Disrupted Synaptic Transmission and Abnormal Short-term Synaptic Plasticity

in an Angelman Syndrome Mouse Model

by

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ABSTRACT

Angelman syndrome (AS) is a neurodevelopmental disorder characterized by developmental delays, intellectual disabilities, impaired language and speech, and movement defects. Most AS cases are caused by dysfunction of a maternally-expressed E3 ubiquitin ligase (UBE3A, also known as E6 associated protein, E6-AP) in neurons. Currently, the mechanism on how loss-of-function of the enzyme influences the nervous system development remains unknown. We hypothesize that impaired metabolism of proteins, most likely those related to E6-AP substrates, may alter the developmental trajectory of neuronal structures including dendrites, spines and synaptic proteins, which leads to disrupted activity/experience-dependent synaptic plasticity and maturation. To test this hypothesis, we conducted a detailed investigation on neuronal morphology and electrophysiological properties in the prefrontal cortex (PFC) layer 5 (L5) corticostriatal pyramidal neurons (target neurons). We found smaller soma size in the maternal *Ube3a* deficient mice (m-/p+; 'AS' mice) at postnatal 17-19 (P17-19), P28-35 and older than 70 days (>P70), and decreased basal dendritic processes at P28-35. Surprisingly, both excitatory and inhibitory miniature postsynaptic currents (mEPSCs and mIPSCs) decreased on these neurons. These neurons also exhibited abnormalities in the local neural circuits, short-term synaptic plasticity and AMPA/NMDA ratio: the excitatory inputs from L2/3 and L5A, and inhibitory inputs from L5 significantly reduced in AS mice from P17-19; Both the release probability (Pr) and readily-releasable vesicle (RRV) pool replenishment of presynaptic neurons of the target neurons were disrupted at P17-19 and P28-35, and the change of RRV pool replenishment maintained through adulthood (>P70). The AMPA/NMDA ratio showed abnormality in the L5 corticostriatal neurons of PFC in AS mice older than P28-35, during which it decreased significantly compared to that of age-matched WT littermates. Western Blot analysis revealed that the expression level of a key regulator of the cytoskeleton system, Rho family small GTPase cell division control protein 42 homolog (cdc42), reduced significantly in the PFC of AS mice at P28-35.These impairments of synaptic transmission and short-term synaptic plasticity may account for the impaired neuronal morphology and synaptic deficits observed in the PFC target neurons, and contribute to the phenotypes in AS model mice. The present work reveals for the first time that the E6-AP

deficiency influences brain function in both brain region-specific and age-dependent ways, demonstrates the functional impairment at the neural circuit level, and reveals that the presynaptic mechanisms are disrupted in AS model. These novel findings shed light on our understanding of the AS pathogenesis and inform potential novel therapeutic explorations.

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CHAPTER 1

INTRODUCTION

Angelman syndrome (AS), a rare monogenic neurodevelopmental disorder, results from the dysfunction/inactivation of the maternal *UBE3A* gene and its surrounding chromosome structures (Wagstaff et al., 1992). The *UBE3A* gene encodes an E3 ubiquitin-protein ligase (UBE3A, also called E6 associated protein, E6-AP. *UBE3A*/*Ube3a* will be used to refer to the gene in human or mouse respectively, whereas E6-AP will be used to represent the protein hereafter) and locates within chromosome 15q11-13 in humans. Due to genetic imprinting of the paternal copy of *UBE3A* gene in many brain regions, loss of function of a single maternal copy of *UBE3A* is highly penetrant and pathogenic. Most of the deficits seen in AS patients have been reproduced in *UBE3A* gene maternal deficiency mice ('AS mouse', *UBE3A*$^{m-/p+}$), thus enabling mechanistic interrogations of AS pathogenesis and therapeutic explorations AS treatment using mice models. The introduction briefly discusses recent advances on AS etiology and identifies some challenges in translating mechanistic insights into potential therapeutic interventions. Experimental evidence collected so far indicate impaired maternal *UBE3A* in neurons may contribute to AS deficiency by influencing multifaceted neural developmental processes including cell survival, synaptic transmission, signal transduction and gene expressions.

1.1 The Clinical Characteristics of Angelman Syndrome

Angelman syndrome is first reported and named thereafter by pediatrician Harry Angelman in 1965 (Angelman, 1965). AS is a debilitating neurodevelopmental disorder characterized by severe mental retardation, speech impairment, seizures, motor dysfunction and a high prevalence of autism (Williams et al., 2010a)(Bird, 2014). Some symptoms such as microcephaly, epilepsy and abnormal electroencephalography (EEG) are usually accompanied deficits found in more than 80% of patients. Other symptoms, for example muscle hypotonia, arrant looking such as flat neck and protruding tongue, and behavioral disorders including food-intake and sleep

disturbances are frequent ones but vary among patients (Angelman, 1965) (Bower and Jeavons, 1967) (Williams et al., 2006) (Pelc et al., 2008a)(Clayton-Smith and Laan, 2003a)(Bruni et al., 2004)(Colas et al., 2005). AS patients show high rate of incontinence although most patients underwent well toilet training in their childhood (Niemczyk et al., 2015) (Zori et al., 1992). Bone abnormalities, such as brachycephaly, microcephaly, osteoporosis and delayed bone development-associated limb deformity and osteopenia are often co-occurring conditions (Õiglane-Shlik et al., 2005)(Williams et al., 2006)(Coppola et al., 2007)(Rusińska et al., 2013)(Kyllerman, 2013)(Thibert et al., 2013). AS influences the general population with an estimated rate of 1:10000 to 1:40000 in U.S. and the United Kingdom, respectively (Buckley et al., 1998)(Clayton-Smith, 2003).

1.2 The Genetic Abnormality and Phenotypic Presentation

Loss of *UBE3A* gene function was identified as the cause of AS by two research groups in 1997 (Kishino et al., 1997)(Matsuura et al., 1997). The human *UBE3A* gene encodes the E6-AP. *UBE3A* gene is normally expressed in neurons only from the maternally inherited allele, while the paternal allele is silenced by epigenetic mechanisms known as imprinting. Therefore, mutation of the single maternal *UBE3A* allele in neurons leads to complete loss-of-function of E6-AP. In the majority of AS patients, *UBE3A* gene is found inactivated by either intragenic mutation, chromosomal microdeletion in the 15q11-13 regions, paternal uniparental disomy (UPD), or a defective imprinting center (IC) that controls UBE3A transcription (for a review, see (Mabb et al., 2011)).

Consistent with this genetic architecture, targeted inactivation of *UBE3A* gene in mice (Jiang et al., 1998) also support the role of E6-AP in AS; upon inheritance of the mutation through the maternal germline, *Ube3a* mutant mice (*Ube3a*$^{m-/p+}$, 'AS mice') display salient pathological features of AS. Critical defects in both morphology and function of neurons was found (Jiang et al., 1998). It is important to note that while deficiency of *UBE3A* causes AS, increased *UBE3A*

gene dosage (from maternal duplications of the *UBE3A*-spanning 15q11–q13 region (Cook et al., 1997)), appears to be associated with intellectual and developmental abnormalities seen in the autism spectrum disorders, and reproduces most autism features in mouse models (Nakatani et al., 2009)(Smith et al., 2011). However, it is currently not clear whether an increase in *Ube3a* dosage alone accounted for the autism phenotypes.

Molecular studies have revealed that knockdown of *Ube3a* in mouse increases neuronal death, which might be due to the accruement of p53 protein, p53-dependent transcription, or deposition of intracellular misfolded polyglutamine proteins (Mishra et al., 2008). In AS mouse models, the deficiency of E6-AP causes a reduction of dendrites spine density and dendritic length in multiple brain areas including the hippocampus, the cortex layer Ⅲ-Ⅴ and the cerebellum (Dindot et al., 2008)(Yashiro et al., 2009). AS mice also show defects of dendrite polarization of pyramidal neurons in the cortex and the hippocampus, decreased dendritic arborization in the somatosensory (S1) cortex layer II/III (Miao et al., 2013), decreased synaptic vesicle density in the hippocampus and abnormal clathrin-coated vesicles accumulation in both the glutamatergic and the GABAergic (γ-aminobutyric acid) neurons synaptic terminals (Su et al., 2011) (Wallace et al., 2012) (Judson et al., 2016). The morphological abnormality seems to be age-dependent as no difference in dendritic arborization in the cerebellum or morphological defects in the hippocampus was observed on animals aged 3-4 months old (Jiang et al., 1998). Interestingly, recent studies found that the glucocorticoid receptor (GR) expression level altered in AS mice. A research showed the GR expression reduced drastically in the hippocampus, but kept normal in the cerebral cortex and the cerebellum in 4-months-old male AS mice (Godavarthi et al., 2012). The reduction of GR in the hippocampus can be partially rescued with fluoxetine treatment (Godavarthi et al., 2014). Controversially, another report illustrated that AS mice have elevated GR expression level and reduced cortical myelin proteins, and the GR expression level can be influenced by maternal status (i.e. GR expression level decreased in pups raised by the carrier (normal) mother compared to those raised by AS mother) (Grier et al., 2015a). Similar

abnormalities of brain structure such as myelination decrease, axonal density or diameter reduction, axonal organization aberrance and reduced cortical/subcortical gray matter volume were also found in AS patients (Peters et al., 2010b) (Aghakhanyan et al., 2016).These morphological changes are consistent with the observed functional deficits. For example, decreased mEPSCs and synaptic plasticity (such as long-term potentiation (LTP) impairment) are found in AS mouse models (Weeber et al., 2003), which involves down-regulated NMDAR function and deficiency of calcium influx. These evidences are indicative that E6-AP is required for normal neuronal activity. On the other hand, cellular E6-AP levels are also affected by neuronal activity. Filonova et al. (Filonova et al., 2014) recently reported that synaptic activation leads to dramatic changes in *Ube3a* neuronal expression. Both increased neuronal activity by depolarization or fear conditioning behavioral paradigm enhanced neuronal E6-AP levels. The authors also found that in the absence of E6-AP, activity-dependent increases in extracellular signal-regulated kinases (ERK1/2) phosphorylation was impaired. This altered mitogen-activated protein kinases (MAPK) pathway may underlie the impaired synaptic plasticity and cognitive function in AS mice.

Intriguingly, experience-dependent maturation of excitatory cortical circuits, and the visual cortex function associated with ocular dominance plasticity were found impaired in AS mice, suggesting E6-AP is necessary for maintaining developmental cortical plasticity and its loss-of-function may contribute to AS pathophysiology (Yashiro et al., 2009). The same research group also reported that dysfunction of *Ube3a* resulted in deficits of fast-spiking inhibitory interneurons in cortex layer Ⅱ-Ⅲ for an abnormality of presynaptic vesicle release and mIPSCs in younger (P25) mice while kept normal in older (P80) ones (Wallace et al., 2012). Consistent with the role of *UBE3A*/*Ube3a* in plasticity, a recent study demonstrated that the type 5 metabotropic glutamate (mGluR5) receptors-dependent LTD was potentiated in the hippocampus in AS mice (Pignatelli et al., 2014). It has been also reported that parvalbumin-positive (PV) interneurons in AS mice are more vulnerable than those of wild type mice in responding to chronic stress. Chronic stress treatment leads to more pronounced decrease of PV neurons in the hippocampus

and the basolateral amygdala of AS mice, a process that can be antagonized with fluoxetine (Godavarthi et al., 2014). AS mouse also show abnormality in behavior related to the malfunction of basal ganglia circuits (e.g. instrumental conditioning). These mice have severe difficulty in initial acquisition of lever pressing, and were more habitual and impervious to changes compared with the wild-type control ones. Electrophysiological results revealed that both amplitude and frequency of mEPSCs are decreased in the dorsomedial striatum in AS mice, suggesting specific impairment of synaptic function in an associative corticostriatal circuit (Hayrapetyan et al., 2014) that is also shared by the autism spectrum (Rothwell et al., 2014). The poorer performance in hidden platform test of Water-maze task in AS mice in contrast to that of the wildtype control demonstrated the learning and memory dysfunction (Miura et al., 2002). The ataxia, a common symptom in AS, is accompanied by the abnormal oscillation of cerebellar cortex which can be inhibited by sensory stimulation, inhibitors of gap junction or $GABA_A$ receptors (Cheron et al., 2005a). Other behavioral abnormalities including increased fluid consumption, defect in rotarod test, vertical rope climbing and raised beam task, and reduction of grip strength in AS model mice were also reported (Heck et al., 2008).

1.3 The E6-AP

1.3.1 The Gene and Protein Structures of the E6-AP (Human and Mouse)

The *UBE3A* (human) or *Ube3a* (mouse) gene is located on chromosome 15 or 7 respectively. The gene DNA sequence includes 10 exons in 60kb length which encodes mRNA of 5kb, and 5 isotypes of mRNA are generated due to alternative splicing. The 5 transcript variants give rise to 3 isoforms of protein products named isoform I, II and III, with an additional 20 or 23 amino acid (aa) on amino terminal of II and III, respectively, in contrast to isoform I. These additional aa do not involve into the E6-AP E3 ligase function, which is mediated by active 833 cysteine residing within the homologus to E6-AP carbonyl terminus (HECT) domain on the C terminal. The protein also contains E6 binding domain which locates closely to the HECT domain

(Yamamoto et al., 1997) (El Hokayem and Nawaz, 2014). The human and mouse E6-AP homologue contains 865 or 885 aa respectively and share 99% similarity in sequence (Huibregtse et al., 1993b).

1.3.2 The Isoforms of the E6-AP

At least three isoforms (I-III) due to alternative splicing of the same gene have been reported in human and mouse. Other species investigated thus far including rat, pig and Rhesus macaque, have different number of isoforms with little being known about functional difference between them (Yamamoto et al., 1997) (LaSalle et al., 2015). Recent studies showed that these isoforms have distinct functions in neurons. For example, Miao et al. found mouse isoforms I&II distributed in the cytosol and dendrite, whereas isoform III restricted within the nucleus. The demolished dendrite phenotype in cultured hippocampal neurons caused by *Ube3a* knockout could only be rescued with isoform II (Miao et al., 2013). A recent discovery revealed that the *Ube3a* isoform I RNA (*Ube3a1*) 3′ untranslated region (UTR) can promote spine formation and suppress dendrite growth by sequestering micro RNA (miRNA) including miR-134 and miR-485 (Valluy et al., 2015). Although RNAs of all three isoforms have similar developmental expression pattern, the RNA of isoform I differs from I&II in spatial expression and responses to stimulation, of having higher expression ratio to isoforms I&II in processes than in cell body, and a drastic increase of expression level after stimulation by brain-derived neurotrophic factor (BDNF) (for 5h) or potassium chloride (KCl, for 6h) compared to isoforms I&II. It seems like that these three isoforms play antagonistic roles in some physiological processes and the coordination of their activity is crucial for neuron function. The mechanism regulating their function still needs to be elucidated.

1.3.3 Substrates and Interacting Proteins of the E6-AP

In mammals, there are more than 600 E3 ligases that have been reported thus far, but most of them are not characterized with substrates (Iconomou and Saunders, 2016). The situation also

apples to E6-AP, whose substrates and interacting proteins remains to be identified although bunch of putative candidates have been proposed (Clayton-Smith, 2003)(Martínez-Noël et al., 2012)(Jensen et al., 2013)(Sell and Margolis, 2015)(LaSalle et al., 2015). It has been reported the E6-AP interacts with the protein-binding domain of the chaperone protein heat shock protein 70 (HSP-70) and its chaperone sacsin (Parfitt et al., 2009) so as to induce the degradation of misfolded substrates (Mishra et al., 2009b) (Greer et al., 2011), then it is not surprising that protein metabolism, especially degradation, is disrupted in AS. For example, p53, activity-regulated cytoskeleton-associated protein (Arc), Ephexin5, tuberous sclerosis complex 2 (TSC2), p27, polyglutamine, and Ube3a itself have been reported increased in AS mice (Jiang et al., 1998)(Greer et al., 2010)(Margolis et al., 2010)(Sun et al., 2015a)(Mishra et al., 2009a) (Mishra et al., 2008)(Mishra et al., 2009a)(Nuber et al., 1998). Also it has been reported that Golgi apparatus deployment is disturbed and Reelin expression level decreases in AS mice (Miao et al., 2013)(Hethorn et al., 2015), which gives rise to the disruption of apical dendrite development (Horton et al., 2005)(Jossin and Goffinet, 2007)(Miao et al., 2013)(Matsuki et al., 2010).

Other substrates of UBE3A include Really interesting gene 1B (Ring1B), sex determining region Y-box 9 (SOX9), multicopy maintenance protein 7 (MCM7), Myc, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2 (HERC2), human homologs of Saccharomyces cerevisiae Rad23 (HHR23A), B lymphoid kinase (Blk), the human homologue of the Drosophila Scribble (Vartul) tumor suppressor protein (hScrib), Peroxiredoxin 1, nuclear transcription factor, X-box binding 1 (NFX1-91), E6 targeting protein 1 (E6TP1), Amplified in breast cancer 1 (AIB1, also known as ACTR/RAC-3/TRAM-1/SRC-3/p/CIP), Rho family guanine nucleotide exchange factor (GEF) Pbl/ECT2 (Pebble, in Drosophila and Epithelial Cell Transforming 2 (ECT2) in mammals), Estrogen receptor (ER), Androgen receptor (AR), protein tyrosine phosphatase H1/N3 (PTPH1/PTPN3), Disks large homolog 4/postsynaptic density 95 (DLG4/PSD-95), Annexin A1, Promyelocytic leukemia (PML) tumor suppressor, GABA transporter 1 (GAT1), small-conductance potassium channel 2 (SK2), human telomerase reverse transcriptase (hTERT), human gene for hypoxia-inducible factor 1-alpha inhibitor (HIF1AN), neuralized E3 ubiquitin

protein ligase 4 (NEURL4), MAPK6, actin, abnormal spindle-like, microcephaly-associated protein (ASPM) and brain and muscle Arnt-like 1 (BMAL1) et al. These substrates or interacting proteins can be categorized into different groups as illustrated in the Table 1. (Zaaroor-Regev et al., 2010)(Hattori et al., 2013)(Kühne and Banks, 1998)(Liu et al., 2007)(Liu et al., 2007)(Kühnle et al., 2011)(Kühnle et al., 2011)(Kumar et al., 1999)(Oda et al., 1999)(Nakagawa and Huibregtse, 2000)(Takizawa et al., 2006)(Nasu et al., 2010)(Gewin et al., 2004)(Gao et al., 2002)(Mani et al., 2006)(Reiter et al., 2006)(Li et al., 2006)(Khan et al., 2006)(Töpffer et al., 2007)(Handa et al., 2007)(Nasu et al., 2010)(Louria-Hayon et al., 2009)(Egawa et al., 2012)(Sun et al., 2015b)(Liu et al., 2005)(Parfitt et al., 2009)(Jensen et al., 2013)(Martínez-Noël et al., 2012)(Singhmar and Kumar, 2011)(Gossan et al., 2014).

Table 1. The Categories of Substrates and Interacting Proteins*

Category	**Substrates and Proteins**
Channels, receptors, synaptic proteins and transporters	ER, AR, SK2, DLG4/PSD95, GAT1
Signal transduction	TSC2, Blk, Reelin
DNA function (duplication, repair segregation)	p53, p27, RingB1, MCM7, HHR23A, hTERT, ASPM
Transcription factor (TF)	SOX9, NFX1-91, BMAL1
Cytoskeleton system related	Arc, Ephexin5, actin
Enzymes	E6-AP, HERC2, E6TP1, pbl/ECT2, PTPH1/PTPN3, NEURL4
Oncogene/tumor suppressor gene	AIB1, PML tumor suppressor
Other	HSP-70, Sacsin, polyglutamine, hScrib, HIF1AN

*Note: the interacting E2 conjugating enzymes of E6-AP are not listed and discussed.

Among these substrates, Arc, Ephexin5, SK2, p27 and TSC2 have been reported involved into synaptic plasticity impairment in AS mice via different mechanisms: Arc accumulation in AS decreases the AMPA receptor expression (Rial Verde et al., 2006); SK2 expression increased so as to affect the activation of NMDA receptors in AS mice; Ephexin5 and p27 promotes and inhibits RhoA function respectively; And the elevation of inhibitory type of TSC2 (p-Thr1462) inhibits mammalian target of rapamycin complex 2 (mTORC2), which regulates synaptic cytoskeleton and spine formation, thus the spine density decrease in AS mice could be restored by Rapamycin administration.

A close observation revealed that substrates and interacting proteins of E6-AP participated in multiple physiological processes including DNA replication, repair and segregation, protein synthesis and transport, signal transduction, synaptic transmission, cell growth and proliferation, et al. This is one of the reasons why a single enzyme disruption can give rise to such a severe neurodevelopmental disorder with various symptoms.

1.4 E6-AP in the CNS and the Potential Function

1.4.1 The Functional Difference of Paternally & Maternally Derived E6-AP

The functions of paternally and maternally derived E6-AP in postmitotic neurons (subgranular zone (SGZ) neural precursors (Judson et al., 2014) and dorsal pallium embryonic neural stem cells (Yamasaki et al., 2003)) are postulated to be different. As the paternal E6-AP expression level reduced with neuronal maturation, its expression level maintained in immature neurons including granule cells in dentate gyrus (DG) and deep layer within the external granule cell layer (GCL) at P7 mouse brain, P28 neural stem cells in DG SGZ. More importantly, the paternal E6-AP expression was found reciprocal to NeuN but colocalized with Nestin and Ki67 (Judson et al., 2014). The expression of paternal E6-AP can be detected in most brain regions including the

PFC, the cortex, the hippocampus, the striatum, the cerebellum from AS model mice aged 2-4 months either (Filonova et al., 2014). Collectively, these observations implicated that the paternal E6-AP may have different function from those of the maternal one, involving in neurogenesis and/or proliferation.

A recent study revealed that the imprinting did not occur in *Dube3a* (*Drosophila melanogaster* (fruit fly, fly hereafter) *UBE3A* homolog) in fly neurons, and that either paternal or maternal *Dube3a* expressed in fly neurons but not from both alleles. This indicated that the imprinting of E6-AP gene is a relatively late evolutionary event and also implied the divergence between paternal/maternal E6-AP may contribute to some advanced brain functions in mammals in which imprinting emerged (Hope et al., 2016).

To sum up, emerging evidences indicate that paternal and maternal E6-AP may play different roles in neurons development. However, the relationship (cooperation, reciprocal action, sequential action, or exclusive function) and underlying mechanisms need further investigations.

1.4.2 The Spatiotemporal Expression and Change with Developmental Stages

In the CNS, the imprinted genes express in a brain region-specific way and the distinctly distributed genes demonstrate differentiation in physiological functions (Gregg et al., 2010), however, for spatially restrictive distribution of imprinted expression of maternal *Ube3a*, in situ hybridization (ISH) revealed the lacking or decreasing expression of imprinted *Ube3a* is confined to the hippocampus, the cerebellum and the olfactory (Albrecht et al., 1997), but immunoblot and immuneohistochemisty detects showed the deficiency of E6-AP distributes more diffusely, including the hippocampus, the hypothalamus, the olfactory bulb, the cerebral cortex, the striatum, the thalamus, the midbrain, and the cerebellum (Gustin et al., 2010). The discrepancy indicated that some unknown mechanisms keep the maternal *UBE3A* gene silent in most of brain areas in AS model animals.

The regulation and spatiotemporal properties of imprinting have been extensively explored. One study revealed that the cis-regulation center of transcription of *Ube3a* antisense RNA transcript (ATS) is located in upstream (U) exon (Landers et al., 2004). The imprinting of paternal allele is found to occur at embryonic 10 in mouse brain by using a model mouse which exons 15 and 16 of *Ube3a* is knocked out (Yamasaki et al., 2003). A recent study showed that the expression level of paternal *Ube3a* is decreased in mouse neurons after the first postnatal week when these neurons are undergoing rapid maturation. At the same time, the decrease of paternal *Ube3a* was accompanied by the nuclei accumulation of maternal *Ube3a*. Interestingly, in contrast to neuron, glia cells (both astrocyte and oligodendrocyte) seem to express *Ube3a* biallelically with the oligodendrocytes became the main cell type to express both alleles in adulthood (Judson et al., 2014). The temporal and spatial imprinting pattern of paternal *Ube3a* was further explored in detail and confirmed by another research. It was found that the imprinting of paternal *Ube3a* occurred incompletely in cortex at P3 when the expression level maintained 25% of the wild type level while it dropped below 10% of that of wild type in other brain regions (Grier et al., 2015b). Other research found that the imprinting was not fully established in the visual cortex until the start of critical period (Sato and Stryker, 2010). So the imprinting of maternal allele of *Ube3a* does not accomplish until late stage of neuron development (Dindot et al., 2008) (Also reviewed by (Philpot et al., 2011)).

The change of maternal E6-AP subcellular distribution and temporal expression level with development stage has long been recognized. Maternal E6-AP illustrated dynamics in spatial distribution. *In vitro* investigation in cultured hippocampal neurons found the E6-AP localized in both cytosol, synapse and nucleus (Dindot et al., 2008). Further study in the same system revealed that isoforms distributed in the cytosol and synapse are I&II, whereas the nucleus is enriched with isoform III (Miao et al., 2013). *In vivo* researches demonstrated E6-AP transferring to nucleus with development while still maintaining expression level in axon terminal (Judson et al., 2014)(Burette et al., 2016). The temporal expression of E6-AP also changed with

development: all the three isoforms of E6-AP expression declined with time in cultured hippocampal neurons (Miao et al., 2013). And the expression level dropped drastically (50-80%) in frontal, auditory and visual (V1, V3 and V4) cortex in human, macaque monkeys and cats with aging, this loss of E6-AP was assumed to contribute to the function alteration in aging brains (Williams et al., 2010b).

