variability of numerical synapse densities appears to be less than 15% (Donohue et al., 2006), which is below the detection limit of our methods. That is why we have been consistently sampling from the entire hippocampus (Hajszan et al., 2004, 2005, 2009, 2010; MacLusky et al., 2006; Baka et al., 2017), following similar approaches in other laboratories (Chen et al., 2008, 2009, 2010, 2018; Ardalan et al., 2017).

A physical disector was prepared at each counting site by taking electron micrographs of matching areas in consecutive ultrathin sections at a final magnification of 11,000×. This sampling technique provided 20 physical disectors (N_D = 20) for each of CA1sr, CA3sl/sr, and DGsm, i.e., 60 disectors per hippocampus altogether. Prior to spine synapse counting, all micrographs were coded for blind analysis. An unbiased counting frame superimposed onto the electron micrographs was utilized to determine the number (ΣS) of asymmetric spine synapses (Fig. 1) within disectors. The estimated quantity of spine synapses in a particular sampling area was then calculated using the formula:

$$\frac{\sum S * V_{SA}}{N_D * V_D}$$

where ΣS = sum of synapse counts, V_{SA} = sampling area volume, N_D = number of disectors, V_D = disector volume (5.94 μm^3).

Long-term potentiation. With the sole purpose of verifying the effectivity of stress training in diazepam-pretreated animals, induction and stability of LTP were examined in hippocampal slice preparations as described earlier (Marosi et al., 2009; Kocsis et al., 2014). Ovariectomized females were randomly selected from the veh/ns (n = 6), veh/is (n = 5), and dz10/is (n = 6) groups to investigate functional neuroplasticity at a timepoint representing the onset of active escape testing (Table 1). Selected rats were rapidly decapitated under isoflurane anesthesia and their brains were removed and placed in an ice-cold artificial cerebrospinal
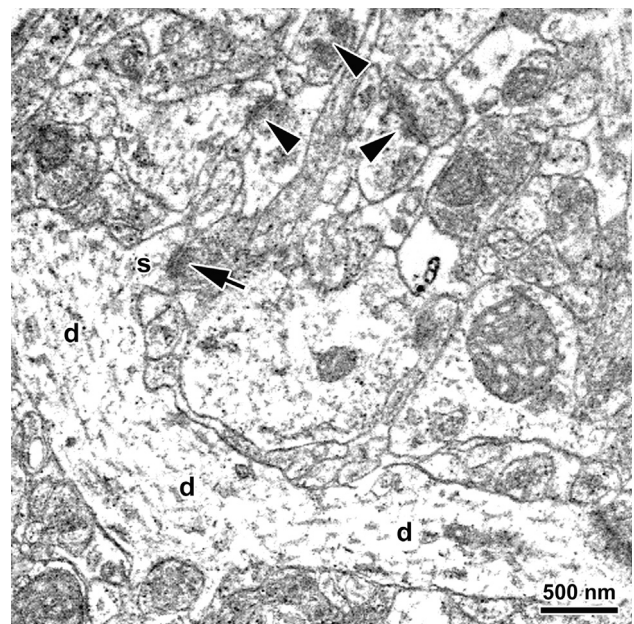
Fig. 1. Representative electron micrograph taken from the dentate gyrus stratum moleculare of a vehicle-pretreated ovariectomized female rat exposed to inescapable stress. The micrograph demonstrates a dendritic segment (d) with an emerging dendritic spine (s) that forms a spine synapse (arrow). Additional spine synapses are labeled by arrowheads. Scale bar = 500 nm.

fluid (aCSF) solution that was composed of 130 mM NaCl, 3.5 mM KCl, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 1 mM $CaCl_2$, 3 mM $MgSO_4$, 10 mM D-glucose, and saturated with 95% O_2 and 5% CO_2. Following brain removal, 350-μm coronal slices were prepared from the middle portion of hippocampus with a vibratome and kept in ice-cold aCSF. After vibratoming, slices were transferred to a Haas-type recording chamber and allowed to recover at room temperature for 1 h. The chamber contained a recording solution that had a flow rate of 1.5–2 ml/min and differed from the aCSF in the $CaCl_2$ (3 mM) and $MgSO_4$ (1.5 mM) concentrations. Following recovery, chamber temperature was set to and maintained at 34 °C during the entire recording procedure.

Orthodromic stimulation of the Schaffer collateral/commissural pathway was achieved via a bipolar, concentric, stainless steel stimulating electrode (Neuronelektrod Ltd, Budapest, Hungary) inserted in the stratum radiatum at the CA1/CA2 border. Evoked field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1sr using aCSF-filled glass micropipettes with a resistance of 1.5–2.5 megaΩ. Constant current stimuli were delivered in 0.2-ms pulses at 0.05 Hz to generate a half-maximal response that was determined from an input/output curve expressing fEPSP amplitudes against gradually increasing stimulating currents (0–100 μA). Employing the half-maximal response, amplitude stability of fEPSPs was monitored for 30 min, and then a baseline recording was acquired for 10 min.

LTP of the Schaffer collateral-CA1 synaptic response was induced by theta-burst stimulation (bursts of four impulses at 100 Hz, 100% stimulus intensity, 350 ms burst interval) (Marosi et al., 2009). After stimulation, fEPSPs were recorded for 60 min, amplified with a neutralized high-input-impedance preamplifier, filtered in the 1 Hz–3 kHz range, and digitized at a sampling rate of 10 kHz. Each animal provided a maximum of two recordings that were saved to a computer and analyzed offline with the Origin 8.0 software (OriginLab Corporation, Northampton, Massachusetts).

Statistical analysis

Means were computed for each experimental outcome in each group of animals. The Shapiro–Wilks and Lilliefors tests were used to explore whether data are normally distributed and to determine a parametric or non-parametric statistical approach. The following sampling and statistical strategy was employed to evaluate the various experimental outcomes.

Behavioral and corticosterone measures. From each group, $n = 10$ rats were randomly selected for behavioral testing. Three measurements were obtained from every single animal: (1) number of escape failures and (2) mean of escape latencies during the active escape task, and (3) serum corticosterone concentrations at the completion of active escape testing. These three measurements were analyzed separately with two-way parametric fixed-effect ANOVA

(drug treatment × stress) followed by the Neuman–Keuls post-hoc test.

Synapse counts. From each group, $n = 4$ rats were randomly selected and sacrificed at a timepoint representing the onset of active escape testing to process their brains for electron microscopic analysis. Three measurements were obtained from every single animal: (1) number of spine synapses in CA1sr, (2) number of spine synapses in CA3sl/sr, and (3) number of spine synapses in DGsm. These three measurements were evaluated collectively with three-way parametric fixed-effect ANOVA (drug treatment, between subjects factor × stress, between subjects factor × sampling area, within subjects factor) followed by the Tukey–Kramer post-hoc test.

fEPSP amplitudes. From the ovariectomized veh/ns, veh/is, and dz10/is groups of the diazepam experiment, $n = 5$–6 rats were randomly selected and sacrificed at a timepoint representing the onset of active escape testing to process their brains for electrophysiological analysis. Every single animal provided two independent time-series of fEPSP amplitude measurements. The amplitude data were evaluated with Scheirer-Ray-Hare two-way non-parametric fixed-effect ANOVA (group × time) followed by the Mann–Whitney U-test.

The significance level was conventionally set at $P < 0.05$.

RESULTS

Diazepam promoted stress resilience, protected hippocampal spine synapses, and muted the stress response during escape testing

Diazepam effects on helpless behaviour. At three randomly selected time-points during stress training, the experimenter briefly observed behavioral responses to inescapable stress, such as vocalizing, assuming evasive postures, and running about the cage trying to escape. Based on these behavioral responses, each animal was confirmed to be awake and perceiving footshocks.

