Chapter 6

General Discussion

Discussion

As discussed in Chapter 1, ubiquitination identifies and tags a protein for degradation. Ubiquitination is also involved in endocytotic trafficking and is associated with the initiation of DNA repair[1]. Proteins must be ubiquitinated in an orderly fashion during development and for the normal homeostasis of the body[2]. Disruption of the ubiquitin proteasome pathway (UPP) has been implicated in the pathogenesis of many disorders including Huntington disease, Alzheimer's disease, Parkinson's disease, prion-like disorders, cystic fibrosis, Liddle syndrome and many kinds of cancers[3-7]. The research in this thesis focused on two neurological developmental disorders in which the UPP is pathogenically associated, namely UBE2A deficiency syndrome and Angelman syndrome (AS)[8-12]. The aim of this thesis is to expand our knowledge on these UPP related disorders. In Chapter 2 we identified new research options for the UBE2A deficiency syndrome. In Chapter 3, we have focused on the involvement of the cerebellum in underlying the motor deficits in AS. We investigated one of the possible targets of UBE3A in Chapter 4. In Chapter 5 we investigated if there is a critical time window in which AS symptoms can be reversed.

The importance of the UPP in neuronal functioning

The UPP seems to be involved in almost all cellular functions such as cellular stress, apoptosis and cell cycle control [13-15]. Even slight disruptions of the UPP have severe consequences, as shown by the many diseases associated with dysfunction in the UPP[7,16,17]. It is thus no surprise that ubiquitination is of great importance to the development and functioning of the nervous system[18-20]. Most of the diseases known to be associated with the UPP result in dysfunction of neurons later in life, as is illustrated by diseases such as Alzheimer's disease, Lewy body dementia and Parkinson disease[4,6,7,21-23]. In this thesis we looked at two developmental disorders related to the UPP.

Since UBE2A deficiency syndrome is X-linked, men display signs of the syndrome[24,25]. Females carrying the mutation are not affected because of the inactivation of the X chromosome carrying the faulty gene[26]. The skewing of the X-inactivation in females most likely represents a survival/proliferation advantage of lymphocytes[26]

The need for strict control for the UPP is also demonstrated in Angelman syndrome. The UBE3A gene (encoding an E3 ligase) is only expressed from the maternal alleles in neurons; all other tissues and cell types in the body express both alleles. Mutations affecting the maternal allele causes loss of or nonfunctional UBE3A protein in the neurons of the brain[27-29]. The lack of paternal UBE3A expression in neurons is due to a long antisense transcript (UBE3A-ATS)[30]. The difference between cell types expressing UBE3A is hypothetically caused by alternate splicing of the IC-UBE3A-ATS transcripts[30]. Why these splice difference exist and why the *UBE3A* gene is silenced might be linked to the finding that multiple active copies of the UBE3A gene are the most common genetic cause of autism[31]. Sno RNAs are believed to play a role in the splicing of the *UBE3A* gene[32,33] and their absence is believed to play a role in the pathogenesis of the Prader Willi Syndrome[34]. The presence of a functional but silenced copy of the *UBE3A* gene creates a potential therapeutic opportunity as explored by Meng and colleagues. They showed that genetically truncating the UBE3A-ATS by inserting a poly-A cassette in the paternal allele resulted in the amelioration of some behavioral deficits in the AS mice[35]. Huang and colleagues were also successful in activating the silent paternal *Ube3a* allele in neurons by down-regulating the UBE3A-ATS with Topotecan[36]. In 2015, the use of anti-sense oligonucleotides (ASOs) to reduce the UBE3A-ATS and subsequently unsilence the UBE3A gene was reported. Injecting ASO's into mature AS mice thus relieved some of the phenotypes displayed by these mice[37]. These studies highlight a possible treatment for AS.

Notably, the distribution of UBE3A undergoes a cytoplasmic to nuclear shift in postnatal development of neurons, suggesting that it may have several distinct functions in the neuron[29]. UBE3A was shown to regulate dendritic arbor growth in fruit flies[38]. Overexpression also reduced dendritic complexity. A similar finding using AS mice showed that UBE3A ligase helps in specifying the polarization and asymmetric outgrowth of pyramidal neurons dendritic arbors and also influencing the distribution Golgi apparatus[39]. Using the fear-conditioning paradigm an up-regulation of maternal UBE3A was demonstrated using the Ube3a-YFP reporter mice. The absence of UBE3A in AS mice led to deficits in activity- dependent ERK1/2 phosphorylation[40], an enzyme which is well known for its role in memory formation and neuronal plasticity[41-43]. The ERK-dependent increase of AMPA receptor insertion was shown to be Ca2+/calmodulin-dependent protein kinase II (CaMK2A) dependent[44]. A previous

study from our own lab showed that AS mice have an increased amount of T305/306-phosphorylated CaMK2A protein[45]. Hence, UBE3A might regulate synaptic plasticity by controlling the amount of inhibited CaMK2A. Increased CaMK2A inhibition leads to deficits in ERK phosphorylation[40], explaining the effects on the synapse in AS syndrome.

