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The role of copper and the copper-related protein CUTA in mediating APP processing and Aβ generation

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ABSTRACT

One major pathologic hallmark and trigger of Alzheimer's disease (AD) is overproduction and accumulation of β-amyloid (Aβ) species in the brain. Aβ is derived from β-amyloid precursor protein (APP) through sequential cleavages by β- and γ-secretases. Abnormal copper homeostasis also contributes to AD pathogenesis. Recently, we find that a copper-related protein, CutA divalent cation tolerance homolog of *Escherichia coli* (CUTA), interacts with the β-secretase β-site APP cleaving enzyme 1 (BACE1) and inhibits APP β-processing and Aβ generation. Herein, we further found that overexpression of CUTA increases intracellular copper level, whereas copper treatments promote CUTA expression. We also confirmed that copper treatments promote APP expression and Aβ secretion. In addition, copper treatments promoted the increase of Aβ secretion induced by CUTA downregulation but had no effect on CUTA-β-site APP cleaving enzyme 1 interaction. On the other hand, CUTA overexpression ameliorated copper-induced Aβ secretion but had no effect on APP expression. Moreover, we found that Aβ treatments can reduce both CUTA and copper levels in mouse primary neurons. Consistently, both CUTA and copper levels were decreased in the hippocampus of APP/PS1 AD mouse brain. Together, our results reveal a reciprocal modulation of copper and CUTA and suggest that both regulate Aβ generation through different mechanisms, although Aβ mutually affects copper and CUTA levels.

1. Introduction

A major pathologic hallmark of Alzheimer's disease (AD) in the brain is the formation of senile plaques whose major components are heterogenous β-amyloid (Aβ, mostly Aβ40 and Aβ42) peptides. Multiple lines of evidence demonstrate that Aβ peptides are neurotoxic and can trigger a cascade of neurodegenerative steps including the formation of neurofibrillary tangles, synaptic deficits, and neuronal loss, indicating that Aβ plays a pivotal role in the pathogenesis of AD (Eimer and Vassar, 2013; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). Aβ is generated from β-amyloid precursor protein (APP) through sequential cleavages first by β-secretase and then by γ-secretase. Excessive Aβ can trigger a cascade of neurodegenerative steps, including the formation of senile plaques and intra-neuronal fibrillary tangles and neuronal loss in susceptible brain regions. Alternatively, APP can be cleaved by α-secretase within the Aβ domain. α-cleavage precludes Aβ generation and releases a large extracellular domain of APP known as soluble APPα (sAPPα) instead (Zhang et al., 2011; Zheng and Koo, 2011).

Environmental factors such as heavy metals also play important roles in the pathogenesis of AD, either as triggers or as modulators of disease progression (Bush, 2003). Copper is indispensable in the human central nervous system and may function as a cofactor for multiple enzymes, activate neuropeptides and hormones, protect against reactive oxygen species, and so forth (Lutsenko et al., 2010). Abnormal homeostasis of copper has been shown to be involved in AD. However, the contribution of copper to disease pathology and development is far from being elucidated. Copper is enriched in amyloid plaques of AD patients compared with age-matched subjects (Bush, 2003). Copper can interact with APP and one of the 2

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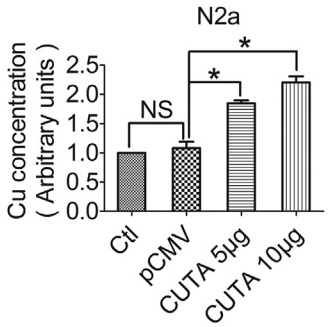

Fig. 1. Overexpression of CUTA increases intracellular copper level. N2a cells were transfected with indicated amounts of CUTA plasmid or pCMV vector for 24 hours. Untransfected cells were also used as control (Ctl). Cellular Cu levels were quantified by ICP-MS. N = 3, $^*p < 0.05$. Abbreviations: CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; ICP-MS, inductively coupled plasma mass spectrometry; NS, not significant.