Considering escape performance, two-way ANOVA (diazepam treatment × stress) demonstrated significant diazepam treatment effects ($F_{2,54} = 4.262$, $P \leq 0.02$ for escape failures; $F_{2,54} = 5.005$, $P \leq 0.01$ for escape latencies), significant stress effects ($F_{1,54} = 4.934$, $P \leq 0.04$ for escape failures; $F_{1,54} = 11.173$, $P \leq 0.002$ for escape latencies), and significant interaction effects ($F_{2,54} = 17.467$, $P \leq 0.001$ for escape failures; $F_{2,54} = 13.453$, $P \leq 0.001$ for escape latencies) on behavioral measures in ovariectomized females; while there were significant stress effects ($F_{1,36} = 41.060$, $P \leq 0.001$ for escape failures; $F_{1,36} = 21.687$, $P \leq 0.001$ for escape latencies) and significant interaction effects ($F_{1,36} = 17.157$, $P \leq 0.001$ for escape failures; $F_{1,36} = 7.927$, $P \leq 0.008$ for escape latencies), with the diazepam treatment effect being significant on escape failures ($F_{1,36} = 21.508$, $P \leq 0.001$) but not on escape

latencies ($F_{1,36} = 1.906$, $P = 0.176$) in simulated proestrus rats. Irrespective of hormonal status, diazepam treatment under non-stressed conditions did not affect escape performance (Fig. 2A, B ovariectomized females, dz5/ns vs. veh/ns, $q_{54} = 2.963$, $P = 0.1004$ for escape failures, $q_{54} = 1.329$,

$P = 0.3515$ for escape latencies, dz10/ns vs. veh/ns, $q_{54} = 2.586$, $P = 0.0731$ for escape failures, $q_{54} = 1.993$, $P = 0.3433$ for escape latencies; Fig. 2G, H simulated proestrus, dz10/ns vs. veh/ns, $q_{36} = 0.496$, $P = 0.7279$ for escape failures, $q_{36} = 1.435$, $P = 0.3172$ for escape latencies). On the other hand,
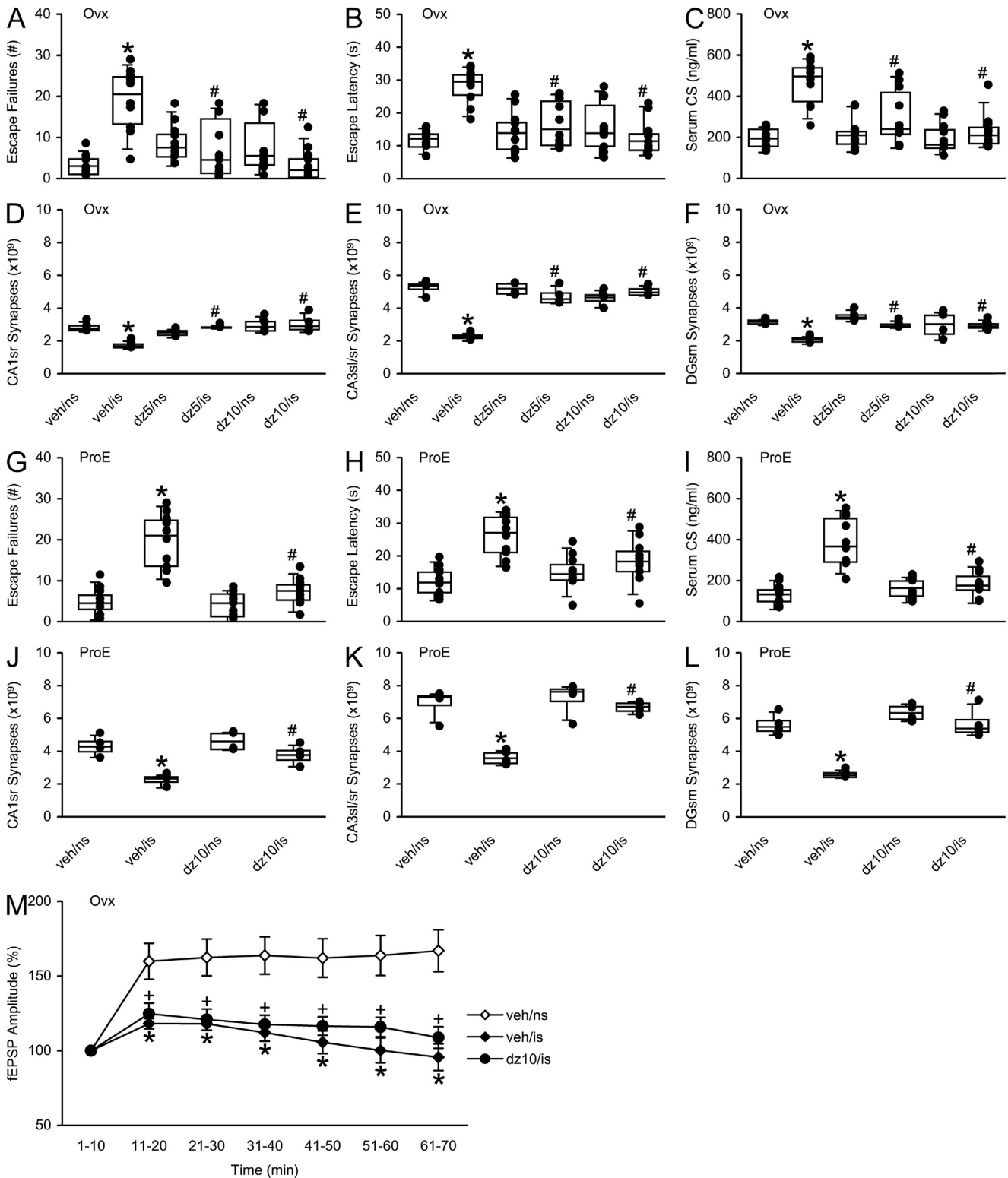
inescapable stress increased escape failures and escape latencies after vehicle treatment, which likewise occurred in both the absence and the presence of ovarian hormones (Fig. 2A, B ovariectomized females, veh/is vs. veh/ns, $q_{54} = 8.566$, $P \leq 0.0001$ for escape failures, $q_{54} = 8.502$, $P \leq 0.0001$ for escape latencies; Fig. 2G, H simulated proestrus, veh/is vs. veh/ns, $q_{36} = 10.550$, $P \leq 0.0001$ for escape failures, $q_{36} = 7.472$, $P \leq 0.0001$ for escape latencies). However, these stress-induced increases were not observed in diazepam-pretreated females (Fig. 2A, B ovariectomized females, dz5/is vs. dz5/ns, $q_{54} = 0.700$, $P = 0.6223$ for escape failures, $q_{54} = 1.226$, $P = 0.3898$ for escape latencies, dz10/is vs. dz10/ns, $q_{54} = 2.424$, $P = 0.0923$ for escape failures, $q_{54} = 1.540$, $P = 0.2811$ for escape latencies; Fig. 2G, H simulated proestrus, dz10/is vs. dz10/ns, $q_{36} = 2.266$, $P = 0.1179$ for escape failures, $q_{36} = 1.842$, $P = 0.2012$ for escape latencies). As a result, diazepam treatment prior to inescapable stress caused both behavioral measures to remain significantly below the increased behavioral measures of stress-exposed controls, facilitating stress resilience in both ovariectomized and simulated proestrus animals (Fig. 2A, B ovariectomized females, dz5/is vs. veh/is, $q_{54} = 6.303$, $P \leq 0.0001$ for escape failures, $q_{54} = 5.947$, $P \leq 0.0002$ for escape latencies, dz10/is vs. veh/is, $q_{54} = 8.404$, $P \leq 0.0001$ for escape failures, $q_{54} = 8.049$, $P \leq 0.0001$ for escape latencies; Fig. 2G, H simulated proestrus, dz10/is vs. veh/is, $q_{36} = 8.780$, $P \leq 0.0001$ for escape failures, $q_{36} = 4.196$, $P \leq 0.006$ for escape latencies).

