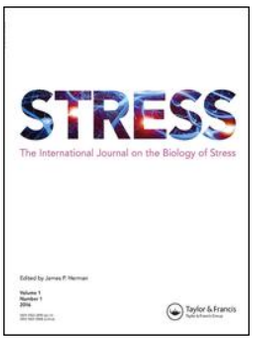

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ORIGINAL RESEARCH REPORT

Differential effects of tianeptine on the dorsal hippocampal volume of rats submitted to maternal separation followed by chronic unpredictable stress in adulthood

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ABSTRACT

Early maternal separation (MS) may produce lasting effects in the dorsal hippocampus (DH) that can change its response to chronic stress in adulthood. Chronic stress affects DH morphology and function, but tianeptine (an anti-depressant) can reverse the stress-induced morphological impairments. Morphologic alterations of hippocampus can affect contextual memory. Therefore, we evaluated the effect of tianeptine in MS and chronically stressed rats on: 1) volume of the DH and its areas using stereology and 2) hippocampal-dependent memory using a fear conditioning test. Male Wistar rats were subjected to daily MS for 4.5 h between postnatal days (PND) 1–21, or to animal facility rearing (AFR). Between (PND) days 50 and 74, rats were exposed to chronic unpredictable stress and were treated daily with tianeptine (10 mg/kg) or vehicle, providing eight groups: AFR-unstressed/vehicle ($n = 5$ for stereology, $n = 18$ for fear conditioning test); AFR unstressed/tianeptine ($n = 6$ and $n = 10$); AFR-chronic stress/vehicle ($n = 6$ and $n = 14$); AFR-chronic stress/tianeptine ($n = 6$ and $n = 10$), MS-unstressed/vehicle ($n = 5$ and $n = 19$), MS-unstressed/tianeptine ($n = 6$ and $n = 10$), MS-chronic stress/vehicle ($n = 6$ and $n = 18$), and MS-chronic stress/tianeptine ($n = 6$ and $n = 10$). MS-chronic stress/tianeptine rats showed a diminished CA1 area than the corresponding MS-unstressed/tianeptine rats. The combination of stressors produced a freezing response similar to those of the control group during postconditioning. During retrieval, MS led to a diminished freezing response compared to the AFR-unstressed groups. Tianeptine had no effect on freezing behavior. Our results show that tianeptine can affect the CA1 area volume differently depending on the nature and quantity of stressors but cannot alter freezing to context.

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Introduction

There is substantial literature on how stress in early life can have mainly adverse neurobiological consequences that last into adulthood (de Kloet et al., 2014; Nishi et al., 2014; Sapolsky, 2015). In rodents, maternal separation (MS) or deprivation has been consistently used as models of exposure to early life stress events (Gutman & Nemeroff, 2002; Levine, 2005; Schmidt, 2010). The hypothalamic–pituitary–adrenal (HPA) axis of rats exhibits a particular maturational profile known as the stress hyporesponsive period (SHRP). The SHRP extends from PNDs 4 to 14 and consists of a period of low activity of the adrenocortical system and refractory responsiveness to stressors that would normally induce a robust stress response in adult animals (Faturi et al., 2010). However, the reduced HPA axis responsiveness observed during this period is not absolute, and can be exceeded by sufficiently potent stressors, including maternal deprivation (Gutman & Nemeroff, 2002). When maternally deprived or separated rodents are tested as adults, they display a heightened stress response (Gutman & Nemeroff, 2002; Suárez et al., 2002) that may induce persistent alterations in brain function, including cellular (Hulshof et al., 2011; Roque et al., 2016), neurochemical (Della et al., 2013; Pinheiro et al., 2015), and behavioral alterations (Pinheiro et al., 2015; Trujillo et al., 2016; Vivinetto et al., 2013).

During adulthood and as a result of chronic social or physical stressors, brain neural architecture is remodeled: dendritic processes expand or retract (depending on the brain region) (Tata & Anderson, 2010; Vyas et al., 2002), the shape of dendritic spines is modified (Conrad et al., 2012; Maras & Baram, 2012), and neurogenesis is inhibited in the dentate gyrus of the hippocampus (Czeh et al., 2001; McEwen, 1999). These transformations may be a sign of successful adaptation, whereas persistence of these changes when stress ends indicates failed resilience (McEwen et al., 2015). Chronic stress paradigms in adult animals recapitulate many of the core neurobiological and behavioral characteristics of depression and are responsive to antidepressant treatment (Willner, 2005; Willner & Mitchell, 2002).

When combined, MS at an early age and chronic stress in adulthood may lead to two possible outcomes: either the effects of stress exposure during a lifetime are cumulative and increase the likelihood of developing a disease

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(Choy et al., 2008; Llorente et al., 2011), or the aversive experiences early in life trigger adaptive processes, thereby rendering an individual better adapted to aversive challenges later in life (Nederhof, 2012; Schmidt, 2010). The first outcome is known as the "cumulative stress" or "two hit" hypothesis, while the second is known as the "mismatch hypothesis" (Nederhof, 2012).

As mentioned above, the hippocampus is one of the structures strongly affected by chronic stress. As a result of stress-induced morphological modifications, hippocampal volume shrinks both in depressed patients (Sheline et al., 1996) and in chronically stressed rats (Liu et al., 2011). This volume reduction may be more closely tied to a reduction in neuropil volume, which contains primarily dendrites and axons and a small proportion of glial processes (Tata & Anderson, 2010). Particularly, in animal studies, the antidepressant tianeptine ameliorates stress-induced morphological sequelae in the hippocampus, enhancing dendritic remodeling (Czeh et al., 2001; Magarinos et al., 1999; Watanabe et al., 1992) and increasing neurogenesis (Czeh et al., 2001; Kuipers et al., 2013).

