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## Corticotrophin Releasing Factor Accelerates Neuropathology and Cognitive Decline in a Mouse Model of Alzheimer's Disease

Hongxin Dong<sup>a,\*</sup>, Keely M. Murphy<sup>a</sup>, Liping Meng<sup>a</sup>, Janitza Montalvo-Ortiz<sup>a</sup>, Ziling Zeng<sup>b</sup>, Benedict J. Kolber<sup>c</sup>, Shanshan Zhang<sup>a</sup>, Louis J. Muglia<sup>d</sup>, and John G. Csernansky<sup>a</sup>

<sup>a</sup>Department of Psychiatry and Behavioral Sciences, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

<sup>b</sup>Department of Neurology, Qilu Hospital, Shandong University, Jinan, People's Republic of China

<sup>c</sup>Pain Center and Department of Anesthesiology, Washington University School of Medicine, St Louis, MO, USA

<sup>d</sup>Departments of Pediatrics and Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, USA

### Abstract

Chronic stress has been suggested to influence the pathogenesis of Alzheimer's disease (AD); however, the mechanism underlying this influence remains unknown. In this study, we created a triple transgenic mouse model that overexpresses corticotrophin-releasing factor (CRF) and human amyloid- $\beta$  protein precursor (A $\beta$ PP), to investigate whether increases in the expression of CRF can mimic the effects of stress on amyloid metabolism and the neurodegeneration. Tg2576 mice that overexpresses human A $\beta$ PP gene were crossbreed with Tetop-CRF (*CRF*) mice and CaMKII-tTA (*tTA*) mice to create a novel triple transgenic mouse model that conditioned overexpresses CRF in forebrain and overexpresses human A $\beta$ PP (called A $\beta$ PP+/CRF+/tTA+, or TT mice). Then we evaluated serial neuro-anatomical and behavioral phenotypes on TT mice using histological, biochemical, and behavioral assays. TT mice showed a Cushingoid-like phenotype starting at 3 months of age. At 6 months of age, these mice demonstrated increases in tissue-soluble amyloid- $\beta$  (A $\beta$ ) and A $\beta$  plaques in the cortex and hippocampus, as compared to control mice. Moreover, TT mice characterized substantial decreases in dendritic branching and dendritic spine density in pyramidal neurons in layer 4 of the frontal cortex and CA1 of the hippocampus. Finally, TT mice showed significantly impaired working memory and contextual memory, with a modest increase in anxiety-like behavior. Our results suggested genetic increases in the brain of CRF expression mimicked chronic stress on the effects of amyloid deposition, neurodegeneration, and behavioral deficits. The novel transgenic mouse model will provide a unique tool to further investigate the mechanisms between stress and AD.

### Keywords

Alzheimer's disease; amyloid- $\beta$ ; cognitive function; corticotrophin-releasing factor; neurodegeneration; stress

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\*Correspondence to: Hongxin Dong, M.D., Ph.D., Assistant Professor, Department of Psychiatry and Behavioral Sciences, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Ward 12-369, Chicago, IL 60611, USA. Tel.: +1 312 503 3433; Fax: +1 312 503 0466; h-dong@northwestern.edu. .

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## INTRODUCTION

There is a growing appreciation of the impact of environmental factors on the pathogenesis of Alzheimer's disease (AD), particularly the late-onset sporadic form of this disease [1-3]. In patients with AD, psychosocial stress, mediated by changes in the hypothalamic-pituitary-adrenal (HPA) axis, has been suggested as one of these environmental factors [3-8]. Changes in the HPA axis in AD patients includes increases in plasma cortisol levels [9-11], correlations between increases in plasma cortisol levels and cognitive decline [3]. Furthermore, the expression of corticotrophin releasing factor (CRF), including reductions in CRF-immunoreactive neurons in the frontal and temporal cortices [12, 13] coupled with increases in the density of postsynaptic CRF receptors in postmortem tissue samples [14, 15]. While these changes are sometimes interpreted as evidence of HPA axis disinhibition triggered by AD-related hippocampal degeneration, the mechanism underlying the relationship between stress and the pathogenesis of AD remains unclear.

Genetically manipulated mice that imitate some of the neuropathological changes of AD provide a unique opportunity to investigate the influence of environmental factors, such as stress, on the pathogenesis of AD at the cellular and molecular levels. The Tg2576 mouse is arguably the most well-known mouse model of AD. This mouse overexpresses human amyloid- $\beta$  protein precursor (A $\beta$ PP) 695 and demonstrates amyloid- $\beta$  (A $\beta$ ) plaque deposition in the cortex and hippocampus and memory-related behavioral deficits at 9-10 months of age [16, 17]. Recent studies indicated that behavioral stressors increase the production of A $\beta$  and A $\beta$  plaques throughout the hippocampus and cortex of stressed Tg2576 and A $\beta$ PP<sub>V717I</sub>-CT100 mice [18, 19]. Furthermore, treatment of triple transgenic A $\beta$ PP/PS1/MAPT mice with dexamethasone, a glucocorticoid receptor agonist, increases brain A $\beta$ PP and A $\beta$  levels as well as BACE ( $\beta$  secretase) and the  $\beta$ -CTF of A $\beta$ PP [20]. Our group reported that behavioral stressors increase interstitial levels of A $\beta$  via the activation of CRF receptors (CRFR1) and concomitant increases in neuronal activity [21, 22].

CRF and its receptors are critical regulators of HPA axis activity in response to stress, and have been shown to modulate neuronal activity throughout the cortex and hippocampus [23, 24]. In order to test the hypothesis that increases in CRF expression may be one of the mechanisms by which environmental stressors influence the behavioral and histological pathogenesis of AD, we created then evaluated a novel triple transgenic mouse model that conditionally overexpresses the CRF and human A $\beta$ PP. These novel mice significantly increased in tissuesoluble A $\beta$  and A $\beta$  plaques, decreased in dendritic branching and dendritic spine density, and impaired working memory. Our findings suggest that conditionally overexpressed CRF mediated amyloid deposition, neurodegeneration, and behavioral deficits, which mimicked chronic stress induced neuropathological changes in the mouse model of AD [22, 25].

## METHODS AND MATERIALS

### Breeding and genotyping of transgenic mouse lines

All animal procedures were done in accordance with the NIH and Animal Studies Committee at Northwestern University guidelines. We currently maintain three single transgenic mouse colonies, Tg2576, Tetop-CRF and CaMKII-tTA. The details of the procedure to create Tetop-CRF and CaMKII-tTA have been described previously [26]. We crossbred Tg2576 mice (A $\beta$ PP+, male) with either a Tetop-CRF+ or CaMKII-tTA+ mouse to obtain either an A $\beta$ PP+/CRF+ or A $\beta$ PP+/tTA+ mouse line. We crossed these mice with either a CaMKII-tTA+ or Tetop-CRF+ mouse to obtain an A $\beta$ PP+/CRF+/tTA+ mouse line (referred to as TT mouse). For control of the inducible tetracycline-off/on system, mice "on

doxycycline” were fed doxycycline chow (200 mg doxycycline/1 kg; Research Diets) from weaning for 6 months to suppress CRF transgene expression.

### Corticosterone assay

Blood was collected by rapid retro-orbital phlebotomy at 5 : 45 AM before light on and 5 : 45 PM before light off. Plasma concentration of corticosterone was measured using ELISA kit according to published methods [26]. The animal number of each group = 5 for this measurement.

