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LETTER TO THE EDITOR

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Astroglial Activation and Tau Hyperphosphorylation Precede to Neuron Loss in a Neurodegenerative Mouse Model

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Neurodegenerative diseases including AD (Alzheimer's disease) display several common neuropathological changes such as neuroinflammation, tau pathology, and neuron loss [1,2]. However, it remained unknown which types of pathology occur as early events during the progression of the disease. This is an important question to be investigated, as it is believed that prevention of early pathological changes is pivotal to developing effective therapies for neurodegenerative diseases. Presenilins, NCT (nicastrin), PEN2 (presenilin enhancer 2), and Aph1 (anterior pharynx defective 1) are four essential subunits of the γ-secretase complex. It is well known that presenilin mutations are the major cause of familial AD [3–5]. Recent evidence has strongly suggested that there is loss of presenilin function mechanism in the pathogenesis of AD [5,6]. Consistent with this notion, a number of recently published γ-secretase subunit-based mouse models were reported to display AD-like neurodegeneration in an age-dependent manner [6–11]. However, as neuron loss takes place in the above models at very young ages, for example, 2–3 months, it is difficult to dissect out early pathological changes.

Findings

In this study, we generated a new line of *NCT* cKO mice by crossing $NCT^{f/f}$ with the T29 *CaMKIIα-Cre* transgenics (Tg), in which Cre recombinase starts to express in excitatory neurons of the forebrain at 1.5–2 months [12,13]. Our biochemical analysis confirmed significantly reduced NCT protein levels in *NCT* cKO mice across ages (Figure 1A. $Ps < 0.01$), for example, there was about 30% of reduction on NCT. Whereas levels of full-length APP (APP-FL) in *NCT* cKO mice were not different, as compared to controls (Figure 1A. $Ps > 0.1$), those for APP C-terminal fragment (APP-CTF) in *NCT* cKOs were massively increased (Figure 1A), confirming decreased γ-secretase activity.

Nissl staining showed no detectable change in brain morphology of *NCT* cKO mice at 6 months (Figure 1B–a,d). In contrast, the cortex size of *NCT* cKO mice became significantly smaller than that of age-matched littermate controls at 10 (Figure 1B–b,e) and 13 (Figure 1B–c,f) months. To determine at which age mutant mice began to exhibit evident neuron loss, Western blotting and IHC (immunohistochemistry) using NeuN, a marker for mature neurons, were conducted. Relative protein levels of NeuN were significantly decreased in the cortex of *NCT* cKO mice at 13 (Figure 1C–c) months but not at 5–6 (Figure 1C–a) or 10 months (Figure 1C–b), suggesting a possibility that there was a loss of the total number of mature neurons. Consistent with biochemical results, immuno-reactivity of NeuN in the cortex of *NCT* cKO mice was unchanged at 6 (Figure 1D–a,b) or 10 months (Figure 1D–c,d), but was reduced at 13 months (Figure 1D–e,f). Using a stereological method, we counted the average number of NeuN-positive (+) cells across a number of brain sections. We found that the