1.4.3 The Postulation of the E6-AP Function in the CNS

E6-AP function in CNS has not been understood although lots of hypotheses have been presented (Mabb et al., 2011)(El Hokayem and Nawaz, 2014). Some hints might be obtained from its original function in carcinogenesis. The E6-AP enhances the tumorigenesis via various mechanisms, for example, it expedites cancer by degrading oncogene suppressor p53 (Huibregtse et al., 1991) and cyclin-dependent kinase inhibitor p27 (Mishra et al., 2009a), or it promotes cell growth in prostate cancer by activating phosphoinositide 3-kinase (PI-3K)-Akt pathway (Srinivasan and Nawaz, 2011). Also it showed the inhibition on interleukin (IL)-1β in immortalized keratinocytes (Niebler et al., 2013). So it seems that the original function of E6-AP is to promote cell proliferation through myriad pathways. This function may still maintain in CNS.

The behavioral deficits ameliorated in adult mice by retrieval expression of *Ube3a* using the antisense oligonucleotide (ASON) of paternal ATS are memory defect and obesity (Meng et al., 2015), neural control centers/brain regions in CNS for both are adjacent to active neurogenesis areas in adulthood, the hippocampus DG and the median eminence (ME) (Lee et al., 2012) respectively. This phenomenon implies that *Ube3a* exerts more important action on neonatal and/or developmental neurons than does on existing and mature ones. Thus processes related closely to the neuronal development, such as cell proliferation, cell cycle, cell growth, cell differentiation, cell migration, growth cone growth, synaptogenesis, dendrite pruning, and apoptosis, et al, should be the most possible target processes regulated by *Ube3a*. In support of this, the BDNF-TrkB pathway was found impaired in AS model mice (Cao et al., 2013). And the

reduction of E6-AP expression resulted in increased cell death, proliferation reduction and decreased NeuN positive neurons (Mishra et al., 2008)(Mishra and Jana, 2008) (Mishra et al., 2009a)(Mardirossian et al., 2009). This possibility also attains evidence from our findings of smaller soma size in AS mice (Refer to Chapter 4). In addition to promoting neuron growth, E6-AP may also promote glia growth and proliferation. It has been reported that the cortical myelin protein expression level declined in AS model mice (Grier et al., 2015a). Both white matter structure including myelination, axonal density or diameter, axonal organization and cortical/subcortical gray matter volume were also found reduced in AS patients (Peters et al., 2010b) (Aghakhanyan et al., 2016) .

1.5 The UPS and Neurodegenerative Disorders

Ubiquitin, a 76-aa-residue protein, is a denominator for protein degradation via proteasome and linked to target proteins by a sequential enzymatic reactions mediated by 3 enzymes, i.e. the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-protein ligases, which catalyze the reactions of activating, conjugating and ligation of ubiquitin, respectively (Ciechanover, 1994)(Hershko and Ciechanover, 1998) (Heinemeyer et al., 2004). The ubiquitin-proteasome system represents one of three main protein degradation pathways and involves into myriad physiological processes as well as many nervous system disorders including the Alzheimer's disease and the Parkinson's disease (Clague and Urbé, 2010) (Iconomou and Saunders, 2016)(Lam et al., 2000)(McNaught et al., 2001)(Zheng et al., 2016). Reciprocally, both β-amyloid and α-synuclein which is the culprit protein characterized in the Alzheimer's disease (AD) and the Parkinson's disease (PD) respectively, were postulated as candidates of E6-AP substrates and found either increased in AS patients' post-mortem brains or colocalized with Lewy bodies in PD patients' post-mortem brains (Erickson et al., 2016)(Mulherkar et al., 2009). These abnormalities of both proteins expressions may contribute in part to the intellectual impairment and ataxia symptoms of AS patients respectively (Williams et al., 2010a).

1.6 The Model Mice

Most, if not all advances in understanding the pathogenesis of AS came from the study on AS model mice. These AS model mice are invaluable and valid because mouse *Ube3a* shares 97% and 99% similarity with human *UBE3A* in DNA and protein sequence respectively (Yamamoto et al., 1997)(El Hokayem and Nawaz, 2014). The AS model mice recapitulated E6-AP expression defect in most brain regions and main symptoms such as memory and motion deficits found in patients while all explorations performed on animals are not possible to be carried out on AS patients (Jiang et al., 1998)(Miura et al., 2002) (Daily et al., 2012).

Many model mice have been created and are commercially available since 1997 when the first one, i.e. a partial paternal UPD line, was produced. This first model demonstrated deficiency of maternal *Ube3a* expression in most brain regions and some symptoms such as ataxia, abnormal EEG, obesity et al., but the low viability limits it utility (Cattanach et al., 1997)(Albrecht et al., 1997). The first widely used strain was generated by Dr. Beaudet's lab in Baylor College of Medicine. The researchers inserted a DNA fragment (targeting vector) containing neomycin resistance cassette (Neo) and hypoxanthine guanine phosphoribosyl transferase (HPRT) into the target genomic DNA on chromosome 7 to replace a sequence starting from exon 2 in 129/SvEv strain. The replacement would induce frame shift which terminated the expression of E6-AP. The E6-AP expression was disrupted in the hippocampus and the cerebellum but no morphological abnormality was found in animals aged 3-4 months. Mice showed motor defect, seizure, impaired LTP and context-dependent learning (Jiang et al., 1998). Later studies performed on this model revealed more brain regions beyond the hippocampus and the cerebellum with E6-AP deficiency (Daily et al., 2012) as well as multiple functional deficits (Refer to section 1.2 for detailed information). This is also the model that has been being used in this study.

Other models were also created to meet different demands. Miura et al. produced a model with a reporter gene *lacZ* which facilitated detection of gene expression. They deleted exon 16 and part of exon 15 to inactivate *Ube3a* (Miura et al., 2002). Similarly, Dindot et al. analyzed the subcellular distribution of E6-AP by using a model expressing E6-AP:YFP (yellow fluorescent protein) fusion protein (Dindot et al., 2008). By adopting Cre-loxp or tetracycline transactivator-Tet operator (tTA-tetO) system, temporal expression control of Ube3a could also be manipulated in model mice (Silva-Santos et al., 2015) (Stock No: 026279 FVB/N-Tg(tetO-Ube3a*2)884Svd/J Jackson lab database).

1.7 The Pathophysiological and Molecular Changes

A number of combined genetic and molecular studies have shed light on AS etiology. The E6-AP, first identified as the mediator of human papillomavirus types 16 and 18 E6 protein and p53 (Huibregtse et al., 1991)(Huibregtse et al., 1993a)(Huibregtse et al., 1993b)(Scheffner et al., 1993) , regulates the ubiquitin-mediated degradation of many proteins (Refer to section 1.3). Many other proteins are regulated by UBE3A protein when expressed in flies (Reiter et al., 2006) (Refer to section 1.3).

It has been found that various genetic mechanisms cause the loss (deletion or UPD), inactivation or mutations of maternal *UBE3A* gene (located in chromosome 15q11–13) (Lossie et al., 2001) (Table 2). The 3' end of intact paternal equivalence where the *UBE3A* gene is located remains silent due to imprinting mediated by a bipartite IC. This IC includes the Angelman syndrome imprinting center (AS-IC) and the Prader–Willi syndrome imprinting center (PWS-IC) (Nicholls et al., 1998)(Chamberlain and Brannan, 2001)(Ramsden et al., 2010). Silencing the paternal copy of *UBE3A* gene is likely through paternal expression of *UBE3A*-ATS and small nucleolar RNAs (snoRNAs) in neurons (Kishino et al., 1997)(Matsuura et al., 1997)(Girardot et al., 2012). It was found that the two types of RNA transcript, sense and antisense, both the products of *UBE3A* gene, are expressed in a cell-type specific way in the brain. Neurons

expressed maternal sense and paternal antisense, whereas glia expressed biallelical sense (Yamasaki et al., 2003). Furthermore, the disruption of maternal *UBE3A* gene resulted in an increase of paternal *UBE3A*-ATS in AS mouse model (Landers, 2005). The *UBE3A*-ATS illustrated the inhibitory effect on the expression of paternal *UBE3A* gene (Meng et al., 2012), and is consistent with a large scale screening that revealed that maternal biased genes are significantly related to the developing brain (Gregg et al., 2010). Another study further support that the function of UBE3A protein impaired in AS patients is related to ubiquitin ligase instead of to its functional coactivator of transcription of the nuclear hormone receptor superfamily, such as the progesterone receptor (PR) (Nawaz et al., 1999). The disturbance of the ubiquitin ligase activity gives rise to the impairment of protein ubiquitination (Cooper et al., 2004).

Table 2. The Ascertained Genetic Abnormalities in AS*

Genetic Abnormality	**Percentage in AS Cases**
Maternal deletion of 15q11-13 (*De novo*)	~70%
Paternal UPD	2-5%
Imprinting defects	2-5%
Mutations/variants of *UBE3A* gene	~5-10 %
Other causes unidentified	~10 %

*Note: According to a report by Ramsden SC, et al. 2010, and also based on the data from the public database Decipher (https://decipher.sanger.ac.uk).

1.8 The Putative Mechanisms Underlying AS Pathology

The detailed mechanisms on how deficiency of *UBE3A* leading to AS are poorly understood. Studies using AS mouse have provided some mechanistic insights by demonstrating that E6-AP plays a pivotal role in the multiple CNS developmental processes, including cell cycle, signal

transduction, transcription and synaptic plasticity (Reviewed by (Girardot et al., 2012)). One possible mechanism may be that changes of *UBE3A* expression can influence the viability and proliferation of neurons. It was reported that the postmitotic neonatal neurons are decreased after maternal *Ube3a* inactivation in AS mouse hippocampus (Mardirossian et al., 2009). The loss of neurons may be due to either impairment of the metabolism or the disturbance of genes involved in cell death process (Low and Chen, 2010), or both. It was also shown that mitochondria in AS mouse exhibited a smaller size in the hippocampus and a partial oxidative phosphorylation defect in the whole brain (Su et al., 2011). Another study revealed that the proliferation of neurons was disrupted in AS mice due to the increased expression of cyclin-dependent kinase inhibitor p27, whose degradation is mediated by the E6-AP (Mishra et al., 2009a). It was found that the α-synuclein degradation is mediated by E6-AP which is a component of Lewy body (Mulherkar et al., 2009). Then the reduction of dopaminergic neurons in substantia nigra caused by E6-AP deficiency is proposed as the underlying reason of the motor impairment in AS model mouse (Mulherkar and Jana, 2010). The involvement of nigrostriatal pathway in AS pathological characteristics is supported by the efficiency of Levodopa in treatment of motor deficits in adult AS patients (Harbord, 2001).

Another potential mechanism is that protein synthesis including receptors expression can be affected by E6-AP dysfunction. A recent study showed that the Golgi apparatus (GA) cistern was swollen and disorganized in AS mice, and the pH in GA lumen is increased in cortical neurons. This has implication that a less acidified GA would result in impaired protein sialylation and secretion mechanisms (Condon et al., 2013). Previous studies also showed that E6-AP regulates the degradation and turnover of RhoA-GEF Ephexin-5, Arc, p53, and p27 via ubiquitination (Margolis et al., 2010)(Greer et al., 2010)(Kühnle et al., 2013) (reviewed by (Mabb et al., 2011)). The fact that neural activities stimulate the *Ube3a* expression implies that *Ube3a* expression is responsible to and regulated by the neurons responses (Reviewed by (El Hokayem and Nawaz, 2014)). Further investigation revealed that both the Arc mRNA and protein can be locally induced at the postsynaptic sites of the activated dendrites (Lyford et al., 1995)(Steward et al., 1998), and

the Arc protein can promote endocytosis of the AMPA-type glutamate receptor (AMPAR), thereby reducing cell surface functional glutamate receptors by facilitating their interaction with dynamin and endophilin (Rial Verde et al., 2006) (Chowdhury et al., 2006). It is not surprising that AMPAR quantity at excitatory synapses was also found decreased, in correlation with an increase of Arc expression after E6-AP function was disturbed in neurons (Yashiro et al., 2009)(Greer et al., 2010). Also as Arc involves in memory consolidation and homeostatic plasticity scaling control (Plath et al., 2006)(Shepherd et al., 2006), so the Arc expression increase in AS model mice may contribute to the abnormalities including learning and memory deficits. In contrast, studies in another AS model mouse showed both c-Fos and Arc expression decreased in DG (Mardirossian et al., 2009). And the activation of mGlu5 did not trigger the expression increase of Arc in brain slice of another AS model mice, but the Homer1a expression decreased in AS model mouse which might attribute to the mGlu5-mediated long-term depression (LTD) enhancement (Pignatelli et al., 2014). So the expression of Arc seems to be regulated differentially by different stimulations and differs from genotype to genotype, which obscures its role in the pathogenesis of AS. But these results still demonstrate that the E6-AP defect may affect synaptic receptors and/or channels expressions, as other E3 ligases regulate G protein-coupled receptor (GPCR) (Kennedy and Marchese, 2015). This postulation has gained support from a recent study. In that study, researchers found that SK2 is the substrate of E6-AP, whose deficiency in AS mice results in membrane overexpression of SK2 which impedes NMDA receptors activation leading to the impairment in synaptic plasticity and memory (Sun et al., 2015b). The potential regulatory role of Ube3a in regulating other receptors and channels awaits further investigations.

Another hypothetical cause is that *UBE3A* abnormality disturbs the regulation of gene expression and signal transduction. A recent study demonstrated that levels of both Ring1B, which ubiquinates nucleosomal histone H2A to regulate gene expression, and histone H2A, are elevated in many tissues in AS mice (Zaaroor-Regev et al., 2010). One recent study found that 7 genes are increased and 57 genes decreased in AS mouse, and these genes are functioning in signal transduction, nervous system development and cell death. Some of those genes (*Fgf7,*

Glra1, Mc1r, Nr4a2, Slc5a7 and *Epha6*) are confirmed of relevant with AS phenotype (Low and Chen, 2010). For signal transduction, multiple signal pathways have been found to be regulated by E6-AP, thus their functional defects in AS may contribute to AS pathogenesis. Protein kinase 3',5'-cyclic adenosine monophosphate (cAMP)-dependent (PKA), protein kinase C (PKC), MAPK and calcium/calmodulin-dependent protein kinase II (CaMKII) pathways have ever been investigated in the hippocampus of AS model mice. Kinases expression levels of these pathways illustrated no change except that the phosphorylation of CaMKII (Pho-Thr^{286} and Pho-Thr^{305}) increased in AS (Weeber et al., 2003). A recent report illustrated that among AS patients caused by *UBE3A* missense mutation, a T485A substitute makes E6-AP lose response to PKA phosphorylation and keep constitutively active (Yi et al., 2015). It has been reported that neuregulin1 expression and phosphorylated Erb-B2 Receptor Tyrosine Kinase 4 (ErbB4) (Pho-Tyr^{1056}, Pho-Tyr^{1166} and Pho-Tyr^{1188}) increased while the total ErbB4 decreased in the hippocampus of AS model mice, the enhanced neuregulin1-ErbB4 pathway is supposed to be responsible to the LTP and fear memory impairment (Kaphzan et al., 2012). The activation of PI-3K-Akt-mTOR pathway is well-known to promote cultured hippocampal neuron growth and dendrite branching (Jaworski et al., 2005). Study performed in prostate cell revealed that E6-AP regulated PI-3K-Akt pathway via both androgen-independent and independent mechanisms (Srinivasan and Nawaz, 2011). The change of PI-3K pathway in AS has been explored in a recent study which illustrated that elevated Arc level in AS mouse disturbs the BDNF to recruit the PSD-95 protein, disrupts association of PSD-95 with TrkB, and the association of phospholipase C (PLCγ) and growth factor receptor-bound protein 2 (Grb2)-associated binder 1 (Gab1) with TrkB, therefore impairing the BDNF, TrkB and PI-3K-Akt pathways (Cao et al., 2013). Other studies demonstrated that the mGlu5-mediated LTD depended on both the MAPK and PI-3K-mTOR pathways, whereas both did not illustrate abnormality in AS model mouse (Pignatelli et al., 2014). Reelin, an extracellular matrix protein regulating myriad neural developments such as neuronal polarization, spine density etc and its defect underlies symptoms in Reeler mice (Rice and Curran, 2001)(Frotscher, 2010)(Wasser and Herz, 2016)(Matsuki et al., 2010)(Rodriguez et al., 2000)(D'Arcangelo et al., 1995), was found reduced in both AS model mice and AS patients

post-mortem brains (Hethorn et al., 2015). The mTOR pathway demonstrated divergent changes in mTORC1 and mTORC2, in that mTORC1 was enhanced whereas mTORC2 was inhibited by the increased inhibitory type of phosphorylated TSC2 in the cerebellum in AS mice. Interestingly, these alterations were not observed in dorsal striatum, showing a brain region-dependent pattern (Sun et al., 2015a). The focal adhesion signaling pathway, which is crucial in neurite contact development, was revealed with impaired function in cultured hippocampal neurons from AS mice, and its functional deficit compromised neurites capability in responding to chemical and physical guidance along their growing path (Tonazzini et al., 2016). These deviant effects of Ube3a deficit on even some same signal pathways showed spatially and receptor dependent patterns so that further investigation are needed to reveal the Ube3a function on signal transductions although, they support the postulation that Ube3a participates into regulation of components of multiple signal transductions and these pathways are impaired in AS.

The defects of neuron intrinsic properties and synaptic transmission might give rise to the AS pathogenesis. It was found that the expression of α1 subunit of sodium/potassium-ATPase (α1-NaKA) is increased in the hippocampus in AS mouse. The abnormal expression likely explain a series of changes such as elevated axon initial segment proteins and altered membrane properties including resting potential, threshold potential and action potential (Kaphzan et al., 2011)(Hallengren and Vaden, 2014). These alterations were corrected by reducing α1-NaKA genetically by breeding *UBE3A*$^{m-/p+}$ female mice with the 1-NaKA$^{m+/p-}$ deletion male mice (Kaphzan et al., 2013). This study suggests the loss of E6-AP leads to changes in neuronal excitability likely through altered membrane biophysical properties. In addition, synapse vesicular release was also influenced by E6-AP deficiency in AS mice. Synaptic vesicle density was found decreased in the hippocampus, whereas clathrin-coated vesicles, a main type of vesicle for receptor-mediated endocytosis (Kandel, 2012a), accumulated abnormally in both the glutamatergic and the GABAergic neurons synaptic terminals (Su et al., 2011) (Wallace et al., 2012) (Judson et al., 2016). The abnormalities of both intrinsic properties and vesicular release of

neurons are supposed to disrupt the synaptic transmission in AS mice. These may provide an explanation for intellectual symptoms in AS.

So the downregulation of maternal *UBE3A* in neurons may contribute to AS deficiency by influencing cell survival, cell proliferation, synaptic transmission, signal transduction, gene expression and neural development process. Extensive investigation is needed to elucidate the linkage of the UBE3A and mechanisms underlying the AS.

1.9 The Therapeutic Explorations

The current efforts in therapeutic exploration for AS have been taken on identified putative pathological basis. A conspicuous idea would be to restore the function of the *UBE3A* in the brain, by retrieval of either maternal or paternal copy of the gene. For restoration of maternal *Ube3a*, one study used recombinant adeno-associated virus (rAAV) to introduce type 2 terminal repeat (TR2) flanked *Ube3a* into the hippocampus of adult AS mice. The study found that rAAV restored the level of E6-AP in AS hippocampus, rescued the impaired LTP, and enhanced the cognitive learning as evaluated by Morris Water Maze (MWM) test (Daily et al., 2011). These rescue experiments suggest that neuronal circuit deficits can arise from lack of E6-AP function per se, and restoration of *UBE3A*/*Ube3a* expression could potentially overcome certain aspects of developmental deficits.

The existence of the intact paternal *UBE3A*/*Ube3a* allele has the intriguing implication that activation of the silent allele may be able to fulfill the functions of the missing maternal ones, in a way analogous to rescuing neural deficits in adult *Mecp2* knockout mice by reinstating the functional *Mecp2* gene (Guy et al., 2007). The first attempt was performed by Carlos A. Bacino and his colleagues in 2010 and 2011. In those clinical trials, researchers tried to resurrect the paternal *UBE3A* by improving DNA methylation using methyl donor dietary (Betaine and folic acid, or betaine, metafolin, creatine, and vitamin B(12)) to decrease AS-ATS expression. The

treatments did have positive effect on some patients although no significance was found (Peters et al., 2010a)(Bird et al., 2011). In an elegant genetic study, Meng et al. showed that inhibition of *Ube3a*-ATS expression both *in vivo* and *in vitro* could elevate expression of paternal *Ube3a* (Meng et al., 2012). The activation of paternal *Ube3a* could be achieved by blocking the paternal *Ube3a*-ATS with poly-adenine cassette insertion in AS mouse models. Many resulting AS deficiencies, such as impaired LTP, cognitive deficits, and motor dysfunction were ameliorated (Meng et al., 2013). Recently, the same group reported that some cognitive deficits such as memory impairment and obesity phenotype in AS model mice have been ameliorated after the paternal *Ube3a* was resurrected by using ASONs of Ube3a-ATS (Meng et al., 2015). This un-silencing paternal *Ube3a* strategy was improved by using optimal delivery system of artificial transcription factors (ATFs) recently, which consists of human immunodeficiency virus (HIV) TAT protein and zinc finger transcription factor, rescues paternal *Ube3a* expression in a spread brain regions in an AS model mice when delivered via intraperitoneal or subcutaneous injection (Bailus et al., 2016). Restoring the paternal *UBE3A*/*Ube3a* expression through non-genetic approaches also seems to hold great promises. Through chemical library screening, Huang et al. had found that several topoisomerase inhibitors, such as topotecan and irinotecan could resuscitate paternal *Ube3a* and rescue cellular function in neurons (Huang et al., 2011). Although topoisomerase inhibitors lack specificity on neurons and are likely to be toxic to many tissue types, this study represents a major conceptual breakthrough by showing that rescuing the dysfunctional *UBE3A*/*Ube3a* gene in the brain can be achieved through bypassing the genetic manipulations. The reason that this strategy could only rescue some rather than all symptoms might be interferences were administrated after the critical period, as is illustrated in a recent work which showed the paternal *Ube3a* resurrected in early developmental stage could ameliorate most symptoms in AS model mice (Silva-Santos et al., 2015).

Maternal *Ube3a* deficiency in mice is known to impair synaptic transmission and interfere with a critical molecular player in synaptic plasticity, CaMKII (Weeber et al., 2003)(Yashiro et al., 2009). Weeber et al. showed that AS mice had impaired hippocampal LTP and reduced context-

dependent learning, which is correlated with an increased CaMKII phosphorylation at Thr305/Thr306 inhibitory sites and a reduced kinase activity (Weeber et al., 2003). In a following study (Woerden et al., 2007), the same group further crossed female AS mice with heterozygous males that carried the targeted CaMKII-T305V/T306A mutation, a genetic manipulation that prevents inhibitory phosphorylation of CaMKII and elevates CaMKII activity. Intriguingly, a reduction of CaMKII inhibitory phosphorylation was able to rescue the motor deficits, seizures, LTP impairment and the hippocampus dependent learning. Collectively, these findings indicate misregulation of CaMKII may be a molecular substrate underlying the neurobehavioral deficits in AS. The notion that restoring affected signal transduction pathways may alleviate AS pathology is also supported by other recent studies (Kaphzan et al., 2012)(Cao et al., 2013). The altered LTP in AS mouse model can be corrected either after the TrkB signal pathway was restored by using a bridged cyclic peptide (CN2097) to interfere the interaction between the increased Arc and PSD-95, or by administering ErbB inhibitors PD158780. As mentioned earlier, Reelin protein expression level decreased in AS mice, the administration of Reelin supplementary rescued spine density reduction and some symptoms such as LTP impairment, associative and spatial learning and memory (Hethorn et al., 2015). Therapeutic effects were also achieved by the restoration of mTOR pathway imbalance in AS model mice by inhibiting mTORC1 or activating mTORC2: the enhanced mTORC1, attenuated mTORC2, impaired LTP in the hippocampal brain slice, actin deploymerization and elevated Arc were reversed after treatments (Sun et al., 2016). However, no matter how exciting these results in AS model mice are, the clinic treatments for AS patients are still in an embarrassing situation (Tan and Bird, 2016) which can be attributed to several reasons. One of them is the stability of neural circuits which might have been influenced in both formation and refinement by the E6-AP deficiency at early development stage, and it is not possible to rearrange and correct these circuits fault in adulthood at all by changing one or some physiological processes, as were demonstrated in several unsuccessful attempts of recovering most symptoms of AS by unsiliencing paternal allele of *Ube3a* in model mice (Meng et al., 2015)(Sun et al., 2016). Further study indicated the intervention time was crucial (Silva-Santos et al., 2015). Another deserved attention is that all higher cognitive functions differing

humans from other species lack in AS model mice, leaves alone detecting or restoring them. Then last but not least, the interactions between signal transduction pathways are of extreme complexity which defies the possibility to point out the changes of the entire system after altering one or a few components of it. Frustrating at first, these works contributed to understanding the AS phathogenesis, which will lead to some potent therapies for AS patients eventually after underlying mechanisms are fully understood.

Drug and diet treatments have also been attractive options for the merits such as convenience, noninvasiveness and safety. A diet treatment tried to resuscitate the paternal *UBE3A* by increasing DNA methylation did not achieve the goal though (Peters et al., 2010a)(Bird et al., 2011), recent advances in the direction are exciting. It has been reported Ampakines, a positive modulator of AMPAR (Arai et al., 1994), can enhance LTP and improve fear-conditioning learning in AS model mice (Baudry et al., 2012). The fluoxetine, a serotonin reuptake inhibitor, can restore PV neurons decrease in the hippocampus and the basolateral amygdala of AS mice induced by chronic stress, the GR and serum corticosterone levels and anxiety behavior (Godavarthi et al., 2014). Ketone ester supplementation R, S-1,3-butanediol acetoacetate diester (KE) significantly improved the motor coordination, early phase LTP and associative learning deficits in AS mice, it also showed effect in controlling epilepsy and obesity (Ciarlone et al., 2016). Other clinical treatments on AS patients aimed to ameliorate an aspect of AS symptoms such as motor deficits, epilepsy have attained promising outcomes. Levodopa treatment in two adult AS patients demonstrated apparent effects in controlling motor deficits including resting tremor and cogwheel rigidity (Harbord, 2001). The epilepsy, a common symptom in more than 90% AS patients (Pelc et al., 2008a), was reported to be controlled effectively with low glycemic index diet, it was effective on 22 of 23 (96%) patients treated (Grocott et al., 2017).

