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Chronic stress accelerates learning and memory impairments and increases amyloid deposition in APP_{V717I}-CT100 transgenic mice, an Alzheimer's disease model

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ABSTRACT

Although chronic stress is known to be linked with memory and other neurological disorders, little is known about the relationship between chronic stress and the onset or development of Alzheimer's disease (AD). In this study, we investigated the effects of long-term stress on the onset and severity of cognitive deficits and pathological changes in APP_{V717I}-CT100 mice overexpressing human APP-CT100 containing the London mutation (V717I) after exposure to immobilization stress. We found that chronic immobilization stress accelerated cognitive impairments, as accessed by the Passive avoidance and the Social Transfer of Food Preference (STFP) tests. Moreover, the numbers and densities of vascular and extracellular deposits containing amyloid beta peptide (Aβ) and carboxyl-terminal fragments of amyloid precursor protein (APP-CTFs), which are pathologic markers of AD, were significantly elevated in stressed animals, especially in the hippocampus. Moreover, stressed animals, also showed highly elevated levels of neurodegeneration and tau phosphorylation and increased intraneuronal Aβ and APP-CTFs immunoreactivities in the hippocampus and in the entorhinal and piriform cortex. This study provides the first evidence that chronic stress accelerates the onset and severity of cognitive deficits and that these are highly correlated with pathological changes, which thus indicates that chronic stress may be an important contributor to the onset and development of AD.

Key words: Long-term stress • cognitive impairments • amyloid plaque

In addition to the several genetic factors that are known to be involved in early-onset familial Alzheimer's disease (AD; i.e., linked missense mutations in amyloid precursor protein [APP] and the presenilin 1 and 2 genes), aging, and other factors, such as, environmental effects, are also believed to influence the pathogenesis and the behavioral disturbances associated with AD (1, 2). For example, a poor early life environment and a lower socioeconomic level may place a

person at higher risk of AD in later life (3, 4), whereas a higher level of education has been reported to significantly reduce the risk of AD (5).

Stress is an unavoidable condition of the human experience and includes both major life events and the problems of daily life, which both elevate the activities of physiological systems and disrupt homeostasis (6, 7). Stress is a risk factor for many diseases, such as depression and cardiovascular disease (7, 8, 9), and extensive research in rodents and humans has demonstrated that stress is a biologically significant factor that can alter brain cell properties, disturb cognitive processes, such as, learning and memory, and consequently limit the quality of human life (10, 11). Moreover, in humans and animals, the offspring of mothers that experience stress during pregnancy have been reported to display life-long cognitive dysfunctions, which can induce structural and morphological changes in the brain (12–14).

The hippocampus is a target for glucocorticoid stress hormones, which promote hippocampal aging (e.g., decreased neuronal density, altered synaptic plasticity, dendritic atrophy, and spatial learning deficits) (15–20). Moreover, the hippocampus is a recognized vulnerable region in AD.

The above-mentioned findings support the hypothesis that chronic stress plays a pivotal role in the development of AD. However, it has not been elucidated as to whether long-term adverse environmental conditions, such as stress, could affect the onset or degree of cognitive dysfunction in AD.

In this study, we investigated the effects of long-term stress (8 months of immobilization) on the onset and degree of cognitive dysfunction and on pathological changes in APP_{V717I}-CT100 mice. These mice overexpress human APP-CT100 containing the London mutation (V717I) and are a recognized transgenic model of AD (21).

Our study provides evidence that chronic stress accelerates the onset and severity of cognitive dysfunctions and that these are highly correlated with increased extracellular amyloid deposits, elevated immunoreactivities of intraneuronal amyloid beta peptide (Aβ) and carboxyl-terminal fragments of APP (APP-CTFs), and increased neurodegeneration and tau phosphorylation in the hippocampus and in the entorhinal and piriform cortex, which suggests that chronic stress is an important contributor to the onset and development of AD.

MATERIALS AND METHODS

Reagents and antibodies

6E10 antibody specifically recognizing the 1–17 amino sequence of human Aβ was obtained from Signet Laboratories (Dedham, MA); C9 antibody specifically recognizing the last 9 amino acids of the C-terminus of APP from Chemicon International (Temecula, CA); and AT8 antibody specifically recognizing the phospho Ser^{202}/Thr^{205} tau from Innogenetics (Gent, Belgium). Both Vectastain Elite ABC obtained from Vector Laboratories (Burlingame, CA) and 3, 3-diaminobenzidine-tetrahydrochloride (DAB) from DAKO Corp. (Carpinteria, CA) were used for immunohistochemistry. FITC- and Cy3-conjugated donkey Ig G antibodies were obtained from Jackson ImmunoResearch (West Grove, PA); both were used for immunohistofluorescence by confocal microscopy.

APP_{V717I}-CT100 transgenic mice

APP_{V717I}-CT100 mice overexpressing familial AD V717I (valine to isoleucine) "London" mutation within the 100 amino acid carboxyl-terminal (C-terminal) of human amyloid precursor protein (APP-CT100), encompassing the Aβ sequence, were prepared as described previously (21). Briefly, site-directed mutagenesis was used to introduce the V717I mutation into cDNA encoding the CT100 fragment of human APP. The mutated cDNA was then subcloned into murine Thy1 genomic expression vectors at an XhoI site, and a Kozak consensus sequence was introduced upstream of the initiation codon. Transgenic mice were produced by the pronuclear injection of (C57BL/6J × CBA/Ca) F1 embryos. Founders were identified by PCR analysis of tail biopsy lysates using the primer pairs 5' TTCCGACATGACTCAGGATATGAAGTTC 3' and 5' CGTTCTGCTGCATCTTGGACAGG 3' (CT100). Founder animals were intercrossed with (C57BL/6J-CBA/Ca) F1 mice to establish lines.

Tg2576 transgenic mice

APPswe Tg2576 mice (22) were obtained from Taconic Farms, Germantown, NY, and were bred by mating male mice with C57Bl6/SJL F1 females, as recommended by the suppliers and as described by others (23). Studies were performed by comparing heterozygous transgenic (Tg2576) mice to age-matched, transgene-negative littermates (non-Tg).

Immobilization stress

Experiments were performed during the light period of the circadian cycle. Control animals were left undisturbed and allowed contact with each other, but stressed animals were housed in isolation and subjected to 6 h/day of immobilization stress in a mouse stress box (4.5W×10L×4.5H cm) in their home cages. This stress was initiated 3 months after birth and was performed 4 days per week in an irregular manner to prevent habituation.

All experiments were performed in accordance with Guidelines for Animal Experiments issued by the Ethics Committee of Seoul National University.

Behavioral tests

The best way found of assessing learning and memory in transgenic or knockout mice involves mouse-friendly tasks that require different sensory and motor abilities (24). Here we used two behavioral tests: the Passive Avoidance Task (24) and the Social Transfer of Food Preference (STFP) task (21).

Passive Avoidance Task

The Passive Avoidance Task determines the ability of a mouse to remember a foot shock delivered 24 h earlier (24). The box used for this purpose was divided into two compartments, one illuminated and one dark; both were equipped with a grid floor. Training begins by placing a mouse in the light chamber for 10 s and then opening the door between the chambers. Most strains of mice are highly exploratory and prefer the dark chamber, and thus mice quickly enter the dark chamber. When a mouse does so, the inter-chamber door is closed and a single foot shock is delivered through the grid floor (0.3 mA, 1 s). The mouse is held in the dark chamber for an

additional 10 s, to allow the animal to form an association between the properties of the chamber and the foot shock. It is then returned to its home cage. During actual testing, performed 1 day after a training trial, a mouse was placed in the light compartment and the time was taken (the step-through latency) for it to enter the dark compartment was recorded. The maximum test latency was set at 300 s, at which the test was discontinued.

