

# Effect of Brain-Derived Neurotrophic Factor Haploinsufficiency on Stress-Induced Remodeling of Hippocampal Neurons

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**ABSTRACT:** Chronic restraint stress (CRS) induces the remodeling (i.e., retraction and simplification) of the apical dendrites of hippocampal CA3 pyramidal neurons in rats, suggesting that intrahippocampal connectivity can be affected by a prolonged stressful challenge. Since the structural maintenance of neuronal dendritic arborizations and synaptic connectivity requires neurotrophic support, we investigated the potential role of brain derived neurotrophic factor (BDNF), a neurotrophin enriched in the hippocampus and released from neurons in an activity-dependent manner, as a mediator of the stress-induced dendritic remodeling. The analysis of Golgi-impregnated hippocampal sections revealed that wild type (WT) C57BL/6 male mice showed a similar CA3 apical dendritic remodeling in response to three weeks of CRS to that previously described for rats. Haploinsufficient BDNF mice (BDNF<sup>±</sup>) did not show such remodeling, but, even without CRS, they presented shorter and simplified CA3 apical dendritic arbors, like those observed in stressed WT mice. Furthermore, unstressed BDNF<sup>±</sup> mice showed a significant decrease in total hippocampal volume. The dendritic arborization of CA1 pyramidal neurons was not affected by CRS or genotype. However, only in WT mice, CRS induced changes in the density of dendritic spine shape subtypes in both CA1 and CA3 apical dendrites. These results suggest a complex role of BDNF in maintaining the dendritic and spine morphology of hippocampal neurons and the associated volume of the hippocampal formation. The inability of CRS to modify the dendritic structure of CA3 pyramidal neurons in BDNF<sup>±</sup> mice suggests an indirect, perhaps permissive, role of BDNF in mediating hippocampal dendritic remodeling. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** CA3; corticosterone; golgi staining; hippocampus; neurotrophins; stress

## INTRODUCTION

In the mammalian brain, the hippocampal formation is a critical component of the cortical-hippocampal circuitry, a network with complex information processing capabilities (Eichenbaum, 2000). The shape of hippocampal dendritic arbors, and the resulting pattern of afferent connections, can be dramatically affected by changes in environmental

cues and by how new information is being processed. For example, during extreme environmental conditions, hibernating species show a temporary synaptic disengagement of intrahippocampal connectivity, probably underlying the resulting unused navigational skills during deep torpor (Popov et al., 1992; Magariños et al., 2006; von der Ohe et al., 2006). Subtler, but equally significant, changes in the pattern of dendritic complexity can be observed in the hippocampus of experimental animals exposed to chronic stress. In rats and primitive primates, repeated chronic restraint stress (CRS) causes morphological rearrangements of the dentate gyrus-CA3 region (McEwen, 1999), a key intra hippocampal connection site for the processing of spatial information (Eichenbaum, 2000). Specifically, exposure to CRS and the administration of glucocorticoids cause, in rats, the reversible retraction and simplification of the distal apical dendritic arbors in CA3 pyramidal neurons (Watanabe et al., 1992; Magariños and McEwen, 1995; Baran et al., 2005; Conrad et al., 2007).

The structure of neurons and the maintenance of their dendritic arborizations and synaptic connectivity require neurotrophic support not only during development but also in adulthood (Huang and Reichardt 2001; Chao, 2003). Among the members of the neurotrophin family, brain derived neurotrophic factor (BDNF) is a growth factor enriched in the rodent hippocampus (Conner et al., 1997) that is released from neurons in an activity-dependent manner and modulates both neuronal morphology and synaptic plasticity (Chao, 2003). Although some reports described that chronic immobilization stress reduces hippocampal BDNF mRNA levels in the hippocampus of adult rats (Smith et al., 1995; Nibuya et al., 1999), other studies have found unchanged levels of the neurotrophin mRNA and protein levels after a less severe chronic restraint stress (CRS) challenge (Kuroda et al., 1998; Reagan et al., 2007) or a chronic variable stress (Isgor et al., 2004), suggesting that the intensity of the paradigm utilized can differentially affect the balance between synthesis and release of BDNF (Marmigere et al., 2003). Given these considerations and the acknowledged importance of BDNF in neuronal function and plasticity, we investigated the role of BDNF deficiency on the stress-induced hippocampal

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Grant sponsor: National Institute of Mental Health; Grant numbers: MH41256 to BMc, MH060478 to KGB, NS052019 to FSL.

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Accepted for publication 14 October 2009

DOI 10.1002/hipo.20744

Published online in Wiley InterScience (www.interscience.wiley.com).

dendritic remodeling and hypothesized that (1) the dendritic arborizations of hippocampal CA3 pyramidal neurons of haploinsufficient BDNF (BDNF<sup>±</sup>) mice would be more vulnerable to CRS due to their partial trophic support, showing inability to remodel in response to the chronic stress challenge, and that (2) the hippocampal volume would be reduced in BDNF<sup>±</sup> mice as a consequence of the diminished CA3 dendritic material.

We report that, similar to rats, stressed wild type mice show CA3 apical dendritic retraction and simplification that is not observed in CA1 pyramidal cells. Stress also alters the density of specific spine subtypes resulting in unchanged and decreased total spine density in CA3 and CA1 apical dendrites, respectively. These responses to chronic stress are absent in BDNF<sup>±</sup> mice, which also have a reduction in dendritic arborizations in CA3, but not in CA1, without prior stress. These results suggest that a deficiency in neurotrophic signaling could play a role in the lack of stress-induced dendritic plasticity in BDNF<sup>±</sup> mice.

## MATERIALS AND METHODS

### Experimental Animals

Wild type (WT) and haploinsufficient BDNF male mice (BDNF<sup>±</sup>, C57BL/6 background) between three and four months of age were provided by Regeneron, Tarrytown, NY. These mice were generated using homologous recombination targeting vectors in which the BDNF coding region was disrupted and deleted (Conover et al., 1995). Since mice homozygous for the BDNF mutation failed to survive beyond the second postnatal week, the experiments were performed using BDNF<sup>±</sup> mice. Animals were kept in temperature-controlled rooms and subjected to a 12/12 light/dark cycle with lights on at 7 am. All procedures were performed in accordance with the National Guidelines on the Care and Use of Animals and an animal study protocol approved by the Rockefeller University Animal Care and Use Committee.

### Experimental Design

Experiment 1 was planned to examine if WT C57BL/6 mice show a similar hippocampal dendritic remodeling in response to chronic restraint stress to that observed and already reported in rats (Watanabe, 1992; Magariños and McEwen, 1995). Mice were randomly assigned to either a control group ( $n = 6$ ) or a restraint stressed group ( $n = 6$ ). Controls were left undisturbed and stressed mice were subjected to 21 days of daily repeated restraint stress in their home cages (6 h/day, from 10 am to 4 pm) using flexible wire mesh restrainers of cylindrical shape (~15 cm in length and 4 cm in diameter). To assess the impact of a single stress session on BDNF protein levels, two additional groups of mice ( $n = 6$  per group) were used. Groups were as follows: a 6-h restraint stress and an unstressed control group. The same procedures as described below were used for

tissue collection, hippocampal lysis, protein estimation, and ELISA.

