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Physiological properties of cerebellar circuits in a mouse model for Angelman Syndrome and Modulation of inhibitory transmission by NMDA receptor activation in the mouse hippocampus

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Abstract

UBE3A encodes the ubiquitin ligase E6-AP and the point mutation of this gene causes severe neurological disorder called Angelman Syndrome (AS) in a young child. To elucidate the molecular basis of AS pathogenesis and promote the design of rational therapies for AS patient, an *Ube3a* null mutation mouse, which recapitulated the development and behavioural defects of human patients, was generated. The mice with maternal deficiency (m-/p+) for *Ube3a* display several features of AS, including microcephaly, motor dysfunction, inducible seizures, abnormal hippocampal EEG and deficits in context-dependent learning. Based on the knowledge of altered *UBE3A* expression profile and uncoordinated movement observed in AS patients, I hypothesized that the normal function and electrophysiological properties of Purkinje neurons (PNs), which provide the sole output of cerebellar cortex might be affected by the deletion of E6-AP. The electrophysiological studies on PNs of AS mouse would be able to provide meaningful information on deciphering the molecular basis for the learning and memory deficit in AS and be helpful for developing effective drugs.

Thus, in the first part of this thesis, the physiological properties of cerebellar cortex in *Ube3a* null mutation mice were thoroughly examined. The data collected from acute cerebellar slices showed that the intrinsic excitability, the inhibitory synaptic transmission and basal excitatory synaptic transmission were normal in the mutant mice. There was also no alteration in a type of long-term potentiation (LTP) that is presynaptically expressed at PF (parallel fiber)-PN synapses. In contrast, another type of LTP, which expressed postsynaptically at the same PF-PN synapses, was impaired, and another type of synaptic plasticity, long-term depression (LTD),

was enhanced in the mutant mice. The Western blotting data showed an up-regulation of β CaMKII expression in the cerebellum, but not in the forebrain area. Taken together, these findings suggest that mutation of E6-AP directly affect neither the functional properties of ion channels underlying the regulation of the intrinsic excitability of neurons nor the basal properties of both inhibitory and excitatory synaptic transmission. The data provide evidence that E6-AP plays a pivotal role in expression of specific forms of activity-dependent synaptic plasticity, such as postsynaptic LTP and LTD at cerebellar PF-PN synapses, and that β CaMKII likely serves as a substrate of E6-AP and E6-AP-induced changes in the β CaMKII or expression level affect synaptic plasticity in the mutant mice.

In the second part, using hippocampal slices I found that brief activation of NMDA receptors (NMDARs) in mouse hippocampal CA1 region profoundly enhances GABAergic inhibitory transmission between interneurons and pyramidal neurons. NMDARs have so far been implicated in synaptic plasticity and modulation of neurotransmission at glutamatergic excitatory synapses. However, a role of NMDARs at inhibitory synapses remains largely elusive. Here I report that a brief application of NMDA could induce two distinct actions in CA1 pyramidal neurons in mouse hippocampal slices: (1) an inward current attributed to activation of postsynaptic NMDARs and (2) fast phasic synaptic currents, namely spontaneous IPSCs (sIPSCs), mediated by GABA_A receptors in pyramidal neurons. The mean amplitude of sIPSCs was also increased by NMDA. This profound increase in the sIPSC frequency and amplitude was markedly suppressed by the sodium channel blocker tetrodotoxin, whereas the frequency and mean amplitude of miniature IPSCs were not significantly affected by NMDA, suggesting that NMDA elicits repetitive firing in GABAergic interneurons, thereby leading to GABA release from multiple

synaptic sites of single GABAergic axons. I found that the NMDAR open-channel blocker MK-801 injected into recorded pyramidal neurons suppressed the NMDA-induced increase of sIPSCs, which raises the possibility that the firing of interneurons may not be the sole factor and certain retrograde messengers may also be involved in the NMDA-mediated enhancement of GABAergic transmission. Our results from pharmacological tests suggest that the nitric oxide signaling pathway is mobilized by NMDAR activation in CA1 pyramidal neurons, which in turn retrogradely facilitates GABA release from the presynaptic terminals. Thus, NMDARs at glutamatergic synapses on both CA1 pyramidal neurons and interneurons appear to exert feedback and feedforward inhibition for determining the spike timing of the hippocampal microcircuit.

Overall, my studies strongly suggest that the normal function of E6-AP is critical for the induction and expression of activity-dependent synaptic plasticity in PF-PN synapse and the deficit of motor coordination observed in AS patients might be caused by the up-regulation of β CaMKII expression level in cerebellar cortex. In addition, the data from hippocampal slices demonstrate that NMDARs in both presynaptic interneurons and postsynaptic pyramidal neurons play a pivotal role in the regulation of GABAergic inhibitory transmission in the CA1 subfield. The activation of postsynaptic NMDARs appears to elicit the liberation of NO, which retrogradely promotes GABA release from interneurons.

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Chapter I Literature reviews

1.1 Angelman Syndrome

Angelman Syndrome (AS) is a severe neurological disorder first documented by a British pediatrician Dr Harry Angelman in 1965 [1]. Now it becomes familiar to most child neurologists as a distinct syndrome characterized by mental retardation and absence of speech, as well as seizure activity and “puppet-like” ataxic gait. AS has a prevalence of 1: 12,000 to 1: 20,000 in general population without geographic clustering [2]. So far, the only treatments available are supportive.

1.1.1 Clinical features and developmental phenotypes

In 1995, a consensus criteria for the clinical diagnosis of AS was developed by the Scientific and Research Advisory Committee of the US Angelman Syndrome Foundation, intending to assist in the evaluation and diagnosis of AS [3]. Ten years later, an updated version of such diagnostic criteria was published based on our increased knowledge about the cellular and molecular mechanism of AS, which is illustrated in Table I [4]. As shown in this table, four characteristics are virtually 100% consistent in all AS patients irrespective of the genetic background. Those are development delay, speech impairment (usually absence of speech), movement and balance disorder and behavioral abnormalities. Other features, such as seizure, abnormal Electroencephalograph (EEG) pattern and microcephaly vary case by case, highly depending on the causation of AS.

The newborn baby shows normal behavior. In the first 6 months, feeding problem and muscle hypotonia are frequently reported. At the age of 6-12 months,

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developmental delay is observable, but keeps on progressing. However, there is no significant deficit of brain structure, although some non-specific changes like mild cortical atrophy or delay in myelination may be seen on magnetic resonance scans. Moreover, the blood test and urine test are also normal. The lack of clinical manifestation makes the diagnosis of AS become very difficult at this stage [5-6]. From the age of 1 year, a child with AS shows more typical AS features such as mental retardation, outbursts of laugh, macrostomia, maxillary hypoplasia and neurological problems with a puppet-like gait. Particularly, the EEG pattern in AS is very abnormal and significantly different from the control group. The three main specific EEG patterns are (1) persistent rhythmic 4-6 Hz high voltage activity (reaching more than 200 μ V) which sustains for most of the record and is unrelated to drowsiness, (2) prolonged rhythmic 2-3 Hz delta activity mainly over the frontal regions with superimposed spikes and sharp waves of moderate amplitude, and (3) spikes and sharp waves mixed with 3-4 Hz high amplitude components which are mainly posteriorly distributed and readily elicited by passive eye closure [7-10]. The EEG findings alone can strongly suggest the AS diagnosis.

With advancing age, the phenotype of AS may change and has pronounced facial characteristics with marked prognathism, macrostomia and prominent lower lip can be noted in adult patients [11]. A stable vision without any type of degenerative retinopathy is observed and optical nerve atrophy and blindness are rarely reported. The patient becomes less active because of limb hypertonicity, development of thoracic scoliosis and a general reluctance to walk. As a result, some patients become wheelchair bound. In some cases, obesity and cardiac rhythm disturbance resulting from vagal hypertonia may occur and can be severe [7, 12-13]. Figure 1.1 shows some AS patients at different ages.

Table I Angelman syndrome: consensus criteria for clinical diagnosis

A. Consistent (100%)	
•	Developmental delay, functionally severe
•	Movement or balance disorder, usually ataxia of gait, and/or tremulous movement of limbs. Movement disorder can be mild. May not appear as frank ataxia but can be forward lurching, unsteadiness, clumsiness, or quick, jerky motions
•	Behavioral uniqueness: any combination of frequent laughter/smiling; apparent happy demeanor; easily excitable personality, often with uplifted hand-flapping, or waving movements; hypermotoric behavior
•	Speech impairment, none or minimal use of words; receptive and non-verbal communication skills higher than verbal ones
B. Frequent (more than 80%)	
•	Delayed, disproportionate growth in head circumference, usually resulting in microcephaly (≤ 2 SD of normal OFC) by age 2 years. Microcephaly is more pronounced in those with 15q11.2-q13 deletions
•	Seizures, onset usually <3 years of age. Seizure severity usually decreases with age but the seizure disorder lasts throughout adulthood
•	Abnormal EEG, with a characteristic pattern, as mentioned in the text. The EEG abnormalities can occur in the first 2 years of life and can precede clinical features, and are often not correlated to clinical seizure events
C. Associated (20%–80%)	
•	Flat occiput
•	Occipital groove
•	Protruding tongue
•	Tongue thrusting; suck/swallowing disorders
•	Feeding problems and/or truncal hypotonia during infancy
•	Prognathia
•	Wide mouth, wide-spaced teeth
•	Frequent drooling
•	Excessive chewing/mouthing behaviors
•	Strabismus
•	Hypopigmented skin, light hair, and eye color compared to family), seen only in deletion cases
•	Hyperactive lower extremity deep tendon reflexes
•	Uplifted, flexed arm position especially during ambulation
•	Wide-based gait with pronated or valgus-positioned ankles
•	Increased sensitivity to heat
•	Abnormal sleep-wake cycles and diminished need for sleep
•	Attraction to/fascination with water; fascination with crinkly items such as certain papers and plastics
•	Abnormal food related behaviors
•	Obesity (in the older child)
•	Scoliosis
•	Constipation

EEG, electroencephalography; SD, standard deviation; OFC, occipitofrontal head circumference. Table adapted from [4]

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Figure 1.1 (A-D) Four AS patients at different ages presenting the typical facial features: round face, microcephaly and happy personality. Figure adapted from [11]

1.1.2 Genetic aetiology

It's well known that the abnormalities of human chromosome 15q11-q13 region give rise to AS. So far, four mechanisms have been illustrated and AS patients are normally categorized into class I to IV based on the genetic ground. In addition, a fifth group of AS patients, named class V have clinical features of AS but have unknown molecular lesion. A summary of the complicated genetic basis of AS are listed in Table II and the genetic map of chromosome 15q11-q13 is shown in Figure 1.2. Clearly, the *de novo* deletion of the maternal chromosome 15q11-q13 region is

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the most frequent causation of AS (65-70% of all AS cases). Most of the deletions show a similar size, approximately 4 Mb and always carry consistent breakpoint in flanking cassettes of repetitive genes (duplicons). Unequal or misaligned crossing-over between these duplicons results in chromosome defect. The common deletion can be readily diagnosed by performing fluorescent *in situ* hybridization (FISH) analysis and by methylation analysis of *SNRPN* (Small Nuclear Ribonucleoprotein N) promoter which locates within a CpG island in chromosome 15q11-q13. In the situation of maternal deletions, only the paternal, unmethylated patterns are detected [7, 14-15].

Approximately 3-5 % of individuals with AS have paternal uniparental disomy (UPD) for chromosome 15 and thereby are lack of the genes inherited from maternal allele within the locus. UPD refers to the situation in which both homologous of a chromosome pair derived from a single parent [16-17]. Multiple mechanisms including 1) *gamete complementation* - fertilization of an egg with a extra chromosome (disomy) by a sperm which is missing the same chromosome (or vice versa); 2) *trisomic zygote rescue* - lose of one of the supernumerary chromosomes in the early development stage and 3) *compensatory UPD* - replacement of an abnormal or missing chromosome with a copy of normal homologue can give rise to UPD. With respect to the maternal UPD in AS, it has been demonstrated that most cases of UPD arise from a mosaic blastocyst, providing a means to rescue an abnormal eggs that is nullisomic. For diagnostic, microsatellite polymorphisim and restriction fragment length polymorphisims (RFLP) analysis of DNA samples from patients and their parents are commonly used to determine the parental contribution for the chromosome 15. The tests are usually employed after the DNA methylation test and FISH assay to differentiate UPD cases with other classes [2, 18-19].

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The third class of AS patients show no evidence of chromosome deletions, or maternal UPD, but have uniparental DNA methylation at several loci along 15q11-q13 including *MKRN3*, *PW71* and 5' *SNURF-SNRPN*, which are typical characteristic of AS [20]. These patients are thought to possess mutations in a short DNA sequence, termed imprinting control center (IC), which regulates DNA methylation and allele-specific gene expression within chromosome 15q11-q13 region in a *cis*-acting manner. Genomic imprinting is a phenomenon that allows some genes to be expressed according to the parental origin of chromosomes or chromosomal subregions. Typically, one allele at an imprinted locus is transcriptionally silent, with all gene products produced from the other allele [21]. In about 50% AS patients with imprinting defect, an identifiable mutation within the IC, locating 35 kb upstream of *SNURF-SNRPN* exon 1 is thought to impair the establishment of maternal imprint and cause AS, while in the remaining patients, no mutation can be detected despite sequencing of the entire IC. It has been proposed that in the later non-IC-mutation cases, the incorrect imprint is possibly caused by a spontaneous pre- or postzygotic error, like a *cis*-acting mutation outside of IC or a mutation in a *trans*-acting factor [22-23].

Class IV patients have been found to have mutations within *UBE3A* gene, the gene encoding an ubiquitin ligase E6-AP (E6-Associated Protein). E6-AP is initially identified as a cellular protein that associates with the E6 oncoprotein of human papillomavirus, type 16 to promote the ubiquitination and degradation of the tumor suppressor p53 [24]. Clinical studies carried out from both sporadic and familial case of AS have shown that the majority of the mutations are nonsense mutations which are predicted to cause a frameshift and/or premature termination of translation, but some are missense mutations that still allow the translation of a full-length protein

[25-27]. Further molecular analysis demonstrated that the point mutations affect the ubiquitin ligase activity of E6-AP in diversified manners. Some mutations map to the conserved residues of catalytic cleft, thereby interfere with the ubiquitin-thioester bond formation. Other mutations retain the capability of forming thio ester intermediate with ubiquitin, but are incapable to transfer the active ubiquitin to a substrate efficiently and the remaining mutations may result in protein misfolding and reduce the half-life *in vivo* [28-30]

Figure 1.2. Schematic drawing of genetic map of human chromosome 15q11-q13 region. BP represents break point relating to duplicons. Paternally expressed genes are depicted in blue and maternally expressed genes in red. Those expressed equally from two alleles are shown in black. See text for further details of AS causation.

The final group of patients (class V) show typical clinical features of AS but have no detectable cytogenetic or molecular abnormalities involving chromosome 15q11-q13. Since there are no identifiable mutations observed, there are arguments that class V AS are likely due to erroneous diagnosis and other syndromes sharing phenotypic overlap with AS like Rett syndrome, Gurrieri syndrome and alpha-thalassemia retardation syndrome (ATR-X). However, the data collected by research groups with significant clinical experience of AS strongly support the idea that class V patients do exist [31-33]. One possible explanation is these patients have yet

undetected lesions in chromosome 15q11-q13 that affects the expression of *UBE3A* gene. Alternatively, these patients may have novel genetic defects upstream of *UBE3A* that alter the expression of additional genes of ubiquitin-mediated protein degradation pathway.

Tabel II Genetic mechanisms giving rise to AS

Class	Mechanism	Methylation analysis	Other diagnostic tests	Frequency
Ia	De novo deletion	Abnormal Paternal band only	High resolution cytogenetics FISH	70%
Ib	Deletion owing to chromosome rearrangement	Abnormal Paternal band only	High resolution cytogenetics FISH	<1%
II	Uniparental paternal disomy	Abnormal Paternal band only	RFLP analysis	2%
IIIa	Imprinting defect owing to imprinting centre mutation	Abnormal Paternal band only	Screening of IC for mutation is positive	2%
IIIb	Imprinting defect without IC mutation	Abnormal Paternal band only	Screening of IC for mutation is negative	2%
IIIc	Mosaic imprinting defect	Abnormal Faint maternal band	Screening of IC for mutation is usually negative	?
IV	<i>UBE3A</i> mutation	Normal Both maternal and paternal bands	Screening of <i>UBE3A</i> for mutations	5-10%
V	No genetic abnormality identified	Normal Both maternal and paternal bands	Consider other diagnosis	12-15%

Table adapted from [7].

1.1.3 Phenotype-genotype correlations

There are some correlations between the phenotypic severity of AS and the genetic mechanism giving rise to it. In general, the patients with large chromosome deletions (class I) are likely to exhibit the most classical and severe features. They have a higher incidence of seizures, microcephaly, absence of speech and hypopigmentation in skin, eyes and hair. In addition, they learn to sit and walk later and achieve the developmental milestones to a less degree compared with the other classes [31, 34-35]. These observations, especially for the severe epilepsy in deletion cases are thought to be the result of haploinsufficiency of additional non-imprinted genes within chromosome 15q11-q13 region. The lack of these genes exacerbates the effect of *UBE3A* mutation and results in much severe behaviour abnormalities. In another words, although the mutations of *UBE3A* are sufficient to lead to the four characteristic phenotypes of AS, other genes must also contribute to the severity of seizures and the development of cognition. In contrast, the individuals with uniparental disomy and imprinting defects (class II and class III) are much less severely affected. They are likely to have no seizures and have a low incidence of microcephaly and hypopigmentation. Many of these patients are able to say a few words and have better cognitive function, although severe mental problems are still present [36-37]. The patients caused by *UBE3A* mutation (class IV) fall somewhere in the middle. They are similar to the deletion patients concerning the incidence and severity of seizures and microcephaly they suffered. But, their motor skills, as well as the communication capability are much better than the deletion group. Overall, it is clear that the AS cases without chromosome deletions appear to show milder phenotypes than deletion patients. A clinical severity scale from high to low can be

described as follows: deletion cases > *UBE3A* mutation cases > imprinting mutation and/or UPD cases.

1.1.4 *UBE3A*, the candidate gene for AS

The *UBE3A* gene was mapped to the AS critical region in 1994, but it was not initially considered a strong candidate as an AS gene because imprinting analysis using RT-PCR from fibroblasts and lymphoblasts of AS patients failed to detect the imprinting expression of this gene [38]. However, the subsequent findings that point mutations in *UBE3A*, mostly truncating mutations were observed in nondeletion/non-UPD/non-imprinting mutation (NDUI) AS patients demonstrate that *UBE3A* mutations are one cause of AS and suggest that *UBE3A* is the AS gene [25-26]. The further evidence that *UBE3A* was specifically imprinted in certain brain area but not in other somatic tissues and the experimental data from the *Ube3A* null mutation mice reinforces and consolidates the idea that loss of *Ube3A* function in the nervous system underlies AS [39-43]. Therefore, to better understand the pathogenesis of AS, it's necessary to take a close view of the gene structure, expression pattern of *UBE3A* and the function of its protein product E6-AP.

1.1.4.1 Organization of *UBE3A* gene

Molecular analysis indicates that the gene encoding *UBE3A* includes at least 16 exons that span approximately 120 kb along genomic DNA, with the transcription starts from telomere towards centromere [44]. Because the 5'- end of the gene displays alternative splicing, the exact number of RNA variants still remains unclear [39-40, 44-45]. But, three predicted protein isoforms differing in the use of initiation codon

are confirmed. Isoform I, which is the shortest one, corresponds to the initially described open reading frame for E6-AP. It contains 865 amino acids with a total predicted molecular mass of 99,289 Da [46]. Isoforms II and III have an additional 20 and 23 amino acids at their amino-terminus, respectively [45]. Given the imprinted and tissue-specific expression pattern of E6-AP, it is of great interest to determine the role of additional amino acids contained in isoform II and III played in physiological conditions. However, the high similarity of predicted molecular weights of the isoforms prevents the unambiguous identification of each isoform by conventional molecular techniques. We can only speculate that these amino acids may be necessary in the recognition of real cellular substrates of E6-AP *in vivo*, thereby conferring diversified substrates specificities to the enzyme.