STFP task

The STFP task is used to assess olfactory memory and spatial memory function (21). This assessment took place over a 7-day period during, which normal home cage food was restricted. Demonstrator animals (120 days old; 129sv*C57BL/6J) were housed in the same holding rooms as the test mice and were maintained on the same food-restricted regime. In keeping with the home office project license conditions, no animal was permitted to lose more than 20% of free-feeding body weight during this experiment, and any mouse exceeding this criterion was removed from the experiment and fed normally. Two habituation sessions were undertaken on consecutive days, during which mice were permitted to freely explore the test arena for 30 min, which contained a single food dispenser filled with 2 g of powdered standard laboratory chow (plain food). A test of basic olfaction was undertaken prior to STFP testing, which involved placing two food dispensers into the test arena. One dispenser contained plain food, and the other contained powdered chow mixed with ginger (10%) or coriander (1%). Equal amounts of each food type were placed in the dispensers, and the mice were allowed to freely explore for 30 min. During the social interaction phase, demonstrator mice were given access to powdered chow mixed with one of four aromas (1% cumin, 5% nutmeg, 10% coffee, or 10% cocoa) for 2 h (the cued food). Food consumption was monitored, and demonstrator mice were only used in the ensuing social interaction if they had consumed more than 0.4 g of cued food. To achieve social interaction, test mice were placed in the test arena with two demonstrator mice (sedated with 15 mg sodium of pentobarbitone-Sagatal/kg of body weight intraperitoneally), and the mice were left to interact for 5 min. The behavior of the test mice during the ensuing social interaction was monitored by video. The time spent by the test mice in close contact with the mouths of demonstrator mice has been shown to be a key factor in the learning process. During this social interaction session, numbers, latencies, and durations of contacts with the mouths of demonstrator mice were determined. Memory tests of 1 and 24 h were conducted after this social interaction, when test mice were presented with a choice of two foods, i.e., a dispenser containing powdered food with the same aroma as that used for the social interaction (the cued food) and another mixed with a novel aroma (uncued food). After 30 min of exploration and food consumption, mice were returned and fed ad libitum in home cages. In sessions when food was present, the amount of food consumed was calculated and plain food preference (olfaction test) and cued food preferences (memory tests) were calculated from total food consumed.

Estimation of plasma corticosterone

After behavioral tests, transgenic animals were sacrificed and blood samples were obtained by heart puncture. The fluorimetric method (25) was used to estimation plasma corticosterone, as an index of hypothalamo-pituitary-adrenal axis (HPA) function. Plasma stored at –70C° overnight was used for these determinations. A reaction mixture consisting of 1 ml plasma and 7.5 ml dichloromethane was shaken for 2 min and centrifuged (in order to separate the phases) and then the plasma layer was removed. At 0 time, 2.5 ml of fluorescence reagent (7 volumes of

concentrated sulphuric acid and 3 volumes of ethanol) was added; after shaking for 2 s, the supernatant was removed. At exactly 12 min, the acid extract was transferred to separate cuvettes for reading. The readings were taken at 530 nm with excitation at 470 nm. Concentrations are expressed as ng/ml of blood.

Histological examination

After behavioral tests, the brains of the transgenic animals were removed and immersed in 4% paraformaldehyde/PBS for histology. After fixation for 48 h at 48C°, brains were sequentially dehydrated in graded alcohols, treated with cedar wood oil and methyl salicylate, and embedded in paraffin. Hippocampal coronal sections (4 μm) were prepared and processed for immunohistochemistry and immunohistofluorescence.

Immunohistochemistry

Prior to immunostaining, sections mounted on slides were deparaffinized by oven heating and immersion in xylene. After dehydration through graded alcohols to tap water, endogenous peroxidase activity was quenched by incubating sections with 1% hydrogen peroxide in methanol. They were then washed in 0.05 M tris-buffered saline (TBS) and incubated with primary antibodies in TBS containing 0.5% BSA and 0.5% Triton-X 100 overnight at 4C°. After being washed three times with TBS, slides were incubated with biotinylated secondary antibodies (Vectastain Elite ABC) for 45 min at room temperature, then for 1 h with avidin-biotin-peroxidase complex (Vectastain Elite ABC). Reaction product was detected using 3, 3-diaminobenzidine-tetrahydrochloride (DAB) as a chromogen (0.05% diaminobenzidine, 0.01% H_2O_2 in PBS). Finally, sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped using Canadian balsam solution. Peroxidase stained sections were examined under a light microscope (Olympus PM-20).

Immunohistofluorescence by confocal microscopy

Tissues were processed prior to primary antibody incubation in immunohistochemistry, as described above, and then incubated with primary antibodies overnight at 4°C. After three washes with TBS, primary antibodies were revealed by incubating the tissues for 1 h with Cy3- or Cy2-conjugated secondary antibodies. Sections were mounted in anti-fade medium (fluorescent mounting medium containing 15 mM NaN_3, Dako) on glass slides, and fluorescence images were obtained using a Zeiss LSM510 confocal laser-scanning microscope mounted on a Zeiss Axiovert 200 inverted microscope.

Hematoxylin and eosin staining

Slides were processed prior to primary antibody incubation in immunohistochemistry, as described above, and then incubated with hematoxylin and eosin. Sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in Canadian balsam solution. Cell loss in brain sections was determined using a light microscope (Olympus PM-20).

Statistical analysis

Results are presented as mean ± SEM. All statistical analyses were performed using Statview statistic software (SAS Institute, Cary, NC). Passive avoidance and STFP comparisons were made using one-way ANOVA, and further examinations of group differences were performed using Tukey's and Duncan's post hoc analyses. The student's *t* test was used to compare plasma corticosterone concentration data. *P* values <0.05 were considered statistically significant.

RESULTS

Long-term stress accelerates behavioral impairments in APP_{V717I}-CT100 transgenic mice

To examine the relationship between chronic stress and cognitive deficits in our AD mice model, we assessed learning and memory ability by using passive avoidance and STFP tests, after administering chronic immobilization stress for 8 months. APP_{V717I}-CT100 mice were housed under stress (CT-S) or non-stress conditions (CT-C), as described above, from 3 months after birth. APP_{V717I}-CT100 mice, 24 months old and housed under non-stress conditions (CT-C old), were used as positive controls. CT-S group animals exhibited significant memory deficits compared with the CT-C group by Passive Avoidance test (Fig. 1*A*) (n=5 per group, $F_{2,12}$=10.26, **P*<0.05, one-way ANOVA) and post hoc (Tukey test) revealed that CT-S animals had a significantly lower latency (52.84 s±28.07) to enter the darkened chamber than CT-C animals (218.83 s±37.93). A significant group difference was also found between the latencies of CT-C (11 months, 218.83 s±37.93) and CT-C old (24 months, 33.93 s±11.48).

In the STFP task, an olfactory memory task based on conspecifics as a source of food choice information, significant differences were found between CT-C and CT-S or CT-C old animals (Fig. 1*B–D*). Olfactory-based behaviors can be confounded by general anosmia and, therefore, the task incorporated a basic olfactory discrimination test in addition to the olfactory memory test component. Mice were presented with a choice between familiar plain and novel aromatic powdered chows. Neophobia and olfactory functioning of APP_{V717I}-CT100 transgenic mice were measured by determining plain food preference (Fig. 1*B*). Plain food preference (%) was assessed by determining the ratio of plain food to total food consumed. In present study, plain food consumption exceeded 50%, which is consistent with the typical neophobic response expected of rodents. These results suggest intact normal olfactory functioning in all experimental groups (n=5 per group, mean±SEM) and that the only discriminating factor was a novel food flavoring.