Experiment 2 was designed to investigate the consequences of chronic stress in the hippocampal dendritic organization and spine density of mice with a partial deficiency in BDNF. Animals were randomly assigned to four experimental groups: (1) Control wild type (WT) mice; (2) Control BDNF<sup>±</sup> mice were left undisturbed; (3) Restraint stressed WT mice; and (4) Restraint stressed BDNF<sup>±</sup> mice were subjected to 21 days of daily repeated restraint stress in their home cages (6 h/day, from 10 am to 4 pm) using wire mesh restrainers. The number of animals per experimental group was five for the dendritic branch point and length analysis and six for the spine density estimation (see details below).

At the end of the experimental period, on Day 22, all experimental mice were euthanized between 9 and 11 am. Animals were anesthetized and transcardially perfused with a fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4). Brains were removed from the skulls and stored in the fixative solution overnight at 4°C. Coronal sections (100 μm thick) through the dorsal hippocampus were cut on a Vibratome and washed in several changes of PB. Sections were processed for Golgi impregnation (see below). A second cohort of mice underwent rapid cervical dislocation, followed by decapitation and collection of trunk blood for corticosterone assays. Brains were removed from the skull on ice and multiple brain regions were dissected and flash frozen by placing into prechilled eppendorf tubes stored on dry ice for later processing.

### Golgi Staining Procedure

Sections were processed according to a modified version of the “single” section Golgi impregnation procedure (see Magariños et al., 2006 for details). Briefly, brain sections were incubated in 3% potassium dichromate in distilled water overnight. The sections were then rinsed in distilled water, mounted onto plain slides and a coverslip was glued over the sections at the four corners. These slide assemblies were incubated in 1.5% silver nitrate in distilled water overnight in the dark. The following day the slide assemblies were dismantled and the tissue sections were rinsed in distilled water and then dehydrated in 95% followed by absolute ethanol. The sections were then cleared in Xylenes (Fisher Scientific) mounted onto gelatinized slides and coverslipped under Permount (Fisher Scientific).

### Estimation of Dendritic Branching Points and Total Length

The Golgi method described in this study has been used in numerous, previous studies in our laboratory (see Introduction) and by others (Bessa et al., 2008) and have yielded consistent results on stress effects that have been corroborated by other methods, e.g., dye filling of neurons in the case of medial prefrontal cortex (Brown et al., 2005; Liston et al., 2006; Bessa et al., 2008). Slides containing brain sections were coded prior to quantitative analysis; the code was not broken until the anal-

ysis was complete. To be selected for analysis, Golgi impregnated neurons had to meet the following characteristics: (1) location in the CA3 or CA1 subregion of the dorsal hippocampus. For the CA3 subregion, the segment examined excluded the CA3 curvature and ended at the imaginary line connecting the ends of the dorsal and ventral blades of the dentate gyrus. This CA3 segment contains characteristic pyramidal neurons with well-defined apical and basal dendritic trees and is devoid of the atypical and more heterogeneous pyramidal cell types mainly observed between the blades of the dentate gyrus. Special care was taken to include the same number of pyramidal neuron subtypes across experimental animals and experimental groups; (2) dark and consistent impregnation throughout the extent of all of the dendrites; (3) relative isolation from neighboring impregnated cells that could interfere with analysis; and (4) a cell body in the middle third of the tissue section in order to avoid analysis of impregnated neurons which extended largely into other sections. For each brain, and therefore for each experimental animal, 14 to 16 pyramidal cells from the CA3 and CA1 subregions of the dorsal hippocampus were selected from both hemispheres. We were able to match neuronal subtypes across the hippocampi of five experimental animals so the final *n* per group was five. Each selected neuron was traced at a final magnification of 560 $\times$  using a Zeiss light microscope with a camera lucida drawing tube attachment. From these drawings the number of dendritic branch (bifurcation) points within a 100  $\mu$ m thick section of each dendritic tree was determined for each selected neuron. In addition, the length of the dendrites present in a 100  $\mu$ m thick section was determined for each dendritic tree using a Zeiss Interactive Digitizing Analysis System. Means were determined for the number of branch points and total dendritic length for each experimental animal and a final average was calculated per experimental group.

### Estimation of Spine Density

The analysis was performed on coded Golgi impregnated brain sections containing the dorsal hippocampus of six mice per experimental group and is based on a method used to demonstrate estrogen effects to increase the number of mushroom type spines in the mouse CA1 hippocampal pyramidal neurons (Li et al., 2004). This method does not assess spine density in a three dimensional manner, but focuses on spines that are parallel to the plane of section and thus underestimates the total number. Nevertheless, it does provide comparisons across treatment groups analyzed in the identical manner. The number of visible spines was counted on apical tertiary dendritic segments of CA1 and CA3 pyramidal neurons. On the basis of their shape, spines were classified in the following categories (Peters and Kaiserman-Abramof, 1970): (1) stubby, very short spines without a distinguishable neck and head, (2) thin, spines with a long neck and a clearly visible small head, and (3) mushroom, big spines with a well-defined neck and a very voluminous head. Other spine shapes considered immature or transitional forms, such as filopodia and branched spines, were

excluded from the analyses because they were rarely observed or, when detected, difficult to be precisely identified at the light microscopic level. All dendritic segments selected for spine density estimations were of similar diameter ( $\sim$ 1  $\mu$ m) to minimize the effect of hidden spines above or below the dendrites. Also, dendritic segments had to remain in a single plane of focus so that the length of the dendrite projected in 2D would approximate the 3D dendritic length. Dendritic segments were traced with the aid of a camera lucida drawing tube at 1,400 $\times$ , and the length of each segment was estimated from the tracings on a digitizing tablet (ZIDAS, Zeiss). Five tertiary dendritic segments, at least 15  $\mu$ m in length, were analyzed per neuron, and six neurons were analyzed per experimental brain. Spine counts were averaged and spine density was expressed as the number of total spines -or spine subtypes- per 10  $\mu$ m of dendritic length.

### Corticosterone Assay

Plasma levels of corticosterone were measured in samples taken from trunk blood collected at time of sacrifice, within 30 s after the animal removal from the home cage. Corticosterone levels were assayed using a commercially available radioimmunoassay kit (ICN Biomedicals, Irvine, CA). The limit of detection for the test was 25 ng/ml of corticosterone and the inter- and intra-assay variability was 7.2% and 7.1%, respectively.