### CRF measurement

Free CRF levels from tissue samples were analyzed using an ELISA kit (COSMO BIO Co., Tokyo, Japan). The brain tissue was prepared by homogenization and centrifuged (15,000 rpm/min, 20 min) at 4°C. Supernatant was transferred into a glass tube in an ice bath. Methanol was applied into an extraction column for conditioning and then drained by aspiration. The column was equilibrated by two washes with distilled water, and the brain supernatant was added to the column with a pipette. The volume of the supernatant applied was recorded. The column was aspirated slowly, washed with distilled water and finally eluted twice with methanol. The eluate was collected in a glass tube, dried in a centrifugal vaporizer, and the resulting residue (sample for assay) was stored at -20°C until the assay is performed. The assay procedure was detailed in the ELISA kit. The animal number of each group = 3–5 for this measurement.

### A $\beta$ PP and A $\beta$ in brain tissue

Mice were rapidly (1 min) perfused with 0.9% saline at 6 months of age. The brain was then dissected, and one hemisphere was frozen at -80°C for biochemical analyses. The remaining hemisphere was fixed in 4% paraformaldehyde for morphological analysis. ELISA kits (Invitrogen) were used to measure soluble and insoluble A $\beta$ <sub>42</sub> in the cortex and hippocampus according to the manufacturer’s instructions. Western blots was performed to measure A $\beta$ PP levels. The primary antibodies were used for immunoblotting at the following dilutions: A $\beta$ PP 22C11 (1 : 8000), A $\beta$ PP C-terminal (2.F2.19B4) (1 : 10000), Beta-tubulin III (1 : 1000). The animal number of each group = 5 for these measurement.

### A $\beta$ plaque identification

The detailed methods have been described in our previous studies [22, 27]. The animals were perfused transcardially with 1% heparinized 0.01 M phosphate buffer (PBS) for 2 min and then 4% paraformaldehyde for 25–30 min. Brains were removed and post-fixed at 4°C using the same fixative with 30% sucrose for 48 h. The brains were dissected and embedded in Tissue-Tek embedding medium (Electron Microscopy Sciences, Hatfield, PA), and cut into 35  $\mu$ m thick sections in the coronal plane using a cryostat (Leica CM 1850 UV, Nussloch, Germany). Selected sections were rinsed with 0.1 M PBS (pH 7.4) and incubated in a blocking solution of 5% normal goat serum for 1 h. Sections were then incubated overnight in the primary antibody for A $\beta$  at 4°C (rabbit polyclonal antibody, 1 : 1000, Biosource, Camarillo, CA). After PBS washing, the sections were incubated in biotinylated anti-rabbit secondary antibody for 2 h at room temperature (RT), then in an avidin-biotin complex for 1 h at RT (Vector Laboratories, Burlingame, CA). A $\beta$ -like immunoreactivity was visualized using a DAB kit (Vector Laboratories, Burlingame, CA). To confirm the presence of compact (fibrillar) A $\beta$  plaques in selected sections, we stained floating sections using a 1% thioflavine S aqueous solution for 5 min and then differentiated in 70% alcohol for 3–5 min. A $\beta$  plaques were determined separately in two brain areas, the cerebral cortex and hippocampal formation. Cortex was defined as all areas dorsal to the rhinal fissure and dorsal or lateral to the corpus callosum and external capsule. The hippocampal formation

was defined as the hippocampus proper plus the dentate gyrus and subiculum. We used two different staining methods to quantify the density of A $\beta$  plaques, and measurement of total A $\beta$  plaque area (i.e., amyloid burden) in each brain was evaluated using the CAST stereological program. The animal number of each group = 5 for A $\beta$  plaque measurement.

### Measurements of dendritic spine size and density

At 6 months of age, mice were sacrificed and their brains were collected and subjected to Golgi staining (FD Rapid GolgiStain Kit; FD Neurotechnologies) according to the manufacturer's instructions. Briefly, the brains were immediately removed and rinsed in 0.1 M phosphate buffer. Brains were immersed in a Golgi-Cox solution. The mixture of solutions was replaced once after 12 h of initial immersion, with storage at room temperature in darkness for 2–3 weeks. After the immersion period in the Golgi-Cox solution, brains were transferred to a cryoprotectant solution and stored at 4°C for at least 48 h in the dark before cutting. Brains were rapidly frozen with dry ice and cut in the coronal plane at approximately 150  $\mu$ m thickness on a cryostat. The sections were transferred onto gelatin-coated slides. Cut sections were air dried at room temperature in the dark. After drying, sections were rinsed with distilled water and were subsequently stained in a developing solution and dehydrated, cleared and coverslipped. The pyramidal neurons in the layer 4 of the frontal cortex (up to dorsal hippocampal area), the pyramidal neurons of CA1 in the dorsal hippocampus and neurons in the medial-dorsal thalamic nucleus were selected for dendritic analysis. Twenty-five neurons (10 from the cortex; 10 dorsal hippocampus, and 5 from thalamus) of each animal were selected by stereological program (CAST) for dendritic branching and spine density measurement. Dendritic branching was evaluated using Sholl analysis, measuring total dendritic length and number of intersections at concentric circles at increasing distance from the soma (1/2 diameter = 150  $\mu$ m) [28, 29]. Spines were identified and characterized [30]. Spine density was expressed as the number of spines per 10  $\mu$ m of dendrite length. Five mice in each group were used for dendrite branching and spine density measurements.

### Behavioral testing

All behavior testing was done by experimenters blinded to genotype and treatment in doxycycline experiments. Eight-ten mice in each group were used for the behavioral tests.

### Spontaneous alternation

The apparatus consists of a three-arm (5  $\times$  21  $\times$  15.5 cm) Y maze. The mouse was placed on the center region facing one of the closed arms where it was allowed to freely explore the arms for 5 min. Each mouse completes one trial and is then placed back in its home cage. An entry was defined as the presence of all four paws of the animal in the given arm. The sequence of arm entries was recorded by two trained observers; a successful alternation was defined as entry into all three arms in consecutive choices. The apparatus was cleaned between trials to remove any olfactory cues.

### Fear conditioning task

Detailed methods have been described in our previous studies [31]. Animals were trained and tested in two Plexiglas conditioning chambers (26  $\times$  18  $\times$  18 cm) (Med Associates Inc., Georgia, VT), with a metal grid floor, within a larger sound-attenuating chamber. On day 1, training took place in the first chamber which contained a cup containing mint extract placed beneath the grid floor. Freezing behavior, defined as no movement (ambulation, sniffing or stereotypy) other than respiration, was recorded every 10 s for 5 min. After the first 2 min, a 20 s, 80 dB, a 2800 Hz tone was presented, and during the last 2 s of the tone, the animals received a 0.5 mA continuous footshock. This procedure was repeated two more times at 1

min intervals. On day 2, animals were returned to the chamber, and the amount of freezing behavior in response to context (i.e., memory for context) was recorded every 10 s for 8 min. On day 3, the animals were placed in a second chamber (scented with coconut with the grid floor covered with polyurethane). Freezing behavior was again recorded for 2 min. Then, the 80 dB, 2800 Hz tone was represented continuously for 8 min, and freezing behavior in response to the cue was recorded.