Both UBE2A and UBE3A have clear effects on neuronal functioning, however many questions remain. The exact function of these proteins in the synapse remains to be elucidated and how exactly the proteasome is recruited and translocated to specific neuronal compartments is also still unclear[46].

Behavior of mice and men

Research using model organisms is extremely valuable where human experimentation is impossible due to ethical or practical reasons. Research using Mus musculus as an animal model is of great importance to further our knowledge of human physiology of all cell (tissue) types including neurons[47-50]. Animal models allow us to study gene defects seen in humans and generate large experimental groups. Scientists try to relate behaviors observed in human patients to the behavioral deficits seen in mouse models of a specific disease. Sometimes this behavior corresponds perfectly to behaviors seen in humans, as exemplified in the tuberous sclerosis complex syndrome (TSC) mouse model[51,52]. However caution is advised when extrapolating the observations made in mouse models to humans, and in particular when such an extrapolation relates to the potential for therapy. This is clearly illustrated by the Neurofibromatosis mouse model research, where the use of statins showed a huge improvement in spatial learning deficits and attention impairments[53]. When testing statins in human trials it did not show any improvement in learning or attention[54]. Likewise a promising drug for treating fragile X syndrome called AFQ056, a new mGluR5 antagonist reduced spine abnormalities and rescued sociability behavior in mice[55-57]. However in humans no improvement in behavior was observed[58].

In Chapter 2 we tested a novel mouse model for UBE2A deficiency syndrome and we showed it to be a good model for learning deficits demonstrated by the patients. The mice, however, did not display an epilepsy phenotype in contrast to what is seen in 80% of the patients[9,10,59]. There are many mouse models that display spontaneous seizures, and these models have been extensively studied to identify and test anti-epileptic drugs [60] [61]. These studies are of great importance

for developing new treatments, as 40% of manifest epilepsy in humans is defined as intractable epilepsy and it does not respond to any anti-epileptic drug treatment[62]. Epilepsy is a key symptom in syndromes like AS and Dravet syndrome and is displayed in the corresponding mouse models, but only in specific background strains.[63,64]. Likewise our findings of lack of epilepsy in the UBE2A deficiency syndrome could very well be a matter of choosing the wrong background for our experiments. Given that the prevalence of epilepsy in the human UBE2A and AS patients is not 100%, there could be modifier genes in certain individuals and mouse strains that make them resistant to epilepsy. More research into these modifier genes could help with new possible treatment avenues for syndrome related epilepsy and intractable epilepsy.

Water maze and fear conditioning (FC) deficits in AS mice have been repeatedly reported[64-68]. As shown in Chapter 5, we did not find such deficits. The *Ube3a* mice used in Chapter 5 are a mix of the 129S1/SvImJ strain and the C57BL/6J strain from the cross with PML mice (P. Pandolf) [69]. The water maze test has been widely criticized as a stressful task [48,49,70]. The differences in stress levels in mice could result in increased water maze learning as seen in the post earthquake water maze in the study of Yanai *et al.* 2011 [71]. Interestingly Huang 2013 *et al.* showed that water maze deficits in AS mice are also strain dependent [64]. The genetic background of the mice used in Chapter 3 are different from the mice described in our water maze study.[68] The same arguments about strain differences hold true for FC where this background difference could explain the specific FC generalization phenotype instead of the previous described context deficit [64].

Marble burying behavior is decreased in AS mice as we show in Chapter 5. What impaired marble burying behavior means in terms of clinical relevance is unclear. In literature it is used to test neophobia, anxiety and obsessive compulsive disorder (OCD)[72-74]. The behavior is probably a type of defensive burying, demonstrated by coating the marbles with Tabasco making them more averse increased the marble burying[75]. Brain areas responsible for marble burying have not been extensively studied but lesions in hippocampus and septum decrease marble burying[49]. Huang *et al.* 2013 suggested that it is just a way to measure motor impairment like the rotarod and the rearing, which is also deficient in these mice[64]. In light of the evidence that we were able to fully rescue the rotarod phenotype but not marble burying phenotype, it appears that the Marble burying deficit in AS mice does not just reflect a motor impairment.