### Hippocampal Volume Estimation

Using Stereo Investigator software (Microbrightfield, Vermont) the entire volume of the hippocampus was measured at 4 $\times$  objective magnification. Total hippocampal volume was chosen, as delineation of a "boundary" between dorsal and ventral hippocampus is difficult to reliably identify from brain to brain. As such, modest fluctuations in plane of section can significantly impact measurements of regional volume and thus obscure instead of enhance group differences. By inclusion of entire hippocampal volume, we have greater confidence in the accuracy of the measurements. The external capsule, alveus of hippocampus, and white matter were used as boundary landmarks. All sections throughout each hippocampus were traced and reconstructed.

### BDNF ELISA

The BDNF protein concentrations in hippocampal lysates were determined using the BDNF Emax immunoassay system with recombinant mature BDNF as a standard (Promega). This ELISA is based on an antibody to the carboxy terminal region of BDNF and can also recognize proBDNF. Total bilateral hippocampus was lysed with TNE Lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% NP-40) using a dounce homogenizer. Lysates were clarified by centrifugation at 4C (13000RPM). Clarified supernatant was collected and total protein concentrations were measured using the Bradford Method. For ELISA, lysates were diluted 1:1 in sample buffer

TABLE 1.

Effect of 21 Days of Repeated Chronic Restraint Stress on Organ Weights and Serum Corticosterone of Wild Type and Haploinsufficient BDNF Mice (BDNF<sup>±</sup>)

	Control		Restraint	
	Wild Type	BDNF <sup>±</sup>	Wild Type	BDNF <sup>±</sup>
Body weight (g)	44.25 ± 1.03	46.00 ± 2.00	30.44 ± 0.56*	30.22 ± 1.08*
Adrenal weight (mg/100 g)	10.92 ± 1.17	10.46 ± 0.93	15.60 ± 0.58*	16.26 ± 1.57*
Thymus weight (mg/100 g)	63.91 ± 6.61	75.30 ± 13.08	13.35 ± 4.37**	15.29 ± 4.78**
Corticosterone (ug/dl)	1.54 ± 0.33	1.37 ± 0.23	1.07 ± 0.25	1.36 ± 0.37

\* $P < 0.05$  and \*\* $P < 0.01$  compared with Control wild type and BDNF<sup>±</sup>, one-way ANOVA, Tukey post hoc test. Values represent the mean ± SEM.

(provided in Promega BDNF ELISA kit). BDNF ELISA is capable of detecting a minimum of 15.6 pg/ml of BDNF (Promega, BDNF Emax immunoassay system technical bulletin). All samples were run in triplicate on the same plate to eliminate any plate-to-plate (interassay) variability.

### Statistical Analysis

For dendritic branch points, dendritic length, spine density and BDNF protein levels, group differences were tested using one-way analysis of variance followed by Tukey HSD post hoc comparisons. Unpaired two-tailed Student's *t*-test was used to establish the statistical significance of the differences in the morphological values between control and restraint groups and the hippocampal volume values between wild type and BDNF<sup>±</sup> groups. A probability level of  $P < 0.05$  was used as the criterion for statistical significance.

## RESULTS

### CRS Induces the Remodeling of the Hippocampal Apical Arborizations of Male C57BL/6 Mice

At the end of the CRS paradigm, both wild type mice showed a significant body weight loss ( $F_{3,20} = 67.29$ ,  $P < 0.001$ ) and a significant thymic atrophy ( $F_{3,20} = 18.97$ ,  $P < 0.001$ ) and adrenal hypertrophy ( $F_{3,20} = 12.01$ ,  $P < 0.001$ ) compared with nonstressed mice, regardless of the genotype (Table 1). The morphological analysis of Golgi-stained hippocampal tissue in wild type animals revealed that C57BL/6 mice subjected to CRS showed a significant retraction and simplification in the CA3 apical dendritic arborizations compared with nonstressed controls ( $t_{10} = 2.51$ ,  $P < 0.05$  and  $t_{10} = 2.98$ ,  $P < 0.05$ , respectively, Fig. 1). However, the total length of basal dendrites, as well as the number of branch points of the basal arborizations, were not different between unstressed and stressed groups ( $t_{10} = 0.80$ ,  $P = 0.44$  and  $t_{10} = 1.37$ ,  $P = 0.20$ , respectively, Fig. 1). These changes recapitulate changes seen many times in the dendritic trees of CA3 neurons in rats

and tree shrews subjected to chronic stress (Watanabe et al., 1992; Magariños et al., 1995; Magariños et al., 1996; McEwen 1999; Baran et al., 2005; Conrad et al., 2007).

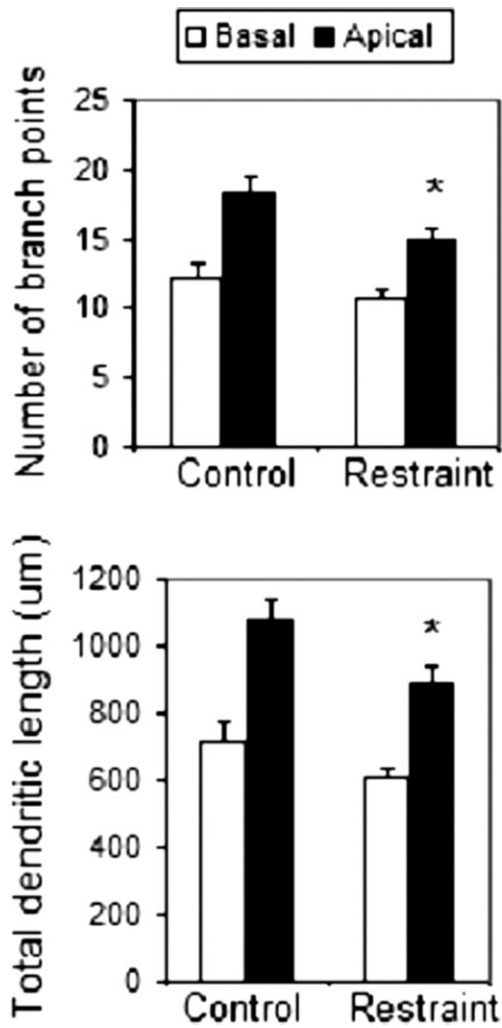
### BDNF<sup>±</sup> Mice Show Reduced Hippocampal Volume and Dendritic Branching and No Effect of CRS

We first set out to verify a morphological alteration reported in BDNF haploinsufficient mice: decreased hippocampal volume (Chen et al., 2006). Using the Cavalieri volume estimation, we detected a decrease in total hippocampal volume in BDNF<sup>±</sup> mice, as compared with WT mice (BDNF<sup>±</sup> =  $19.73 \pm 0.34$  mm<sup>3</sup> vs. WT =  $22.90 \pm 0.34$  mm<sup>3</sup>, Fig. 2; Student's *t*-test,  $P < 0.01$ ). To further investigate if the reduced hippocampal volume was associated with a decrease in dendritic material, we analyzed the morphology of CA1 and CA3 pyramidal neuron dendritic arbors in BDNF<sup>±</sup> and WT mice. Analysis of Golgi-stained sections containing the dorsal hippocampus showed that unstressed BDNF<sup>±</sup> mice presented a similar apical dendritic debranching to that observed in stressed WT, namely, reduced apical, but not basal, dendritic branching and length in CA3 pyramidal neurons ( $F_{3,16} = 5.48$ ,  $P < 0.01$ , Figs. 3 and 4). However, stressed BDNF<sup>±</sup> mice were unresponsive to the CRS challenge and no evidence of further decrease in the number of apical dendritic branch points was observed (Figs. 3 and 4). Similarly, BDNF<sup>±</sup> mice showed a significant decrease in total CA3 apical dendritic length compared with unstressed WT mice ( $F_{3,16} = 4.89$ ,  $P < 0.05$ ).

