12th National Meeting of the French Angelman Syndrome Association

Paris, 18-19 October 2014

Contents

News and Views on UBE3A/E6AP and its Role in Ubiquitin Conjugation

Martin SCHEFFNER, Ph.D.

Good morning. I would like to thank the organisers for having invited us to the beautiful City of Paris. I decided not to repeat the presentation I delivered yesterday to an audience of scientists, some of whom in fact told me that they did not understand everything I said. Instead, I would like to present you an introductory overview of the complexity of biology. I will not go into much detail about Angelman Syndrome, since my colleagues are much more qualified to do so and I am sure they will provide you very interesting insights and results concerning AS.

The ability to rapidly sequence whole genomes has been one of the major breakthroughs of the past twenty years. It is now possible to sequence a genome in just a few days' time and find out a lot about what is going on in our bodies, including what kind of disorders such as cancer we may develop during our lifetime. When we take a look at the number of genes present in the genomes of different organisms, we actually experience quite a surprise. Baker's yeast is a single-cell organism and has approximately 6,000 genes. We consist of about 10^{13}-10^{14} cells but our genome contains just about 20,000 genes, in other words, not more than a nematode - which is a small worm - or than a chicken. How is it, then, that we are so different from worms or from mice that with respect to genes, are almost 90 percent similar to us?

The central dogma in molecular biology goes like this: every gene is transcribed into RNA and then translated into a protein. Given that an individual has about 20,000 genes, does it follow that these give rise to about 20,000 different proteins? No, the situation is far more complex: for example, a given gene can give rise to different RNAs via a process termed splicing and each of these transcripts produces a different protein. Furthermore, proteins are subjected to so-called posttranslational modifications, i.e. they can be modified by the attachment of different chemical groups, which alter the properties/functions of the respective protein. Remarkably, more than 200 different types of such modifications are known. Thus, mechanisms like splicing and posttranslational modification generate a huge complexity of proteins in our cells so that a single human cell does not contain 20,000 different proteins (as expected from the gene number), but rather hundreds of thousands of different proteins.

The human body is made up of many different tissues with different functions, however all cells in our organism contain the identical genetic information. So, how come that for example, intestinal cells function very differently as those of the brain? The solution is that a given cell does not express all 20,000 genes but only a distinct subset of it. In other words, there must be mechanisms/tools that allow cells to differentiate into different tissues by expressing different genes (i.e. only a subset of genes is expressed in a given cell). In recent years, much progress has been made in this research area, and two major processes have been identified that govern tissue-specific gene expression. One of these is termed Epigenetics and the other deals with so-called non-coding RNAs; both of these processes play a major role in determining which genes are expressed in a given cell or tissue and which not. Consequently, epigenetics and/or non-coding RNAs are nowadays one of the most interesting and most intensely studied research areas. A special example for tissue-specific expression is the UBE3A gene, which you are all familiar with. Our genome contains two copies of each gene and these copies are termed allele. One allele comes from the father and one from the mother. Both alleles of the UBE3A gene are expressed in most tissues, except for certain areas in the brain. In these areas, the paternal allele is silent (i.e. it is not or only poorly expressed), while the maternal allele is expressed.

My research group has been studying the biochemical functions of the UBE3A protein since about two decades. So, what does the UBE3A protein do in a cell? Every protein in a cell is degraded at a certain stage in its life. This may sound like a relatively simple task, however it is actually one of the most complex processes. You have to imagine a cell as a heavily crowded place containing millions of different molecules. Within this crowd of molecules, the task of UBE3A is to identify those that shall be degraded. This search can be compared to looking for Waldo in one of those "*Where in the World is Waldo?"* books which our children so love. When UBE3A recognizes such a protein, it modifies it with another protein termed ubiquitin. The latter is then recognised by a specific degradation complex.

So, UBE3A is part of the "ubiquitin-conjugation system". The importance of this system for our cells is illustrated by the fact that the human genome contains approximately 600 genes encoding proteins, whose function - like UBE3A - is to identify proteins that shall be modified by ubiquitin. Thus, this system is one of the most complex enzymatic systems in our cells. Furthermore, the ubiquitin system is highly conserved in eukaryotic evolution from yeast over nematods (small worms) to humans, and many, if not all, fundamental biological processes are in part controlled by this system. Thus, it is not surprising that deregulation of this system has been associated with the development of various diseases including cancer and neurological disorders such as Parkinson's disease or Angelman syndrome.

UBE3A is a prime example for the notion that deregulation of the ubiquitin system contributes to the development of human diseases. When I was a postdoc in the early 1990s, we originally discovered UBE3A as a cellular protein that is hijacked by an oncoprotein of cancer-associated human papillomaviruses, and this interaction is a key event in the development of cervical cancer. A few years later, as you all know, it has been shown that loss of expression of UBE3A causes the development of Angelman syndrome, and just a few years ago, it has been reported that overexpression of UBE3A contributes to Autism Spectrum Disorders. Thus, UBE3A is highly interesting protein to scientists in different fields since it is a putative target for the development of therapeutic strategies for different disorders (cervical cancer, Angelman syndrome, autism spectrum disorder.