Sensitivity to the footshock was tested on the day following completion of the cued conditioning evaluation. The animals were returned to the first conditioning chamber for 2 min and exposed to a series of 2 s shocks, beginning with an intensity of 0.05 mA. The shock intensity was increased by 0.05. The level of shock required to evoke flinching, running, vocalization, and jumping will be determined, once the threshold for each of these responses has been determined the exposures will stop, or if the threshold reaches 1.0 mA, for example, if all behavioral responses are observed at a level of 0.25 mA, that is determined to be the threshold for that animal and no further shocks are administered. This measure is used to determine if perception of the shock stimulus is consistent across experimental groups.

### Light-dark preference

The apparatus consists of a shuttle box separated into two chambers by a guillotine door. One compartment is dark and the other is illuminated with a bright stimulus light (500 lux); both chambers contain a metal grid floor. Photobeams installed in each chamber detect the location and locomotor activity of the mice and report that activity to the Graphic State computer program, which controls the experimental processes. All of the testing equipment is housed within a sound-attenuating chamber, which contains a fan that provides 68 dB of background noise. On testing day, the animal was placed in the dark chamber with the sliding door closed and allowed to explore for 1 min. At this point, the sliding door opened and the animal was allowed to explore both the dark and light chambers for 10 min.

### Data analysis

We used two-way ANOVA or an unpaired two-way Student-Test to assess measures of A $\beta$ , plaque deposition, spine density, and biochemical and immunohistochemical indices ( $p < 0.05$ ). When genotype (i.e., TT, CT, A $\beta$ PP versus WT), drug condition (i.e., doxycycline versus vehicle), genotype \* drug interactions were found, post-hoc analyses were performed using Fisher's Protected Least Squares Design (PLSD) tests.

## RESULTS

### Establishing a AD mouse model with conditional CRF overexpression

A triple transgenic mouse that overexpresses human A $\beta$ PP as well as CRF in the forebrain in the absence of doxycycline administration was produced by first crossbreeding Tg2576 males (referred to as A $\beta$ PP+) with Tetop-CRF+ or CaMKII-tTA+ females to obtain either A $\beta$ PP+/CRF+ or A $\beta$ PP+/tTA+ mice. These two lines of mice were then crossed with either a CaMKII-tTA+ or Tetop-CRF+ mouse to produce the triple transgenic mouse line (A $\beta$ PP+/CRF+/tTA+ mouse line, referred to as TT). The electrophoresis gel pictured (Fig. 1A) verifies that these triple transgenic mice contained the human A $\beta$ PP, tetop-CRF and CaMKII-tTA genes. CRF+/tTA+ (referred to as CT) mice were produced as previously described [26].

By 3–4 months of age, a Cushingoid phenotype was observed in both CT and TT mice, characterized by hair loss and an olive-shaped body (Fig. 1B and C). In addition, TT mice had significantly reduced body weights (Fig. 1C). Levels of both CRF and CRFR

expression, as measured by enzyme-linked immunosorbent assay (ELISA) and Western blot, were increased in the cortex and hippocampus of both TT and CT mice (Fig. 1D-F). Increases in basal plasma corticosterone levels were also detected in CT and TT mice (Fig. 1G).

### Increases in tissue A $\beta$ levels and A $\beta$ plaque deposition

Before investigating whether CRF forebrain overexpression exacerbates pathological and behavioral outcomes, we first evaluated A $\beta$ PP and A $\beta$  expression in TT mice. Western blot analysis showed that A $\beta$ PP levels were not increased in TT mice as compared to levels in A $\beta$ PP+ mice (Fig. 2A). ELISA analysis indicated that levels of tissue soluble (Fig. 2B, upper panel) and insoluble A $\beta$ <sub>42</sub> (Fig. 2B, lower panel) were significantly increased in the cortex and hippocampus of TT mice as compared to the levels in A $\beta$ PP+ mice at 6 months of age. Using immunohistochemical staining, we found that these A $\beta$  plaques were distributed predominately in the cortex of TT mice (Fig. 2C, two upper panels). Thioflavin S staining confirmed that the observed plaques were composed of amyloid (Fig. 2C, two lower panels). A $\beta$  plaques were less commonly observed in the hippocampus of TT mice, but not in any other brain areas. Quantitative analysis of A $\beta$  plaque deposition indicated that both A $\beta$  plaque number and burden were increased in the cortex and hippocampus of TT mice as compared to A $\beta$ PP mice (Fig. 2D-G). These results suggest that CRF overexpression increases A $\beta$  production from A $\beta$ PP and/or slows A $\beta$  degradation without affecting A $\beta$ PP expression in TT mice. Since CT and wild type mice were absent of human A $\beta$ PP gene, there were no A $\beta$  plaques in these mice therefore we excluded these two groups in the results.

### Decreases in dendritic numbers, branching, and spine density

As CRF has been reported to affect neuronal development and survival [28], we investigated the effect of CRF overexpression on neuronal architecture in the presence or absence of A $\beta$ PP production. We compared dendritic branch number, length, and spine density across the groups of animals using Golgi staining in forebrain regions where CRF expression was increased (e.g., cortex and hippocampus) as well as non-forebrain regions (e.g., thalamus). We found a significant decrease in the number of cortical dendritic branch terminals (but not in primary or secondary branches) (Fig. 3A-C) in TT, CT and A $\beta$ PP+ mice as compared to wild type mice. In addition, dendritic spine density was significantly decreased in the cortex and hippocampus but not in the thalamus of TT, CT, and A $\beta$ PP+ mice as compared to those in wild type mice. In both of these comparisons, TT mice showed the significant lowest values compared to all other genotypes (Fig. 3D-G).

### Exacerbation of cognitive function deficits and increases in anxiety-like behavior

To assess whether changes in A $\beta$  production, A $\beta$  plaque deposition, and neuronal architecture induced by forebrain CRF overexpression were accompanied by changes in memory-related behavior, we compared performance on tests of spontaneous alternation and contextual fear conditioning across the groups of mice at 6 months of age. The spontaneous alternation, measured by a Y-arm maze testing an animal's tendency to enter novel arms, is commonly used to test spatial working memory. In our study, TT, CT, and A $\beta$ PP+ mice demonstrated significant decreases in the percentage (%) of correct alternations as compared to alternations in WT mice at 6 months of age (Fig. 4A). The largest decreases in spontaneous alternation performance were observed in TT mice and were significantly different as compared to other genotypes at 6 months of age (Fig. 4A). Similarly, we observed significant deficits in contextual memory but not cued memory in the fear-conditioning test (i.e., freezing behavior in response to context and cue) in TT and A $\beta$ PP+ mice as compared to CT and WT mice at 6 months of age (Fig. 4B, data not shown the

cue-induced memory results), with TT mice again showing the largest decrease in freezing behavior.

To assess the effect of CRF overexpression on anxiety-like behavior, we measured behavioral responses in the light:dark (L:D) preference test. Consistent with an anxiogenic phenotype, at 6 months of age we observed increases in latency to enter the illuminated compartment in both CT and TT mice, as compared to A $\beta$ PP+ and WT mice (Fig. 4C). In addition, CT, but not TT mice, spent longer time in the dark chamber compared to A $\beta$ PP+ and WT mice (Fig. 4D).