The functional significance of the morphological changes will depend on the region of the hippocampus, since although the internal circuitry of the hippocampus is regular along its septo-temporal axis, the extrinsic connectivity is different for the dorsal and ventral sub-regions (Moser et al., 1993; Swanson & Cowan, 1977). Functionally, this is reflected in dissociation between the effects of selective fiber-sparing dorsal and ventral hippocampal lesions. Dorsal lesions impair performance across a wide range of spatial memory tasks. Particularly, two groups (Kim & Fanselow, 1992; Phillips & LeDoux, 1992) confirmed that dorsal hippocampal lesions made after tone–shock conditioning interfere with the display of fear to the context but not to the tone. In contrast, ventral hippocampal lesions have been found to reduce anxiety on a number of unconditioned tests including the elevated place maze (Bannerman et al., 2003). When a chronic stress protocol is applied that is unpredictable, it can also impair contextual or spatial reference memory assessed through aversive learning (Das et al., 2005; Ricon et al., 2012; Zalosnik et al., 2014).

We based the present study on the following previous findings: (1) MS has enduring neurobiological consequences for stress responses; (2) hippocampal volume is reduced in chronically stressed rats; (3) tianeptine reverses the stress-induced reduction of hippocampal volume; and (4) the stress-induced reduction of hippocampal volume is associated with stress-induced deficits in spatial and contextual memory. Hence, we hypothesized that administering tianeptine to adult male rats that were maternally separated in early life and/or chronically stressed in adulthood will prevent the decrease of dorsal hippocampal volume and associated deficits in contextual fear conditioning.

Methods

Animals

All rat handling and experimental procedures were approved by the Animal Care and Use Committee of the National University of Córdoba, in accordance with the NIH Guide on Care and Use of Laboratory Animals.

Wistar-derived rats were bred and reared in our colony. Rats were housed in a temperature-controlled room (21 ± 1 °C) under artificial illumination (12 h:12 h light/dark schedule; lights on at 07:00 h). Except when required by the stress paradigm the rats had ad libitum access to food (standard lab chow) and tap water. Two non-related females were mated with a male and they remained together until there was physical evidence of pregnancy. After that the male was removed, the females were housed together until around gestation day 15 when they were put in separate cages. The day of birth was designated as postnatal day (PND) 0. On PND 1 litters were culled, from an average of 12 pups per litter, to 10 pups per dam (4–5 males, 5–6 females). Whole litters were randomly assigned to one of two rearing conditions: MS or standard animal facility rearing (AFR).

Early maternal separation

The MS procedure was based on a previously standardized protocol. For MS litters, rats were separated from the mother for 4.5 h every day from PND1 to PND 21 (Ogawa et al., 1994) (Figure 1). Separation consisted of removing the dam from the home cage and placing it alone into an adjacent cage while the litter was kept together in the nest. After the separation period, the dam was returned to the home cage. Separations were carried out between 09:00 h and 13:30 h. In AFR litters, pups remained with the dams undisturbed until weaning age at PND 22, except for routine cage cleaning twice a week.

Post-weaning housing conditions

From PND 22 until PND 49, male rats were selected and housed in standard cages in groups of four. They were handled daily by the same researcher to minimize stress reactions to manipulation at the time of treatment application. At PND 49, male offspring from both rearing conditions (MS and AFR) were randomly subdivided into four treatments, non-stressed with vehicle or with tianeptine, and unpredictable chronic stress with vehicle or with tianeptine (Figure 1), yielding eight experimental groups: 1 – AFR-unstressed/vehicle (control) ($n = 5$ for stereology, $n = 18$ for fear conditioning test); 2 – AFR-unstressed/tianeptine ($n = 6$ for stereology, $n = 10$ for fear conditioning test); 3 – AFR-chronic stress/vehicle ($n = 6$ for stereology, $n = 14$ for fear conditioning test); 4 – AFR-chronic stress/tianeptine ($n = 6$ for stereology,

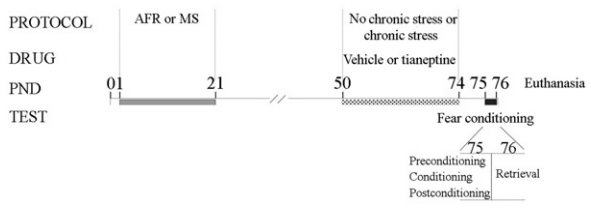

Figure 1. Timeline diagram of the stress protocols, tianeptine administration, and the fear conditioning test performed. AFR: standard animal facility rearing; MS: maternal separation; PND: postnatal day.

$n = 10$ for fear conditioning test), 5 – MS-unstressed/vehicle ($n = 5$ for stereology, $n = 19$ for fear conditioning test), 6 – MS-unstressed/tianeptine ($n = 6$ for stereology, $n = 10$ for fear conditioning test), 7 – MS-chronic stress/vehicle ($n = 6$ for stereology, $n = 18$ for fear conditioning test), and 8 – MS-chronic stress/tianeptine ($n = 6$ for stereology, $n = 10$ for fear conditioning test) . To control for litter effects, each experimental group was made up of rats from different litters (one rat per litter per condition). Male offspring were housed in standard cages in groups of four until the end of the experimental period.