As indicated, UBE3A was identified more than 20 years ago, before it was known that it is causally involved in the development of Angelman Syndrome. However, there is still a lot to be learned about UBE3A. For example, we still do not know the target proteins of UBE3A that are relevant for its function in the brain or how the activity of UBE3A is regulated. In the past few years, we have embarked on the latter issue and obtained evidence that UBE3A on its own is only poorly active and that its activity can be stimulated by other proteins. Since Angelman patients are assumed to contain residual UBE3A protein, we are presently setting up a screening system that should allow us to identify small compounds that can activate UBE3A by so-called high-throughput screening. Such molecules may eventually - in 10-15 years' time maybe - contribute to activate UBE3A in Angelman children.

Characterisation of UBE3A Mutations in Angelman Patients

Silvia RUSSO, Ph.D.

Many thanks to the organisers for having invited me; thanks, also, to Martin Scheffner, whose explanations will make my job a bit easier. Lastly, a special greeting to Jonas, who is sitting in the last row. My presentation today will deal with UBE3A mutations and, specifically, those mechanisms that lead to Angelman Syndrome.

The loss of UBE3A protein function in the brain is a problem. The main UBE3A mutation is a deletion, which occurs on maternal chromosome 15. The mutation is the cause of 70% of all cases of Angelman Syndrome. Some patients have inherited two copies of paternal chromosome 15. However, paternal chromosome 15 is inactive and cannot express the protein, thus leading to Angelman Syndrome. The regulating region, which is found in the terminal part of the gene, does not function and causes methylation, which then triggers sequencing errors. Proteins are made based on DNA. The long base sequences are translated into messenger RNA. When one letter is missing or two letters are switched around, the final resulting word is different and a genetic error has occurred. The percentage of patients with two copies of paternal chromosome 15 varies within cohorts, but it can reach 15%.

Angelman Syndrome can have varying degrees of severity. When a patient shows parental disomy, the effects are less severe and communication is easier. When there is a lesion, the surrounding genes can be affected, making the phenotype more severe. In such patients, the resulting epilepsy is exacerbated and language almost non-existent. By studying the most common mutations, we can better understand the different functions of UBE3A.

I would like to emphasise the importance of UBE3A, which varies in activity depending on the bodily tissues in which it is found. Angelman Syndrome can also occur when the protein is present only in low quantities. The presence of UBE3A in overly large quantities brings about other neurological phenotypes, such as autism.

UBE3A is a ligase protein that serves a variety of necessary functions. The HECT domain activates the proteins that transport deteriorated ubiquitin. Neurons communicate at the synaptic level thanks to UBE3A. The latter are very important in cerebral function. Up to this point, we have been able to identify only a few of UBE3A's target proteins.

The RNA transcription process and the subsequent translation into proteins can be likened to a puzzle. It is vital that everything be absolutely perfect, though a variety of configurations are possible, as UBE3A occurs in three different isoforms. A single molecule is not enough for the protein to function properly. There must be interaction between the three molecules, attached to one another. In a study published last September, Professor Feng put together all UBE3A mutations reported in the literature up to the present time. In only 4.41% patients, the error occurs at the level of the UBE3A gene. In our Italian cohort, the error was found in half of the mutations. The difference may, however, result from distinct geographical features, which should be studied in depth.

Several mutations affect the last part of the protein. This part is crucial, in that it contains amino acids that contribute to the protein's catalytic function. The UBE3A mutation affects 27% of patients in the Italian cohort. However, our institute was the first to sequence UBE3A, so we put together all of the methylation-negative patients. In our cohort, there found 3 recurring mutations and 41 private mutations. Out of 36 mothers' patients, 15 were carriers of mutations. The frequency of mother-child transmission was found to be 40%.

Mosaicism can occur and even if the mothers are not carriers they can be at risk to have other babies with UBE3A mutation. This proves the importance of the genetic counselling that could be provided to parents.

Mutations result in errors into genetic coding. Sometimes the variations are non pathogenetic, they are only variations which do not cause the disease, in that it is tolerated by the body. Sometimes we need to study parents or brother to understand if they are cause of Angelman syndrome or not. False-positive mutations of this kind lead to loss of protein function. We have also seen cases of letter insertion. Cases of substitution can have the effect of a "stop sign" in the sequence.

In most cases of mutation, UBE3A is truncated. Mutations that affect splicing then have an impact on the puzzle to which I was referring previously.

Patients with different mutations also differ significantly from one another. Half of the patients with uniparental paternal disomy may be able to utter or even put together various words and form short sentences. Consequently, it is important to identify which functions are preserved, making it possible for them to communicate, when other patients are not able to do so. Many UBE3A patients can walk at 2 years. In addition, in half of the cohort, there was no scoliosis..

Missense mutations differ one from the other. We are unable to establish a correlation here. The missense mutation can have impacts on major protein functions. Other effects, however, are relatively well-tolerated by the body. For instance, if an amino acid or a specific region is replaced, the target proteins will form a connection. In some patients, a specific amino acid is missing: this is considered a false-positive insofar as the protein is being produced, but lacks one letter. All amino acids are essential. Whenever one is missing, the protein's shape is modified and the case becomes more severe.

Through bioinformatics, it has become possible to better understand the mechanisms underpinning this, in particular within families. We can now state whether a missing letter or two permutated letters will lead to a mutation or not. Familial studies help us to understand if the variant is damaging or not. Usually, we study father and mother, and if necessary the grandfather and the grandmother. Bioinformatics tools make it possible for us to look more closely at such cases, in order to identify the underlying problems. Through functional studies, we will be able to confirm or disprove this impression.