### **Doxycycline prevents CRF overexpression-induced A $\beta$ levels and cognitive deficits**

To confirm that increases in A $\beta$  plaque deposition and behavioral deficits could be reversed in part or in whole by decreases in forebrain CRF expression, we administered doxycycline in the chow of TT mice from weaning until 6 months of age. In the CT model, we previously demonstrated that doxycycline effectively inhibits CRF overexpression [26]. Here, we found TT and CT mice treated with doxycycline fail to express the Cushingoid-like phenotype (i.e., mice no longer have the hair loss and change in body shape) and have normal body weights compared to wild type mice. Moreover, A $\beta$  deposition was reversed in TT mice treated with doxycycline (Fig. 5A and B). Finally, doxycycline treatment improved performance with variable results on tests by significantly increasing the % of corrected alternation in TT mice in spontaneous alteration test (Fig. 5C), and significantly increasing the freezing time in CT mice in contextual memory test (Fig. 5D). Moreover, the anxiety like behavior was reversed in CT mice by significantly decreasing the latency to enter the light field (Fig. 5E) and decreasing the time spent in the dark field (Fig. 5F).

## **DISCUSSION**

Previous studies using different transgenic mouse models of AD indicate that behavioral stressors can increase the production of A $\beta$  and accelerate its incorporation into A $\beta$  plaques [18-20]. Moreover, CRF has been implicated in mediating the effects of behavioral stressors [18, 20-22, 32, 33]. However, research in this area has been studied with exogenously administered CRF because of the limitation that chronic CRF administration cannot pass through the blood brain barrier. To our knowledge, our present studies are the first to use a genetic approach to investigate the effects of consistent increases in the release of CRF on A $\beta$  metabolism, A $\beta$  plaque deposition, neurodegenerative process and their behavioral consequences. Creating a novel mouse model with a Tg2576 genetic background that conditionally overexpresses CRF in the forebrain (referred to A $\beta$ PP+/CRF+/tTA+ mice or TT mice) allows us to directly control intrinsic increases in CRF and linked such increases with changes in A $\beta$  metabolism and deposition.

Using this mouse model, we found that overexpression of CRF increased tissue levels of A $\beta$ , increased A $\beta$  plaque deposition, and accelerated dendritic degeneration. These pathological changes were accompanied by the appearance of behavioral deficits related to working memory induced by Y-maze and contextual memory induced by fear conditioning tests. The TT mice showed Cushingoid-like phenotypes with increased plasma corticosterone, suggesting that genetically overexpressed CRF made an impact on the function of the HPA axis. However, the reduced body weight in the TT mice is unusual. Perhaps overexpression of CRF in hypothalamic regions that regulate appetite/food intake are responsible for this effect. The TT mouse exhibited characteristics highly analogous to the effects of behavioral stressors, in particular chronic isolation stress, on the A $\beta$ PP+ mouse model (Tg2576 mouse line). Table 1 summarizes the shared characteristics of TT mice and stressed A $\beta$ PP+ mice [18, 22, 25]. Increased tissue A $\beta$  levels and accelerated A $\beta$ -plaque deposition appeared in both TT and stressed A $\beta$ PP+ mice at 6 months of age, which

is 2-3 months earlier than regular A $\beta$ PP+ mice. The distribution of amyloid plaques was somewhat different in that cortical plaques were most prominent in TT mice while plaques were more evenly distributed throughout the cortex and hippocampus as well as the striatum and corpus callosum in stressed A $\beta$ PP+ mice [18]. This difference in plaque distribution may be due to the forebrain-specific expression of the CaMKII $\alpha$  promoter in the TT mouse, which was used to drive the overexpression of CRF. Next, we will investigate patterns of the effect of CRF on tissue A $\beta$  and A $\beta$ -plaque deposition with increasing age up to 18 months. The results further support the hypothesis that stress and stress factors, particularly CRF, will influence neuropathogenesis and cognitive function in AD [18, 20-22, 31, 32].

Our findings provide a plausible basis for the regulation of A $\beta$  metabolism by CRF. Since our data suggests that A $\beta$ PP is not increased in TT mice, it is likely that CRF increases A $\beta$  by increasing the conversion rate of A $\beta$ PP to A $\beta$  and/or decreasing A $\beta$  degradation. Further investigating C-terminals fragments could help determine whether  $\beta$ -secretase or  $\gamma$ -secretase cleavage of A $\beta$ PP is affected. CRF-induced increases in A $\beta$  and CRF expression itself may cause synapse loss, which may be one of the biological bases of the behavioral impairment. CRF and its receptors are critical regulators of HPA axis activity in response to stress and widespread neuromodulators, particularly in the cortex and hippocampus [23, 34]. Therefore, overexpression of CRF in the brain may influence A $\beta$  metabolism either directly through neuromodulator activity or indirectly through downstream changes in HPA axis activity. In future experiments, we will perform adrenalectomies to block downstream changes and to distinguish between these two pathways.

It has been accepted that A $\beta$  metabolism is regulated by neuronal activity, which may be increased by CRF [21]. CRF exerts its cellular effects by activating one of its two known G-protein-coupled receptors (GPCRs), CRFR1 and CRFR2 [35]. In fact, CRF binds to CRFR2 with ~40-fold lower affinity; therefore, most of its activity can be attributed to activation of CRFR1 [36]. CRFR stimulation primarily activates the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling cascade in neuronal systems [37, 38], though CRF receptors also can couple to the phospholipase C (PLC)-protein kinase C (PKC) pathway [38, 39]. Stressors induce the release of CRF from the hypothalamus, which in turn initiates the stress response pathway [40]. Also, stressors can induce a large increase in catecholamine release in the prefrontal cortex, which in turn increases cAMP and protein kinase C (PKC) intracellular signaling. This can lead to reduced prefrontal neuronal firing and impairments in working memory [41, 42]. In addition, elevated CRF receptor binding during periods of increased activity can raise PKC levels above the threshold for synaptic modification.

Recent studies indicate that PKC plays an important role in the pathophysiology of AD [43-50]. For example, increases in PKC activity stimulate A $\beta$  peptide production and tau protein hyperphosphorylation [50]. Dysregulation of PKC-MAPK signaling increases A $\beta$  level in the brain [48]. However, PKA/PKC and other GPCRs signaling pathways might regulate  $\alpha$ -secretase-mediated cleavage of A $\beta$ PP [49], and a novel type of PKC, PKC $\epsilon$ , activates the A $\beta$  degrading activity of endothelin converting enzyme type 1 (ECE-1), which might be mediated via the mitogen-activated protein kinase (MAPK) pathway as well. The highest level of adenylate cyclase activation is in the cerebral cortex, an area that is profoundly involved in AD [51]. This neuroanatomical specificity supports the hypothesis that CRFR1 receptors play a role in the pathogenesis of AD [49]. Future studies using our model to investigate cAMP-PKA and/or PLC/PKC signaling pathways involved in CRF-mediated effects on AD-like pathology are necessary.