In contrast to CA3 dendritic debranching and shorter length, CA1 pyramidal neurons showed no dendritic simplification or retraction either in the BDNF<sup>±</sup> mice or after CRS in either group (Fig. 5). Thus the effects of BDNF haploinsufficiency and CRS on dendritic remodeling are specific within hippocampus to the apical dendrites of CA3 pyramidal neurons.

### Chronic Restraint Stress Decreases Hippocampal Spine Density in Wild Type But Not in BDNF<sup>±</sup> Mice

To further investigate the consequences of chronic stress and the deficiency of BDNF on dendritic structure, we estimated



**FIGURE 1.** Effect of chronic restraint stress (Restraint) on the number of dendritic branch points (upper panel) and total dendritic length (lower panel) of pyramidal neurons from mouse hippocampal CA3 subregion. \* $P < 0.05$ , compared with controls, unpaired two-tailed Student's  $t$ -test. Bars represent means + SEM.

total spine density in tertiary apical dendrites of CA1 and CA3 pyramidal neurons. In both hippocampal subregions, and regardless of the genotype, we found a similar distribution of spine subtypes, thin spines being the most abundant, followed by the mushroom subtype and the minority fraction represented by stubby spines (Fig. 6). CRS consistently induced a decrease in the density of the thin spine subtype in both CA1 and CA3 pyramidal apical dendrites of wild type mice only ( $F_{3,20} = 6.80$ ,  $P < 0.005$  and  $F_{3,20} = 12.03$ ,  $P < 0.001$ , respectively, Fig. 6). This effect was balanced out by an increase in the density of stubby spines in CA3 dendrites ( $F_{3,20} = 14.47$ ,  $P < 0.001$ ), resulting in no changes of total spine density ( $F_{3,20} = 2.89$ ,  $P = 0.06$ ) among experimental groups. On the other hand, there was a further decrease in the density of mushroom spine subtypes in CA1 dendrites of stressed wild type mice ( $F_{3,20} = 6.73$ ,  $P < 0.01$ ) that was accompanied by a reduction in the density of thin spines ( $F_{3,20} = 6.80$ ,  $P <$

0.005), resulting in a net significant decrease in total spine density ( $F_{3,20} = 6.80$ ,  $P < 0.01$ , Fig. 6).

CRS had no effects on CA1 or CA3 spine morphology or number in BDNF<sup>±</sup> mice. For all spine subtypes, the hippocampal spine density of BDNF<sup>±</sup> mice, whether stressed or control, remained at similar levels to those in unstressed wild types. Furthermore, no alterations in the distribution pattern of spine morphology were evident after stress in the BDNF insufficient mice (Fig. 6).

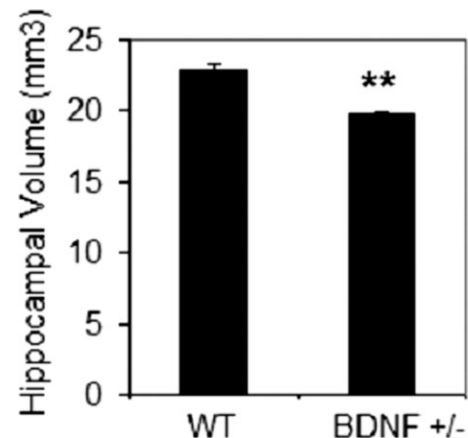
### Basal Corticosterone Plasma Levels Are Genotype Independent and Are Not Elevated 24 h After CRS and Both Genotypes Respond Equally to CRS

Twenty four hours after the last restraint stress session, at the time of euthanasia for collection of brain tissue, no significant differences were detected in corticosterone plasma levels among experimental groups ( $F_{3,20} = 0.61$ ,  $P = 0.61$ ) and BDNF<sup>±</sup> mice showed similar corticosterone plasma levels to those of WT mice regardless of being stressed or not (Table 1).

Moreover, both genotypes responded to chronic stress, since, after CRS, haploinsufficient BDNF mice showed a significant decrease in body weight ( $F_{3,20} = 67.13$ ,  $P < 0.001$ ), thymus weight ( $F_{3,20} = 18.97$ ,  $P < 0.001$ ), as well as an increase in adrenal weights compared with non stressed mice ( $F_{3,20} = 18.97$ ,  $P < 0.001$ , Table 1).

### Chronic Restraint Stress Does Not Alter BDNF Protein Levels in the Hippocampus

Twenty four hours after the last restraint stress session, no significant differences were detected in hippocampal BDNF protein levels among nonstressed and stressed WT mice (Fig. 7). However, it should be noted that WT mice did show a significant decrease in BDNF protein levels after a single 6-h restraint stress session compared with nonstressed controls



**FIGURE 2.** Effect of a partial deficiency in BDNF on the total hippocampal volume of male C57BL/6 mice. Volume was estimated using the Cavalieri method (see Material and Methods for details). \*\* $P < 0.01$ , unpaired two-tailed Student's  $t$ -test. Bars represent means + SEM.