copper-binding domains on APP is within the Aβ region (Stefansson et al., 2005). On binding to APP and Aβ, Cu(II) can be reduced to Cu(I), which may generate reactive oxygen species during this process (Multhaup et al., 1996) and contribute to the oxidative stress observed in AD brain (Eskici and Axelsen, 2012). However, although copper overload has been shown to promote APP expression (Armendariz et al., 2004; Borchardt et al., 1999) and copper depletion can downregulate APP expression (Bellingham et al., 2004), the effect of copper on Aβ is controversial: one study found that copper can reduce Aβ level (Borchardt et al., 1999), whereas another study showed that copper can enhance APP dimerization and promote Aβ production (Noda et al., 2013). Results from in vivo studies are also controversial: one study showed that copper level is decreased in APP23 transgenic mice and dietary copper can reduce Aβ production and stabilize brain superoxide dismutase 1 activity (Bayer et al., 2003). Copper level was also found to be increased in APP knockout mice (White et al., 1999). However, other studies found that copper exposure may cause Aβ plaques and learning deficits in a rabbit model of AD possibly through affecting Aβ clearance and exacerbate both amyloid and tau pathology in APP/PS1/tau triple transgenic AD mice by upregulating β-site APP cleaving enzyme 1 (BACE1), the essential β-secretase (Chami and Checler, 2012; Kitazawa et al., 2009).

We recently found that a copper-related protein, the mammalian CutA divalent cation tolerance homolog (*Escherichia coli*), CUTA, can modulate Aβ generation. Human CUTA has several variants that differ in their amino-terminal length and can be separated as heavy and light components. We demonstrated that the heavy component (but not the light component) of CUTA can interact with BACE1 and mediates its intracellular trafficking, therefore affecting β-processing of APP and Aβ production (Zhao et al., 2012a). CUTA can form trimers through a region of about 100 residues that is conserved from bacteria to vertebrates (Savchenko et al., 2004). In bacteria, CutA is involved in copper tolerance and some mutations in the *cutA* gene have been found to lead to copper sensitivity because of its increased uptake (Fong et al., 1995). Additional studies show that many CutA proteins have a high copper-binding capacity and that copper could induce reversible aggregation of the CutA protein (Arnesano et al., 2003; Tanaka et al., 2004). Therefore, in the present study, we further investigated the correlation between CUTA and copper and any potential interplay between the two during their modulating APP processing and Aβ generation.

2. Methods

2.1. Cell culture

Mouse neuroblastoma N2a cells, N2a cells stably expressing human APP695 (N2a-APP695), and HEK293T cells were cultured as previously described (Zhao et al., 2012b). Primary neurons derived from embryonic day 14.5–16.5 C57BL/6 wild type or APP/PS1 mouse embryos were maintained in neurobasal medium supplemented with B27 (Life Technologies). All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Xiamen University.

2.2. Antibodies

The rabbit polyclonal antibody R-CUTA against CUTA (Zhao et al., 2012a) and the rabbit polyclonal antibody Ru369 against APP (Xu et al., 1997) were developed in our laboratories. The mouse monoclonal antibody 22C11 was from Millipore. Mouse anti-α-tubulin, rabbit anti-β-actin, and mouse anti-HA antibodies were from Sigma. Mouse anti-Myc, mouse anti-GAPDH, and mouse anti-PARP antibodies were from Santa Cruz Biotechnology.

2.3. Cell manipulation

For copper treatments, cells were incubated with various amounts of $CuSO_4$ for different time periods. In some experiments, cells were transiently transfected with pCMV, BACE1-HA, and CUTA plasmids, using Turbofect reagent (Fermentas). For RNA interference to downregulate CUTA expression, cells were transiently transfected with a scrambled control shRNA and a shRNA targeting CUTA (piLenti-siRNA 494-GFP: 5′-TCACAGAATCGGTTTCAAATTCTGGCACA-3′), using Lipofectamine2000 reagent (Invitrogen).

For Aβ treatments, primary neurons from wild-type C57BL/6 mice were insulted with 40 μM Aβ42 for 24 hours. Cell lysates were measured for CUTA and copper levels.