A recent study also indicated that stress in Tg2576 mice markedly increased metabolic oxidative stress and down-regulated the expression of matrix metalloproteinase-2 (MMP-2),



a potent A $\beta$  degrading enzyme, in the brain. These stress effects were reversed by CRFR1 antagonist NBI 27914 [33]. Therefore, we need elucidation of whether high reactive oxygen species levels are regulated in the brain of TT mice and to test whether CRFR1 antagonists could reverse stress or CRF induced neuropathological changes. CRFR1 antagonists are currently being evaluated in clinical trials for their efficacy in the treatment of major depression, anxiety, post-traumatic stress disorder, and substance abuse disorders [52]. However, their potential therapeutic value for neurodegenerative disorders has not yet been explored. The effects of CRFR1 antagonists on the metabolism of A $\beta$  and A $\beta$  plaque deposition will be facilitated by the availability of our novel TT animal model.

Dendritic spines are critical structural and functional units of neurons. In our prior studies of stressed A $\beta$ PP+ mice, we found that neuronal degeneration was related to plaque deposition [27]. There is also increasing evidence that both behavioral stressors and stress-related hormones can induce the disintegration of dendritic arborization [53-55] and dendritic spine density [28, 29, 56]. In addition, a direct effect of CRF on spine density was found to be selective to the area of CA3 apical dendritic spines in the hippocampus, associated with a loss of excitatory synapses, the site of long-term potentiation and learning and memory function deficits [56]. In the present study, we found either A $\beta$ PP or CRF overexpression influenced dendritic architecture by decreasing the terminal branches and spine densities but not the primary and secondary branches in the layer 4 of the frontal cortex and the pyramidal cells of CA1 of dorsal hippocampus. In TT mice with combined expression of A $\beta$ PP and CRF, the degree of spine loss in the cortex and hippocampus was much more prominent. Spine loss and regression have been reported in human studies in the context of normal aging [57] and neurodegenerative disorders, including AD [58, 59]. The integrity of dendrites and their arborization are regulated by a variety of extracellular factors including neurotransmitters, growth factors, and hormones [29, 60]. Reduced spine number would result in the reduction of the total postsynaptic area of excitatory synapses, which in turn would influence receptor density, synaptic signaling [61], and the number of functional synapses [60]. Eventually, these changes may influence overall synaptic plasticity and function [62, 63]. Finally, synaptic plasticity is believed to underlie learning and memory functions [63, 64]. Thus, decreases in spine number induced by changes in CRF and A $\beta$ PP/A $\beta$  release may serve as conceivable substrates for the deficits in working and contextual memory function detected in the TT, CT and A $\beta$ PP+ mice.

In summary, the results of the present study suggest that intrinsic increases in CRF may be the key regulator by which behavioral stressors influence A $\beta$  metabolism and plaque deposition in mouse models of AD and perhaps in patients with sporadic AD as well. The TT mice we created provide a unique opportunity to further investigate the link between CRF stimulation and A $\beta$  production through specific signal transduction pathways, which would offer insights into the CNS mechanisms that govern A $\beta$  regulation and amyloid plaque production. In future studies, we will use the TT animal model to address such questions and to test the efficacy of CRFR antagonists as potential therapeutics for AD.