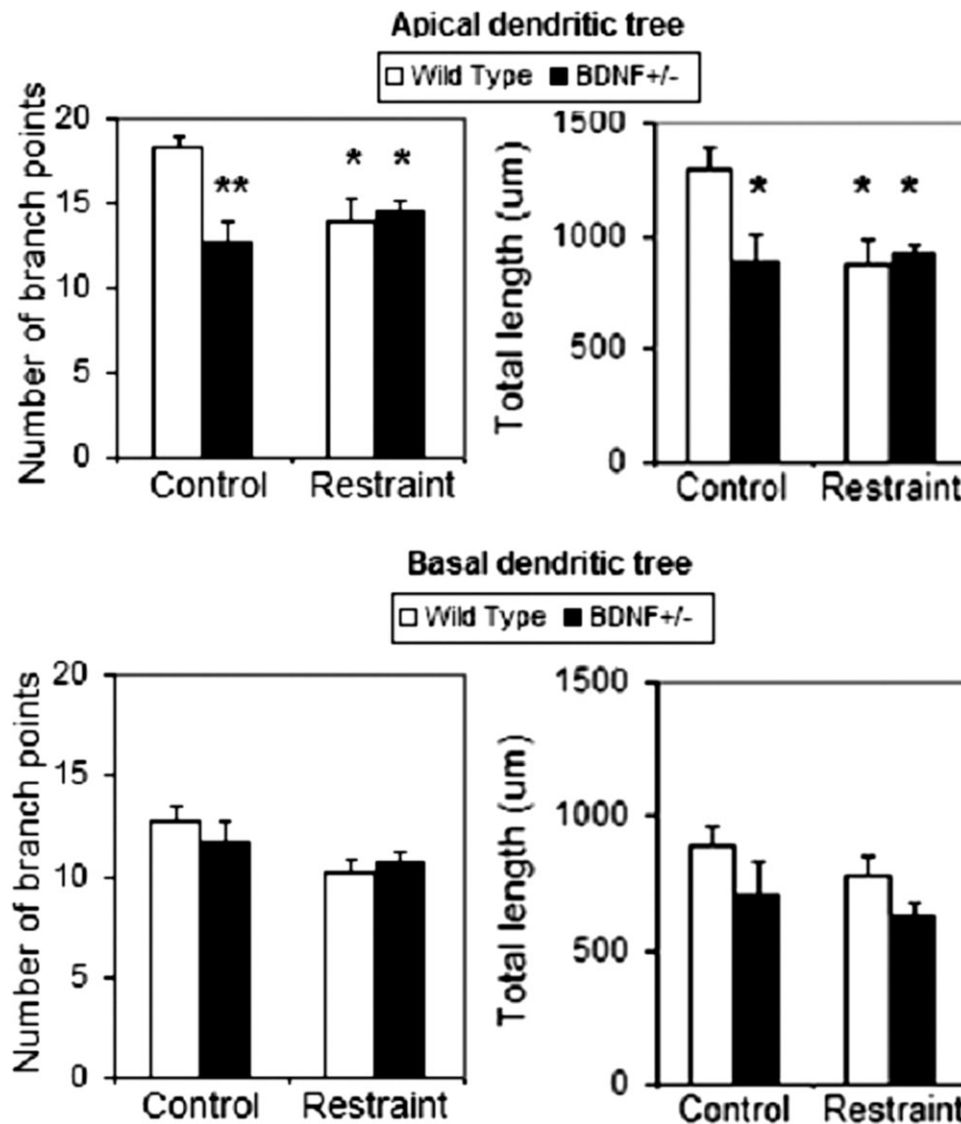


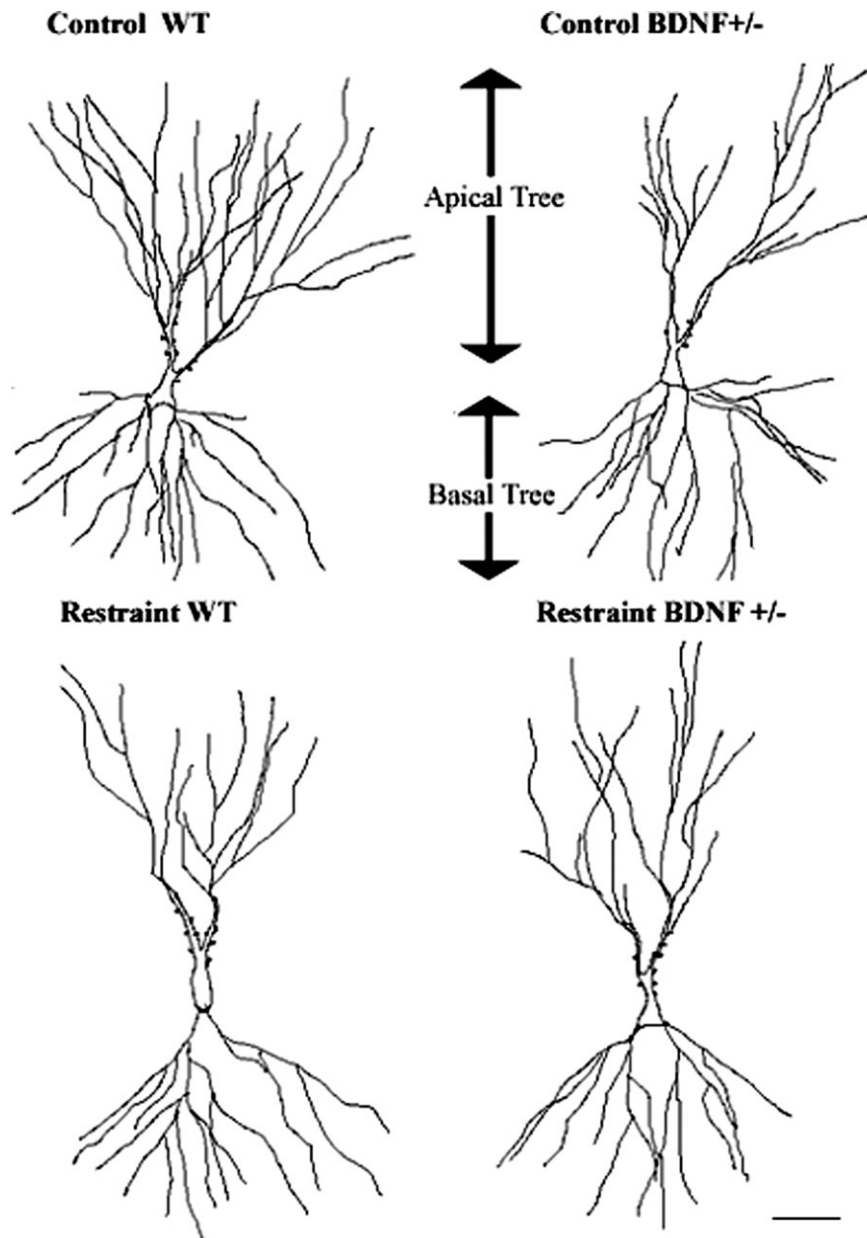
FIGURE 3. Effect of chronic restraint stress on the number of apical and basal (upper and lower panel on left) dendritic branch points and total dendritic length (upper and lower panel on right) of CA3 pyramidal neurons from wild type and haplodeficient BDNF (BDNF<sup>±</sup>) mice. \*\* and \**P* < 0.01 and *P* < 0.05 respectively, compared with control wild types. One-way ANOVA, Tukey post hoc test. Bars represent means + SEM.

(6-h restraint WT =  $59.69 \pm 7.84$  pg/mg vs. cont WT =  $75.58 \pm 2.81$  pg/mg; Student's *t*-test, *P* < 0.05). As expected, BDNF<sup>±</sup> mice showed approximately a 50% lower hippocampal BDNF protein levels compared with WT mice, regardless of whether the animals were undisturbed or given CRS (Fig. 7).

