Both pre- and post-synaptic alterations contribute to aberrant cholinergic transmission in superior cervical ganglia of APP^{-/-} mice

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ABSTRACT

Though amyloid precursor protein (APP) can potentially be cleaved to generate the pathological amyloid β peptide ($A\beta$), APP itself plays an important role in regulating neuronal activity. APP deficiency causes functional impairment in cholinergic synaptic transmission and cognitive performance. However, the mechanisms underlying altered cholinergic synaptic transmission in APP knock-out mice (APP^{-/-}) are poorly understood. In this study, we conducted *in vivo* extracellular recording to investigate cholinergic compound action potentials (CAPs) of the superior cervical ganglion (SCG) in APP^{-/-} and littermate wild-type (WT) mice. Our results demonstrate that APP not only regulates presynaptic activity, but also affects postsynaptic function at cholinergic synapses in SCG. APP deficiency reduces the number of vesicles in presynaptic terminals and attenuates the amplitude of CAPs, likely due to dysfunction of high-affinity choline transporters. Pharmacological and biochemical examination showed that postsynaptic responses mediated by $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptors are reduced in the absence of APP. Our research provides evidences on how APP regulates cholinergic function and therefore may help to identify potential therapeutic targets to treat cholinergic dysfunction associated with Alzheimer's disease pathogenesis.

1. Introduction

Alzheimer's disease (AD) is threatening the health of aged population worldwide. Over three decades of research, it is well accepted that AD results from the detrimental aggregation of β -amyloid peptides ($A\beta$) derived from proteolytic processing of the amyloid precursor protein (APP) (Benilova et al., 2012; Bignante et al., 2013; Tanzi and Bertram, 2005). Unfortunately, anti- $A\beta$ therapeutics in AD is much less effective or even ineffective on clinical trials so far (Callaway, 2012; Golde et al., 2011). Nevertheless, the cognitive impairment of AD is considered more correlated with synaptic dysfunction (Selkoe, 2002), especially cholinergic deficits (Mufson et al., 2008; Rakonczay et al., 2005). APP is a conserved type I membrane protein and highly expressed in neurons. Apart from producing $A\beta$, APP plays a critical role in the regulation of neural activity including cholinergic synaptic function (Wang et al., 2012). It has been reported that APP deficiency causes high-affinity choline transporters (CHT) dislocation and choline uptake reduction (Wang et al., 2007; Wang et al., 2009). APP deficiency leads to impaired synaptic transmission in neuromuscular junction (NMJ) (Yang et al., 2009). However, the precised mechanism underlying altered cholinergic synaptic transmission in APP knock-out mice ($APP^{-/-}$) remained unclear.

Acetylcholine (ACh) is the neurotransmitter for cholinergic synapses including connections between preganglionic and postganglionic neurons of superior cervical ganglia (SCG) (Alkadhi et al., 2005). SCG is the upmost ganglia of the paravertebral sympathetic chain. Within SCG, the preganglionic input fibers ascending in the sympathetic trunk synapse with the postganglionic neurons which innervate the neck, face and intracranial structures (Savastano et al., 2010). As one largest ganglia of peripheral autonomic nervous system, SCG is frequently used for research as synaptic connections show many similarities to the central nervous system and is relatively accessible (Jackson and Tourtellotte, 2014; Klimaschewski et al., 1996). Also, SCG has been implicated in neuropathies including Horner's syndrome, stroke and epilepsy (Ladd et al., 2014).

ACh is synthesized and packaged to synaptic vesicles in presynaptic compartment. ACh released from presynaptic terminal binds to acetylcholine receptors (AChRs) to elicit postsynaptic responses. The majority of ACh is otherwise hydrolyzed by acetylcholinesterase (AChE) to choline which can be recycled by CHT from synaptic cleft back to presynaptic terminal for ACh resynthesis (Sarter and Parikh, 2005). Furthermore, as for postsynaptic events, nicotinic AChRs (nAChRs) mediate fast

synaptic transmission in the form of compound action potentials (CAPs) evoked by electrical stimulation of the preganglionic nerve of the autonomic nervous system (David et al., 2010; Gingras et al., 2007). In SCG, nAChRs consists of dominant $\alpha 3\beta 4$ hetero-pentamers and $\alpha 7$ homo-pentamers (David et al., 2010; Putz et al., 2008), while $\alpha 4\beta 2$ nAChRs was only detected in SCG of early postnatal mice (Scholze et al., 2011).