In closing, I would like to thank all those who worked with us, including the clinicians who advised our patients. Clinical data make it possible for us to understand the severity of the syndrome.

A Critical Role for UBE3A in Brain Development

Ype ELGERSMA, Ph.D.

Good morning, everyone. It is a pleasure for me to be in Paris. Yesterday's international scientific meeting was a success. Today, I would like to tell you about our recent studies and put forth the following question: is there a critical window in brain development? I would also like to thank the parents who donated money to support our AS research the past years. Our laboratory has now been working on Angelman Syndrome for ten years.

Approximately 1% of new-borns suffer from intellectual disability. In addition, 25 to 50% of these children carry a genetic mutation which causes the intellectually disability. UBE3A is one of the 500 genes can result in intellectual disability.

We started a clinical expertise center for these children at the Erasmus Medical Center in Rotterdam, in order to better help your children. The center is called the ENCORE expertise centre for neurodevelopmental disorders. Hundreds of children come to us, including from France. We want to provide the best clinical care possible to your child.

Our laboratories are engaged in research to understand Angelman Syndrome. Like the AS children, AS mice lack a functional UBE3A gene. We can, in fact, extract the brain of the mouse in order to study how it works. We observe the ways in which proteins impact neuronal function and, in so doing, hope to better understand Angelman Syndrome and identify treatment in order to serve you better. Four clinical trials are now taking place at our centre. Our goal is ultimately to enable better quality of living for both the children and families affected.

Our laboratory is focused in particular on understanding the role of UBE3A, in order to shed light on what it regulates. We are studying the ways in which the treatment of symptoms, including in epilepsy treatment, can be improved. Lastly, we are trying to activate the paternal gene.

DNA comes from both the father and the mother, each contributing a copy of the chromosome. This means that there are two chromosomes, when everything goes according to plan. Chromosome 15 is distinct in that only the maternal copy is used, leaving the paternal copy inactive. This process is known as imprinting. In Angelman Syndrome, the maternal gene is mutated resulting in the absence of the UBE3A protein. Our research is aimed at enabling the paternal gene to express itself, by activating it.

The paternal chromosome is inactive because there an RNA molecule is blocking the UBE3A gene from being transcribed. As researchers, we need to investigate how we can remove the RNA block so that the paternal gene can be activated. The laboratories of Ben Philpot and Art Beaudet have been able to activate the paternal Ube3a gene and we have replicated their important findings. One approach uses Topotecan, a drug currently used to treat cancer, and which has been shown to stop the synthesis of the RNA blocking. Another angle of research involves using an anti-sense oligonucleotide, which will cut the transcript and, in so doing, make it possible to use the paternal gene. Importantly, this promising approach will now be throught to clinical trials through a very experienced company.

For the time being, we do not know what kinds of patients could benefit from activation of the paternal gene in humans, whether in the foetus, new-born, 10-year-old or 20- to 40-year-old patient. We have now tried to reactivate the gene in mice at different ages. Our

brain goes through different stages. For instance, it is difficult to learn a language in adulthood. Incidentally, a language learned in adulthood is not stored in the same place as a language learned as a child. In order for an individual to be able to see with both eyes, the relevant part of the brain needs to be developed by the age of eight. A lazy eye cannot be repaired in an individual aged 15 to 20, as part of the brain is shut-off to that activation. In mice, the critical window for binocular vision is just five weeks. Hence, the mouse's brain development is very quick. A similar critical window occurs with hearing: a child who cannot hear well must be equipped with a hearing aid very early. Similarly, a child who does not have any social activities or interaction will not be able to regain such activity in adulthood.

When the brain of a mouse born with Angelman Syndrome is injected with the drug, it begins to reactivate the Ube3a gene. Once the gene has been activated, the genetic cause of Angelman Syndrome disappears. Through a series of experiments, we tried to determine whether the mouse could walk regularly, behave and learn normally.

First of all, we activated the gene in the embryo. At the time of birth, the mouse does not show Angelman Syndrome and does not have trouble moving around, unlike the untreated mice. In another test, the mice tried to bury marbles in the sand. The Angelman mouse responds differently from a healthy mouse. When the gene is reactivated early, the Angelman mouse's anxiety disappears. In another open-field test, Angelman mice are placed in the fields and, as a result, grow nervous. We observed that the anxiety faded away in those Angelman mice in which the gene had been activated. As part of a social test, the mice were expected to build their nest from accessories we provided. Unlike healthy mice, the Angelman mice did not build their nest, or did so extremely slowly. Once the gene was activated, the Angelman mice recovered fully normal behaviour and built their nest. In other words, when activated early, the gene enables the return of normal social behaviour. The positive effects can also be seen with epilepsy: the epilepsy goes away in Angelman mice in which the gene was reactivated at the embryo stage.

In other words, early gene activation during embryo development can be used to treat and care for symptoms in mice. What happens if we activate the gene at a later time? A series of experiments was carried out to ensure that the therapeutic action is also effective on young mice and adult mice.

Juvenile mice are three weeks old, and do no longer need the mother as they can eat on their own. The 6-week-old mice are considered adolescent and are able to reproduce. The adult mice are 14 weeks old. We studied the behaviour of all three categories of mice after gene insertion and a set waiting period. The motor skills of the juvenile mice can be fully restored, as we were glad to see. The results are not as good in the adolescent mice, but are still resulting in a significant improvement. While they are not as good as healthy mice, the gene-reactivated mice still significantly outperform the Angelman mice. However, the effects of adult treated mice are relatively limited. We did not note a difference between gene-activated mice and the Angelman mice. This shows that it is crucial to administer the treatment as early as possible. The later the treatment comes, the less the chance that the mouse's condition will improve.