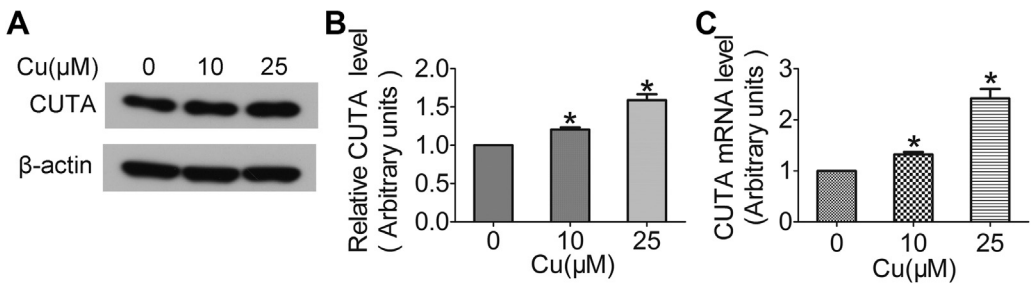

Fig. 2. Copper treatments induce CUTA expression. N2a cells were treated with indicated amounts of Cu for 24 hours. (A) Cell lysates were assayed for CUTA by Western blot. (B) CUTA levels were quantified by densitometry for comparison. (C) The mRNA level of CUTA was determined by quantitative real-time PCR for comparison. N = 3, $^*p < 0.05$. Abbreviations: CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; mRNA, messenger RNA; PCR, polymerase chain reaction.

2.4. Copper level measurement

To measure intracellular copper levels, treated cells were digested in 1 mL HNO_3 (65%–68%, Sinopharm, China) overnight at room temperature. Samples were diluted to a final concentration of 3.5% HNO_3 and then filtered through a 0.45 filter membrane for analysis by inductively coupled plasma mass spectrometry (ICP-MS), using Agilent 7500ce.

For mouse brain samples, they were digested in HNO_3 at 40 °C overnight and then added with H_2O_2 (Sinopharm, China) until the solution became colorless and clear. Solution was diluted to a final concentration of 3.5% HNO_3, filtered and analyzed by ICP-MS as described previously.

2.5. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) and subjected to reverse transcription using SuperScript First-Strand kit (Invitrogen). Quantative real-time polymerase chain reaction was carried out with an ICycler instrument (ABI).

Mouse CUTA primers were as follows:
CUTA-forward: 5′-TTGTCACTTGTCCCAACGAAA-3′
CUTA-reverse: 5′-ATCTGCGGGATGAGGTTGAC-3′
Mouse APP primers were as follows:
APP-forward: 5′-ACCGTTGCCTAGTTGGTGAG-3′,
APP-reverse: 5′- GAATCCACGCTGTCGCTTT-3′.
Mouse CPH primers were as follows:
CPH-forward: 5′-CACCGTGTTCTTCGACATC-3′
CPH-reverse: 5′-ATTCTGTGAAAGGAGGAACC-3′.

2.6. Coimmunoprecipitation

Cells were co-transfected with Myc-CUTA and BACE1-HA for 24 hours and treated with copper for another 24 hours. Treated cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.5% Nonidet P-40, supplemented with a protease inhibitor mixture). Equal protein amounts of cell lysates were incubated with normal rabbit IgG or indicated antibodies together with rProtein A sepharose (GE) at 4 °C overnight. Immunoprecipitated proteins were analyzed by Western blot.

2.7. β-Amyloid ELISA

Human Aβ40 and Aβ42 were measured by using commercial ELISA kits (Life Technologies), following the manufacturer's protocols. In some experiments, human Aβ42 was also measured following a previously described method (Li et al., 2012).

2.8. Statistical analysis

Data were analyzed by Student t test and $p < 0.05$ was considered to be statistically significant. Data in all figures are expressed as mean ± standard error of the mean.