Similarly, the forced swim test outcome where AS mice spend more time floating than WT mice and the lack of a recue with gene reactivation also suggests this task does not reflect a motor impairment. The forced swim test has been associated with depression when mice float more[76-78]. This test is very sensitive to antidepressants, suggesting a depression like phenotype in AS mice. Surprisingly however reactivation of the gene does not rescue this phenotype. Therefore I would suggest more assessment into his inhibited behavior seen in these mice. Testing anti-depressants on both the forced swim test and the marble burying test would add greatly to our understanding of the tasks and the possibility of reversing this phenotype in the AS mice.

Validating targets

UBE3A, the E3 ligase mutated in Angelman syndrome has been associated with many targets as shown in table 1 of the introduction. However, little work has been done on independently validating targets. The need for this is illustrated by the high-profile reports on ARC (Activity-Regulated-Cytoskeleton associated protein). In 2010 Greer *et al.* found ARC to be tagged and ubiquitinated by the UBE3A protein. This resulted in de hypothesis that there would be higher levels of ARC protein present in AS.[79]. This would result in increased internalization of AMPA receptors and thus cause weakening of the synapse ultimately resulting in leaning deficits in AS. However, Kuhnle *et al.* in 2013 showed that ARC was not a direct substrate of UBE3A[80], and they hypothesized that ARC protein levels are controlled at the transcriptional level by UBE3A. Like ARC, the PML protein described in Chapter 4 proved an equally exciting target protein based on the paper by Regad *et al.* 2009 where PML was shown to be important for the development of the neocortex[81,82]. However we demonstrated in Chapter 4 that PML is not increased in the AS brain using western blot, questioning the relevance of this target in underlying AS. Moreover, loss of PML did not cause any behavioral deficits, questioning the importance of PML in brain development. In the introduction we report a list of the targets described in literature. Like Kuhnle and colleagues, we propose that in addition to in vitro ubiquitination studies, additional criteria are needed before calling a protein it a physiologically relevant target[80].

A critical window for rescuing AS phenotypes?

In Chapter 5 we looked into the reversibility of AS phenotypes in mice. A previous study from our own lab showed that AS mice have reduced CaMK2A activity due to increased phosphorylation at amino acids TT305/306 of the CaMK2A protein[45]. CaMK2A is expressed postnatally reaching its maximal expression 2 weeks after birth[83]. If changes to a postnatally expressed protein can rescue all of the AS phenotypes displayed by the mouse model, then there is probably not a large developmental component to the AS phenotype. In Chapter 5 we looked into the effects of temporally-controlled *Ube3a* gene reactivation. We showed that motor deficits of the *Ube3a* mice could be rescued by gene reinstatement in adolescent mice, whereas anxiety, repetitive behavior, and epilepsy could only be rescued by gene reinstatement very early in life. Failure to rescue a majority of the Angelman phenotypes is quite disappointing. This would actually indicate that AS is mostly an early-developmental disorder, which appears to be at odds with our data obtained with the *Camk2a* mutation. Our lab might have overestimated the rescue seen in the mice with the *Camk2a* counter mutation. The effect we saw might have been in part an effect of the *Camk2a* mutation also seen in WT mice. Homozygous *Camk2a*-TT305/6VA mice were shown to have some deficits in watermaze reversal and LTP as shown by Elgersma *et al.* 2002 [84]. However *Camk2a-TT305/6VA* heterozygous mice do significantly better in rotarod, show a tendency for lower water maze latencies and a tendency for increased fear conditioning as shown by van Woerden *et al.* 2007[68], suggesting that the improvement of the *Camk2a* mutation may act as a general enhancer of these behaviors. This idea is strengthened by our findings in Chapter 2, where the *Camk2a* counter mutation could not rescue the cerebellar deficit seen in AS mice.

There is however a positive note in Chapter 5. We see that LTP phenotype has no critical window in which it can be rescued. Since LTP is required for normal learning, there is some optimism that cognitive fuction can be improved at any time point[85]. Secondly as we point out in the discussion of Chapter 5, the difference between a critical period in humans and mice might be completely different. Mice have rapid development and an embryo matures in 21 days while this takes 9 months in humans. The same can be said for the brain development and the critical window in humans, which might be a lot longer than in mice[86,87]. We might have a bigger window to rescue certain behavioral phenotypes in humans than we find in our mice.

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