Diazepam effects on escape-evoked corticosterone levels. Two-way ANOVA (diazepam treatment × stress) found a significant diazepam treatment effect (ovariectomized females, $F_{2,54} = 7.799$, $P \leq 0.001$; simulated proestrus, $F_{1,36} = 12.831$, $P \leq 0.001$), a significant stress effect (ovariectomized females, $F_{1,54} = 27.402$, $P \leq 0.001$; simulated proestrus, $F_{1,36} = 31.353$, $P \leq 0.001$), and a significant interaction effect (ovariectomized females, $F_{2,54} = 8.703$, $P \leq 0.001$; simulated proestrus, $F_{1,36} = 22.726$, $P \leq 0.001$) on corticosterone measurements in both ovariectomized and simulated proestrus animals. Irrespective of hormonal status, diazepam treatment under non-stressed conditions did not affect escape-

evoked corticosterone release (Fig. 2C ovariectomized females, dz5/ns vs. veh/ns, $q_{54} = 0.783$, $P = 0.8451$, dz10/ns vs. veh/ns, $q_{54} = 0.030$, $P = 0.9833$; Fig. 2I simulated proestrus, dz10/ns vs. veh/ns, $q_{36} = 1.185$, $P = 0.4074$). On the other hand, inescapable stress potentiated escape-evoked corticosterone secretion after vehicle treatment, which likewise occurred in both the absence and the presence of ovarian hormones (Fig. 2C ovariectomized females, veh/is vs. veh/ns, $q_{54} = 8.997$, $P \leq 0.0001$; Fig. 2I simulated proestrus, veh/is vs. veh/ns, $q_{36} = 10.367$, $P \leq 0.0001$). However, this stress-induced potentiation was not observed in diazepam-pretreated females (Fig. 2C ovariectomized females, dz5/is vs. dz5/ns, $q_{54} = 2.737$, $P = 0.0582$, dz10/is vs. dz10/ns, $q_{54} = 1.088$, $P = 0.4448$; Fig. 2I simulated proestrus, dz10/is vs. dz10/ns, $q_{36} = 0.832$, $P = 0.5598$). As a result, diazepam treatment prior to inescapable stress prevented escape-evoked corticosterone release to attain the high levels of corticosterone secretion detected in stress-exposed controls, mitigating the stress response in both ovariectomized and simulated proestrus animals (Fig. 2C ovariectomized females, dz5/is vs. veh/is, $q_{54} = 5.476$, $P \leq 0.0004$, dz10/is vs. veh/is, $q_{54} = 7.879$, $P \leq 0.0001$; Fig. 2I simulated proestrus, dz10/is vs. veh/is. $q_{36} = 8.349$, $P \leq 0.0001$).

Diazepam effects on hippocampal synapse counts. Three-way ANOVA (diazepam treatment × stress × sampling area) revealed a significant diazepam treatment effect ($F_{2,18} = 34.700$, $P \leq 0.001$), a significant stress effect ($F_{1,18} = 51.713$, $P \leq 0.001$), a significant diazepam treatment × stress interaction effect ($F_{2,18} = 49.522$, $P \leq 0.001$), a significant sampling area effect ($F_{2,36} = 136.063$, $P \leq 0.001$), a significant diazepam treatment × sampling area interaction effect ($F_{4,36} = 3.107$, $P \leq 0.03$), and a significant stress × sampling area interaction effect ($F_{2,36} = 5.352$, $P \leq 0.009$) on synapse counts in ovariectomized females, while there was a significant diazepam treatment effect ($F_{1,12} = 31.168$, $P \leq 0.001$), a significant stress effect ($F_{1,12} = 41.435$, $P \leq 0.001$), a significant diazepam treatment × stress interaction effect ($F_{1,12} = 15.299$, $P \leq 0.002$), a significant sampling area effect ($F_{2,24} = 96.285$, $P \leq 0.001$), a significant diazepam treatment × sampling area interaction effect

Fig. 2. Protective actions of diazepam treatment 30 min prior to inescapable stress in ovariectomized (Ovx, panels **(A–F, M)**) and simulated proestrus (ProE, panels **(G–L)**) animals: Effects on escape performance (panels **(A, B, G, H)**), escape-evoked corticosterone levels (panels **(C, I)**), synapse counts (panels **(D–F, J–L)**), and long-term potentiation of the Schaffer collateral-CA1 synaptic response (panel **(M)**). Box plots depict the 25th, 50th, and 75th percentiles, while whiskers show the 5th and 95th percentiles. Panels **(A, G)**: Numbers of failed trials during the 30-trial active escape task that was performed 24 h after inescapable stress. Panels **(B, H)**: Average escape latencies of all 30 escape trials. Panels **(C, I)**: Stress responses to the active escape task, which were measured immediately after the completion of escape testing by assaying serum corticosterone (CS) levels. Panels **(D–F, J–L)**: Numbers of hippocampal spine synapses, which were determined immediately before the onset of active escape testing. Panel **(M)**: Changes in the amplitude of evoked field excitatory postsynaptic potentials (fEPSP), which were recorded at a time representing the onset of active escape testing. Each tracing point shows the average of measurements from a 10-min interval (mean ± SEM) normalized to the baseline recording (first tracing point). Treatment and stress codes: veh, vehicle-pretreated; dz5, low-dose diazepam-pretreated (5 mg/kg); dz10, high-dose diazepam-pretreated (10 mg/kg), ns, exposed to sham stress; is, exposed to inescapable stress. Significance markers ($P < 0.05$): (*) Significant stress effect between groups treated with the same drug; (+) significantly different from the veh/ns group; (#) Significant diazepam treatment effect among stress-exposed groups (vs. veh/is). Area abbreviations: CA1sr, CA1 stratum radiatum; CA3sl/sr, CA3 stratum lucidum/radiatum; DGsm, dentate gyrus stratum moleculare.