Anxiety phenotypes are the most difficult to treat. Gene reactivation in juvenile mice did not cause the anxiety to go away during the marble test and the open field test. Likewise, the nest building test results were not conclusive. The mice treated for three weeks also show poor response to these tests. What's more, the epilepsy was not treated. However, epilepsy drugs are effective on these mice. As a result, it is essential that symptomatic treatments are developed, next to working on gene reactivation.

We cannot inject the new born mice with the drug. Therefore we gave the drug to the mother mice instead, who would transmit it to the pups during nursing. As a consequence of this, 30% of the cells undergo gene reactivation. This was sufficient to rescue not only the motor problems but also the anxiety phenotype.

Neuronal plasticity is vital to learning. When we learn, we improve our neuronal plasticity, as our neurons are constantly in connection. The connections can be measured using

electrophysiology. Gene reactivation in young mice enables the brain to recover its plasticity. Similarly, plasticity can be fully-recovered in adult mice when the gene is activated. This is an important discovery.

In summary, gene reactivation has limited effect on anxiety behaviours, but can rescue the motor deficits, in particular in young and adolescent mice. Lastly, gene reactivation makes it possible to recover neuronal plasticity, even in adult mice. Gene reactivation is thus very promising, but other avenues should still be explored, as reactivation alone will probably not be able to address all issues. Thank you.

Monamine Transporters as Targets of CamKIIalpha: Implications for Angelman Syndrome

Harald H. SITTE, Ph.D.

[The recording starts midway through the presentation.]

We move through different stages of sleep, from deep sleep to REM sleep, during which eye movement can be seen beneath the eyelids. REM sleep is interesting in that it is the dream stage. Sleep is a cycle consisting of different stages and, once we have gotten enough sleep, we awaken. Sleep regulation requires balance between a number of factors and appropriate activity in various neurotransmitters. Some neurotransmitters push us toward wakefulness, while others pull us away from it. Serotonin is one such neurotransmitter. It can therefore be important to study it in connection with Angelman Syndrome.

As a pharmacologist by training, I am interested in the way drugs work. I have studied in particular cocaine and other illegal drugs because they target neurotransmitter transporters and change the synaptic concentrations of neurotransmitters. Over the course of a study on transporters, we found out that the action of amphetamines was impacted by an intracellular kinase. We identified it as Calcium-Calmodulin-dependent kinase II alpha.(CamKII) Amphetamines operate by inducing reverse transport at the monoamine transporters – and thereby elevate the synaptic concentrations of monoamines. We can easily measure this in a functional study and therefore, we can look closely at the transporter function and how it gets affected by the interaction with CamKII.

I met Ype Elgersma, who was studying CaM kinase as part of his post-doctoral studies in California. He told me that our observations were remarkable and he would hypothesize that it would be interesting to see amphetamine-effects in Angelman Syndrome mice. As a result, we embarked on a joint research project, looking first at the ex vivo effects and then at the behaviour of CaM kinase knock-out mice and Angelman Syndrome, in the presence or absence of amphetamines. The results were promising: the effect had been significantly impaired in the Angelman Syndrome mice. The serotonin transporters are possible to be targeted by medication or a drug. Consequently, if we are able to hit a target using a drug, we can take on which may have an impact on Angelman Syndrome.

This project is a real challenge because there are conflicting data from different laboratories. Other studies, such as the study of Farouk and colleagues, published two years ago, showed is not in line with the results of other laboratories. The disparity between our own findings calls for further research on the issue. An article by Malanga and Philpot looks at the dopamine levels and found no change. Therefore, the studies need tp be repeated, in the hopes of achieving significant progress in understanding the disease mechanisms. Thank you for your attention.

Using Human-Induced Pluripotent Stem Cells to Model Angelman Syndrome

Stormy J. CHAMBERLAIN, Ph.D.

Our laboratory uses human stem cells to study Angelman Syndrome. The body contains different types of cells, from epidermal cells to neurons. Stem cells are central to the system in that they can turn into any type of cell in the body. Pluripotent stem cells are induced, meaning that they can be created in a laboratory setting. You have probably heard of embryo stem cells, which can be created in laboratories through in vitro fertilisation. The embryo is placed in a culture medium until the stem cells develop. The use of stem cells raises issues, in particular due to the low availability of embryos affected by Angelman Syndrome.

We use blood and skin samples from Angelman patients to grow stem cells. We have stem cells from patients who have undergone all kinds of mutations, and using these, we are able to develop stem cell lines. Stem cells produced in a laboratory setting were subject to a methylation test in order to ensure that they all carry Angelman Syndrome.

These fine stem cells, all properly diagnosed, were then converted into neurons similar to human neurons. The neuron is Angelman Syndrome's site of action. However, these cells were not produced for the purpose of serving as replacement cells, which could worsen your children's condition. The aim is, rather, to understand the disease, to find out how it functions and to test potential therapies. For instance, in our laboratory, we are able to test the effectiveness of the different therapies discussed by my colleagues on the human neuron.