1.1.4.2 Neuron-specific imprinting of *UBE3A*

Initially, the reverse transcription (RT)-PCR results from lymphocytes and fibroblasts of AS patients indicate that *UBE3A* is equally expressed from both alleles and there is no imprinting in these cell types [38]. Later, the same experiments carried out in brain preparation reveal that *UBE3A* displayed maternal-specific expression in brain but not in other somatic tissues [39-40]. Further *in situ* hybridization data from the paternal UPD mouse demonstrate that the imprinting of *Ube3a* in brain is region-specific, with silencing of the paternal allele in hippocampus and cerebellum but not in anterior commissure, optic chiasma and other parts of the brain [41]. Recently, a new expression pattern of *Ube3a* is reported by Kishino's group. Using the primary cortical cell cultures, they found that the sense transcript of *Ube3a* gene is expressed maternally in neurons but biallelically in glia cells, while the antisense transcript is

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expressed only in neurons and only from the paternal allele [47]. Most likely, the previously described brain region-specific imprinting of *Ube3a* is the result of cell-type imprinting, i.e., the brain areas with higher density of neurons, such as hippocampus and cerebral cortex, will show high specific imprinting of *Ube3a*. In contrast, those regions contain less number of neurons will show low specificity of *Ube3a* imprinting. Figure 1.3 illustrated the relationship between genomic imprinting and *UBE3A*.

Figure 1.3. Schematic drawing of imprinting expression of *UBE3A*. In normal individuals, *UBE3A* is expressed equally from both parental alleles (maternal chromosome in pink; paternal chromosome in blue) in most tissue types. In neurons, the paternal *UBE3A* allele is silenced by the process of genomic imprinting and only the maternal *UBE3A* allele is active. In the case of AS, the maternal *UBE3A* allele is either absent (due to chromosome deletion) or inactivated (caused by null mutation), thereby leading to no functional expression of *UBE3A* in the brain. Figure adapted from [48].

The molecular mechanism of tissue and cell type specific expression of *UBE3A* has been extensively investigated and many hypotheses have been advanced. One simplified mechanism is differential DNA methylation. The methylation might

directly modify one parental allele, allowing the binding of *trans* factors which induce a heterochromatic state, thereby suppresses the transcription initiation. On the other hand, that unmodified allele can be normally transcribed, results in the monoallelic expression of imprinted gene. Another much more complicated model is expression-competition model. In this model, the methylation acts indirectly on the imprinted gene via modifying a flanking imprinting control element which plays a regulatory role. The nature of interaction between imprinting control gene and the target imprinted gene is not specified [49]. In terms of imprinting of *UBE3A*, the discovery of *UBE3A*-ATS, a paternally expressed antisense RNA transcript (ATS) of *UBE3A*, and the existence of imprinting control centre in AS region strongly argue for this hypothesis. *Ube3a*-ATS spans more than 460 kb and includes at least 148 exons. It is alternatively spliced and serves as the host for multiple types of small nucleolar RNA species [50]. It is generally regarded that antisense transcript suppress the expression of sense transcript in a *cis* manner with three possible silencing mechanisms including *transcriptional interference* due to an inhibition in transcription elongation, *RNA masking*- antisense-mediated alteration of transcript structure and *RNA interference*-double strand RNA dependent degradation [51-52]. However, the recent report that maternal disruption of *Ube3a* results in increased expression of *Ube3a*-ATS argues for the *trans* interaction between *Ube3a* and *Ube3a*-ATS, not in a *cis* manner as have been observed in other cases [53]. This finding suggests that the imprinting mechanism of *UBE3A* is quite unique and much more complicated as we ever expected. Further experiments are required to clearly delineate the mechanism of silencing of paternal allele of *UBE3A*. Elucidating the role of *UBE3A*-ATS played in the imprinting of *UBE3A* will give us important insight into the regulation of genomic imprinting.

1.1.4.3 Function of *UBE3A* protein product E6-AP

E6-AP is the founding member of the HECT (homologous to E6-AP C terminus) domain family of E3 ubiquitin ligase that plays important role in the ubiquitin-mediated protein degradation pathway [54]. The ubiquitin system is a hierarchical enzymatic cascade involving the cooperation of three sets of enzymes called E1, E2 and E3. E1, the ubiquitin activating enzyme activates ubiquitin in an ATP-dependent manner and transfers it to a cysteine residue of an E2, the ubiquitin conjugating enzyme. The ubiquitin-bound E2 then interacts with the E3, ubiquitin ligase to form the polyubiquitin chain onto the target protein. The ubiquitinated substrate is subsequently shuttled to the 26S proteasome and is degraded to peptides and amino acids [55-56]. It is generally believed that E3 has a more critical role in this process since it specifically recognizes the target protein and thereby determines the specificity of the reaction.

The first substrate of E6-AP we have known is cell cycle regulator p53. In high risk HPV (human papillomavirus)-infected cells, E6-AP degrades p53 with the aid of E6 oncoprotein in an ubiquitination-dependent manner, resulting in rapid growth of the cervical cancer [24, 57]. In normal cells, E6-AP is also reported to be involved in p53 degradation and regulation of p53 gene transcription [58]. In addition to p53, HHR23A, a protein homologous of yeast Rad23 which is involved in DNA repair and cell cycle progression; MCM-7, a subunit of the DNA replication licensing factor-M; Blk, a nonreceptor kinase of Src family and Annexin A1, a member of the annexin superfamily which plays important role in the inhibition of cell proliferation, anti-inflammatory effect and control of cell differentiation all have been identified as potential targets of E6-AP in non-neuronal cells [59-62]. Moreover, yeast-two hybrid screening study shows E6-AP can also function as a coactivator for the nuclear

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hormone receptor superfamily and coactivates the hormone-dependent transcriptional activity of progesterone receptor. Further analysis demonstrates that mutant forms of E6-AP isolated from AS patients possess the capability of being a coactivator while the ubiquitin-protein ligation function is impaired, indicating that the defect in ubiquitin-proteasome pathway is the sole cause of AS phenotype [63].

In neuronal cells, with the aid of YFP labelling, Dindot et al reported that E6-AP: YFP construct was differentially distributed between the nucleus and cell body soma, extending to the growth cone and synaptic compartments in cultured hippocampal neurons [64]. The deletion of maternal E6-AP had no effect on the dendritic density and branching of Purkinje neurons, whereas the spine density and spine length were significantly reduced, suggesting that E6-AP may function locally to regulate spine development [64]. Consistent with this observation, Lu et al found that in *Drosophila*, *Drosophila UBE3A (dUBE3A)*-null mutation reduced the dendritic branching of sensory neurons and slowed the formation of terminal fine dendritic branches [65]. Interestingly, overexpression of *dUBE3A* also caused a reduction of dendritic branching, a phenotype similar to that caused by deletion of *dUBE3A*, implicating that the proper level of *dUBE3A* is critically important for the neuronal differentiation [65-66]. Collectively, these data provide strong evidences for the hypothesis that normal function of E6-AP is indispensable for the neuronal patterning. Abnormal dendritic structure of neuronal cells resulted from loss of function of E6-AP may contribute to the neurological deficits of AS patients. However, the substrates that are under the control of *UBE3A* to exert their functions on the development of neuronal dendrite are largely unknown.

1.1.5 Mouse models of AS

The complex phenotypes and genetic backgrounds of AS make it very important to generate mouse models that recapitulate the developmental and behavioural defects of human patients. Such models will help to elucidate the molecular basis of AS pathogenesis and promotes the design of rational therapies for AS. The first reported mouse model for AS is a model harbouring uniparental disomy of AS homologous region [67]. Although the behaviours of this mouse closely mimick the phenotypes of human patients, the large region of uniparental disomy make it impossible to evaluate the contribution of individual gene to the phenotype of AS.

Given the physiological function of GABA_A receptor, which is the major receptor type mediating fast inhibitory transmission in central nervous system, and the close location of a cluster GABA_A receptor subunits $\alpha 5$, $\beta 3$ and $\gamma 3$ to the AS core region (Fig 1.2), the $\beta 3$ subunit of GABA_A receptor (GABARB3) gene had been considered as an AS candidate gene. Supporting this hypothesis, disruption of the GABARB3 gene produces electroencephalographic abnormalities and seizure in mice. Moreover, GABARB3 mutation mice exhibit the learning and memory deficits, poor motor skills, and hyperactivity features that parallel those observed in AS [68-70]. However, the fact that about 30% of AS patients including patients with imprinting defect and uniparental disomy do not have a detectable deletion of GABARB3 gene and the lacking of allele-specific expression pattern of GABARB3 strongly argues against the above hypothesis. One explanation to reconcile the discrepancy is that the alteration of GABA_A receptor-mediated inhibitory transmission alone is not the cause of AS, but the deletion of GABARB3 will exacerbate the consequence of lack of other AS candidate gene, resulting in more severe phenotypes.

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The finding that about 20% AS patients carry the point mutation or truncating mutation within the open reading frame of *UBE3A* gene and its maternal allele-specific expression pattern consolidate the hypothesis that *UBE3A* is the candidate gene for AS. Since then, two mouse models carrying a null mutation of *Ube3a* have been generated. One model was developed by Miura et al in 2002 [43]. They selectively inactivated the *Ube3a* gene by inserting a *lacZ* reporter gene which helps to facilitate the tracing of E6-AP expression. The result that only maternal but not paternal transmission of targeted allele gives rise to the β -galactosidase activity confirms the previous data that the *UBE3A* is exclusively expressed from the maternal allele. Behaviour studies show the maternal deficient mice have no obvious phenotypic abnormalities but exhibit deficit in the fine tests of motor function and spatial learning compared with control siblings [43]. Using this model, Mardirossian et al demonstrated that the basal expression of *c-fos* and *Arc* genes which are required for synaptic plasticity and memory formation is reduced in dentate gyrus (DG) of mutant. Additionally, the adult neurogenesis is also affected by *Ube3a* deletion, suggesting the disruption of immediately-early genes is involved in the cognitive impairments and behaviour abnormalities of AS [71].

Another *Ube3a* null mutation mouse was described by Jiang et al in 1998 [42]. The mice with maternal deficiency (m-/p+) for *Ube3a* display several features of AS, including microcephaly, motor dysfunction, inducible seizures, abnormal hippocampal EEG and deficits in context-dependent learning [42]. Electrophysiological studies on this model have revealed that long term potentiation (LTP), a well-characterized phenomenon whereby conditioning stimulation of presynaptic axons enhances the strength of synaptic connections is impaired in hippocampal neurons of maternal mutation mice. Subsequent work finds that the

phosphorylation state of hippocampal calcium/calmodulin-dependent protein (CaM) kinase II (CaMKII), specifically at sites of Thr286 and Thr305 is significantly up-regulated, while no change is observed in other downstream effectors of LTP process such as protein kinase C and cAMP-dependent protein kinase A [72]. Consistent with these findings, an additional mutation at the inhibitory phosphorylation site (Thr305/306) of CaMKII is found to be able to rescue the molecular and behavioural deficits seen in AS mice. In *Ube3a*/CaMKII double mutant, the LTP can be normally induced as the control littermates. The cognitive deficits such as defects in spatial learning and fear conditioning associated with AS mutation are also rescued by introducing this CaMKII mutation [73]. Together, these results demonstrate that the increased inhibitory phosphorylation of CaMKII associated with *Ube3a* maternal deficiency may underlie the deficits in synaptic plasticity observed in AS mouse model and potentially underlies the etiology of human AS. However, how the E6-AP links with CaMKII remains unclear. Further research is needed to establish such connections.

1.1.6 The synaptic transmission of cerebellar cortex

Traditional lesion studies have established the classical concept that the cerebellum is the site of motor control. It does not initiate the movement, but functions as a neuronal machine to combine simple elements of movement into more complex coordinated act, enabling animals and humans to carry out smooth and accurate movement, even at a high speed and without visual feedback [74-75]. In addition, recent functional neuroimaging studies suggest that cerebellum may also be involved in higher cognitive function [76-77], although the mechanism of how cerebellum processes the information beyond the domain of motor control is largely unknown.

1.1.6.1 Cerebellum architecture

At the level of large-scale anatomy, the cerebellum can be viewed as a cauliflower-shaped brain structure consisting of tightly folded cerebellar cortex, white matter and a fluid-filled ventricle in the middle. The cerebellum connects to the brain stem with large bundle of nerve fibers called cerebellar peduncles which are made up by numerous afferent and efferent tracts. The structure of cerebellum can be further divided into 3 divisions according to the phylogenetical criteria: the flocculonodular lobe, the one locates at the back of cerebellum and mainly participates in balance and eye movement control; the anterior lobe, which is the most front part of cerebellum and functions in the control of proximal muscles, posture and locomotion; and posterior lobe, the one separated from anterior lobe by primary fissure and plays a important role in the motor coordination [75, 78-79].

Functionally, the cerebellar cortex can be subdivided into a series of parallel longitudinal zones, extending across the cerebellar lobules in the rostro-caudal orientation. There are seven morphologically distinct sagittal zones in each hemisphere, designated as zone A, B, C1, C2, C3, D1 and D2 respectively [80]. The Purkinje neurons in an individual zone receive climbing fiber inputs from a distinct region of inferior olive and send the outputs to a particular area of cerebellar nuclei, thereby creating special olivo-cortico-nuclear loop. A longitudinal zone can be further divided into microzones. A microzone consists of a small cluster of neurons in vestibular or cerebellar nucleus, a group of neurons in inferior olive and a strip of Purkinje cells in the cerebellar cortex. It is generally regarded as the least functional unit of the cerebellum and is supposed to have a similar internal structure, but with different inputs and outputs. Estimated from the size of individual microzone and entire surface area of cerebellar cortex, a total of 5000-15 000 microcomplexes are thought

to exist *in vivo* [81]. Recently, a new concept, named multizonal microcomplex (MZMC) is used for the model study of cerebellum function. MZMC is defined as more than one microzones in different area of cerebellar cortex that receive the climbing fiber inputs with similar receptive field and that converge their corticonuclear outputs onto same group of cerebellar nuclei. Such a functional arrangement of cerebellar cortex is thought to reflect the spinal withdraw reflex organization and has large contributions to the movement control [82].

1.1.6.2 Control theory of cerebellum

Differing from most other parts of the brain especially the cerebral cortex where numerous excitatory recurrent inputs occur within local circuit, the signal processing in cerebellum is almost entirely feedforward. The signals go unidirectionally from input site to output site with very small portion of recurrent internal connection, although there is the existence of mutual inhibition. This feedforward mode of operation implies that the cerebellum functions as a neuronal machine to integrate the external inputs rather than a self-sustained neuronal structure as the cerebral cortex is thought to be [80].

To fully explain the contribution of cerebellum to motor control and to delineate the internal information processing of cerebellar cortex, many theoretical models have been proposed. Over recent years, the concept of internal models has been extensively studied and has been regarded as the major theories in motor control and learning in cognitive neuroscience. Basically, internal models are neural representations that mimic the behaviour of a natural process such as body movements. By repeated trials, they continuously refine input-output relationships between motor commands and their consequences [83-85]. There are two classes of

internal models, forward and inverse models. The forward models capture the current transition behaviour of the motor apparatus in response to motor command and aim to predict the next state of behaviour [84]. Such models have been successfully applied to explain certain types of behaviour such as motor learning, state estimation and motor control [86-88]. In contrast, the inverse models invert the causal flow of the motor system. They aim to estimate the motor command that causes the particular movement, not to predict the next movement state following a specific motor command [89]. It is believed that the forward models match the organization of cerebrocerebellar connection formed between the motor cortex and the intermediated part of cerebellar hemisphere well, whereas the parallel connections between the cerebellar hemisphere to the cerebral association cortex can be well simulated by inverse models [90]. The combination of these two forms of internal models has been proven to be able to create a system with high learning capabilities [91].

1.1.6.3 Microcircuit of cerebellar cortex

Viewed from the molecular level, the cerebellum is constructed with stereotyped and relatively simple neuronal arrangements. These arrangements shown in Figure 1.4 can be regarded as a neuronal machine designed to process input information and to transmit it over output fibers in a unique way. Purkinje neuron (PN), the sole principle neuron in cerebellar cortex, receives two types of excitatory input originating from different sites. The first one is from mossy fibers (MFs), which passes sensory and motor information from the brainstem and spinal cord, and activates PNs via parallel fibers (PFs), the axons of intermediary granule cells (GCs). The second one is from climbing fibers (CFs), the axon of inferior olive. A unique feature of this arrangement is the high number of GCs; over 100 000 granule cell axons (parallel fibers) synapse onto each PN, whereas PN receives only single CF [80, 92-94]. In physiological

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experiments, these two types of excitatory input can be clearly discriminated by their identifying characteristics. The CF response is usually large and shows an all-or-none aspect. For the threshold stimulation, it is either full amplitude when the input fiber is successfully stimulated, or completely absent. On the other hand, the parallel fiber response is smoothly graded as a function of the stimulation intensity and shows paired pulse facilitation [95].

Figure 1.4. Synaptic transmission in mammalian cerebellum. The excitatory synapses are denoted by red color, and inhibitory synapses are denoted by blue. ML: molecular layer; PL: Purkinje cell layer; GL: granule cell layer; WM: white matter; PN: Purkinje cell; CF: climbing fiber; PF: parallel fiber; MF: mossy fiber; GOC: Golgi cell; DCN: deep cerebellar nuclei. Figure adapted from [94].

A peculiar feature of excitatory synaptic transmission at PN synapses is lack of functional expression of N-methyl-D-aspartate (NMDA) receptors in mature PN.

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The fast excitatory transmission is exclusively mediated by DL- α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)-selective glutamate receptor and there is no contribution from NMDA receptors [96-97]. Immunostaining studies show that the GluR2 and GluR3 subunits are dominant at the postsynaptic sites, while minor GluR1 expression is also observed [98-99]. In addition to ionotropic AMPA receptor, metabotropic glutamate receptor (mGluR) is also involved in the excitatory synaptic transmission of PN synapses. Among eight subtypes of the mGluR family that has been identified to date, mGluR1 and mGluR7 are found to be expressed in perisynaptic or extrasynaptic site outside of the postsynaptic density of PN synapses, while mGluR4 is reported to act as an autoreceptor at the presynaptic site of PF-PN synapses to modulate the synaptic transmission [100]. Puff applied specific agonist or repetitive PF stimulation evokes mGluR-mediated slow inward current in PN which is resistant to AMPA receptor blocker but sensitive to mGluR1 selective inhibitor CPCCOET [101-104]. However, the mechanism for generating the mGluR-mediated current has not been clearly identified. One hypothesis is that mGluR1 links with a nonspecific cation channel [101]. Alternatively, mGluR1 may associate with an electrogenic $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger. The Ca^{2+} flux driven by the activation of $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger gives rise to the slow current [81].

Another important feature of glutamate-mediated transmission in PNs is the regulation of intracellular Ca^{2+} signalling. Dynamics of local calcium concentration play pivotal roles in the regulation of many neuronal activities. Phenomena ranging in time scale from the submillisecond triggering of transmitter release at presynaptic terminals to the long-lasting changes in gene expression all require the action of Ca^{2+} [105]. In PNs, local calcium signal can be generated through two distinct pathways: ionotropic receptor activation and metabotropic receptor activation induced calcium

influx. In spines, because the absence of functional NMDA receptor and the presence of GluR2 subunit in postsynaptic site, which is barely permeable to Ca^{2+} , the Ca^{2+} influx following the glutamate binding to receptor channels is negligible [106]. In contrast, the postsynaptic membrane depolarization caused by the Na^+ influx through AMPA receptors is sufficient to activate local voltage-gated Ca^{2+} channels (VGCC), most of which are P/Q type, high-voltage-activated channels, resulting in fast Ca^{2+} transient [107]. In dendrite shafts to which the spines are attached, Ca^{2+} entry through VGCCs can further trigger Ca^{2+} -induced Ca^{2+} release (CICR) through ryanodine receptors (RyRs) [108]. With repetitive synaptic activity, a more complex Ca^{2+} signal consisting of two discrete temporal components can be elicited. The initial rapid component depends on the AMPA receptor activation, while the slower one is produced by Ca^{2+} release from smooth endoplasmic reticulum (ER) via mGluR1-PLC-IP3 pathway [109-110]. In PNs, mGluR1 tightly couples to G proteins of Gq family, which also link with PLC β (phospholipase C β). Thereby, the activation of mGluR1 by glutamate spillover during repetitive stimulation initiates the synthesis of IP3 (inositol trisphosphate) by PLC β . Upon IP3 binding, the opening of IP3 receptor, with IP3R1 as the predominant form, produces large Ca^{2+} release from the internal Ca^{2+} stores [111].