We report here that APP deficits attenuate cholinergic synaptic transmission in SCG that involves reduction in both presynaptic CHT levels and sequential ACh containing-vesicle numbers; and postsynaptic $\alpha 4\beta 2$ and $\alpha 7$ nAChRs mediated activities. Our *in vivo* experiments provide evidences in supporting the role of APP in regulating cholinergic synaptic function, and therefore help to locate the presynaptic and postsynaptic signaling pathways by which APP contributes to cholinergic synaptic transmission.

2. Materials and Methods

2.1. Animals and Reagents

The APP^{-/-} mice, purchased from the Model Animal Research Center of Nanjing University, were generated by intercrossing the APP heterozygous males and females as described previously (Wang et al., 2007; Yang et al., 2007; Yang et al., 2009; Zheng et al., 1995). All mice were housed at room temperature with a 12:12 hour light-dark cycle and provided with food and water. Animals were used for all experiments in this study at the age of 2-month old. All animal experiments were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, published by the Ministry of Science and Technology of China. All experimental procedures were approved by the South China Normal University Animal Care and Use Committee.

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The drugs used in this study were diluted with physiological saline which was given in equivalent volume in vehicle controls.

2.2. Electrophysiological Procedures

Surgical procedures were adapted from the operation of SCG in rats (Savastano et al., 2010). Two-month-old mice (APP^{-/-} and littermate WT) were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and fixed supinely in a stereotaxic frame. After cutting the ventral skin of the neck vertically about 2 cm and displacing glands and muscles laterally, the Y-shaped carotid bifurcation was identified. SCG was exposed

after pulling carotid bifurcation slightly with a micro glass dissecting needle. A bipolar electrode (twist-insulated Ni chrome wire) was placed on the preganglionic nerve of SCG to deliver pulse stimulations and the evoked CAPs were extracellularly recorded with a monopolar electrode (Teflon-coated stainless-steel pin, 0.1 mm diameter) positioned at the postganglionic trunk of SCG. A clip connected to the skin nearby served as a ground (Liu et al., 2014).

Data were obtained and analyzed by PowerLab/8sp system (ML785, AD Instrument, Australia). The CAP amplitude was determined as the vertical distance between the peak and a tangent constructed between the onset and termination of the CAP. The input-output test was conducted with a series of current stimuli: 0.5, 1, 1.5, 2, 3 and 4 mA, and then adjusted at an intensity to produce about 50 % of maximal CAP as the test pulse for further experiments. Paired-pulse stimulation was performed at the inter-pulse interval of 50 ms. Sustained firing was evoked by a train of repetitive stimulation at 10 Hz for 30 s (Krishnaswamy and Cooper, 2009). Evoked synaptic responses were delivered at 0.05 Hz and 3 consecutive responses were averaged to estimate the CAP components of postsynaptic nAChRs.

To evaluate the function of synaptic signal molecules, antagonists and one agonist (to enhance CHT) were applied onto the exposed SCG at the speed of 10 μ L / min with a 10 μ L pipette. Antagonists were as follows: hemicholinium-3 (HC3, 10 μ M, to inhibit CHT) (Rassadi et al., 2005), mecamylamine hydrochloride (Mec, 1 μ M, to inhibit $\alpha 3\beta 4$ nAChR; 10 μ M, to inhibit $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$ nAChR) (Papke et al., 2001), dihydro- β -erythroidine hydrobromide (DH β E, 3 μ M, to selectively inhibit $\alpha 4\beta 2$ nAChR) (Zhou et al., 2013), methyllycaconitine (MLA, 0.1 μ M, to selectively inhibit $\alpha 7$ nAChR) (Singh et al., 2013). The CHT enhancer used in this study was coluracetam (MKC-231) (1 μ M) (Akaike et al., 1998). In addition, exogenous ACh (3 mM) was applied to SCG at the speed of 5 μ L / sec with a 10 μ L pipette to examine the postsynaptic function (Tochitsky et al., 2012). These drugs were dissolved in physiological saline.

2.3. Western blotting

The western blotting was performed as described previously (Yang et al., 2009) with minimal modification. For either APP^{-/-} or WT, a total of 12 SCGs were excised from 6 mice and combined according to genotypes. Then SCGs were homogenized by mechanical disruption in lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 % sodium dodecyl sulfate (SDS), 5 mM EDTA and protease inhibitors (complete Min; Roche). Homogenate was then centrifuged at 14,000 rpm for 10 min,

and supernatant was collected. Tissue lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and probed with specific antibodies against the $\alpha 4$ subunit (Millipore, 1: 1000 dilution) and $\alpha 7$ subunit (Abcam, 1: 1000 dilution) of nAChR. Anti- β -actin antibody was used as loading control. Bound antibodies were detected using horseradish peroxidase-coupled secondary antibodies (Abcam, 1: 5000 dilution) and enhanced chemiluminescence. The relative optical density of immunoreactive bands was quantified using Gel-Pro Analyzer software.