Other progress is that some activities, such as depolarization in cultured neurons and fear condition for live animals, changed biallelical E6-AP expression *in vitro* and *in vivo*. That might in

part rescue the elevation of the activity-dependent ERK1/2 phosphorylation (Filonova et al., 2014).

1.10 Outstanding Questions and Major Challenges

Despite these emerging successes in restoring neural functions in AS mouse models, outstanding questions and challenges remain in the field. For example,

1. What is the definite role of E6-AP in neural connections or circuits within and between many brain regions, and in what molecular context is E6-AP involved to regulate synaptic development, transmission, and plasticity? Why increased *UBE3A* dosage is more represented in autism spectrum disorders (Nakatani et al., 2009)(Smith et al., 2011)? The molecular mechanisms by which *UBE3A*/*Ube3a* deficiency lead to AS remain enigmatic.

2. How does E6-AP differentially affect both excitatory and inhibitory synapses, favoring an enhanced local circuit hyper-excitability (Yashiro et al., 2009)(Wallace et al., 2012)?

3. The protein substrates of E6-AP in neurons remain to be identified (Clayton-Smith and Laan, 2003a)(Sell and Margolis, 2015). p53, Arc, HSP70, Sacsin, HHR23A and Ephexin 5 et al. represent only a small number of proteins known to be directly regulated by E6-AP in neurons. Revealing more neuronal molecular substrates or interactomes and how deficiency of maternal *UBE3A* disrupts cellular homeostasis can be illuminating for AS pathogenesis and molecular interventions.

4. The dramatic variations of symptoms among AS patients imply the contributions of other elusive and perhaps much more complex causes other than maternal *UBE3A* dysfunction. For example, other genes such as $GABA_A$ receptor β3 subunit (*GABRB3*) gene which locates within

the chromosome 15q11-13 locus have been proved to play a role in the AS development. The impaired expression of *GABRB3* can render featured phenotypes of AS in mice. These results raised the questions of the definite role of the *GABRB3* and its relation to *UBE3A* in AS genesis (DeLorey et al., 1998). Also other genes including *ATP10A* are supposed to involve in the process (haploinsufficiency hypothesis) (Reviewed by (Lalande and Calciano, 2007)).

5. Some discrepancies exist between AS model mouse behavior and AS patient clinical features. AS mice showed normal social seeking and activity level in contrast to the frequently observed behavioral deficits of AS patients (Allensworth et al., 2011). This may be explained by the larger size of genetic defect in patients than that of the AS mice. Further observations on the variations in eating behavior and body growth among patients with different genetic deficits, specifically patients with big deletion or ones with UPD implied that other factors within the 15q11-13 locus may play a role in the pathogenesis too (Lossie et al., 2001)(Mertz et al., 2014).

6. On the forefront of AS therapeutic endeavors, the potential of topoisomerase inhibitors in restoring *UBE3A* expression and correcting AS pathophysiology as well as its side effect on synaptic transmission awaits more experimental validation (Huang et al., 2011)(Mabb et al., 2014).

1.11 The Specific Aims (SA) and Hypothesis of This Study

Specific aims and hypothesis: current literatures support that two major neurodevelopmental modalities are impaired in AS mice: 1. Impaired neural circuits assembly and plasticity as a result of impaired activity dependent changes(Yashiro et al., 2009) (Wallace et al., 2012); and 2. Impaired cellular protein homeostasis (Greer et al., 2010) (Margolis et al., 2010), possibly due to aberrant turnover of substrates of E6-AP (Mabb et al., 2011) (Upadhyay et al., 2015). However, the molecular and circuitry mechanisms of AS pathogenesis are still unclear.

We hypothesize that attenuated synaptic transmission and neurites growth may alter the developmental trajectory of PFC L5 pyramidal neurons structures including dendrites and synaptic proteins in both brain region specific and development stage dependent ways, which leads to disrupted activity/experience-dependent synaptic plasticity and maturation. This hypothesis will be tested in three specific aims:

SA1. To test the hypothesis that synaptic transmission and circuit function in the prefrontal cortex (PFC) neurons in AS mice are impaired, which may be dependent on developmental stages.

SA2. To test the hypothesis that presynaptic vesicular release and short-term plasticity at the layer 2/3 (L2/3) to layer 5 (L5) PFC circuits are altered in AS mice. These alterations may contribute to synaptic transmission and circuit abnormalities in SA1.

SA3. To explore the morphological and molecular changes underlying the defects described in SA1 and SA2. Dendritic and synaptic proteins changes in the PFC of AS mice of different ages are detected by Western Blot analysis, and are compared to littermate controls.

The study will investigate the novel effects of E6-AP deficiency on synaptic structure and neural activity in the PFC of AS mouse, an important yet unexplored brain region in the AS pathogenesis, on both single neuron and circuit level. The results will help to reveal the brain region-specific role of E6-AP playing in the neural development, and to reveal potential novel substrates and pathways that may be targeted for interventions.

CHAPTER 2

THE SYNAPTIC TRANSMISSION DEFICIENCIES OF THE PFC L5 CORTICOSTRIATAL PYRAMIDAL NEURONS

2.1 Introduction

Synapse, the special contact site between neurons for communicating, is the structural basis of nervous system function (Kandel, 2012b). Synaptic transmission dysfunction has disastrous consequences and involves in multiple CNS diseases such as cognitive diseases and neurodegenerative diseases (Südhof, 2008)(Palop and Mucke, 2010). The synaptic transmission defects including impaired mEPSCs, LTP and LTD, have also been reported in the visual cortex, the dorsal striatum and the hippocampus of AS model mice (Weeber et al., 2003)(Yashiro et al., 2009)(Greer et al., 2010)(Hayrapetyan et al., 2014)(Pignatelli et al., 2014). The mIPSCs of the L2/3 and L5/6 fast-spiking interneurons in the visual cortex, are also found deminished in P80 but kept comparable to WT in P25 AS mice (Wallace et al., 2012).

However, the change of synaptic transmission has not been explored in other brain regions including the PFC in AS model mice. The PFC comprises almost one third of neocortex (Fuster, 2001), with the L5 pyramidal neurons projecting to several other regions including contralateral striatum, contralateral cortex, superior colliculus, pons (Wang and McCormick, 1993)(Molnár and Cheung, 2006)(Brown and Hestrin, 2009a) (Shepherd, 2013), thalamic pulvinar nuclei (White and Hersch, 1982), the PFC L2, L3, L5 and L6 (Yamamori and Rockland, 2006), and receiving inputs from multiple other brain regions including the agranular insula, the dorsal polymodal thalamic nuclei, the contralateral PFC, motor areas, the claustrum, the hippocampus, the basolateral amygdala (BLA), the dorsal thalamus and the anterior cingulate area (ACA), et al (Hoppa et al., 2015), is believed to participate in multiple pivotal brain functions including cognition, decision-making and working memory (Goldman-Rakic et al., 2011)(Fuster, 2001)(Euston et al., 2012)(Bicks et al., 2015)(Kandel, 2012c). These functions of the PFC are closely related to

intellectual capabilities and sociality, both demonstrated abnormal phenotypes in AS (Bird, 2014). This makes the PFC deserve extensive investigation in AS model mice.

Moreover, little is known about the relation between the functional changes and development stages as no systematic comparison between animals of different ages has been performed. The investigation of mIPSCs change in the visual cortex revealed difference between younger (P25) and older (P80) AS mice (Wallace et al., 2012). Also the AMPA/NMDA ratio recorded in hippocampal slice of AS model mice demonstrated an age-dependent pattern: the ratio decreased in P15-18 mice but kept normal in adults aged 10-16 weeks (Greer et al., 2010) (Kaphzan et al., 2012). Thus comparison of changes in AS model mice between different ages is needed to figure out those morphological and functional defects due to the deficits of synapse formation, refinement or both.

In addition, much efforts have been exerted on neuron functional changes in AS model mice though, no study has been carried out to examine neural circuit change thus far, whereas this question is of extreme importance because it is well known that it is different neural circuits that signals transduced rather than either neurons or signals itself are the basis of normal function of nervous system (Kandel, 2012d). When and how connections of a specific type of neuron are affected by E6-AP deficiency needs to be determined in AS model mice.

Glutamate receptors including AMPAR and NMDAR play a key role in synaptic transmission, plasticity and neuron development (Meldrum, 2000)(Camilli, 2001). Both receptors' activity were found disturbed in AS model mice, and that the internalization of AMPAR is enhanced by the increased Arc (Greer et al., 2010) while NMDAR activation is impaired due to elevated membrane expressed SK2 (Sun et al., 2015b). BDNF, the major neurotrophic factor in the PFC and the hippocampus (Hofer et al., 1990) which regulates both the excitatory and the inhibitory synaptic transimssion in the hippocampal culture (Bolton et al., 2000), its singaling was revealed impaired in E6-AP deficient cells and AS mice (Cao et al., 2013).

We hypothesize that synaptic transmission is affected in the PFC at early developmental stage, and this disruption at synapse level will interfere neural circuit formation and function. Indeed, both mEPSCs and mIPSCs showed decline in frequency in AS mice aged P17-19 and P28-35. The neural circuit, as the PFC L5 corticostriatal pyramidal neurons' inputs were measured by using laser scanning photostimulation (LSPS), demonstrated abnormalities either: the inputs from L2/3 and L5A reduced significantly in AS mice as young as P17 and kept the same abberant pattern till adulthood (older than P70 (>P70)). These defects in the PFC from early developmental stage would have impacted not only the relavant functions of the PFC but also the communication between the PFC with various brain regions where the PFC projects to or receives inputs from, thus influenced spread brain areas and circuits as well as corresponding functions undertaken by these circuits.

2.2 Materials and Methods

2.2.1 Animals and Habituation

A well-established AS model mice (Jiang et al., 1998) with their both age-matched and sex-matched wild-type (WT) littermates were used for all experiments. Unless otherwise noted, three groups of AS mice aged postnatal 17-19 days (P17-19), P28-35 or older than P70 (>P70), representing neonatal, adolescent and adult developmental stages, respectively, were studied as listed in table 3. Both male and female mice were included.

Table 3. The Animal Groups and Numbers (Pairs).

Age / Genotype	P17-19	P28-35	>P70
WT	3-5	3-5	3-5
AS	3-5	3-5	3-5

2.2.2 Stereotactic Injection and Handling

Animals were raised with food and water *ad libitum* in a temperature-controlled room (23±0.5°C) with a 12 h light/dark cycle (light on 8:00 A.M.-8:00 P.M.). All experiments were performed during the light phase of the cycle with the approved protocol by the Institutional Animal Care and Use. Stereotactic surgery, postsurgical handling, and habituation were performed as reported by Cetin A and colleagues (Cetin, Komai et al. 2006). In brief, the mice were kept anesthetized by isoflurane/oxygen mix (2-4% isofluorane, NDC 14043-225-06, Webster Veterinary Supply Inc.) provided by anesthetic equipment (Vet Equip) during the whole procedure, and mounted on researcher-built stereotactic apparatus. After the skull was disinfected with Betadine solution (NDC 67618-155-16, Purdue Products L.P.), brain was exposed and positioned in horizontal level, the target areas were gauged using bregma and lambda as landmarks, and coordinates were determined according to the mouse brain in stereotaxic coordinates third edition (Keith B.J. Franklin & George Paxinos. 2007) as following: contralateral (left side) dorsal striatum (DS) (0.75 mm posteroanterior, 2.0 mm lateral to bregma and -2.75 mm ventral). For mice aged P17-19, brains have similar coordination in dorsoventral orientation, but a 1 mm or 0.5 mm shortage in anteroposterior and median lateral orientations respectively in comparison to those of adults (Aggarwal et al., 2009), coordinations were adjusted correspondingly as following: (0.5 mm posteroanterior, 1.8 mm lateral to bregma and -2.6 mm ventral). 50-100 nano liters (nl) of retrograde red fluorescent latex microspheres ('micro beads', Red 1× Retrobeads™, LumaFluor Inc.) were injected. Post-operative analgesia were performed

with Bupivicaine/epinephrine 0.25% (NDC 0409-132159-01, Hospira Inc.), 0.1ml applied to skull as local anesthetic, then animals were returned and housed individually after they fully recovered from unconsciousness, one injection of Carprofen 5-10 mg/kg subcutaneous (S.C.) after 24 hr. And a minimum of 2-3 days were given for corticostriatal PFC L5 pyramidal neurons to be labeled antidromiclly before electrophysiology experiments were performed. Meanwhile the animals were checked daily and Carprofen was supplied if necessary.

2.2.3 Patch Clamp Whole-cell Recordings

2-3 days after stereotactic injection, mice were anesthetized with isoflurane and were decapitated, and sagittal slices (350 µm thick) were dissected by using vibrotome (Model LEICA VT1200S. Leica company), transferred into artificial cerebral spinal fluid (ACSF) which containing (in mili molar (mM)) 0.25 KCl, 1 Glucose, 12.6 NaCl, 0.125 NaH_2PO_4, 0.2 $MgCl_2$–$6H_2O$, 0.2 $CaCl_2$–H_2O, 2.6 $NaHCO_3$. The ACSF was saturated with 95% oxygen/5% carbon dioxide mixture (95% O_2/5% CO_2) and preheated to 32°C. After 30 minutes recovery at 32°C, the slices were kept at room temperature and recorded within the following 6 h. For old age (>P70) group, mice were intracardially perfused with ice cold sucrose cutting solution containing (in mM): 85 NaCl, 75 Sucrose, 2.5 KCl, 25 Glucose, 1.25 NaH_2PO_4, 4 $MgCl_2$, 0.5 $CaCl_2$, and 24 $NaHCO_3$ before decapitation.

Data were collected by using MultiClamp 700B amplifier and acquired via a Digidata 1440A device (Molecular Devices). Brain slices were being perfused with ACSF, which was aerated with 95% O_2/5% CO_2 and maintained at room temperature (RT), during the whole experiment procedure. The perfusion rate was 2ml/min. The whole-cell recordings were used in the voltage-clamp mode. Recording electrodes were made from premium standard wall with filament borosilicate glass capillary (640792; Warner Instruments LLC; PRE SW FILA BORO CP 1.5MM X 3") and filled with either the potassium-gluconate internal solution containing (in mM): 126 potassium-Gluconate, 4 KCl, 10 HEPES, 4 ATP (magnesium salt), 0.3 GTP (sodium salt), 10

Phosphocreatine, adjusted to pH ~7.2 with KOH, 270-290 mOsm (for mEPSCs recording), or KCl containing (in mM): 125 KCl, 2.8 NaCl, 2 $MgCl_2$, 2 Mg^{2+}-ATP, 0.3 Na^{3+}-GTP, 0.6 EGTA and 10 HEPES, pH adjusted to 7.3 with KOH (for mIPSCs). The series resistance (Rs) was not compensated during the recording. Recordings with the Rs changed by 20% or Rs>30 MΩ were discarded. And responses were recorded with Gap-free protocol held at -70mV. Signals were amplifiedand digitized (10 kHz), and stored on disk for off-line analysis. For mEPSCs recording, 50 mM picrotoxin (PTX) and 1 micro molar (μM) tetradotoxin (TTX) were added into ACSF to eliminate IPSCs and action potentials respectively. For mIPSCs recording, 20 mM CNQX, 50 mM AP-5 and 1μM TTX were administered into ACSF to exclude mEPSCs and spontaneous action potentials. The responses were analyzed by using the template search of Clampfit (version 10.4; Molecular Devices). The recording-specific template was created by selecting at least 20 typical events for individual recording. Only recordings with stable baselines were included in the analysis.

2.2.4 Laser Scanning Photostimulation (LSPS)

Local circuit connections of the PFC L5 pyramidal neurons were investigated by using LSPS as reported previously (Peng et al., 2016). In brief, brain slices were perfused with ACSF containing 0.2 mM 4-methoxy-7-nitroindolinyl-caged glutamate (Canepari et al., 2001), then laser pulses (20 mW, 1-ms UV laser, 355 nm; DPSS Lasers) were used to activate glutamate at selected sites in a random sequence. The active glutamate excited neurons located nearby, the activated presynaptic neuron released neurotransmitter (either glutamate or γ-aminobutyric acid (GABA) depending on neuron type) onto the target neuron innervated which was recorded with whole-cell patch clamp, and gave rise to the monosynaptic excitatory (holding potential (V_H)=-70 mV) or inhibitory (V_H=0 mV) currents. The connections of the target neuron (mapping of inputs) was thus generated and analyzed with Ephus and Matlab software respectively (Peng et al., 2016) (Suter et al., 2010).

2.2.5 Labeling of the PFC L5 Corticostriatal *Pyramidal Neurons (Target Neurons).*

Retrograde micro beads injection site was examined when brain slices were dissected for electrophysiological experiments (patch clamp recordings and LSPS). Only brain slices (from the right hemisphere) from animals with correct injection site (left DS) and labeled target neurons were used. Target neurons labeled with retrograde micro beads were recorded in both patch clamp recordings and LSPS experiments, filled with 1% biocytin contained in electrode internal solutions and revealed with fluorescence-conjugated streptavidin (Alexa Fluor® 488 Streptavidin, S32354, ThermoFisher Scientific) after recordings following protocol as previous report (Peng et al., 2016). Double-labeled neurons were used for morphological analysis.

All chemicals were purchased from Sigma-Aldrich or TOCRIS unless were indicated specifically.

2.2.6 Data Analysis

Data were expressed as the mean ± S.E.M and the change in mean mEPSC frequency and amplitude were analyzed and assessed via either One-way ANOVA or two-tailed unpaired Student's test (t test hereafter unless specified) with $p < 0.05$ as standard of statistical significance. For cumulative frequency and amplitude analysis, Kolmogorov-Smirnov (K-S) test was used, and a cutoff value of 0.05 was used as significant threshold.

The linear distribution of inputs onto target neuron in LSPS was analyzed by using Two-way ANOVA and $p < 0.05$ is considered significant.

2.3 Results

2.3.1 The PFC L5 Corticostriatal Pyramidal Neurons Selection for Electrophysiological Experiments

Retrograde micro beads-positive target neurons were clustered mainly in PFC L5 on brain slices (Figure 2.1C) from animals with correct injection site in left DS (Figure 2.1B). Fluorescent microscope revealed that the locations of all target neurons recorded (biocytin-positive) were comparable and concentrated within L5A (Figure 2.1C), and all biocytin-positive (green) neurons were also retrograde micro beads-positive (red) with the typical PFC pyramidal morphology (triangle soma, single thin-tufted apical dendrite and multiple short basal dendrites (Molnár and Cheung, 2006)) (Figure 2.1C, high magnification).

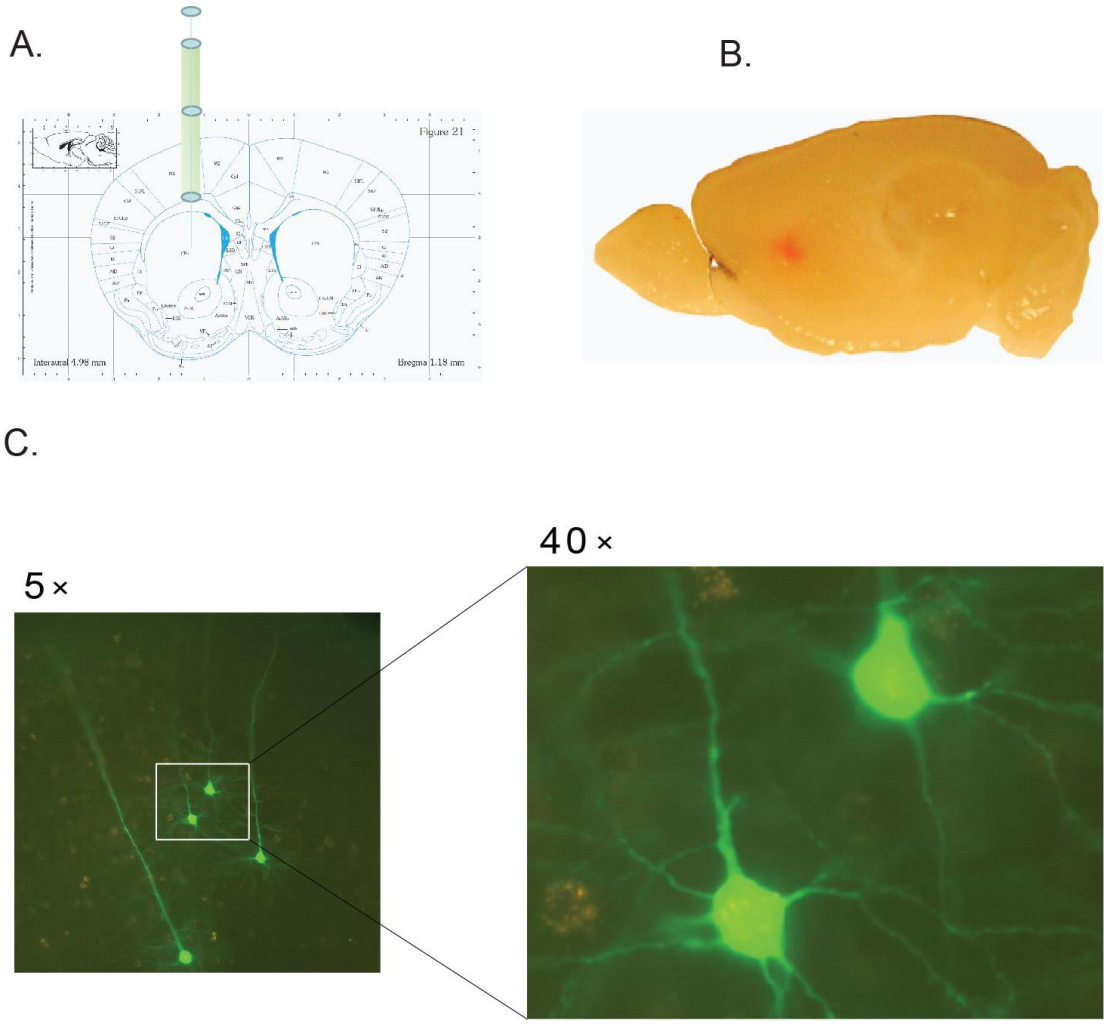

Figure 2.1. The Retrograde Beads Labeling of the PFC L5 Corticostriatal Pyramidal Neurons. A. Stereotactic injection into contralateral DS; B. The injection site in contralateral DS; C. Double-labeled neurons distribution in PFC L5 (Red spots: retrograde microbeads. Green: biocytin-streptavidin staining).