The glutamatergic excitatory input from parallel fibers also stimulates two types of local interneurons, stellate and basket cells, which in turn suppress the excitability of PNs by release of GABA. Basket cells mainly forms inhibitory synaptic contact with the bottleneck of a PN soma and initial axon segments, whereas the stellate cells supply inhibitory synapses onto PN dendrites [92]. In physiological conditions, the vesicular GABA release from presynaptic terminals activates postsynaptic GABA_A receptors which are permeable to chloride and bicarbonate. Due

to the low intracellular chloride concentration in mature neurons, the activation of GABA_A receptors usually causes a net entry of anion, thereby hyperpolarizing the membrane and reducing the probability of action potential firing [112-113]. By this means, the local interneurons participate in the generation of Na⁺ and Ca²⁺-dependent action potential, shape the input-output relationship of target neuron and regulate the generation and pacing of synchronous oscillatory activities of neuronal network [114-115]. Another inhibitory effect of GABA is mediated by GABA_B receptor. Contrast to GABA_A receptor mediated fast chloride current, the GABA_B receptor mediated postsynaptic response is potassium dependent. Although these slow outward potassium currents produce less change in membrane conductance compared to GABA_A receptor mediated currents, they are still strongly inhibitory because they hyperpolarize the neuron and keep the membrane potential further away from the threshold of firing [116-117].

1.1.6.4 Long term synaptic plasticity in cerebellum

Synaptic plasticity refers to the process by which the synaptic connections are structurally and functionally modified in response to external stimuli or environmental cues [118]. Activity-dependent long-term synaptic plasticity are widely believed to be the cellular basis of learning and memory and are proven to be important in neuronal development and pathological states of neuronal excitability [119]. During the past three decades, the most well characterized examples of such synaptic plasticity in central nervous system (CNS) are long-term potentiation (LTP) and long-term depression (LTD), whereby a brief period of conditioning stimuli induces persistent changes in the strength of synaptic transmission lasting at least several hours [120-121]. In cerebellum, the contribution of use-dependent synaptic plasticity to the

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associative motor learning was initially predicted by an elaborate computational model proposed by Marr and later developed by Albus [122]. According to this model, the PF inputs of PN could provide contextual information, while the CF input carries an instructive “error” signal that requires alteration of subsequent behaviour. The conjunctive activation of these two synaptic inputs could decrease the efficacy of PFs-PN synapses to create a memory trace for motor learning. As a result, the inappropriate motor signals conveyed by PFs are selectively weakened through their repeated, temporal association with CF activity [122-124].

One decade later, the experimental supports for Marr-Albus model were eventually provided by the findings that LTD at PFs-PN synapses (referred as LTD hereafter) could be consistently induced by conjunctive stimulation of PFs and CF both *in vivo* and in slice experiment [125-126]. Since that, extensive investigations concerning the cellular and molecular aspects of LTD have been carried out and the mechanisms underlying LTD induction, expression and maintenance are now well established. Essentially, there are three indispensable elements involved in the initial phase of LTD induction: calcium influx provided by CF activation or membrane depolarization, activation of AMPAR expressed at PFs-PN synapses and activation of mGluRs. The signal transduction of LTD induction is initiated by the AMPAR activation. AMPAR activation induced by glutamate release from both CF and PF terminals provides sufficient depolarization to activate VGCCs. Consequently, large amount of Ca^{2+} influx is produced by VGCCs activation. Simultaneously, glutamate in a high concentration also reacts with mGluR1s, which in turn activate PLC via $\text{G}\alpha_q$ protein, causing the production of diacylglycerol (DAG) and IP3. IP3 functions to induce IP3 receptor-dependent CICR from ER, while DAG activates protein kinase C (PKC). The high intracellular Ca^{2+} concentration produced by above mentioned

coincidental events changes the output of phosphorylation-dephosphorylation (PD) system, which is composed of protein kinases and protein phosphatases and functions to control the balance between phosphorylation and dephosphorylation of AMPARs, resulting the phosphorylation of AMPARs in PF synapses. Finally, phosphorylation at Ser880 in the PDZ-binding domain of AMPAR causes the release of AMPARs from their synaptic anchor protein, glutamate receptor interacting protein (GRIP). The free form AMPAs are further removed via clathrin-mediated endocytosis [81, 124, 127-128].

Theoretically, to maintain the stability of learning mechanisms in cerebellum, an opposing process must be present to reverse the LTD. Otherwise, the accumulation of synaptic depression will finally saturate, with no capability of further change [129]. Indeed, two forms of LTP have been identified at PFs-PN synapses so far. One is typically evoked by 120 PF-stimuli at 4-8 Hz (“4Hz” LTP), without CF activation. In contrast to LTD which is induced and expressed postsynaptically, such a 4Hz LTP is thought to result from an increase in glutamate release from presynaptic terminals and is therefore named presynaptic LTP (pre-LTP) [130-131]. Another one is found to be induced by PF stimulation alone at 1 Hz for 5 minute (“1Hz” LTP) and is thought to be postsynaptically expressed via N-ethylmale-imide-sensitive factor (NSF)-dependent GluR2 trafficking (post-LTP) [132-133]. Interestingly, it is post-LTP but not pre-LTP that is capable to reverse LTD, suggesting post-LTP is the real counterpart of LTD. Thus, the synaptic plasticity at PFs-PN synapses operates bidirectionally. That is, PF stimulation alone elicits LTP, while paired PF and CF stimulation induces LTD. The direction of synaptic change is thought to be determined by intracellular Ca^{2+} level. A high Ca^{2+} concentration produces LTD, whereas a low Ca^{2+} level produces LTP [134-136].

1.2 Modulation of GABAergic inhibitory transmission by NMDAR activation

Inhibitory GABAergic interneurons play a crucial role in the information processing of hippocampal neuronal network. They regulate the dendritic electrogenesis and spike generation of pyramidal neurons, and control the maintenance of rhythmic oscillations associated with specific behavioural states [137-140]. Moreover, interneuron activity also modulates the development of cortical circuitry and governs the long term synaptic change of excitatory synapses [114, 141]. In this respect, understanding the regulation of presynaptic GABA release would be critical and informative.

1.2.1 Interneuron diversity

Compared to the pyramidal neuron — the principle neuron in hippocampus and cerebral cortex, the local circuit inhibitory interneuron exhibits far more morphological diversities. Anatomically, they can be classified into distinct groups based on the following principles in their organization. First, the brain area- and cell domain-specific afferent inputs have strong influences on the dendritic structure of interneurons. Second, the axonal projections of interneurons selectively make contacts with different subcellular domain of postsynaptic target cells. Third, the axonal terminals of local GABAergic neurons may be well co-aligned with respect to the arrangement of their excitatory inputs [115, 142]. With the aid of development of novel techniques, including infrared visualization, single-cell anatomical reconstruction, electron microscopy, patch-clamp recording and single-cell RT-PCR, as well as the application of genetically modified animal models, 21 distinct classes of

interneurons have been identified so far [138]. It is believed that more types of interneurons exist and each type of interneuron has specific function in the control of hippocampal network activity.

1.2.2 Spatial and temporal information processing of interneurons

Functionally, local interneuron-mediated fast GABAergic inhibition can be divided into two classes: feedforward inhibition and feedback inhibition. Feedforward inhibition refers to the synaptic arrangement that excitatory afferent fibers innervate both pyramidal cells and inhibitory interneurons [143-144]. Such a disynaptic feedforward inhibition provides strong inhibition in the somatic area of pyramidal cells to shorten the duration of excitatory postsynaptic potential (EPSP), thereby limits the time window within which EPSP summates to reach the threshold for spike generation. This minimization of time window allows the pyramidal cells to precisely detect the coincidence inputs and process the information with a high degree of temporal fidelity [140, 145]. In contrast, feedback inhibition refers to the situation in which the interneuronal excitation is provided by the recurrent collaterals of local pyramidal neurons, not by the extrinsic origination. Because the feedback inhibition temporally overlaps with local recurrent excitation, it therefore functions to limit the lateral spread and the duration of recurrent excitation. Moreover, the widely spread of local circuit inhibition may contribute to the synchronization of pyramidal neurons discharges by generating a synchronous relative refractory period [146-147]. One relevant example of feedforward interneurons in hippocampus is represented by interneurons located in the stratum-lacunosum molecular (O-LM) of CA1 region which are mainly innervated by extrinsic afferent originating from the entorhinal cortex [148]. Conversely, one class of interneurons located in stratum oriens (SO),

named O-LM cell is thought to mediate feedback inhibition, because it receives most of its excitatory inputs from the axon of same population of pyramidal neurons with which it forms the inhibitory synaptic contacts in the distal dendrites [149].

Addition to the feedforward-feedback dichotomy, another striking morpho-functional dichotomy of cortical interneurons is the targeting of the perisomatic versus the dendritic domain of pyramidal neurons [150]. In hippocampus, some types of GABAergic interneurons, such as basket cells and axo-axonic cells project their axon terminals specifically onto the somata and axon initial segments of target neurons, forming the perisomatic inhibition. By contrast, other types of interneurons such as O-LM cells and bistratified cells mainly innervate the dendritic area of pyramidal neurons, providing the dendritic inhibition [151]. Electrophysiological recordings show that these two types of interneurons appear to play distinct roles in postsynaptic electrogenesis. Single inhibitory postsynaptic potential (IPSP) mediated by perisomatic inhibitory cells could suppress the repetitive firing of sodium spikes, whereas dendritic IPSPs could inhibit the discharge of calcium-dependent action potential [137]. In addition, the perisomatic inhibition is thought to regulate the synchrony of action potentials of large population of pyramidal neurons and to be involved in the generation of theta oscillation, while the dendritic inhibition is responsible for the control of the efficacy and plasticity of excitatory inputs of principle neurons and is implicated in gamma oscillation [139, 152-153]. The perisomatic-dendritic dichotomy is further complicated by the recent findings that within the perisomatic inhibition, the basket cell subtypes subserve subtle tasks in regulating the outputs of pyramidal cells. Basket cells expressing the cholecystokinin (CCK) and CB1 endocannabinoid receptors tend to integrate over long time windows and across many afferent inputs, making them ideally suited to function as a plastic

fine-tuning device. On the other hand, the parvalbumin (PV)-containing and CB1 negative basket cells appear to respond reliably and immediately to subtle and repetitive excitation, thereby faithfully reporting the timing of ongoing hippocampal activity [154-155].

1.2.3 Modulation of GABAergic transmission

A variety of neurotransmitters including dopamine, serotonin, glutamate and GABA itself have been reported to modulate the GABA release from presynaptic terminals [156-159]. Particularly, the modulatory action of glutamate is of great interest, since it directly links the excitation and inhibition together. In hippocampus, ambient glutamate released by basal activity increases the efficacy of GABAergic synapses by activating presynaptic kainate receptors (KR) [160]. In contrast, postsynaptic KRs activation by high a concentration of KR agonists markedly increases the firing frequency of interneurons, leading to massive GABA release and hence a use-dependent depression of evoked response [161]. The modulation effect of metabotropic glutamate receptor (mGluR) on GABA release is also well documented. Bath application of specific group II mGluR agonist DCG IV selectively inhibits the inhibitory transmission originating from stratum radiatum but not stratum oriens interneuron [162]. Moreover, group III mGluR activation by synaptically released glutamate could disinhibit interneurons and thereby dampen hippocampal excitability [163-164]. The effect of NMDA receptor activation on inhibitory transmission has been studied in cultured and isolated hippocampal neurons. In tissue culture, brief NMDA application can induce the reduction of surface AMPARs expression while simultaneously increasing the expression of surface GABA_ARs via NSF-dependent exocytosis [165]. In acutely dissociated hippocampal neurons, Ca²⁺ influx through

NMDA receptors activates the Ca^{2+} -dependent phosphatase which in turn suppresses the GABA_A Rs mediated response through dephosphorylation [166].

1.2.4 NMDARs and nitric oxide (NO) signalling

NMDARs are a critical component of fast excitatory synaptic transmission in central nervous system (CNS), playing pivotal roles in controlling a wide range of neuronal activities from synaptogenesis to long term synaptic plasticity. Distinct from other types of ligand-gated ionotropic receptors, NMDARs exhibit several unique biophysical properties. First, the NMDARs-mediated current flow is blocked by extracellular magnesium in a voltage-dependent manner. That is, at hyperpolarizing membrane potential, the channel pore is blocked by Mg^{2+} , while brief membrane depolarization removes Mg^{2+} block, allowing ion flow [167-168]. Second, NMDARs are highly permeable to Ca^{2+} ions. Transient Ca^{2+} influx through activated NMDARs is a key factor for both LTP and LTD inductions in cerebral cortex and therefore contributes to activity-dependent learning and memory [169]. Third, the gating kinetics of NMDARs is very slow. In slice recording, the EPSC component mediated by NMDARs activation (NMDR-EPSC) has a slow rise-time and a decay of several hundred milliseconds, 100 times longer than the mean open time of AMPARs [170-171]. Structurally, native NMDARs appear to be heteromers composed of two NR1 subunits and two NR2 subunits. To date, one NR1 subunit, four NR2 subunits (NR2A, -2B, -2C and -2D) and two NR3 subunits (NR3A and NR3B) have been identified. Work on recombinant and native NMDARs has shown that the functional and pharmacological properties of NMDARs are highly determined by the identity of NR2 subunit. In general, the NR2A/2B-containing receptors have relative fast kinetics and display a high sensitivity to divalent cations block, especially for Zn^{2+} and Mg^{2+} .

By contrast, the NR2C/D-containing receptors exhibit the slow rising and decay time constant and show a low sensitivity to Mg^{2+} block [172-173].

The rising of intracellular Ca^{2+} following NMDARs activation may induce the synthesis of nitric oxide (NO)-a multifunctional messenger characterized by low molecular weight, hydrophobic nature and high diffusion constant via a Ca^{2+} /calmodulin complex-dependent pathway [174]. In vertebrate, NO acts as a gaseous transmitter to regulate the function of CNS in many aspects, including neurogenesis, neuronal differentiation and development, learning and memory formation, as well as sleeping, feeding and other behaviours [175-177]. Generally, the modulation effect of NO is mediated by two cascades. The first one is NO-cyclic guanosine-3',5'-monophosphate (cGMP) pathway. In this scheme, NO activates soluble guanylyl cyclase (sGC), which in turn converts the GTP into cGMP. An increase of cGMP level activates cGMP-dependent kinase (cGKs, also called PKG) to modify the phosphorylation state of ion channels, thereby altering the excitability of target neurons. Additionally, cGMP can directly bind to cyclic-nucleotide gated channels and can activate cyclic-nucleotide phosphodiesterase (PDE), potentially raising the intracellular cAMP concentration [178-179]. Another NO cascade supplanting to the classical NO-cGMP pathway is S-nitrosylation-a chemical reaction by which NO covalently binds to the thio side-chains of cysteine residues of target protein without the assistance of enzymes. This cGMP-independent action of NO has been proved to have important implications in regulating the function of transcription factors, ion channels and structural proteins [180].

1.3 Objectives

Based on the knowledge of altered *UBE3A* expression profile and uncoordinated movement observed in AS patients, I reasoned that the normal function and electrophysiological properties of Purkinje cell, which provides the sole output of cerebellar cortex might be affected by the deletion of E6-AP. The electrophysiological studies on PN of AS mouse would be able to provide meaningful information on deciphering the molecular basis for the learning and memory deficit in AS and be helpful for developing effective drugs. For this purpose, the intrinsic excitability, the basal and activity-dependent modification of inhibitory transmission, as well as the basal and long-term synaptic plasticity of excitatory inputs of PN were carefully examined. Moreover, the immunostaining technique was applied to detect the expression of three GABA_A receptor subunits in cerebellar cortex to discriminate the roles played by *UBE3A* and GABA_A receptor-mediated inhibition in AS pathology. Furthermore, the candidate protein underlying the deficit of long-term synaptic plasticity observed in PN of E6-AP mutant was investigated by Western blot.

In the second part, the modulatory effect of NMDA receptor activation on GABAergic transmission was examined in hippocampal neurons with whole-cell patch clamp configuration. The spontaneous IPSCs were recorded in CA1 pyramidal neurons and the NMDA receptors were activated by transient NMDA puff application. To elucidate the possible mechanism underlying the potentiating effect of NMDA on sIPSCs, series of pharmacological tools including the NO scavenger, the NMDA receptor open-channel blocker and specific voltage-gated calcium channel blocker were applied. Particularly, a VGAT-Venus transgenic mouse strain in which the GABAergic interneuron was easily identified under fluorescence microscope was used for direct interneuron recording.

Chapter II Materials and Methods

2.1 Materials

2.1.1 Mouse strains

Mutant mice deficient in E6-AP were produced as described previously [42]. Briefly, a targeting vector designed to delete a 3 kb fragment which contains exon 2 (299 bp) of *Ube3a* was constructed and electroporated into AB2.2 ES cells (see Figure 2.1 for the preparation of targeted construct). The G418-resistant clones were screened and the positive clones were confirmed by Southern blotting using various probes prior to injection. Then, the targeted clones were injected into C57BL/6J blastocysts and multiple high percentage chimeras were bred to C57BL/6 to score the germline transmission that was obtained for two clones. These mice were maintained on a hybrid (C57BL/6 and 129/SvEv) background. Chimeras were bred to 129/SvEv mice to establish the targeted allele on 129/SvEv inbred backgrounds.

Figure 2.1. Genomic structure, targeting construct and targeted allele for deletion of exon 2 of *Ube3a*. P1, P2, and P3 are the primers designed for PCR genotyping of mice. Abbreviations: X, Xba I; B, Bam HI; H, Hin dIII; S, Sac I; R, Eco RI; Int, internal probe; HSV, herpes simplex virus; TK, thymidine kinase; Neo, neomycin

resistance cassette; and HPRT, hypoxanthine guanine phosphoribosyl transferase. Figure adapted from [42].

Both wild type and mutant mice were kept in the same room at the animal facility at the Nanyang Technological University. For the hippocampal slice preparation, control C57BL/6 mice and VGAT-Venus transgenic mice [181] were kept in animal room of Takushima Bunri University. All experiments were carried out according with the National Advisory for Laboratory Animal Research guideline of Singapore and Guidelines for Proper Conduct of Animal Experiment of Japan to minimize the suffering of animals.

2.1.2 PCR primers

The primer sites were as diagrammed in Figure 2.1. The primer sequences were specified below: P1: genomic forward, 5'-ACTTCTCAAGGTAAGCTGAGCTTGC-3'; P2: reverse, 5'-GCTCAAGGTTGTATGCCTTGGTGCT-3'; P3: HPRT forward, 5'-TGCATCGCATTGTGTGAGTAGGTGTC-3'.

2.1.3 Chemicals for Molecular biology

Table III. List of Chemicals for Molecular biology

Chemicals	Usage	Company
Proteinase K	DNA extraction	Roche (Basel, Switzerland)
Phenol, Phenol/Chloroform	DNA extraction	Bio-Rad (California, USA)
DNA polymerase, dNTP	PCR	Bio-Rad (California, USA)
Polyacrylamide, SDS running buffer, Transferring buffer	Western blotting	Bio-Rad (California, USA)
BCA protein kit	Western blotting	Pierce (Illinois, USA)
ECL detection kit	Western blotting	Amersham (New Jersey, USA)

2.1.4 Antibodies

The antibodies used in this study are listed below and the dilutions times used in the Western blotting (WB) and immunostaining (IHC) experiments are also specified.