## DISCUSSION

We report that male mice subjected to CRS results in a significant decrease in the complexity and length of CA3 apical dendritic arbors, thus extending our previous reports of a simi-

lar result in male rats and tree shrews (see McEwen, 2007 for review). We also show that transgenic mice lacking a single BDNF allele (BDNF<sup>±</sup>) fail to respond to CRS with dendritic remodeling and yet, without stress, BDNF<sup>±</sup> mice showed a simplification and retraction of CA3 apical dendrites comparable with that seen in stressed wild types. The actions of stress and deficient neurotrophic support on the dendritic architecture of hippocampal pyramidal neurons are subregion specific, since the remodeling is observed in CA3 and not in CA1 subfields. Furthermore, while WT mice showed a CRS-induced decrease in total dendritic spine density in CA1, but not in CA3 apical dendrites, BDNF<sup>±</sup> mice showed no stress-induced changes in spine density in either hippocampal field. Overall, these results suggest that BDNF may exert a permissive role in



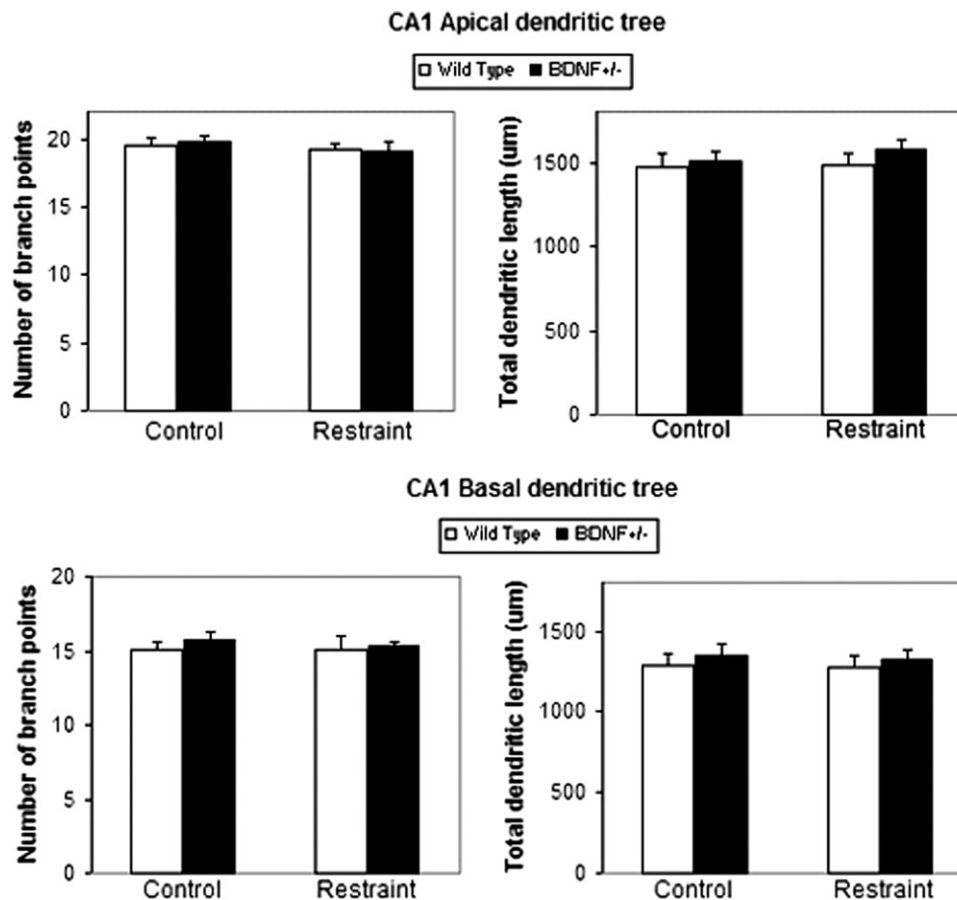
**FIGURE 4.** Camera lucida drawings of representative Golgi-impregnated CA3 pyramidal neurons from control and chronically restrained wild type (WT) and BDNF deficient ( $BDNF^{\pm}$ ) mice. Notice the more elaborated apical dendritic tree in control WT compared with the other experimental groups. Scale bar = 20  $\mu$ m.

the stress-induced changes on dendritic spine density, as well as dendritic length and complexity.

### Role of BDNF in Maintaining Dendritic Morphology

These results may be viewed in light of literature showing that the morphology of neuronal dendritic trees appears to be an early target of BDNF, as demonstrated in BDNF null mutants which, soon after birth, show an impaired maturation of Purkinje cell dendritic trees (Schwartz et al., 1997). However,

these mice do not survive long enough to test if those neurons ultimately die. Indeed, total deletion of the BDNF gene before the onset of its expression leads to early postnatal death (Snider, 1994). Instead, heterozygous mice lacking a single BDNF allele (this study) survive and attain an almost normal life span. Since  $BDNF^{\pm}$  mice are partially deficient for their entire lives, developmental processes may largely determine the role of BDNF in dendritic structure. Indeed, the forebrain-restricted deletion of BDNF during embryogenesis results in a normal dendritic development of cortical dendrites initially, but only after three weeks of age, dendritic retraction becomes



**FIGURE 5.** Chronic restraint stress does not affect the apical or basal branching pattern (panels on left) nor the total dendritic length (panels on right) of CA1 pyramidal neurons from wild type and haplodeficient BDNF (BDNF<sup>±</sup>) mice. Bars represent means + SEM.

apparent (Gorsky et al., 2003). These results suggest that BDNF is required during development for the maintenance of cortical dendritic structures. In this report, however, we show that the retraction of dendritic arborizations in the hippocampus of adult BDNF<sup>±</sup> mice is not a global phenomenon, but region and stratum specific; that is, whereas apical, but not basal, CA3 dendrites display a significant simplification and shrinkage, neither apical nor basal CA1 dendritic trees show signs of debranching or retraction.

Taken together, these results support both developmental and nondevelopmental roles of BDNF on dendritic morphology. By circumventing the effects of developmental abnormalities caused by early BDNF deficiency, Hill et al. reported no changes in dendritic morphology in the prefrontal cortex of a mouse model with an induced knockout of the BDNF gene in early adulthood (Hill et al., 2005); however, based on the differences between CA1 and CA3 dendritic morphology in this study, one cannot predict what would happen with an inducible knock-down of BDNF in CA3 neurons. Further studies (e.g., using either inducible KO mice lines in which genes that code for BDNF or its high affinity receptor TrkB are turned off later in life, or using BDNF/TrkB gene silencing by RNA interference) are needed to investigate the morphology of hippocampal dendritic fields that are affected by

the loss of BDNF signaling in the adult mice. In contrast to the BDNF deficiency-induced retraction and simplification of hippocampal dendritic fields, life-long BDNF overexpression increases dendrite elaboration in DG granule cells (Tolwani et al., 2002) and the transgenic overexpression of BDNF prevents stress-induced hippocampal dendritic simplification (Govindarajan et al., 2006).