2.3.2 The mIPSCs and mEPSCs Changes of the Target Neurons in AS Mice

2.3.2.1 The mIPSCs Changes

The mIPSCs of PFC L5 corticostriatal pyramidal neurons in AS mice and WT littermates from three age groups were measured. Compared to the frequency of age-matched WT littermates, the frequency of mIPSCs in AS mice decreased at P17-19 (Figure 2.2A, WT = 2.69 ± 0.24 Hz, n=13 cells/3 mice; AS = 1.13 ± 0.19 Hz, n = 14 cells/3 mice; $p < 0.01$, t test) and P28-35 (Figure 2.2C, WT=4.56 ± 0.61 Hz, n=11 cells/mice; AS=2.50 ± 0.46 Hz, n=9 cells/mice; $p < 0.05$, t test), but kept similar to WT at age >P70 (Figure 2.2E, WT=2.03 ± 0.43 Hz, n=7 cells/3 mice; AS=1.72 ± 0.28 Hz, n = 11 cells/3 mice; $p > 0.05$, t test). And the decrease demonstrated a trend of decrement with age: the frequency decreased 138% in P17-19 group, 82% in P28-35 group and 17% in >P70 group. But the cumulative amplitude increased at P17-19 (Figure 2.2B, $p < 0.05$, K-S test), decreased at P28-35 (Figure 2.2D, $p < 0.05$, K-S test). No amplitude difference of mIPSCs between AS and WT mice observed at >P70 (Figure 2.2F, $p > 0.05$, K-S test). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

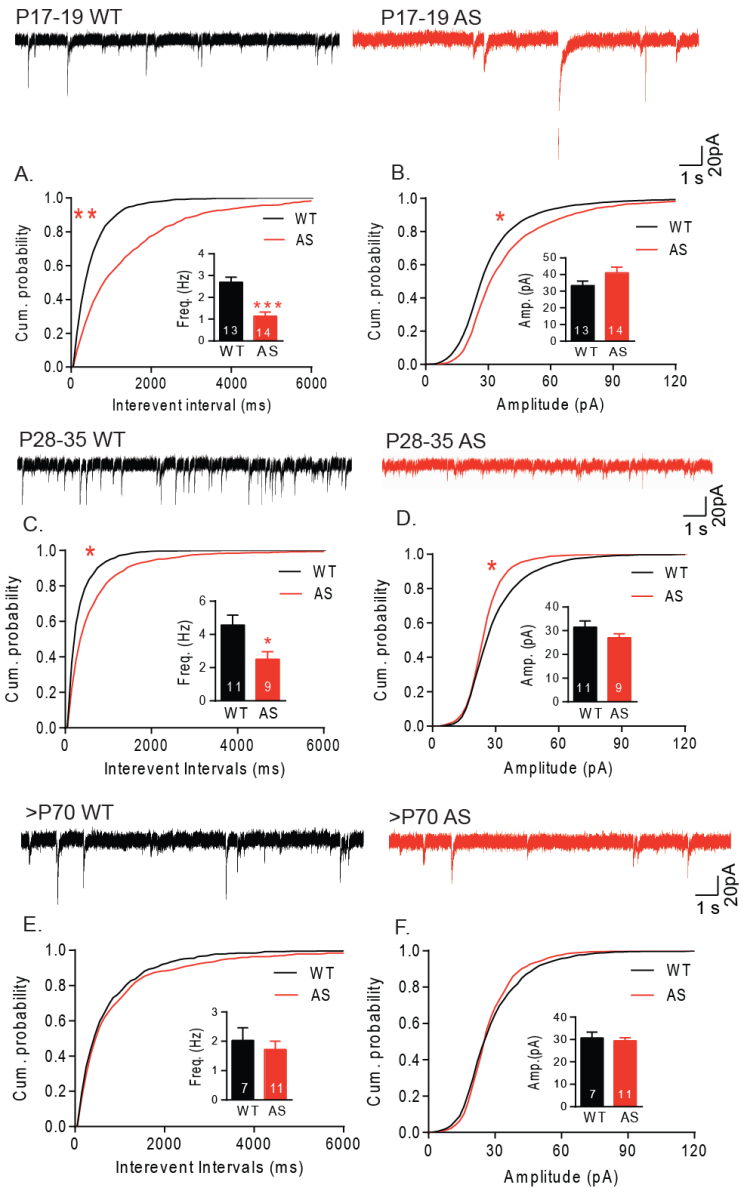

Figure 2.2. The Inhibitory Synaptic Transmission Deficiency of the PFC L5 Pyramidal Neurons in AS Mice. The mIPSCs frequency showed significant decrease in AS mice at P17-19 (A, $p < 0.001$, t test), P28-35 (C, $p < 0.05$, *t* test) but kept comparable to that of WT mice at >P70 (E, $p > 0.05$, *t* test). But the amplitude increased in AS at P17-19 (B, $p < 0.05$, K-S test), decreased at P28-35 (D, $p < 0.05$, K-S test) and did not show difference between AS and WT at > P70 (F, $p > 0.05$, K-S test). (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.3.2.2 The mEPSCs Changes

At three developmental stages examined, the mEPSCs of the PFC L5 corticostriatal pyramidal neurons in AS mice showed similar changes like mIPSCs: the frequency of AS mice also reduced at P17-19 (Figure 2.3A, WT=3.43 ± 0.49 Hz, n=14 cells/3 mice; AS=1.40 ± 0.25 Hz, n=14 cells/3 mice; $p < 0.001$, t test), P28-35 (Figure 2.3C, WT=3.79 ± 0.64 Hz, n=11 cells/3 mice; AS=1.73 ± 0.44 Hz, n=10 cells/3 mice; $p < 0.05$, t test), but did not change at >P70 (Figure 2.3E, WT=0.66 ± 0.17 Hz, n=10 cells/4 mice; AS=0.56 ± 0.09 Hz, n=10 cells/4 mice; $p > 0.05$, t test). And the decrease illustrated an age-dependent pattern either: the frequency decreased 145% in P17-19 group, 119% in P28-35 group and 19% in >P70 group. The cumulative amplitude increase was observed in AS mice at P28-35 (Figure 2.3D, $p < 0.05$, K-S test), but not at P17-19 (Figure 2.3B, $p > 0.05$, K-S test) and >P70 (Figure 2.3F, $p > 0.05$, K-S test). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The decrease of frequency of mPSCs (mIPSCs and mEPSCs) indicated that either synapses or their release probability (Pr) decreased. The increase of amplitude suggested that the sensitivity of postsynaptic receptors to neurotransmitter impaired in AS mice (Fatt and Katz, 1952) (Cummings et al., 1996).

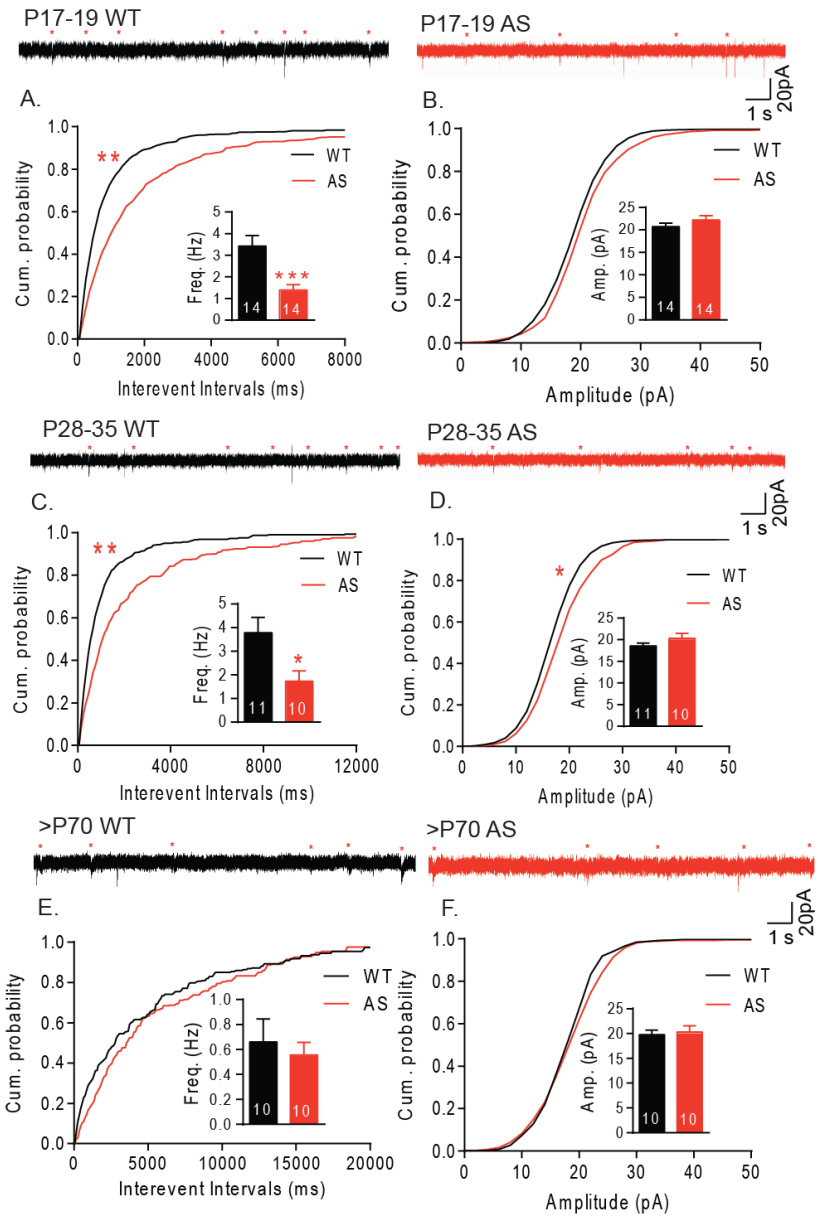

Figure 2.3. The Excitatory Synaptic Transmission Deficiency of the PFC L5 Pyramidal Neurons in AS Mice. The mEPSCs frequency demonstrated significant decrease in AS mice at P17-19 (A, $p < 0.001$, t test), P28-35 (C, $p < 0.05$, t test) but ketp indistinguishable to that of WT mice at >P70 (E, $p > 0.05$, t test). The cumulative amplitude increased in AS mice at P28-35 (D, $p < 0.05$, K-S test), but not at P17-19 (B, $p > 0.05$, K-S test) and >P70 (F, $p > 0.05$, K-S test). (Small red asterisks above typical recordings indicate mEPSC responses). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.3.3 The Neural Circuit Alteration

The synaptic connectivity of the PFC L5 corticostriatal pyramidal neurons was investigated by using LSPS as previously reported (Peng et al., 2016) (Suter et al., 2010). The L5 neurons in WT mice received excitatory synaptic inputs mainly from L2/3 and L5A, and the circuit pattern maintained through developmental stages, wheareas in AS mice the primary excitatory inputs onto these neurons derived from L5B instead of L2/3 and L5A, and these abnormal connectivity emerged from early postnatal age, kept with little change to adulthood (Figure 2.4A and 2.4B, P17-19, $F_{(15, 144)} = 5.378$, $p < 0.0001$, Two-way ANOVA; 2.4C and 2.4D, P28-35, $F_{(15, 320)} = 56.88$, $p < 0.0001$, Two-way ANOVA; 2.4E and 2.4F, >P70, $F_{(15, 176)} = 29.05$, $p < 0.0001$, Two-way ANOVA.), the difference in the pooled excitatory synaptic inputs of the PFC L5 neurons in relation to their laminar location is genotype dependent. The similar changes were also found for the inhibitory inputs (Figure 2.5A and 2.5B, P17-19, $F_{(15, 80)} = 7.805$, $p < 0.0001$, Two-way ANOVA; 2.5C and 2.5D, P28-35, $F_{(15, 288)} = 10.90$, $p < 0.0001$, Two-way ANOVA; 2.5E and 2.5F, > P70, $F_{(15, 160)} = 8.526$, $p < 0.0001$, Two-way ANOVA).

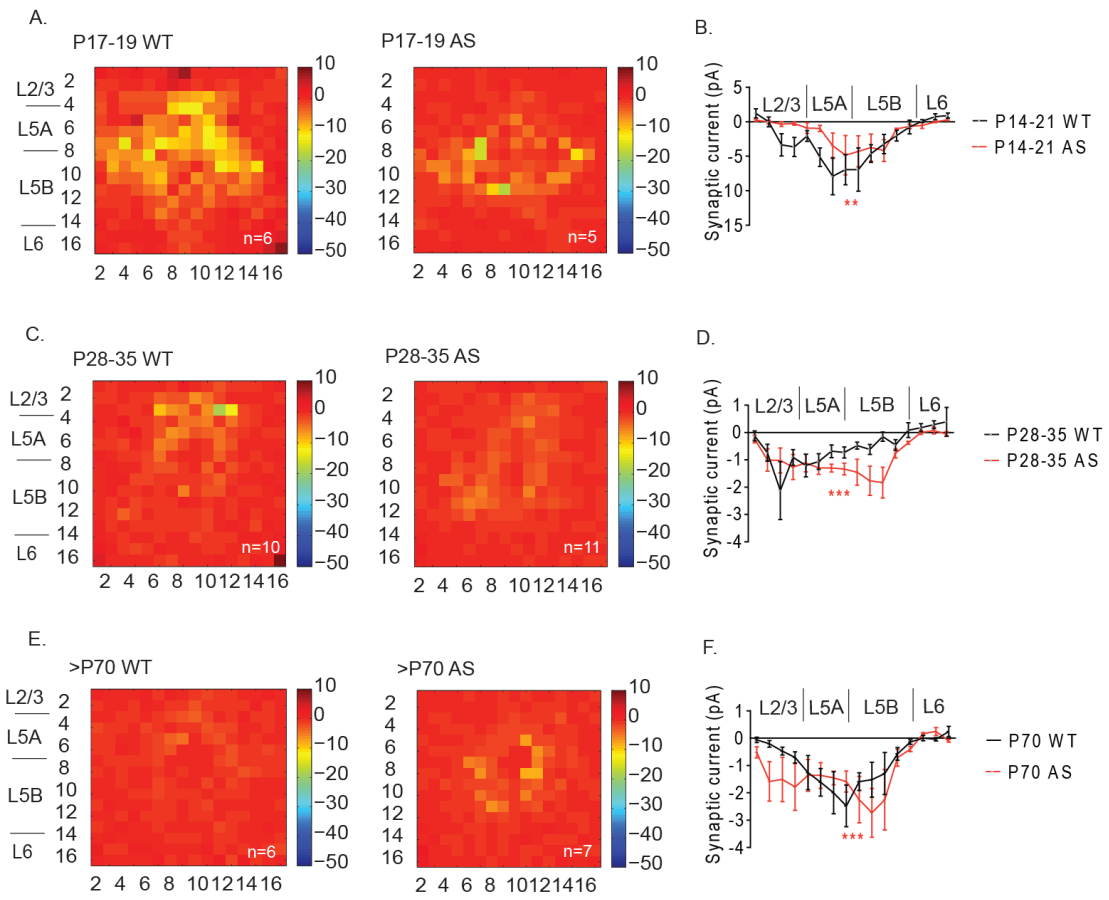

Figure 2.4. The Change of Excitatory Presynaptic Inputs of the PFC L5 Pyramidal Neurons. The synaptic responses strength were illustrated by pseudo-color representation (A, C and E) or inputs currents to location (B, D and F). Circuit connectivity showed significant decrease of inputs from L5A at P17-19 (A and B), P28-35 (C and D) and >P70 (E and F) in AS mice. The excitaory inputs of these neurons derived from the PFC L5A in WT, whereas from the PFC L5B mainly in AS mice. (A, C and E: averaged excitatory synaptice inputs; B, D and F: pooled excitatory synaptic inputs of the PFC L5 neurons in relation to their laminar location).

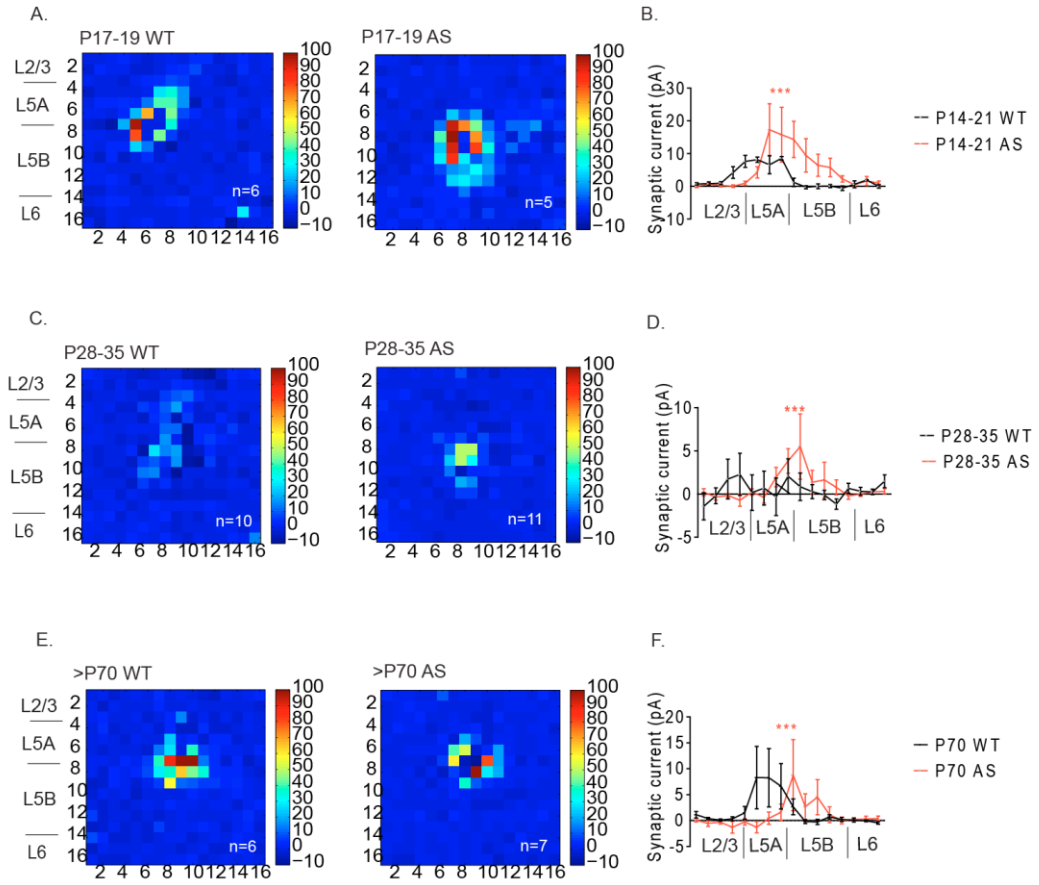

Figure 2.5. The Alteration of Inhibitory Presynaptic Inputs of the PFC L5 Pyramidal Neurons. The synaptic responses strength were illustrated by pseudo-color representation (A, C and E) or inputs currents to location (B, D and F). Circuit connectivity from L2/3 and L5A showed significant decrease at P17-19 (A and B), P28-35 (C and D) and >P70 (E and F) in AS mice. The major inhibitory inputs of these neurons derived from the PFC L5B in AS mice. (A, C and E: averaged excitatory synaptice inputs; B, D and F: pooled excitatory synaptic inputs of the PFC L5 neurons in relation to their laminar location).

2.4 Discussion

Our work showed that synaptic transmission including mEPSCs and mIPSCs were impaired in the PFC L5 corticostriatal pyramidal neurons in AS mice, and that frequencies of both reduced at different scales with a slight bias toward mIPSCs (82-119% (mEPSCs) *vs* 138-145% (mIPSCs), in contrast to the level in WT) which suggests an excitation/inhibition imbalance observed in the visual cortex in previous report (Wallace et al., 2012), illustrating the similarity in consequences of E6-AP deficiency in these two brain regions. The biased reduction of inhibition in cerebellum has been concerned extensively in the deficits of locomotion (Egawa et al., 2012), and the contribution of decreased inhibition in neocortex to epilepsy, a common symptom in about 90% AS patients (Pelc et al., 2008b), has also been investigated (Dan and Boyd, 2003)(Judson et al., 2016). The unequal decrement of mIPSCs and mEPSCs observed in the PFC L5 corticostriatal pyramidal neurons, might perturb the function of these neurons in regulating the planned actions and being executed ones, so as to give rise to motor deficits in AS (Shepherd, 2013)(Williams et al., 2010a).

The defects of synaptic transmission occurred very early in development as was shown in our data from ages as early as P17-19, maintained through later developmental stages with significant improvement with age (frequency decline rate compared to age-matched WT: 119% at P17-19, 82% at P28-35 (mEPSCs), 145% at P17-19, 138% at P28-35 (mIPSCs)). At adulthood (>P70), both mIPSCs and mEPSCs of AS mice could not be distinguished from those of WT ones, in contrast to the mIPSCs alteration with age was reported in the visual cortex in another study (Wallace et al., 2012).

Neural circuit in the PFC of AS mice demonstrated dramatic changes as is revealed by the LSPS measurement of excitatory and inhibitory local inputs onto L5 corticostriatal pyramidal neurons. These neurons receive inputs mainly from L2/3 and L5A, with nearly equal (18-27%) and a bias toward L5 inhibitory inputs from those aforementioned layers in normal WT mice

(Hoppa et al., 2015), as is also shown in our data from WT mice. However, the excitatory inputs of the L5 corticostriatal pyramidal neurons in P17-19 AS mice come from L5B mainly, and the abnormal pattern keeps till adulthood (>P70). Similar inhibitory inputs pattern alteration and quantity reduction are also found in AS at the three ages tested. This is the first investigation in neural connectivity on AS model mice.

The heterogeneity in responding to the same external stimuli or insults of different subset neurons has long been recognized (Kandel, 2012e). Thus it is not surprising that the synaptic transmission deficits in the PFC demonstrated some brain region specific characteristics along with some features in common with other brain areas such as the striatum and the visual cortex (Hayrapetyan et al., 2014) (Wallace et al., 2012). The PFC has not drawn enough attention in AS perhaps because it was considered that the hippocampus and the cerebellum are the only two brain regions where *UBE3A*/*Ube3a* is imprinted (Albrecht et al., 1997)(Jiang et al., 1998)(Miura et al., 2002)(Gustin et al., 2010). It is recently realized as an area with maternal *UBE3A*/*Ube3a* expression pattern (Dindot et al., 2008)(Yashiro et al., 2009)(Daily et al., 2012)(El Hokayem and Nawaz, 2014)(Judson et al., 2014)(Grier et al., 2015b), and the E6-AP expression showed a similar pattern in the neocortex like that in the hippocampus (Burette et al., 2016). Our data demonstrate that the L5 pyramidal neurons in the PFC differ from those in other brain regions such as the striatum, the hippocampus, the cerebellum and the visual cortex in responding to the E6-AP deficiency in AS mice, which may result from the possibility that their relatively late generation and functional maturation in comparison to those in other brain regions, rendering them more susceptible to E6-AP deficiency (Elston and Fujita, 2014). The embryonic cortex neuron generation booms in the late stage of embryonic development, in the last 5 days of embryonic development in mice for example, after the imprinting of *UBE3A*/*Ube3a* (Qian et al., 1998)(Gupta et al., 2002)(Yamasaki et al., 2003)(Kandel, 2012f)(El Hokayem and Nawaz, 2014)(Ueda et al., 2015)(Silbereis et al., 2016). Notably, the parvalbumin gene expression also reaches a peak during this time (Ueda et al., 2015). This may explain why these neurons were found deficient in the visual cortex (Wallace et al., 2012). It may be also true for the vulnerability

of the PV-positive interneurons in the hippocampus and basolateral amygdala of AS mice from P10 to P120. These cells were more vulnerable than those of WT mice in responding to chronic stress, which decreased the number of these neurons (Godavarthi et al., 2014), and also the cortex and the cerebellum have different developmental process which is regulated by different factors respectively (Kandel, 2012g). Therefore, the lag between neurogenesis of the most PFC neurons and *UBE3A*/*Ube3a* imprinting makes the PFC an ideal area to investigate the E6-AP effects on most of neural development events including neuron generation, migration, neural process outgrowth and pruning. This will provide a more comprehensive understanding of E6-AP function, especially during critical period and on synaptic plasticity.

As the PFC L5 pyramidal neurons in adult animals demonstrate distinct diversity in properties in a projecting-pathway manner (Molnár and Cheung, 2006)(Brown and Hestrin, 2009a), neurons recorded in our work were retrograde-labeled with microbeads injected into contralateral dorsal striatum (corticostriatal neurons)(Shepherd, 2013). Corticostriatal pathway involves into movement, learning and intentional behaviors (Graybiel, 1995)(Schultz et al., 2003)(Kreitzer and Malenka, 2008)(Goldman-Rakic et al., 2011), closely related functions are impaired in AS patients as shown like intellectual retardation and ataxia (Buiting et al., 2016). In addition, some studies showed that the striatum is also affected in AS model mice. For example, mEPSCs frequency was reported decreased in the dorsomedial striatum in AS mice, implicating synaptic malfunction in the corticostriatal circuit (Hayrapetyan et al., 2014), while another study showed the striatum is exempted from mTOR pathway deficiency which was found in the cerebellum in AS mice (Sun et al., 2015a). These intrigued us to select the corticostriatal pathway as the target area for our study.

The relation between pathological modalities of AS and developmental stages or ages has not been extensively explored so far. The temporal effect of E6-AP in neuronal development, its relation to critical period and its effect on synaptic plasticity are elusive. Currently, neither the

reason why the imprinting of *UBE3A*/*Ube3a* occurs selectively in neurons nor the interactions of E6-AP with other proteinss during brain development is fully understood (Rougeulle et al., 1997)(Yamasaki et al., 2003)(Mabb et al., 2011). Neuronal development is characterized by crucial temporal course when different genes expressions turn on/off in a strict sequence. The circuits are refined constantly in an experience-dependent manner, especially during the critical period. The critical period varies in different brain region (Meredith, 2015)(Kandel, 2012g). This variability in development stages including critical period may be the reason why the full establishment of *UBE3A*/*Ube3a* imprinting differs in different brain regions (Sato and Stryker, 2010)(Judson et al., 2014)(Grier et al., 2015b)(Dindot et al., 2008)(Philpot et al., 2011). Reports in patients show that difference between different ages also exists: it was reported that a 3-year-old boy AS patient had disorganized frontal and temporal lobe, irregular gyri and normal cerebellum (Kyriakides et al., 1992), whereas a 21-year-old woman AS patient showed normal gyri and decreased dendrite arborization in the visual cortex (L3 and L5) and the cerebellum (Jay et al., 1991). Morphology and function alteration has also been investigated in AS model mice of different ages. Wallace et al. found the mIPSCs defect in the visual cortex of P80 AS mice could not be detected on young ones (P25) (Wallace et al., 2012). Jiang et al. reported that morphology of the cerebellum in old mice (3-4 months) is normal (Jiang et al., 1998). Dindot et al. also showed that Purkinje cells in old mice (7-8 weeks) are not affected by E6-AP deficiency (Dindot et al., 2008). However, more studies showed morphological abnormalities in many brain areas (Refer to chapter 1 for details). Disappointedly, the pathology of AS at different ages has not been studied intentionally. Here we examined the synaptic transmission and circuit change in AS model mice at three ages, i.e. P17-19, P28-35 and older than P70, represent neonatal, adolescent and adult ages respectively. Our data demonstrated that impairment in both synaptic transmission and connectivity of the PFC L5 pyramidal neurons established in early developmental stage, i.e. P17-19, these abnormalities will be kept unchanged through adulthood. The similar synaptic transmission between AS and WT mice at adult (>P70) does not mean equal function because neural circuits have been established, so activity amount equity does not execute normal function as the connectivity is aberrant. It is worth a mention that E6-AP

expression level declines in the cortex (frontal, auditory and visual) in normal humans, monkeys and cats with age, and the decrement is supposed to related to the vision acuity decline in aged animals (Williams et al., 2010b). It is unknown if the age-dependent decrease of E6-AP expression contributes to the decline of other functions in aging brains or not, thus further exploration in the relation between E6-AP and age is needed to elucidate the role of E6-AP in CNS. This is of important clinic meaning for determining the optimal invention timing as illustrated in a recent report (Silva-Santos et al., 2015).