In order to ascertain that our cells did carry Angelman Syndrome, we conducted a further test. We wondered whether the UBE3A gene had been inactivated. In patients with Angelman Syndrome, UBE3A is put out of working order by RNA. The copy of Chromosome 15 is functional on it, but inactivated. Consequently, we wanted to know whether the same mechanism was in play in neurons derived from stem cells.

A description of the sequencing showed IPS cells, neuro-progenitors and embryo stem cells. We looked at each part of the transcription unit, weaving in those that are specific to the brain. The imprinting process by which UBE3A can be inactivated also works on the stem cells. The protein is, as a result, absent from the neurons, as is the case in your children.

This means that skin cells and blood cells can be used as stem cells. The methylation test then makes it possible to ensure that the cells do resemble those of our Angelman patients. The cells only produce anti-sense when they become neurons.

In my laboratory, researchers are also working to understand the ways in which anti-sense works, as this differs depending on whether the cell is a stem cell or a neuron. The stop sign holding up the anti-sense could mark a new therapeutic avenue for us in Angelman Syndrome treatment. In mice without a stop sign, the regulation process is different. It is important that we continue research on humans.

We believe that the CTCF protein could be the stop sign we have been seeking. We thus looked to see whether it could be found in the region of the existing stop sign. This is a protein that shows lesions only in the paternal allele. It is not found in neurons at all, and logically so, in that it's very absence is what enables the anti-sense to emerge.

It is also possible to study the appearance of neurons in patients with Angelman Syndrome. A synapse is the interface between two neurons. The neurons of Angelman mice show fewer dendritic spines than those of normal mice. One of my colleagues in Connecticut is currently conducting research on this.

Another question we are exploring has to do with whether neurons can send out signals. Research in this area cannot be conducted on post-mortem tissues; it has to be performed on living neurons. These studies will enable us to understand what is not working and to develop treatment options.

Tests can also be conducted to check the effectiveness of the drug on neurons. A topoisomerase inhibitor can be used to deactivate the anti-sense, so as to trigger the production of UBE3A. In order to be reactivated, human neurons require lengthier treatment than do those of mice, at higher doses. Whereas UBE3A concentration in mice rises in 3 days, the process takes 6 days in humans. Thanks to this information, we can identify better therapeutic compounds. The drugs already available for other indications may prove effective on UBE3A.

Our stem-cell neurons can be used to determine whether the mechanism works in the same way on mice and on humans. Ben Philpot has shown that topotecan works on long genes. We also use neurons to identify biomarkers, in order to determine whether the treatment works. It is important to understand whether the drug works on the brain, without having to measure its actual results. For instance, you must be able to ascertain that the drug is working without having to check that your child is progressing in all areas. Furthermore, it should be noted that a lack of conclusive results in adults might jeopardise trials on children. This explains why the bio-marker is so important.

We are also developing new research models. We have grown a brain in a Petri box, in order to study neurons more efficiently.

The stem cells are used to study Angelman Syndrome in living human neurons. While the mouse is a good tool, there are notable differences between humans and mice. We need to understand what human Angelman neurons look like and how they behave. These neurons can then be used to test existing drugs and develop new ones.

My laboratory works with other laboratories, students, post-doctoral students, the NIH, the state of Connecticut, the Raymond and Beverly Sackler Foundation and the Angelman Foundation, which made it possible for me to be with you today.

Model Systems to Study UBE3A/E6AP Function

Rossella AVAGLIANO TREZZA, Ph.D.

First of all I would like to thank the AFSA to give me the opportunity to be here today at this outstanding seminar.

Today I will be discussing with you the model systems and the techniques used in our laboratory to study Ube3a function and its role in Angelman Syndrome. I work in Amsterdam, at the Academic Medical Center in the Department of Medical Biochemistry with Dr. Ben Distel. As you probably guessed by now from my accent, I'm Italian.

When the AFSA asked me to give this talk, they told me I had to speak to parents, so I imagined how it would have been to explain my work to my parents, and the task turned out to be more difficult than expected.

Therefore, I decided to make it simple and start with a very well-known video game figure from the 1980s: Pac Man.

Pac Man moves around a maze, trying to gobble down all of the possible monsters, but can only do so under certain circumstances. Now imagine that the maze is a cell, the monsters are proteins and Pac Man is a complex in charge of protein "elimination": the proteasome. Pac Man, though, cannot eat the monsters until they are marked for elimination, or better said "degradation". Such a mark is called "ubiquitin" and it's placed on the monsters by an E3 ligase enzyme: E6AP (Ube3a as most of you know it). This is an enzyme in charge of binding to other proteins, which must be eliminated from the cell. The monsters at this point are ubiquitinated and become "targets", therefore they can be degraded (eaten) by the proteasome (Pac Man).

The ubiquitination process has been very well described this morning by Dr. Martin Scheffner. It involves three enzymes:

- Protein E1, responsible for ubiquitin activation;
- Protein E2, which accepts the ubiqutin from the E1 and binds the E3;
- Ligase E3, which is responsible for the target ubiquitination

There are two types of E3 ligases: RING E3 ligases and HECT E3 ligases. The RING E3 ligase binds to the E2, which transfers the ubiquitin directly on the target protein. The HECT E3 ligase first binds to the E2, then accepts the ubiquitin on its active domain before transferring it on the target protein.