### Possible Contribution of Shrunken Hippocampal Dendritic Fields to the Decreased Hippocampal Volume in BDNF<sup>±</sup> Mice

Very much like the reduced dendritic complexity in the CA3 apical dendrites described in this study, a recent report shows that hippocampal dentate gyrus granule neurons from adult BDNF<sup>±</sup> mice also show decreased dendritic elaboration (Chen et al., 2006). Since reduced BDNF levels has also been shown to interfere with neurogenesis in the dentate gyrus (Lee et al., 2002; Sairanen et al., 2005), the resulting insufficient integration of new neurons into the hippocampal circuitry may contribute, together with the decrease in dendritic material in pre-existing granule neurons, to the decrease in hippocampal



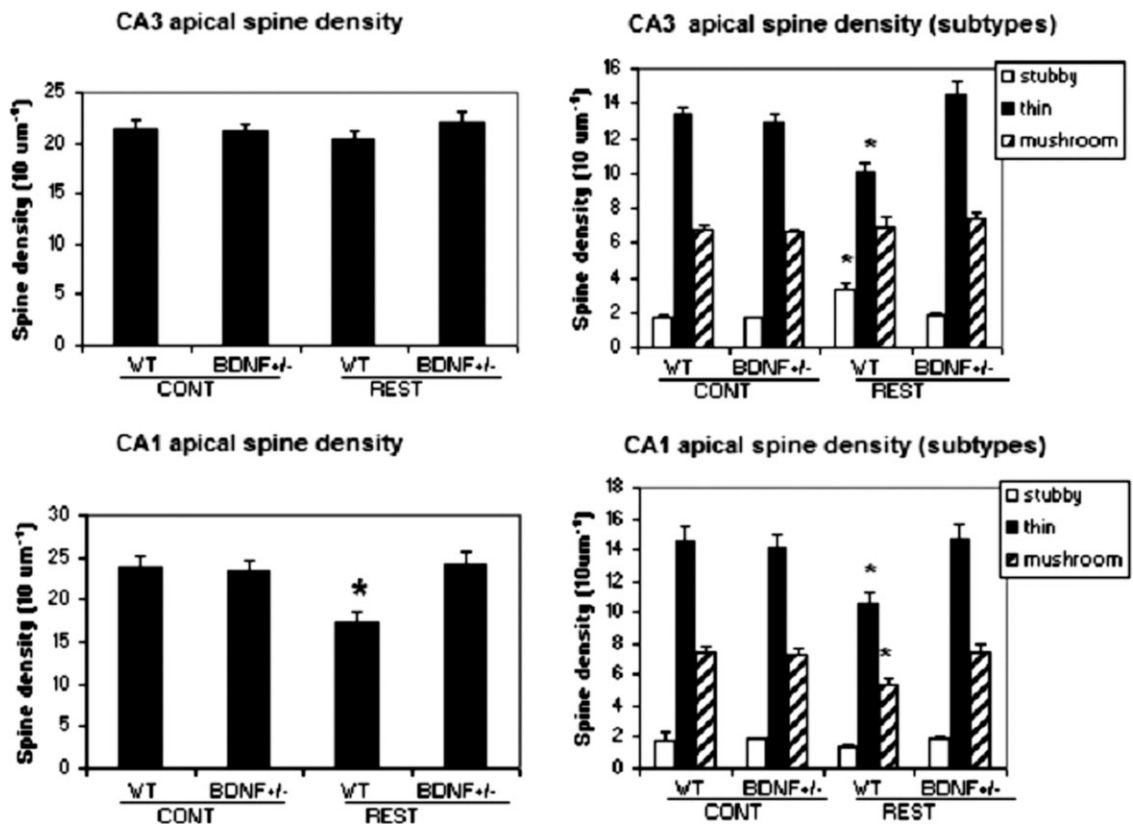


FIGURE 6. Chronic restraint stress decreases density of spine subtypes of CA1 and CA3 apical dendrites of wild type mice. BDNF<sup>±</sup> mice, whether stressed or not, did not show changes in total dendritic spine density or spine morphology. \* $P < 0.01$ , compared with control wild types. One-way ANOVA, Tukey post hoc test. Bars represent means + SEM.

volume via a dentate gyrus volume decrease (Chen et al., 2006).

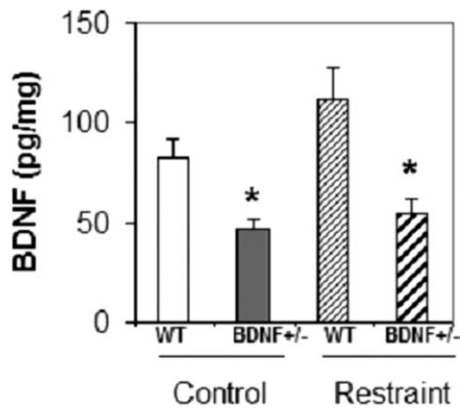
The hippocampal volume loss in BDNF<sup>±</sup> mice described here echoes that observed in patients undergoing stress-related disorders associated with deficient neurotrophic support, such as post-traumatic stress disorder, Cushing's syndrome and major depression (for reviews see Duman et al., 1997; Sapolsky, 2000; McEwen, 2007). However, it is important to keep in mind that heterozygous BDNF mice have not been shown to consistently exhibit depression-like or anxiety behavior compared with their WT littermates (McQueen et al., 2001; Chourbaji et al., 2004; Chen et al., 2006) suggesting that reduced BDNF expression per se is insufficient to induce mood disorders. On the other hand, the finding that the loss of forebrain BDNF in adult mice interferes with antidepressant efficacy in the force swim test may indicate that the loss of BDNF could point to a vulnerable phenotype when confronted with chronic challenges (Monteggia et al., 2004) and it supports the hypothesis that adequate BDNF promotes adaptive plasticity of the brain.

### Stress Effects on Spines Also Depend on BDNF Neurotrophic Support

Dendritic spines are highly dynamic structures and undergo constant remodeling as a function of neuronal activity (Kasai

et al., 2003; Yuste and Bonhoeffer, 2004) and the different geometry that spines adopt can affect calcium compartmentalization and synaptic plasticity (Nimchinsky et al., 2002). Here we show that CRS induces a significant decrease in total dendritic spine density in CA1 apical dendrites of WT mice as a result of the diminished density of both thin and mushroom-shaped spines. From a mechanistic point of view, it has been previously shown that the chronic stress-induced decreases in CA1 spine density is associated with a decrease in NMDA receptor subunit NR1. These receptor changes are dependent on the extracellular serine protease tissue plasminogen activator (tPA), a modulator of hippocampal plasticity that, through the activation of plasmin, converts proBDNF to mature BDNF in the hippocampus (Pang et al., 2004). These morphological and molecular effects are accompanied by behavioral changes, such as the impairment of acquisition, but not retrieval, of hippocampal-dependent learning tasks (Pawlak et al., 2005).