2.4. Immunohistochemistry

The procedures for immunohistochemistry were conducted as described previously (Liu et al., 2013) with minimal modification. The SCGs were dissected immediately and washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 3 h at 4 °C, and submerged in 30% sucrose overnight. The SCGs were then dehydrated in graded ethanol, embedded in paraffin and cut with a slice thickness of 4 μ m. The paraffin sections were baked and de-waxed. Immunostaining for $\alpha 4$ and $\alpha 7$ were carried out as follows. After wash with PBS for three times, the sections were incubated in 1 % H_2O_2 for 8 min to block the endogenous peroxidase. Following rinses in PBS for 10 min, sections were incubated with 1 % bovine serum album (BSA) for 30 min, then the sections were incubated either with rabbit anti-nAChR $\alpha 4$ (Millipore, 1: 500) or anti-nAChR $\alpha 7$ (Abcam, 1: 500) diluted in BSA for overnight at 4 °C. After primary antibody incubation, sections were rinsed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam, 1: 1000) diluted in BSA for 2 h at room temperature. The sections were washed in PBS for 10 min and reacted with diaminobenzidine chromogen for 20 min in darkness. The sections were examined under optical microscope (BX51TF, Olympus, Japan). The changes of intensity of optical density (IOD) for nAChR subunits $\alpha 4$ and $\alpha 7$ were analyzed by Image Pro-Plus software.

2.5. Ultrastructural studies

As described in previous reports (Krishnaswamy and Cooper, 2009); (Cai et al., 2013), the SCGs were rapidly dissected from APP^{-/-} and WT mice, placed directly into 2.5 % glutaraldehyde in 0.2 M PBS at room temperature, and fixed for 24 h in darkness. The tissue was then rinsed three times in 0.2 M PBS at room temperature (15 min each), post-fixed in 1 % OsO_4 for 1 h at 4 °C, rinsed briefly with 0.2 M PBS, dehydrated in graded series of acetone concentrations up to 100 %, placed in 100 % epoxy resin for 24 h, and embedded in EPON812 and polymerized for 8 h in an oven at 70 °C. The

tissue was sectioned into 60 nm per micrograph with LKB ultramicrotome (LKB Corporation, Switzerland), and stained with uranyl acetate followed by lead citrate, then observed and photographed using a Hitachi-7500 transmission electron microscope. Number of presynaptic vesicles and dimensions of postsynaptic density, synaptic cleft and interface curvature were measured using Image Pro-Plus software. Presynaptic vesicle density was determined as the number of synaptic vesicles per square micrometer of profile area (Wang et al., 2005). Synaptic cleft was estimated as the mean of three values including the largest, middle, and smallest (Xu and Zhang, 2006). PSD thickness was measured at the thickest part of PSD (Xu et al., 2013). PSD length was measured from the full-length PSD along postsynaptic membrane boundary (Fukunaga et al., 2015). Curvature of synaptic interface was calculated as the arc length of synaptic interface divided by the straight length between two ends of PSD (Xu et al., 2013).

2.6. Statistical analysis

All data were expressed as mean $\pm$ SEM. Origin 8.0 software was employed for generating graphs. Statistical analyses were performed with Prism6 (GraphPad Software). Input-output curves and sustained firing tests were analyzed using 2-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparisons. All other data were analyzed using unpaired t-test if values complied with Gaussian distribution tested by D'Agostino & Pearson omnibus normality test, or using the nonparametric Mann-Whitney test otherwise. $p < 0.05$ was considered as significant difference.

3. Results

3.1. APP deficiency resulted in impaired CAP in SCG *in vivo*

The efficiency of synaptic transmission measured by recording of CAP can be reflected by the analysis of input-output relationship (Diogenes et al., 2011; Oddo et al., 2003). Therefore, we first evaluated the relationship between the intensity of electrical stimulation and CAP amplitude *in vivo* in APP^{-/-} and WT SCG. As shown in Figure 1A, at low-intensity stimuli (less than 2.0 mA), CAP amplitude was identical between APP^{-/-} and WT. However, high intensity of electrical stimulation (2.0~4.0 mA) differentiated the reduced CAP amplitude in APP^{-/-} from that in WT (at 2.0, 3.0 and 4.0 mA, CAP amplitude: 1.50 ± 0.20 , 2.00 ± 0.28 and 2.59 ± 0.46 mV in APP^{-/-} vs. 2.96 ± 0.53 , 4.20 ± 0.59 and 4.93 ± 0.74 mV in WT, respectively, 2-way ANOVA, $F(1,18) = 10.75$, $p < 0.01$, $n = 10$), implicating an impaired cholinergic

transmission in SCG in the absence of APP.