($F_{2,24} = 4.872$, $P \leq 0.02$), but no stress × sampling area interaction effect ($F_{2,24} = 1.031$, $P = 0.372$) in simulated proestrus rats. Irrespective of hormonal status, diazepam treatment under non-stressed conditions did not change synapse numbers (Fig. 2D–F ovariectomized females, dz5/ns vs. veh/ns, $q_{18} = 1.928$, $P = 0.3804$ for CA1sr, $q_{18} = 0.285$, $P = 0.9779$ for CA3sl/sr, $q_{18} = 1.704$, $P = 0.4655$ for DGsm, dz10/ns vs. veh/ns, $q_{18} = 0.670$, $P = 0.8843$ for CA1sr, $q_{18} = 3.021$, $P = 0.1105$ for CA3sl/sr, $q_{18} = 0.924$, $P = 0.7928$ for DGsm; Fig. 2J–L simulated proestrus, dz10/ns vs. veh/ns, $q_{12} = 1.098$, $P = 0.4525$ for CA1sr, $q_{12} = 0.805$, $P = 0.5796$ for CA3sl/sr, $q_{12} = 2.233$, $P = 0.1404$ for DGsm). On the other hand, inescapable stress caused a decline in the number of spine synapses in all three hippocampal areas after vehicle treatment, which likewise occurred in both the absence and the presence of ovarian hormones (Fig. 2D–F ovariectomized females, veh/is vs. veh/ns, $q_{18} = 6.132$, $P \leq 0.0005$ for CA1sr, $q_{18} = 14.226$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 5.221$, $P \leq 0.002$ for DGsm; Fig. 2J–L simulated proestrus, veh/is vs. veh/ns, $q_{12} = 7.254$, $P \leq 0.0004$ for CA1sr, $q_{12} = 8.614$, $P \leq 0.0002$ for CA3sl/sr, $q_{12} = 9.197$, $P \leq 0.0001$ for DGsm). More importantly, these stress-induced declines were not observed in diazepam-pretreated rats (Fig. 2D–F ovariectomized females, dz5/is vs. dz5/ns, $q_{18} = 2.025$, $P = 0.1693$ for CA1sr, $q_{18} = 2.177$, $P = 0.1413$ for CA3sl/sr, $q_{18} = 2.580$, $P = 0.0848$ for DGsm, dz10/is vs. dz10/ns, $q_{18} = 0.531$, $P = 0.7117$ for CA1sr, $q_{18} = 2.010$, $P = 0.1724$ for CA3sl/sr, $q_{18} = 0.108$, $P = 0.9400$ for DGsm; Fig. 2J–L simulated proestrus, dz10/is vs. dz10/ns, $q_{12} = 3.047$, $P = 0.0522$ for CA1sr, $q_{12} = 1.415$, $P = 0.3369$ for CA3sl/sr, $q_{12} = 1.945$, $P = 0.1942$ for DGsm). As a result, diazepam treatment prior to inescapable stress forced synapse counts in all areas to remain significantly above the reduced synapse levels of stress-exposed controls, protecting synapses in both ovariectomized and simulated proestrus animals (Fig. 2D–F ovariectomized females, dz5/is vs. veh/is, $q_{18} = 6.230$, $P \leq 0.001$ for CA1sr, $q_{18} = 11.764$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 4.345$, $P \leq 0.02$ for DGsm, dz10/is vs. veh/is, $q_{18} = 7.333$, $P \leq 0.0002$ for CA1sr, $q_{18} = 13.215$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 4.189$, $P \leq 0.03$ for DGsm; Fig. 2J–L simulated proestrus, dz10/is vs. veh/is, $q_{12} = 5.304$, $P \leq 0.003$ for CA1sr, $q_{12} = 8.004$, $P \leq 0.0002$ for CA3sl/sr, $q_{12} = 9.485$, $P \leq 0.0001$ for DGsm).

Diazepam effects on LTP. The Scheirer-Ray-Hare two-way non-parametric ANOVA (group × time) showed a significant group effect ($H_{2,180} = 86.224$, $P \leq 0.001$), but no time effect ($H_{5,180} = 3.666$, $P = 0.598$), and no interaction effect ($H_{10,180} = 3.164$, $P = 0.977$) on the Schaffer collateral-CA1 synaptic response in ovariectomized females. Inescapable stress in vehicle-pretreated ovariectomized animals decreased theta-burst-stimulated fEPSP amplitudes during the entire 60-min recording period (Fig. 2M, veh/is vs. veh/ns, $U_{11,10} = 7$, $P \leq 0.0004$ for 11–20 min, $U_{11,10} = 8$ $P \leq 0.0005$ for 21–30 min, $U_{11,10} = 6$ $P \leq 0.0003$ for

31–40 min, $U_{11,10} = 10$, $P \leq 0.0008$ for 41–50 min, $U_{11,10} = 6$ $P \leq 0.0003$ for 51–60 min, $U_{11,10} = 4$ $P \leq 0.0002$ for 61–70 min). Inescapable stress similarly reduced stimulated fEPSP amplitudes in ovariectomized rats pretreated with high-dose diazepam (Fig. 2M, dz10/is vs. veh/ns, $U_{11,12} = 109$, $P \leq 0.005$ for 11–20 min, $U_{11,12} = 113$, $P \leq 0.002$ for 21–30 min, $U_{11,12} = 117$, $P \leq 0.0008$ for 31–40 min, $U_{11,12} = 116$, $P \leq 0.001$ for 41–50 min, $U_{11,12} = 114$, $P \leq 0.002$ for 51–60 min, $U_{11,12} = 120$, $P \leq 0.0004$ for 61–70 min). As a result, diazepam pretreatment did not modify the effects of inescapable stress on fEPSP measurements (Fig. 2M, dz10/is vs. veh/is, $U_{10,12} = 55$, $P = 0.3708$ for 11–20 min, $U_{10,12} = 62$, $P = 0.4475$ for 21–30 min, $U_{10,12} = 56$, $P = 0.3960$ for 31–40 min, $U_{10,12} = 48$, $P = 0.2144$ for 41–50 min, $U_{10,12} = 44$, $P = 0.1457$ for 51–60 min, $U_{10,12} = 43$, $P = 0.1312$ for 61–70 min).

Raw datasets are available in Mendeley Data (Huzian et al., 2021).