We assessed short-term and long-term olfactory memory retrieval ability by using the time difference cue test 1 and 24 h after demonstrator-observer interaction. Cued food preference (%) was assessed by determining the percentage of cued food consumed versus total food consumed. One hour after the demonstrator-observer interaction, significant group differences were observed in terms of cued food preference (%) (Fig. 1*C*) (n=5 per group, $F_{2,12}$=6.20, **P*<0.05, one-way ANOVA). Post hoc analysis using Duncan's test revealed that preference for cued food (%) was significantly lower in the CT-S group (45.31%±4.6) than in the CT-C group (64.28%±5.1), and a significant difference was observed between the CT-C (64.28%±5.1) and CT-C old groups (43.94%±3.8). We found that, 24 hours after demonstrator-observer interactions, cued food preference was significantly lower in the CT-S group than in the CT-C group (Fig. 1*D*) (n=5 per group, $F_{2,\ 13}$=5.15, **P*<0.05, one-way ANOVA), and post hoc analysis using Duncan's test revealed that the CT-S group (35.63%±6.9) had a significantly lower cued food preference (%)

than the CT-C group (60.76%±4.8). In addition, the CT-C old group (35.98%±8.3) showed a significantly lower cued food preference (%) than the CT-C group (60.76%±4.8).

Plasma corticosterone concentrations were determined as a means of quantifying stress, and immobilization stress caused a significantly greater in blood corticosterone in the CT-S group than in the CT-C group (Fig. 1*E*) (n=10 per group, $F_{1, 18}$=3.8, *P<0.05, student's t test).

Another series of experiments was conducted to confirm whether these behavioral changes were specific to AD Tg mice using another AD Tg mice model, namely, Tg 2576 and non-Tg mice. STFP test was performed after administering chronic stress, precisely as described above, for 6 months beginning from 3 to 9 months after birth. Tg 2576 mice housed under stress (Tg-S) showed a lower cued food preference than non-stressed Tg 2576 mice (Tg-C), and stressed (N-S) and non-stressed (N-C) non-Tg mice in the 1 h and 24 h cue tests (Fig. 2). Moreover, non-Tg mice were unaffected by chronic stress, indicating that the observed behavioral changes caused by chronic stress were specific in AD Tg mice.

Long-term stress increased extracellular amyloid plaques and intraneuronal Aβ and APP-CTFs depositions in the hippocampus and cortex in APP_{V717I}-CT100 transgenic mice

To investigate the possibility of links between memory impairment severity and amyloid deposition, we examined extracellular amyloid plaque load and intraneuronal Aβ and APP-CTFs depositions in 11-month-old APP_{V717I}-CT100 transgenic mice (i.e., after behavioral tests) by using 6E10 antibody, which specifically recognizes the 1–17 amino acids sequence of the Aβ region, and by using C9 antibody, which specifically recognizes the last 9 amino acids of the APP-C terminus (Fig. 3).

Dense cored plaques (represented by short-tailed arrow) and vascular deposits (represented by long-tailed arrow) of amyloid were detected immunohistochemically using 6E10 (Fig. 3*A–C*) and C9 antibodies (Fig. 3*D–F*) in the serial brain paraffin sections of the CT-C, CT-S, and CT-C old groups. In brain sections, extracellular Aβ immunoreactive plaques and vascular deposits, as detected by 6E10 antibody, were highly stained in the hippocampal region (Fig. 3*A–C*) and cortex (data not shown). However, extracellular Aβ plaques in the hippocampal region of the CT-S group (Fig. 3*B*) were significantly more numerous than in the CT-C group (Fig. 3*A*). In the dentate gyrus (DG) of the hippocampus (Fig. 3*D–F*) and in the cortex (data not shown) of serial brain sections, some APP-CTFs immunoreactive plaques and vascular deposits, stained by C9 antibody, were found to concur with extracellular Aβ immunoreactive plaques and vascular deposits (Fig. 3*A–C*), although Aβ immunoreactive plaques and vascular deposits predominated extracellularly in the hippocampus and cortex.

And then, serial brain sections were also labeled with 6E10 and C9 antibodies conjugated with FITC (green) and Cy3 (red), respectively, and were visualized by confocal microscopy. Interestingly, the intraneuronal immunoreactivities of Aβ and APP-CTFs were highly elevated in the CT-S and the CT-C old group versus the CT-C group, especially in the hippocampal CA3 region (Fig. 3*J–L*).

The intraneuronal immunoreactivities of Aβ and APP-CTFs were markedly increased in the CT-S group (Fig. 4*B*, *E*) versus the CT-C group (Fig. 4*A*, *D*) in the entorhinal and piriform cortex. In the

CT-C old group, the immunoreactivities of Aβ and APP-CTFs were significantly more increased in the entorhinal cortex than in the CT-C and CT-S groups (Fig. 4*A–C*), but in the piriform cortex, immunoreactivities were similar in the CT-C old and CT-S group (Fig. 4*E*, *F*).

Neuronal degenerative changes were observed by hematoxylin and eosin (H&E) staining. In serial brain sections, degenerating neurons (eosinophilic pyknotic neuron; Fig. 3*M–R*) were observed in hippocampal C9 antibody positively stained regions (Fig. 3*D–I*). Profoundly degenerated neurons were remarkably more increased in the hippocampal CA3 region of the CT-S group (Fig. 3*Q*) than in the CT-C group (Fig. 3*P*). However, no large difference was observed between the CT-S (Fig. 3*N*) and CT-C (Fig. 3*M*) groups in terms of the numbers of eosinophilic neurons in the hippocampal DG.

Degenerating neurons (Fig. 4*G–L*) were also observed in regions stained by 6E10 and C9 antibody (Fig. 4*A–F*) in the entorhinal and piriform cortex. Moreover, in accordance with Aβ and APP-CTFs immunoreactivities, higher numbers of degenerating neurons were observed in the entorhinal cortex of the CT-S and CT-C old groups (Fig. 4*H*, *I*) and in the piriform cortex of the CT-S group (Fig. 4*K*) than in the CT-C group (Fig. 4*G*, *J*).

Long-term stress increased tau phosphorylation in the hippocampus and cortex in APPV_{717I}-CT100 transgenic mice

Tau phosphorylation was observed in serial brain sections showing Aβ and APP-CTFs immunoreactivities. Brain sections were probed with AT-8 antibody specifically recognizing phospho Ser^{202}/Thr^{205} tau and with C9 antibody conjugated with FITC (green) and Cy3 (red), respectively. Tau phosphorylation levels were low in the hippocampal DG and CA1 regions, and no significant differences were observed between the CT-C, CT-S, and CT-C old groups in this respect. However, tau phosphorylation levels were much higher in the hippocampal CA3 region and entorhinal and piriform cortex of CT-S and CT-C old group animals (Fig. 5*K*, *L*, *O*, *P*, *S*, *T*) than in CT-C group animals (Fig. 5*J*, *N*, *R*). Interestingly, phosphorylated tau and APP-CTFs immunoreactivities substantially overlapped in these regions.