We thus used a factorial ($2 \times 2 \times 2$) experimental design, with separation (AFR or MS), stress (nonstressed or chronically stressed), and drug (vehicle or tianeptine) as between-subjects factors.

Unpredictable chronic stress

At PND 50, rats in the stressed groups were exposed to an unpredictable 24-day chronic stress paradigm (Table 1): 4 h of noise produced by an alarm bell (85 dB; 2.5 Hz) (randomly applied 6 times during stress protocol); loss of consciousness by ether anesthesia and subsequent ether exposure for 2 min (5 times); two intraperitoneal (i.p.) injections of 0.5 ml isotonic (0.9%) saline solution at 4 h intervals (4 times); restraint for 1 h by placement inside a 6-cm-diameter metal grid cylinder (4 times); and food deprivation for 24 h (3 times). There were also days without stress (3 times) (Suárez et al., 1999; Trujillo et al., 2016). Only one stressor was applied per day. To maximize the unpredictability, the stressors were applied in random order and at varying times during the light phase, except on day 24 when noise was used as the last stressor. All rats received the same sequence of stressors in each replication of the experiments. The unstressed control group was not exposed to any stressor and these rats remained in their home cages until euthanasia for brain processing. The stress paradigm used does not affect body weight (Suárez et al., 1996).

Table 1. Chronic stress model.

Day	Stressor	Hour
1	Ether anesthesia	16:30 h
2	Two 0.9% saline injections	10:00 and 14:00 h
3	Restraint	11:30–12:30 h
4	Noise	12:00–16:00 h
5	Fasting	For 24 h
6	Rest day	–
7	Ether anesthesia	12:00 h
8	Noise	13:00–17:00 h
9	Restraint	8:30–9:30 h
10	Ether anesthesia	16:30 h
11	Noise	10:00–14:00 h
12	Fasting	For 24 h
13	Rest day	–
14	Immobilization	11:30–12:30 h
15	Noise	13:00–17:00 h
16	Two saline injections	9:30 and 13:30 h
17	Ether anesthesia	15:00 h
18	Noise	8:30–12:30h
19	Fasting	For 24 h
20	Rest day	–
21	Ether anesthesia	12:00 h
22	Two saline injections	10:00 and 14:00 h
23	Restraint	16:00–17:00 h
24	Noise	8:00–12:00 h

Rats were subjected to the various stressors for 24 days.

Tianeptine and vehicle administration

Starting at PND 50 rats were treated daily during 24 days with either tianeptine (commercial name: Stablon; from Servier laboratories of France) or vehicle according to the designated group (Figure 1). Tianeptine (10 mg/kg) was prepared diluted in 0.9% NaCl solution (vehicle). Both tianeptine and vehicle were administered i.p. in an end volume of 0.5 ml, between 12:00 h and 13:00 h.

Fear conditioning test

Twenty-four hours after the last stress session (PND 75), each rat was tested for hippocampus-dependent contextual memory by a fear conditioning test (Rudy et al., 2002). During the first day, each rat was left in the box for 3 min, allowing it to explore freely and form a hippocampal representation of the context (preconditioning) (Rudy et al., 2002). Next, four electric shocks were applied (intensity 0.41 mA for 2 s) separated by intervals of 64 s (conditioning). Finally, the rat was left in the box for 3 min with no electrical stimulus (postconditioning). On the next day, the rats were reexposed to the same environment as on the first day for 8 min, without applying the electric shocks (retrieval) (Figure 1). The percentage of freezing to context was calculated each day during preconditioning, conditioning (discarding the first 10 s of the inter-trial period of unconditioned response to the electrical stimulus) and postconditioning (day 1), and during retrieval (day 2). All testing took place between 09:00 h and 12:00 h, and the assessor was blind to the experimental condition of each rat.

Stereology

At PN 77, each rat was deeply anesthetized with chloral hydrate (540 mg/kg, 6%, i.p.) and transcardially perfused with heparinized 0.9% saline followed by a 4% paraformaldehyde phosphate buffered saline (PBS) solution. Brains were removed and left overnight in 4% paraformaldehyde–PBS solution and then stored at 4° C in 20% sucrose-PBS solution until sectioning.

Serial bilateral coronal sections (40 μm thick) were subsequently obtained from the dorsal hippocampus (DH) (from Bregma −1.72 mm to Bregma −3.84 mm) using a freezing microtome. The starting point for slicing was set at Bregma −1.72 mm (Paxinos & Watson, 2007). The first appearance of the ventral hippocampus (Bregma −3.84 mm) was considered the caudal limit of the DH (Slomianka & West, 2005). In each rat, the complete rostro-caudal set of sections through the dorsal hippocampus was sequentially ordered into multidish wells using five series. Using a random starting point, all the sections in a series were mounted onto glass slides and Nissl-stained. At least 9–11 sections through the entire length of the dorsal hippocampus were analyzed per rat.

The volumes of the hippocampal formation were estimated on the basis of the Cavalieri principle (Gundersen et al., 1988).