Several factors may contribute to the defects in synaptic transmission and neural circuits in AS mice. First, receptors including glutamate receptors AMPAR, NMDAR and GABAR functions may be affected due to various reasons. It was found that Arc expression and degradation is regulated by E6-AP, and E6-AP deficiency in AS mice results in increased Arc which enhances AMPAR internalization, correspondingly, mEPSCs and AMPA/NMDA ratio decreased significantly in the hippocampus of AS mice (Greer et al., 2010). AMPAR delivery and distribution in synapse as well as new AMPAR insertion into membrane is controlled by NMDAR activation (Shi et al., 1999)(Lu et al., 2001) which was found impaired in the hippocampus of AS mice due to elevated SK2 (Sun et al., 2015b). Both AMPAR and NMDAR are regulated by CaMKIIα which function was revealed disrupted for abnormal phosphorylation at Thr^{286} and Thr^{305} in AS mice (Colbran and Brown, 2004)(Weeber et al., 2003). For GABAR, there is indication of impaired GABAergic neuron function in AS. For example, the ataxia, a common symptom in AS, is accompanied by the abnormal oscillation of cerebellar cortex, which can be inhibited by sensory stimulation, inhibitors of gap junction, or GABAA receptor blockers (Cheron et al., 2005b). The loss of GABAergic neurons is also found in the neocortex (Judson et al., 2016). Second, BDNF is an important factor regulating both excitatory and inhibitory synatic transmission (Bolton et al., 2000), either by recruiting sypnatic receptor clustering (Elmariah, 2004) or by enhancing neurotranmitter release (Li et al., 1998), its signaling pathway was deminished in AS mice (Cao et al., 2013). Third, the imbalance in response to E6-AP between neurons in a cluster can result in developmental distinction and different activity, these discrimination in activity suppression between a single neuron and a neurons population

might be a reason that gives rise to the defect of circuit development (Kandel, 2012e) (Buffelli et al., 2003)(Potjans and Diesmann, 2014). The structure of neural circuits determines functions (Kandel, 2012h), and neural activities and experience refine the circuits, especially during the critical period (Alberts, 2008a)(Shulz and Jacob, 2010)(Kandel, 2012i)(Silbereis et al., 2016). The role of experience-dependent synaptic plasticity in neurodevelopmental disorders has long been proposed and gradually accepted (Zoghbi, 2003)(Kubota et al., 2015)(Wei et al., 2015). Interestingly, E6-AP expression itself can be regulated by neuronal activity (Greer et al., 2010), so defects of both morphology and function in AS may result from a reciprocal interaction (Mabb et al., 2011). That is, the proceeding protein homeostasis disturbance impairs every aspects of development including the neuron generation, migration, neurites outgrowth and/or refining/pruning, so as to induce the failure in forming normal circuits. The situation is aggravated by the defects in synaptic plasticity. One more factor involved into the synaptic transmission and circuit deficit is the abnormal reduction in neuron structures including dendritic branching, spine and synaptic vesicle (Dindot et al., 2008)(Su et al., 2011)(Wallace et al., 2012). The aberrant CCV accumulation was also reported in both glutamatergic and GABAergic neuron terminals (Wallace et al., 2012)(Judson et al., 2016). As the NMDAR activation was reported disturbed by elevated SK2 expressed in membrane (Sun et al., 2015b), the spine density and structure may be also affected by malfunction of NMDAR (Lüthi et al., 2001)(Ultanir et al., 2007). These disruptions in both pre- and postsynaptic structures will considerably impair neuron signaling capability.

A lot of open questions still leave not answered. For example, 1. Whether or not the functions of interneurons in the PFC are disrupted and if so, how this influences the PFC L5 pyramidal neurons (Wallace et al., 2012); 2. Whether any presynaptic properties such as calcium signal (Yashiro et al., 2009), presynaptic NMDA receptors (Kunz et al., 2013)(Banerjee et al., 2016) are changed or not; 3. How the excitation/inhibition balance is impacted in the PFC; and 4. The influence of activity/experience on synaptic transmission in AS mice needs to be pinpointed.

Here for the first time the impact of E6-AP expression deficiency in AS on: 1.Synaptic transmission; 2. The neural signaling at circuit level in the PFC is investigated and compared at different developmental stages. It will benefit the field in three ways by 1. Helping to understand the temporal function of E6-AP in neural system development; 2. Providing information about potential targets if effects on different stages can be determined; and 3. Helping to determine the proper intervention timing in a brain region-specific way (Kolb and Gibb, 2015).

CHAPTER 3

THE SHORT-TERM SYNAPTIC PLASTICITY DEFICITS OF THE PRESYNAPTIC NEURONS AND AMPA/NMDA RATIO CHANGE OF THE PFC L5 CORTICOSTRIATAL PYRAMIDAL NEURONS

3.1 Introduction

Synaptic plasticity, a physiological process that synaptic strength is regulated by specific synaptic activities and its formation involves both presynaptic and postsynaptic mechanisms, is believed to contribute to memory and learning (Zucker and Regehr, 2002)(Ho, 2011). Long-term synaptic plasticity such as LTP, LTD have been well studied and reported impaired in multiple brain areas in AS model mice (Jiang et al., 1998)(Weeber et al., 2003)(Daily et al., 2011)(Meng et al., 2013)(Sun et al., 2016)(Pignatelli et al., 2014). In contrast, the effects of E6-AP deficiency on short-term synaptic plasticity has not drawn extensive attentions still, and has been just investigated in the visual cortex where the neocortical plasticity and the excitatory cortical circuits were found impaired in AS mice, and the inhibitory inputs onto pyramidal neurons from interneurons drastically reduced (Yashiro et al., 2009)(Wallace et al., 2012). Little is known about the change of short-term plasticity in other brain areas including the PFC in AS model mice.

Compared to L2/3 neurons in the primary visual cortex (V1), the pyramidal neurons in the PFC is characterized with a unique property in short-term plasticity: they showed more augmentations in response to train stimulus (Hempel et al., 2011). Furthermore, even different subsets of the PFC neurons projecting to different brain regions demonstrate high level of heterogeneity in electrophysiological features (Brown and Hestrin, 2009b). The heterogeneity is also reflected by the distinctively diverse responses of the PFC neurons to even a single type of external stimulus in adult animals (Kolb and Gibb, 2015). Thus results or conclusions gained from not only other brain areas but also neurons in different circuit or neural pathways of the PFC do

not apply to all PFC neuron populations, where changes of synaptic plasticity needs exclusive investigation.

Previous studies revealed that synaptic structures such as spine density, synaptic vesicle of neurons in the hippocampus and the visual cortex also disturbed by E6-AP deficiency in AS model mice (Dindot et al., 2008)(Su et al., 2011)(Wallace et al., 2012)(Judson et al., 2016). These morphological abnormalities contribute to the functional defects such as impairments in mEPSCs, mIPSCs, LTP and LTD in multiple brain areas and short-term plasticity deficit in the visual cortex. Besides, the intrinsic properties of the neurons in AS model mice were found impacted due to the abnormal expression sodium channel NaV1.6 and Na/K ATPase at the axon initial segment (AIS). The affected neurons showed elevated threshold potential, depolarized resting membrane potential and longer AIS compared to those of unaffected neurons in WT mice (Kaphzan et al., 2011). These alterations in the intrinsic properties will influence neuron activities including the synaptic plasticity.

AMPAR and NMDAR, the main glutamate receptors, play different roles in the synaptic transmission and plasticity, with AMPAR regulating short-term synaptic strength while NMDAR controlling the maintenance of short-term changes by influencing gene expression (Rat, 1990)(Dodt et al., 1998)(Rao and Finkbeiner, 2007). At excitatory synapse, AMPAR and NMDAR are organized and distributed orderly by different postsynaptic proteins (Sheng and Hoogenraad, 2007)(Kandel, 2012j). The two type receptors regulate each other's activities, with respect that the AMPAR activation depolarizes neuron to expel magnesium blocking the NMDAR (Kandel, 2012j); NMDAR activation regulates AMPAR delivery and distribution at the synapse, the insertion into and removal from cell membrane (Shi et al., 1999)(Lu et al., 2001) (Brown et al., 2005). Moreover, the signaling via NMDAR plays an important role in promoting dendrite growth and new spine formation (Dalva et al., 2000), and AMPAR mediated activity is potent in stabilizing new spines (McKinney et al., 1999). Thus the coordination between AMPAR and NMDAR is crucial for normal excitatory synapse function (Rao and Finkbeiner, 2007) and the currents

through NMDAR and AMPAR are regulated proportionally by the neural activity as is indicated by a fixed AMPA/NMDA ratio (Watt et al., 2000) (Saal et al., 2003).

AMPAR and NMDAR alteration has been investigated in the hippocampus in AS model mice with controversial results. Both the AMPAR expression level and the AMPA/NMDA ratio was found decreased inn animals aged P15-18 (Greer et al., 2010), whereas both AMPAR and NMDAR expression levels as well as the AMPA/NMDA ratio were also reported normal in older mice aged 10-16 weeks (Kaphzan et al., 2012). Although the AMPA/NMDA ratio of L2/3 neurons is conserved between the PFC and the visual cortex under normal conditions (Myme et al., 2003), it showed a brain region-specific pattern in one autism model mice (Won et al., 2012). In addition, the PFC expresses higher NMDAR mRNA than other cerebral cortices in humans as shown in immunohistochemistry study in postmortem brains (Kosinski et al., 1998), suggesting that the glutamate receptors might be affected in a unique manner in the PFC of AS model mice.

These unknown questions led us to examine the possibility that the short-term synaptic plasticity alteration in L2/3 PFC neurons and AMPA/NMDA ratio change in L5 PFC corticostriatal pyramidal neurons of AS model mice may illustrate both the PFC-specific and age-dependent features. These assumptions were confirmed by our investigations in three age groups. The data showed: 1. The synaptic release probability (Pr) of L2/3 PFC neurons is impaired in AS mice at P17-19 and P28-35 but is comparable to age-matched WT mice at >P70; 2. The AS mice demonstrated deficit of readily-releasable vesicle (RRV) pool replenishment of L2/3 PFC neurons at the three ages detected; and 3. The AMPA/NMDA ratio L5 PFC corticostriatal pyramidal neurons is similar between AS and WT mice at P17-19, but decreased dramatically after P28. Our results presented the changes of short-term plasticity and the AMPA/NMDA ratio with age in the PFC of AS model mice for the first time.

3.2 Materials and Methods

3.2.1 Animals and Habituation

As was described in Chapter 1.

3.2.2 The Stereotactic Injection and Handling

As was described in Chapter 1.

3.2.3 Patch Clamp Whole-cell Recordings

Patch clamp whole-cell recording technique was performed on PFC L5 pyramidal neuron as was described in Chapter 1. Recording electrodes were filled with cesium-gluconate (Cs-gluconate) internal solution containing (in mM): 125 cesium-gluconate, 5 TEA-Cl, 10 HEPES, 8 NaCl, 2.5 Cs-Cl, 5 QX314-Cl, 4 ATP (magnesium salt), 0.3 GTP (sodium salt), 1 EGTA, 10 Phosphocreatine, adjusted to pH ~7.2 with CsOH, 290 mOsm. Recordings were carried out at the presence of 50 mM picrotoxin (PTX) in ACSF to inhibit GABA current.

The PFC L5 pyramidal neuron intrinsic properties such as resting potentials, input resistance were detected. Presynaptic input efficacy and short-term synaptic plasticity alteration including the neurotransmitter release probability (Pr), synaptic RRV pool replenishment rate, which were measured by paired-pulse facilitation (PPF) and high frequency depletion (HFD), respectively, and AMPA/NMDA ratio of presynaptic neurons innervating L5 pyramidal neuron were investigated on brain slice. A dual-pole stimulating electrode was placed on PFC L2/3, 150-200 μm away from the target L5 neuron and the evoked AMPA EPSCs (eEPSCs) were recorded at the holding potential of -70 mV (Bellingham and Walmsley, 1999). Electrical stimuli were generated by a researcher self-built stimulus generator ISO which was controlled by the

command from digitizer. For PPF, a serial of pulse-pair with different inter-stimulus intervals (ISI, starting from 500 mili seconds (ms), ending at 50 ms with 50 ms decrement) were administered (McKernan and Shinnick-Gallagher, 1997)(Lane-Donovan et al., 2016). The Pr was valued with the ratio of $eEPSC_2/eEPSC_1$. For HFD, a train of 40 stimulations with either 5 Hz or 10 Hz which do not exceed physiological firing frequency range for PFC pyramidal neurons (Wang, 1999) was applied subsequently. The ratio of $eEPSC_{40}/eEPSC_1$ was used to indicate the RRV depletion rate. For AMPA/NMDA ratio, AMPA and NMDA eEPSC were recorded when membrane potential was held at -70 or 40 mV respectively. The average amplitude of 10 responses was used to compare and the NMDA eEPSC amplitude was measured after 50 ms from the onset of response to exclude AMPA current component (Etherton et al., 2009).

3.2.4 Data Analysis

Data were shown as mean ± S.E.M and analyzed by using One-way ANOVA or t test. $p < 0.05$ was taken as significant threshold.

3.3 Results

3.3.1 The Alteration of Release Probability (Pr) of the Presynaptic Neurons of the Target Neurons

Neurotransmitter Pr of L2/3 presynaptic neurons of PFC L5 corticostriatal pyramidal neurons were tested by using PPF to record eEPSCs. The $eEPSC_2/eEPSC_1$ ratios to stimuli with ISI within 100 ms showed less facilitation in AS compared to that in WT at P17-19 (Figure 3.1A, n=3 mice/genotype, $p < 0.05$ (50 ms), t test) and P28-35 (Figure 3.1B, n=3 mice/genotype, $p < 0.05$ (100 ms), t test), but comparable between AS and WT at age >P70 (Figure 3.1C, n=3 mice/genotype, $p > 0.05$, t test. Representative responses to paired-pulse stimuli of different ISI were illustrated on the left). At the ISI of 50 ms (P17-19) or 100 ms (P28-35), the mean $eEPSC_2/eEPSC_1$ ratios were: WT = 1.61 ± 0.20, n=5 cells/3 mice; AS = 1.11 ± 0.10, n = 7 cells/3

mice (ISI = 50 ms, P17-19); WT=1.58 ± 0.22, n=10 cells/3 mice; AS = 0.94 ± 0.11, n = 8 cells/3 mice (ISI=100 ms, P28-35), and WT=1.77 ± 0.21, n=13 cells/2 mice; AS=1.39 ± 0.12, n=10 cells/2 mice (ISI=50 ms). WT=1.15 ± 0.10, n=13 cells/2 mice; AS=1.22 ± 0.15, n=10 cells/2 mice (ISI=100 ms) (>P70) respectively.

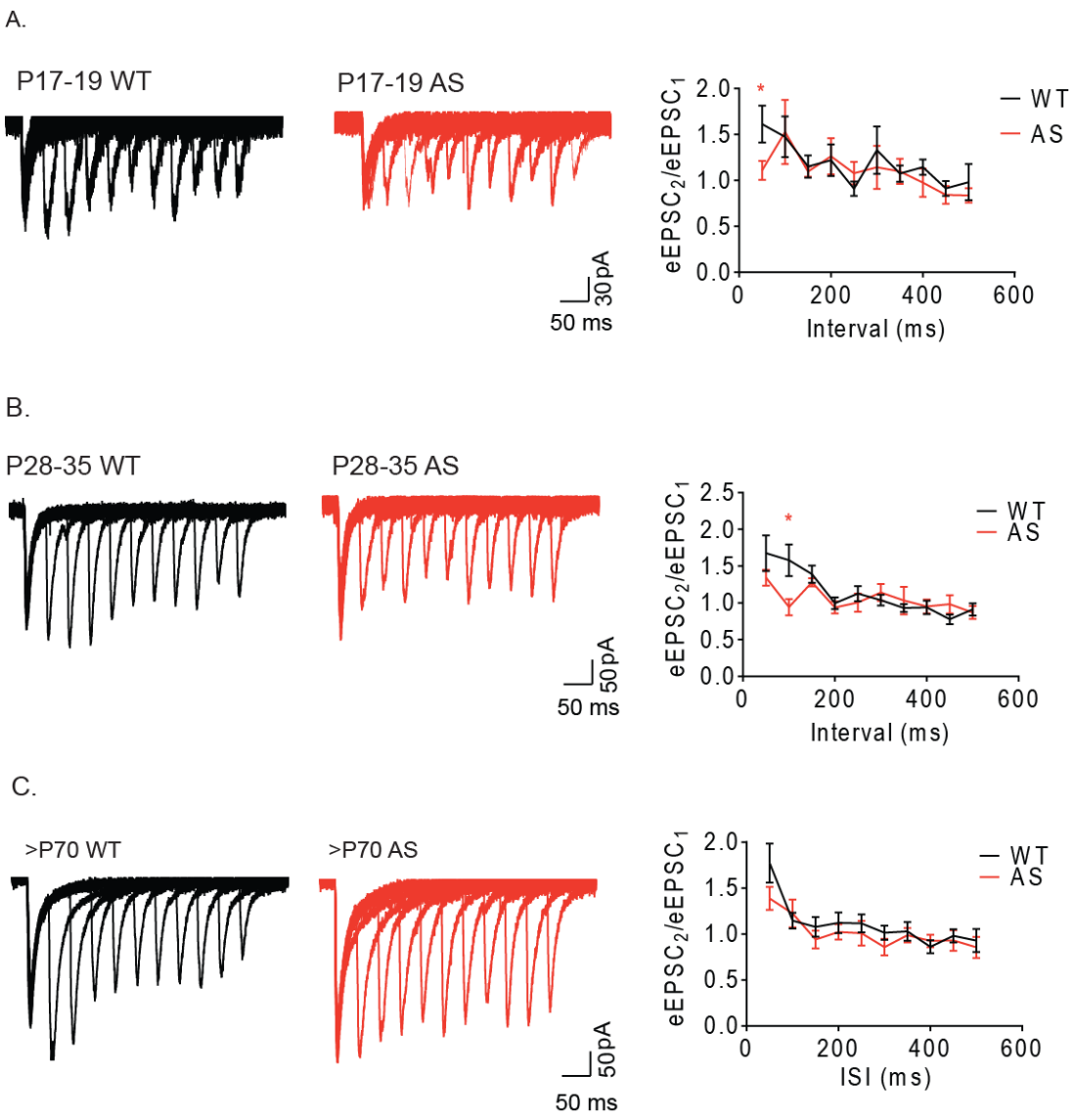

Figure 3.1. The Change of Release Probability (Pr) of the Presynaptic Neurons Innervating the PFC L5 Corticostriatal Pyramidal Neurons. The PPF test showed that the second eEPSC facilitation was less in AS mice than that in WT mice at P17-19 (A, n=5 (WT) or n=7 (AS), $p < 0.05$, t test) and P28-35 (B, n=10 (WT) or n=8 (AS), $p < 0.05$, t test), indicating that the Pr of these presynaptic neurons increased in AS mice. No difference in PPF between WT and AS mice at >P70 (C, n=13 (WT) or n=10 (AS), $p > 0.05$, t test).

3.3.2 The RRV Pool Change of the Presynaptic Neurons Innervating the Target Neurons

The eEPSCs of presynaptic L2/3 PFC neurons to a train of 40 stimulations at either 5 Hz (Figure 3.2) or 10 Hz (Figure 3.3) demonstrated distinctive characteristics in both genotype- and age-dependent manners. In P17-19 and P28-35 WT mice, all 3 age groups of AS mice, eEPSCs induced by the stimuli both frequencies declined gradually with stimulus increase (depression), and those of AS mice dropped more drastically compared to those of age-matched WT mice: at P17-19, WT=1.42 ± 0.17, n=5 cells/3 mice; AS=0.91 ± 0.07, n=6 cells/3 mice, $p < 0.05$, t test (5Hz HFD). WT=0.96 ± 0.18, n=5 cells/3 mice; AS=0.48 ± 0.10, n=6 cells/3 mice, $p < 0.05$, t test (10Hz HFD). The $eEPSC_{40}/eEPSC_{1}$ ratio reduced 56% (5Hz HFD) or 100% (10Hz HFD). At P28-35, WT=0.88 ± 0.09, n=9 cells/3 mice; AS=0.61 ± 0.09, n=13 cells/3 mice, $p < 0.05$, t test (5Hz HFD). WT=0.87 ± 0.08, n=9 cells/3 mice; AS=0.49 ± 0.07, n=13 cells/3 mice, $p < 0.01$, t test (10Hz HFD). The ratio decreased 46% (5Hz HFD) or 76% (10Hz HFD). At >P70, WT=0.93 ± 0.13, n=9 cells/2 mice; AS=0.76 ± 0.12, n=9 cells/2 mice, $p > 0.05$, t test (5Hz HFD). WT=0.85 ± 0.14, n=9 cells/2 mice; AS=0.36 ± 0.04, n=9 cells/2 mice, $p < 0.01$, t test (10Hz HFD). The ratio declined 23% (5Hz) or 137% (10Hz).

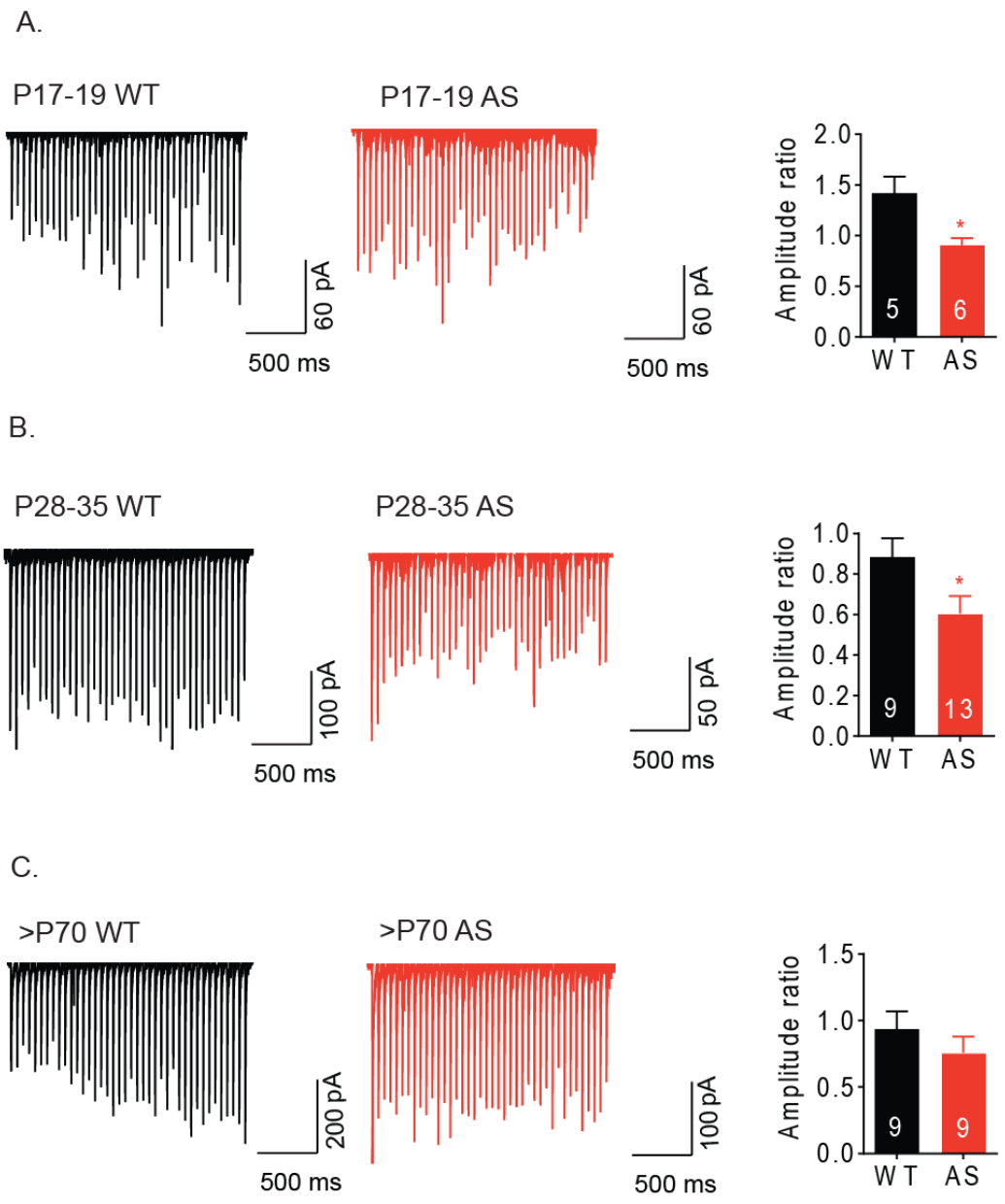

Figure 3.2. The RRV Pool Replenishment Rate Impaired in the Presynaptic Neurons of the PFC L5 Corticostriatal Pyramidal Neurons. 5Hz HFD (40 stimulations) inhibited the eEPSCs amplitude significantly in AS mice at P17-19 (A, n=5 (WT) or n=6 (AS), $p < 0.05$, t test) and P28-35 (B, n=9 (WT) or n=13 (AS), $p < 0.05$, t test), but not at >P70 (C, n=9, $p > 0.05$, t test). (The EPSCs amplitudes are indicated with lines as the time scale is highly compressed).