Did the reduction in CAP amplitude result from pre- or postsynaptic changes? With regard to this concern, we performed paired-pulse experiment, a protocol commonly used to determine presynaptic function (Yang et al., 2007; Yao et al., 2013), by applying two stimulation pulses at 50 ms interval. As shown in Figure 1B, the paired-pulse ratio (PPR), which was determined by the ratio of second CAP amplitude over the first one, was significantly enhanced in APP^{-/-} compared with WT (APP^{-/-} 1.04 ± 0.03 vs. WT 0.82 ± 0.08 , unpaired t-test with Welch's correction (two-tailed), $p < 0.05$, $n = 8$), suggesting that attenuated synaptic transmission in APP^{-/-} SCG involves, at least in part, presynaptic defects.

3.2. Recession of sustained firing was associated with aberrant activities of CHT in APP^{-/-} SCG

High quality of presynaptic activity permits the persistence of high-frequency synaptic transmission. Particularly, choline uptake by CHT is a rate-limiting step during cholinergic sustained firing (Krishnaswamy and Cooper, 2009; Wang et al., 2007). Although impaired CHT expression and function had been reported in the NMJ of APP^{-/-} mice (Wang et al., 2007), if CHT deficit occurred in SCG of APP^{-/-} mice remained unknown. We then tested if blockade of CHT activity by HC3 would normalize the CAP differences in between WT and APP^{-/-} mice (Figure 2). Interestingly, HC3 only normalized the PPR difference in between APP^{-/-} and WT SCG as PPR was identical in the presence of HC3 (APP^{-/-} 0.93 ± 0.04 vs. WT 0.93 ± 0.07 , unpaired t-test (two-tailed), $p > 0.05$, $n = 8$) (Figure 2B). However, defect on CAP amplitude remained in the presence of HC3 in between APP^{-/-} and WT (Figure 2A). The results indicate that presynaptic deficit in APP^{-/-} SCG involves altered CHT activity, while the reduction on CAP amplitude may be attributed to different mechanisms.

Altered CHT activity in APP^{-/-} SCG was further confirmed by experiments with train stimulation. Sustained firing in SCG was elicited by a train of repetitive stimulation at 10 Hz for 30 s as previously described by other groups (Krishnaswamy and Cooper, 2009; Rassadi et al., 2005). The results showed that the firing in APP^{-/-} SCG was dramatically declined to 57.0 ± 6.3 % of initial level after 30 sec, which was significantly lower compared with WT (2-way ANOVA, $F(1,14) = 6.605$, $p < 0.05$, $n = 8$) where the firing sustained at 81.6 ± 7.9 % of initial level (Figure 2C & D). Under the presence of HC3 (10 μ M), the firing was sharply attenuated by a similar degree in

both genotypes (APP^{-/-} 45.7 ± 8.4 % of initial level vs. WT 51.9 ± 7.3 % of initial level, 2-way ANOVA, F(1,14) = 0.1307, p > 0.05, n = 8). In addition, we applied coluracetam (MKC-231) (1μM) (Akaike et al., 1998), a choline uptake enhancer, to APP^{-/-} SCG, which in turn increased the firing activity to the level of WT (2-way ANOVA, F(1,12) = 0.0940, p > 0.05, n = 7) (Figure 2C & D). Our results suggested that APP deficiency led to the firing decline of cholinergic synaptic transmission by diminishing CHT.

The disruption of CHT abolished the difference in sustained firing between APP^{-/-} and WT. However, the original amplitude of CAP was decreased in APP^{-/-} compared with WT even in the presence of HC3 (APP^{-/-} 0.56 ± 0.08 mV vs. WT 1.20 ± 0.12 mV, Mann-Whitney test (two-tailed), p < 0.05, n = 7) (Figure 2C & E). Again, these results suggested that there would be other signal pathways, likely postsynaptic mechanisms involved in cholinergic transmission dysfunction with APP deficits.

3.3. Postsynaptic nAChR alterations contributed to abnormal CAP in APP^{-/-} SCG

To examine if impaired CAP seen in APP^{-/-} SCG involves postsynaptic alterations as well, exogenous ACh at a concentration of 3 mM was delivered onto SCG to induce postsynaptic responses (Tochitsky et al., 2012). Indeed, the postsynaptic potential was significantly decreased in APP^{-/-} compared with WT SCG (APP^{-/-} 0.605 ± 0.110 mV vs. WT 1.29 ± 0.203 mV, Mann-Whitney test (two-tailed), p < 0.01, n = 7) (Figure 3), suggesting postsynaptic function was also impaired possibly due to alterations on AChRs in APP^{-/-} SCG.