As field CA2 is not identifiable in Nissl-stained sections, this area was analyzed together with CA3 (Tata & Anderson, 2010). For CA1 and CA2/CA3, pyramidal cell layer volumes were measured. Apical dendrites from pyramidal cells that project through the strata lucidum, radiatum, and lacunosum-moleculare were considered as the apical layer and the dendrites that project into the stratum oriens as the basal layer (Tata & Anderson, 2010) (Figure 2). Thus, the volumes that were measured were: 1) the total dorsal hippocampus (DH); 2) the total areas CA1, CA2/3, and dentate gyrus; 3) the apical, pyramidal, and basal layers of CA1 and CA2/3 areas; and the granular, molecular, and polymorphic layers of dentate gyrus. The coefficient of error (CE) of the individual estimates was calculated according to Slomianka and West (2005) and in all cases it was <0.5.

Statistical analysis

To test whether hippocampal volume variation was correlated with body weight, group differences were determined by two-way analyses of covariance, with rearing condition and stress as factors and body weight as a covariate. As covariance was not significant in any group, a three-way ANOVA with maternal separation (AFR or MS), chronic stress (nonstressed or chronic stress), and drug (vehicle or tianeptine) as factors was used for statistical comparisons. For the percentages of freezing assessed during the fear conditioning test, a three-way ANOVA with the same factors and levels was also performed. Data are presented as mean ± standard error of the mean (SEM). The Tukey's post hoc test was performed for further examination of group differences. Significance was set at $p \leq .05$. When a three-way ANOVA was significant, the data were split by MS factor, and then separate two-way ANOVAs were performed on the split data. All analyses were conducted by using Infostat software (www.infostat.com.ar).

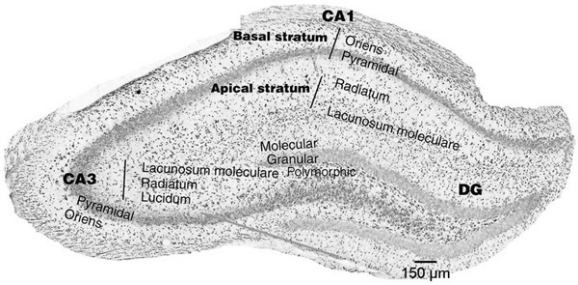

Figure 2. Cytoarchitecture of the dorsal hippocampus. Coronal photomicrograph which shows its areas and layers. The CA areas contain the pyramidal cell layers, an apical portion (which includes the stratum lucidum, radiatum, and lacunosum-moleculare), and basal portion (includes the stratum oriens). In dentate gyrus (DG), granular, molecular, and polymorphic strata are identified.

Results

Stereology

DH, CA1, CA2/3, and dentate gyrus volumes

A significant triple interaction was found between the effects produced by MS, chronic stress in adulthood, and drug on volumes of the DH and CA1, CA2/3, and dentate gyrus areas (DH: $F_{1,38} = 5.52$, $p = .02$; CA1: $F_{1,38} = 4.03$, $p = .05$; CA2/CA3: $F_{1,38} = 5.59$, $p = .02$; dentate gyrus: $F_{1,38} = 4.63$; $p = .04$). As the triple interaction was significant, a two-way ANOVA with the split data of the AFR and maternally separated groups was performed. In the AFR groups, the two-way ANOVA revealed no interaction or main effect produced by chronic stress or drug on dorsal hipocampal volume or its areas (Table 2). In the maternally separated groups, the two-way ANOVA revealed a significant double interaction between the effects produced by chronic stress and drug on volumes of the DH, CA1, and dentate gyrus areas (DH: $F_{1,19} = 4.66$, $p = .04$; CA1: $F_{1,19} = 4.42$, $p = .05$; dentate gyrus: $F_{1,19} = 4.87$; $p = .04$) (Table 2). Post hoc analysis showed that in MS-chronic stress/tianeptine rats the CA1 area decreased significantly compared with MS-unstressed/tianeptine rats ($p \leq .05$) (Figures 3 and 4).

Layers from CA1, CA2/3, and dentate gyrus

A significant triple interaction was found between the factors (MS, chronic stress, and drug) on volumes of apical layers of the CA areas (CA1: $F_{1,38} = 5.38$, $p = .03$; CA2/CA3: $F_{1,38} = 6.54$,

Table 2. Results of two-way ANOVA for the volumes of the dorsal hippocampus and its areas and layers.

	Chronic stress (df 1,19)		Drug (df 1,19)		Interaction (df 1,19)	
	F	*p*	*F*	*p*	*F*	*p*
Animal facility reared						
Dorsal hippocampus	1.25	.28	1.40	.25	1.48	.24
CA1 area	1.77	.20	0.02	.90	0.86	.37
Apical stratum	3.46	.08	0.09	.76	1.36	.26
CA2/3 area	1.09	.31	4.35	.05	2.89	.11
Apical stratum	3.67	.07	1.79	.20	1.91	.18
Dentate gyrus	0.56	.47	2.04	.17	0.76	.39
Maternal Separated						
Dorsal hippocampus	2.79	.11	0.21	.65	4.66	.04
CA1 area	4.96	.04	0.02	.89	4.42	.05
Apical stratum	5.85	.03	0.13	.72	5.72	.03
CA2/3 area	1.78	.20	0.42	.52	2.96	.10
Apical stratum	2.03	.17	0.01	.91	5.55	.03
Dentate gyrus	1.14	.30	0.51	.48	4.87	.04

F and *p* values corresponding to main effects (rearing condition and stress) and their interaction for hippocampal volumes from all eight experimental groups. df: degrees of freedom.