Why it is so difficult to find new targets of E6AP? The reason is that among all the E3 ligases belonging to the HECT family, E6AP is the only one that doesn't contain known protein-protein interaction domains in its structure. This makes identification of binding partners very challenging, since there is no indication of where and how these proteins could bind.

Until now very few proteins have been described to physically bind E6AP and of only a few the binding site is known: HERC2 and E6, two regulating proteins, bind in the N-terminal region of E6AP, while Ubch7, a specific E2 enzyme, binds in the C-terminal part (on the HECT domain).

To identify more targets we used several techniques. The first I will describe involves a very famous organism: Bakers yeast (S. cerevisiae).

In our laboratory yeasts are used to perform a technique called yeast two-hybrid assay (Y2H) which is very similar to fishing: we toss out a bait in the hope to catch a fish (prey).

We used this technique on a mouse brain library (our fishes) having E6AP as the bait. We managed to identify six different proteins, four of which were confirmed to be direct interactors of E6AP: UIP2, UIP3, UIP4 and Ubch7 (previously known as the specific E2 of E6AP).

Another model system we use in our laboratory is the reason why sometimes we get sick: bacteria. They are not always bad, in the lab they can be pretty useful. Bacteria can produce proteins for us and we can use them as our biological background. Thanks to a vector system obtained from the laboratory of Dr. Gali Prag (Israel), which we have optimised for our purposes, we are able to "make" in bacteria everything we need to reproduce exactly the ubiquitination system and/or to study more in detail the interaction of E6AP with its targets.

The last model system we are currently working with are mammalian cells. In particular we are using HEK293T cells, generously provided by Dr. Martin Scheffner, which have very low levels of E6AP. In these cells it is possible to observe the effect of E6AP on target degradation. With increasing levels of E6AP the proteins are degraded faster (eg. Ring1b, a very well established target of E6AP). In absence of E6AP the proteasome (Pac Man) cannot degrade target proteins because they are not marked with ubiquitin.

Once UIP2, UIP3 and UIP4 were identified we asked ourselves where these proteins were binding on E6AP. To answer this question we made several deletion constructs of E6AP, deleting parts of the protein starting from the N-terminal domain. Using these deletion constructs in a Y2H, we were able to identify three different binding domains on E6AP for UIP2, UIP3 and UIP4. Some of these domains contain AS mutations.

We hope that studying these mutations in the complete E6AP and investigating their effects on target interaction will help us to uncover the molecular basis of some of the symptoms observed in Angelman Syndrome.

Lastly, we will look at the role of the newly-identified targets in mice in collaboration with Ype Elgersma at the Erasmus Medical Center in Rotterdam.

I would like to thank my supervisor Dr. Ben Distel and the whole team back in Amsterdam, and of course the AFSA to give me and my fellow scientists the possibility to meet the people we are really working for: you and your children.

Mechanisms of UBE3A Imprinting

Angela MABB, Ph.D.

I would first like to thank the organisers for inviting me. I will be presenting a summary of my research and that of my colleagues in the laboratories of Ben Philpot and Mark Zylka. I will conclude by discussing some recent therapeutic developments.

As a post-doctoral student in the laboratories of Dr. Ben Philpot and Dr. Mark Zylka, I was awarded a fellowship from the Angelman Syndrome Foundation to identify potential therapeutic treatments for Angelman Syndrome. We are trying to understand the basic functions of UBE3A and, in particular, how it contributes to brain function and organisation.

This morning, many of the presentations dealt with Angelman Syndrome. I will only briefly go into the murine model and strategies being considered for therapeutic treatment. I will discuss more specifically how we identified topotecan, a topoisomerase inhibitor being considered as a potential treatment for Angelman syndrome. In closing, I will briefly summarise the treatments currently being used by other laboratories.

The genome contains some 25,000 different genes. Most neurological disorders are the result of genetic mutations. Angelman Syndrome results from a problem with UBE3A, which plays a fundamental role. The prevalence of Angelman Syndrome is approximately 1:15 000. Angelman Syndrome and autism are two facets of the same problem, regarding UBE3A concentration.

Stormy Chamberlain made an excellent presentation about imprinting mechanisms. The mouse model was very helpful to us in that imprinting mechanisms in mice and humans are similar. UBE3A is expressed from the maternal allele in the central nervous system. The transcribed anti-sense blocks transcription and knocks the paternal allele out of working order. We put together a murine model that makes use of the heritability of UBE3A mutations. We cross a normal male mouse that has normal gene copies of UBE3A with a mouse carrying a mutation on one of the copies of UBE3A. The offspring can either be a healthy mouse that inherited two perfect copies of UBE3A, known as the wild type or neurotypical mice, or a mouse that inherited a mutation from the mother. Both the neurotypical and the Angelman syndrome model mice carry an intact but inactivated paternal allele of UBE3A, due to the genomic imprinting phenomenon. Let me remind you that this phenomenon occurs only in neuronal tissue. In other tissues of the body, both alleles are expressed. The phenotypes observed in individuals with Angelman Syndrome are due to the fact that UBE3A is not expressed in the neurons.

In the murine model which I am presenting today, UBE3A is expressed in the neurons of the brain of a healthy mouse. In Angelman syndrome mice, the paternal allele is intact but inactivated and we see loss of UBE3A expression in neurons and residual expression of UBE3A due to the brain's nonneuronal cells called glial cells. Angelman Syndrome is due to a single gene which has been identified. Unlike in Alzheimer's disease where the brain cells deteriorate, the brain's structure in Angelman Syndrome is largely intact but neurons do not properly communicate with one another. A pharmacological approach can thus be taken to improve brain functions in Angelman syndrome.