The protective actions of fluoxetine are limited

Fluoxetine effects on helpless behavior. Two-way ANOVA (fluoxetine treatment × stress) demonstrated significant fluoxetine treatment effects ($F_{2,54} = 15.611$, $P \leq 0.001$ for escape failures; $F_{2,54} = 19.697$, $P \leq 0.001$ for escape latencies), significant stress effects ($F_{1,54} = 100.551$, $P \leq 0.001$ for escape failures; $F_{1,54} = 121.662$, $P \leq 0.001$ for escape latencies), and significant interaction effects ($F_{2,54} = 9.040$, $P \leq 0.001$ for escape failures; $F_{2,54} = 4.618$, $P \leq 0.02$ for escape latencies) on behavioral measures in ovariectomized females, while there were significant fluoxetine treatment effects ($F_{1,36} = 15.884$, $P \leq 0.001$ for escape failures; $F_{1,36} = 11.309$, $P \leq 0.002$ for escape latencies), significant stress effects ($F_{1,36} = 38.219$, $P \leq 0.001$ for escape failures; $F_{1,36} = 12.433$, $P \leq 0.001$ for escape latencies), but no interaction effects ($F_{1,36} = 3.428$, $P = 0.072$ for escape failures; $F_{1,36} = 1.051$, $P = 0.312$ for escape latencies) in intact rats. Fluoxetine treatment under non-stressed conditions improved escape latencies in ovariectomized females (Fig. 3B, fx5/ns vs. veh/ns, $q_{54} = 4.553$, $P \leq 0.003$, fx20/ns vs. veh/ns, $q_{54} = 5.580$, $P \leq 0.001$) but not in intact rats (Fig. 3H, fx20/ns vs. veh/ns, $q_{36} = 2.337$, $P = 0.1071$), whereas the same treatment did not affect escape failures irrespective of ovarian status (Fig. 3A ovariectomized females, fx5/ns vs. veh/ns, $q_{54} = 3.249$, $P = 0.0647$, fx20/ns vs. veh/ns, $q_{54} = 2.830$, $P = 0.0504$; Fig. 3G intact rats, fx20/ns vs. veh/ns, $q_{36} = 2.134$, $P = 0.1401$). On the other hand, inescapable stress increased escape failures and escape latencies after vehicle treatment, which occurred in both the absence and the presence of ovaries (Fig. 3A, B ovariectomized females, veh/is vs. veh/ns, $q_{54} = 8.875$, $P \leq 0.0001$ for escape failures, $q_{54} = 7.915$, $P \leq 0.0001$ for escape latencies; Fig. 3G, H intact rats, veh/is vs. veh/ns, $q_{36} = 8.034$, $P \leq 0.0001$ for escape failures, $q_{36} = 4.551$, $P \leq 0.003$ for escape latencies). These stress-induced increases persisted in ovariectomized females pretreated with low-dose
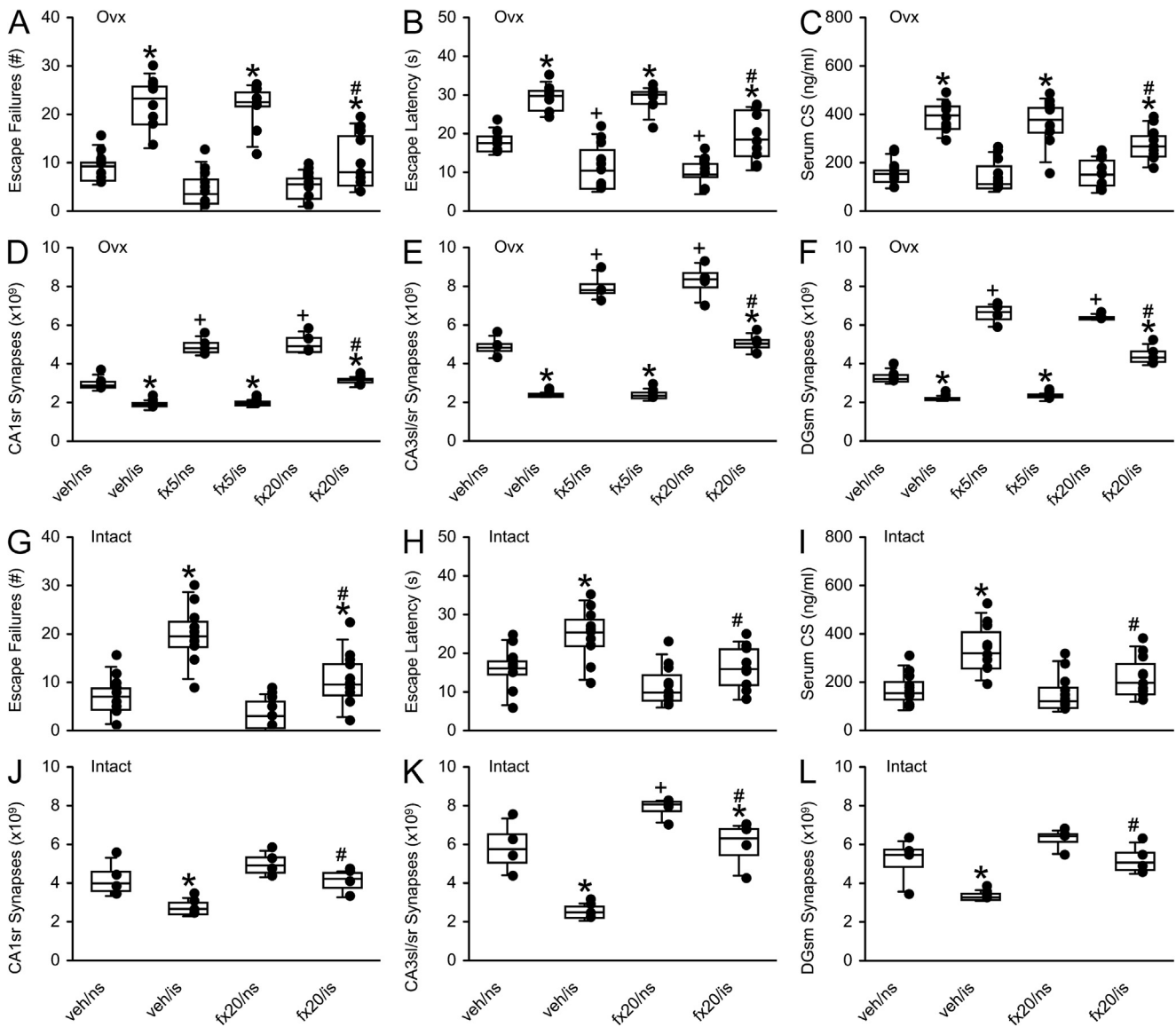
Fig. 3. Protective actions of a two-week fluoxetine treatment prior to inescapable stress in ovariectomized (Ovx, panels **(A–F)**) and intact (panels **(G–L)**) animals: Effects on escape performance (panels **(A, B, G, H)**), escape-evoked corticosterone levels (panels **(C, I)**), and synapse counts (panels **(D–F, J–L)**). Box plots depict the 25th, 50th, and 75th percentiles, while whiskers show the 5th and 95th percentiles. Panels **(A, G)**: Numbers of failed trials during the 30-trial active escape task that was performed 24 h after inescapable stress. Panels **(B, H)**: Average escape latencies of all 30 escape trials. Panels **(C, I)**: Stress responses to the active escape task, which were measured immediately after the completion of escape testing by assaying serum corticosterone (CS) levels. Panels **(D–F, J–L)**: Numbers of hippocampal spine synapses, which were determined immediately before the onset of active escape testing. Treatment and stress codes: veh, vehicle-pretreated; fx5, low-dose fluoxetine-pretreated (5 mg/kg); fx20, high-dose fluoxetine-pretreated (20 mg/kg); ns, exposed to sham stress; is, exposed to inescapable stress. Significance markers ($P < 0.05$): (*) Significant stress effect between groups treated with the same drug; (+) Significant fluoxetine treatment effect among sham-stressed groups (vs. veh/ns); (#) Significant fluoxetine treatment effect among stress-exposed groups (vs. veh/is). Area abbreviations: CA1sr, CA1 stratum radiatum; CA3sl/sr, CA3 stratum lucidum/radiatum; DGsm, dentate gyrus stratum moleculare.

fluoxetine (Fig. 3A, B, fx5/is vs. fx5/ns, $q_{54} = 12.054$, $P \leq 0.0001$ for escape failures, $q_{54} = 12.440$, $P \leq 0.0001$ for escape latencies, fx5/is vs. veh/is, $q_{54} = 0.070$, $P = 0.9608$ for escape failures, $q_{54} = 0.029$, $P = 0.9840$ for escape latencies). Although inescapable stress increased both escape failures and escape latencies in high-dose fluoxetine-pretreated ovariectomized females as well (Fig. 3A, B, fx20/is vs. fx20/ns, $q_{54} = 3.634$, $P \leq 0.02$ for escape

failures, $q_{54} = 6.663$, $P \leq 0.0001$ for escape latencies), these increases were observed only in escape failures (Fig. 3G, fx20/is vs. fx20/ns, $q_{36} = 4.331$, $P \leq 0.005$) but not in escape latencies of similarly-treated intact rats (Fig. 3H, fx20/is vs. fx20/ns, $q_{36} = 2.501$, $P = 0.0856$). Importantly, treatment with high-dose fluoxetine prior to inescapable stress caused both behavioral measures to remain significantly below the increased behavioral measures of stress-exposed controls, facilitating stress

resilience in both ovariectomized and intact animals (Fig. 3A, B ovariectomized females, fx20/is vs. veh/is, $q_{54} = 8.071$, $P \leq 0.0001$ for escape failures, $q_{54} = 6.832$, $P \leq 0.0001$ for escape latencies; Fig. 3G, H intact rats, fx20/is vs. veh/is, $q_{36} = 5.837$, $P \leq 0.0003$ for escape failures, $q_{36} = 4.388$, $P \leq 0.004$ for escape latencies).