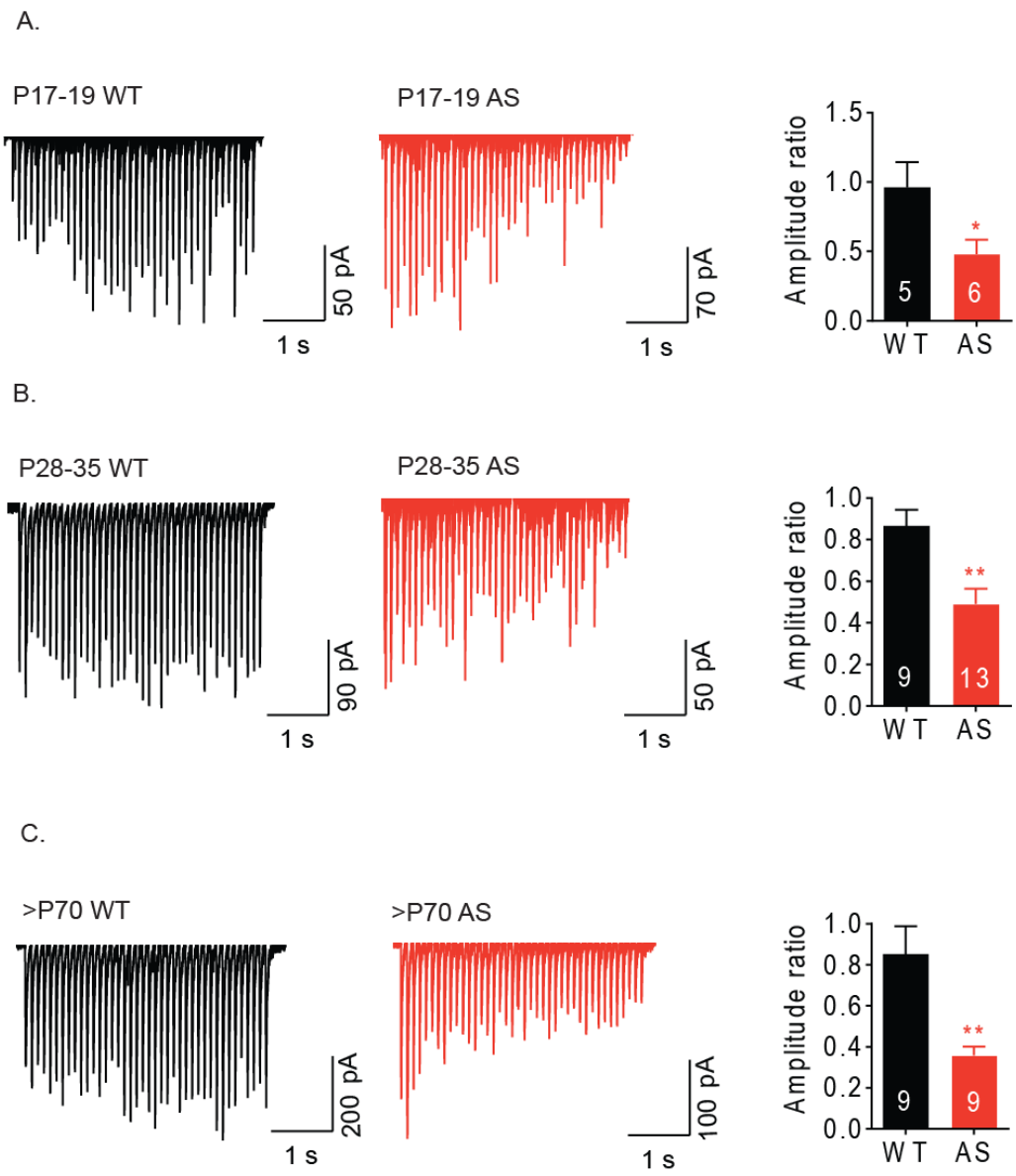

Figure 3.3. The RRV Pool Replenishment Rate Attenuated in the Presynaptic Neurons of the PFC L5 Corticostriatal Pyramidal Neurons. 10Hz HFD (40 stimulations) inhibited the eEPSCs amplitude considerably in AS mice P17-19 (A, n=5 (WT) or n=6 (AS), $p < 0.05$, t test) and P28-35 (B, n=9 (WT) or n=13 (AS), $p < 0.01$, t test), and >P70 (C, n=9, $p < 0.01$, t test). (The EPSCs amplitudes are indicated with lines as the time scale is highly compressed).

3.3.3 The AMPA/NMDA Ratio Change of the PFC L5 Corticostriatal *Pyramidal Neurons*

The AMPA/NMDA ratio kept comparable between WT and AS mice at P17-19 (Figure 3.4A, WT=1.58 ± 0.39, n=4 cells/3 mice; AS=1.84 ± 0.18, n=8 cells/3 mice, $p > 0.05$, t test). In comparison to that of WT neurons, AMPA/NMDA ratio of AS neurons showed dramatic reductions at both P28-35 (Figure 3.4B, WT=2.63 ± 0.17, n=7 cells/3 mice; AS=1.15 ± 0.11, n=10 cells/3 mice, $p < 0.001$, t test) and >P70 (Figure 3.4C, WT=2.86 ± 0.59, n=6 cells/2 mice; AS=0.98 ± 0.06, n=4 cells/2 mice, $p < 0.05$, t test).

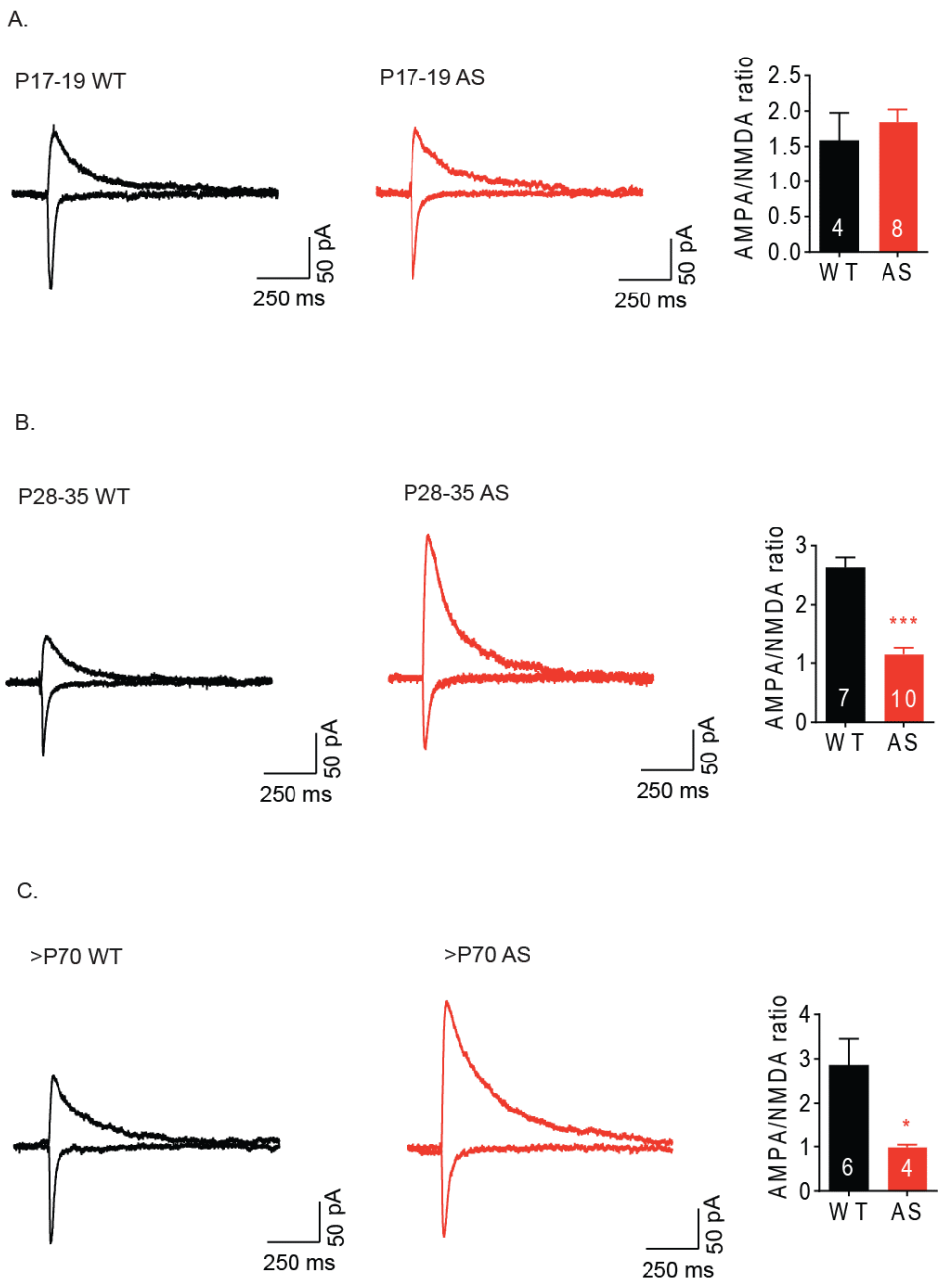

Figure 3.4. The AMPA/NMDA Ratio Alteration in the PFC L5 Corticostriatal Pyramidal Neurons. The AMPA/NMDA ratio did not demonstrate change in AS mice at P17-19 (A, n=4 (WT) or n=8 (AS), $p > 0.05$, t test), but decreased significantly in AS mice at P28-35 (B, n=7 (WT) or n=10 (AS), $p < 0.001$, t test) and >P70 (C, n=6 (WT) or n=4 (AS), $p < 0.05$, t test).

3.4 Discussion

Our results of short-term plasticity and the AMPA/NMDA ratio in the PFC slice of AS mice demonstrate different characteristics from other brain regions ever studied. Firstly, the PPF test shows the second eEPSCs at 50 ms interval is facilitated in both AS and WT at P17-19 with different rates, which is significantly higher in WT; at P28-35, the second eEPSCs at 100 ms interval is facilitated in WT mice but depressed in AS ones; No difference can be found between WT and AS mice aged >P70. This data show that the synaptic release probability (Pr) increases in AS mice at P17-19 and P28-35 but returns to similar level of age-matched WT mice in >P70 group. Secondly, the HFD test shows the replenishment rate of the RRV pool is deficient in AS mice at the three ages detected. Finally, the AMPA/NMDA ratio reduced drastically in AS mice after P28, while it keeps similarly between AS and WT mice at P17-19. This is the first report about the short-term plasticity and the AMPA/NMDA ratio deficits with both brain-region specific and age-dependent features in the PFC of AS model mice at three representative developmental stages: neonatal, adolescent and adult.

Impairment in synaptic transmission including mEPSCs and mIPSCs, long-term plasticity such as LTP and LTD, is a common phenomenon in the hippocampus, the visual cortex and the striatum in AS model mice (Jiang et al., 1998)(Yashiro et al., 2009)(Greer et al., 2010)(Hayrapetyan et al., 2014)(Pignatelli et al., 2014)(Cheron et al., 2014). The deficiency of both mEPSCs and mIPSCs are also shown in the PFC L5 I pyramidal neurons of AS model mice (Refer to chapter 1). For synaptic transmission deficiency, the reduction of frequency indicates the possibility of presynaptic release probability decrement (Fatt and Katz, 1952)(Cummings et al., 1996). Our LSPS results also demonstrate that the inputs of L5 pyramidal neurons from L2/3 decreased (Refer to chapter 1). However, a previous study illustrate that the neurons in AS mice may have higher release probability than those of WT, because the changed intrinsic properties including depolarized resting potential, threshold potential and longer AIS due to the elevated expressions of NaV1.6 and Na/K ATPase make the neuron hyper-excitable (Kaphzan et al.,

2011). Our PPF data also show that the L2/3 neurons in PFC of AS mice are less facilitated (P17-19) or depressed (P28-35) compared to those of WT mice, suggesting that the neurons of AS mice have higher release probability (Debanne et al., 1996). This discrepancy forces us to test other reasons contributing to the presynaptic release deficiency.

The presynaptic release probability is affected by RRV pool (Stevens and Williams, 2007), another important factor which determines both neurotransmitter vesicle release process and transmission strength (Südhof, 2004)(Rosenmund and Stevens, 1996) and may be affected in AS mice by several causes. First, the neurotransmitter release and vesicle number docking can be potentiated by BDNF (Tyler and Pozzo-Miller, 2001), while the BDNF signaling is impaired in AS mice which may influence the quantal release efficiency in addition to signal transductions including PI-3K-Akt and CaMKII (Cao et al., 2013). Second, the reduction of presynaptic inputs may attribute in part to the reduction of synaptic vesicle density and abnormal accumulation of CCV, which result in the disruption of neurotransmitter release (Su et al., 2011)(Wallace et al., 2012)(Judson et al., 2016). This reduction of vesicle density and aberrant accumulation indicate the disturbance in vesicle cycle process (Südhof, 2004) which consumes most ATP demanded by the synapse (Rangaraju et al., 2014). The ATP is synthesized by both glycolysis and oxidative phosphorylation in the mitochondrion (Alberts, 2008b), whereas the function of latter is disrupted in AS mice (Su et al., 2011). The deficit of vesicular release may make a considerable proportion of release invalid leading to the reduced synaptic transmission. This possibility is also supported by our HFD data. The HFD data show that the L2/3 PFC neurons of AS mice illustrate deficiency in RRV pool replenishment when a high frequency stimulation of 10 Hz which does not exceed the physiological stimulus (Wang, 1999) is administered. We also examined other components involving the vesicular release process including cell division control protein 42 homolog (cdc42) and RIM1α/2α. The cdc42 controls the exocytosis by increasing membrane tension (Bretou et al., 2014). RIM1α/2α are key presynaptic proteins regulating synaptic vesicular release through interacting with both Rab3 and Munc13-1 (Wang et al., 1997)(Schoch et al., 2002), and they may have broader effects in regulating synaptic release in sense of stabilizing RRV and modulating

calcium channel localization via its binding proteins as revealed by recent studies (Müller et al., 2015) (Grauel et al., 2016). Western Blot data demonstrate that cdc42 expression level decreased significantly in the PFC of AS mice aged P28-35 (Refer to chapter 4). However, RIM1α/2α expression levels did not change in AS mice aged P28-35 (Refer to chapter 4). Taken into account the complexity of presynaptic components regulating vesicle release and their interactions, further investigations on disruption of other presynaptic proteins in AS mice is needed.

The AMPAR and NMDAR mediate fast or long-lasting currents respectively. Both receptors are regulated by myriad factors through extremely complicated mechanisms in the neuron (Rao and Finkbeiner, 2007). First, the postsynaptic receptor numbers of both AMPAR and NMDAR can be modulated proportionately by long-lasting activity alteration (Watt et al., 2000) or the CaMKIIα activity (Colbran and Brown, 2004). The AMPAR membrane-expression is regulated by Arc, PSD-95, PI-3K and Na/K ATPase et al (Shepherd et al., 2006)(Béïque et al., 2006)(Man et al., 2003)(Zhang et al., 2009). The NMDAR activation is regulated by SK, PKA, PKC and Src et al (Ngo-Anh et al., 2005) (Dongen, 2009). Second, The AMPAR and NMDAR coordinate by regulating each other reciprocally, and that the NMDAR can only be activated after the blocking magnesium is expelled by depolarization induced by AMPAR activation (Kandel, 2012j), while AMPAR's redistribution and cycling at synapse is regulated by NMDAR activation (Shi et al., 1999)(Lu et al., 2001) (Brown et al., 2005). Third, AMPAR and NMDAR expression demonstrate development-dependent pattern, in sense of absence of AMPAR at early stage (Isaac et al., 1995) and the NMDAR subunits NR2B replacement by NR2A with development (Cull-Candy et al., 2001).

With different roles in the excitatory synapses plasticity, i.e. the NMDAR signaling promotes more synapse formation (Dalva et al., 2000) and AMPAR signaling stabilizes new synapses (McKinney et al., 1999), the NMDAR and AMPAR coordinate in the synaptic scaling (Turrigiano et al., 1998). The expression levels of NMDAR and AMPAR are proportionately scaled so that a

fixed AMPA/NMDA ratio is achieved to help preserving transmission information (Watt et al., 2000). The AMPA/NMDA ratio which regulates synapse time course and current summation is synapse-specific (Watt et al., 2000). Little is known about the AMPA/NMDA ratio change in AS mice, except that in the hippocampus where the ratio was found decreased in P15-18 mice (Greer et al., 2010) while kept unchanged in postnatal 10-16 weeks animals (Kaphzan et al., 2012). CaMKII malfunction due to abnormal phosphorylation increase of inhibitory form and SK elevated expression in membrane have been found in AS mice (Weeber et al., 2003) (Sun et al., 2015b), both attenuate NMDAR activation. However, it is still not possible to predict the change of AMPA/NMDA ratio in AS mice because regulating factors alterations potentiated or attenuated the AMPAR have been reported. For example, Arc which increases AMPAR internalization increased (Greer et al., 2010) and PI-3K which promotes AMPAR insertion into membrane impaired in AS mice (Cao et al., 2013), whereas Na/K ATPase which enhances AMPAR membrane-expression elevated (Kaphzan et al., 2011), thus the AMPAR change depends on the outcome of the war-of-tug of these antagonistic regulators. Our findings of the decrement of AMPA/NMDA ratio in the PFC of AS mice only after P28 is exactly opposite to previous reports in the hippocampus (Greer et al., 2010)(Kaphzan et al., 2012). Further study awaits to explain the phenomenon although, there are some possibilities that caused it. First, the PFC expresses higher level of NMDAR mRNA than other brain regions so the reduction of NMDAR activation might be compensated in part by the receptor number increase (Kosinski et al., 1998). Second, the maturation process of NMDAR in the cortex does not complete till P28 (Reyes and Sakmann, 1999) and subunits NR2B replacement with NR2A switch occurs around P12 in the cortex whereas NR2A is absent in the hippocampus (Monyer et al., 1994). It is possible that these subunits have different sensitivities to the E6-AP deficiency insult. Third, the AMPARs and NMDARs are found to have different distribution in the neocortical neurons, and that NMDARs cluster near the soma and proximal dendrite while the AMPARs concentrate on the distal dendrite (Dodt et al., 1998). The basal dendrite morphology keeps normal on the PFC L5 corticostriatal pyramidal neurons in AS mice at P17-19 but decrease dramatically after P28 as revealed by our biocytin-avidin stain (Refer chapter 4). This may give rise to receptor, especially AMPAR, number

decrement in AS mice after P28 when the AMPA/NMDA ratio decrease emerges. Finally, it is possible that the silent excitatory synapses, which contain NMDAR but no AMPAR, increase on the PFC L5 corticostriatal pyramidal neurons due to E6-AP deficiency in the AS mice (Kerchner and Nicoll, 2008), whereas the activation of the silent synapse by activity-dependent AMPAR recruitment (Liao et al., 2001) is impaired in AS mice for the elevated expression of Arc which promotes AMPAR internalization (Greer et al., 2010). Thus this disproportionate expression of NMDAR and AMPAR may contribute to the decrease of AMPA/NMDA ratio.

It is worth noting that the method used in our short-term synaptic plasticity study, i.e. the recordings of eEPSCs induced by extracellular stimulation, has the limitation in identifying the type and location of presynaptic neurons excited. In addition, the possibilities of the neuromodulatory fibers excitation and the involvement of polysynaptic network could not be excluded (Hempel et al., 2011). Thus further investigation by using dual-patch clamp technique is needed to address the aforementioned questions.

CHAPTER 4

MECHANISMS EXPLORATION

4.1 Introduction

The mechanisms underlying the AS are still elusive although a lot of substrates or interacting proteins candidates and some hypothesis have been proposed (Mabb et al., 2011)(Sell and Margolis, 2015)(Buiting et al., 2016). Among the many potential substrates and interacting proteins of E6-AP, Arc and a RhoA GEF Ephexin5 have drawn the intensive attention in the etiology of AS (Greer et al., 2010)(Cao et al., 2013)(Kühnle et al., 2013)(Mandel-Brehm et al., 2015)(Margolis et al., 2010). The effects of increased Arc and Ephexin5 in the impairment of both structure and function of synapse in AS model mice have been developed into a current prevailing hypothesis: the overactive RhoA and the reduction of membrane-expressed AMPAR caused by accumulation of Ephexin5 and Arc respectively, disrupt spine formation and experience-dependent pruning in AS (Mabb et al., 2011) (Buiting et al., 2016). This hypothesis can explain most morphological and functional deficits such as the decreased dendrites and spine density, the impaired synaptic transmission and plasticity found in AS model mice (Dindot et al., 2008)(Jiang et al., 1998)(Weeber et al., 2003)(Yashiro et al., 2009)(Greer et al., 2010)(Hayrapetyan et al., 2014), also the hypothesis does not exclude roles of other substrates and mechanism possibilities, expecially in a brain region specific and developmental stage dependent way, in the AS (Mabb et al., 2011). Meanwhile some studies have different even contrary findings about the expression level of Arc (Mardirossian et al., 2009) (Pignatelli et al., 2014). This discrepancy suggests that further investigation on other factors except for Arc and Ephexin5 is needed.

Previous reports demonstrated that the substrates and interacting proteins of E6-AP are considerably enormous and complicated (Sell and Margolis, 2015), and the diversity of functions of these candidates and physiological processes they involved indicates that it is possible that

every aspect of neuron including generation, growth, differentiation, migration and connection in neural circuit are influenced by the E6-AP deficiency in AS (Mardirossian et al., 2009)(Cao et al., 2013)(Tonazzini et al., 2016)(Jaworski et al., 2005). Besides, neuron structures including neurites quantity and morphology, spine density and types, synaptic proteins, vesicles and receptors were reported impaired in AS model mice (Jiang et al., 1998)(Dindot et al., 2008)(Miao et al., 2013)(Kim et al., 2016)(Cao et al., 2013)(Wallace et al., 2012)(Judson et al., 2016) (Greer et al., 2010). In addition, most signal transduction pathways such as CaMKII, neuregulin-ErbB4, BDNF-TrkB, MAPK, PI-3K-Akt-mTOR, and Reelin-Dab1 have been revealed abnormal in AS model mice (Weeber et al., 2003)(Kaphzan et al., 2012)(Cao et al., 2013)(Filonova et al., 2014)(Sun et al., 2015a) (Hethorn et al., 2015).

These abnormalities in signal transductions entangle, complicate the situation and bring big challenges for elucidating the possible mechanisms underlying the AS. For example, 1. There are complex interactions between autophagy, UPS, apoptosis and PI-3K-Akt-mTOR pathway (Khaminets et al., 2015)(Korolchuk et al., 2009)(Wang et al., 2013)(Oral et al., 2015)(Zhao et al., 2015). The PI-3K-Akt-mTOR pathway is in the central intersection of several physiological processes (Ikenoue et al., 2009)(Zhang et al., 2014); 2. The Reelin signaling pathway interacts with two other pathways in regulating neuron development: 1). The balance between LKB1-Stk25-GM130 and Reelin-Dab1 signaling pathways determines neuron process polarity (Matsuki et al., 2010) (Huang et al., 2014); and 2). Reelin signaling regulates both cortical development and dendrite growth via PI-3K-Akt-mTOR pathway (Jossin and Goffinet, 2007); and 3. Autophagy disruption (Tang et al., 2014) and caspase-3 increase (Erturk et al., 2014) enhance dendrite arborization development. As the disrupted PI-3K-Akt-mTOR and Reelin pathway antagonize the impaired autophagy and apoptosis in neurite development, other factors or pathways may contribute to the dendrite decrement in the hippocampus, the visual cortex and the cerebellum.

Like synaptic transmission and plasticity, heterogeneity of protein homeostasis changes in different brain regions has also been presented in AS model mice. For example, imbalanced

mTOR signaling is found in the cerebellum but not in the striatum (Sun et al., 2015a). GR expression reduces in the hippocampus but does not change in the cerebral cortex and the cerebellum (Godavarthi et al., 2012). These implicates that the PFC neurons may respond to E6-AP deficienty in AS in a different way from the hippocampal, cerebellar and visual cortical neurons do.

In addition to spatial change, temporal alteration is another important issue needs to be taken into account in biology study. The age dependence has been shown in synaptic transmission strength: the reduction of mIPSCs in the visual cortex showed on P80 AS mice was not found on young ones (P25) (Wallace et al., 2012); whereas both membrane-expressed AMPAR and AMPA/NMDA ratio decreases found in the hippocampus of P15-18 AS mice kept comparable to those of WT mice at postnatal 10-16 weeks (Greer et al., 2010)(Kaphzan et al., 2012). The age-dependent distinction in the brain structure also demonstrated in AS patients postmortem brains, and that the frontal and temporal lobe, gyri have abnormal structures, but the cerebellum is normal in a 3-year-old AS boy (Kyriakides et al., 1992), while the contrary is true for a 21-year-old woman AS patient (Jay et al., 1991). However, all studies for mechanisms exploration were performed on adult mice (at least 2 months old) so far, thus the protein homeostasis change with age in AS mice is not known.

Here we tested the possibility that both the morphology of the PFC L5 pyramidal neurons and protein homeostasis in AS mice may illustrate an age-dependent pattern in response to E6-AP deficiency. Moreover, some components of other pathways such as Rho family small GTPases and presynaptic proteins may also affected in AS model mice. Our data show that morphological defect including basal dendrite decrease is present in the PFC L5 corticostriatal pyramidal neurons of P17-19 and P28-35. The branching pattern is indistinguishable between adult (>P70) AS and WT mice. The cdc42 expression level reduced significantly in the PFC of AS mice at P28-35. These results provide implications for disruption of vesicular release and change of morphological deficits in AS.

4.2 Materials and Methods

4.2.1 Animals and Habituation

As was described in Chapter 1.

4.2.2 Primary Antibodies

Activated (GTP-bound) cdc42 or Rac1 (cdc42 activation assay kit (17-441, Millipore)); Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5174, Cell Signaling Technology); Polyclonal rabbit antibody 1 against RIM1α (140003, Synaptic Systems (SYSY)); Synapsin-1 (D12G5) XP® Rabbit mAb (5297, Cell Signaling Technology); Rabbit anti-Glutamate receptor 1 Antibody (GluR1)(AB1504, Millipore); Mouse anti-NMDAR1 Antibody (NR1)(MAB1586, Millipore); Mouse anti-NR2B Antibody, clone BWJHL (05-920, Millipore); Mouse anti-PSD-95 (AB9708, Millipore); Rabbit anti-Homer1 Antibody (ABN37, Millipore); Rabbit anti-Bassoon (D63B6) Rabbit mAb (6897, Cell Signaling Technology); p62, Atg5, Atg7, Atg12, Atg16L1, LC3A/B (Rabbit IgG) (Autophagy Antibody Sampler Kit (4445, Cell Signaling Technology)); Caspase-3 Antibody (9662, Cell Signaling Technology); mTOR (mTOR Substrates Antibody Sampler Kit (9862, Cell Signaling Technology)); Cofilin (Cofilin Activation Antibody Sampler Kit (8354, Cell Signaling Technology)); Purified Mouse anti-GM130 Clone 35 (610822, BD Biosciences); NCAM (CD56) (123C3) Mouse mAb (3576, Cell Signaling Technology); Rabbit anti-Growth Associated Protein 43 Antibody (GAP-43) (AB9854, Millipore).