DISCUSSION

The presence of amyloid plaques containing predominantly Aβ and neurofibrillary tangles is believed to be a pathological feature of AD (1, 2). Moreover, the APP_{V717I} point mutation (numbering based on APP 770), which was identified in the London pedigree, is known to be associated with aggressive early-onset dementia. This APP mutation shows characteristic increases in the long amyloidogenic form of Aβ ($Aβ_{1–42}$) in the AD brain (26). In a previous study, the expression of the APP_{V717I}-CT100 transgene was found to lead to the productions of Aβ and APP-CTFs and the deposition of diffuse, noncongophilic extracellular Aβ plaque in APP_{V717I}-CT100 mice beginning at 6 months of age (21), and immunohistochemistry using Aβ-specific antibodies revealed its widespread distribution and accumulations in the brain parenchyma and cerebral blood vessels. Moreover, this distribution of Aβ plaque was detected most extensively in the cortex and in hippocampal regions, although it was also found in other brain regions, including the olfactory bulbs and hindbrain. However, in APP_{V717I}-CT100 mice, spatial olfactory memory deficits occurred from the age of 12 months (21).

In the present study, we investigated the effects of chronic stress on the onset and severity of cognitive deficits and on pathological changes in APP_{V717I}-CT100 transgenic mice. It was found that exposure of these mice to immobilization stress for 8 months (from age 3 to 11 months) resulted in severe learning and memory impairments and that it increased extracellular amyloid plaque deposition, intraneuronal Aβ, and APP-CTFs immunoreactivities, neurodegeneration, and tau phosphorylation.

The Passive Avoidance test has been widely used to evaluate rodent working memory ability in association with cortical and hippocampal functions (24). In the present study, Passive Avoidance test showed that latency times were not recovered in the CT-S group at 24 h after electric shock, suggesting that memory retrieval ability was more severely impaired by chronic stress. The STFP task assesses social olfactory and paired associate learning (27), which are hippocampus-dependent (28–31). In previous studies, olfaction ability was impaired in the early stages of AD; moreover, the olfactory memory test is an accepted highly sensitive test for detecting cognitive deficits in the early stages of AD (32–36). In the present study, 1 and 24 h after demonstrator-observer interaction, short-term and long-term memory retrieval abilities were found to be more severely impaired in the CT-S group than in the CT-C group. In the CT-C old groups, the olfactory memory was greatly impaired by aging.

Overall, as accessed by the Passive avoidance and STFP tests, memory dysfunction was found to be more severe in the CT-S group and CT-C old group than in the CT-C group. In a previous study (21), APP_{V717I}-CT100 mice were first found to show memory impairments at 12 months. Interestingly, in the present study, chronic stress not only induced an earlier onset of learning and memory impairments, but also accelerated the severity and progress of memory deficits, suggesting that the progression of memory impairments in AD is greatly affected by chronic stress.

The amount of extracellular amyloid plaque was greater in the hippocampus and cortex of the CT-S group than in the CT-C group. In serial brain sections, APP-CTFs immunoreactive plaques largely overlapped with Aβ immunoreactive plaques, indicating that most Aβ immunoreactive plaques contained APP-CTFs.

Many studies have reported upon the neurotoxicity of APP-CTFs in AD development. APP-CTFs, the carboxy-terminal fragments of APP, have been identified in the AD patients' brains, and have been reported to be substantially more neurotoxic in a variety of preparations than Aβ (37–42). Furthermore, APP-CTFs are known to impair calcium homeostasis (43, 44, 45), learning, and memory by blocking LTP and thus trigger inflammatory reaction through MAPKs- and NF-κB-dependent astrocytosis and iNOS induction (46–48). Recently, it was reported that APP-CTFs translocate into the nucleus, where they bind Fe65 and CP2, which affects glycogen synthase kinase-3beta transcription, tau hyperphosphorylation, and cell death (49).

In addition, it was reported that intraneuronal Aβ accumulation is correlated with long-term synaptic plasticity deficits and earliest cognitive impairments in 3× Tg-AD mice (50, 51).

Interestingly, significant increases of intraneuronal Aβ and APP-CTFs immunoreactivities were observed in the CT-S group than in the CT-C group, especially in the CA3 region of the hippocampus. In addition, also high levels of neurodegeneration were in the CA3 region of serial

brain section, which suggests that increased intraneuronal Aβ and APP-CTFs levels might significantly be correlated with neurodegeneration.

The hippocampus is a target of stress hormones, and it is an especially plastic and vulnerable region of the brain. Chronic repeated psychosocial or restraint stress, or chronic treatment with corticosterone or adrenal steroids along with excitatory amino acids, especially causes apical dendrites atrophy in CA3 pyramidal neurons and specific cognitive deficits in spatial learning and memory and alters mossy fiber synaptic terminal structure (52–55). However, dendritic atrophy was not observed in granule cells of the dentate gyrus, or in CA1 or CA2 pyramidal cells affected by these treatments (56). This structural plasticity and atrophy are mediated by glucocorticoid and excitatory amino acids, through NMDA glutamate receptors, which also participate in this plasticity (57).

Taken together, it appears that chronic stress increases extracellular plaque formation, the intraneuronal expressions of Aβ and APP-CTFs, and neurodegeneration in the hippocampus, especially in the CA3 region of the hippocampus, a specific target region of stress hormones, by increasing the production and processing of APP-CTFs via hormonal stress mechanisms.

The adult piriform cortex shows abundant glucocorticoid receptor expression (58, 59) and is a limbic area activated by acute restraint stress (60). Moreover, patients with posttraumatic stress disorder are less proficient at odor identification, which involves the piriform cortex (61). Recently, it has been reported that the piriform cortex is targeted by stress and stress-related hormones and that chronic restraint stress and chronic corticosterone treatment induce differential changes in the expression of molecules related to structural plasticity in the piriform cortex (62). Numerous studies have also reported that AD patients exhibit olfactory dysfunction, particular-olfactory identification impairment (32, 35, 36).

In our study, the immunoreactivities of Aβ and APP-CTFs in the piriform cortex were found to be extensively elevated in the CT-S group, and this group was also found to have an impaired olfactory memory versus the CT-C group. Bearing in mind that the piriform cortex is targeted by stress hormones, this result suggests that Aβ and APP-CTF might also be upregulated by hormonal stress.

One of the hallmarks of AD, neurofibrillary tangles (NFTs) are formed by insoluble intracellular polymers of hyperphosphorylated tau (63). Hyperphosphorylated tau is considered to be one of the earliest signs of neuronal degeneration. Previously, we demonstrated that AICD and C31 exerted neurotoxicity by inducing GSK-3β expression and increased tau phosphorylation through the formation of a ternary complex between Fe65 and CP2/LSF/LBP1 transcription factor in the nucleus of differentiated PC12 cells (49). In addition, the hyperphosphorylated tau is the major component of paired helical filaments (PHFs) and NFTs and is believed to cause apoptosis by disrupting cytoskeletal and axonal transport (63). Furthermore, it has been reported that there was enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP and that the injection of β-amyloid Ab42 fibrils into the brains of P301L mutant tau transgenic mice increased neurofibrillary tangle formation (64, 65).

The present study shows that tau phosphorylation was significantly higher in the CA3 region of the hippocampus and in the entorhinal and piriform cortex of the CT-S group than in those of the

CT-C group. We suggest that this high level of intraneuronal abnormal tau phosphorylation might be caused by the up-regulation of intraneuronal Aβ and APP-CTFs and that it may cause cellular death and cognitive deficits.