Fluoxetine effects on escape-evoked corticosterone release. Two-way ANOVA (fluoxetine treatment × stress) found a significant fluoxetine treatment effect ($F_{2,54} = 3.448$, $P \leq 0.04$), a significant stress effect ($F_{1,54} = 110.346$, $P \leq 0.001$), and a significant interaction effect ($F_{2,54} = 4.111$, $P \leq 0.03$) on corticosterone measurements in ovariectomized females, while there was a significant fluoxetine treatment effect ($F_{1,36} = 6.030$, $P \leq 0.02$), a significant stress effect ($F_{1,36} = 18.255$, $P \leq 0.001$), but no interaction effect ($F_{1,36} = 3.587$, $P = 0.066$) in intact rats. Irrespective of ovarian status, fluoxetine treatment under non-stressed conditions did not affect escape-evoked corticosterone release (Fig. 3C ovariectomized females, fx5/ns vs. veh/ns, $q_{54} = 0.666$, $P = 0.6395$, fx20/ns vs. veh/ns, $q_{54} = 0.012$, $P = 0.9933$; Fig. 3I intact rats, fx20/ns vs. veh/ns, $q_{36} = 0.562$, $P = 0.6935$). On the other hand, inescapable stress potentiated escape-evoked corticosterone secretion after vehicle treatment, which likewise occurred in both the absence and the presence of ovaries (Fig. 3C ovariectomized females, veh/is vs. veh/ns, $q_{54} = 10.486$, $P \leq 0.0001$; Fig. 3I intact rats, veh/is vs. veh/ns, $q_{36} = 6.167$, $P \leq 0.0002$). This stress-induced potentiation persisted in ovariectomized females pretreated with low-dose fluoxetine (Fig. 3C, fx5/is vs. fx5/ns, $q_{54} = 9.966$, $P \leq 0.0001$, fx5/is vs. veh/is, $q_{54} = 1.186$, $P = 0.4053$). Although inescapable stress potentiated escape-evoked corticosterone release in high-dose fluoxetine-pretreated ovariectomized females as well (Fig. 3C, fx20/is vs. fx20/ns, $q_{54} = 5.280$, $P \leq 0.0005$), this potentiation was not observed in similarly-treated intact rats (Fig. 3I, fx20/is vs. fx20/ns, $q_{36} = 2.379$, $P = 0.1013$). Importantly, treatment with high-dose fluoxetine prior to inescapable stress prevented escape-evoked corticosterone secretion to reach the extents of corticosterone release encountered in stress-exposed controls, mitigating the stress response in both ovariectomized and intact animals (Fig. 3C ovariectomized females, fx20/is vs. veh/is, $q_{54} = 5.194$, $P \leq 0.002$; Fig. 3I intact rats, fx20/is vs. veh/is, $q_{36} = 4.349$, $P \leq 0.004$).

Fluoxetine effects on hippocampal synapse counts. Three-way ANOVA (fluoxetine treatment × stress × sampling area) revealed a significant fluoxetine treatment effect ($F_{2,18} = 106.089$, $P \leq 0.001$), a significant stress effect ($F_{1,18} = 394.252$, $P \leq 0.001$), a significant fluoxetine treatment × stress interaction effect ($F_{2,18} = 33.850$, $P \leq 0.001$), a significant sampling area effect ($F_{2,36} = 129.389$, $P \leq 0.001$), a significant fluoxetine treatment × sampling area interaction effect ($F_{4,36} = 6.959$, $P \leq 0.001$), and a

significant stress × sampling area interaction effect ($F_{2,36} = 32.444$, $P \leq 0.001$) on synapse counts in ovariectomized females; while there was a significant fluoxetine treatment effect ($F_{1,12} = 34.075$, $P \leq 0.001$), a significant stress effect ($F_{1,12} = 33.306$, $P \leq 0.001$), a significant sampling area effect ($F_{2,24} = 16.962$, $P \leq 0.001$), a significant fluoxetine treatment × sampling area interaction effect ($F_{2,24} = 5.201$, $P \leq 0.02$), a significant stress × sampling area interaction effect ($F_{2,24} = 4.109$, $P \leq 0.03$), but no fluoxetine treatment × stress interaction effect ($F_{1,12} = 2.262$, $P = 0.158$) in intact rats. Fluoxetine treatment under non-stressed conditions elicited synaptogenesis all along the trisynaptic loop in ovariectomized females (Fig. 3D–F, fx5/ns vs. veh/ns, $q_{18} = 10.053$, $P \leq 0.0001$ for CA1sr, $q_{18} = 10.252$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 17.356$, $P \leq 0.0001$ for DGsm, fx20/ns vs. veh/ns, $q_{18} = 10.867$, $P \leq 0.0001$ for CA1sr, $q_{18} = 11.232$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 16.371$, $P \leq 0.0001$ for DGsm), whereas the same treatment increased synapse numbers only in CA3sl/sr (Fig. 3K, fx20/ns vs. veh/ns, $q_{12} = 3.953$, $P \leq 0.02$) but not in CA1sr and DGsm of intact rats (Fig. 3J, L, fx20/ns vs. veh/ns, $q_{12} = 2.156$, $P = 0.1533$ for CA1sr, $q_{12} = 2.710$, $P = 0.0795$ for DGsm). On the other hand, inescapable stress caused a decline in the number of spine synapses in all three hippocampal areas after vehicle treatment, which occurred in both the absence and the presence of ovaries (Fig. 3D–F ovariectomized females, veh/is vs. veh/ns, $q_{18} = 5.696$, $P \leq 0.0009$ for CA1sr, $q_{18} = 8.144$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 5.931$, $P \leq 0.0006$ for DGsm; Fig. 3J–L intact rats, veh/is vs. veh/ns, $q_{12} = 4.109$, $P \leq 0.02$ for CA1sr, $q_{12} = 6.509$, $P \leq 0.0007$ for CA3sl/sr, $q_{12} = 4.287$, $P \leq 0.02$ for DGsm). These stress-induced declines were not affected by pretreatment of ovariectomized females with low-dose fluoxetine (Fig. 3D–F, fx5/is vs. fx5/ns, $q_{18} = 15.408$, $P \leq 0.0001$ for CA1sr, $q_{18} = 18.396$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 22.662$, $P \leq 0.0001$ for DGsm, fx5/is vs. veh/is, $q_{18} = 0.341$, $P = 0.9686$ for CA1sr, $q_{18} = 0.000$, $P = 1.0000$ for CA3sl/sr, $q_{18} = 0.625$, $P = 0.8985$ for DGsm). Although inescapable stress induced synapse losses all along the trisynaptic loop in high-dose fluoxetine-pretreated ovariectomized females as well (Fig. 3D–F, fx20/is vs. fx20/ns, $q_{18} = 10.132$, $P \leq 0.0001$ for CA1sr, $q_{18} = 10.631$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 10.467$, $P \leq 0.0001$ for DGsm), these losses were observed only in CA3sl/sr (Fig. 3K, fx20/is vs. fx20/ns, $q_{12} = 3.743$, $P \leq 0.03$) but not in CA1sr and DGsm of similarly-treated intact rats (Fig. 3J, L, fx20/is vs. fx20/ns, $q_{12} = 2.527$, $P = 0.0993$ for CA1sr, $q_{12} = 2.506$, $P = 0.1018$ for DGsm). Most importantly, treatment with high-dose fluoxetine prior to inescapable stress forced synapse counts in all areas to remain significantly above the depressed synapse levels of stress-exposed controls, protecting synapses in both ovariectomized and intact animals (Fig. 3D–F ovariectomized females, fx20/is vs. veh/is, $q_{18} = 6.431$, $P \leq 0.0007$ for CA1sr, $q_{18} = 8.745$, $P \leq 0.0001$ for CA3sl/sr, $q_{18} = 11.836$, $P \leq 0.0001$ for DGsm; Fig. 3J–L intact rats, fx20/is vs.

veh/is, $q_{12} = 3.738$, $P \leq 0.03$ for CA1sr, $q_{12} = 6.719$, $P \leq 0.0006$ for CA3sl/sr, $q_{12} = 4.491$, $P \leq 0.008$ for DGsm).

Raw datasets are available in Mendeley Data (Huzian et al., 2021).