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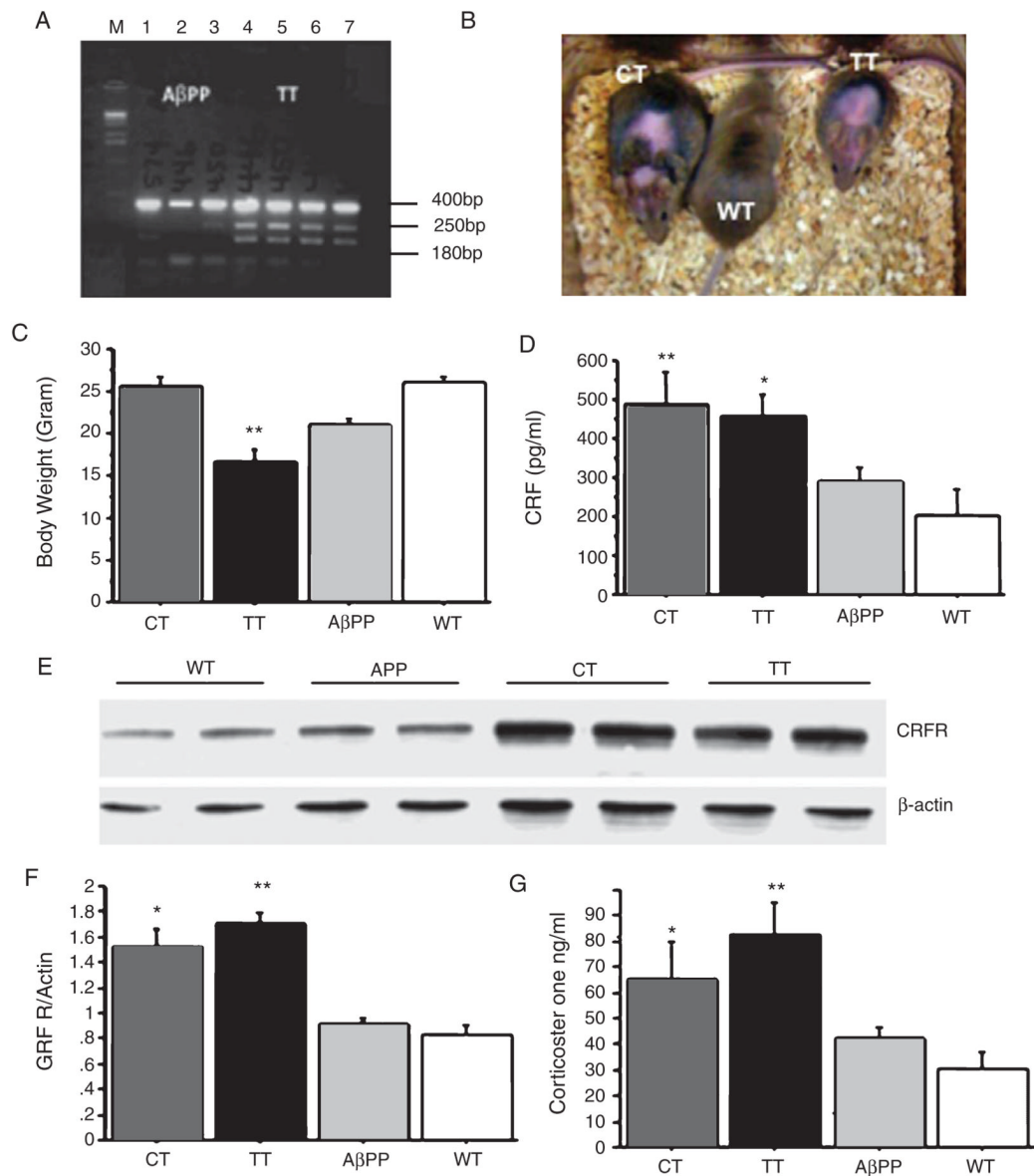
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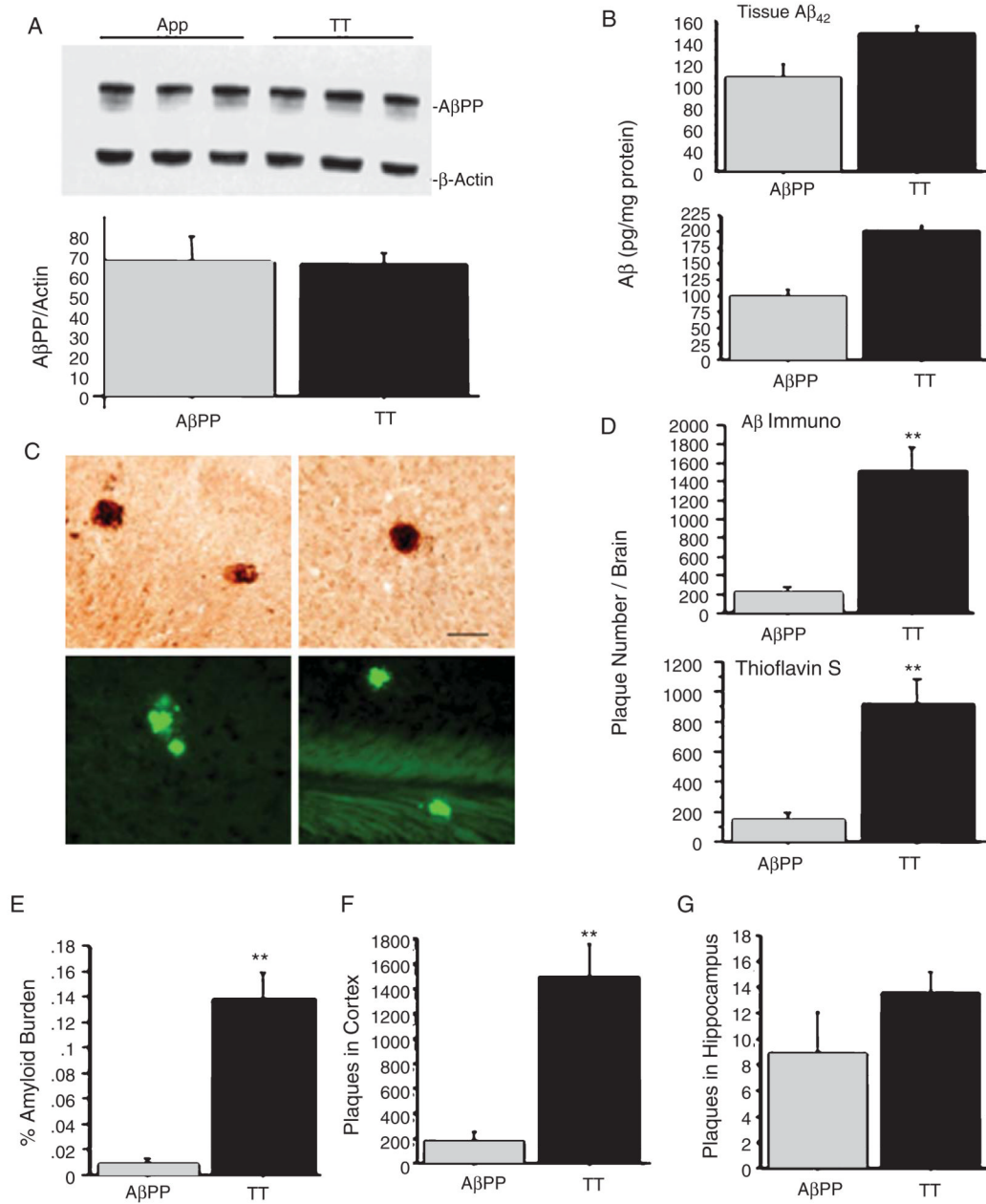
**Fig. 1.** Characterization of the novel triple transgenic mouse (TT). A) Electrophoresis gel analysis indicating the presence of *AβPP*, *Tetop-CRF*, and *CaMKII-tTA* DNA in TT mice. B) At 6 months of age, a Cushingoid-like phenotype with hair loss and an olive-shaped body was observed in both CT and TT mice. C) TT mice had significantly reduced body weights, starting from weaning (21 days) to 6 months of age ( $p < 0.001$ ,  $n = 8-10$  for each group). D) A significant effect of genotype on CRF protein levels in the cortex and hippocampus was observed ( $F(3,18) = 4.48$ ,  $p = 0.03$ ;  $n = 3-5$  for each group); with post-hoc testing (corrected for multiple tests) suggesting that both CT and TT mice had significant increases in CRF levels compared to AβPP+ and wild type mice (post-hoc  $p < 0.05$  in both cases). E, F) A significant effect of genotype on CRF receptor 1 (CRFR1) protein levels in the cortex and hippocampus was observed ( $F(3,18) = 4.48$ ,  $p = 0.04$ ,  $n = 3-5$  for each group), with both CT and TT mice again showing significant increases as compared to AβPP and wild type mice (post-hoc  $p < 0.05$  in both cases). G) A significant effect of genotype on basal plasma

corticosterone at 6 months of age (\* $p < 0.05$ , and \*\* $p < 0.01$ ). A $\beta$ PP (or APP): amyloid- $\beta$  protein precursor; CRF: corticotrophin releasing factor; Tetop-CRF: corticotrophin releasing factor gene under the control of tetracycline/doxycycline operator, CaMKII-tTA: tetracycline transactivator gene under the control of Ca<sup>2+</sup>/calmodulin-dependent protein kinases II promoter; CT: Tetop-CRF+/CaMKII-tTA+ mice or CRF+/tTA+ mice; TT: A $\beta$ PP+/Tetop-CRF+/CaMKII+ mice or A $\beta$ PP+/CRF+/tTA+ mice; WT: wild type control mice.

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**Fig. 2.**

Analysis of AβPP and Aβ deposition in TT and AβPP+ mice. A) AβPP levels were similar in TT and AβPP+ mice (an unpaired two way Student-Test,  $p = 0.93$ ) at 6 months of age. B) ELISA analysis shows that TT mice had higher levels of both soluble ( $p = 0.033$ ) (upper panel) and insoluble ( $p = 0.0024$ ) (lower panel) Aβ<sup>42</sup> levels at 6 months of age compared to levels in AβPP+ mice. C) Aβ immunohistochemical staining revealed substantial Aβ plaque deposition in the cortex of TT mice at 6 months of age (upper panels, Bar = 50 μm), but not at 3 months of age. Thioflavine staining revealed a similar distribution of plaque deposition (lower panels). D, E) Quantitative analysis of Aβ plaque deposition indicates a significant effect of genotype on Aβ plaque number ( $p < 0.0001$ ) by immunostaining and thioflavine S staining, D) and amyloid burden ( $p < 0.0001$ , E). F, G) TT mice showed dramatic increases