$p = .01$). As the triple interaction was significant, a two-way ANOVA with the split data of AFR and maternally separated groups was performed. In the AFR groups, the two-way ANOVA revealed no interaction or main effect produced by chronic stress or drug on apical layers of the CA areas (Table 2). In the maternally separated groups, the two-way ANOVA revealed a significant double interaction between the effects produced by chronic stress and drug on volumes of the apical layers of CA1 and the CA2/3 areas (CA1: $F_{1,19} = 5.72$, $p = .03$; CA2/CA3: $F_{1,19} = 5.55$, $p = .03$) (Table 2). Post hoc analysis showed that in the MS-chronic stress/tianeptine rats the total volume of the apical layers of the CA1 area decreased significantly compared with the MS-unstressed/tianeptine rats ($p \leq .05$) (Figure 5).

Fear conditioning test

No experimental group showed freezing behavior during the preconditioning. All rats showed exploratory behavior, indicating the absence of alterations by the treatments in their ability to explore the context.

During the conditioning stage, when the series of shocks were given to the rats, for freezing behavior we found no interactions (MS × chronic stress: $F_{1,102} = 3.48$; $p = .07$; MS × drug: $F_{1,102} = 0.09$; $p = .77$; chronic stress × drug: $F_{1,102} = 0.02$; $p = .90$; MS × chronic stress × drug: $F_{1,102} = 0.001$; $p = .97$) or main effects (MS: $F_{1,102} = 0.79$; $p = .38$; chronic stress: $F_{1,102} = 0.07$; $p = .80$; drug: $F_{1,102} = 1.7$; $p = .20$). Hence, all rats showed similar freezing responses to context (Figure 6(A)).

During the postconditioning stage, there was a MS × chronic stress interaction ($F_{1,102} = 6.37$; $p = .01$) on freezing behavior. Post hoc analysis revealed that MS-chronically stressed rats showed a greater freezing response compared with AFR-chronically stressed rats ($p \leq .05$). A significant main effect of MS ($F_{1,102} = 4.05$; $p = .05$) was found on freezing to context (Figure 6(B)).

Twenty-four hours later, during retrieval, a significant MS × chronic stress interaction ($F_{1,102} = 5.11$; $p = .03$) was found on freezing behavior. Post hoc analysis revealed that MS-unstressed rats showed a lower freezing response than AFR-control rats ($p \leq .05$) (Figure 6(C)).

No effects of tianeptine were found in any of the stages of the fear conditioning test, so it could not reverse the changes observed after MS or chronic stress protocols in freezing to context.

Discussion

To study the effects of tianeptine in an animal model of MS followed by subsequent unpredictable adult chronic stress,

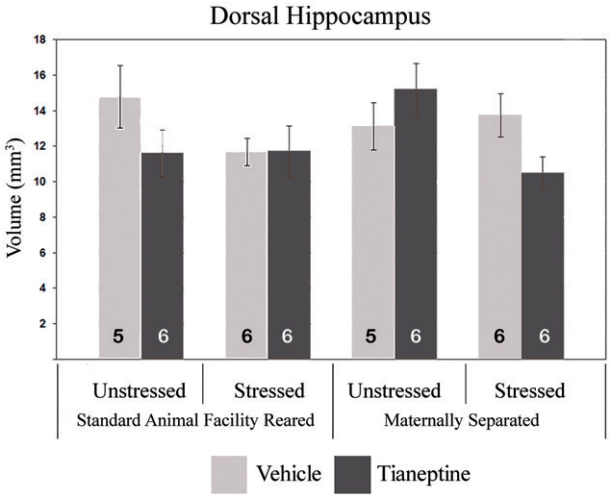

Figure 3. Effect of tianeptine on the volume of the dorsal hippocampus in rats exposed to early maternal separation and chronic variable stress in adulthood. Values are mean ± SEM for standard animal facility rearing (AFR) and maternally separated (MS) rats submitted to chronic stress or unstressed under tianeptine (dark gray bars) or vehicle (light gray bars) treatment. The number of rats per group is included inside each bar. ANOVA revealed a significant maternal separation × chronic stress × drug interaction ($p < .05$).