The aim of our research is to restore UBE3A to working order by reinstating expression of the paternal UBE3A allele. We first carry out high-speed, high-volume screening using imaging. We place murine neurons in a Petri dish, in which connections are made. A robotic system can generate various drug series, and hundreds of different compounds can be screened concurrently. We are doing this work in conjunction with Dr. Bryan Roth, whose stellar laboratory facilities are on par with those of a major pharmaceutical company. We are fortunate to have such a resource at our disposal.

The neurons are placed on a 384-well drug screening plate. Using this method, the normal imprinting process of UBE3A is preserved such that the paternal allele of UBE3A is not active normally. We use neurons from a mouse that is genetically modified such that its neurons turn fluorescent green when the paternal allele of UBE3A becomes activated. For instance, when a compound activates paternal UBE3A transcription, the green colour appears. We screened some 2,500 compounds, only one of which caused the green to appear: it was a topoisomerase inhibitor called irinotecan, which is currently in use to treat cancer. In addition to irinotecan, we performed dose-response curves for a number of topoisomerase inhibitors. The curves identify drug concentrations needed in order to achieve paternal UBE3A unsilencing. Topotecan clearly stands out as more efficient than irinotecan. In addition, we noted lower toxicity with topotecan. Topotecan is already being used to treat certain forms of cancer so we deduced that it was a good therapeutic avenue.

While the mouse's imprint mechanism is similar to that of humans, differences are found in other areas. This caused us to raise the question of how relevant our results would be if applied to humans. One question is how long the unsilencing of paternal UBE3A will last. To examine this, we used intrathecal drug administration (that is, applying drug at the spinal cord), as this is a possible route for drug delivery in humans. We accordingly administered topotecan to mice for two weeks. We found that topotecan could unsilence paternal UBE3A in a small neuron sub-set. Excitingly, this effect could last up to a year in the spinal cord, which was as long as we looked.

We wanted to gain some perspective and look also at how topoisomerase works as well as identify its side effects. Oncology and neurosciences have looked extensively at this substance, in that it is used in chemotherapy. In contrast, its action on the nervous system is still unknown and studies on the topic need to be continued in order to ensure that the treatment is both tolerated and lasting.

Topotecan is a Type-1 inhibitor. Topoisomerase is the protein that maintains the structure of DNA, which is a twisted ladder. In order to reach the DNA, the DNA structure has to be unfurled, through small cuts made by the topoisomerase enzymes. Topoisomerase inhibitors are interspersed between the cuts and limit DNA repair from taking place. As the cells can respond poorly to this process, it is important to carry out tests beforehand.

Topoisomerases play a very important part in maintaining gene expression and in DNA replication in dividing cells. They also play a fundamental part in regulating gene expression, as we showed during one study. Topoisomerase inhibition downregulates the expression of long genes, which include the UBE3A anti-sense normally responsible for silencing the paternal allele of UBE3A. In order to study topotecan function in neurons, we started out by asking a number of questions. Is topoisomerase found in the brain? If so, where is it expressed? What are its primary targets? To answer these, we looked at expression in different regions of the brain. We know that Angelman mice show highly-disrupted cortical function. Some of the symptoms observed in Angelman cases might in fact be due to changes in cortical function. All neurons express topoisomerase 1 in the adult brain. This means that the therapeutic target does exist and comprises all cells in the cortical zone. Proper product administration thus implies targeting all neurons in the brain in order to activate them.

We also looked at the hippocampus, where memory is stored. All of the neurons in this zone expressed topoisomerase. All neurons in the cerebellum, which controls coordination and motor skills, also expressed the protein. In other words, in adults, topoisomerase is expressed by all neurons. This means that all of the neurons in the brain can be activated if the therapeutic target can be delivered there – a very promising discovery.

We studied neuron survival in the event of long-term application of topotecan. No toxicity was identified in the neurons treated in vitro with topotecan for seven days. This means that topotecan does not kill neurons at the doses we used.

In the brain, neurons are connected by synapses that send out signals from one neuron to another. These synapses form a complex network which enables the brain to function. This

complex network can be reproduced very simply, in a Petri dish. By using different drugs, we are able to trigger changes in communication between neurons. In the topotecan-treated neurons, the communication between neurons was severely limited. While this suggests that there might be transient cognitive impairments seen with topotecan treatment, as sometimes seen with cancer treatments, our results were exciting because synaptic communication returned to normal soon after topotecan was removed. This suggests that the negative effects of topotecan on synaptic function may be rapidly reversed. Overall, the results are promising. In some tissues, activation can last up to one year. If synaptic activity is compromised for a short period of time, it can be recovered.

Alternative drug strategies also need to be studied. Working in cooperation with Mark Cushman and Yves Pommier, we looked at new classes of topoisomerase inhibitors that had yet never been used. We noted that UBE3A disinhibition could be triggered by this class of topoisomerase inhibitors, which is very promising. These compounds are currently being studied to determine whether they cross the blood brain barrier and whether they make it possible to activate UBE3A in vivo, in animals. Many pharmaceutical companies are currently struggling with the problem of how to cross the blood brain barrier. Several studies are thus underway on the topic. In addition, the research performed by Arthur Beaudet on anti-sense oligonucleotides have given rise to excellent results, although these compounds would likely have to be delivered quite frequently to achieve therapeutic benefit. Lastly, adenovirus treatments might make it possible to reintroduce UBE3A into the brain. However, the use of a virus for therapeutic purposes raises the problem of virus dissemination and how it can be controlled. Similarly, we do not know how many neurons we need to activate to restore function.