Table IV. List of antibodies

Primary antibody	Dilution	Company
Rabbit polyclonal to UBE3A	1:250 for IHC 1:1000 for WB	Abcam, UK
Rabbit polyclonal to GABA _A α5	1:250 for IHC	Abcam, UK
Rabbit polyclonal to GABA _A β3	1:250 for IHC	Abcam, UK
Rabbit polyclonal to GABA _A γ3	1:250 for IHC	Abcam, UK
Rabbit polyclonal to pT286	1:500 for WB	Promega, USA
Mouse monoclonal to GAD65	1:100 for IHC	Abcam, UK
Mouse monoclonal to β-CaMKII	1:500 for WB	Invitrogen, USA
Mouse monoclonal to α-CaMKII	1:500 for WB	Upstate, USA
Secondary antibody	Dilution	Company
Goat to Rabbit IgG (Biotin)	1:250 for IHC	Sigma, USA
Goat to Rabbit IgG (HRP)	1:1000 for WB	Abcam, UK
Goat to Rabbit IgG (Cy5)	1:250 for IHC	Abcam, UK
Goat to Rabbit IgG (FITC)	1:200 for IHC	Abcam, UK
Rabbit to Mouse IgG (HRP)	1:1000 for WB	Pierce, USA

2.1.5 Chemicals for electrophysiology

Unless otherwise stated, all the chemicals were of at least analytical grade. NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), Picotoxin (1:1 mixture of picrotoxinin and picrotin), TTX (Tetrodotoxin), D-APV (D-2-Amino-5-phosphonovalerate), NMDA (N-methyl-D-aspartate), SR95531 hydrobromide (2-(3-Carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide), QX-314 (N-(2,6-Dimethylphenylcarbamoymethyl)triethylammonium bromide) and CPCCOET

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(7-Hydroxyiminocyclopropan[b]chromen-1 α -carboxylic acid ethyl ester) were obtained from Tocris Bioscience (Bristol, UK); (+)-MK-801 hydrogen maleate ((5S,10R)-(-)-5-Methyl-10,11-dihydro-5H-dibenzo-[a,d]-cyclo-hepten-5,10-imine hydrogen maleate), DEANO (diethylamine nitric oxide, sodium salt) and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) from Sigma (St. Louis, USA); ω -Agatoxin TK and ω -Conotoxin GVIA from Peptide Institute (Osaka, Japan). All drugs were prepared in stock solution and were diluted into working concentration immediately before each experiment. 0.25-0.5 mg/ml BSA was co-applied when testing the effect of calcium channel blockers to prevent the absorption to perfusion line [182].

2.1.6 Solutions

Table V. List of solutions

Buffers for	Usage	Composition (mM)
Molecular biology		
NTES	DNA extraction	50 Tris, 50 EDTA, 100 NaCl, 5 DTT, 0.5 Spermidine and 2% SDS
TE	Dissolving DNA	0.1 Tris-HCl and 10 EDTA
PBS	IHC	20 phosphate, 150 NaCl
Lysis buffer	Protein extraction	100 NaF, 150 NaCl, 1 PMSF, 50 Hepes, 1% Triton X-100, 5 μ g/ml Pepstatin A, 5 μ g/ml Leupeptin, 2 μ g/ml Aprotinin, and 1 Na_3VO_4
6 X Loading dye	Electrophoresis	125 Tris-HCl, 2% SDS, 20% glycerol and 0.2% bromophenol blue
TBST	WB	20 Tris-HCl, 500 NaCl and 0.1% Tween 20

Buffers for Electrophysiology	Usage	Composition (mM)
ACSF [183]	External solution	138.6 NaCl, 3.4 KCl, 2.5 CaCl ₂ , 1.0 MgCl ₂ , 21.0 NaHCO ₃ , 0.6 NaH ₂ PO ₄ and 10 glucose (bubbled with 95% O ₂ and 5% CO ₂)
Cutting solution 1 [184]	Cerebellar slice preparation	299.2 Sucrose, 3.4 KCl, 0.3 CaCl ₂ , 3.0 MgCl ₂ , 10 Hepes, 0.6 NaH ₂ PO ₄ and 10 glucose (constant bubbled with pure O ₂)
Cutting solution 2 [185]	Hippocampal slice preparation	110 Choline chloride, 2.5 KCl, 2.0 NaH ₂ PO ₄ , 25 NaHCO ₃ , 0.5 CaCl ₂ , 7 MgCl ₂ , 20 Glucose, 1.3 Sodium ascorbate and 0.6 Sodium pyruvate (constant bubbled with pure O ₂)
Internal solution 1 [186]	For WCR except membrane excitability measurement	140 CsCl, 10 Hepes, 2 QX-314, 3 MgATP, 0.1 EGTA and 0.4 Na ₃ GTP (pH 7.4 adjusted with CsOH)
Internal solution 2 [187]	Membrane excitability measurement	136.5 KCl, 10 EGTA, 10 Hepes, 4 MgATP, 0.4 Na ₃ GTP (pH 7.4 adjusted with KOH)

2.2 Methods

2.2.1 Genotype identification

On 7-10 postnatal days, 1-2 cm tail was cut and the pup was clearly labelled. The tails were thoroughly digested in 0.5mg/ml proteinase K containing NTES buffer by overnight shaking at 55 °C. On the following day, equal volume of phenol (equilibrated with Tris, pH 8) was added and the microfuge tubes were shaken vigorously for 1 hr at RT, ensuring phases could be mixed completely. The phases were separated by centrifuging at 15, 000 rpm for 15 min and the upper layer was

gently transferred to a fresh tube. 700 μ l Phenol/chloroform (1:1) was added and the tube was shook again. After centrifuge, the organic phase was discarded and 1 ml ethanol and 70 μ l 3 M sodium acetate (pH 7.0) were added to precipitate the DNA. After washing with 70% ethanol, the pellet was dissolved in 100 μ l TE buffer and the DNA concentration was measured with NanoDrop spectrophotometer (Thermo Scientific, DE, USA).

The 20 μ l PCR reaction mixture contained the following component: 2 μ l of 10 X enzyme buffer, 2 μ l of 10 μ M primer 1, 0.4 μ l of 10 μ M primer 2, 2 μ l of primer 3, 0.1 μ g genomic DNA, 0.5 μ l of 10 mM dNTP mix, 0.2 μ l of 5 U Taq DNA polymerase and appropriated volume of dd H₂O. The PCR reaction was performed as following: 95 °C for 5 min, 95 °C for 30 seconds, 65 °C for 1 min, 72 °C for 1 min and cycled 35 times for optimal amplification of the target fragments. The PCR product was examined by agarose gel electrophoresis and the image was captured with Syngene Bio Imaging System (Syngene, Cambridge, UK). The samples showed only one band at approximately 700 bp were identified as control mouse, while the samples had one more band at approximately 300 bp were regarded as E6-AP mutant [42].

2.2.2 Immunostaining

Mice of both wild-type and mutant (20-28 days old) were deeply anesthetized with halothane and transcardially perfused with 0.1 M PBS, pH7.3 at room temperature (RT) followed by ice-cold 4% paraformaldehyde. After perfusion, the cerebellum was removed and post-fixed in the same fixative for at least 2 hr. The fixed tissue was then immersed in 10% sucrose containing PBS buffer at 4 °C overnight for cryoprotection.

On next day, the sucrose concentration was increased to 20% for another overnight incubation. After dehydration, the blocks were embedded in O.C.T compound (Sakura Finetek, CA, USA) and frozen in liquid nitrogen. Frozen sections were cut sagittally by a freezing Microtome (Leica, Nussloch, Germany) with 15 μ m thickness and mounted onto histidine-coated glass slides (Menzel GmbH, Braunschweig, Germany).

For staining of E6-AP and α 5, β 3, γ 3 subunit of GABA_A receptor individually, sections were first blocked with 5% normal goat serum in 0.1 M PBS for 1 hr at RT and then incubated in rabbit polyclonal anti-E6-AP or anti- α 5, β 3, γ 3 antibody at 4°C overnight. After 3 times rinsing with 0.1 M PBS, slides were incubated with FITC conjugated goat anti-rabbit IgG at 37 °C for 1 hr inside dark box. After rinsing several times, sections were coverslipped and observed with a Nikon TIRF microscope equipped with differential interference contrast optics and a Retiga EXI digital camera (Qimaging, BC, Canada).

For double staining of 65 KDa isoform of glutamate decarboxylase (GAD65) and E6-AP, sections were incubated with these two antibodies sequentially. Briefly, sections were first stained with rabbit polyclonal anti-E6-AP and Cy5 conjugated goat anti-rabbit IgG to visualize E6-AP expression pattern. After that, sections were stained with mouse monoclonal anti-GAD65 and FITC-labelled goat anti-mouse IgG to visualize the distribution of GABAergic interneurons in cerebellar cortex.

2.2.3 Western blot

After decapitation, mouse brain was immediately removed and the cerebellum and forebrain were dissected. The tissues were homogenized in 400 μ l lysis buffer. Protein concentrations were determined by using BCA protein kit. The sample was first

mixed with 6 X loading buffer and heated at 100 °C for 7 min. After that, total 20 µg of protein sample was loaded into each lane of 12% SDS-polyacrylamide gel and the electrophoresis was carried out at constant voltage of 100 mV till the tracking dye running out of the separating gel.

After running, the gel was directly processed for electroblotting. In this step, the Hybond-ECL nitrocellulose membrane (Amersham Biosciences, NJ, USA) and the gel were overlaid on each other and sandwiched between 4 filter papers inside the transfer cassette. The transfer cassette was then placed inside the transfer cell in the right orientation ensuring the proteins could be transferred from the gel onto the nitrocellulose membrane. The transfer process was conducted at a constant voltage of 100 V for 2 hrs in cold room with constant stirring to disperse the heat.

After transfer, the membrane was first treated with 5% milk containing TBST solution for 1 hr at RT to block the non-specific binding. Then the membrane was incubated in primary antibody (appropriate antibody with proper dilution) for overnight at 4 °C. The membrane was then washed with TBST solution for 3 times, 15 minutes per wash. After incubation with the appropriate secondary antibody (with proper dilution) the signals were detected by ECL advance Western Blotting Detection Kit.

2.2.4 Slice preparation

Sagittal cerebellar slices (250µm thickness) were prepared from the cerebellum of wild-type and E6-AP mutant mice (16-26 days old) following standard protocol [158]. Briefly, animal of either strain was deeply anaesthetized with halothane inhalation and their brains were rapidly removed. Sagittal slices were cut by using a linear slicer pro7

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(DSK, Osaka, Japan) at 4 °C in a Na⁺-deficient saline (cutting solution formula 1). The slices were incubated in a humidified and oxygenated chamber with an interface of artificial cerebrospinal fluid (ACSF) at least for 1 hr at RT before making recording.

C57BL6/J mice with the age of postnatal 11-16 days were adapted for hippocampal slice preparation. Transverse slices of the right hemisphere were cut at 300 µm thickness in ice-cold cutting solution (formula 2, see materials) by using a VT1000S microtome (Leica, Nussloch, Germany).

2.2.5 Whole-cell patch clamp recording (WCR) from Purkinje cells

Visualized whole-cell patch clamp recordings were conducted at room temperature following conventional method [188]. After incubation, the slices were transferred to a recording chamber mounted on the stage of BX51WI microscope (Olympus, Tokyo, Japan) and continuously perfused with the oxygenated ACSF at a flow rate of 1.0-1.5 ml/min. Patch electrode had resistances of 3-4 MΩ when filled with intracellular solution. PNJs were visually identified under Nomarski optic with a water-immersion objective (Olympus, Tokyo, Japan). Membrane currents and potentials were recorded with a Multiclamp 700B amplifier (Axon Instrument, CA, USA). The accessing resistance monitored through experiments was in the range from 15-25 MΩ and was not compensated. Data were discarded if this value changed by more than 20%. All signals were filtered at 1k Hz and sampled at 5k Hz. All data were reported as the mean ± standard error of the mean (SEM). The statistical analysis was performed using unpaired Student-test for two independent means, with p values of less than 0.05 being considered statistically significant.

2.2.6 Membrane excitability evaluation

A series of current steps, each lasting 500 ms, were delivered to the PNs at intensities from -600 to +600 pA with 100 pA step increment and 5 s step interval when the PN soma was constantly injected with -200 pA current. Input resistance was determined by measuring the maximal voltage deflection (the difference between the baseline and the maximal voltage reached during the hyperpolarizing current injection) [189]. The activity of the inward rectifying cationic current channel (I_h) was measured as the difference between the maximal negative voltage and the plateau voltage deflection [190]. The maximal firing frequency (MMF) was calculated by measuring the spike frequency at the highest depolarizing current injection at which the PN fired throughout the step duration. From the same step, the first inter-spike interval (ISI) was measured. The threshold for action potentials (APs) was measured from the first spike of the step to -100 pA current injection. The threshold for APs firing was defined as the first point of rising phase where the velocity entered within the range 30-60 mV/ms. The AP amplitude was measured from the same spike as the difference between the peak and the threshold voltage. The amplitude of the afterhyperpolarization following action potential (AP-AHP) was measured as the difference between the AP threshold and the negative peak of AHP [187].

2.2.7 sIPSC and mIPSC recordings

Cerebellar interneuron-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 10 μ M NBQX. These IPSCs were completely abolished by 100 μ M picrotoxin, indicating that they were mediated by GABA_A receptors. Miniature IPSCs (mIPSCs) were recorded in the presence of both 10 μ M NBQX and 0.2 μ M TTX to block the action potential dependent GABA release from

presynaptic sites. The IPSC events were detected using Minianalysis (Synaptosoft, NJ, USA). The threshold value for detecting IPSCs was set as twice the baseline noise (root-mean-square, RMS) and the events detected by the software were visually checked to minimize errors. A fixed number of mIPSCs from each neuron (i.e., the 1st 100 mIPSCs) were pooled for conducting histograms for amplitude and inter-events interval cumulative probability and the Kolmogorov-Smirnov (K-S) two samples, two-tailed test was used to compare the cumulative probability of mIPSCs between control and mutant group. For kinetic analysis, the 10-90% rising time and decay time of IPSCs were automatically measured by Minianalysis.

2.2.8 Synaptic plasticity of inhibitory transmission

The conditioning protocol for depolarization-induced suppression of inhibition (DSI) and rebound potentiation (RP) induction consisted of 10 pulses membrane depolarization from -70 mV to 0 mV at 1 Hz with each pulse lasting 500 ms. DSI was quantified as the mean sIPSC frequency change and RP was expressed as the mean sIPSC amplitude change. The timing for DSI calculation initiated from 10 sec after depolarization to avoid the interference of large calcium-activated chloride conductance [191] and ended 100 sec after depolarization.

2.2.9 Long-term synaptic plasticity of excitatory transmission

A stimulating pipette filled with ACSF was positioned about 200 μm away from the recorded cell in the molecular layer and was used to stimulate parallel fibers (PFs). PF elicited excitatory postsynaptic current (EPSC) was identified by its normal amplitude and a marked facilitation of successive response: for two consecutive stimuli, the second response is bigger than the first one [95]. Test responses were evoked at a

frequency of 0.05 Hz using 10-40 V pulses with 80 μ s duration. After at least 5 min stable baseline recording, 120 PF stimulation repeated at 4 Hz was applied to induce presynaptic PF-LTP [192] and 300 PF stimulation was applied at 1 Hz to induce postsynaptic LTP [133, 135].

To monitor long-term depression (LTD) of PF-PNs, two distinct conditioning protocols were adapted. In protocol one, the stimulus intensity was adjusted to evoke PF-EPSC whose basal amplitude was 100-200 pA. After stable recording, conjunctive membrane depolarization (from -70 mV to -20 mV, lasting 200 ms) and PF stimulation were applied 20 times at 1 Hz [193]. The other LTD induction protocol consisted of 300 times PF stimulation in conjunction with depolarization, repeated at 1 Hz [194-195].

2.2.10 WCRs from hippocampal slice

CA1 hippocampal pyramidal neurons were identified under infrared-differential interface contrast (IR-DIC) video microscopy at room temperature and WCRs from pyramidal neurons were achieved using a Multiclamp 700B amplifier (Axon Instruments, CA, USA). When tested the effects of intracellularly applied NMDA channel blocker, 1 mM MK-801 was included in the pipette [196]. The accessing resistance monitored by a constant membrane test pulse throughout experiments was in the range from 25–35 M Ω and was not compensated. CA1 pyramidal neurons were held at -60 mV to record membrane currents and synaptic currents. Signals were digitized by the pClamp9 program through an A/D converter, Digidata 1322A (Axon Instruments, CA, USA). NMDA (100 μ M) prepared in Mg²⁺-free ACSF was puff-applied through the micropipette placed in the vicinity of recorded pyramidal neurons.

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To investigate the effects of NMDA on interneurons, hippocampal slices were prepared from VGAT (vesicular GABA transporter)-Venus transgenic mice [181] whose GABAergic interneurons could be clearly identified under fluorescence microscope and recordings were made from interneurons under current-clamp mode without current injection. Because puff application of NMDA induced highly frequent firings in interneurons, signals were analyzed by the Clampfit program (Axon Instruments) to measure the peak amplitude and decay time of postsynaptic NMDA response. Basal and NMDA-induced synaptic events were analyzed by the MiniAnalysis program (Synaptosoft, NJ, USA). The paired two-tailed Student's t-test was used for comparisons between two groups. Data are expressed as means $\pm$ SEM in all cases.

Chapter III Physiological properties of cerebellar circuit in a mouse model for Angleman Syndrome

As has been described in the literature review part, the main purpose of this study is to systematically determine how deletion of E6-AP affects the physiological properties of cerebellar circuit and to elucidate a possible mechanism for this alteration. Therefore, this chapter contains three parts: 1) evaluation of intrinsic properties of PN including its response to hyper- and depolarizing currents; 2) examination of basal inhibitory synaptic transmission of PNs and activity dependent synaptic plasticity of inhibitory transmission, as well as the GABA_A subunit expression and 3) study of basal excitatory synaptic transmission of PN and the long term synaptic change of PFs-PN synapses responding to the conditioning stimuli.

3.1 Results

3.1.1 Intrinsic excitability of PN

Neuronal excitability is defined as a propensity of the neuron to generate the action potential from a given input signal. At the mechanism level, this process requires the opening of voltage-gated ion channels located in the neuronal membrane when an excitatory synapse is activated [197-198]. PN action potential is generated at the soma-axon hillock [199], where all input signals converge and are integrated, determining the final output signal of the cerebellar cortex. Our hypothesis is that the deletion of E6-AP in the PN possibly affects the function or expression of ion channels which may be involved in the generation of action potential, thereby alters the intrinsic excitability of PN. To address this hypothesis, the passive and active membrane properties of PNs were examined.

Figure 3.1. Purkinje neuron responses to hyperpolarizing currents. (A) Representative traces of voltage deflection evoked by different current steps (-100, -200, -300, -400 and -500 pA). IR: inward rectification. PA: peak amplitude. (B) *Left*: plots of peak amplitude versus injected current in control. *Middle*: amplitude-current injection curve in mutant. *Right*: histogram of input resistance. (C) *Left*: Plots of IR amplitude versus current in control. *Middle*: same curve drawn from mutant. *Right*: histogram of IR index.

3.1.1.1 PNs response to hyperpolarizing currents

PNs are known to respond in a complex fashion to negative current injection, mainly because of the inward rectifier non-selective cationic current— I_h expressed on the soma [200]. The voltage deflection responding to hyperpolarizing current injection is comprised of a negative voltage jump followed by a short rising phase and remains stable on a plateau (Fig 3.1A). The negative deflection is caused by the passive membrane response and it changes the direction when the I_h current is activated to depolarize the membrane. In this respect, I used the peak of the negative voltage deflection to calculate the input resistance to minimize the interference of I_h current [190] and used the amplitude shift from the peak to the steady level of potential to estimate the intensity of I_h current. For better measurement, five different intensities of current injection were used to draw a voltage deflection-current injection curve and the slope of the best-fitting line was regarded as an index of inward rectification (Fig 3.1B), an index of input resistance (Fig 3.1C), respectively. Measured in this way, the input resistance of mutant was $99.05 \pm 4.0 \text{ M}\Omega$, and $97.52 \pm 3.31 \text{ M}\Omega$ in control ($n=18$, $p>0.05$, Fig 3.1C). For the I_h channel, this value was $34.45 \pm 0.62 \text{ mV}$ in mutant and $35.38 \pm 1.76 \text{ mV}$ in control group ($n=18$, $p>0.05$, Fig 3.1B).