DISCUSSION

This study demonstrates in ovariectomized female rats that acute treatment with diazepam prior to inescapable stress promotes stress resilience in the learned helplessness paradigm, prevents the loss of hippocampal spine synapses, and reduces corticosterone secretion during escape testing. By contrast, chronic treatment with fluoxetine prior to inescapable stress, at a dose that is effective in a chronic unpredictable stress paradigm (Valentine et al., 2008), does not show any preventive effects, requiring a much higher dose of fluoxetine to partly reproduce the above-mentioned responses to diazepam. Importantly, the protective actions of both diazepam and high-dose fluoxetine are retained in the presence of ovarian hormones. Under non-stressed conditions, on the other hand, diazepam does not affect any of the analyzed endpoints irrespective of hormonal status. Fluoxetine, by contrast, improves escape performance and exerts a powerful hippocampal synaptogenic response in non-stressed ovariectomized females, but these effects become masked and almost completely disappear in the presence of ovarian steroids. Moreover, in line with its limited preventive capabilities, exposure to stress mostly destroys the hippocampal synaptogenic power of fluoxetine, especially when lower doses are applied.

Our results concerning the remodeling of spine synapses need to be highlighted as the main novel findings of this study. There is a remarkable contrast between diazepam and fluoxetine in their hippocampal synaptic actions. Diazepam elicits robust synaptoprotective and absolutely no synaptogenic effects, while fluoxetine shows strong synaptogenic and only limited synaptoprotective actions. These behavioral and synaptic observations are in alignment with the tenets of the synaptogenic hypothesis of major depression.

The protective actions of diazepam

Our present observations with diazepam confirm and extend earlier findings most notably by providing conclusive ultrastructural evidence. This study demonstrates that diazepam, when given prior to inescapable stress, promotes stress resilience in learned helplessness, which is in agreement with a line of earlier studies (Drugan et al., 1984; De Pablo et al., 1991; Petty et al., 1992). We extend this finding by unveiling a synaptoprotective action of diazepam that preserves connectivity along the hippocampal neural circuitry during stress exposure. Other laboratories similarly report that benzodiazepines counter stress-induced alterations in other modalities of neuroplasticity in the hippocampus (Magarinos et al., 1999; Leussis et al., 2008; Giachero et al., 2015). By contrast, we also show that diazepam, when given without stress exposure, does not influence

any experimental outcomes, which includes the lack of effect on hippocampal synaptogenesis. Other laboratories demonstrate that benzodiazepines reduce or even completely halt various modalities of synaptic plasticity as well (Evans and Viola-McCabe, 1996; Seabrook et al., 1997; Tampellini et al., 2010; Curto et al., 2016). Considering the fact that an antidepressant response requires generation of new synapses (Li et al., 2010), the lack of synaptogenic power contributes to the inability of improving escape performance, i.e., benzodiazepines possess no antidepressant efficacy (Sherman et al., 1982; Drugan et al., 1984; Maier, 1990; Naruo et al., 1993; Martin and Puech, 1996).

It has been argued that the protective actions of benzodiazepines may be attributed to their anxiolytic and hypnotic effects, causing animals not to perceive and/or not to remember stress exposure, thereby mitigating stress and negating the effectivity of stress training. Although it is not possible to discount this argument, each diazepam-pretreated rat in this study provided behavioral responses to inescapable stress, indicating that animals were awake and perceiving footshocks. The above argument is also based on the assumption that the development of helpless behavior requires *stress experience and learning*. Interestingly, several studies show that helpless behavior is readily induced by simple procedures, such as ovariectomy in females, and treatment with high-dose corticosterone or benzodiazepine receptor antagonists in males (Drugan et al., 1985; Hajszan et al., 2009, 2010). These simple procedures are completely devoid of stress experience and of learning the context of stress training, yet they are comparable to inescapable stress in evoking severe escape deficits. We also performed a limited LTP experiment in this study, which demonstrates a stress-induced LTP damage in agreement with previous reports (Kim et al., 2006). This LTP damage is not rescued by diazepam, indicating that a certain level of stress effectively reaches diazepam-pretreated animals (see Fig. 2M). Our LTP experiment additionally suggests that successful coping in the active escape task does not require impeccable functional plasticity.

Nevertheless, benzodiazepines are among the most potent suppressors of the stress response irrespective of what mechanisms are involved (File, 1982; Pohorecky et al., 1988; de Boer et al., 1990; Matar et al., 2009). Further studies are needed, however, to reveal whether the protective actions of diazepam are mediated exclusively by mitigating stress, or additional mechanisms are also involved. Our present observation of hippocampal synaptoprotection may be especially useful by providing guidance for these future studies. Loss of spine synapses appears to be a neuronal defense mechanism against glutamatergic insults, because eliminating these excitatory synapses reduces the excitatory load of neurons (Sapolsky, 2000b; McCall et al., 2013; McEwen et al., 2016; Hajszan, 2020). In light of this theory, it is noteworthy that benzodiazepines also block evoked glutamate release in the hippocampus (Bagley and Moghaddam, 1997; Khan et al., 1999) and decrease the excitatory load of neurons by potent hyperpolarization

(Iversen et al., 2009). These effects mitigate the need for neuronal defenses and may contribute to the protective actions of diazepam.

The conflict between stress and fluoxetine

Our findings with fluoxetine are a bit difficult to place into perspective, as fluoxetine is rarely investigated in this "preventive" setting of learned helplessness. Nevertheless, this study shows that fluoxetine, in the *absence* of stress, stimulates escape performance and hippocampal synaptogenesis, which is in line with earlier results (Hajszan et al., 2005; Reines et al., 2008; O'Leary et al., 2009; McAvoy et al., 2015; Wainwright et al., 2016). The major benefit of applying fluoxetine prior to stress exposure was gaining the insight that inescapable stress practically wipes out almost all responses to fluoxetine, paralleling clinical and preclinical findings about stress interfering with antidepressant efficacy (Heuser et al., 1996; Appelhof et al., 2006; Valentine et al., 2008; Branchi et al., 2013; Khemissi et al., 2014). This interference from stress may underlie the growing number of studies showing SSRI antidepressants being ineffective in a wide variety of paradigms (Magarinos et al., 1999; Kobayashi et al., 2008; Valentine et al., 2008; Venzala et al., 2012; Kokras et al., 2015; Workman et al., 2016). It may also explain why fluoxetine is usually found effective only in less stressful versions of learned helplessness (Ferguson et al., 2000; Iwamoto et al., 2005; Zazpe et al., 2007; Yamada et al., 2014) and in less stressful models of the antidepressant response, such as the chronic unpredictable/mild stress paradigm (Valentine et al., 2008; Padilla et al., 2011; El Yacoubi et al., 2013). In order to make fluoxetine effective under more stressful conditions, earlier learned helplessness studies used higher doses of the drug (Malberg and Duman, 2003; Ruedi-Bettschen et al., 2004; Song et al., 2006; Fernandez Macedo et al., 2013; Holanda et al., 2016). In line with these findings, we also needed a significantly increased dose of fluoxetine to counter stress effects, and even this higher dose failed to fully reproduce the strong protective actions of diazepam.

The responses to fluoxetine may be explained by the hypothesis that stress reduces drug efficacy, requiring much higher doses under stressful conditions. Reversing this concept, it is more likely that the stress-mitigating effect of fluoxetine is dose-dependent. It is well known that fluoxetine in the higher dose ranges induces the release of noradrenaline in addition to serotonin (Vizi et al., 2004; Kobayashi et al., 2008; Kamińska et al., 2013). As a result, the protective actions of the higher fluoxetine dose may mimic those of tricyclic antidepressants that strongly suppress hypothalamic–pituitary–adrenal axis activity (Willner et al., 1987; Shimoda et al., 1988; Centeno and Volosin, 1997). By contrast, the non-protective lower fluoxetine dose, that probably elicits only serotonin release, fails to counter the stress-induced changes (Duncan et al., 1998; Marcilhac et al., 1999; Zhang et al., 2000) (Ferrero et al., 2005).