3. Results and discussion

3.1. Overexpression of CUTA increases intracellular copper levels

Several studies have found that bacterial CutA has high copper-binding capacity and is involved in copper tolerance (Fong et al., 1995). In addition, copper can induce reversible aggregation of the CutA protein (Arnesano et al., 2003; Tanaka et al., 2004). The high conservation of CUTA from mammalian to bacteria suggests that mammalian CUTA might also interplay with copper. However, study regarding this is scarce. One work reported that overexpression of the mammalian CUTA isoform 2 may enhance the cytotoxicity of copper (Yang et al., 2008). Mammalian CUTA has several variants that differ in their amino-terminal length and can be separated as heavy and light components (Liang et al., 2009; Zhao et al., 2012a). CUTA isoform 2 is a light component and cannot interact with BACE1 to affect APP processing and Aβ generation. To study the interplay between copper and CUTA during their mediating APP processing and Aβ generation, we focused on the role of the CUTA heavy component and referred it to CUTA hereafter. We first studied whether CUTA affects uptake of copper by cells. When mouse neuroblastoma N2a cells were

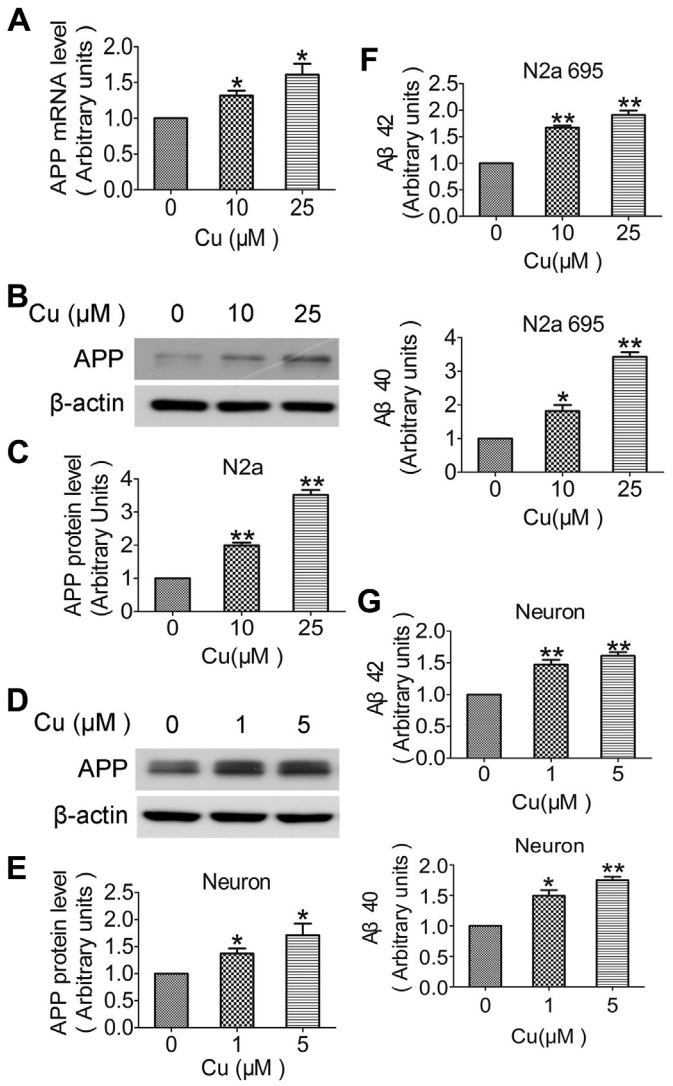

Fig. 3. Copper treatments enhance APP expression and Aβ secretion. N2a cells were treated with 10 μM and 25 μM Cu for 24 hours. Cells treated with 0 μM Cu were used as controls. (A) The mRNA level of APP was determined by quantitative real-time PCR, normalized to that of CPH, and compared with that of controls (set as 1 arbitrary unit). (B) APP protein was detected by Western blot. (C) APP protein levels were quantified by densitometry, normalized to those of β-actin, and compared with that of controls. (D) Mouse neurons were treated with 0 μM, 1 μM, and 5 μM Cu. Cell lysates were assayed for APP protein by Western blot. (E) Neuronal APP levels were quantified by densitometry for comparison. (F) N2a695 cells and (G) primary neurons derived from APP/PS1 mice were treated with indicated concentrations of Cu for 24 hours. Levels of Aβ40 and Aβ42 in conditioned media were quantified by ELISA and compared with those of controls. N = 3, $^{*}p < 0.05$; $^{**}p < 0.01$. Abbreviations: Aβ, β-amyloid; APP, β-amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; PCR, polymerase chain reaction.