3.1.1.2 PNs response to depolarizing currents

Active membrane properties were evaluated by delivering steps of depolarizing current into the PN soma. As shown in Fig 3.2A, the firing of action potential (AP) was proportionate to the injected current and showed significant adaptation at high current steps. Here, I adapted two parameters, named maximal firing frequency (MFF) and inter-spike interval (ISI) to measure the firing capability of PNs. MFF was referred to the spiking frequency at the highest current injection step that evoked a PN

discharge throughout the current step [187], and ISI was regarded as the first inter-spike interval. Two-tailed Student's-test showed no significant difference between normal and mutant group for both of these parameters (for MFF, 131.66 ± 4.79 Hz in control, $n=18$, and 125.35 ± 5.37 Hz in mutant, $n=22$, $p>0.05$; for ISI, 4.43 ± 0.26 ms in control, $n=18$, and 4.84 ± 0.33 ms in mutant, $n=22$, $p>0.05$, Fig 3.2B).

Figure 3.2. Measurement of active membrane properties. (A) Representative traces of PN response to depolarizing current steps (0, +400 and +600 pA). The MFF and ISI

were measured from the middle row where PN firing extended throughout the step duration. (B) The values of MFF and ISI were not significantly different between two groups. (C) Sample traces showing the action potential evoked by depolarizing current injection. *Upper*: trace from control. *Bottom*: trace from mutant. (D) No difference was detected for the AP amplitude and AP-AHP between control and mutant group.

Next, I examined a variety of parameters of action potential evoked by depolarizing current injection in mutant mice. Firstly, the threshold of AP which was defined as the membrane potential at which the rate of depolarization entered within the range of 30-60 mV/ms was measured (Fig 3.2C). In mutant group, this value was -56.45 ± 0.73 mV and was similar to the value of -58.10 ± 0.85 mV in control group ($n=18$ for control and $n=20$ for mutant, $p>0.05$). Secondly, the mean amplitude of AP which was calculated as the voltage deflection from the resting membrane potential to the peak of AP was compared. Statistic analysis showed there was no significant difference in terms of this parameter ($P>0.05$, Fig 3.2C and D). Finally, the amplitude of the after-hyperpolarization following each action potential (AP-AHP) was measured. Also, no significant difference was observed for this value in mutant relative to the control mice ($p>0.05$, Fig 3.2D).

3.1.2 Inhibitory synaptic transmission

Inhibitory GABAergic interneurons play a crucial role in the information processing of neuronal network. They regulate the dendritic electrogenesis and spike generation of principle neurons, and control the maintenance of rhythmic oscillations associated with specific behavioural states [137-140]. In cerebellar cortex, local GABAergic interneurons provide powerful inhibitory inputs onto Purkinje neurons to regulate its excitability and play pivotal roles in the regulation of long term synaptic change of

excitatory synapses. From this point of view, it would be informative to know how the deletion of E6-AP affects the properties of inhibitory inputs of PN, thereby indirectly affects the output of PN. For this purpose, the sIPSC, mIPSC, as well as the short term and long term plasticity of inhibitory transmission were examined.

3.1.2.1 Spontaneous IPSC recordings

When recorded under voltage clamp mode and hold the membrane at -70 mV, with a Cs-filled pipette and normal ACSF outside, frequently occurring spontaneous synaptic currents with variable amplitude were observed (Fig 3.3A and B). The complete blockade of spontaneous activities by addition of 100 μ M picrotoxin in the external solution confirmed that these currents were mediated by synaptic GABA_A receptors and were inhibitory in nature (Fig 3.3B). Statistic analysis showed that the mean amplitude of sIPSC in mutant was slightly but not significantly high than that in control group (122.86 ± 12.49 pA, $n=30$ for control; 143.72 ± 15.54 pA, $n=30$ for mutant, $p>0.05$, Fig 3.3C). The same situation happened for sIPSC frequency measurement. The mean frequency was 5.45 ± 0.39 Hz in mutant and was not significantly different from 4.97 ± 0.43 Hz in control mice ($p>0.05$, Fig 3.3C).

The inhibitory synaptic inputs of PN are mainly originated from local GABAergic interneurons — basket cells and stellate cells. Both of these two types of interneurons are located in the molecular layer, but the former is more close to the Purkinje cell layer. Histologically, the inhibitory synapses between basket cells and PNs are densely formed at the bottleneck of PN soma while synapses between stellate cells and PN are concentrated at PN dendrites [80, 92]. Such a position specific distribution pattern of inhibitory synapses are thought to associated with specific

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actions and could be discriminated by measuring the rise time of synaptic events [201-202]. Therefore, I compared the kinetics of sIPSC to see whether the composition of sIPSC was altered due to E6-AP deletion. Statistic analysis showed that no significant difference were detected (for 10-90% rising time, 4.38 ± 0.41 ms, $n=30$ for control, 4.60 ± 0.36 ms, $n=30$ for mutant, $p>0.05$; for decay time, 16.83 ± 0.87 ms for control, 17.68 ± 0.78 ms for mutant, $p>0.05$, Fig 3.3D), suggesting the distribution of inhibitory synapses was normal in mutant.

Figure 3.3. Spontaneous IPSC recorded in PN. (A) and (B) Examples of sIPSC recordings from control (A) and mutant (B) mice. The boxed parts of the recordings were shown in expanded scale accordingly. (C) Summary data of the frequency and amplitude of sIPSC. No difference was observed for both parameters between control and mutant ($p>0.05$). (D) Pooled data of the kinetic of sIPSC. Also, no statistic difference was detected ($p>0.05$).

3.1.2.2 Miniature IPSC recordings

Spontaneous IPSC recorded in the absence of tetrodotoxin (TTX) consists of two different components: action potential-dependent IPSC and action potential-independent IPSC — mIPSC. The mIPSC is caused by the randomly single vesicular release and is insensitive to TTX, while the action potential-dependent IPSC is driven by the activation of voltage gated calcium channels expressed at the presynaptic terminals and is produced by the synchronous GABA release [203-204]. To examine the effect of E6-AP on mIPSC, I recorded inhibitory synaptic currents in the presence of 0.2 μ M TTX. As shown in Fig 3.4A and B, addition of TTX largely decreased the frequency and amplitude of spontaneous events in both control and mutant slices (see Fig 3.3 for the difference). The mean amplitude of mIPSCs was 37.96 ± 2.58 pA in control and 39.0 ± 3.86 pA in mutant, with no difference observed ($p > 0.05$). Similarly, the mean frequency of mIPSCs was also not changed in mutant (3.27 ± 0.61 Hz, $n = 15$ for control; 3.79 ± 0.44 Hz, $n = 16$ for mutant, $p > 0.05$). The cumulative curves of inter-events interval and amplitude of mIPSCs were also not significant different ($p > 0.05$, K-S test, Fig 3.4C and D), providing further evidence that the basal inhibitory synaptic transmission between local GABAergic interneurons and PN functioned normally.

3.1.2.3 DSI and RP

Activity dependent change of synaptic efficacy is considered to be the basis for learning and memory in the brain and has been extensively studied [118, 205-206]. As for the inhibitory synapses formed between local interneurons and PNs, two forms of synaptic plasticity — DSI and RP have been well documented and are examined here. DSI is referred to the phenomenon that membrane depolarization induced transient

inhibition of sIPSCs and is expressed presynaptically as a suppression of transmitter release [207-209]. To examine DSI, I first made 2 min baseline recording and the mean frequency of sIPSC was calculated. Then, the PN was subjected to depolarization for total 5 sec (500 ms duration, 10 pulses), and recording continued for another 2 min. As can be seen in Fig 5A, DSI was clearly observed in both mutant and control group in terms of sIPSC frequency and amplitude reduction after depolarization. To quantify DSI, I measured the mean sIPSC frequency for each 10 sec epoch during the trials and drew the time course with the pooled data. In addition, the normalized sIPSC frequency at 30 sec after depolarization was calculated to evaluate the extent of DSI occurred in each group. Measured in this way, the time course and extent of DSI were almost identical between mutant and control group ($54.45 \pm 4.7\%$, $n=10$ for control; $51.38 \pm 5.3\%$, $n=10$ for mutant, $p>0.05$, Fig 3.5B and C), implying the normal induction and expression of short-term synaptic plasticity of inhibitory transmission in PNs of mutant mice.

In contrast to DSI, RP is thought to be a long-term phenomenon as the increase of sIPSC amplitude induced by membrane depolarization could last for long time and is regarded to be expressed postsynaptically as an increase of postsynaptic GABA_A receptor sensitivity [210-212]. For this reason, at least 15 min recording was made in each trial and the normalized sIPSC amplitude was used to measure the extent of RP. Shown in Fig 3.6A and B, after the cessation of DSI, the amplitude of sIPSC gradually increased and remained at a steady level for several min. Calculated at 10 min after conditioning, the RP was slightly low in mutant compared to that in control group ($154.47 \pm 9.87\%$, $n=4$ for control, $149.04 \pm 5.54\%$, $n=5$ for mutant, $p>0.05$, Fig 3.6C and D), indicating that the long term synaptic plasticity of inhibitory synapses was inducible but with a relative low extent.

Figure 3.4. Quantitative analysis of miniature IPSC. (A) and (B) Representative traces of miniature IPSC recorded in wild-type (A) and mutant (B). The left traces were 45 s recordings of mIPSC from normal and mutant mice, respectively. Right traces were expansion of the boxed parts of recordings (1 and 2) in left traces. (C) *Left*: cumulative curve for the mIPSC amplitude in control. *Right*: histogram for inter-event intervals of mIPSC in control. (D) Same analysis in mutant. Note, the amplitude and the inter-event curves of mutant mice were similar to their controls ($p > 0.05$, K-S test).

Figure 3.5. Depolarization induced suppression of inhibition (DSI) analysis. (A) *Left*: demo trace showing the change of sIPSC induced by depolarization in wild-type. *Right*: sample traces recorded in mutant. The fragment indicated by the *bar* in top traces of both control and mutant were exemplified in low time scale in bottom traces. (B) The time course of sIPSC frequency change. Each dot represented the mean sIPSC frequency within 10 sec epoch. (C) Bar chart of the DSI calculation measured 30 sec after depolarization.

Figure 3.6. Rebound potentiation (RP) of sIPSC in PNs. (A) and (B) Representative traces of sIPSC change induced by depolarization in control (A) and mutant slices (B). Note, the increase of mean amplitude of sIPSC was persistent in both groups. (C) Normalized mean sIPSC amplitude before and after the conditioning stimuli (100 ms duration, 1 Hz, repeated 5 times). The mean amplitude of sIPSC recorded 2 min before the conditioning was taken as reference. (D) Bar graph of the relative sIPSC amplitude enhancement (RP, %) over baselines (set as 0%) in both control and mutant groups.

3.1.2.4 E6-AP and GABA_A receptor subunits expression

Because of their locations within the AS-deletion region and close proximal to *UBE3A* gene, a cluster of GABA_A receptor subunit genes, named $\alpha 5$, $\beta 3$ and $\gamma 3$, which encoded the GABA_A receptor protein subunits $\alpha 5$, $\beta 3$ and $\gamma 3$ respectively, are thought to be involved in the severe epilepsy observed in AS cases and their expression level are proposed to be under the control of E6-AP [70, 213]. In this part, I took the advantage of E6-AP mutant mice to directly examine the distribution of these three subunits in cerebellar cortex to prove above hypothesis.

I first checked the E6-AP expression level in mutant. The Western blot and immunofluorescence data shown in Fig 3.7A-D was consistent with the initial report that E6-AP was abundantly expressed in Purkinje cell layer and granule cell layer of control slice while there was no detectable signal in mutant mice [42]. Interestingly, the fluorescence signal exhibited in the molecular layer of control mice suggested that only certain cell types, most likely to be GABAergic interneuron expressed E6-AP. To confirm this, I used 65 kDa isoform of glutamate decarboxylase (GAD65) which was specifically distributed at the presynaptic inhibitory synaptic terminals as a neuronal marker to identify the cell type. The image data from Fig 3.7E-F clearly showed that E6-AP colocalized well with GAD65 in the Purkinje cell soma, dendrites and GABAergic interneuron in molecular layer. Interneurons lack E6-AP staining also had no detectable GAD65 signal, suggesting that E6-AP was specifically expressed in GABAergic inhibitory neurons, not glutamatergic neurons in the molecular layer of cerebellar cortex.

Next, I examined the distribution of $\alpha 5$, $\gamma 3$ and $\beta 3$ subunits of GABA_A receptor in mutant slices individually. In cerebellum, total 13 GABA_A receptor subunits ($\alpha 1$ - $\alpha 6$, $\beta 1$ - $\beta 3$, $\gamma 1$ - $\gamma 3$ and δ) are found to be heterogeneously expressed [214],

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and the majority of GABA_A receptors are composed of $\alpha 1$, $\alpha 6$, βx , $\gamma 2$ or δ subunits [215]. Consistent with these findings, my image data showed that all of the three specific subunits were detectable in cerebellar cortex, with $\alpha 5$ subunit had the faintest immunoreactivity and the $\beta 3$ subunit exhibited the most intensive fluorescence (Fig 3.8A-F). The expression of $\alpha 5$ subunit in mutant appeared to be identical as that in control slices (Fig 3.8A and B). However, slightly difference was observed for $\gamma 3$ and $\beta 3$ subunits, especially in the granule cell layer (Fig 3.8 C-F). The filament staining pattern of $\beta 3$ subunit in granule cell layer was absent in mutant. Instead, the $\gamma 3$ subunit showed filament staining in granule cell layer of mutant which was not observed in control slide. Possibly, there was a homeostatic up-regulation of $\gamma 3$ subunit to compensate the low expression of $\beta 3$ subunit. Taken overall, these data demonstrated that there were no significant differences in terms of expression of these three subunits between mutant and control mice in cerebellar cortex.

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Figure 3.7. Expression of E6-AP in cerebellar cortex. (A) A typical genotyping result. Neg: negative control without DNA template. M: 100 bp DNA ladder. Sample exhibited 2 bands was regarded as E6-AP mutant (labelled as m-/p+). (B) The expression of E6-AP detected by Western blot. (C) and (D) The comparison of E6-AP expression by immunofluorescence staining. (E) and (F) The staining of E6-AP and GAD65 expression individually. (G-H) Double-staining of E6-AP and GAD65 in cerebellum of wild-type. Arrows (G and H) indicated E6-AP and GAD65 positive interneuron, and triangle represented E6-AP and GAD65 negative neurons in molecular layer. Scale bar: 150 μ m.

Figure 3.8. The expression of GABA_A receptor subunits in cerebellar cortex. (A) and (B) Almost identical expression level of $\alpha 5$ subunit between control and mutant. (C) and (D) Distribution of $\gamma 3$ subunit in cerebellar cortex. Note: the mutant slide showed slightly stronger signal in granule cell layer than that in control. (E) and (F) The $\beta 3$ subunit expression pattern. Note: the signal in granule cell layer was relatively stronger in control than that in mutant. Scale bar: 20 μm .

3.1.3 Properties of excitatory synaptic transmission

In this part, the functional consequence of deletion of E6-AP in cerebellar cortex was examined by measuring the basal excitatory activities and activity-dependent synaptic change of excitatory input of PN. Because previous reports have demonstrated that

both the hippocampal long term potentiation and the neocortical plasticity associated with experience-dependent maturation of neocortical circuit were profoundly impaired in E6-AP mutant mice [42, 216], much more attention was paid here to check various forms of long term synaptic plasticity to see whether similar deficits occurred in cerebellum.

3.1.3.1 Properties of PFs-PN synapses

I first examined the synaptic transmission at parallel fiber-Purkinje neuron (PF-PN) synapses by measuring the input-output relationship of PFs. To do this, the PFs were stimulated by different stimulus intensities through a pipette placed at molecular layer and the amplitude of PF-EPSCs were plotted as a function of stimulus intensity. Shown in Fig 3.9A and B, the peak amplitude of PF-EPSC linearly increased with the strength of stimuli intensity in both group, and no statistic difference was observed in the slope of amplitude-intensity curve between wild-type and mutant ($n=13$, $p>0.05$, Fig 3.9C). I also examined the kinetics of PF-EPSCs. The 10-90% rising time of PF-EPSC in mutant was 2.70 ± 0.92 ms and was similar to that (2.74 ± 0.86 ms) in control ($n=18$, $p>0.05$, Fig 3.9D). Similarly, the decay time of PF-EPSC appeared to be similar between mutant and control (13.75 ± 4.08 ms in control, 14.48 ± 0.28 ms in mutant, $n=19$, $p>0.05$, Fig 3.9D).

Next, the short term plasticity of PF-EPSC was examined by measuring the paired-pulse facilitation (PPF) which referred to the situation that the second of two pulses delivered with a short interval elicited a stronger synaptic response [217], and by measuring the synaptic fatigue responding to repetitive stimulation. The PF-EPSC displayed prominent facilitation and the amplitude of PPF in mutant was not

significantly different from those of the control mice at all interpulse intervals (Fig 3.9A, B and E). The application of repetitive PF stimulation at 20 Hz resulted in synaptic fatigue in both experimental groups. The ratio of amplitude of the 3rd, 5th, 10th, 15th and 20th of EPSC to the 1st EPSC in mutant was similar to those in control (Fig 3.9A, B and F), suggesting the transmitter release probability at presynaptic parallel fiber terminals was not affected by E6-AP deletion.

3.1.3.2 mGluR1 response

At PFs-PN synapses, glutamate released from presynaptic terminals exerts two distinct effects: it evokes fast excitation by binding to glutamate-gated ionotropic receptor (AMPA) receptors and produces a slower excitation through metabotropic glutamate receptors (mGluRs), particularly the group I mGluRs [218-219]. The activation of group I mGluR triggers calcium release from internal IP₃-sensitive stores and is considered to be indispensable for the induction and expression of certain forms synaptic plasticity [109-110, 123]. To specifically measure the mGluR-mediated response, the PFs-EPSC was first identified by its characteristic PPF. Then, the AMPAR-mediated fast synaptic current was completely blocked by the addition of 10 μ M NBQX to allow me to isolate the slow synaptic current. After that, the stimulus intensity and the stimulus number were adjusted to evoke the maximum mGluR response and the nature of mGluR-mediated slow inward current was confirmed by 100 μ M CPCCOET, a specific group I mGluR antagonist (Fig 3.10A). Measured in this way, the peak amplitude of mGluR elicited by repetitive PFs stimulation (100 Hz, 20 pulses) was 97.44 ± 14.34 pA in control slices and 120.22 ± 21.47 pA in mutant ($n=9$, $p>0.05$). Furthermore, the kinetics of mGluR response were also examined and no significant difference was observed (for rise time: 0.42 ± 0.02 s for control, $0.51 \pm$

0.04 s for mutant; for decay time: 6.53 ± 0.55 s for control, 6.90 ± 0.5 for mutant, $p > 0.05$, Fig 3B).

3.1.3.3 Climbing fiber response

The synaptic transmission between climbing fiber and Purkinje neurons is very powerful and reliable, due to the large number of synaptic contact sites and the high release probability of each climbing fiber terminal [220-221]. Under current clamp mode, stimulation of climbing fiber induces an all-or-none “complex” spike in Purkinje cells, which is composed of a fast sodium spike, followed by a series of spikelets superimposed on the depolarizing plateau [222]. To measure the strength of climbing fiber-mediated synaptic transmission in mutant mice, I first identified CF response by its characteristic all-or-none feature. Then the area under the complex spike representing the charge transferred by CF was measured. Statistic analysis showed there was no difference concerning this value (1323 ± 134.56 mVms, $n=8$ for control; 1103.10 ± 126.50 mVms, $n=7$ for mutant, $p > 0.05$, Fig 3.10C and D). Next, I measured CF response under voltage clamp mode. As shown in Fig 3.10E, the activation of CF elicited large EPSCs, which sequentially evoked the dendritic action potential. To improve the quality of voltage clamp, I partially blocked the AMPA receptor activity by adding 1 μ M NBQX in the external solution [223]. Under this condition, the CF response was distinguished by the feature of paired pulse depression and the all-or-none behaviour responding to gradually increased stimulation intensity. Measured in this way, the peak amplitude of CF response was 161.4 ± 12.53 pA in control group and 164.81 ± 10.66 pA in mutant ($p > 0.05$, Fig 3.10F). Moreover, the paired pulse ratio of CF response calculated with 125 ms stimulation interval was

almost same between the two groups (0.41 ± 0.02 , $n=11$ for control; 0.46 ± 0.02 , $n=15$ for mutant, $p>0.05$, Fig 1.10F).