Nicotinic and muscarinic receptors jointly give rise to cholinergic activities (Beierlein, 2014; Sun et al., 2013). However, cholinergic synaptic transmission is mainly mediated by nAChR and the possibility of APP's role in regulating mAChR was ruled out because our preliminary experiment turned out no significant change in atropine-sensitive component between APP^{-/-} and WT (Figure 4, Mann-Whitney test (two-tailed), p > 0.05, n = 7 for all 2, 5 and 10 μM atropine). In SCG, dominant subtype of nAChR is α3β4 hetero-pentamer (David et al., 2010). Herein, the contribution of α3β4 nAChR was evaluated by using its antagonist, 1 μM Mec. At this does Mec selectively blocks α3β4 nAChR (Eguchi et al., 2007). Our results showed that evoked CAP amplitude in WT declined from 3.00 ± 0.16 mV to 2.44 ± 0.37 mV 30 sec after Mec application, while CAP amplitude in APP^{-/-} was decreased from 2.02 ± 0.22 mV to 1.49 ± 0.26 mV under Mec (1 μM) application (Figure 5A). These

reductions reflected the component of cholinergic synaptic transmission sensitive to 1 μ M Mec. However, these decrements were not significantly different between APP^{-/-} and WT (APP^{-/-} 0.41 $\pm$ 0.17 mV vs. WT 0.56 $\pm$ 0.14 mV, Mann-Whitney test (two-tailed), $p > 0.05$, $n = 7$) (Figure 5C), suggesting similar $\alpha 3\beta 4$ nAChR mediated responses in between APP^{-/-} and WT SCG.

When given a higher dose of Mec (10 μ M) which can block nAChR subtypes including $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$ (Papke et al., 2001), the experiments showed that the component of evoked CAP sensitive to 10 μ M Mec was significantly reduced in APP^{-/-} compared with WT (APP^{-/-} 0.15 $\pm$ 0.12 mV vs. WT 1.00 $\pm$ 0.36 mV, Mann-Whitney test (two-tailed), $p < 0.01$, $n = 7$) (Figure 5B & C). These results suggested that APP deficiency may impair postsynaptic $\alpha 4\beta 2$ and/or $\alpha 7$ nAChR rather than $\alpha 3\beta 4$ nAChR. CAP was not completely blocked by 10 μ M Mec, which suggested other subtype receptors mediated CAP in both WT and APP^{-/-} SCG. However, these non-identified components were unaltered since the remaining CAP was identical between WT and APP^{-/-} 7 min after drug application (Figure 5B).

Further experiments were performed with the application of DH β E (3 μ M, a selective antagonist for $\alpha 4\beta 2$ nAChR) (Zhou et al., 2013) and MLA (0.1 μ M, a selective antagonist for $\alpha 7$ nAChR) (Singh et al., 2013), respectively. As speculated, both DH β E- and MLA-sensitive components of evoked CAP were significantly decreased in APP^{-/-} compared with WT (DH β E-sensitive: APP^{-/-} 0.50 $\pm$ 0.08 mV vs. WT 0.99 $\pm$ 0.20 mV, Mann-Whitney test (two-tailed), $p < 0.05$, $n = 7$; MLA-sensitive: APP^{-/-} 0.19 $\pm$ 0.06 mV vs. WT 0.44 $\pm$ 0.12 mV, Mann-Whitney test (two-tailed), $p < 0.05$, $n = 7$) (Figure 6A). These results indicated that SCG cholinergic transmission mediated by $\alpha 4\beta 2$ and $\alpha 7$ nAChRs was impaired due to missing APP.

We next performed western blotting and immunohistochemical staining to further investigate the cellular and molecular profile of $\alpha 7$ and $\alpha 4\beta 2$ nAChR underlying altered synaptic transmission. Our results revealed that the level of nAChR subunit $\alpha 7$ was dramatically reduced in APP^{-/-} compared with WT in both western blotting and immunohistochemical assay (Mann-Whitney test (two-tailed), $p < 0.05$, $n = 5$), however the level of nAChR subunit $\alpha 4$ was identical in both genotypes (Mann-Whitney test (two-tailed), $p > 0.05$, $n = 5$) (Figure 6B & C). Together with functional measurement, these data suggested that APP deficits impaired $\alpha 4\beta 2$ nAChR activity rather than expression level.