It is noteworthy that synaptic responses to fluoxetine are not uniform across the trisynaptic loop in intact rats,

as certain changes emerge only in CA3sl/sr (see Fig. 3J–L). The functional consequences of this finding can be discerned from the anatomical properties of hippocampal circuitry. The trisynaptic loop connects the entorhinal cortex to the subiculum (Amaral and Witter, 1995), as signals from the entorhinal cortex enter the dentate gyrus, then propagate through CA3 toward CA1 (Stepan et al., 2012, 2015). One may thus reasonably expect that synaptic damage even in a single area may be sufficient to compromise signal flow along the trisynaptic loop and affect behavior. In line with this theory, stress-induced synapse loss in fluoxetine-treated intact rats occurs in CA3sl/sr but not in CA1sr and DGsm, yet a weakness appears in escape behavior (see Fig. 3K, G). These findings also agree with the results of our earlier study, where a synaptic deficit apparent only in CA1sr was sufficient to impair escape performance (Hajszan et al., 2009). Conversely, fluoxetine treatment of sham-stressed intact rats elicited significant synaptogenesis only in CA3sl/sr, but behavioral and corticosterone measures in these animals did not improve (see Fig. 3K, G–I), suggesting that synaptogenesis needs to take place along the entire trisynaptic loop in order to improve signal flow and behavior.

The influence of ovarian hormones

Aside from modified baselines, responses to diazepam remain basically unaltered in simulated proestrus rats. This baseline change is best seen in the synapse counts, as synapse levels shift generally higher in the presence of ovarian hormones, which is in line with the well-known hippocampal synaptogenic power of estradiol (Woolley and McEwen, 1992). Importantly, the hormone-induced baseline shift also occurs in stress-exposed controls, indicating that ovarian steroids possess their own preventive effects. We have repeatedly demonstrated these preventive effects of ovarian hormones in learned helplessness (Hajszan et al., 2010; Baka et al., 2017), matching earlier findings of other laboratories (Rachman et al., 1998; Frye and Wawrzycki, 2003). Moreover, combining the forces of ovarian steroids and fluoxetine in this study yielded improved protective actions that were comparable with those of diazepam, which parallels synergism between fluoxetine and estrogens observed in other models of major depression (Estrada-Camarena et al., 2004). The protective actions of ovarian hormones may be based on multiple mechanisms, including neurosteroid effects on GABA receptors (mimicking diazepam pharmacology) (Bitran and Dowd, 1996; Stell et al., 2003), stress mitigation (Young et al., 2001; Lunga and Herbert, 2004), neurotrophic stimulation (Scharfman et al., 2007), and neurosteroid control of hippocampal long-term potentiation/depression (Tozzi et al., 2019, 2020).

Interestingly, while ovarian hormones improve the protective actions of fluoxetine under stressful conditions, the robust hippocampal synaptogenic response to fluoxetine observed under non-stressed conditions largely disappears when ovarian steroids are introduced. This phenomenon is probably based on a ceiling effect in synapse numbers. There appear to be well defined upper and lower bounds on synapse sizes

in the hippocampus, and synapse sizes strictly correlate with the sizes of pre- and post-synaptic structures (Bartol et al., 2015). These bounds define an obvious limit to how many synapses can be crammed into a certain volume. Indeed, the highest estimates of numerical synapse densities range between 1.8 and 2.3 synapses/μm^3 in the *rat* hippocampus, even after widely varying methods of synaptogenic stimulation, such as water maze training (Sandi et al., 2003; Geinisman et al., 2004), LTP generation (Stewart et al., 2000; Dhanrajan et al., 2004), monoaminergic antidepressants (Hajszan et al., 2005, 2009; Chen et al., 2008, 2010, 2018), ketamine (Ardalan et al., 2017), electro convulsive seizures (Chen et al., 2009), ovarian steroids (MacLusky et al., 2005; Hajszan et al., 2010), and drugs of abuse (Scallet et al., 1987; Heidari et al., 2013). As a result, the hormone-induced substantial shift of baseline synapse numbers toward this ceiling drastically reduces the potential of fluoxetine actions.

Stress resilience, hippocampal synaptoprotection, and antidepressant therapy

Because successful coping in the active escape task is thought to require hippocampus-dependent functions, such as motivation and cognitive capabilities (Thiebot et al., 1992; Vollmayr and Henn, 2001; Cryan et al., 2002), preserving spine synapses under stressful conditions to maintain proper connectivity along the hippocampal neural circuitry may significantly contribute to determining the outcomes of both learned helplessness and antidepressant therapy. In light of the central role that the hippocampus also plays in regulating the stress response (Sapolsky, 2000a; Sala et al., 2004; McEwen et al., 2016), corticosterone release in response to stress may represent an indirect indicator of hippocampal functionality. Our present results combined with our earlier study (Baka et al., 2017) demonstrate that three distinct pharmacological interventions, i.e., the pregnancy levels of female reproductive hormones, the GABA-A receptor benzodiazepine site agonist anxiolytic diazepam, and high-doses of the classic SSRI antidepressant fluoxetine, all promote stress resilience and protect hippocampal spine synapses in the rat learned helplessness paradigm, which is consistently associated with reduced secretion of corticosterone during escape testing. Development of helpless behavior and loss of spine synapses in the hippocampus, on the other hand, lead to a runaway stress response during the escape task. The context of our findings and current knowledge suggests that the protective actions of these three interventions most likely hinge upon their capabilities to mitigate stress. As we mentioned above, however, the possibility can not be excluded that the protective actions are, at least partly, independent of the stress-mitigating effect. Considering their distinct pharmacological properties, it is even conceivable that each intervention requires unique mediators to achieve the same protective outcome, calling for extensive studies in the future to ascertain the underlying mechanisms.

Stress resilience and hippocampal synaptoprotection may be critical factors in determining the outcome of antidepressant therapy, and as such, they could be highly relevant to the major clinical problem of antidepressant resistance. The importance of stress resilience is demonstrated by clinical and preclinical studies reporting that persistent hyper-responsiveness of the hypothalamic–pituitary–adrenal axis is associated with antidepressant resistance and recurring depression (Heuser et al., 1996; Appelhof et al., 2006; Khemissi et al., 2014), while curbing the stress system seems to improve the antidepressant response (Barden et al., 1995; Jahn et al., 2004; Nemeroff and Owens, 2004; Rogoz et al., 2005). A principal objective of the present study was highlighting the significance of hippocampal synaptoprotection in antidepressant therapy as a potential mediator of stress resilience. Although earlier work shows that there is no antidepressant response in the absence of synaptogenic power (Sherman et al., 1982; Li et al., 2010), our fluoxetine experiment demonstrates that even a strong hippocampal synaptogenic power may not always be sufficient, as protecting the newly-generated synapses may be likewise required for a *sustained* antidepressant response. As a result, synaptoprotective mechanisms may provide useful guidance for future studies to establish a causal relationship between synaptoprotection and stress resilience and to better understand the mechanisms of antidepressant therapy.

DECLARATIONS OF INTEREST

None.

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Orsolya Huzian: Investigation, Writing - original draft, Funding acquisition. **Judith Baka:** Investigation. **Eszter Csakvari:** Investigation. **Nikoletta Dobos:** Investigation. **Csaba Leranth:** Investigation, Writing - review & editing. **Laszlo Siklos:** Investigation, Writing - review & editing, Funding acquisition. **Ronald S. Duman:** Conceptualization, Writing - review & editing. **Tamas Farkas:** Conceptualization, Methodology, Supervision, Investigation, Formal analysis, Writing - original draft. **Tibor Hajszan:** Conceptualization, Methodology, Supervision, Investigation, Formal analysis, Writing - review & editing, Visualization, Funding acquisition.

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