Figure 3.9. Characterization of parallel fibers response in PN. (A) Demo traces showing the PF-EPSC evoked by different stimulation intensity (*left*), paired pulse response (*middle*) and synaptic fatigue induced by 20 Hz stimulation (*right*) in wild-

type. (B) Demo trances from mutant. (C) Plot of PF-EPSC amplitude at different stimuli intensity. (D) Kinetics of PF-EPSC. (E). Pooled data of paired pulse ratio. (F) Summary diagram showed the ratio of amplitude of EPSC evoked by the 3rd, 5th, 10th, 15th, and 20th stimulus versus the first EPSC amplitude.

Figure 3.10. The properties of mGluR and climbing fiber response. (A) Sample traces showing the maximum mGluR response evoked by repetitive PF stimulation and the inhibitory effect of CPGCOET in control (*left*) and mutant slices (*right*). (B) Kinetics of mGluR response. (C) CF response under current clamp mode in control (*left*) and

mutant (*right*). (D) Summary of CF strength measured by the area under complex spike. (E) *Left*: CF response under voltage clamp mode and the effect of 1 μ M NBQX. *Right*: demo traces showing the CF response in the presence of NBQX. (F) No difference was detected regarding the peak amplitude and paired pulse ratio of CF response between control and mutant.

3.1.3.4 Normal presynaptic LTP induction and impaired postsynaptic LTP

In the cerebellar cortex, two forms of LTP have been characterized at PF-PN synapses so far: a presynaptic form that involves modification of the secretory machinery resulted from repetitive stimulation of PFs at 4 Hz for 30 sec, and a postsynaptic form that requires the up-regulation of AMPA receptor trafficking caused by 1 Hz 5 min PFs tetanization [133, 192]. To fully assess the effect of E6-AP deletion, both of these two forms LTP were examined. As shown in Fig 3.11A, after 4 Hz PF conditioning, the PF-PN synaptic strength was enhanced both in control and mutant and the time course of PF-EPSC was also similar. The overall potentiation extent calculated at 20 min after conditioning was similar between mutant and wild-type ($152.91 \pm 10.09\%$, $n=7$ for control, $150.33 \pm 23.24\%$, $n=11$ for mutant, $p>0.05$, Fig 3.11B). The PPF ratio change, which was defined as the PPF ratio of post tetanization recording divided the PPF ratio of baseline recording, was also not statistically different ($90.35 \pm 6.46\%$ in control, $91.58 \pm 5.3\%$ in mutant, $p>0.05$, Fig 3.11B).

Figure 3.11. The induction of presynaptic form LTP. (A) Presynaptic PF-LTP was induced by PF stimulation alone at 4 Hz for 30 sec both in control and mutant mice. Inset showed PF-EPSC before (*dashed*) and after the application of condition stimuli in control (*left*) and mutant (*right*). (B) The mutant mice showed same extent of potentiation of PF-EPSC amplitude as their controls (*top*) and showed same level of PPF change as that in control mice (*bottom*).

Next I applied standard induction protocol to induce postsynaptic LTP. Following acquisition of a stable baseline, 1 Hz 300 PF stimulations were applied. This treatment persistently potentiated the synaptic strength of PF-PN synapses in control cerebellar slices, whereas only a small degree of potentiation was observed in mutant (Fig 3.12A). Statistic analysis showed that the potentiation extent was significantly low in mutant than that in control ($149.45 \pm 20.8\%$, $n=4$ for control, $107.90 \pm 16.8\%$, $n=6$ for mutant, $p<0.001$, Fig 3.12C), suggesting the impairment of induction of postsynaptic LTP in Purkinje neurons of E6-AP mutant.

The nitric oxide has been reported to be involved in the postsynaptically expressed LTP signalling pathway. The blockade of NO generation by NO synthase inhibitor substantially prevents the postsynaptic LTP induction, while directly

delivered NO via an NO donor is sufficient to induce LTP [132-133]. Therefore, it was logical to test whether the deficiency of LTP observed in mutant could be rescued by direct NO application. As shown in Fig 3.12B, superfusion with 10 μ M DEANO, a potent NO donor which smoothly decomposes at physiological pH to release two moles of NO [224] for 5 min, reliably increased the amplitude of PF-EPSCs, and this potentiation was able to last thereafter as long as the cell could be recorded (up to 50 min) in control slices. However, the augmentation of EPSC amplitude was transient, and was entirely absent 15 min after DEANO application in mutant slices. The overall potentiation extent was significantly lower in mutant ($146.48 \pm 3.17\%$, $n=10$ for control, $96.70 \pm 2.25\%$, $n=8$ for mutant, $p<0.001$, Fig 3.12C), with no difference observed in terms of PPF change between control and mutant (Fig 3.12D). Cumulatively, these data showed that the deletion of E6-AP in Purkinje neurons altered NO mediated postsynaptic LTP, whereas the cAMP mediated presynaptic LTP pathway was unaffected.

3.1.3.5 Enhanced LTD in mutant

The impairment of postsynaptic LTP induction mentioned above prompted me to examine the LTD in mutant, since the postsynaptic LTP and LTD are regarded as opposite process, and each could reverse the other at the same PF-PN synapses [134, 225]. Here, two distinct induction protocols were applied. The first one was composed of 300 single PF stimulation in conjunction with a depolarizing pulse (200 ms, 1 nA current injection) repeated at 1 Hz [194-195]. As there was the report that the incidence and degree of LTD was dependent on the baseline PF-EPSC amplitude, I carefully adjusted the PF stimulation intensity to induce the baseline PF-EPSC with the amplitude ranging from 500 pA to 700 pA, with which the outcome of pairing

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protocol was supposed to be variable [226]. Indeed, in control group with mean PF-EPSC amplitude of 554.65 ± 86.92 pA, after the application of conditioning stimulation, only 1 of 7 recordings showed depression, 2 cells showed potentiation and the 4 remaining cells displayed no overall change in amplitude with respect to baseline. In contrast, the proportion of cells that underwent LTD increased dramatically in mutant group. Among total 13 recordings with mean PF-EPSC amplitude of 579.37 ± 79.90 pA, 8 cells showed depression, 1 cell showed potentiation and the 4 remaining cells exhibited no obvious inflection (Fig 3.13A and C). The overall depression calculated from all pooled data was $108.76 \pm 6.3\%$ in control and $81.4 \pm 13.0\%$ in mutant ($p > 0.05$, Fig 3.13A and D), suggesting the LTD could be relatively easily induced in mutant mice.

I next monitored LTD with a relative moderate induction protocol, consisting of 200 ms depolarization of PN membrane from -70 mV to -20 mV coupled with paired PF stimulation with 50 ms interval, repeated 20 times at 1 Hz [193]. As could be seen from the time course, the amplitude of PF-EPSC was largely reduced after the application of LTD induction protocol in slices of both control and mutant, but the later showed a higher depression (Fig 3.13B). Calculated at 20 min after induction, the value of depression in mutant was significantly lower than that in control ($76.34 \pm 1.42\%$, $n=5$ for control, $64.58 \pm 3.36\%$, $n=6$ for mutant, $p < 0.05$, Fig 3.13B and D). Taken together, the data suggested that LTD was enhanced in mutant mice.

Figure 3.12. Stimulation induced postsynaptic LTP and chemical induced LTP. (A) 1 Hz 300 PF stimulation reliably potentiated the PF-EPSC in wild-type but not in mutant. *Left*: super-imposed PF-EPSC. (B) DEANO application failed to enhance PF-EPSC in mutant. *Left* were the demo trances showing the change of PF-EPSC before and after conditioning. (C) Bar graph showing the potentiation of PF-EPSC induced by either PF stimulation or DEANO superfusion. Both 1 HZ PF conditioning and DEANO application failed to induce LTP in mutant mice ($P < 0.01$) (D) No difference was observed for PPF change between control and mutant.

Figure 3.13. Induction and expression of PF-LTD. (A) Conjunctive 1 Hz 300 times PF stimulation and membrane depolarization preferentially induced LTD in mutant, but not in control slices, when the baseline PF-EPSC amplitude was within the range of 500-700 pA. (B) The mutant showed a high degree of reduction of PF-EPSC after 1 Hz 20 times conjunctive stimulation than that in control group. (C) Summary of the outcome of LTD induction protocol consisting of 1 Hz 300 times PF stimulation and membrane depolarization. (D) Histograms showing depression averaged 20-25 min

after tetanization. Note, an induction protocol consisting of 20 times membrane depolarization and simultaneous PF stimulation induced higher degree of depression in mutant ($P<0.05$).

3.1.4 Up-regulation of β CaMKII in cerebellar cortex

The alteration of long term synaptic plasticity of PF-PN synapses in mutant suggested certain downstream factors of LTP and LTD cascade was regulated by E6-AP in either a direct or indirect manner. To date, numerous signalling molecules, such as protein kinase and protein phosphatase have been found to play pivotal roles in the induction and expression of activity-dependent synaptic plasticity [127, 227-228]. Among many factors, the α and β forms of Ca^{2+} /calmodulin-dependent kinase II (CaMKII) was particularly relevant to my study for several reasons. First, activation of α CaMKII by calcium influx is a prerequisite for the induction of LTP and is sufficient to maintain LTP expression at most excitatory synapses in the hippocampus and neocortical structure. In addition, α CaMKII have been shown to function as a molecular memory switch to control the direction of synaptic change, and was essential for motor learning [229]. α CaMKII^{-/-} mice showed an impairment of parallel fiber LTD and showed abnormal gain-increase adaptation of both the vestibular ocular reflex and optokinetic reflex [230]. Second, both α CaMKII and β CaMKII were highly distributed in cerebellum with β form in predominant position [231]. The function of β CaMKII in cerebellar cortex was reported to control the direction of plasticity at PF-PN synapses. β CaMKII modulated the polarity of synaptic plasticity in two functionally distinct ways; specifically, β CaMKII was required for driving the synaptic depression pathway under high-calcium conditions. However, under low calcium conditions, β CaMKII prevented activation of this pathway [232]. Finally, the

phosphorylation state of α CaMKII was misregulated in hippocampal slices *Ube3a* maternal null mutant mice. Mutant animals exhibited a significant increase in hippocampal CaMKII phosphorylation state, specifically at sites Thr286 and Thr305, with no corresponding change in the levels of total CaMKII. In addition, mutants show a reduction in CaMKII activity, autophosphorylation capability, and total CaMKII associated with postsynaptic density [72].

In this regard, the expression of α and β CaMKII in cerebellar cortex was examined. Moreover, I also analyzed their expression in forebrain area as a control. Shown in Fig 3.14A, a significant increase of β CaMKII in cerebellum was detected (Fig 3.14C), with no much difference for α CaMKII (data not shown). Moreover, the alteration of β CaMKII expression level appeared to be tissue specific as there was no such change occurred in forebrain homogenate (Fig 3.14B and C).

Figure 3.14. Western blotting analysis of α and β CaMKII. (A) Representative data of β CaMKII expression in cerebellum. (B) The data of α and β CaMKII expression in forebrain area. (C) Pooled data showed that β CaMKII was highly up-regulated in cerebellum of E6-AP mutant.

3.2 Discussion

In this study, I used the *Ube3a* null mutation mice, a gene-specific mouse model for Angelman Syndrome, to investigate the functional consequence of E6-AP deletion on the physiological properties of cerebellar cortex and try to establish the link between the behaviour phenotypes of AS and the molecular and cellular phenotypes at the neuronal circuits level. The main experimental findings are as following: (1) the intrinsic excitability and basal excitatory synaptic transmission of PN are normal; (2) the GABA_A receptor-mediated basal inhibitory synaptic transmission, as well as the short-term and long-term synaptic plasticity of inhibitory transmission of PN are normal; (3) the postsynaptic, NO mediated LTP at PF-PN synapses is impaired, while the LTD at PF-PN is enhanced; (4) the β CamKII is up-regulated in cerebellum, but not in forebrain area. To our knowledge, this study provides for the first time evidences that the long term synaptic change in Purkinje cells is associated with ubiquitin pathway and the β CamKII might be a candidate of E6-AP.

3.2.1 Normal intrinsic excitability of PNs

The ubiquitin protein-ligases have been identified as key regulators involved in various cellular processes of neurons, such as proteasome-mediated cytoplasmic protein degradation [55], regulation of presynaptic and postsynaptic protein turnover [233-234], and the development and remodeling of synapses [235-236]. Particularly, the recent reports by Abriel's group demonstrated that the surface expression of voltage-gated sodium channel, which is responsible for action potential initiation and essential for the conduction of electrical pulses in most excitable cells is under the regulation of Nedd4-2, a member of HECT family (homologous to E6-AP protein C-terminal). The ubiquitination of Na_v1.5 by Nedd4-2 dramatically reduced the surface

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expression of Na_v1.5, with no impact on the single channel biophysical properties, thereby largely decreased the current density of Na_v1.5-transfected cell [237-238]. In addition, Ekberg et al. reported that the voltage-gated potassium channels, which are known to be important regulators of neuronal excitability, are also ubiquitinated by Nedd4-2 in vivo [239], suggesting that the members of HECT family might play pivotal roles in controlling the traffic and turnover of ion channels, especially for those channels implicated in the membrane excitability of neurons.

As the founding member of HECT family, E6-AP is proposed to have similar regulatory effects on the ion channels expressed in the surface of neuronal membrane, thereby produces severe neuronal deficits in AS patients. However, there have no such candidates been identified to date. In this study, I take the functional approach to examine the intrinsic excitability of Purkinje neurons, to detect the consequence of E6-AP deletion. Whole-cell recordings on PN soma from acute slices of 16-26 days old mice showed that passive membrane properties including the input resistance and the hyperpolarization-activated cationic current (I_h) were not altered in the E6-AP mutant. Input resistance is determined by the size, morphology and resting conductance of PN and has been reported to be modified in a synaptic activity dependent manner [240]. I_h has been shown to act as a pacemaker current to support the generation of spontaneous firing in hippocampal interneuron and to be indispensable for maintaining the membrane potential of PN [190, 241]. The lack of changes in passive membrane properties suggests that the development of PN and the surface expression of I_h channel are normal in mutant. In addition, no alterations in the active membrane physiological features like the threshold of action potential generation and AP-AHP were founded. The firing threshold and the amplitude of AP are related to fast sodium conductance and a reduction of AP threshold is observed in

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cerebellar granule cells after repetitive mossy fiber stimulation [240, 242]. In PN, AP-AHP is produced by the activation of two types of potassium channels — voltage-gated Kv3 channel and large conductance Ca^{2+} -activated K^{+} channel [243-244]. The observation that normal active membrane properties were presented in mutant mice implies that voltage-gated sodium and potassium channels which are expressed at PN soma and are involved in action potential generation are not directly regulated by E6-AP.

Regarding the active membrane properties, it is necessary to contrast my current findings with those of Cheron et al.[245], who reported that the simple spike spontaneous firing rate of PN was higher in a kind of E6-AP mutant mouse strain than in wild-type animals. Several reasons might exist to account for the discrepancy. First, the mouse strain they applied is different from the one I used. Although both strains possessed the mutation at the coding region of *Ube3a* gene and have exhibited the phenotypes mimicking the AS patients, slightly differences were reported especially concerning the accumulation of p53, a well known target of E6-AP [42-43]. Second, they made the recordings on the live animals, not from the slice preparation. In my experimental conditions, the firing frequency of PN is assessed by measuring the PN responses to stepwise increased depolarizing current injection. Holding the membrane at -70 mv by constantly injected hyperpolarizing current prevented the spontaneous action potential occurring. Therefore, it would be helpful to examine the spontaneous firing by other method, such as cell-attach clamp to see the difference. Besides these, the age of mice is also different. They used 10-13 months old adult mice, while the one I used is around 16-26 day old. It is possible that the neuronal deficit arising from E6-AP is development-dependent. That is, the alteration of firing properties of PN is

not detectable at developing stage, but it will be exacerbated and become dominate at a relative old age.

3.2.2 Normal inhibitory synaptic transmission of PNs

In PN, the sIPSC events recorded in the absence of TTX is the sum of the response evoked by action potential-dependent GABA release and the response induced by action potential-independent GABA release (mIPSC). The action potential-dependent GABA release refers to the synchronous GABA release at the presynaptic terminals following the calcium influx through voltage-gated calcium channels (VGCC), which are activated by the firing of interneuron. In contrast, the action potential-independent transmitter release is generated by randomly occurred exocytosis of a GABA-containing vesicle, which is insensitive to the inhibition of presynaptic VGCC [113]. Thus, the strength of sIPSC is determined by both the firing properties of presynaptic interneurons and the nature of single synaptic contact, such as the transmitter release probability and the number of GABA_A receptors located at the postsynaptic site. The lack of changes in the biophysical features of mIPSC (the mean amplitude and frequency, the kinetics of mIPSC) implies that the inhibitory synapses formed between local GABAergic interneurons and PN are normally and functionally developed in E6-AP mutant. Moreover, the normal amplitude and frequency of sIPSCs suggested that the intrinsic excitability of interneurons is also not affected by E6-AP deletion, which in turn provide evidence to support the notion that E6-AP is not associated with the ion channels involved in action potential generation either in principle neurons or interneurons.

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Transient elevation of intracellular Ca^{2+} concentration in postsynaptic PNs could trigger two distinct signalling pathways to modify the strength of inhibitory inputs — DSI and RP. The DSI is mediated by endogenous cannabinoids, most likely to be 2-AG (2-arachidonoylglycerol), a lipid-soluble second messenger, that is generated by PN during transient Ca^{2+} increase and activates presynaptic CB1 receptors (cannabinoid receptor 1) in a retrograde manner [155, 246]. The activation of CB1 receptors mobilizes the $\beta\gamma$ subunits of G_i protein, which suppress the calcium influx through VGCC at presynaptic terminals, thereby almost entirely block the transmitter release at GABAergic buttons within a short time window [207-209, 247]. On the other hand, a rise of Ca^{2+} following either direct membrane depolarization or climbing fiber activation leads to the formation of a Ca^{2+} /calmodulin (Ca^{2+} /CaM) complex, which activates CaMKII. The activated CaMKII then produces long lasting potentiation effect on the amplitude of sIPSCs, possibly by direct phosphorylation of GABA_A receptors, which might increase its affinity for GABA and increase its single channel properties [211, 248]. The results that both DSI and RP could be normally induced in PNs of mutant suggest that there is no direct linkage between E6-AP and endocannabinoid system and the signalling pathway for RP expression is well preserved in the case of E6-AP deletion.

In this study, I further examined the inhibitory synaptic transmission by measuring the distribution of three GABA_A receptor subunits $\alpha 5$, $\beta 3$ and $\gamma 3$ individually. Previously, Sinkkonen et al. have reported that the binding of GABA_A receptor channel site ligands was normal in E6-AP deficient mice, suggesting there is no alteration in the GABA_A receptor expression in brain sections [249]. Keeping in line with that report, I also did not find significant difference regarding the distribution of GABA_A receptor subunits in cerebellar cortex between wild-type and

mutant, except that slightly high expression of $\gamma 3$ and relative low expression of $\beta 3$ was observed in the granule cell layer of mutant slices. However, such subtle changes have only negligible effect on the function of inhibitory transmission of PNs, as no difference was detected for the mIPSC events between control and mutant. Direct recording from granule cell might be able to provide more information about the functional consequence of such minor modification in GABA_A receptor expression. Additionally, my immunostaining results showed that E6-AP exhibits neuron type specific expression pattern in molecular layer of cerebellar cortex. Namely only GABAergic interneuron, most likely to be basket cell and stellar cell express E6-AP, while the GAD65-negative neurons do not express it. This finding is really meaningful as it provides compelling evidence to demonstrate that E6-AP shows cell specific imprinting not only in the cultured neurons as has been reported before [47], but also in the brain section preparations. To my knowledge, this is the first time to report the cell type specific expression of E6-AP in brain.