3.4 APP^{-/-} SCG demonstrated abnormal ultrastructure

Synaptic transmission relies on the structural basis of presynaptic and postsynaptic compartments (Harris and Weinberg, 2012). Potentially, APP may serve as an adhesion molecule or trophic factor in synapse formation and modification (Muller and Zheng, 2012). To reveal changes in synaptic ultrastructure of APP^{-/-} SCG, morphological experiments were conducted with transmission electron microscope (TEM). The density of synapses in sympathetic ganglia was too low to qualify the credit of synapses amount with TEM as described previously (Krishnaswamy and Cooper, 2009). However, we observed remarkable changes in presynaptic vesicle quantity, postsynaptic density (PSD), synaptic cleft and interface curvature of synapses.

As shown in Figure 7 and Table 1, a striking reduction of synaptic vesicle number occurred in presynaptic terminals of APP^{-/-} SCG. Quantitatively, the vesicle density (number of synaptic vesicles per square micrometer of profile area) in APP^{-/-} SCG was approximately reduced by half (Mann-Whitney test (two-tailed), $p < 0.05$, $n = 5$). PSD length in APP^{-/-} was shorter than that in WT (Mann-Whitney test (two-tailed), $p < 0.05$, $n = 5$), though PSD thickness kept constant (**Mann-Whitney test (two-tailed)**, $p > 0.05$, $n = 5$). These ultrastructural results revealed that APP deletion indeed impaired the morphology of cholinergic synapses. On the other hand, we found decreased synaptic cleft (Mann-Whitney test (two-tailed), $p < 0.05$, $n = 5$) but increased interface curvature of synapses (Mann-Whitney test (two-tailed), $p < 0.05$, $n = 5$) in APP^{-/-} SCG. Both narrower synaptic cleft and larger interface curvature of synapses were of benefit for synaptic transmission (Di and Zheng, 2013; Hu et al., 2014), which probably compensated presynaptic and postsynaptic impairments to help synapses survive under APP deficiency.

Table 1. Comparison in synaptic ultrastructure

	WT	APP ^{-/-}
Vesicle density (N/ μm^2)	370.6 $\pm$ 96.7	185.5 $\pm$ 27.7 *
Synaptic cleft (nm)	20.65 $\pm$ 2.27	11.62 $\pm$ 3.47 *
PSD length (nm)	525.1 $\pm$ 57.5	324.0 $\pm$ 55.2 *
PSD thickness (nm)	27.88 $\pm$ 2.92	20.47 $\pm$ 4.10
Curvature of synaptic interface	1.097 $\pm$ 0.038	1.235 $\pm$ 0.018 *

Values are shown as mean $\pm$ SEM. Mann-Whitney test (two-tailed), * $p < 0.05$ vs WT. $n = 5$ for both groups.

3.5 Asymmetric eyes in APP^{-/-} mice

As we found in the current study, cholinergic synaptic transmission was impaired in APP^{-/-} SCG, which is related to Horner's syndrome characterized by myosis, ptosis, enophthalmos, prolapse of the nictitating membrane and conjunctival and facial congestion (Viscasillas et al., 2013). As shown in Figure 8A, APP^{-/-} mice presented drooping of the one side eyelids randomly and visible white discoloration and inflammatory discharge around the eyelids. Then we compared the differences between bilateral eyes by detecting delta diameter of eyeballs, which was significantly increased in APP^{-/-} mice (APP^{-/-} 110.47 ± 38.14 pixels vs. WT 29.86 ± 2.51 pixels, $p < 0.05$, $n = 7$) (Figure 8B), suggesting that the abnormal eye conditions could be due to SCG sympathetic attenuation.

4. Discussion

In current study, we took advantage of APP gene knock-out mice as loss of function to investigate how the APP plays a role in cholinergic synaptic transmission. However, APP gene is deleted in whole body of the mouse. Experimental research in central nervous system cannot exclude crosstalk from other affected inputs, such as glutamatergic and GABAergic neurons. In this concern, we performed experiments in SCG which receives a prominent cholinergic innervation from the preganglionic sympathetic trunk (Mao et al., 2006). The synaptic alterations as we observed in SCG are promising in cholinergic system due to APP deficits. For further study on the role of APP in collaboration between cholinergic, glutamatergic and GABAergic system, SCG will not meet our requirements for experiments and we will move on to prefrontal cortex. Nevertheless, we report here that APP deficiency results in both presynaptic and postsynaptic defects, leading to impaired SCG cholinergic synaptic transmission. In addition to presynaptic regulation of choline uptake and vesicle pool as shown here and by others (Wang et al., 2007; Wang et al., 2005; Wang et al., 2009), our research reveals that APP is critically involved in modulating postsynaptic nAChR-mediated components. This altered cholinergic synaptic transmission in SCG of APP knockout mice attenuates sympathetic innervation to face and eyes, which could contribute to abnormal eye conditions as we observed.