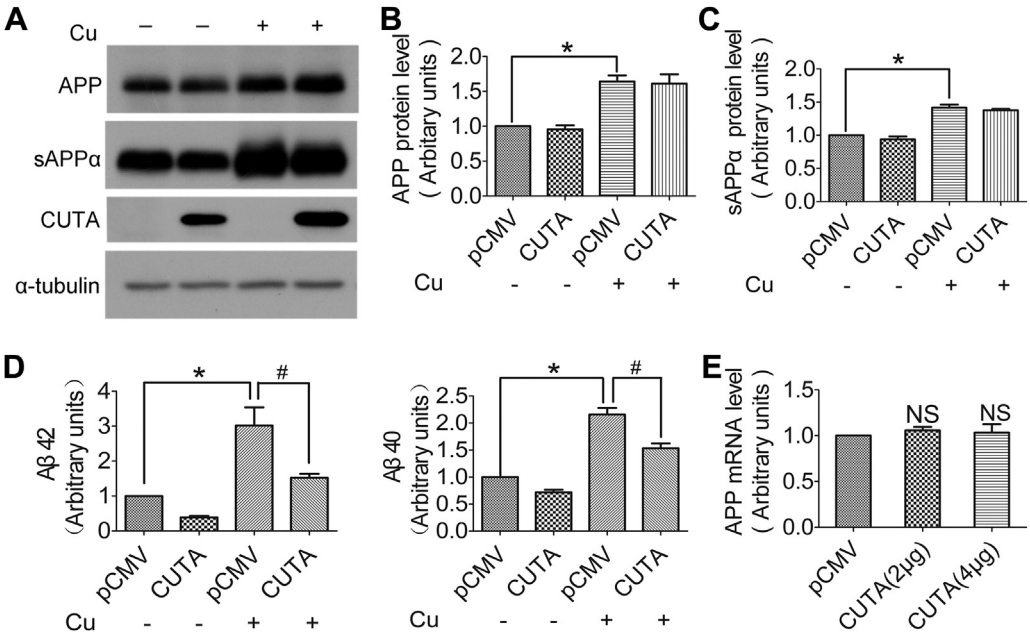

Fig. 4. CUTA overexpression ameliorates copper-induced Aβ production but does not affect APP expression. N2a-APP695 cells were transfected with control pCMV vector or CUTA plasmid, and then treated with (+) or without (−) 25 μM Cu for 24 hours. (A) Cell lysates were assayed for APP, CUTA and α-tubulin, and conditioned media were assayed for sAPPα by Western blot. APP (B) and sAPPα (C) levels were quantified by densitometry for comparison with those of controls (set as 1 arbitrary units). (D) Levels of Aβ40 and Aβ42 in conditioned media were quantified by ELISA for comparison. (E) N2a cells were transfected with indicated amounts of CUTA plasmid or control pCMV vector for 24 hours. The mRNA level of APP was determined by quantitative real-time PCR for comparison. N = 3, $^{\#}p < 0.05$; $^{*}p < 0.05$. Abbreviations: Aβ, β-amyloid; APP, β-amyloid precursor protein; CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; NS, not significant; PCR, polymerase chain reaction; sAPPα, soluble APPα.

transfected with CUTA, we found that intracellular copper levels were increased in a dose-dependent manner (Fig. 1).

3.2. Copper treatments induce CUTA expression

Next, we studied whether copper reciprocally affects CUTA expression. When N2a cells were treated with copper, we found that both CUTA protein (Fig. 2A and B) and messenger RNA (mRNA) (Fig. 2C) levels were increased in a dose-dependent manner, implying a feedback to CUTA expression on copper overload.