Antibodies were diluted between 1:1,000 and 1:10,000. Bands of interested proteins were quantified by using Image J.

4.2.3 Neuron Soma Size Measurement and Comparison

The PFC L5 pyramidal neuron soma sizes were measured on live brain slices from P17-19, P28-35 and >P70 animals for the patch clamp recodings experiments.

4.2.4 Neuron Dendritic Structure Analysis

The morphology of the PFC L5 pyramidal neurons, mainly the apical and basal dendrites, was examined by using both Golgi impregnation and biocytin-filling during brain slice recordings. The Golgi impregnation was performed following the protocol of the assay kit (FD Rapid GolgiStain™ Kit (large) (PK401, FD NeuroTechnologies, Inc.)). Mouse brains were dissected coronally with a thickness of 250 μm before examined under light microscope and neurons were reconstructed by using Neurolucida. For biocytin-filling method, neurons which were recorded in whole-cell patch clamp mode were filled with 0.2% biocytin administered into electrode internal solution during recording. These neurons were revealed with fluorescence conjugated strepavidin staining and reconstructed in Neurolucida. Reconstructed neurons morphology was analyzed with Sholl analysis by using Neurolucida exploror. Morphological changes with developmental stages (P17-19, P28-35 and >P70) were investigated by comparing neurons' structures of AS and WT at the different ages. In Golgi staining test, striatal neurons and hippocampal granule neurons were also analyzed to detect the effect of E6-AP dificiency on different types of neurons.

4.2.5 Western Blot (WB) Analysis

4.2.5.1 The Protein Samples Preparation

Brain tissue including the PFC and the hippocampus were isolated and underwent different treatments for either entire tissue lysate preparation or subcellular components (synaotosome and cytosolic component) separation by using Syn-PER™ Synaptic Protein Extraction Reagent

(87793, Thermo Scientific). Entire tissue lysate was prepared as reported previously (Qiu et al., 2014). Briefly, brain tissues were homogenized in ice-cold NP40 cell lysis buffer (FNN0021, Life Technologies) containing 1 mM phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma-Aldrich) and 1:50 protease inhibitor cocktail (P8340, Sigma-Aldrich) with ultrasonic liquid processor (Q125 sonicator, Qsonica, LLC.). For subcellular compartmentalization, brain tissue was treated following the manufacture's protocol, then synaptosomal and cytosolic fractions were administered 5% dimethyl sulfoxide (DMSO, D8418, Sigma-Aldrich). Protein samples were used for WB analysis immediately or aliquoted and kept at -80℃ for later use.

4.2.5.2 WB

Western Blot analysis was performed as previously reported (Qiu et al., 2014). In brief, equal loading amount of sample total proteins were achieved by using Thermo Scientific™ Pierce™ Micro BCA™ Protein Assay (PI23235, Thermo Fisher Scientific). Protein samples were separated on 8% Sodium dodecyl sulfate (SDS)-polyacrylamide gels (SDS-PAGE, 30% Acrylamide/Bis Solution (1610156, BIO-RAD)), transferred to Immobilon-P membrane (PVDF, IPVH00010, EMD Millipore), blocked in blocking buffer (5% fat-free dry milk in PBST (PBS+0.05% Tween-20 (P1379, Sigma-Aldrich))) at RT for 1 h, incubated with primary antibodies diluted in blocking buffer at 4 °C overnight, washed with PBST to remove unbound primary antibodies before incubated with Horseradish Peroxidase (HRP)-Conjugated polyclonal secondary antibodies (W4011, Goat anti-Rabbit IgG (H+L), HRP Conjugate; W4021, Goat anti-Mouse IgG (H+L), Promega) at RT for 2 h. Signals were detected by using enhanced chemiluminescence (ECL) method (Amersham ECL Prime Western Blotting Detection Reagent, RPN2232, GE Healthcare Life Sciences). The protein bands were analyzed by using Epson Perfection desktop scanner and Image J. Target proteins expression levels will be quantified by comparing band intensity with that of the internal control GAPDH (cytosolic component or total protein) or Homer1 (synaptosomal fraction) levels.

4.2.7 Data Analysis

Dendrite arborization will be reconstructed by using Neurolucida and data will be analyzed with Sholl analysis..

For WB, data were collected from at least 3 independent experiments. Protein band was quantified and analyzed with Image J.

Data were shown as mean ± S.E.M. Either One-way ANOVA or t test was used, and significance will be assessed at $p < 0.05$.

4.3 Results

4.3.1 The Morphological Changes

4.3.1.1 The Soma Size Change of the PFC L5 Pyramidal Neurons

Most of the PFC L5 pyramidal neurons in AS mice were smaller than those of WT littermates on freshly prepared brain slices from 3 age groups (Figure 4.1). The majority of these L5 cells in AS had much smaller soma sizes than those from WT at P17-19 (diameter (unit μm), WT=14.586± 0.201; AS 12.275 ± 0.772. n=3. 10 cells/mouse, 3 mice, $p < 0.01$, t test.), P28-35 and >P70 (data not shown).

The underdeveloped neuron soma in AS was confirmed by using Thy1-EGFP labeling also (data not shown).

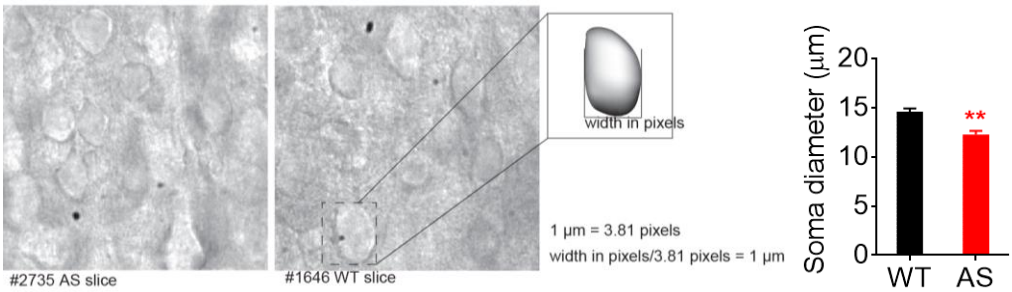

Figure 4.1. The Reduction of the Soma Size of the PFC L5 Pyramidal Neurons in AS Mice. The PFC L5 pyramidal neurons soma size is smaller than those of WT mice at P17-19 (n=30, $p < 0.01$, t test).

4.3.1.2.1 The Dendritic Structures Change-the Results of Biocytin-filling Method

The PFC L5 corticostriatal pyramidal neurons double-labeled by retrograde beads and biocytin (Figure 2.1) were used for morphology analysis. The reconstructed structure was analyzed by using sholl analysis and demonstrated abnormalities in a age-dependent manner. Basal dendritic branching pattern of neurons from AS was similar like those of neurons from WT mice at P17-19 (Figure 4.2A, n=6 mice, 5 cells/mouse, $p > 0.05$, t test) and >P70 (Figure 4.2C, n=4 mice, 2 cells/mouse, $p > 0.05$, t test), then showed decreased basal dendrites in contrast to those corresponding parts of neurons from WT mice after P28 (Figure 4.2B, n=3 mice, 4 cells/mouse, * $p < 0.05$; ** $p < 0.01$, t test).

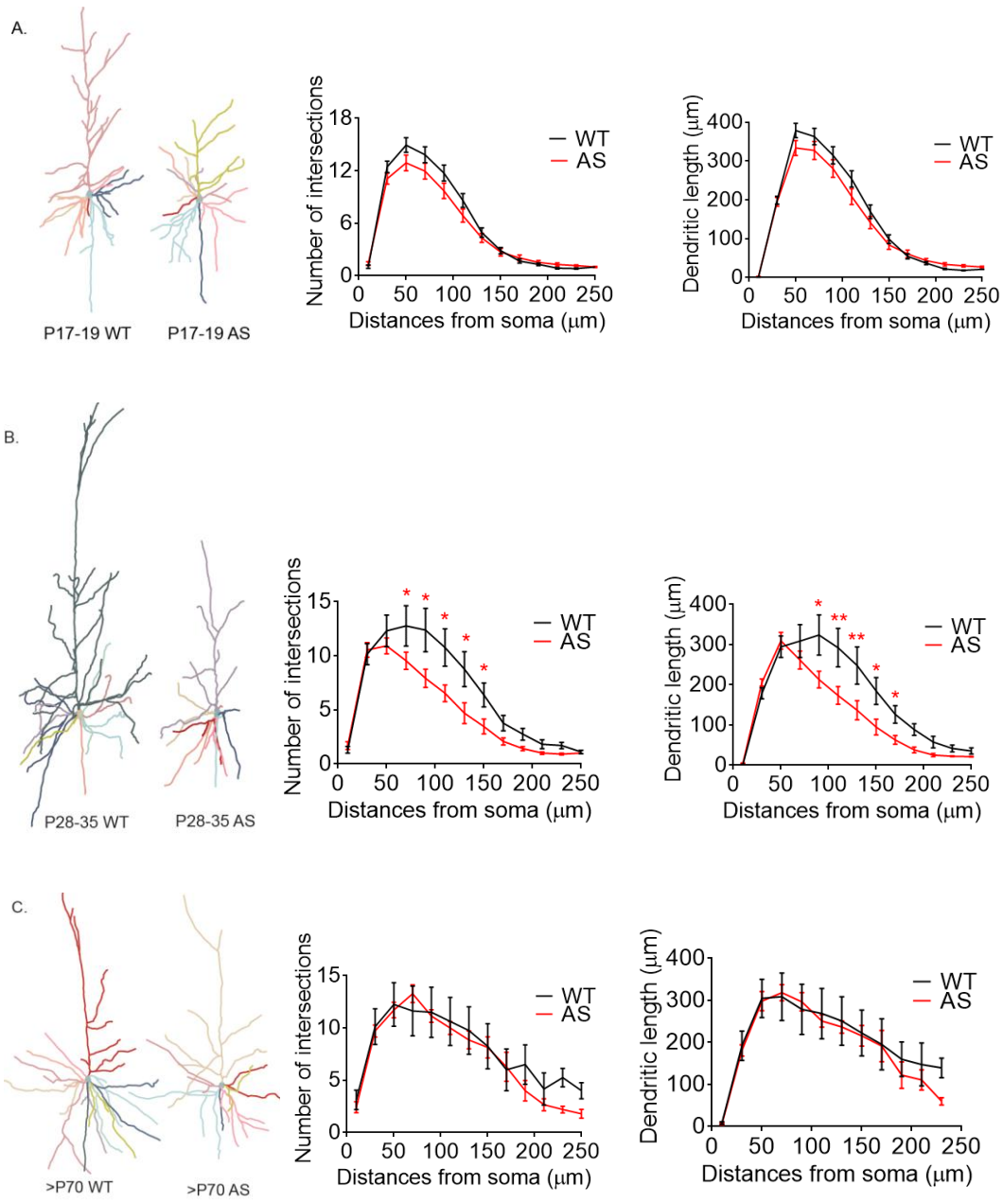

Figure 4.2. The Dendritic Structure Deficits of the PFC L5 Corticostriatal Pyramidal Neurons in AS Mice Revealed by Biocytin Stain. The PFC L5 corticostriatal pyramidal neurons in AS mice showed similar basal dendritic branching like those of WT at P17-19 (A, n=30, $p > 0.05$, t test) and >P70 (C, n=8, $p > 0.05$, t test), whereas had less basal dendritic branches compared to those of WT mice at P28-35 (B, n=12, * $p < 0.05$; ** $p < 0.01$, t test).

4.3.1.2.2 The Dendritic Structures Change-the Results of Golgi Impregnation

Sholl analysis of the PFC L5 pyramidal neurons on the brain slices stained by Golgi impregnation method revealed that basal dendrite arborization increased significantly in AS mice at P28-35 (Figure 4.3B, n=3 mice, 10 cells/mouse, 10 cells/mouse, * $p < 0.05$, ** $p < 0.01$, t test), in contrast to the decrease found in neurons in the hippocampus and the cerebellum reported previously (Dindot et al., 2008)(Miao et al., 2013). This change demonstrated in both an age-dependent and a cell-type specific ways: the basal dendrite arborization was found significant reduced in the PFC L5 pyramidal neurons of AS mice at P17-19 (Figure 4.3A, n=3 mice, $p < 0.05$, t test), but no difference between two genotypes was revealed at >P70 (Figure 4.3C, n=3 mice, 10 cells/mouse, $p > 0.05$, t test). No significant change of the basal dendrite was found on the hippocampal granule neurons from both P17-19 and P28-35 animals (data not shown).

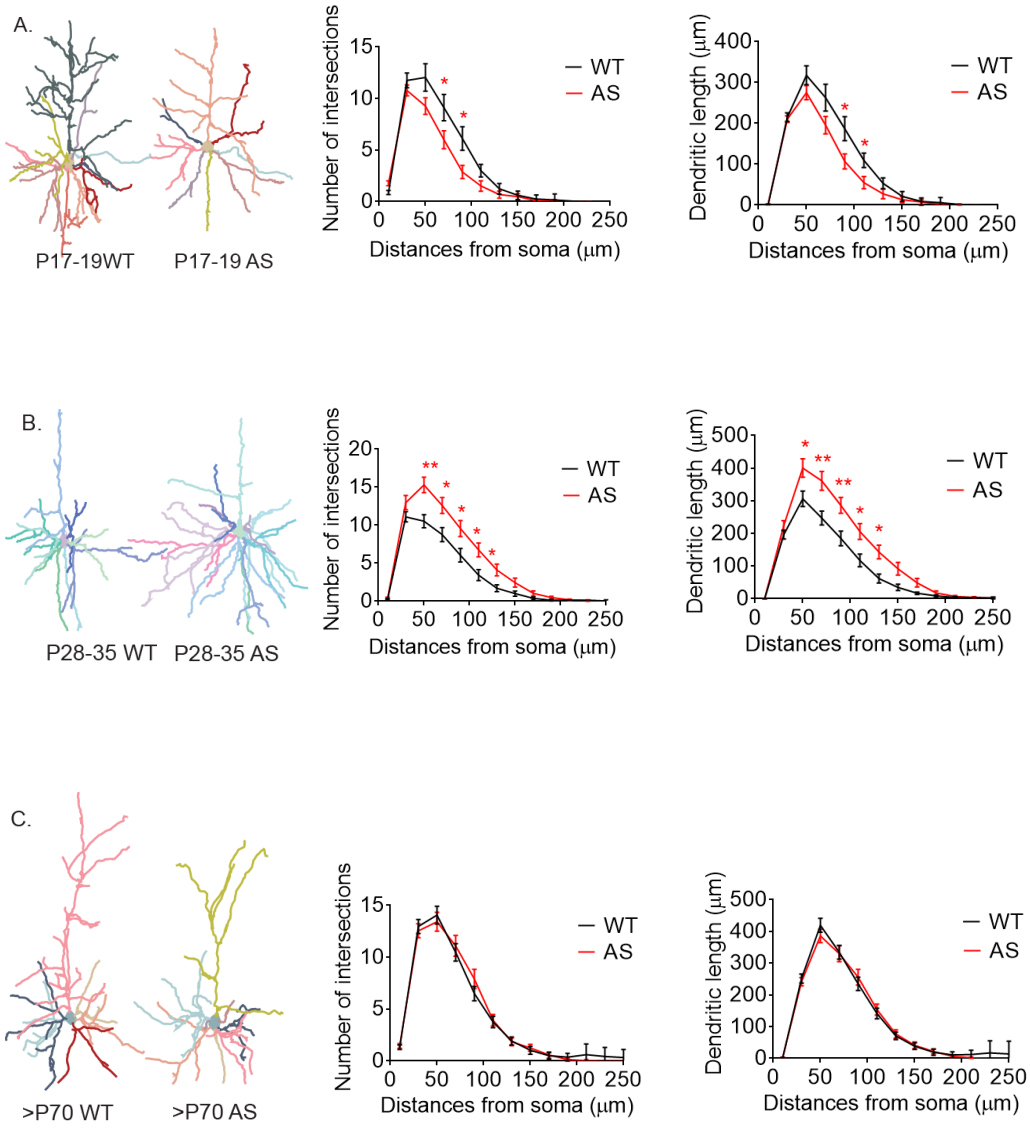

Figure 4.3. The Abnormalities of the PFC L5 Pyramidal Neurons Dendritic Structure in AS Mice (Golgi Stain). The neurons of AS mice have less basal dendrites at P17-19 (A, n=30, $p > 0.05$, t test) but exuberant basal dendrites at P28-35 (B, n=30, * $p < 0.05$; ** $p < 0.01$, t test) compared to those of age-matched WT littermates. No morphological difference in dendrites between AS and WT mice was observed at >P70 (C, n=30, $p > 0.05$, t test).

4.3.2 Results of WB

4.3.2.1 The Expression Levels of Cytoskeleton Regulating Components

Some key regulators of cytoskeleton including two Rho family small GTPases, Rac1 and cdc42 were detected. The cdc42 expression level decreased significantly in the PFC of AS at P28-35 (Figure 4.4A, n=3, $p < 0.05$, t test), but did not change in the PFC and the hippocampus (HIP) of AS at P17-19 or in the HIP at P28-35 (Figure 4.4A, n=3, $p > 0.05$, t test). No change of Rac1 expression level was observed in the PFC and the HIP of AS at P17-19 and P28-35 (Figure 4.4B, n=3, $p > 0.05$, t test).

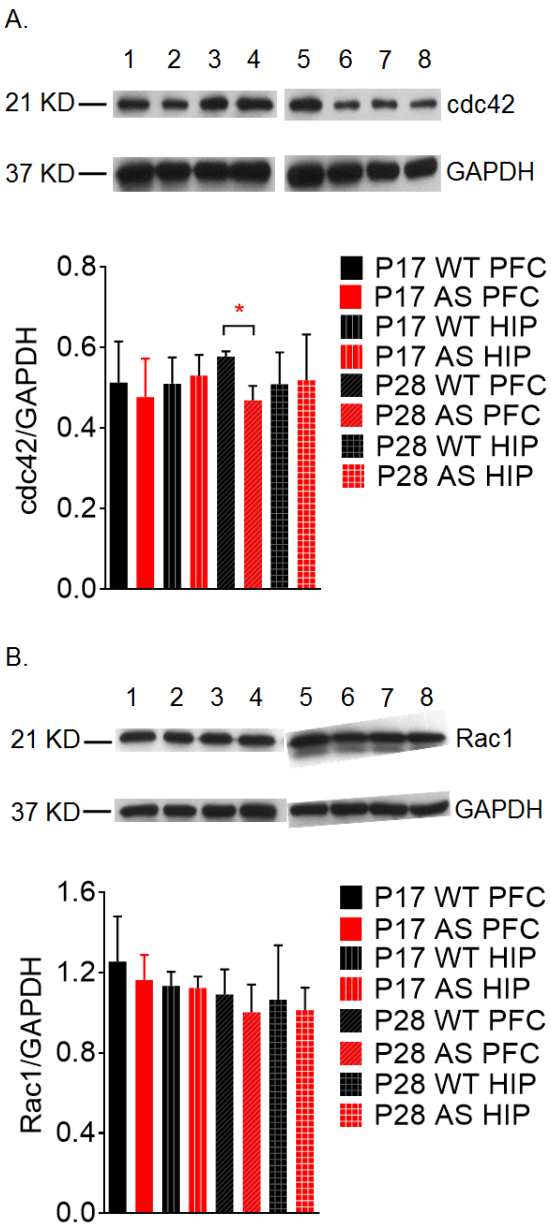

Figure 4.4 The Cdc42 and Rac1 Expression Levels in the PFC and the Hippocampus (HIP). The cdc42 (A, n=3, $p > 0.05$, t test) and Rac1 (B, n=3, $p > 0.05$, t test) expression levels were similar in both brain regions between AS and WT mice at P17-19 and P28-35, except that in the PFC of AS mice at P28-35, the cdc42 expression decreased drastically (A, n=3, $p < 0.05$, t test) (Lanes: 1. P17-19 WT PFC; 2. P17-19 AS PFC; 3. P17-19 WT HIP; 4. P17-19 AS HIP; 5. P28-35 WT PFC; 6. P28-35 AS PFC; 7. P28-35 WT HIP; 8. P28-35 AS HIP).

4.3.3.2 The Expressions of Presynaptic Proteins Related to the Synpatic Vesicular Release

Three presynaptic proteins involving in vesicular release, synapsin Ⅰ and RIM1α/2α, were investigated in synaptosomal fractions of both the PFC and HIP. In both brain areas RIM1α/2α expression levels showed no significant change at P28-35 (Figure 4.5, n=3, $p > 0.05$, test). Synapsin Ⅰ had comparable expression level in the PFC and HIP of AS and WT mice at P28-35 (data not shown).

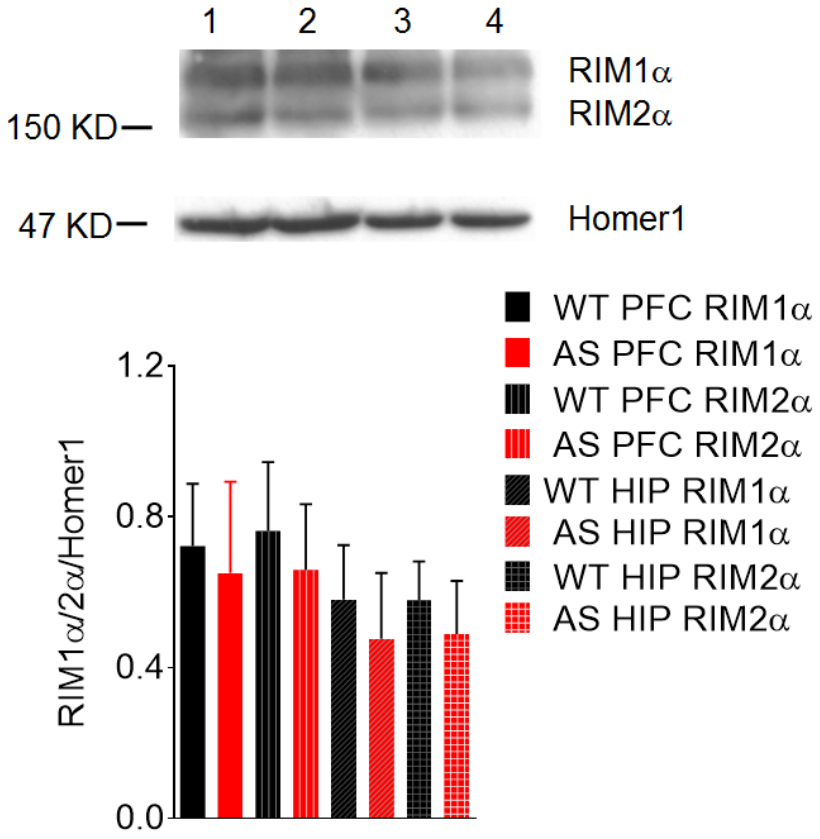

Figure 4.5. The RIM1α/2α Expression Levels in the PFC and the Hippocampus (HIP). RIM1α/2α expression levels were comparable to those of WT mice in both brain areas of AS mice at P28-35 (n=3, $p > 0.05$, t test). (Lanes: 1. WT PFC; 2. AS PFC; 3. WT HIP; 4. AS HIP).

4.3.3.3 The Expressions of Glutamate Receptors and Synaptic Proteins

Some synaptic proteins and receptors including PSD-95, Homer1, Bassoon, GluR1, NR1 and NR2B expression levels in the PFC were examined (Yashiro et al., 2009)(Rial Verde et al., 2006)(Chowdhury et al., 2006)(Greer et al., 2010), and no difference of expression levels between AS and WT was found at P17-19 and P28-35 (data not shown).

4.3.3.4 The Expressions of Other Pathways Components: NCAM, GAP-43, mTOR, Akt, Apoptosis and GM-130

Some proteins of extracellular matrix and other signal transduction pathways such as NCAM (Condon et al., 2013)(Sheng et al., 2015), GAP-43 (Kelly et al., 2010), mTOR (Tang et al., 2014), both the total and active (phosphorylated, Thr^{308} and Ser^{473}) Akt (Cao et al., 2013)(Jossin and Goffinet, 2007), Caspase-3 (uncleaved and cleaved) and GM-130 (Hethorn et al., 2015)(Jossin and Goffinet, 2007) were tested in both the PFC and the hippocampus at P17-19 and P28-35. All these proteins did not illustrate change in expression level in AS (data not shown).

4.4 Discussion

This work constitutes the first study about the mechanisms exploration of AS in the PFC with three developmental stages. We find that soma size of the PFC L5 neuron are smaller in AS mice than that of WT littermates at three ages. The dendritic structures demonstrate divergence in different tests: the PFC L5 pyramidal neurons stained by using Golgi stain show less basal dendrites at P17-19 but more at P28-35 in AS mice compared to those of age-matched WT littermates; however, biocytin-avidin stain shows that the PFC L5 corticostriatal pyramidal neurons have similar basal dendritic branching pattern between two genotypes at P17-19, then neurons of AS mice have less basal dendrites than those of WT mice at P28-35. At adulthood (>P70), neither staining methods reveal difference in morphology of the PFC L5 pyramidal neurons from both AS and WT mice. Furthermore, further investigation shows that cdc42 expression level decreases in the PFC of AS mice at P28-35.