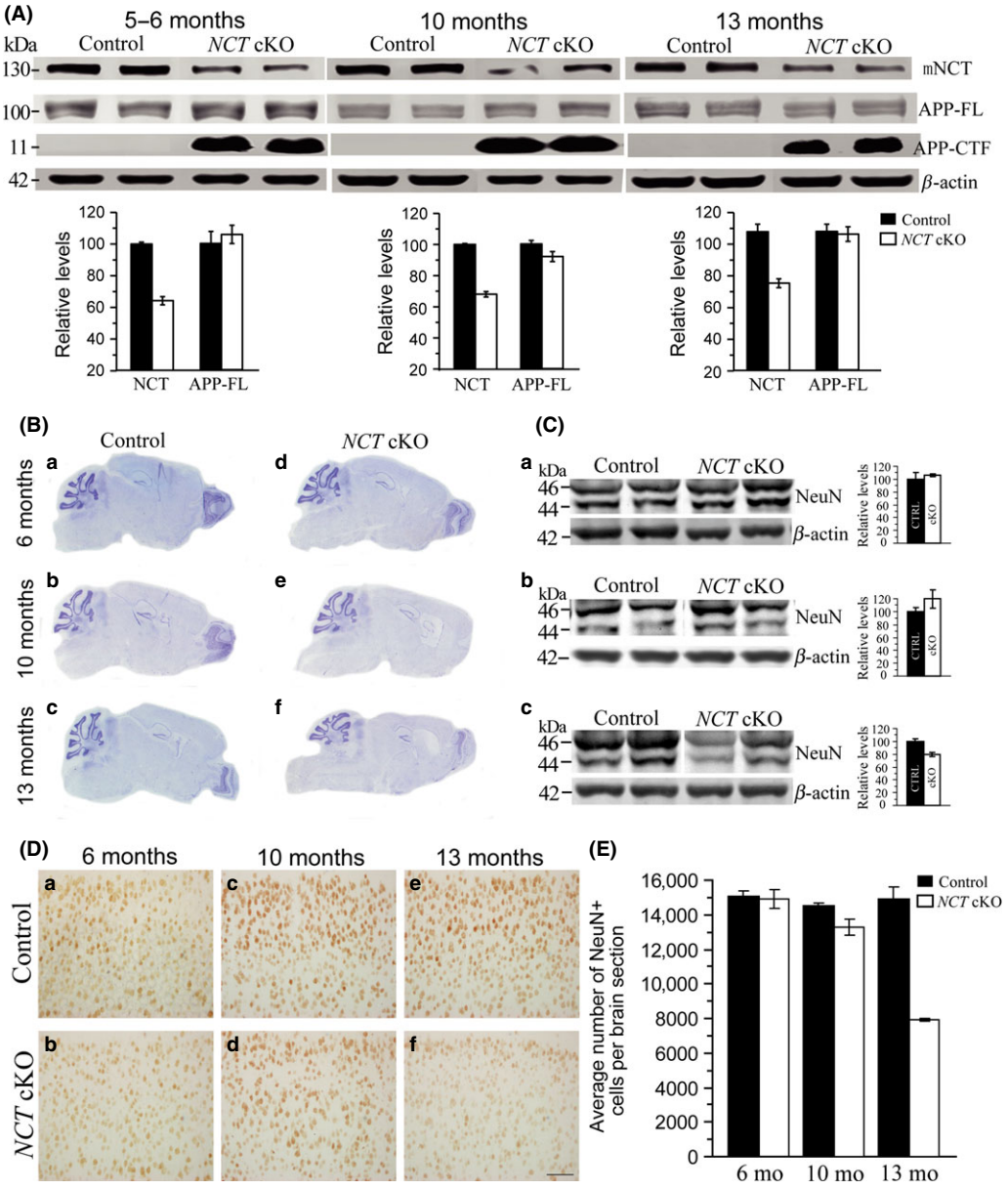

Figure 1 Age-dependent loss of mature neurons in forebrain-specific *NCT* cKO mice. (**A**) Biochemical analyses on NCT, APP-FL, and APP-CTF in *NCT* cKO mice across ages (n = 3–4/group). Western blotting confirmed significantly decreased levels of NCT in three cKO groups (5–6 months: control = 100 ± 1.2%, cKO = 64.3 ± 2.6%, $P = 0.00009$, two-tailed Student t-test; 10 months: control = 100 ± 0.6%, cKO = 68.0 ± 1.7%, $P = 0.003$; 13 months: control = 100 ± 4.4%, cKO = 69.9 ± 4.2%, $P = 0.007$). Western blotting on APP-FL showed unchanged levels in *NCT* cKO groups at 5–6 ($P = 0.56$), 10 ($P = 0.11$), or 13 ($P = 0.81$) months of age. Western results for APP-CTF indicated massive accumulation in *NCT* cKO mice aged at 5–6, 10, or 13 months. (**B**) Nissl staining for *NCT* cKO mice using brain sections aged at 6, 10, and 13 months. The cortex morphology and the cortex size were normal in *NCT* cKOs at 6 months (a,d). The cortex became smaller in *NCT* cKOs at 10 months (b,e). The cortex size was further reduced, and the lateral ventricle became bigger in *NCT* cKOs at 13 months (c,f). (**C**) Western analysis on NeuN. There were no significant differences on protein levels of NeuN in *NCT* cKO brains at 5–6 (a: $P = 0.55$) or 10 (b: $P = 0.10$) months of age. NeuN levels were significantly decreased in *NCT* cKO mice at 13 months, as compared to age-matched littermate controls (c: $P = 0.01$). (**D**) NeuN immunostaining. There was no difference on immuno-reactivity of NeuN in *NCT* cKO brains at 6 (a–b) and 10 (c–d) months. Immuno-reactivity of NeuN in *NCT* cKO brains was decreased at 13 months (e–f). Scale bar=100 μm. (**E**) Quantification data on the average number of cortical NeuN+ cells per brain section. There was no significant difference between *NCT* cKOs and controls at 6 ($P = 0.82$) or 10 ($P = 0.13$) months. However, the average number of cortical NeuN+ cells was significantly decreased in *NCT* cKO mice at 13 months ($P = 0.009$).

average number of cortical NeuN+ cells per brain section was significantly decreased in *NCT* cKO mice at 13 months but not at 5–6 or 10 months (Figure 1E).