In conclusion, this study demonstrates that chronic stress accelerates cognitive impairments and increases extracellular amyloid deposition, intraneuronal Aβ and APP-CTFs immunoreactivity, tau phosphorylation, and neurodegeneration in an AD model, and suggests that disease onset, cognitive impairment, and neurodegeneration are correlated with depositions of Aβ and APP-CTFs. Overall, our study suggests that chronic stress is an important pathogenic factor in the onset and development of AD.

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Fig. 1

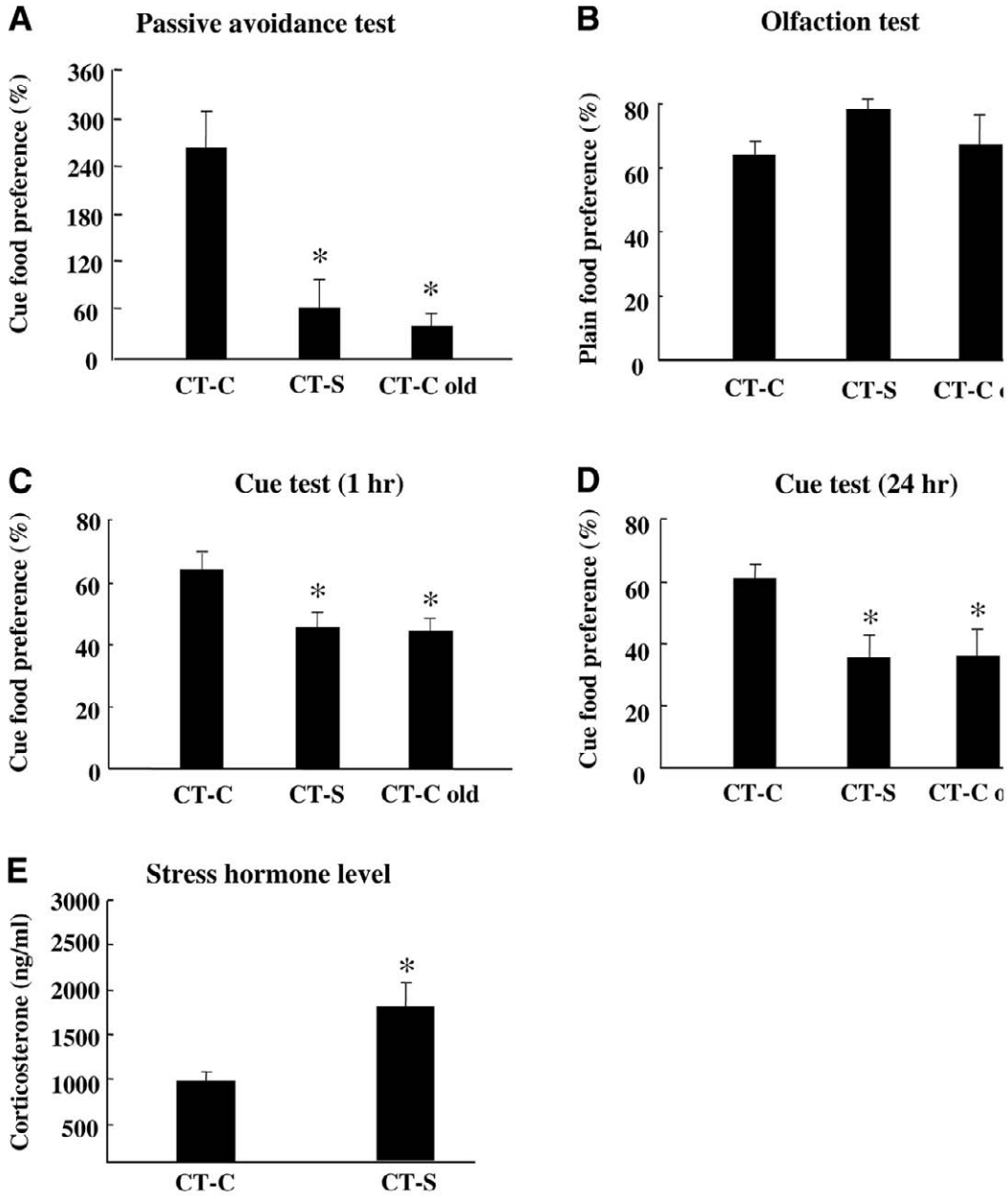

Figure 1. Long-term stress accelerates behavioral impairments in APP_{V717I}-CT100 transgenic mice. Passive avoidance and STFP tests were performed after administering chronic immobilization stress for 8 months. APP_{V717I}-CT100 mice were housed either under stressed (CT-S) or non-stressed conditions (CT-C) from 3 months after birth; 24-month-old APP_{V717I}-CT100 mice housed under non-stressed conditions (CT-C old) were used as positive controls. After 8 months of stress, passive avoidance test was performed and the CT-S group showed significant memory deficits compared with the CT-C group. ***A*)** Data presented are latencies in seconds (mean±SEM, n=5 per group) taken to enter a darkened chamber (*P<0.05; CT-S vs. CT-C and CT-C old vs. CT-C, by post hoc analysis via Tukey test). ***B*)** Neophobia and olfactory functioning as determined using the STFP task. Mice were presented with a choice between familiar "plain" food and the same food mixed with a novel odor to assess neophobic and discriminatory responses. Data are presented as plain food preference (%) (n=5 per group, mean±SEM). ***C*)** One hour cued STFP task. Data are presented as cued food preferences (%) (n=5 per group, $F_{2,12}$=6.20, *P<0.05; CT-S vs. CT-C and CT-C old vs. CT-C, by post hoc analysis via Duncan's test). ***D*)** Twenty-four hours cued STFP task. Figure shows cued food preferences (%) (n=5 per group, $F_{2,12}$=5.15, *P<0.05; CT-S vs. CT-C and CT-C old vs. CT-C, by post hoc analysis via Duncan's test). ***E*)** Plasma corticosterone concentrations were measured as measures stress. Chronic immobilization stress caused a significantly greater elevation in blood corticosterone levels in the CT-S group than in the CT-C group (n=10 per group, $F_{1,18}$ = 3.8, *P<0.05, student's t test).