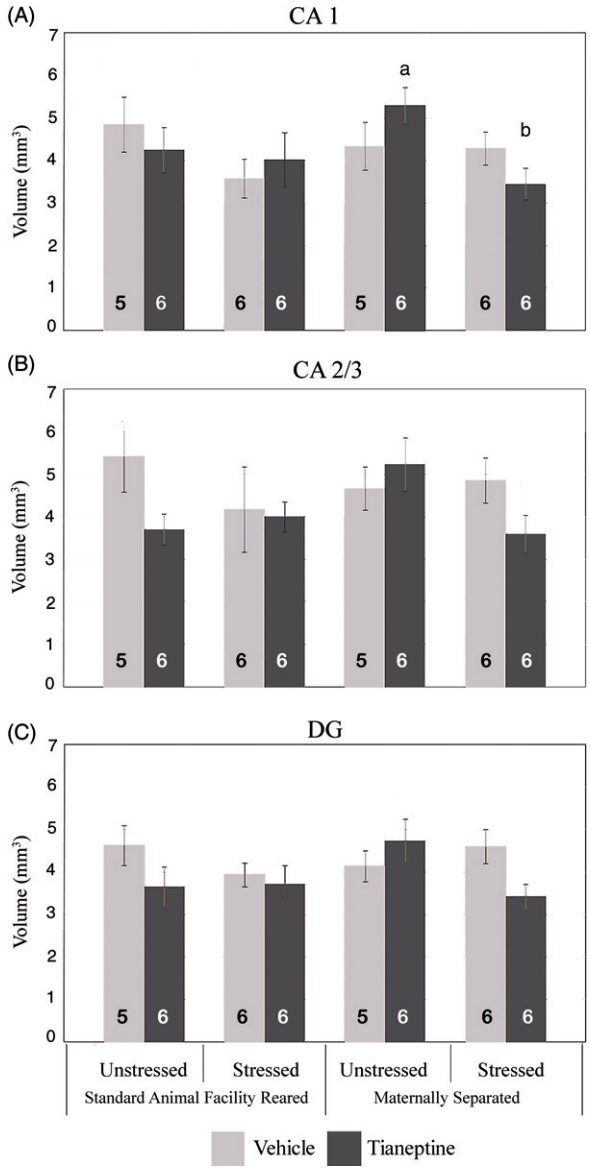

Figure 4. Effect of tianeptine on the volumes of hippocampal regions in rats exposed to early maternal separation and chronic variable stress in adulthood. (A). CA1, (B) CA2/CA3, (C) dentate gyrus (DG). Values are mean ± SEM of standard animal facility rearing (AFR) and maternally separated (MS) rats submitted to chronic stress or unstressed under tianeptine (dark gray bars) or vehicle (light gray bars) treatment. The number of rats for each treatment is included inside each bar. ANOVA revealed a significant maternal separation × chronic stress × drug interaction. Different letters indicate significant differences between groups ($p \leq .05$), Tukey's post hoc test.

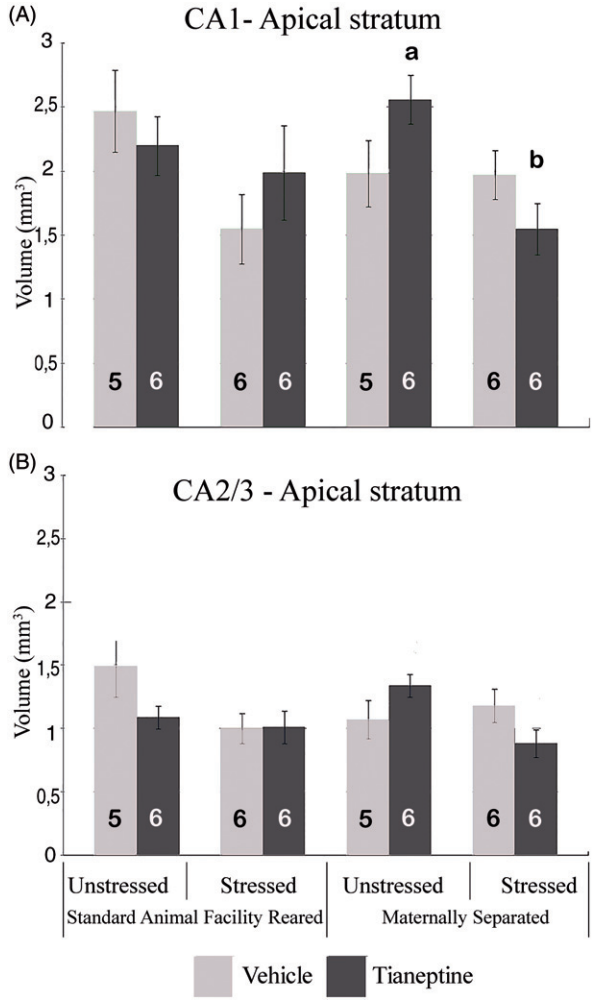

Figure 5. Effect of tianeptine on the volumes of apical layers of CA areas in rats exposed to early maternal separation and chronic variable stress in adulthood. (A) CA1 apical layer, (B) CA2/3 apical layer. Values are mean ± SEM of standard animal facility rearing (AFR) and maternally separated (MS) rats submitted to chronic stress or unstressed under tianeptine (dark gray bars) or vehicle (light gray bars) treatment. The number of rats for each treatment is included inside each bar. ANOVA revealed a significant maternal separation × chronic stress × drug interaction. Different letters indicate significant differences between groups ($p \leq .05$), Tukey's post hoc test.