Neuroinflammation is often associated with neurodegeneration. To determine at which age inflammatory responses would appear in *NCT* cKO mice, GFAP IHC was conducted.

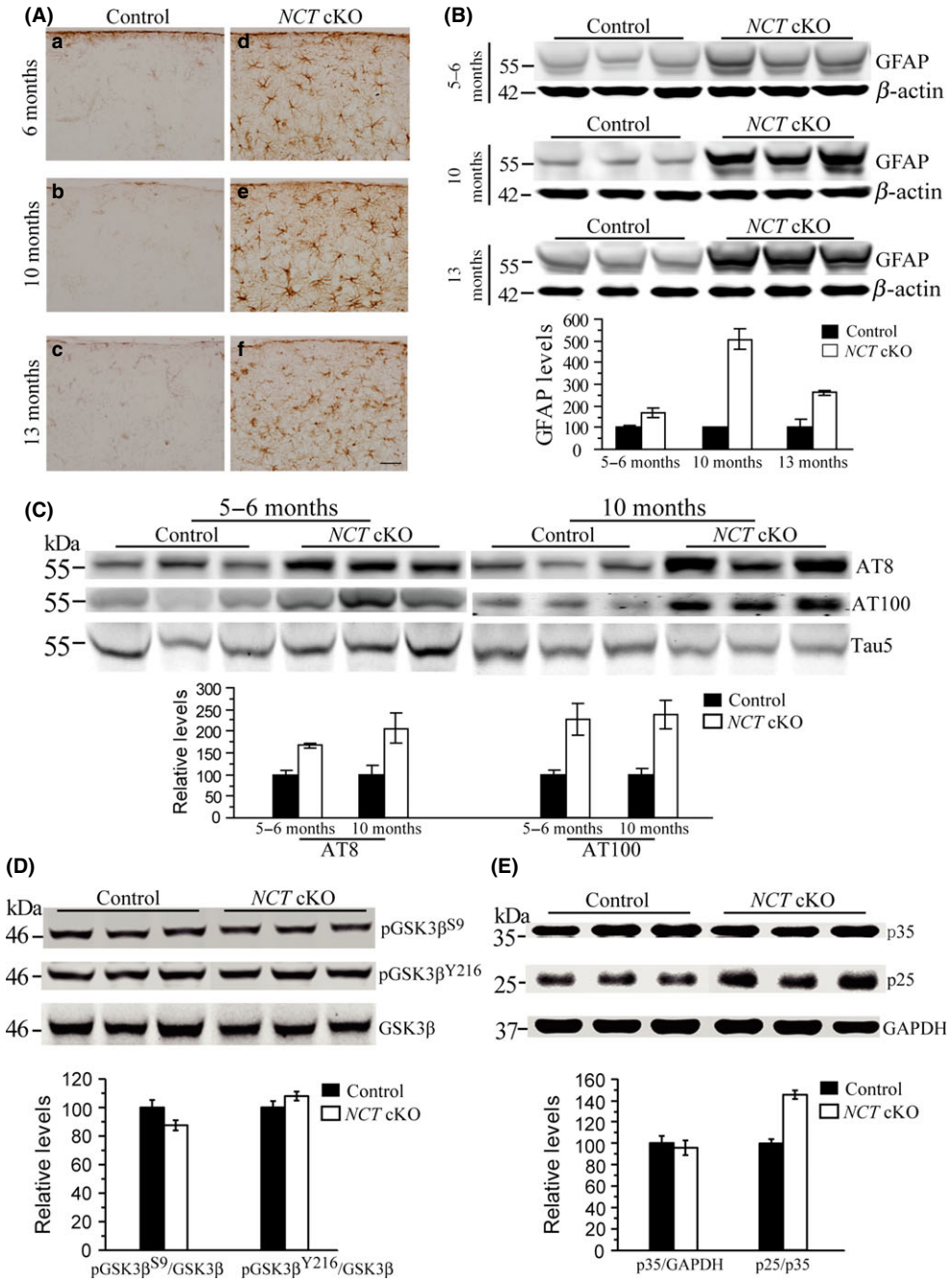

Figure 2 Astroglial activation and tau hyperphosphorylation in *NCT* cKO mice. (**A**) Immunostaining of GFAP. GFAP+ cells were intensively seen in the cortex of *NCT* cKO mice at 6 (a,d), 10 (b,e), and 13 (c,f) months of age, but were hardly detected in age-matched controls. Scale bar = 40 μm. (**B**) Western analysis on GFAP. Protein levels of GFAP were significantly increased in *NCT* cKO mice at 5–6 (P = 0.008, two-tailed Student t-test), 10 (P = 0.00002), and 13 months (P = 0.00001), as compared to age-matched littermate controls. (**C**) Western blotting on p-tau using antibodies of AT8 and AT100. Levels of p-tau were significantly increased in the cortex of *NCT* cKO mice at 5–6 (For AT8: P = 0.004; For AT100: P = 0.04) and 10 months (For AT8: P = 0.031; For AT100: P = 0.02). (**D**) Western analyses on GSK3β and p-GSK3β. Relative levels of p-GSK3β^{S9} (P = 0.7) and p-GSK3β^{Y216} (P = 0.4) were not decreased in the cortex of *NCT* cKO mice. (**E**) Western analyses on p25 and p35. Levels of p25 but not p35 were increased in *NCT* cKO mice (P = 0.0006).

Highly increased immuno-reactivity for GFAP was readily observed in the cortex (Figure 2A–a,d) and the hippocampus (data not shown) of *NCT* cKO mice at 6 months. There was massive elevation of GFAP immuno-reactivity in *NCT* cKOs at 10 (Figure 2A–b,e) or 13 months (Figure 2A–c,f), suggesting severe astroglial activation. Western analysis confirmed increased levels of GFAP in *NCT* cKOs at each age tested (Figure 2B).

Tau hyperphosphorylation is also believed to be a driving force for neuro-degeneration [2]. To study at which age levels of phosphorylated tau (p-tau) began to increase in *NCT* cKO mice, antibodies of AT8 (against p-tau at epitopes of Ser202/Thr205) and AT100 (against p-tau at epitopes of Thr212/ Ser214) were used. AT8 or AT100 Western blotting revealed significantly increased p-tau levels in *NCT* cKO mice at ages such as 6 or 10 months (Figure 2C), suggesting that tau hyperphosphorylation takes place prior to neuron loss. To investigate which type of tau kinases