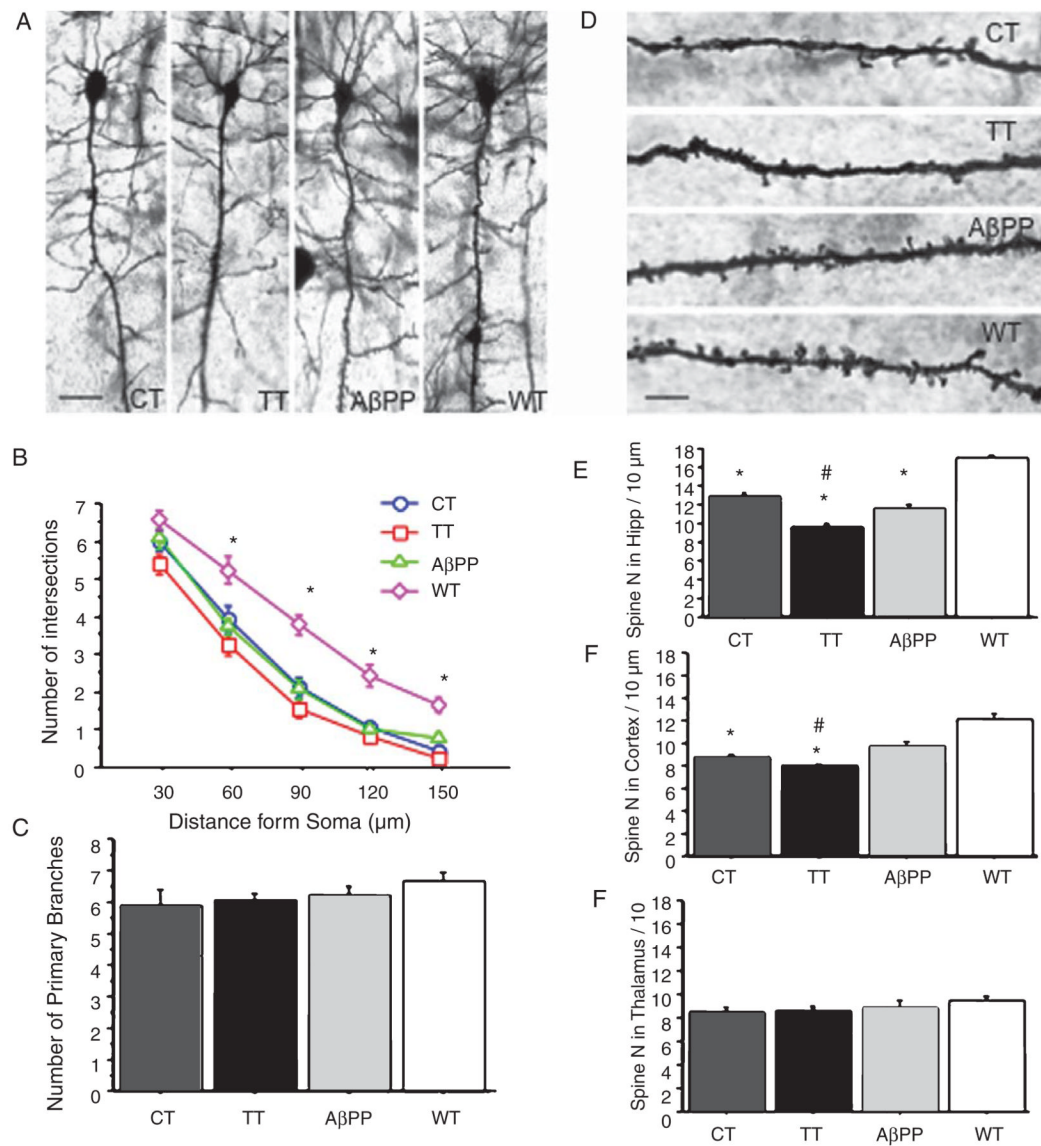


in plaque numbers and burden in the cortex, but not in the hippocampus, as compared to A $\beta$ PP+ mice (\*\* $p < 0.01$ ).

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**Fig. 3.**

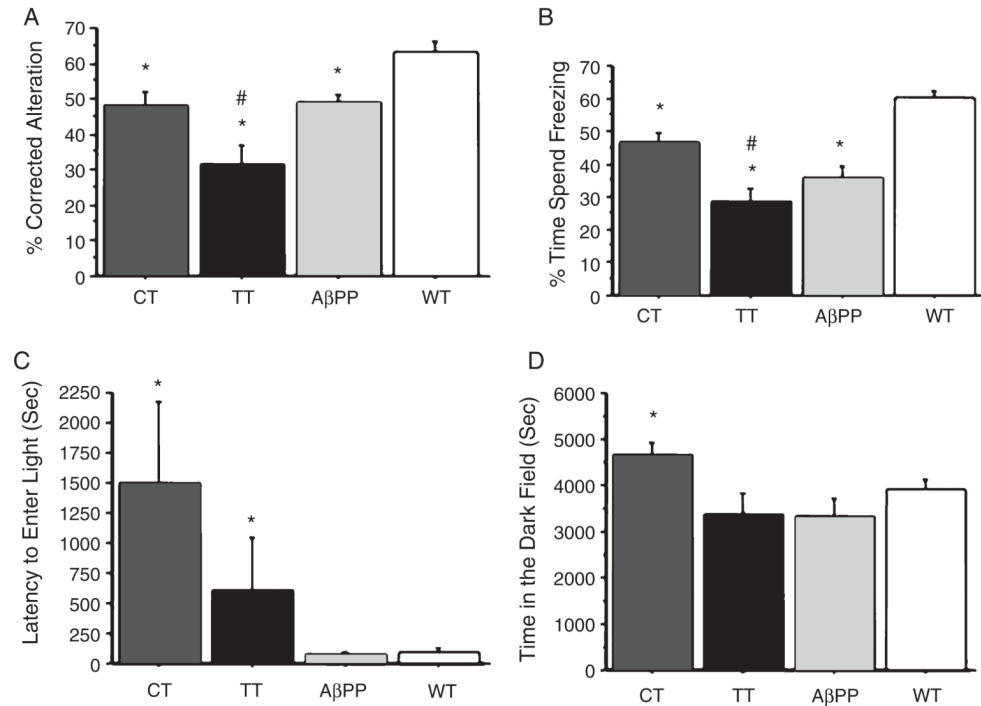
Evaluation of the dendrites spines in TT, CT, AβPP+, and WT mice. A) Golgi staining shows similar gross visual morphology of neuron and dendritic branching for TT, CT, AβPP+ and WT mice at 6 months of age. Scale bar = 50 μm. B) Sholl analysis (i.e., method of concentric circles) for pyramidal neurons in the cortex reveals a significant effect of genotype ( $F(3,61) = 15.84, p < 0.000$ ), distance from the soma ( $F(4,244) = 535.23, p < 0.0001$ ), and a genotype x distance interaction ( $F(12,2444) = 1.83, p = 0.044$ ) on dendritic arborization. The number of dendrite branches in the intersections was significantly decreased in TT mice starting at 60 μm from the soma. C) There is no difference in the number of the primary branches. D) Representative images of pyramidal neuron spines in the cortex of CT, TT, AβPP+ and WT mice from individual spines identified and counted under 100X magnification using stereological methods. Scale bar = 10 μm. E, F) A significant effect of genotype was found on spine density in the frontal cortex (E) ( $F(3,278) = 22.07, p < 0.0001$ ), and hippocampus (F) ( $F(3,136) = 71.28, p < 0.0001$ ). Spine density is expressed as number of spines per 10 μm dendrite length. Post-hoc testing showed

significant decreases spine density in CT, TT, and A $\beta$ PP+ mice as compared to WT mice ( $p < 0.01$ ) in the frontal cortex and hippocampus, with TT mice having lower values as compared to CT and A $\beta$ PP+ mice ( $p < 0.05$ ) (\* $p < 0.05$  compared to WT; # $p < 0.05$  compared to CT and A $\beta$ PP+ mice). G) No effect of genotype was observed on spine density in the thalamus.