Fig. 2

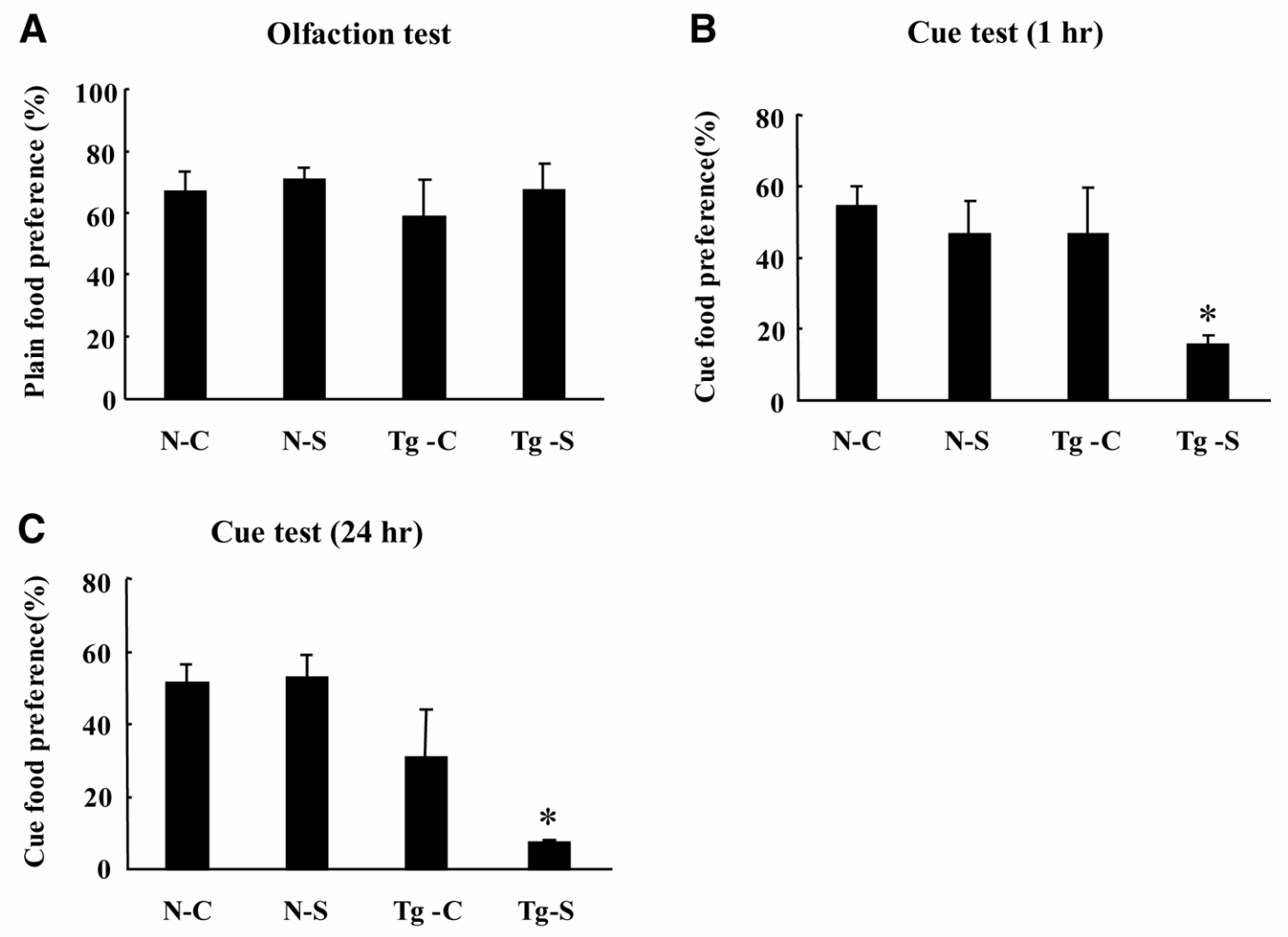

Figure 2. Long-term stress accelerates behavioral impairments in Tg2576 mice. STFP tests were performed after administering chronic immobilization stress for 6 months. Tg2576 and non-Transgenic mice were housed either under stressed (Tg-S and N-S) or non-stressed conditions (Tg-C and N-C) from 3 months after birth. ***A)*** Neophobia and olfactory functioning as determined by STFP. Data are presented as plain food preferences (%) (n=5 per group, mean±SEM). ***B)*** One-hour cued STFP task. Data are presented as cued food preferences (%) (n=5 per group, $F_{3, 16}$=2.87, *P<0.05; Tg-S vs. N-C, N-S, and Tg-C, by post hoc analysis via Duncan's test). ***C)*** Twenty-four-hour cued STFP task. The figure shows cued food preferences (%) (n=5 per group, $F_{3, 16}$=4.73, *P<0.05; Tg-S vs. N-C, N-S, and Tg-C, by post hoc analysis via Duncan's test).

Fig. 3

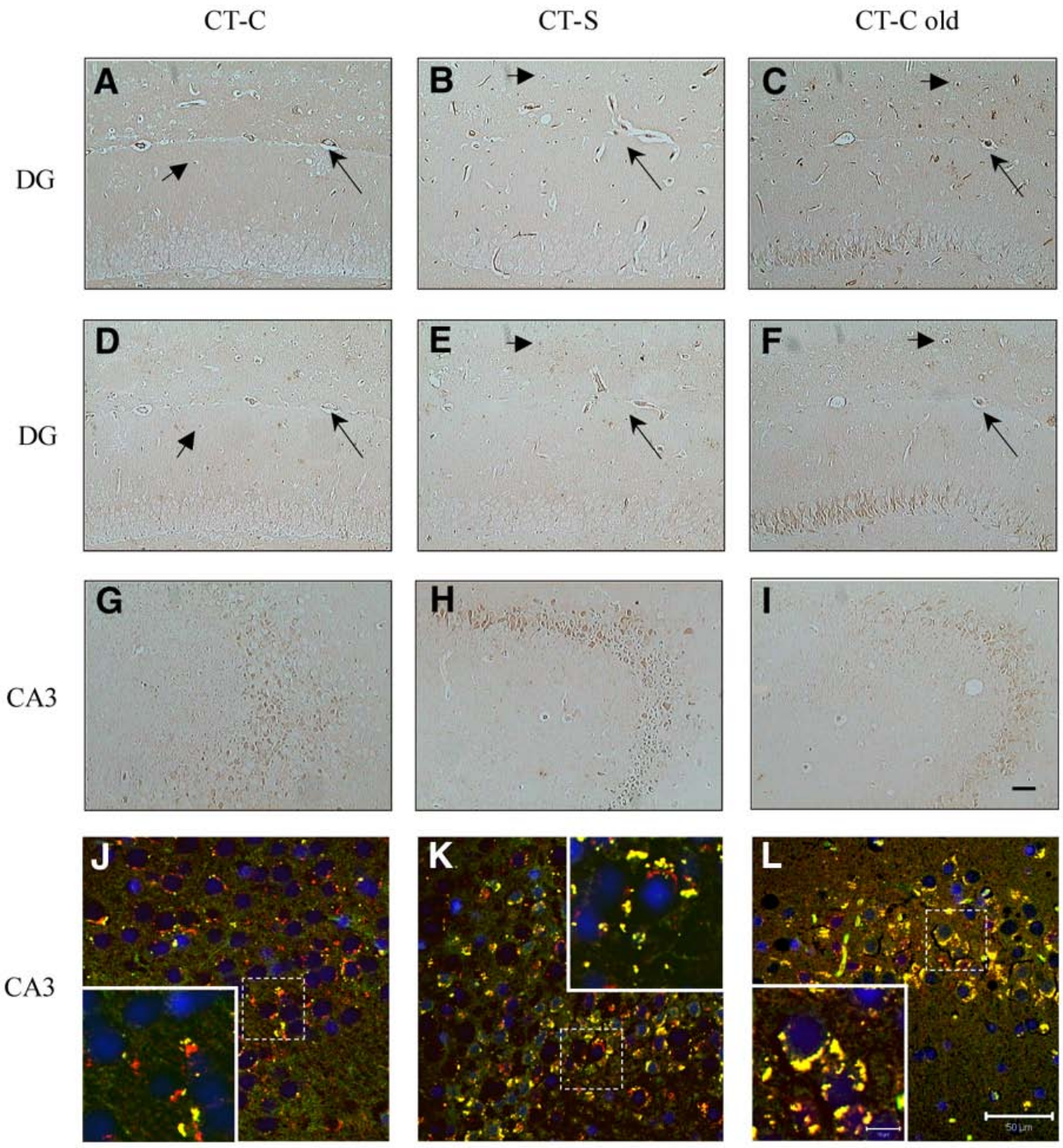

Fig. 3 (cont)