was responsible for the change of p-tau, we analyzed GSK3β and CDK5 [14]. Whereas relative levels of pGSK3β^{Y216} and pGSK3β^{S9} were not significantly reduced (Figure 2D), those of p25 were increased in *NCT* cKO mice (Figure 2E). In contrast, levels of total GSK3β and total p35 were unchanged in *NCT* cKOs as compared to age-matched littermate controls, suggestive of enhanced CDK5 activity.

Discussion

Compared to 50% reduction on NCT protein levels in the Tabuchi line (2009) of *NCT* cKO, the inactivation efficiency of NCT was low in this line (e.g., ~30% reduction on NCT levels). This is likely due to the use of the T29 line of *CaMKIIα-Cre*, which starts to express Cre recombinase at relatively late stage, as compared to the lines of *CaMKIIα-Cre* reported previously [8,9]. Interestingly, this line of *NCT* cKO exhibited significantly reduced cortical neuron number at 13 months. In contrast, dramatic neuron loss was reported in other lines of *NCT* cKO mice at very young ages [8,9]. However, due to the late age by which evident cortical neuron loss takes place in this line of *NCT* cKO, this could allow us to dissect out sequential pathological events during the progression of neurodegeneration. Indeed, astroglial activation and p-tau elevation were already detected in *NCT* cKO at as early as 3.5 months (data not shown), which is much earlier than the age when prominent neuron loss occurred. Overall, these findings strongly suggest that both tau hyperphosphorylation and neuroinflammation may be early pathological events in neurodegenerative diseases. We observed elevated p-tau levels in *NCT* cKO mice and have demonstrated that changes on p-tau were likely caused by enhanced activity of CDK5 but not GSK3β. Tau hyperphosphorylation in *NCT* cKO mice may act as a driving force for neurodegeneration. Although the exact role of neuroinflammation observed in *NCT* cKO mice remains to be investigated, it may also be a trigger to neuron death. Given that there is no effective treatment for neurodegenerative diseases, prevention of early neuropathology should be considered as potential strategies for the treatment of neurodegenerative diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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