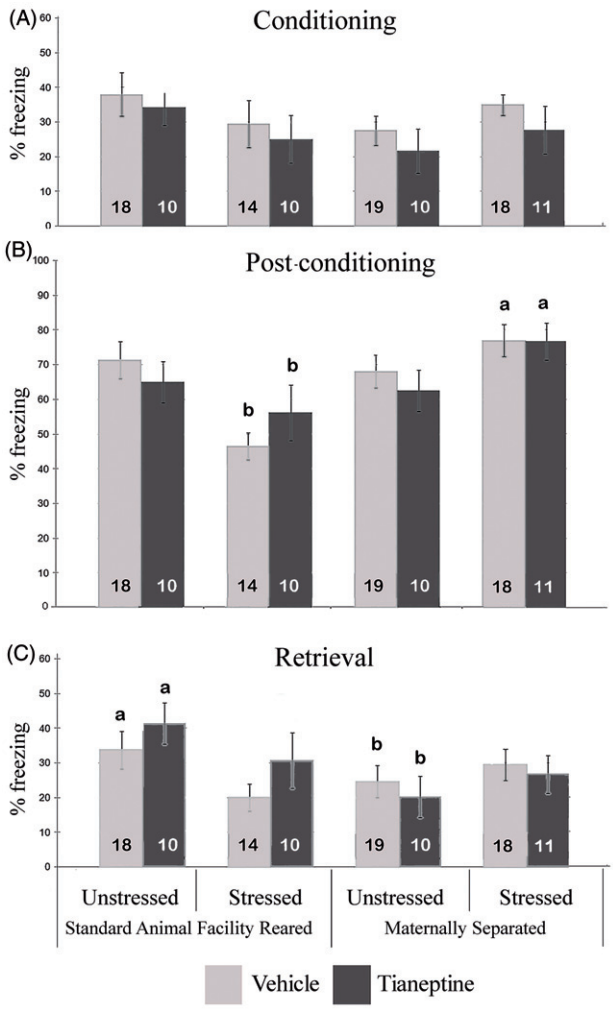

Figure 6. Effect of tianeptine on the freezing response in a fear conditioning test in rats exposed to early maternal separation and chronic variable stress in adulthood. Percentage of time spent freezing is shown during (A) conditioning, (B) postconditioning, and (C) retrieval. Values are mean ± SEM of standard animal facility rearing (AFR) and maternally separated (MS) rats submitted to chronic stress or unstressed under tianeptine (dark gray bars) or vehicle (light gray bars) treatment. The number of rats for each treatment is included inside each bar. ANOVA revealed a significant maternal separation × chronic stress interaction. Different letters indicate significant differences between groups ($p \leq .05$), Tukey's post hoc test.

we determined the volume of the DH, CA1, CA2/3, and dentate gyrus, and hippocampal-dependent memory in adulthood.

We found a triple interaction between MS, chronic stress, and drug on the volumes of DH, CA1, CA2/3, dentate gyrus, apical layer of CA1, and apical layer of CA2/3. The difference in these volumes between tianeptine-treated rats and vehicle-treated rats was greater in maternally separated rats compared to the AFR groups. In the MS-chronic stress group, tianeptine tended to reduce the volume of the DH, CA1, and dentate gyrus areas relative to the vehicle-treated group, while in MS-unstressed rats tianeptine tended to increase the volume of the DH, CA1, and dentate gyrus relative to the vehicle-treated group. Similar results were found in the apical layers of the CA1 and CA2/3 areas. The principal neuronal cell type of the hippocampus is the pyramidal cell, constituting the vast majority of neurons in the pyramidal cell layer. Pyramidal cells have a basal dendritic tree and an apical dendritic tree (Paxinos, 2004). As the decrements that we report were in the apical layers of CA1 and CA2 areas, tianeptine might have affected the patterns of dendritic remodeling in these regions in a different fashion depending on the type and quantity of stressors applied.

In MS-unstressed rats, the tendency of tianeptine to increase the volume of the DH is what we expected of the drug, based on previous reports (Czeh et al., 2001; Liu et al., 2011; Lucassen et al., 2004). There are at least two possible explanations for the actions of tianeptine in MS-unstressed rats. First, tianeptine has been shown to normalize stress-induced alterations in glutamate function (McEwen & Chattarji, 2004); second, tianeptine has been shown to reverse the stress-induced decrease in expression of nerve growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Alfonso et al., 2006). In adult MS rats, the levels of several glutamate receptors are lower relative to controls (O'Connor et al., 2013; Pickering et al., 2006). The reduction in receptor levels may lead to a

reduction in negative feedback, which regulates glutamate release, so potentially leading to excessive glutamate release and resultant hyper-excitability, and in turn causing debranching of apical dendrites in the hippocampus (McEwen & Chattarji, 2004). Hence, tianeptine could act by normalizing the alterations induced in glutamate function (McEwen & Chattarji, 2004). Alternatively, as the differentiation and survival of hippocampal neurons *in vitro* are responsive to the action of factors including BDNF (Cheng & Mattson, 1994) and NGF (Frielingsdorf et al., 2007), a second hypothesis is that tianeptine might normalize the levels of these factors (among others) in MS rats. However, Della et al. (2013) observed a significant decrease of NGF activity in the hippocampus of maternally deprived rats after the administration of tianeptine.

The combination of the two stressors (MS and chronic unpredictable stress) and the administration of tianeptine resulted in a significantly lower volume of the CA1 area and the CA1 apical layer than in the MS-unstressed/tianeptine group. The decreased volumes of the apical layers of CA1 found in the MS-chronic stress group after treatment with tianeptine differed from our expectations based on the properties of the antidepressant described above. This led us to an important question: Is the tianeptine-associated decreased volume in the specific apical layers a protective adaptive response to both stressors or a marker of vulnerability to damage? Indeed, in this study, the AFR-unstressed/tianeptine group displayed a similar DH volume to that of the MS-chronic stress/tianeptine group.

Given the uncertainty regarding the role of apical dendritic remodeling in tianeptine-treated rats, we investigated whether the structural alterations might be related with hippocampal-associated contextual memory. Employing a contextual fear memory test, we found this not to be the case.