3.3. Copper treatments enhance APP expression and Aβ secretion in cells

Although copper overload has been consistently found to promote APP expression (Armendariz et al., 2004; Borchardt et al., 1999), its effect on mediating Aβ generation and changing AD-like phenotypes remain controversial (Borchardt et al., 1999; Noda et al., 2013). Here, we confirmed in N2a cells that copper treatments promote both APP mRNA (Fig. 3A) and protein (Fig. 3B and C) levels in a dose-dependent manner. Consistently, copper treatments also resulted in a dose-dependent increase of APP protein levels in mouse primary neurons (Fig. 3D and E).

Moreover, we found that although copper treatments should promote CUTA expression (Fig. 2) that may inhibit Aβ generation through interacting with BACE1 and interfering with APP β-processing (Zhao et al., 2012a), in both N2a cells stably expressing human APP695 (N2a-APP695) and primary neurons derived from APP/PS1 mice, copper treatments actually dose-dependently increased levels of secreted Aβ40 and Aβ42 (Fig. 3F and G). This is possibly because that CUTA-mediated inhibition of Aβ generation cannot completely counteract APP upregulation-caused excessive

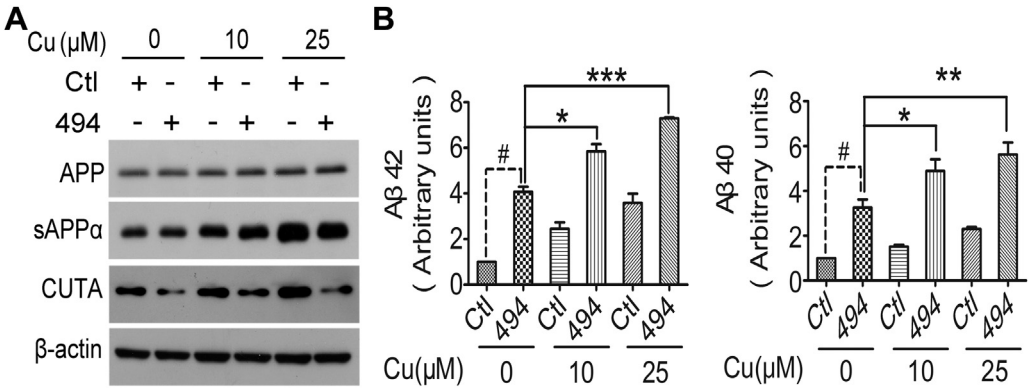

Fig. 5. Copper treatments promote CUTA downregulation induced Aβ secretion. N2a-APP695 cells were transfected with scrambled control shRNA (Ctl) or shRNA targeting CUTA (494) for 48 hours. Cells were then treated with indicated amounts of Cu for 24 hours. (A) Cell lysates were assayed for APP, CUTA and β-actin, and conditioned media were assayed for sAPPα by Western blot. (B) Levels of Aβ40 and Aβ42 in conditioned media were assayed by ELISA. N = 3, $^{\#}p < 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Abbreviations: Aβ, β-amyloid; CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; shRNA, small hairpin RNA.

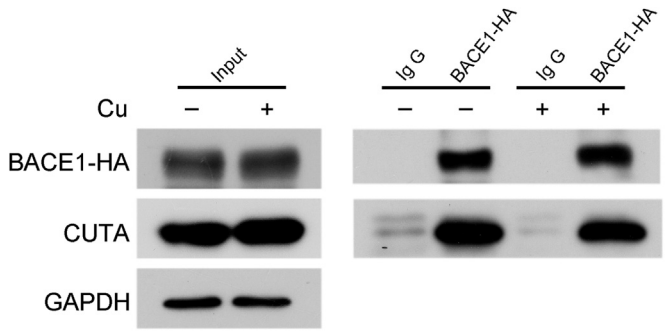

Fig. 6. Copper does not affect the interaction between CUTA and BACE1. HEK293T cells were cotransfected with CUTA and BACE1-HA for 24 hours and then treated with (+) or without (−) Cu for another 24 hours. Equal amounts of cell lysates were incubated with normal rabbit IgG or HA antibody. Immunoprecipitated proteins and input were subjected to Western blot to detect BACE1 and CUTA. Abbreviations: BACE1, β-site APP cleaving enzyme 1; CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*.