4.1. Presynaptic deficits in APP^{-/-} SCG

Focus of presynaptic mechanisms has been given to CHT and choline uptake for effects of APP on cholinergic synaptic activity (Wang et al., 2007; Wang et al., 2009).

It is reported that APP regulates CHT expression and function through protein-protein interaction (Wang et al., 2007) and that the interactions of presynaptic and postsynaptic APP contributed to cholinergic synaptogenesis (Wang et al., 2009). The current study further demonstrated that APP play a crucial role in SCG presynaptic cholinergic function as we observed abnormal PPR which might be resulted from CHT defect and fewer presynaptic vesicles in APP^{-/-} mice. Abrogation by a CHT inhibitor of PPR and repetitive stimulation induced CAP decline, but not the amplitude of CAP, in WT and APP^{-/-} suggests that presynaptic deficits in the SCG of APP^{-/-} mice might be attributed to impaired CHT activities which resulted in impaired choline uptake and sequential ACh synthesis as well as a pitfall of ACh-containing vesicle numbers.

4.2. Postsynaptic defects in APP^{-/-} SCG

A novel finding of the current study is that we demonstrated a critical role of APP in postsynaptic $\alpha 4\beta 2$ and $\alpha 7$ nAChRs mediated transmission. Cholinergic synaptic transmission has been shown to be mainly mediated by nAChR in sympathetic ganglia (David et al., 2010), we observed a significant reduction in exogenous ACh-mediated response and a reduction in both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs function. Though the expression level of $\alpha 7$ nAChR was decreased in APP^{-/-} SCG, the expression level of $\alpha 4\beta 2$ nAChR was unchanged. These findings suggest that APP may regulate $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in different manners. The implication of our findings will drive further research on the role of APP in $\alpha 4\beta 2$ and $\alpha 7$ nAChR-mediated components in central nervous system, since both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs modulate the neurobiological processes underlying learning and memory in brain (Kutlu and Gould, 2015).

4.3 Synaptic morphology deficits in APP^{-/-} SCG

We found in the present study that presynaptic and postsynaptic membranes were more closed as synaptic cleft became narrower in APP^{-/-} SCG. Meanwhile, curvature of synaptic interface was increased in APP^{-/-} SCG. Though these ultrastructural changes are in favor of synaptic transmission (Di and Zheng, 2013; Hu et al., 2014), impaired cholinergic transmission observed indicates that these might be a compensatory effect to cheer up the reduced SCG synaptic transmission in the absence of APP.

In conclusion, our findings demonstrate that APP is critically required in maintaining normal cholinergic transmission in the SCG via presynaptic and postsynaptic mechanisms, and provide profound perspectives to understand the role of APP in cholinergic synaptic activities.

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Legends

Figure 1. The input-output curves and paired-pulse test. (A) Sample traces (top) show representative CAPs recorded at the stimulation intensity of 3 mA in WT and APP^{-/-}. The measurement for CAP amplitude was shown as *P* in WT trace, briefly, which is the vertical distance between the peak and a tangent constructed between the onset and termination of the CAP. Compared with WT, the input-output curve was significantly reduced in APP^{-/-} (***p* < 0.01, *F* (1,18) = 10.75, *n* = 10 for both WT and APP^{-/-}, 2-way ANOVA). The reduction reflects a defective synaptic transmission in APP^{-/-} SCG. (B) The PPR at the inter-pulse interval of 50 ms was significantly increased in APP^{-/-} compared with WT (*n* = 8 for all groups), implicating changes in presynaptic activity of APP^{-/-} SCG. Data are presented as mean ± SEM. ***p* < 0.01.

Figure 2. Effects of CHT on synaptic transmission and sustained firing. (A) Under HC3 (10 μM) application, CAP amplitude in APP^{-/-} was still less than WT (Mann-Whitney test (two-tailed), *p* < 0.05, *n* = 7). (B) PPR difference was normalized with HC3 (10 μM) treatment. (C) Sample traces for each sustained firing group at 1, 15 and 30 s. (D) The onset response was recorded at 1 s and defined as 100 % for data normalization in each group. Sustained firing in APP^{-/-} was decreased sharply, compared with WT (2-way ANOVA, *p* < 0.05, *F* (1, 14) = 6.605, *n* = 8). Furthermore, inhibiting CHT by HC3 (10 μM) depressed the activity of sustained firing to a similar level in APP^{-/-} and WT. In addition, with MKC-231 (1 μM) application, sustained firing in APP^{-/-} was increased and comparable to WT. These results suggested that APP deficits attenuated cholinergic firing, at least in part, by disturbing CHT. (E) The difference of original value of CAP amplitude at 30 s still persisted between APP^{-/-} and WT (unpaired *t* test (two-tailed), *p* < 0.05, *n* = 8), even under the presence of HC3 (unpaired *t*-test (two-tailed), *p* < 0.05, *n* = 8), demonstrating other mechanisms more than presynaptic pathways due to APP deletion. HC3: hemicholinium-3. Values are shown as mean ± SEM. **p* < 0.05.