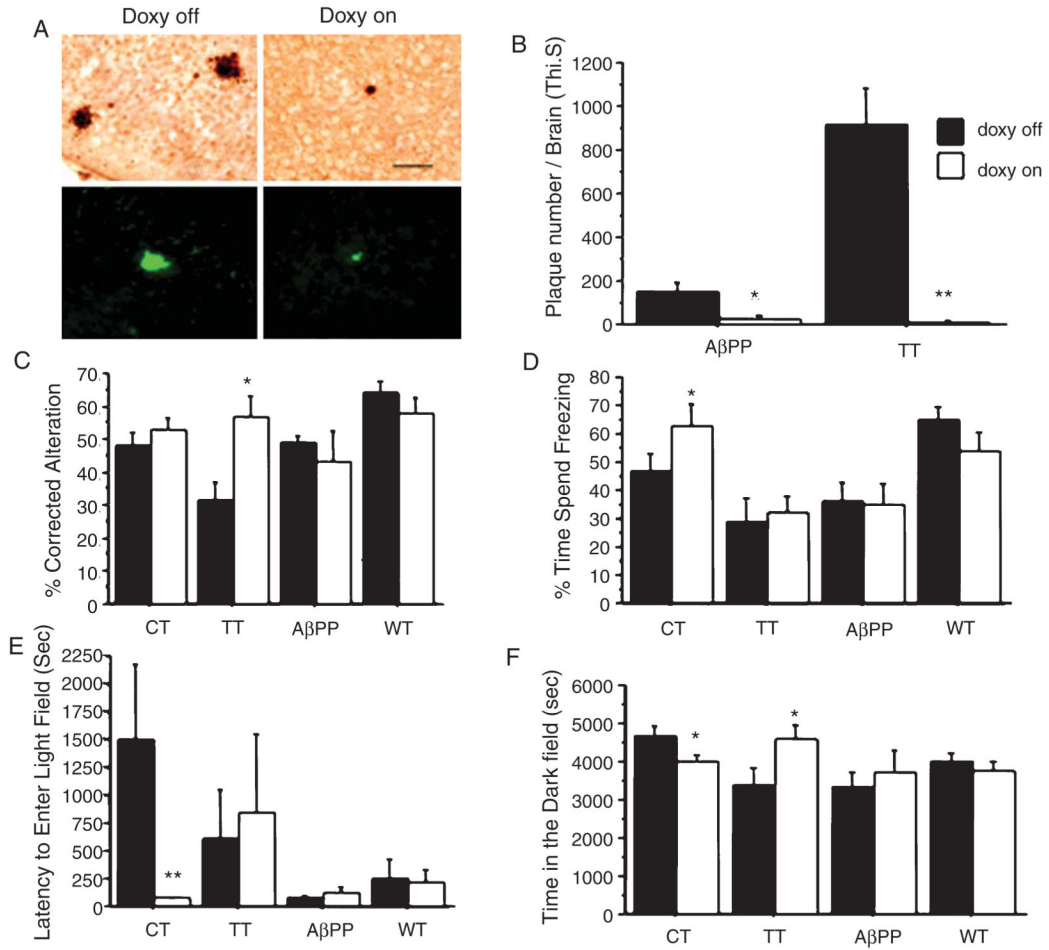
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**Fig. 4.** Memory and anxiety behavioral analysis of TT mice. In the spontaneous Y-maze alternation task, there was a significant effect of genotype on the percentage (%) of correct alternations at 6 months of age ( $F(3,32) = 6.92$ ;  $p = 0.001$ ). A) Post-hoc testing showed that CT, TT, and AβPP+ mice demonstrated decreases in correct alternations as compared to WT mice ( $*p < 0.05$ ), while TT mice demonstrated significant decreases in correct alternations as compared to CT, AβPP+, and WT mice ( $#p < 0.05$ ). Similarly, in conditioned fear testing, there was a significant effect of genotype on freezing behavior in response to context ( $F(3,62) = 5.352$ ,  $p = 0.0024$ ) at 6 months of age. B) Post-hoc tests again showed that CT, TT, and AβPP+ mice demonstrated less freezing behavior than WT mice ( $*p < 0.05$ ), while TT mice demonstrated less freezing behavior than CT and WT mice ( $#p < 0.05$ ), but not AβPP+ mice. The light-dark (L:D) preference test showed a significant effect of genotype on the latency to enter the light chamber ( $F(3,32) = 3.08$ ,  $p = 0.04$ ) and overall time in the dark chamber ( $F(3,32) = 3.84$ ,  $p = 0.02$ ). C) Post-hoc testing indicated that CT and TT mice had longer latencies to enter the light chamber as compared to AβPP+ and WT. D) CT mice spent more time in the dark chamber as compared to TT, AβPP+, and WT mice ( $*p < 0.05$  as compared to WT mice;  $#p$  values  $< 0.05$  as compared to CT and AβPP+ mice).

**Fig. 5.**

Doxycycline reverses the stress induced pathological and behavioral phenotypes in TT mice. A) Fewer Aβ plaques in the cortex and hippocampus were detected by Aβ immunohistochemistry and thioflavine S staining in TT mice fed doxycycline in their chow from weaning until 6 months of age, (scale bar = 50 μm). B) A quantitative analysis of thioflavine S stained sections shows significant effects of genotype ( $F(1,21) = 17.93, p = 0.0004$ ), drug ( $F(21,1) = 33.8, p < 0.0001$ ), and a genotype X drug interaction ( $F(1,21) = 19.09, p = 0.0003$ ). Post-hoc testing indicated that doxycycline almost-completely blocked Aβ plaque deposition in TT mice. Doxycycline also influenced Aβ plaque deposition in AβPP+ mice. C) We also observed a significant effect of genotype ( $F(3,66) = 4.6, p = 0.005$ ) and a genotype \* drug interaction ( $F(3,66) = 4.46, p = 0.0065$ ) on spontaneous alternation behavior, with post-hoc testing indicated that TT mice improved their performance during doxycycline administration. D) A genotype effect ( $F(3,72) = 6.659, p = 0.0005$ ), but neither a drug effect nor genotype \* drug interaction was observed for contextual memory behavior. Post-hoc analysis of freezing behavior indicates that CT but not the TT mice improved their contextual memory test after 6 months of doxycycline administration. E, F) Doxycycline also reversed the anxiety phenotype as measured by decreased the latency to enter light field and decreased time in the dark field in CT mice. (\* $p < 0.05$ ; \*\* $p < 0.01$  compared to the groups with doxycycline off. Dox: doxycycline.

**Table 1**

Comparison of TT mice and chronic isolation stressed Tg2576 mice

Animal (6 months of age)	Cognitive deficits	Anxiety	Tissue Ap	Ap plaques	Synaptic loss
TT (ApPP+/CRF+/fTA)	++++	+	++++	++++	++++
CT (CRF+/fTA+)	+++	++++	-	-	++
Stressed ApPP (iso-Tg2576)	++++	+	++++	++++	+++
Regular ApPP (reg-Tg2576)	++	±	++	±	+

±: Few animals showing the phenotype. +: Some animals showing the phenotype.

++, +++: Most animals; moderate behavioral deficits, tissue Ap levels, Ap plaque and synaptic loss.

++++, +++++: All animals; severe behavioral deficits, Ap levels, Ap plaque and synaptic loss.