Aβ generation, leading to a net effect of increased Aβ secretion. Hence, our results support a harmful role of copper overload in AD.

3.4. Overexpression of CUTA ameliorates copper-induced Aβ secretion but does not affect APP expression

Because copper and CUTA reciprocally modulate each other, there is a possibility that CUTA may also affect APP expression and Aβ generation through affecting copper uptake. On the other hand, the regulation of copper on APP expression might be mediated by CUTA. To determine these possibilities, we first overexpressed CUTA in N2a-APP695 cells and then treated cells with copper. The results showed that CUTA overexpression did not affect APP protein level and sAPPα secretion nor did it affect copper-induced increase of APP protein level and sAPPα secretion (Fig. 4A–C). Consistent with our previous findings (Zhao et al., 2012a), overexpression of CUTA reduced Aβ40/42 secretion. In addition, we found that the induced increase of Aβ40/42 secretion by copper treatments was attenuated by CUTA overexpression (Fig. 4D).

Furthermore, we compared endogenous APP mRNA levels in N2a cells transfected with CUTA and with control vector. We found that overexpression of CUTA does not affect endogenous APP gene expression (Fig. 4E). Because overexpression of CUTA promotes uptake of copper by cells (Fig. 1), the regulation of APP expression by copper should be independent of copper localization, no matter it is in or out of the cell.

3.5. Copper treatments promote downregulation of CUTA induced Aβ secretion

We also downregulated CUTA level through transfecting cells with CUTA shRNA (sh494) and found that CUTA knockdown did not affect APP protein level and sAPPα secretion (Fig. 5A). When these cells were treated with copper, we found that downregulation of CUTA did not affect the induced increase of APP protein level and sAPPα secretion (Fig. 5A). As expected, downregulation of CUTA increased Aβ40/42 secretion (Fig. 5B). Interestingly, we found that copper treatments further promoted the increase of Aβ40/42 secretion induced by CUTA knockdown (Fig. 5B). These results further indicate Aβ generation is differently mediated by copper and CUTA.

3.6. Copper does not affect the interaction between CUTA and BACE1

Because CUTA can interact with BACE1 and affects BACE1-mediated APP processing, we asked whether copper can affect the interaction between CUTA and BACE1. We co-transfected BACE1-HA and CUTA into HEK293T cells and treated them with copper. When these cells were used for coimmunoprecipitation study, we found that HA antibody immunoprecipitated similar levels of CUTA in both copper-treated and untreated cells (Fig. 6), suggesting that copper treatments did not affect BACE1-CUTA interaction.

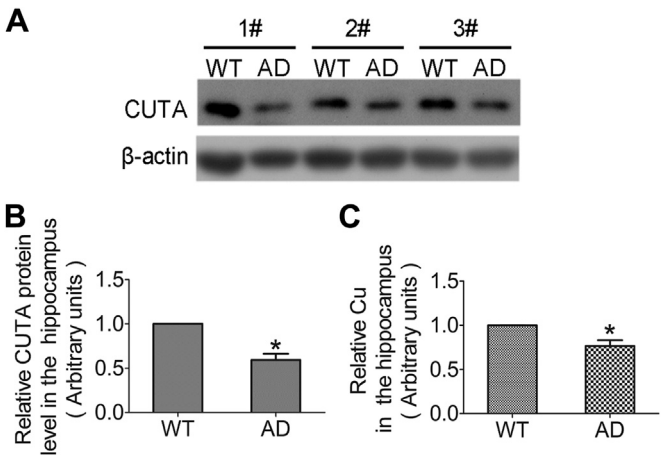

Fig. 7. CUTA and copper levels are decreased in the hippocampus of APP/PS1 AD mice. (A) Equal amounts of lysates of hippocampus from 3 pairs of 7- to 8-month-old APP/PS1 AD mice and wild-type (WT) littermate controls were subjected to Western blot to detect CUTA. (B) CUTA levels were quantified by densitometry for comparison. (C) Hippocampal Cu levels were quantified by ICP-MS for comparison. N = 3, $^*p < 0.05$. Abbreviations: AD, Alzheimer's disease; CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; ICP-MS, inductively coupled plasma mass spectrometry.