Certain manipulations, like enriched environment or extensive training (Hutchinson et al., 2012; Vivinetto et al., 2013; Yau et al., 2011), can increase dendritic complexity in the hippocampus, which can influence function. Consequently, we expected that the positive influence that tianeptine exerted on the volume of apical dendrites in the CA areas of MS-unstressed rats would manifest in enhanced performance of these rats measured by the fear conditioning test. However, contrary to our expectations, the tianeptine-treated groups and the vehicle-treated groups had similar behavior. Particularly, the MS-unstressed groups showed less freezing response during retrieval compared to AFR-unstressed controls, indicating that freezing to context reported in the MS-unstressed/tianeptine group is independent of apical dendritic hippocampal volume. Many studies have demonstrated long-term spatial memory impairment in rats exposed to early life stress. Maternally separated rats showed significant impairment of acquisition in the Morris water maze task (Huot et al., 2002), and decreased mossy fiber density in the stratum oriens region of the hippocampus in adults but no change in volume of the dentate gyrus. MS can decrease the levels of neurotrophic factors in the hippocampus and amygdala (Aisa et al., 2009; Della et al., 2013). In particular, due to the highly protective properties of BDNF for different neuronal phenotypes, a reduction in the expression of hippocampal BDNF after MS may influence vulnerability to memory impairments later in life under challenging situations (Aisa et al., 2009). MS can also diminish the length of ventral hippocampal neurons (Monroy et al., 2010) involved in emotional responses such as fear conditioning (Hunsaker & Kesner, 2008; Zhang et al., 2014).

The hippocampus has two interrelated roles: supporting aspects of spatial and contextual memory and regulating HPA activity. Stimulating the hippocampus generally inhibits the HPA axis and destroying part of the hippocampus or its efferents enhances HPA axis activity. In this study, chronic unpredictable stress tended to decrease volume of apical layers of CA1 and CA2/3, which might have affected freezing to context, and also might have been associated with HPA deregulation. When the HPA axis is deregulated, glucocorticoids can exacerbate effects of stress on contextual memory (Conrad, 2005).

We reported a tendency to decreased volume of the CA1 and CA2/3 areas in the groups submitted to the chronic unpredictable stress protocol (AFR-chronic stress rats), so one hypothesis regarding the reduced freezing to context could be that the dorsal hippocampus was affected. Similarly, social isolation decreased the CA1 volume and the freezing response of female rats in a contextual fear conditioning test (Pereda-Perez et al., 2013). However, our data differ from some previous reports that have found increments in freezing behavior after chronic stress protocols (Conrad et al., 1999; Sandi et al., 2001). The differences in stress protocols may account for this disparity, particularly the unpredictability component which is not present in those previous protocols.

As regards the freezing response of rats submitted to the two stressors (MS-chronic stressed rats), during the postconditioning stage, they showed an increased freezing to context compared to AFR-chronic stress rats. This result may be attributable to the activation of the amygdala in tasks such as fear conditioning (Conrad et al., 2004). Other results from our laboratory showed an increase in medial amygdala activity, assessed by Fos immunocytochemistry, of MS-chronically stressed rats compared to AFR-chronically stressed rats (Trujillo et al., 2016). Chronic stress enhances dendritic arborization in the amygdala (Vyas et al., 2002). As the amygdala has a rich supply of glucocorticoid receptors, and glucocorticoids within this structure are important in regulating contextual conditioning (Conrad et al., 2004), amygdala neurons of MS-chronically stressed rats may be more sensitive to elevated levels of these hormones. Regarding the hippocampus, Trujillo et al. (2016) also reported that the MS-chronic stressed group had a decrease in glucocorticoid receptor expression in the hippocampus. Under these circumstances, the HPA axis could be deregulated, as in the AFR-chronic stress group. During emotionally arousing situations such as fear conditioning, we can hypothesize that both AFR-chronically stressed and MS-chronically stressed groups secreted more glucocorticoids than did AFR-controls (Eiland & McEwen, 2012), so the increased dendritic arborization of amygdala neurons in MS-chronically stressed rats may contribute to facilitation of freezing to context. This may mask any memory impairment produced by the hippocampus that is typically observed under non-emotionally arousing

conditions. Supporting this idea, Choy et al. (2008) employed a maternal deprivation rat model combined with chronic treatment of young adults with corticosterone, and reported impairments in a Y-maze, a contextual memory test without an aversive stimulus.

During retrieval, however, freezing to context of rats exposed to the two stressors in the present study would seem to be influenced by the impact of chronic unpredictable stress on the dorsal hippocampus. The MS-chronically stressed rats did not exhibit an increased freezing response compared to the AFR-chronic stress group, as in the postconditioning stage. In line with our previous explanation, as the retrieval phase was conducted in the absence of shocks, glucocorticoid secretion might be reduced compared to the conditioning stage, and hence the amygdala may be less susceptible to its effects and the hippocampus-dependent memory deficit may be unmasked in these rats.

Another interpretation of our results arises from the possible dissociation between the freezing response and learning to context. We did not assess freezing response in a different context from the context in which we conditioned the rats (Rudy et al., 2002), so the freezing behavior observed might not be closely related with contextual memory. According to this argument, the reduced freezing response obtained in the AFR-chronic stress and the MS-chronic stress groups during retrieval could be attributed to an increase in hyperactivity (Anagnostaras et al., 2001). Thus, their locomotor activity may have been enhanced due to chronic stress-induced morphological changes that affected the hippocampus. Hyperactivity may influence the freezing response, although it is not clear if the deficit in conditional freezing produced by hippocampal lesions is caused by lesion-induced increases in locomotor activity or if increased locomotor activity and decreased conditional freezing are symptomatic of a common syndrome, such as contextual learning deficit (Maren & Fanselow, 1997).

Conclusions

Here we reported that MS and unpredictable chronic stress separately had deleterious effects on freezing to context. We also reported that tianeptine affected the volume of the dorsal hippocampus differently when only MS was applied and when MS and chronic unpredictable stress were applied together. Contrary to our expectations, alterations in the volume of the dorsal hippocampus produced by the antidepressant were independent of freezing to context behaviors.

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