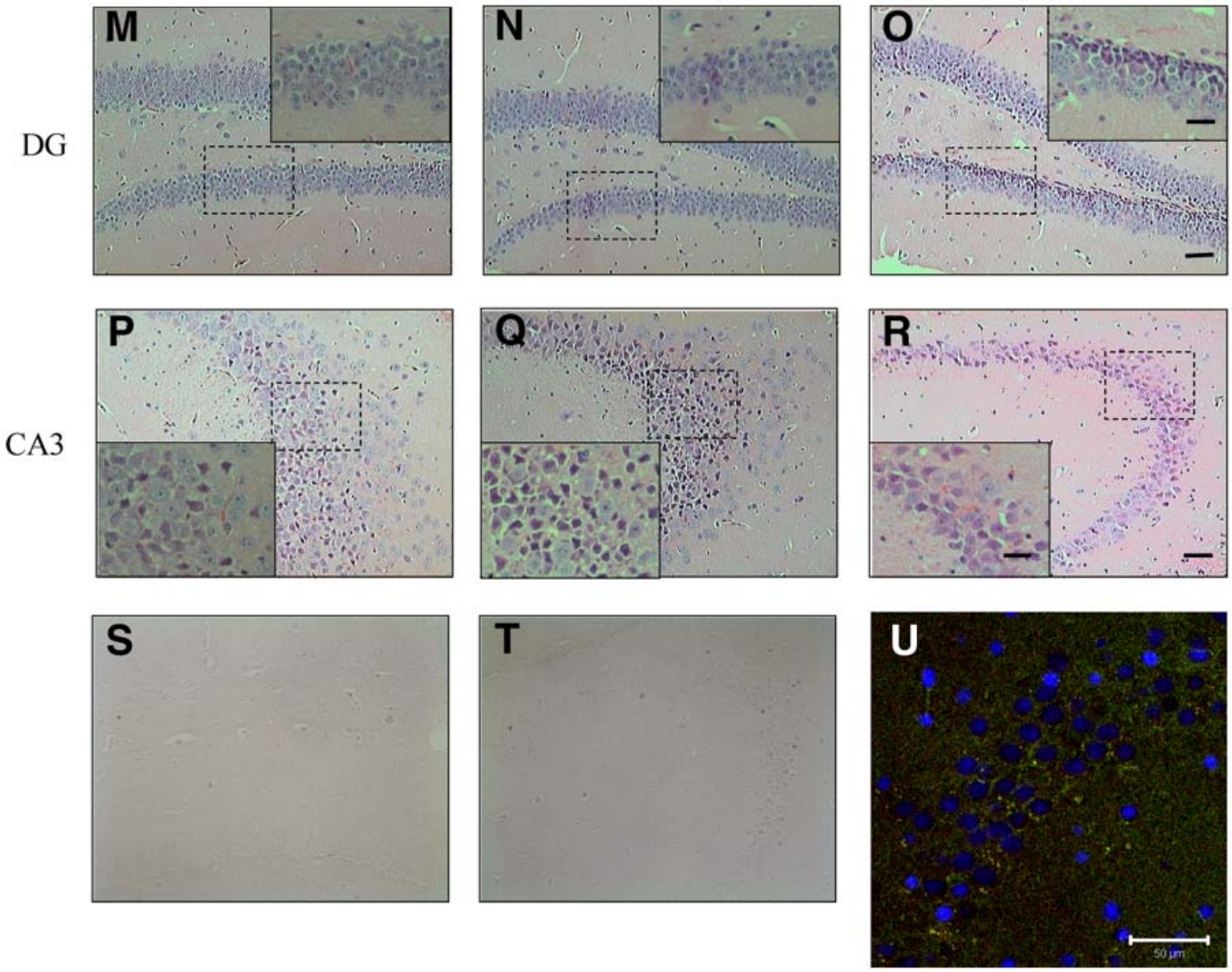

Figure 3. Long-term stress increased extracellular amyloid plaques and intraneuronal depositions of Aβ and APP-CTFs in the hippocampus of APPV717I-CT100 transgenic mice. Extracellular amyloid plaque load and intraneuronal Aβ and APP-CTFs deposition levels were examined in APP_{V717I}-CT100 transgenic mice at 11 months old (i.e., after behavioral testing) using 6E10 antibody, which specifically recognizes the 1–17 amino acid sequence of the Aβ region, and using C9 antibody, which specifically recognizes the last 9 amino acids of the APP-C terminus. Degenerating neurons were examined in the hippocampal dentate gyrus (DG) and in the CA3 region in serial brain sections by hematoxylin and eosin staining. ***A–F***) Immunohistochemical comparisons of extracellular amyloid plaque levels in the CT-C, CT-S, and CT-C old group immunolabeled with 6E10 antibody (***A–C***) and C9 antibody (***D–F***) in the hippocampal DG region in serial brain sections. The long arrow indicates vascular deposits, and the short arrow, dense cored plaque. ***G–I***) Immunohistochemical comparisons of intraneuronal APP-CTFs immunolabeled with C9 antibody in hippocampal CA3 regions. (***A–I***; scale bar indicates 100 μm). ***J–L***) Confocal microscopic analysis of intraneuronal Aβ and APP-CTFs immunoreactivities in the hippocampal CA3 regions (green; 6E10, red; C9, blue; DAPI, scale bar indicates 50 μm, insert, 10 μm). ***M–R***) Hematoxylin and eosin staining in the hippocampal DG and CA3 region (scale bar indicates 100 μm, insert, 50 μm). ***S–U***) Negative controls were examined using anti-mouse Ig G antibody (S; DG, T; CA3) and anti-mouse FITC- and anti-rabbit Cy3-conjugated donkey Ig G antibodies (U; CA3, scale bar indicates 50 μm).