Figure 3. Postsynaptic response in SCG evoked by exogenous ACh (3 mM). Sample traces (left) was displayed individually (light grey) and superimposed to average level for WT (black) and APP^{-/-} (grey). Compared with WT, ACh-mediated response was significantly reduced in APP^{-/-} (Mann-Whitney test (two-tailed), *p* < 0.01, *n* = 7), indicating cholinergic postsynaptic function loss due to APP deficits..

Figure 4. Atropine-sensitive component of CAP is rare and not significantly different between APP^{-/-} and WT. (A) On the top panel, sample traces showed CAPs recorded before and after 5 μ M atropine (a competitive antagonist of the mAChR) application in WT and APP^{-/-}, respectively. On the bottom, CAP amplitude was plotted to the concentration of atropine (2, 5 and 10 μ M), whereas 0 meant only vehicle (saline) treatment. (B) The histogram represented the total CAPs, as the initial level, and the components sensitive to atropine (2, 5 and 10 μ M). n=7 for all groups.

Figure 5. Nicotinic AChR-mediated components of evoked CAP in SCG. (A) The components sensitive to low dose (1 μ M) Mec, which could completely inhibit nAChR subtype α 3 β 4, were similar between APP^{-/-} and WT (Mann-Whitney test (two-tailed), $p > 0.05$, $n = 7$). (B) The component was significantly reduced in APP^{-/-} compared with WT (Mann-Whitney test (two-tailed), $p < 0.01$, $n = 7$), under the application of high dose (10 μ M) Mec, which was strong enough to inhibit nAChR α 4 β 2 and α 7 subtypes. (C) The histogram showed intact CAPs and components sensitive to low and high doses of Mec between APP^{-/-} and WT. These results suggested that the APP deletion caused postsynaptic functional loss, likely due to nAChR α 4 β 2 and/or α 7 impairment rather than α 3 β 4 alteration. Mec: mecamylamine.

Figure 6. Function and expression of nAChR α 4 β 2 and α 7. (A) The components of CAP sensitive to DH β E (3 μ M, selectively inhibit nAChR α 4 β 2) and MLA (0.1 μ M, selectively inhibit nAChR α 7) were decreased in APP^{-/-} compared with WT (for both DH β E and MLA treatment, Mann-Whitney test (two-tailed), $p < 0.05$, $n=7$), indicating α 4 β 2 and α 7 nAChR function loss due to APP deletion. Sample traces (top) showed intact CAPs and the components sensitive to DH β E or/and MLA by subtracting intact CAPs' counterparts under the presence of DH β E or/and MLA, respectively. DH β E: dihydro- β -erythroidine, MLA: methyllycaconitine. (B) The results showed an obvious reduction of nAChR subunit α 7 level in APP^{-/-} compared with WT, while the subunit α 4 level is similar between both genotypes. Statistical histogram (right) was derived from images at the left. (C) Brown shows α 7- or α 4-positive soma of neurons; Blue shows nuclei stained by hematoxylin. The percentage of α 7- or α 4-positive neurons is determined by the number of brown cells divided by blue nuclei amount. The results revealed an unchanged α 4 level (Mann-Whitney test (two-tailed), $p > 0.05$, $n = 5$) and a significant decline of α 7 level compared with WT (Mann-Whitney test (two-tailed), $p < 0.01$, $n = 5$). Scale bars: 20 μ m. Statistical histogram (right) was derived from 5

images of immunoassaying.

Figure 7. Synaptic ultrastructure in WT and APP^{-/-} SCG. Image samples of transmission electronic microscope (TEM) were shown for WT (top) and APP^{-/-} (bottom) at the magnification of 70,000. Scale bars: 200 nm.

Figure 8. Abnormal bilateral eyes in APP^{-/-} mice. (A) APP^{-/-} mice exhibited observable differences between bilateral eyes sensitivity as evidenced by wincing and closure of the eyelid. Visible white discoloration and inflammatory discharge around the eyelid of the right or left eye were also observed. (B) Delta diameter between bilateral eyes was significantly increased in APP^{-/-} compared with WT (n=7 for all groups), implicating damage in APP^{-/-} SCG.

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