3.7. CUTA and copper levels are decreased in the hippocampus of APP/PS1 AD mice

To have better insight of CUTA and copper in AD pathology, we studied the levels of CUTA and copper in the hippocampus of the

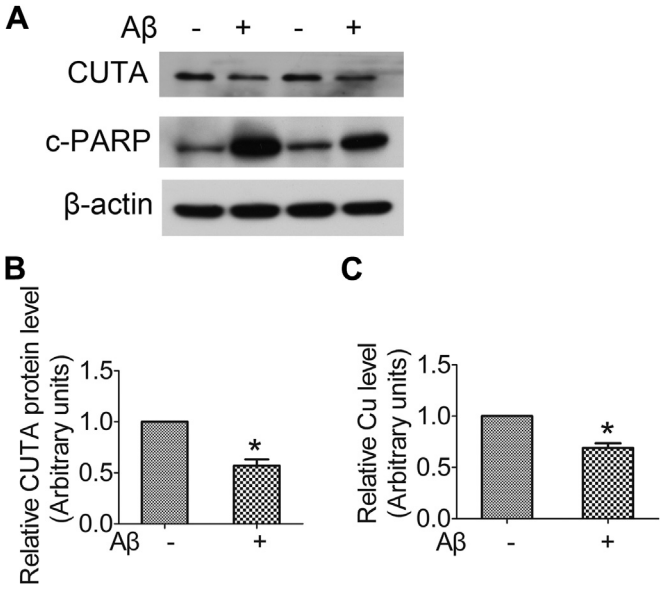

Fig. 8. Aβ insults reduce CUTA and copper levels. Mouse primary neurons were treated with Aβ42 for 24 hours. (A) Cell lysates were subjected to Western blot to detect CUTA and cleaved PARP (c-PARP, indicating Aβ toxicity). (B) CUTA levels were quantified by densitometry for comparison. N = 4, $^*p < 0.05$. (C) Cu levels in treated neurons were quantified by ICP-MS for comparison. N = 3, $^*p < 0.05$. Abbreviations: Aβ, β-amyloid; CUTA, CutA divalent cation tolerance homolog of *Escherichia coli*; ICP-MS, inductively coupled plasma mass spectrometry.

APP/PS1 AD mice. The results showed that compared with those of littermate control mice, both CUTA protein (Fig. 7A and B) and copper (Fig. 7C) levels were dramatically decreased in the hippocampus of APP/PS1 AD mice. The observation of a reduction of copper levels in APP/PS1 mouse hippocampus is consistent with previous reports showing that copper level is decreased in APP23 transgenic mice (Bayer et al., 2003) and increased in APP knockout mice (White et al., 1999). The reduction of CUTA may reflect its association with copper levels in vivo.

3.8. Aβ treatments reduce CUTA and copper levels

To determine whether the reduction of CUTA and copper levels in AD mouse brain is attributed to Aβ accumulation, we treated mouse primary neurons with Aβ. The results showed that both CUTA protein (Fig. 8A and B) and copper (Fig. 8C) levels were markedly reduced on Aβ insults. Hence, Aβ can mutually affect CUTA and copper levels.

4. Conclusion

Taken together, our findings suggest that copper and CUTA reciprocally regulate each other. However, copper and CUTA mediate APP processing and Aβ generation largely through different pathways, that is, copper increases both Aβ and sAPPα secretion through stimulating APP expression, whereas CUTA only affects Aβ generation through affecting BACE1-mediated APP processing. Moreover, Aβ can mutually affect both CUTA and copper levels. Our study provides new insight into the mechanism underlying copper- and CUTA-mediated AD pathology.

Disclosure statement

None of the authors has a conflict of interest to declare in relation to the present research.

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