While stress also causes a decrease in the density of thin spines in CA3 pyramidal apical dendrites, it promotes the induction of stubby-shaped spines as well. Stubby spines do not have a distinguishable neck and they are thought to engage in widespread calcium signaling because they are less effective in isolating Ca<sup>2+</sup> transients from the parent dendrite compared with thin or mushroom spines which have a constricted neck that support electrical compartmentalization (Yuste and



**FIGURE 7.** Hippocampal BDNF protein levels of wild type (WT) and BDNF haploinsufficient mice (BDNF<sup>±</sup>) exposed to 21 days of daily restraint stress. \* $P < 0.01$ , compared with controls. One-way ANOVA, Tukey post hoc test. Bars represent means + SEM.

Majewska, 2001; Kasai et al., 2003; Tyler and Pozzo-Miller, 2003).

Using an automated 3D morphometric analysis, Radley et al., recently reported that, in the medial prefrontal cortex, a similar stress paradigm to the one used in this study induced a decrease in large spines and a shift towards small spines (Radley et al., 2008). These results support the notion that chronic stress modulates synaptic plasticity in different brain areas by changing the balance among spine subtypes. Moreover, as demonstrated in the present study, the somewhat different effects of chronic stress on spine density in CA1 and CA3 apical dendrites depend on the presence of adequate levels of BDNF.

### Relationship of Stress Effects on Morphology to Glucocorticoids

The effects of CRS on dendritic structure are mediated, at least in part, by adrenal steroids, in that either systemic or oral chronic administration of corticosterone decreases the complexity and length of CA3 apical dendrites (McEwen, 1999) and glucocorticoid synthesis blockage prevents the stress-induced remodeling (Magariños et al., 1995). Thus, in the present study, an important factor in explaining differences in response between WT and BDNF<sup>±</sup> mice would be differences in adrenal output and resulting cumulative measures of stress responsiveness. Yet, both WT and BDNF<sup>±</sup> mice showed a distinct lack of habituation to CRS, as shown by decreases of body weight gain, and comparable CRS-induced adrenal hypertrophy and thymus atrophy at the end of the experiment. Moreover, corticosterone levels at the end of the experiment were not different across genotype and stress condition compared to the respective control groups. Thus the differences in the effects of CRS in WT and BDNF<sup>±</sup> mice cannot be attributed to differences in the HPA response to the stress.

The relationships between corticosterone, stress and BDNF are complex, but provide some further clues. As noted, severe stressors such as immobilization have been reported to reduce

hippocampal BDNF mRNA in rats (Smith et al., 1995; Nibuya et al., 1995), whereas a less severe, chronic episode of repeated restraint stress, like the one used in this study, does not result in decreased hippocampal BDNF mRNA levels (Kuroda and McEwen, 1998; Reagan et al., 2007). A similar, negative finding, namely, hippocampal morphological changes in the absence of reduced BDNF, was reported for peripubertal rats using chronic physical and social stressors (Isgor et al., 2004). This argues that a depletion of BDNF expression is not a necessary condition for dendritic remodeling.

Corticosterone is a major factor in stress actions on the hippocampus. Besides causing dendritic remodeling (Watanabe et al., 1992; Magariños et al., 1995), and mediating the effects of stress on remodeling (Magariños and McEwen, 1995), chronic corticosterone injections have been reported to decrease hippocampal BDNF mRNA and protein levels (Jacobsen and Mork, 2006). Other reports have shown that glucocorticoids lower BDNF, but not NT-3, mRNA levels in the hippocampus and other brain areas (Barbany and Person, 1992; Schaaf et al., 2000), as well as depressing the activity-dependent expression of BDNF mRNA within culture hippocampal neurons (Cosi et al., 1993). However, at the same time, a recent report indicates that corticosterone activates the TrkB receptor independently of BDNF (Jeanneteau et al., 2008). Therefore, the corticosterone requirement for CRS-induced dendritic remodeling previously reported (Magariños and McEwen, 1995), could be explained, at least in part, by an activation of the TrkB receptor, although the consequences of ligand-independent activation of TrkB for functions other than neuroprotection (Jeanneteau et al., 2008) remain to be determined.

Despite the clearly demonstrated role of glucocorticoids in structural remodeling in hippocampus, the primary receptor mechanism for these effects is unclear. Although the CA1 and dentate gyrus neurons have abundant glucocorticoid receptor (GR: Type 2) and mineralocorticoid receptor (MR: Type 1) mRNA and immunoreactivity, CA3 pyramidal neurons express abundant MR mRNA but much less GR mRNA and only some cytoplasmic immunoreactivity for GR (Fuxe et al., 1985; Herman et al., 1989). Because there are no studies demonstrating the efficacy of the GR antagonist, Ru486, for hippocampal structural plasticity, it remains to be seen whether cytoplasmic GR in CA3 neurons or nuclear GR in CA1 or dentate gyrus neurons plays a role in glucocorticoid actions on this process. The recent demonstration that MR can play a nongenomic role in glucocorticoid actions (Karst et al., 2005) and the finding that glucocorticoids exert nongenomic actions to elevate glutamate levels (Venero et al., 1999) make a role for MR well worth exploring. In addition, GR also shows a non-nuclear localization, e.g., in postsynaptic densities (Johnson et al., 2005) and the role of non-nuclear GR in CA3 (Fuxe et al., 1985) should also be considered.

The role of hippocampal corticosterone releasing factor (CRF) needs also to be considered (Rice et al., 2008), particularly in the CA1 spine remodeling effects of stress, along with the possible role of tissue plasminogen activator, tPA (Pawlak et al., 2005), since CRF has been found to promote tPA release

(Matys et al., 2004). Indeed, studies by Baram and coworkers have demonstrated the importance of CRF in hippocampus in age-dependent stress-induced structural plasticity (Chen et al., 2004; Fenoglio et al., 2006). In particular, Chen et al. recently reported that CRF-CRFR1 signaling mediates the rapid decrease of spine density in the mouse hippocampal CA3 apical third- and fourth-order dendrites after a single variable stress paradigm and linked this effect to the destabilization of filamentous actin of mature spines (Chen et al., 2008).

In conclusion, haploinsufficiency of BDNF reveals the important role that BDNF plays in establishing and maintaining the dendritic and spine structure of hippocampal neurons. The finding that CA3 dendritic morphology and CA1 and CA3 spine morphology of BDNF<sup>±</sup> mice are unresponsive to CRS suggests that BDNF may have a permissive role in how stress affects the hippocampus and that sufficient levels of BDNF must be present for this neuronal remodeling to occur. Collectively, the findings discussed above indicate that, while BDNF is undoubtedly a significant factor in structural and functional adaptations of the brain to stressors and stress related vulnerability to conditions such as depression, they are also consistent with the notion that BDNF may not determine the absolute nature and direction of structural changes, but rather be permissive for these changes.

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