3.2.3 Altered long-term synaptic plasticity of PFs-PN synapses

In the cerebellar cortex, the glutamatergic synapses formed between parallel fibers and Purkinje cells undergo three types of long-term synaptic modulation: LTD, characterized by conjunctive climbing fiber and parallel fiber stimulation induced clathrin-mediated endocytosis of postsynaptic AMPA receptors [250]; postsynaptic LTP, characterized by nitric oxide induced N-ethylmaleimide-sensitive factor (NSF)-dependent GluR2 exocytosis [132]; and presynaptic LTP, characterized by RIM1 α phosphorylation triggered transmitter release [251]. In this study, all of these three forms of long-term synaptic plasticity were carefully examined and the data showed that E6-AP differentially involved in the signalling pathways of LTP/LTD induction

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as only presynaptic LTP was normally induced, while postsynaptic LTP was severely attenuated and LTD was enhanced in PNs of E6-AP mutant.

It has been demonstrated that the cascade of presynaptic LTP expression initiates from the Ca^{2+} influx at the presynaptic parallel fiber terminals and is independent on the postsynaptic Ca^{2+} increase in PNs. In this scenario, a high frequency (above 4 Hz), repetitive parallel fiber stimulation activates presynaptic VGCC, which sequentially activates a Ca^{2+} /CaM-sensitive adenylyl cyclase, leading to the production of cAMP and the activation of cAMP-dependent protein kinase K (PKA). Activated PKA directly phosphorylates RIM1 α , a central component of presynaptic active zone which interacts with multiple synaptic vesicle components. This phosphorylation triggers an overall restructuring of the active zone, resulting in a long-lasting increase in transmitter release [131, 192, 251-252]. My result that PF-EPSC amplitude persistently increased after 4 Hz 30 s PFs stimulation in mutant slices indicates that E6-AP exerts negligible effect on the activity-dependent modification of transmitter release machinery. Also, the normal basal excitatory synaptic transmission (the PF and CF responses) suggests that the glutamatergic synapses are normally developed in the absence of E6-AP. Moreover, the data suggest that the motor incoordination and deficit in motor learning observed in mutant mice are not attributed to the change of presynaptic LTP. The alteration of other forms of long-term synaptic plasticity must be considered (see below discussion).

In the initial report on the generation of E6-AP mutant mice, Jiang et al. found that the mutant mice exhibited deficits in both fear conditioning paradigm, a test evaluating the associative learning capability and bar-crossing and rotating rod test, tests measuring the motor coordination and motor learning. Further studies suggested that the deficiency of context-dependent learning in mutant could be explained by the

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impairment of hippocampal LTP, the best candidate for a cellular mechanism contributing to the mammalian learning and memory [42]. Here, I provided compelling evidences to show that similar to the situation in hippocampus, the motor dysfunction of mutant mice could be attributed to the abnormal induction of cerebellar LTP at PF-PN synapses. Moreover, my finding that it is postsynaptic LTP, but not LTD is impaired in the E6-AP mutant challenges the classical view of cerebellum-dependent learning, in which the LTD was thought to be the basis of motor learning [80, 90]. On the contrary, my result strengthens the idea that synaptic reversibility is essential for the learning and memory storage in a neuronal network [134], and is consistent with the hypothesis that the behavioural changes are implemented by different plasticity mechanisms, which reverse each other with unequal efficacy [253].

The impaired postsynaptic LTP in PNs of mutant mice suggests that E6-AP is necessary to maintain normal synaptic plasticity and the machinery for plasticity induction including the calcium dynamic, AMPA receptor trafficking or signalling molecules is compromised due to the E6-AP deletion. Particularly, the nitric oxide (NO)-mediated signalling cascade is of great relevance. NO is an indispensable messenger for both LTP and LTD induction in cerebellar PF-PNs synapses. In the presence of a temporally coincident high Ca^{2+} elevation, presynaptic parallel fiber released NO facilitates the removal of surface AMPA receptors in postsynaptic PNs through classical soluble guanylyl cyclase (sGC)- protein kinase G (PKG) pathway [127]. On the other hand, under the condition of weak Ca^{2+} rise, NO promotes the surface expression of AMPA receptor by activating soluble NSF attachment protein receptor (SNARE)-dependent exocytosis of GluR2 cascade [132-133]. The facts that direct delivered NO (by NO donor application) failed to rescue the impairment of LTP and the LTD was relatively easily induced argue for the possibility that alteration

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of NO-mediated receptor trafficking occurred in mutant mice. It is most likely that the lack of E6-AP shifts the direction of NO mediated AMPA receptors trafficking from exocytosis towards endocytosis, thereby producing enhanced LTD and severely attenuated LTP.

My Western blot result that β CaMKII is up-regulated in cerebellar cortex might account for the enhanced LTD in mutant mice. The β CaMKII activity has been thought to contribute to the synaptic depression pathway and the conditioning protocol that normally produces LTD yield LTP in β CaMKII deletion mice [232]. In this regard, it is reasonable to speculate that the β CaMKII is one substrate of E6-AP and the deletion of E6-AP causes the accumulation of β CaMKII, which consequently facilitates the expression of activity-dependent depression of synaptic transmission. However, the result that highly expressed β CaMKII was only detected in cerebellar cortex, but not in forebrain area complicates situation. Right now, I do not have any clues to explain such a tissue-specific regulation of β CaMKII by E6-AP. Possibly, the ubiquitin-proteasome system is differentially expressed in distinct brain area. The role of E6-AP could be replaced by other ubiquitin ligase in forebrain, whereas no such kind of compensating mechanism occurred in cerebellar cortex, resulting in the over-expression of β CaMKII in cerebellum. On the other hand, the enhanced LTD observed in my study could also be explained by the misarrangement of Arc-mediated AMPA receptor endocytosis. Recently, Greer et al. reported that Arc, a synaptic protein which interacts with dynamin and endophilin and regulates the internalization of surface AMPA receptor is a substrate of E6-AP and elevated levels of Arc accumulates in the absence of E6-AP, leading to the excessive endocytosis of AMPA receptors [254]. Thus, the alterations in intracellular Arc and β CaMKII concentration

collectively changed the direction of synaptic plasticity, leading to the impaired synaptic function.

3.3 Summary

Overall, my electrophysiological studies demonstrate that E6-AP does not directly regulate the function of ion channels which determine the intrinsic excitability of neurons and the basal properties of both inhibitory and excitatory synaptic transmission. The deletion of E6-AP affects the normal expression of activity-dependent synaptic plasticity and β CaMKII appears to be a substrate of E6-AP.

Chapter IV Modulation of inhibitory transmission by NMDAR activation in hippocampal pyramidal neurons

In this part, acute hippocampal slices were used to examine how pyramidal neurons and interneurons respond to brief application of NMDA. A profound increase in GABA_A receptor-mediated spontaneous inhibitory postsynaptic current (sIPSC) responding to puff applied NMDA was found and the mechanism underlying the NMDAR activation induced sIPSC enhancement in pyramidal neurons were reported.

4.1 RESULTS

4.1.1 NMDA increases GABA_A-receptor mediated IPSCs in CA1 pyramidal neurons

When recorded from hippocampal CA1 pyramidal neurons, application of NMDA (100 μ M) from the micropipette by a brief pressure pulse to the vicinity of the recorded site elicited not only an inward current but also fast phasic synaptic responses superimposed on the current response, which lasted several seconds (Fig. 4.1; see also Fig. 4.2, *A* and *B*). There were two distinct types of NMDA-induced responses, which we termed type I and II. The type I response was observed in a majority of recordings (94 out of 100 cells): in this group, the amplitude of NMDA-induced phasic responses was small (Fig. 4.2C, mean amplitude change, $227 \pm 21\%$, $n = 13$), and the increase in the frequency was marked ($524 \pm 48\%$, $n = 13$). In contrast, in a minority of neurons observed (6 out of 100 neurons), NMDA elicited a type II response: the mean amplitude of sIPSCs was markedly increased by NMDA (Fig. 4.2C, $1091 \pm 74\%$, $n = 6$, $p < 0.01$ as compared to the type I response), while

the frequency increase was in the same extent as the type I response ($402 \pm 58\%$, $n = 6$, $p > 0.05$ between type II and I responses).

Figure 4.1. NMDA-induced phasic inward currents superimposed on a inward current in a CA1 pyramidal neuron. *A*: a representative trace from a pyramidal neuron displayed on a slow time base. NMDA ($100 \mu\text{M}$) was puff-applied adjacent to the recorded neuron during the period indicated by a horizontal line. *B*: expanded traces obtained in the record shown in A: 1, before NMDA application; 2, during the NMDA-induced inward current response; and 3, after returning to the baseline level.

Note that NMDA induced not only an inward current but also a burst of phasic inward synaptic currents.

Because both excitatory postsynaptic currents (EPSCs) and IPSCs were in an inward direction under my recording conditions, I then examined whether NMDA increases either EPSCs or IPSCs. As illustrated in Fig. 4.2, *A* and *B*, addition of the GABA_A receptor antagonist SR95531 (30 μ M) completely abolished NMDA-induced increases in phasic synaptic activities in both type I and II responses, indicating that NMDAR activation increases the frequency of GABA_A receptor-mediated IPSCs. Moreover, basal spontaneous synaptic events were also markedly inhibited by SR95531 (Fig. 4.2*D*): the frequency of spontaneous events was 2.02 ± 0.28 Hz in control solution ($n = 9$) and 0.11 ± 0.02 in the presence of SR95531 ($p < 0.0001$). Spontaneous activities recorded in CA1 pyramidal neurons thus appeared to be mostly GABAergic in nature.

On the other hand, the AMPA/KA receptor antagonist NBQX exhibited variable effects on the NMDA-induced phasic inward current responses. In most cells examined (12 out of 15 cells), NBQX exhibited no significant effect on the frequency and the amplitude of NMDA-induced phasic synaptic currents: the frequency changes were $593 \pm 143\%$ in control ACSF and $513 \pm 168\%$ in NBQX (10 μ M) containing ACSF ($n = 12$, $p > 0.05$); the amplitude changes were $434 \pm 98\%$ in control ACSF and $391 \pm 117\%$ in NBQX containing ACSF ($n = 12$, $p > 0.05$). In 3 out of 15 cells, NBQX markedly suppressed the NMDA-induced phasic synaptic currents without affecting the tonic inward current (data not shown). The observations suggest that AMPA/KA receptors do not play a substantial role in the NMDA-induced increase in the GABAergic synaptic activity in most CA1 pyramidal neurons and that a minority of pyramidal neurons might make synaptic contacts with GABAergic interneurons via

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axon collaterals to induce AMPAR-mediated feedback GABAergic IPSCs as demonstrated in the neocortical pyramidal neurons-interneuron interaction [255].

Application of either specific NMDAR antagonists, D-APV or Mg^{2+} -rich ACSF, markedly suppressed the inward current responses and the increase in spontaneous GABAergic sIPSCs produced by puff-applied NMDA (Fig. 4.3, *A* and *B*). Summary data depicted in Fig. 4.3*C* show that 50 μ M D-APV completely blocked the NMDA-induced increases in the frequency as well as the amplitude of sIPSCs: for the frequency change, $553 \pm 111\%$ in control and $97 \pm 8.2\%$ in the presence of D-APV ($n = 5, p < 0.05$); for amplitude change, $220 \pm 19\%$ in control and $123 \pm 6.9\%$ in the presence of D-APV ($n = 5, p < 0.01$). Switching from normal ACSF that contained 1 mM Mg^{2+} to high Mg^{2+} (8 mM)-containing ACSF also almost completely abolished the NMDA-induced responses: for the frequency change, $629 \pm 71\%$ in control and $103 \pm 22\%$ in high Mg^{2+} ACSF ($n = 4, p < 0.01$); for the amplitude change, $257 \pm 73\%$ in control and $98 \pm 8.3\%$ in high Mg^{2+} ACSF ($n = 4, p > 0.05$).

Figure 4.2. Two types of responses induced by puff applications of NMDA in CA1 pyramidal neurons in mouse hippocampal slices. *A* and *B*: representative traces of NMDA-induced type I (*A*) and type II (*B*) responses in control ACSF (*top*) and in SR95531 (30 μ M)-containing ACSF (*bottom*). During the period indicated by a horizontal bar, NMDA (100 μ M) was applied by a brief puff given to the micropipette placed near the recorded pyramidal neurons. NMDA induced an inward current and phasic inward synaptic currents with small amplitudes in type I response (*A*) and large amplitudes in type II responses (*B*). The GABA_A receptor blocker SR95531 completely abolished phasic synaptic currents without significantly affecting the

inward currents. *C*: summary graph for changes in the frequency (open column) and amplitude (closed column) of sIPSC in NMDA-induced type I and II responses. There was no significant difference in the extents of sIPSC frequency changes between the Type I and II responses ($n = 6$, $p > 0.05$), whereas the amplitude increase was much larger in the Type II response than in the type I response ($n = 6$, $p < 0.01$). *D*: blockade of basal sIPSCs by bath application of SR95531 ($30 \mu\text{M}$; $n = 9$, $p < 0.0001$).

Figure 4.3. Effects of the NMDAR antagonist D-APV and high Mg²⁺ on NMDA-induced responses in CA1 pyramidal neurons. *A* and *B*: NMDA-induced responses were recorded from pyramidal neurons in control ACSF (*top* traces), D-APV (50

μM)-containing (*bottom* trace in A) and 8 mM Mg^{2+} -containing ACSF (*bottom* trace in B). NMDA (100 μM) was puff-applied adjacent to the recorded neuron during the period indicated by a horizontal line. The NMDA-induced responses were almost completely abolished in the presence of either D-APV or high Mg^{2+} . C: summary graph for the effects of D-APV and Mg^{2+} on NMDA-induced changes in the sIPSC frequency (open column) and amplitude (closed column). D-APV significantly suppressed the NMDA-induced increases in the sIPSC frequency and amplitude ($n = 5$, $p < 0.05$ and $p < 0.01$, respectively), whereas high Mg^{2+} significantly suppressed only the NMDA-induced increase in the sIPSC frequency ($n = 4$, $p < 0.01$) but not in the amplitude ($n = 4$, $p > 0.05$).

4.1.2 Characterization of NMDAR activation responsible for the increase in sIPSCs

Repeated activation of NMDARs has been known to undergo a progressive and use-dependent decline in either a calcium-dependent or a calcium-independent mechanism [256-258]. It is therefore needed to optimize the way of NMDA applications for reproducible responses. For this purpose, I examined the effects of changing the duration of puffs applied to the NMDA-filled pipette (0.5 to 20 sec). Both the amplitude and the decay time of NMDA-induced inward currents in pyramidal neurons increased in a puff-duration dependent manner, with the latter more linearly relating to the puff duration (Fig. 4.4A). The peak amplitude of NMDA-induced currents showed a biphasic increase: it gradually increased in the amplitude with puffs less than 10 sec, then declined with 10 sec-puff and increased again with puffs more than 10 sec, which suggests that there could be two components of NMDA-induced responses, one being postsynaptic direct action and the other a trans-synaptic action (data not shown). Figure 4.4C illustrates the effects of changing the puff duration on the amplitude and the frequency of NMDA-induced sIPSC increases. There was little difference in the amplitude of sIPSCs when the NMDA puff durations were changed,

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and the amplitude increase stayed around 200% of the control with individual puff durations ($n = 5$). In contrast, the frequency change of sIPSCs induced by NMDA puffs reached a maximal increase of $519 \pm 114\%$ at the duration of 3 sec with a gradual decline following further increase in the duration. Thus, it seemed to be appropriate to use the puff duration of 3 to 5 sec. Indeed, the NMDA-induced increases in the amplitude and frequency of sIPSCs remained almost constant when NMDA applications were repeated with an interval of 180 sec throughout the period of experiments (Fig. 4.4, *B* and *D*). In the following experiments, I therefore decided to apply NMDA under these conditions to perform further pharmacological experiments for exploring the mechanisms underlying the NMDA-induced enhancement of GABAergic sIPSCs.

Figure 4.4. Characterization of NMDA-induced responses in CA1 pyramidal neurons. *A*: effects of changing the puff duration on NMDA-induced responses. Upper and bottom pairs of traces; monitoring puff durations (0.5s, 1s, 3s, 5s in *top* trace of the *upper* pair; and 10s, 15s, 20s in *top* trace of the *lower* pair), and superimposed NMDA-induced current responses (*bottom* trace of each pair) produced in response to individual puffs in a single pyramidal neuron. *B*: the NMDA-induced responses in response to the first (1st, *top* trace) and 10th NMDA puff (10th, *bottom*). Both the first and 10th NMDA puffs (during the period indicated by horizontal bars) produced almost reproducible current responses. *C*: effects of changing the NMDA puff duration on the NMDA-induced changes in the sIPSC frequency (open circles) and amplitude (closed circles). *D*: time-course change in the NMDA-induced increases in

the sIPSC frequency (open circles) and amplitude (closed circles). Each value of increase in the sIPSC frequency and amplitude induced by successive NMDA puffs was expressed as a ratio of that induced by the first puff.

4.1.3 NMDAR activation induces interneuron firing to enhance TTX-sensitive IPSCs in CA1 pyramidal neurons

The NMDA-induced increases in the frequency and amplitude of sIPSCs in CA1 pyramidal neurons could be attributed to an increase in the firing of presynaptic GABAergic interneurons or partly to an increase in the sensitivity of postsynaptic GABA_A receptors in pyramidal neurons. To distinguish the two different mechanisms, I examined the effects of a sodium channel blocker, TTX, on the NMDA-induced responses. In the presence of TTX (0.5 μ M), puff application of NMDA gave rise to the NMDAR-mediated inward current, whereas TTX almost completely abolished the NMDA-induced increase in synaptic activities (Fig. 4.5, *A* and *B*): the frequency changes were $394 \pm 59\%$ in the control ACSF and $83 \pm 6.2\%$ in the presence of TTX ($n = 6$, $p < 0.01$); and the amplitude changes were $223 \pm 34\%$ in the control medium; $118 \pm 16\%$ in the TTX-medium ($n = 6$, $p < 0.05$). The result is consistent with the possibility that the firing of presynaptic GABAergic interneurons increases during NMDA application.

To obtain further support for this notion, I next attempted to record from GABAergic interneurons in CA1 subfield and examined the effects of NMDA on them. For this purpose, I recorded from fluorescent GABAergic interneurons in hippocampal slices cut from VGAT-Venus transgenic mice [181]. Puff application of NMDA induced an intense discharge of action potentials during the rising phase of depolarization under current clamp mode in 1 out of 9 Venus-positive interneurons

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(Fig. 4.5D). In interneurons with spontaneous firing, NMDA initially increased the rate of action potential generation and ceased it when the depolarizing action reached a plateau (Fig. 4.5C). Taken together, it is most likely that brief application of NMDA not only induces an inward current in CA1 pyramidal neurons but also activates NMDARs located at soma/dendrites of interneurons to increase their firings, thereby trans-synaptically enhancing GABAergic sIPSCs in pyramidal neurons. The fact that there were at least two types of interneurons in terms of the spontaneous firing and the NMDA sensitivity (see Fig. 4.5, *C* and *D*) could explain the observations that NMDA induced two distinct (type I and II) changes in sIPSCs in pyramidal neurons: the NMDA-induced type I response (namely relatively small sIPSCs evoked in pyramidal neurons) could be produced by interneurons shown in Fig. 4.5C, and interneurons with the firing property shown in Fig. 4.5D could be responsible for the type II response with large sIPSCs in pyramidal neurons.

Figure 4.5. The effects of TTX on NMDA-induced responses in a pyramidal neuron, and the effects of NMDA on interneurons in the CA1 region. *A*: representative traces of NMDA-induced responses in a pyramidal neuron before (*top*) and after treatment with TTX (0.5 μ M, *bottom*). *B*: Summary graph for the effects of TTX on the NMDA-induced changes in the sIPSC frequency (open column) and amplitude (closed column). TTX markedly suppressed both the increases in the sIPSC frequency (($n = 6$, $p < 0.01$) and amplitude ($n = 6$, $p < 0.05$). *C* and *D*: effects of NMDA on two different interneurons. NMDA, applied by puff during the period indicated by a horizontal bar, first increased spontaneous firing of an interneuron together with a

large depolarization and subsequently made it quiescent, possibly because of depolarizing blockade of sodium channels (C) and elicited action potentials superimposed on a depolarizing action in another silent interneuron (D).