Smaller soma size of the PFC neurons in AS model mice is novel but not surprising. As the microcephaly is a main symptom showing in over 80% of AS patients, may result from the decrease in either cell number or cell size, or both (Williams et al., 2010a). Consistently, reduction in volumes of both white matter and gray matter were also found in AS patients (Peters et al., 2010b) (Aghakhanyan et al., 2016). The neuron growth and survival depend on neurotrophic factors and PI-3K pathway (Kandel, 2012k). In the PFC and the hippocampus the major neurotrophic factor is BDNF (Hofer et al., 1990), and the BDNF-TrkB and PI-3K pathways are disrupted in AS model mice which may attenuate both the growth and survival (Cao et al., 2013). Cell proliferation is suppressed and cell death is enhanced after E6-AP is knocked down in cell lines such as Cos-7, Neuro 2a and Cos-1 (Mishra et al., 2008)(Mishra and Jana, 2008) (Mishra et al., 2009a). In addition, the mTORC1 pathway which promotes metabolism is found impaired in the cerebellum of AS mice (Sun et al., 2015a).These may consist the basis of causes of small cell size (Refer figure 4.6).

The conflicting results about dendritic structure from the Golgi stain and the biocytin-avidin stain are puzzling. One possibility is that neurons revealed in the biocytin-avidin staining method are L5 corticostriatal pyramidal neurons, less heterogenous than those stained by Golgi impregnation method, which has limitations including: 1. It stains limited number of neurons by an unknown mechanism; and 2. The neuron type and projection can not be determined. Another explanation is that substrates and interacting proteins of E6-AP have antagonistic effects on neurite development, and these rivals may win either in different neuron subsets or at different developmental stages. For example, p27 and Ephexin 5, both are E6-AP substrates and elevated in AS, suppresses and enhances RhoA function respectively (Mishra et al., 2009a)(Margolis et al., 2010). So do the E6-AP isoforms I and II/III: the isoform I inhibits whereas the isoforms II/III promotes dendrite development (Valluy et al., 2015).

The decrease in dendrite branching and spine density can be explained by the disturbance in signal transduction pathways, neural activity and synaptic protein expression in AS model mice reported by previous reports. The neurite development is regulated by myriad factors and signaling pathways, these include PI-3K-Akt-mTOR pathway (Jaworski et al., 2005), the mTOR-dependent macroautophagy (aforementioned as autophagy) (Tang et al., 2014), Reelin-Stk25 signaling pathway (Matsuki et al., 2010), apoptosis (Erturk et al., 2014) and neural activity et al. First, the activation of PI-3K-Akt-mTOR pathway is well-known to promote cultured hippocampal neuron growth and dendrite branching (Jaworski et al., 2005), and the activation of PI-3K via BDNF-TrkB pathway is shown impaired in the hippocampus of AS mice as illustrated by the decrease of phosphorylated (active) Akt (Cao et al., 2013). The reduced function of Akt will be expected to decrease dendrite branching and to relieve the inhibition on both TSC2 and Bad protein (Alberts, 2008c). Second, TSC2 is the inhibitor of ras homolog enriched in brain (Rheb), which is a small GTPase and can activate mTOR pathway, TSC2 is also a substrate of E6-AP (Zheng et al., 2008) and thus may be increased in AS. This increase and disinhibition due to Akt attenuation of TSC2 in AS will cause mTOR signaling reduction, which should alleviate the inhibition on the mTOR-dependent autophagy. It is known that neuronal autophagy promotes

spine pruning (Tang et al., 2014). As such, the alteration of mTOR-autophagy pathway will decrease spine density in AS. Third, by interacting with PI-3K-Akt-mTOR pathway, Reelin signaling regulates both dendrite growth and neuronal polarization (Jossin and Goffinet, 2007) (Matsuki et al., 2010). Reelin expression deficiency in AS (Hethorn et al., 2015) will disturb dendrite development and lift the ban on Bad protein activation, the enhanced Bad protein promotion effect on caspase-3 in the PFC may be one of reasons that cause the dendrites reduction (Ohkubo et al., 2007) (Erturk et al., 2014) (Refer figure 4.6 for a broader view of the signaling network). The last but not least, the structure of neural circuits determines functions (Kandel, 2012h), and neural activities and experience refine the circuits, especially during the critical period (Alberts, 2008a)(Shulz and Jacob, 2010)(Kandel, 2012i)(Silbereis et al., 2016). Interestingly, E6-AP expression itself can be regulated by neuronal activity (Greer et al., 2010), so defects of both morphology and function in AS may result from a reciprocal interaction (Mabb et al., 2011). The possibility that reduction of neural activity results in more synapse elimination was supported by the recent observation of spine dynamics *in vivo* in the visual cortex of AS mice: the data showed that the thin and stubby types of spines decreased drastically in AS mice at the P30 (Kim et al., 2016), although the synaptic anchoring protein ankyrinG was reported elevated which may give rise to the increase in spine formation in AS mice (Kaphzan et al., 2011).

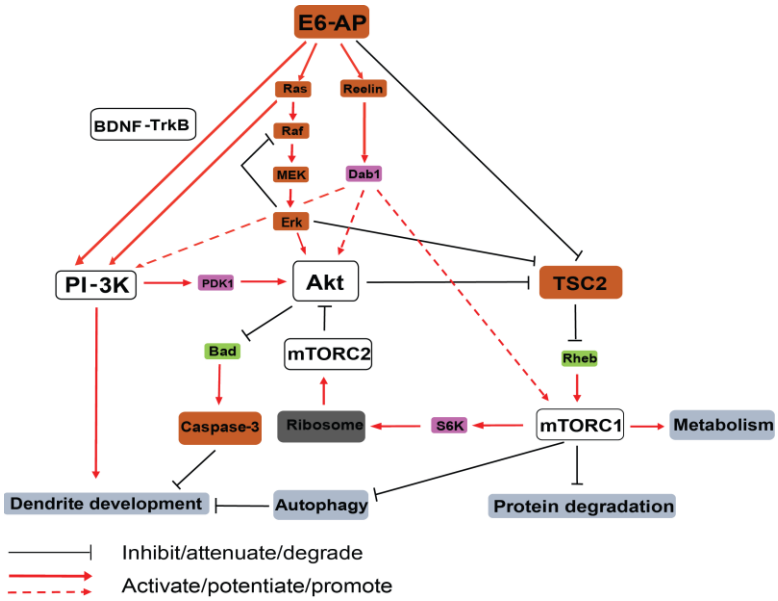

Figure 4.6. The Putative Mechanisms Underlie the Deficiencies of Dendrite and Neuron Soma Development in AS Model Mice. The current information available is simplified in this schematic picture.

Factors interacting with both autophagy and apoptosis in AS may be more complicated than those under physiological conditions as figure 4.6 illustrated. There are several possibilities: 1. E6-AP itself may be among these unknown components potentially participating in the two physiological processes as it has been found that some E3 ligases involved in autophagy (Pandey et al.)(Kuang et al., 2013)(Khaminets et al., 2015), and the proteasomal degradation pathway is enhanced after mTOR inhibition (Zhao et al., 2015), that means the UPS and autophagy interact and coordinate in protein degradation (Wang et al., 2013); and 2. The PI-3K-Akt pathway is also activated by MAPK (Alberts, 2008d), and previous study showed the activity-dependent activation of MAPK is impaired in the hippocampus of AS mice (Filonova et al., 2014). Therefore, autophagy and apoptosis pathways may be interrupted differently in the PFC of AS mice and give rise to the exuberant dendrites of the unidentified population neurons revealed by the Golgi stain.

Comprising both structural and motion machinery of the growth cone, cytoskeleton system plays a key role in regulating neurite development, synaptic transmission, plasticity and release (Kim and Lisman, 1999)(Kandel, 2012l)(Xiao et al., 2016) (Wu et al., 2016). As it has been found that actin is a potential substrate of Dube3a in the fly (Jensen et al., 2013), previous studies have revealed that actin is disturbed in AS (Baudry et al., 2012), which is may be due in part to the expression deficiency of Reelin, an important interacting partner of actin (Chai et al., 2009), in both AS patients and model mice (Hethorn et al., 2015). The cytoskeleton system is regulated by Rho family small GTPases in neuron, with cdc42 promoting dendrite formation (Threadgill et al., 1997). The cdc42 also regulates synaptic release process by influencing vesicle pore enlargement through controlling membrane tension (Bretou et al., 2014). Correspondingly, the disruption of cdc42 expression results in impairment of synaptic plasticity in the PFC (Kim et al., 2014). Conversely, overactive cdc42 gives rise to the increase of dendrites in the PFC (Peng et al., 2016). Our finding of the reduction of cdc42 expression in the PFC of AS at P28-35 may contribute to the defects of synaptic transmission, plasticity and release in the AS mice, although the change at other ages are needed to get a bigger view of the whole situation.

Synaptic vesicular release is a crucial step for neuron communication and regulated by myriad proteins including RIM1α and synapsin I (Kandel, 2012m) (Südhof, 2004). Previous studies have found that AS mice hippocampal neuron terminals have lower synaptic vesicle density (Su et al., 2011), in contrary, aberrant accumulation of CCVs are found in both glutamatergic and GABAergic neurons synaptic terminals in the visual cortex (Wallace et al., 2012) (Judson et al., 2016). The mechanisms underlying these deficits are not understood. Our data show that the expressions of RIM1α/2α and synapsin I do not change in both the PFC and the hippocampus of AS mice at P28-35. Other factors that play a role in the synaptic release deficit (Refer to chapters 2 and 3) and the abnormality of vesicle recycling aforementioned need to be determined.

Expression levels of glutamate receptors including AMPAR and NMDAR are found not changed in PFC of AS mice at P17-19 and P28-35. The AMPA/NMDA ratio reduction (Refer to chapter 3) may results from the abnormality of AMPAR recycling or/and NMDAR activation in AS mice as were reported before (Greer et al., 2010)(Sun et al., 2015b).

CHAPTER 5

CONCLUSIONS AND PROSPECTIVES

5.1 Summary of Main Results

In the present work we test the hypothesis that E6-AP deficiency results in the impairment of both functions and structures in the PFC L5 corcitoctriatal pyramidal neurons of AS model mice, and these abnormalities may demonstrate a developmental stage dependent pattern.

Our data show that synaptic transmission including both mEPSCs and mIPSCs are disturbed in the PFC L5 corticostriatal pyramidal neurons as are indicated by the decrease in frequencies and amplitudes, implicating that both pre- and postsynaptic factors are affected (Fatt and Katz, 1952). The biased reduction level of mIPSCs over mEPSCs demonstrate an equilibrium loss of the excitation and inhibition, as the excitation/inhibition imbalance reduction is found in the visual cortex in the previous study (Wallace et al., 2012), representing the similarity between two brain regions in response to E6-AP absence in AS mice. Moreover, the dynamic alteration of the synaptic transmission at three developmental stages illustrates age-dependent characteristics, in sense of the decrement in the reduction rate with age. On the contrary, neural connectivity of the PFC L5 corticostriatal pyramidal neurons is impaired at early stage as young as P17-19, as is indicated by the reduction of the excitatory inputs from L2/3 and the inhibitory inputs from L5/6, and the impairment is maintained through to the adulthood.

Short-term synaptic plasticity and the AMPA/NMDA ratio results show both presynaptic release and postsynaptic receptors functions are affected in the PFC of AS mice. Both the release probability and RRV pool replenishment of presynaptic neurons of the PFC L5 pyramidal neurons are disturbed from early development stage. The release probability recovers while the RRV pool replenishment keeps disrupted at adulthood (>P70). In contrast to the RRV pool

replenishment, the AMPA/NMDA ratio does not demonstrate abnormality until P28-35 in the PFC L5 corticostriatal pyramidal neurons of AS mice, when it decreases considerably compared to that of age-matched WT littermates.

The mechanisms exploration reveals that the dendritic branching of the PFC L5 corticostriatal pyramidal neurons decreases in AS mice at P28-35, but no difference between AS and WT can be found at other ages. However, unidentified PFC neuron subsets stained by Golgi impregnation method show reduced dendritic branching in AS mice at P17-19, increased branching at P28-35. These abnormality of branching pattern is indistinguishable between AS and WT at adulthood. Interestingly, the PFC neurons of AS mice have smaller soma size compared to those of WT ones, and this may be one of reason caused microcephaly found in over 80% of AS patients (Williams et al., 2010a). Further investigation reveals that the expression levels of cdc42 decreased significantly in the PFC of AS mice at P28-35, which may contribute to the deficits of synaptic transmission, plasticity and vesicular release (Su et al., 2011)(Wallace et al., 2012)(Judson et al., 2016).

5.2 Contributions of Current Work to the Field

This study has several innovations. Firstly, we investigated both structural and functional abnormalities of the PFC L5 corticostriatal pyramidal neurons in AS mice. The PFC represents one of brain areas unexplored so far. Secondly, we tested the neural connectivity deficit at circuit level for the first time by using LSPS technology. Thirdly, we detected and compared the defect resulted from E6-AP deficiency systematically at three representative developmental stages: neonatal, adolescent and adult. Fourthly, we found the AMPA/NMDA ratio decreased in AS mice after adolescence (P28-35). Finally, we reported for the first time that presynaptic release process is disrupted in AS mice as is showed with the increased release probability and impaired RRV pool replenishment.

5.3 Remarks of the Study

The role of experience-dependent synaptic plasticity in neurodevelopmental disorders has long been proposed and gradually accepted (Zoghbi, 2003)(Kubota et al., 2015)(Wei et al., 2015).The efficacy of the epigenomics in treating neurodevelopmental disorders is also supported by the success in treating the depression by using the deep brain stimulation (DBS) or the transcranial magnetic stimulation (TMS) in patients and the optogenetics stimulation on the PFC L5 pyramidal neurons in animal models (Kumar et al., 2013a). In support to this, it has been reported that the anxiety-like behavior in mice can be reversed by the optogenetics stimulation of Thy-1 neurons in the PFC (Kumar et al., 2013b). The striking finding in the V1 that the critical period can be manipulated by regulating even a single type of neurons such as GABAergic neurons (Krishnan et al., 2015), makes the epigenomics more attractive and promising for practical use. The epigenomics have not been explored in AS up to the present date (LaSalle et al., 2015). However, previous research works have demonstrated that the environmental influences can change physiological processes in body including the CNS. For example, the GR expression level in the cortex of AS mice can be regulated by maternal status: AS pups raised by carrier (*Ube3a* m+/p-) mothers have higher GR expression level than that of littermates raised by affected (AS) mothers (Grier et al., 2015a). Another study finds that the long term environmental enrichment (EE) housing (4 months) can ameliorate many behavioral deficits of AS mice as measured by novel objective recognition, light/dark box and rotarods tests (Jamal et al., 2016). These results provide hope for the epigenemics interventions to treat AS.

However, the distinctive responses of different subsets PFC neurons to single external stimulus demand pinpointing the altered neurons and the changes of their properties such as the intrinsic properties, synaptic transmission and short-term synaptic plasticity in AS in both projecting-pathway specific and age-dependent way. The revelation of specific type of neurons and their properties changes will benefit the searching for the epigenetomics therapeutics or interventions in AS patients after birth or after diagnosis.

5.4 Prospectives and Potential Future Directions

5.4.1 The Roles of the Cdc42 and the Cytoskeleton System Components in AS

The functions and changes of the cdc42, its various modified types and microtubule (Stankiewicz and Linseman, 2014)(Roberts et al., 2008)(Xiao et al., 2016) in the AS deserve further investigation. There are many questions are awaiting for answers, for example, is cdc42 the novel substrate of E6-AP or the target of E6-AP substrates? When and how its expression level changes in multiple brain areas of AS mice? What are other consequences except for vesicle release disruption?

5.4.2 The Immune System Components Changes and the Effects in AS

It has been reported that the protein sialylation reduced due to the less acidified PH in the Golgi apparatus (GA) in maternal *Ube3a* knockout neurons (Condon et al., 2013). And it is also well known that the normal mammal cells are coated with specific glycoproteins, especially sialic acid, whereas low or lacking expression level of sialylated protein is a property of pathogens which can be recognized and eliminated by the immune system (Charles A. Janeway Jr, 2007)(Alberts, 2008e). Other membrane glycoproteins changes need to be determined because the substrates of E6-AP remain unknown (Clayton-Smith and Laan, 2003b). The change of cell surface glycoproteins will result in antigen epitope alteration which will exert influences on the immune system in two different ways:

In the first place, autoimmune response involving complement system may be an important factor affecting the neuron viability except other reasons mentioned in the Chapter 1 ‘Putative mechanisms underlying AS pathology’ section. As there is no report about the scanty of sialic acid on neurons in AS and the immune system, except that only one observation about the alteration of the immune system in AS, in that report, researchers found that the natural killer

(NK) cell quantity decreased and immunoglobulin E (IgE) expression level increased (Grzmil et al., 2009), the pressure from the immune response on neurons survival in AS needs to be determined.

On another hand, the immune system, specifically the CNS-specific T lymphocytes and microglia, has been reported regulating neurite pruning (Paolicelli et al., 2011) and neurogenesis including both neuron proliferation and differentiation in adulthood and early postnatal period (Ziv et al., 2006)(Shigemoto-Mogami et al., 2014), while the antigenic specificity is required for the protective effect of T cells under some conditions such as trauma and degeneration (Moalem et al., 1999)(Yoles et al., 2001), so this raises the possibility that the antigen presentation of microglia is impaired so that the immunological responses and regulations to the sialytated cell surface marker proteins, and maybe various other ones, on neurons in AS are abnormal or attenuated, and this issue deserves an intensive investigation either.

The last but not the least, the sialytated proteins on neuron membrane perform functions in the cell-recognition and interaction (Kandel, 2012n). So it also raises the possibility that the down-regulated sialytic acid expression level of neurons in AS may give rise to the defects in the neural circuits formation which depends on the robust cell-recognition and synapse formation between neurons.

5.4.3 Glia and the *UBE3A*/*Ube3a Gene Dosage*

The influences of E6-AP deficiency on glial cells (glia) has not been investigated (El Hokayem and Nawaz, 2014). Glia plays an important role in synapse development via communication with neurons. For example, glia secrets the complex of cholesterol-apolipoprotein E–containing lipoproteins and thrombospondin to promote synapse development (Mauch et al., 2001)(Christopherson et al., 2005), enhances synaptic transmission efficacy by increasing membrane-expressed AMPAR via the secretion of tumor necrosis factor α (TNFα) (Beattie et al.,

2002). Glia also increases the postsynaptic efficacy by secreting ATP (Gordon et al., 2005). The astrocytes have been reported recently to communicate with neurons via calcium signaling (Ma et al., 2016).

The *UBE3A*/*Ube3a* gene imprinting does not occur in glia though, the dosage is crucial for the nervous system development as are implicated by AS or autism caused by overexpression of *UBE3A* (Cook et al., 1997)(Smith et al., 2011). Further study also shows that *Dube3a* is not imprinted but the haploinsufficiency also causes symptoms in the fly (Hope et al., 2016). The facts that E6-AP expression itself can be regulated by neuronal activity (Greer et al., 2010) and E6-AP is the substrate of itself, indicate that the expression of E6-AP must be under strict control. Whether the gene dosage and E6-AP quantity influence the glia function or not is still an open question.

5.4.4 The E6-AP Functions beyond the UPS

What are else functions of E6-AP in the CNS other than E3 ligase? The meaning of ubiquitin message depends on both the number of ubiquitin molecules linked with the target proteins and the way in which they are concatenated together (Chau et al., 1989). For example, monoubiquitination means histone regulation while the monoubiquitylation has not been investigated in AS (Buiting et al., 2016). Polyubiquitination via lysine 63 instead of 48 represents a signal for DNA repair (Alberts, 2008f). Although the coactivator function in transcription of the N terminus of E6-AP remains intact in some AS patients (Lalande and Calciano, 2007), as some of the substrates interacting with E6-AP involve in DNA expression, replication and repair: for example, MCM7 participates in DNA replication; MeCP2 is related to histone methylation; UBE2D1 and UBE2L3 regulate p53, c-Fos and NF-κB; UBE2D1 interacts with BRCA1 which regulates DNA repair, gene activity and embryo development, the possibility that E6-AP may play a critical role in regulation of DNA replication, DNA repair and transcription as well as protein degradation cannot be eliminated (Lonard and O'Malley, 2006). Thus abnormalities in AS may be

attributed to deficient DNA duplication process caused by E6-AP dysfunction (Buiting et al., 2016). This possibility has not been investigated so far.

5.5 Conclusions

The present work supports the hypothesis that E6-AP deficiency impairs both structural and functional properties of the PFC L5 corticostriatal neurons in AS model mice. The reductions of soma size and dendritic branching of the PFC L5 corticostriatal neurons, the abnormal higher release probability and disturbed RRV pool replenishment of their presynaptic neurons due to decreased expression level of cdc42 at P28-35, give rise to the impairments of synaptic transmission and neural connectivity. The impairments of neuron morphology, synaptic transmission, short-term plasticity and vesicular release demonstrate brain region specific and age-dependent characteristics.

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APPENDIX A

LIST OF ABBREVIATIONS

AS: Angelman syndrome

UBE3A (Ube3a): Ubiquitin E3 ligase type A

E6-AP: E6 protein associated protein

PFC: prefrontal cortex

L5: layer Ⅴ

mEPSCs: miniature excitatory postsynaptic currents

mIPSCs: miniature inhibitory postsynaptic currents

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

NMDA: N-methyl-D-aspartic acid

RIM1α: Rab3-interacting molecule subunit 1α

CNS: central nervous system

UPS: Ubiquitin proteasome system

EEG: electroencephalography

UPD: paternal uniparental disomy

IC: imprinting center

S1: somatosensory

GABA: γ-aminobutyric acid

GR: glucocorticoid receptor

LTP: long-term potentiation

ERK: extracellular signal-regulated kinases

MAPK: mitogen-activated protein kinases

mGluR5: type 5 metabotropic glutamate receptor

PV: parvalbumin

aa: amino acid

HECT: homologus to E6AP carbonyl terminus

UTR: untranslated region

miRNA: micro RNA

BDNF: brain-derived neurotrophic factor

KCl: potassium chloride

HSP-70: heat shock protein 70

Arc: activity-regulated cytoskeleton-associated protein

TSC2: tuberous sclerosis complex 2

Ring1B: Really interesting gene 1B

SOX9: sex determining region Y-box 9

MCM7: multicopy maintenance protein 7

HERC2: HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2

HHR23A: human homologs of Saccharomyces cerevisiae Rad23

Blk: B lymphoid kinase

hScrib: the human homologue of the Drosophila Scribble (Vartul) tumor suppressor protein

NFX1-91: nuclear transcription factor, X-box binding 1 isoform 91

E6TP1: E6 targeting protein 1

AIB1: amplified in breast cancer 1

GEF: guanine nucleotide exchange factor

Pbl/ECT2: Pebble (in Drosophila) and Epithelial Cell Transforming 2 (in mammals)

ER: Estrogen receptor

AR: Androgen receptor

PTPH1/PTPN3: protein tyrosine phosphatase H1/N3

DLG4/PSD-95: Disks large homolog 4/postsynaptic density 95

PML: Promyelocytic leukemia

GAT1: GABA transporter 1

SK2: small-conductance potassium channel 2

hTERT: human telomerase reverse transcriptase

HIF1AN: human gene for hypoxia-inducible factor 1-alpha inhibitor

NEURL4: neuralized E3 ubiquitin protein ligase 4

ASPM: abnormal spindle-like, microcephaly-associated protein

BMAL1: brain and muscle Arnt-like 1

TF: transcription factor

mTORC2: mammalian target of rapamycin complex 2

ISH: in situ hybridization

ATS: antisense RNA transcript

SGZ: subgranular zone

DG: dentate gyrus

GCL: granule cell layer

PI-3K: phosphoinositide 3-kinase

IL: interleukin

ASON: antisense oligonucleotide

ME: median eminence

AD: Alzheimer's disease

PD: Parkinson's disease

Neo: neomycin resistance cassette

HPRT: hypoxanthine guanine phosphoribosyl transferase

YFP: yellow fluorescent protein

tTA-tetO: tetracycline transactivator-Tet operator

snoRNAs: small nucleolar RNAs

PR: progesterone receptor

GA: Golgi apparatus

LTD: long-term depression

GPCR: G protein-coupled receptor

cAMP: 3',5'-cyclic adenosine monophosphate

PKA: protein kinase cAMP-dependent

PKC: protein kinase C

CaMKII: calcium/calmodulin-dependent protein kinase II

ErbB4: Erb-B2 Receptor Tyrosine Kinase 4

PLCγ: phospholipase C γ

Grb2: growth factor receptor-bound protein 2

Gab1: Grb2-associated binder 1

NaKA: sodium/potassium-ATPase

rAAV: recombinant adeno-associated virus

TR2: type 2 terminal repeat

MWM: Morris Water Maze

ATFs: artificial transcription factors

HIV: human immunodeficiency virus

KE: S-1,3-butanediol acetoacetate diester

DBS: deep brain stimulation

TMS: transcranial magnetic stimulation

V1: primary visual cortex

GABRB3: $GABA_A$ receptor β3 subunit gene

BLA: basolateral amygdala

ACA: anterior cingulate area

LSPS: Laser scanning photostimulation

WT: wild-type

K-S test: Kolmogorov-Smirnov test

Pr: release probability

RRV: readily-releasable vesicle

PPF: paired-pulse facilitation

HFD: high frequency depletion

eEPSCs: evoked AMPA EPSCs

ISI: inter-stimulus intervals

cdc42: cell division control protein 42 homolog

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GluR1: glutamate receptor 1

NR1: NMDA recepter 1

EGFP: Enhanced Green Fluorescent Protein

DAPI: 4,6-Diamidino-2-phenylindole, dihydrochloride

PMSF: phenylmethanesulfonyl fluoride

DMSO: dimethyl sulfoxide

SDS: sodium dodecyl sulfate

PAGE: polyacrylamide gels

PVDF: immobilon-P membrane

HRP: horseradish peroxidase

ECL: enhanced chemiluminescence

GA: Golgi apparatus

NK: natural killer

IgE: immunoglobulin E

TNFα: tumor necrosis factor α