Fig. 4

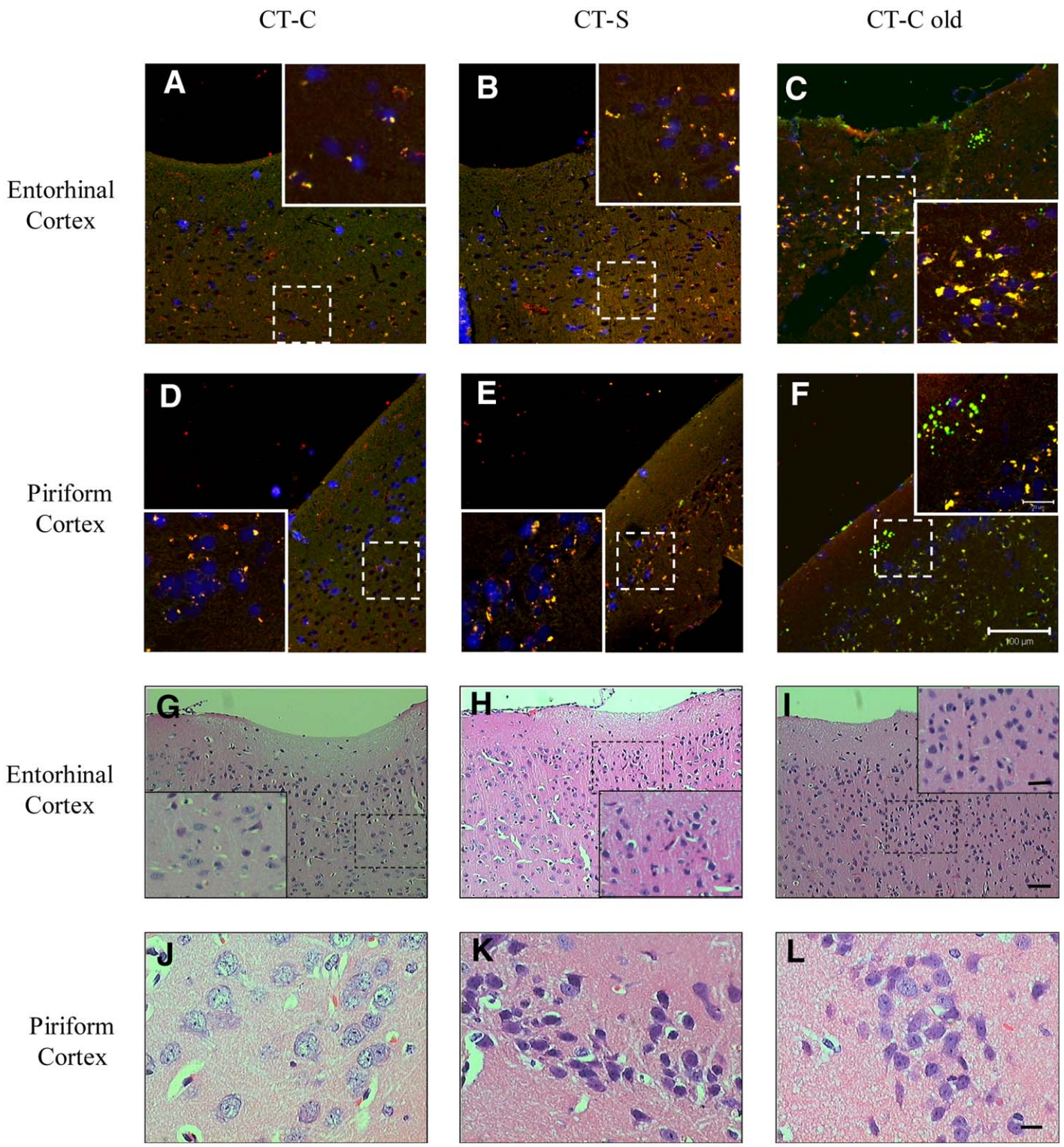

Figure 4. Long-term stress increased intraneuronal Aβ and APP-CTFs depositions in the entorhinal and piriform cortex in APPV717I-CT100 transgenic mice. Intraneuronal Aβ and APP-CTFs levels were examined by immunolabling with 6E10 antibody and C9 antibody in the entorhinal and piriform cortex in the CT-C, CT-S and CT-C old groups. Degenerating neurons were also examined in the entorhinal cortex and piriform cortex in serial brain section. ***A–F***) Confocal microscopic analysis of intraneuronal Aβ and APP-CTFs immunoreactivities in the entorhinal and piriform cortex (green; 6E10, red; C9, blue; DAPI, scale bar indicates 100 µm, insert, 20 µm). ***G–L***) Hematoxylin and eosin staining in the entorhinal and piriform cortex (***G–I***; scale bar indicates 100 µm, insert, 50 µm: ***J–L***; 5 µm).

Fig. 5

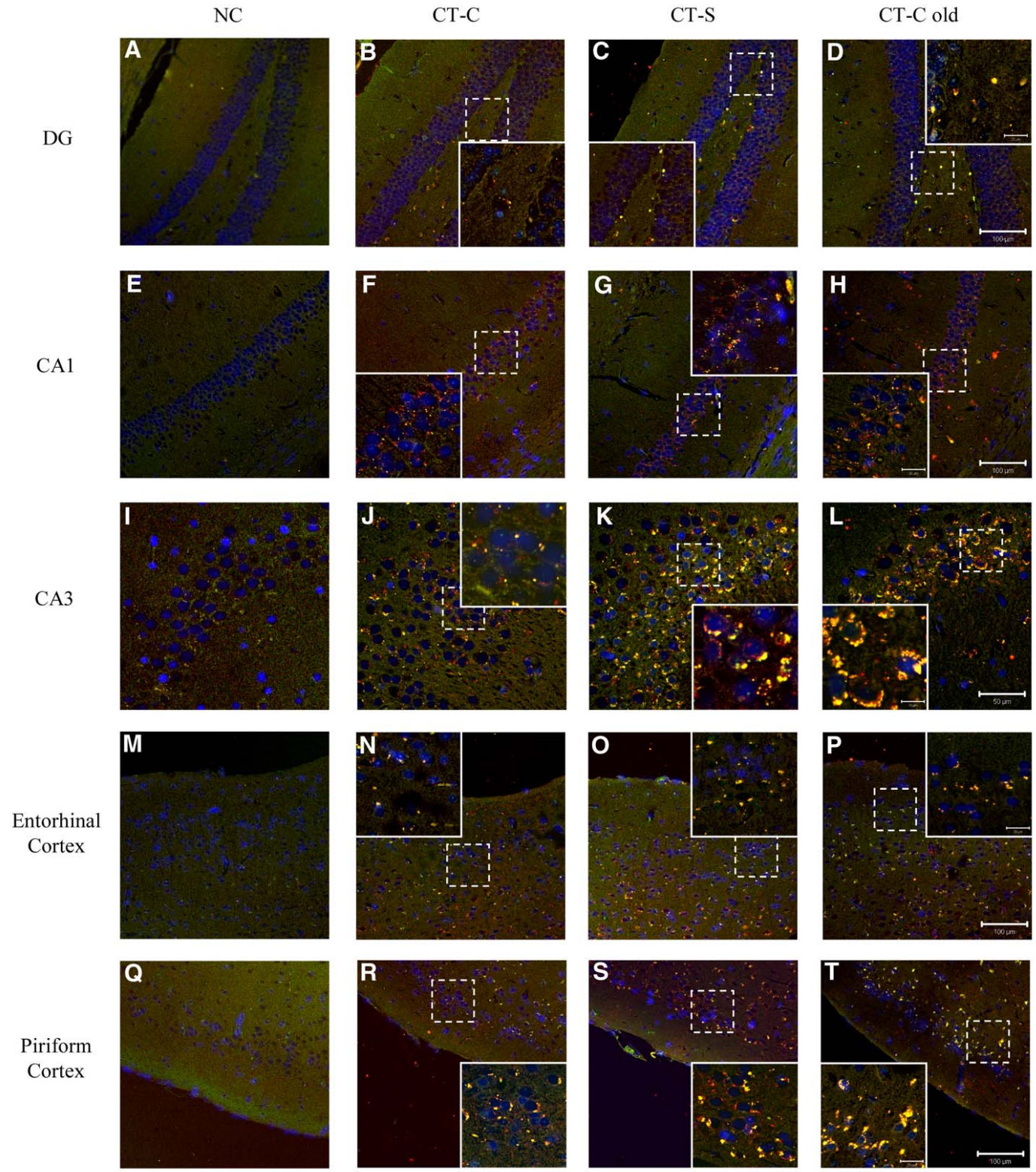

Figure 5. Long-term stress increased tau phosphorylation in the hippocampus and cortex in APPV717I-CT100 transgenic mice. Tau phosphorylation at Ser^{202}/Thr^{205} and intraneuronal APP-CTFs levels were examined in the DG (***B–D***), CA1 (***F–H***), and CA3 (***J–L***) hippocampal regions, and in the entorhinal cortex (***N–P***), and piriform cortex (***R–T***). Brain sections were probed with AT-8 antibody specifically recognizing phospho Ser^{202}/Thr^{205} tau and with C9 antibody specifically recognizing the last 9 amino acids at the APP-C terminus (green; AT-8, red; C9, blue; DAPI, A-H, M-T; scale bar indicates 100 μm, insert, 20 μm: I-L; 50 μm, insert, 10 μm). Negative controls were examined using anti-mouse FITC- and anti-rabbit Cy3-conjugated donkey Ig G antibodies (***A, E, I, M, Q***).