4.1.4 NMDAR activation mobilizes retrograde nitric oxide-mediated signaling from pyramidal neurons to interneurons

The data presented above clearly demonstrate that NMDARs in presynaptic GABAergic interneurons play a major role in the NMDA-induced enhancement of sIPSCs in CA1 pyramidal neurons. In the following experiments, I further examined whether postsynaptic NMDARs in pyramidal neurons could be involved in the NMDA-induced IPSC acceleration. To test this possibility, I attempted to selectively block the postsynaptic NMDAR activation by loading pyramidal neurons with an irreversible NMDAR blocker, MK-801 (1 mM), through the recording patch-pipette under the whole-cell configuration [259-260]. To my surprise, the inclusion of MK-801 in the pipette not only gradually reduced the amplitude of NMDA-induced inward currents as I expected, but also markedly inhibited the NMDA-induced increases in the frequency and the amplitude of sIPSCs in all of 4 pyramidal neurons tested (data not shown). However, I wondered if the blocking action could be ascribed to the leakage of MK-801 by positive pressure applied to the patch-pipette and blockade of NMDARs in interneurons by its diffusion from the pipette before making tight seal for whole-cell recordings in pyramidal cells. In control experiments, perfusion of MK-801 at a relatively low concentration of 40 μ M indeed significantly suppressed the NMDA-induced actions: the frequency increases induced by NMDA were $594 \pm 185\%$ in control ACSF and $185 \pm 40\%$ in MK-801-containing ACSF, respectively ($n = 4, p < 0.01$); the amplitude increases were $209 \pm 25\%$ in control and $122 \pm 21\%$ in MK-801-containing ACSF, respectively ($p < 0.05$).

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Thus, I modified the way of getting into the whole-cell mode, so that the amount and time of positive pressure addition to the patch-pipette were reduced and pressure was applied immediately before approaching onto the cell membrane: as a consequence, the duration of positive pressure application was significantly reduced from 30-60 s to less than 10 s. Under these conditions where cares were taken to minimize the leakage of MK-801, there were still marked reduction of the NMDA-induced increases in the sIPSC frequency and amplitude in 5 out of 8 pyramidal neurons recorded with MK-801 containing internal solution (Fig. 4.6, *A* and *C*), while the postsynaptic NMDAR-mediated inward current was progressively suppressed as expected (Fig 4.6*B*). As described in the control experiments without including MK-801 in the patch-pipette, repeated applications of NMDA produced reproducible responses without any significant sign of rundown (see Fig. 4.4*D*). Thus, the findings strongly suggest that postsynaptic NMDARs also play a role in the NMDA-induced enhancement of sIPSCs. One plausible consequence would be that postsynaptic NMDAR activation could recruit a retrograde messenger that diffuses from pyramidal neurons to the presynaptic terminal of GABAergic interneurons to facilitate GABA release. Several lines of evidence support nitric oxide (NO) as a candidate for retrograde messenger: (1) NO increases the GABA release at several central nervous system (CNS) synapses [261-262] and induces long term potentiation of inhibitory GABAergic synapses in the ventral tegmental area [263-264]; and (2) Ca^{2+} /calmodulin-dependent neuronal nitric oxide synthase (nNOS) occurs in hippocampal pyramidal neurons where NO could be produced following NMDAR activation [265-266]. I, therefore, tested the effect of a commonly used NO scavenger, PTIO, on the NMDA-induced GABAergic synaptic responses. As can be seen from the experiments shown in Fig. 4.7, the addition of 100 μM PTIO reversibly inhibited

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the NMDA-induced increase in the sIPSC frequency (8 out of 14 pyramidal neurons tested), while the increase in the sIPSC amplitude remained unaltered (Fig. 4.7, A and C): NMDA increased the frequency of sIPSCs to $1196 \pm 268\%$ in control ACSF and $711 \pm 140\%$ in the presence of PTIO, respectively ($n = 8$, $p < 0.05$). Together, these data supported the notion that NMDA enhances GABAergic inhibitory transmission through the activation of both presynaptic NMDARs in GABAergic interneurons and postsynaptic NMDARs in pyramidal neurons, the latter leading to mobilization of the retrograde messenger NO signaling pathway to enhance GABA release from interneuron terminals. The NMDA-induced inward current was also truncated by PTIO, which might suggest that NO could act on pyramidal neurons in an autocrine manner to enhance the NMDAR activity as well.

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Figure 4.6. Effects of the NMDAR open channel blocker MK-801 on the NMDA-induced responses in CA1 pyramidal neurons. *A*: representative traces of current responses produced by the first (*left*) and eighth NMDA puffs (*right*) in the same pyramidal neuron into which MK-801 (1 mM) was injected through the recording patch pipette. *B*: time-course change in the amplitude of inward current responses in pyramidal neurons produced in response to repeated puff applications of NMDA with an interval of 3 min. In these neurons, the recordings were made with the patch pipette filled with a MK-801-containing internal solution, and the amplitude of NMDA-induced currents was expressed as a ratio of that produced by the first NMDA puff. *C*: time-course changes in the NMDA-induced increases in the sIPSC frequency (open circles) and amplitude (closed circles). These experiments were performed in the same manner as described in B.

Figure 4.7. Effects of the NO scavenger PTIO on the NMDA-induced increases of sIPSCs in CA1 pyramidal neurons. **A**. time-course changes in the NMDA-induced increases in the sIPSC frequency (open circles) and amplitude (closed circles). The extent of NMDA-induced changes in sIPSC frequency and amplitude was expressed as a ratio of that produced by the first NMDA puff. PTIO (100 μ M) was applied by perfusion during the period indicated by a horizontal line. **B**. sample traces of NMDA-induced responses before (1), during PTIO application (2), and after washing out the drug (3). Each trace was obtained at the time point labeled by 1 to 3 in the graph (A). **C**: summary graph for the effects of PTIO on the NMDA-induced increases in sIPSC

frequency (open column) and amplitude (closed). The value after PTIO treatment was expressed a ratio of the NMDA-induced increase in control ACSF.

4.1.5 Differential involvements of presynaptic calcium channel subtypes in NMDAR-mediated increase in GABAergic transmission

Previous studies have shown that GABA release from hippocampal interneurons is exclusively mediated by either N-type or P/Q-type voltage gated calcium channels (VGCCs) depending on the cell type of interneurons [182, 267]. I therefore asked whether or not subtype of presynaptic VGCCs is involved in the NMDA-induced enhancement of GABAergic transmission from interneurons to pyramidal neurons. Application of a selective N-type VGCC blocker, ω -conotoxin GVIA (ω -CTx-GVIA, 1 μ M), caused little effect on the increases in the frequency and amplitude of sIPSCs following NMDAR activation (Fig. 4.8, *A* and *D*): the NMDA-induced increases in the sIPSC frequency and amplitude were 654 ± 95 and $383 \pm 79\%$ in control ACSF, and 574 ± 76 and $339 \pm 69\%$ in ω -CTx-GVIA-containing ACSF, respectively ($n = 7$, $p > 0.05$).

In contrast, a selective P/Q-type VGCC blocker, ω -Agatoxin TK (ω -Aga-TK, 0.25 μ M), almost completely suppressed the NMDA-induced increases of the sIPSC frequency in 5 out of 8 pyramidal neurons examined (Fig. 4.8, *B* and *D*): $527 \pm 84\%$ in control ACSF and $140 \pm 37\%$ in ω -Aga-TK-containing ACSF ($n = 5$, $p < 0.01$). ω -Aga-TK was less effective on the sIPSC amplitude: $295 \pm 31\%$ in control ACSF and $189 \pm 37\%$ in the toxin-containing ACSF, $n = 5$, $p > 0.05$). In the other 3 pyramidal neurons, ω -Aga-TK strongly inhibited a small component of sIPSCs during the rising phase of NMDA-induced inward current with little effect on a much larger component

of sIPSCs observed during the late phase of NMDAR-mediated current (Fig. 4.8C, *middle trace*).

Interestingly, further addition of 1 μ M ω -CTx-GVIA completely abolished the NMDA-induced larger sIPSCs (Fig. 4.8C, *bottom trace*; Fig. 4.8E). It is therefore likely that CA1 pyramidal neurons are connected by at least two different populations of interneurons selectively expressing either P/Q- or N-type VGCCs at their nerve terminals and that the P/Q-type VGCC expressing type is dominantly involved in the NMDAR-mediated enhancement GABAergic transmission between hippocampal CA1 interneurons and pyramidal neurons. Furthermore, the N-type VGCC in certain interneurons appeared to be very efficient in synchronous release of GABA from their terminals to produce much larger IPSCs in pyramidal neurons. In this context, it seems that interneurons with P/Q-type VGCC could be involved in the NMDA-induced type I response where sIPSCs with relatively small amplitude increased following NMDAR activation, whereas N-type VGCC-expressing interneurons may contribute to type II response with NMDA-induced sIPSCs with large amplitude.

Figure 4.8. Differential effects of the VGCC blockers on the NMDA-induced increases in sIPSCs in CA1 pyramidal neurons. *A*: the NMDA-induced type I response in control ACSF (*top*) was not significantly affected by treatment with the N-type VGCC blocker ω -CTx-GVIA (1 μ M, *bottom*). *B*: the NMDA-induced type I response in control ACSF (*top*) was almost completely abolished by treatment with the P/Q-type VGCC blocker ω -Aga-TK (0.25 μ M, *bottom*). *C*: NMDA puff induced a mixed type I and II response in control ACSF (*top*) of which the initial phase of small sIPSC increase (type I response) was selectively suppressed by ω -Aga-TK (0.25 μ M, *middle*), and the later phase of large IPSC increases (type II response) was totally abolished by ω -CTx-GVIA (1 μ M, *bottom*). *D*: summary graph for the effects of ω -CTx-GVIA (1 μ M, open column) and ω -Aga-TK (0.25 μ M, closed column) on the NMDA-induced increases in the frequency of small IPSCs (type I response). The type I response was selectively suppressed by ω -Aga-TK ($n = 5$, $p < 0.01$), but not by ω -CTx-GVIA ($n = 5$, $p > 0.05$). *E*: summary data for the NMDA-induced changes in sIPSC frequency in control ACSF (NMDA) and in the presence of ω -Aga-TK (0.25 μ M) or ω -Aga-TK (0.25 μ M) plus ω -CTx-GVIA (1 μ M). The data in E were derived from the experiment shown in C.

4.2 DISCUSSION

The main finding of this study is that focal application of NMDA could elicit marked enhancement of GABA_A receptor-mediated inhibitory transmission between hippocampal CA1 interneurons and pyramidal neurons with two distinct presynaptic and postsynaptic mechanisms. First, activation of NMDARs in presynaptic interneurons appears to contribute to the NMDA-induced enhancement of GABAergic transmission. Second, NMDA is likely to act on postsynaptic NMDARs in pyramidal neurons, linking to liberation of the retrograde messenger NO, thereby enhancing GABA release from presynaptic terminals of interneurons.

4.2.1 Postsynaptic expression of NMDAR in hippocampus

A previous study has reported that incubation of a highly purified nerve terminal preparation with NMDA caused an increase in the extracellular GABA concentration, and proposed the existence of presynaptic interneuronal NMDARs responsible for modulation of GABA release from the cytoplasmic pool, presumably through the action of reversing Na^+ -dependent glutamate transporters [268]. Recent histological studies, however, have shown that NMDAR labeling in the hippocampus mostly derived from postsynaptic receptors, but not from presynaptic sites [269-270]. In line with these immunohistochemical data, I find that NMDAR activation elicited the increases in the frequency and amplitude of sIPSCs in a TTX-sensitive manner, but did not alter mIPSCs, arguing against the possible role of presynaptic NMDARs in modulation of spontaneous GABA release from interneuron terminals.

Although the role of NMDARs in long-term synaptic plasticity has been well delineated [271-272], relatively little was known about how NMDARs could be involved in short-term modulation of neurotransmission at CNS synapses. The results of present study reveal that NMDARs in both hippocampal interneurons and pyramidal neurons play a pivotal role in the information flow through glutamatergic transmission within the hippocampal neuronal circuits. Following activation of AMPA-type receptors (AMPA) by the excitatory transmitter released by afferent inputs at excitatory synapses on CA1 pyramidal neurons, there could be a time window in which activation of presynaptic and postsynaptic NMDARs provides a profound inhibitory influence for the processing of afferent signals to hippocampal pyramidal neurons. The fact that diffusible retrograde messengers like NO could be recruited by activation of postsynaptic NMDARs in pyramidal neurons suggests that signal flows through hippocampal CA1 pyramidal neurons may require a period of

feedback inhibition provided by a neuronal circuit connecting pyramidal neurons to certain interneurons and back to pyramidal neurons to prevent overexcitation following transient point-to-point transmission through activation of fast-acting AMPARs in postsynaptic pyramidal neurons.

4.2.2 Heterogeneity of hippocampal interneurons

The results of this study may also support the heterogeneity of hippocampal interneurons [138, 273]. Puff application of NMDA indeed elicited in pyramidal neurons two distinct responses that we referred to as type I and II responses. In the type I response, NMDA recruited GABAergic inputs that elicited IPSCs with relatively small quantal contents, whereas sIPSCs evoked by NMDA in the type II response surprisingly had much larger amplitude. These observations suggest that the properties of GABAergic synapses made by interneurons onto pyramidal neurons might be related to the types of interneurons with distinct properties of GABA release machinery. This possibility is further supported by the finding that distinct VGCC blockers differentially affected the NMDA-induced enhancement of GABA release from interneurons onto pyramidal neurons. The P/Q-type VGCC blocker ω -Aga-TK selectively suppressed the NMDA-induced increase in sIPSCs with small amplitude, whereas sIPSCs with large amplitude produced in response to NMDA application was specifically sensitive to the N-type VGCC blocker ω -CTx-GVIA. Thus it appears that one subclass of interneurons that express N-type VGCCs at their terminals provide a powerful inhibition via synchronous GABA release with large quantal size. Heterogeneous GABAergic interneurons are likely to express distinct calcium channel subtypes and to be endowed with different release machinery at their presynaptic

terminals, thereby differentially contributing to modulation of the excitability in hippocampal CA1 pyramidal neurons. It appears therefore that hippocampal GABAergic interneurons are heterogeneous in terms of not only spiking properties [267] but also the release machinery characteristics.

4.2.3 The involvement of NO signaling

Intracellular injection of the NMDAR open channel blocker MK-801 has been successfully employed in a number of studies to discriminate the roles of presynaptic and postsynaptic NMDARs in long-term synaptic plasticity [259, 274-275] as well as the processes mediated by synaptic and extrasynaptic NMDARs [276]. In this study, MK-801 (1 mM, a commonly used concentration for blockade of postsynaptic NMDARs) included in the patch-pipette, however, appeared to cause a blocking action through the leakage from the recording pipette before the establishment of the whole-cell mode. I therefore adopted a modified protocol in which the amount and the duration of positive pressure applied to the patch-pipette were reduced before forming G Ω seal. With this method, intracellularly applied MK-801 blocked both postsynaptic NMDA response and NMDA-induced increase of sIPSCs simultaneously, pointing to the possible involvement of retrograde messengers in the NMDAR-mediated enhancement of GABAergic transmission from interneurons to CA1 pyramidal neurons. This possibility is consistent with previous findings that the NO synthesizing enzyme nNOS is densely expressed in hippocampal pyramidal neurons and tightly linked to NMDARs at glutamatergic synapses [277-278]. The role of NO has been well documented in modulation of neurotransmitter release at excitatory synapses. For example, repetitive stimulation of glutamatergic afferents has been shown to induce calcium influx through NMDAR channels and activate nNOS coupled to NO

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generation in postsynaptic neurons, thereby retrogradely regulating the release of glutamate to induce long term potentiation [178, 279]. The NO-mediated pathway is also implicated in modulation of GABA release from interneurons onto magnocellular neurons in the paraventricular nucleus [178, 280]. Furthermore, it has recently been reported that NO could be involved in depolarization-induced suppression of inhibition (DSI) following activation of muscarinic receptors in hippocampal pyramidal neurons where NO has been suggested to exert an inhibitory effect on GABA release [281]. In contrast to their report, the results from this study and other studies on other brain regions including the thalamus, ventral tegmental area and paraventricular nucleus [261-262, 264, 282] show the enhancement of GABAergic transmission by NO-mediated signaling pathway. Thus, NO appears to be involved in diverse modes of modulation of neurotransmission at a number of CNS excitatory and inhibitory synapses.

4.3 Summary

Overall, my findings support the notion that NMDARs in both presynaptic interneurons and postsynaptic pyramidal neurons play a pivotal role in the regulation of GABAergic inhibitory transmission in the CA1 subfield. The activation of postsynaptic NMDARs appears to elicit the liberation of retrograde messenger like NO, and profoundly promote GABA release from interneurons, thereby resulting in feedback inhibition to determine a precise timing of action potentials in hippocampal projecting neurons. By this way, NMDARs likely enhance the computational capacity of the hippocampal microcircuit.

Summary

The principle findings of the first part of this thesis are:

- 1) The intrinsic excitability of Purkinje neurons were not affected by E6-AP deletion. The passive and active membrane properties including the input resistance, the hyperpolarization-activated current, the threshold of action potential generation and AP-AHP were not altered in the *Ube3a* null mutation.
- 2) The basal inhibitory synaptic transmission was normal in mutant mice. There were no changes in the amplitude and frequency of sIPSC and mIPSC and the distribution of $\alpha 5$, $\gamma 3$ and $\beta 3$ subunits of GABA_A receptors in cerebellar coretex.
- 3) The immunostaing results showed that E6-AP exhibits neuron type specific expression pattern in molecular layer of cerebellar cortex. Namely only GABAergic interneuron, most likely to be basket cell and stellar cell express E6-AP, while the GAD65-negative neurons do not express it.
- 4) Both DSI and RP were normally induced in mutant mice, indicating that there is no direct linkage between E6-AP and endocannabinoid system and the signalling pathway for RP expression is well preserved in the case of E6-AP deletion.
- 5) The basal excitatory synaptic activities of PNs were normal in mutant. No significant differences were observed for the strength and the transmitter release probability of both PF and CF inputs and the expression of mGluR.
- 6) 4 Hz PF conditioning normally induced LTP at PF-PN synapses in mutant. The overall potentiation extent and time course of PF-EPSC was also similar to the control.

Reference

- 7) A postsynaptic LTP was severely attenuated at PF-PN synapses of mutant. Normal postsynaptic LTP induction protocol and direct NO application failed to potentiate the amplitude of PF-EPSC.
- 8) LTD was enhanced in mutant. Both two distinct protocols induced stronger LTD expression at PF-PN synapses of mutant compared control group.
- 9) Western blotting data showed tissue specific up-regulation of β CaMKII in cerebellar cortex.

The principle findings of the second part of this thesis are:

- 1) Puff application of NMDA in CA1 subfield of hippocampal region profoundly enhanced GABAergic inhibitory transmission between interneurons and pyramidal neurons.
- 2) The increase in the sIPSC frequency and amplitude was markedly suppressed by the sodium channel blocker tetrodotoxin, whereas the frequency and mean amplitude of miniature IPSCs were not significantly affected by NMDA application, suggesting that NMDA elicits repetitive firing in GABAergic interneurons, thereby leading to GABA release from multiple synaptic sites of single GABAergic axons.
- 3) NMDAR open-channel blocker MK-801 suppressed the NMDA-induced increase of sIPSCs, which raises the possibility that certain retrograde messengers may be involved in the NMDA-mediated enhancement of GABAergic transmission.

Reference

- 4) Nitric oxide scavenger PTIO inhibited the NMDAR activation induced increase of sIPSC, suggesting NO was mobilized by NMDAR activation in CA1 pyramidal neurons, which in turn retrogradely facilitates GABA release from the presynaptic terminals.
- 5) Application of a selective N-type VGCC blocker caused little effect on the increases in the frequency and amplitude of sIPSCs following NMDAR activation. In contrast, a selective P/Q-type VGCC blocker almost completely suppressed the NMDA-induced increases of the sIPSC, demonstrating the differential involvements of presynaptic calcium channel subtypes.

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