Doctor Segal will discuss his nucleases with you and, in particular, the TALES, which activate transcription for the paternal allele. Nuclease administration in the brain can cause problems and no clinical trials yet exist on the topic. In addition, the nucleases may trigger an immune response.

A new technology known as CRISPR deletes certain DNA segments. This technology has not been subject to any clinical trials and its action appears to be irreversible.

I would like to thank our laboratory and the members of the public who helped us. As the screening process is extremely costly, we would not have been able to conduct such experiments without the support of our sponsors, whom I also thank.

Targeted Reaction of UBE3A in a Mouse Model of Angelman Syndrome

David SEGAL, Ph.D.

Many thanks to the organisers for giving me this opportunity to talk before an audience of parents and families. Thanks also to Angela, for having presented some of the factors which will be addressing in turn.

I am neither a neurologist, nor a pharmacologist, nor even a specialist of Angelman Syndrome, but rather a protein engineer. I was contacted approximately five years ago by a group of parents and researchers who asked me whether it might be possible to use zinc finger technologies to treat Angelman Syndrome. At that time, having never heard of the syndrome, I looked into the matter and quickly came to see that my technology could be of use in this area.

This morning, we heard that there are critical treatment periods. It is also important that action be taken at a very early age, when the patients are children. This emphasises the role of parents and their families. Everything you do is important, even if you do not notice it: when you talk to scientists and you ask questions, you are inspiring the scientist. I hope I will be able to make a positive difference.

In patients with Angelman Syndrome, the second part of the UBE3A protein is inactive, due to a long RNA transcriber. By reactivating the silent copy of the gene, we may be able to achieve results. This morning's presentations showed that a variety of promising new strategies are starting to emerge, each of which has its own benefits and challenges. I will closely track the development of each of these approaches, none of which were around when I first started taking an interest in the topic. Research is progressing at breath-taking speed.

Our approach is aimed at studying how nature regulates genes in the body. Gene regulation is very complex and sets different components in motion, each of which will enable the cells to determine which genes to activate and which not to activate. The transcription factors are proteins produced by the cells, which indicate to the genes whether they should activate or not. The DNA binding causes imprinting to take place in an effector domain.

Genomes contain common transcription factors. The most frequent of these is the zinc class. We are trying to understand how nature does its job, in order to artificially recreate the same mechanisms ourselves. The zinc proteins wrap themselves around the various DNA groups. The protein sub-units then form a match with the DNA base. In other words, the zinc fingers manage to recognize base pairs. The three amino acid sub-units in the protein recognize the DNA. We could thus change these three components so that they recognize other DNA sequences.

Laboratory research has put this idea to use and managed to give rise to manipulated proteins. Many developments have since come about, including the identification of different binding domains, and have made it possible for me to go about my work more efficiently.

The zinc figure protein occurs in nature. Using recent technologies, it is possible to manipulate this protein to make it bind to a chosen sequence, in one of two ways. We can come up with an artificial transcription factor, which makes it possible to activate the UBE3A gene directly. We can also target a regression factor along the RNA transcriber in

order to make it inactive or decrease its production. If we can stop the transcription of a given strand, the other UBE3A genes would be expressed naturally.

Thanks to several different studies, we have been able to activate the UBE3A gene on a murine model. We were thus faced with the classic question in gene therapy: we now have a tool, but how can we make it enter the cell? The tool needs to make its way into the cell's nucleus, which can be triggered by a variety of factors. A large number of developments have come about in viral factors. However, when a viral factor is brought into the picture, this generally limits the effects of the introduction zone. As UBE3A is expressed across the entirety of the brain, we took an approach based on the cell's penetration peptides. The protein to which the peptide is attached crosses the cell membrane.

The second barrier to be crossed is the haemato-encephalic barrier. Our brains have a barrier that separates the brain's blood from the blood in the rest of the body: it is vital that some of the substances found in blood do not enter the brain. As the barrier is made up of a series of cells, it should be possible to cross it.

We inject the S1 therapeutic protein into the mouse's stomach. Under UV light, we were able to study the protein's presence in the mouse's brain and found that the protein injected into the mouse's stomach can reach the brain. By sacrificing the mouse, we were able to study the brain in greater detail. Those mice that received the protein appear to have it throughout their brain. The protein can cross the haemato-encephalic barrier and affect the entirety of the brain. In addition, we noted that it caused UBE3A expression.

We then studied the effects of the gene expression in the mouse. Our murine model actually provides but little information about the protein's actual action. In addition, the pathology is very deep-set in children, when it is relatively subtle in mice. We are thus currently trying to develop a new murine model in order to test our assessments and assumptions. I hope to be able to come before you again soon to present a behavioural model and report that the protein is indeed effective in mice.

We should also look into the issues of safety and individual response to treatment. We would like to move forward very quickly, but not hurrying the process. It is important that we ensure all treatment is safe and that the side effects are innocuous. Our new murine model will probably make it possible to identify new prospects for measurement. Last but not least, like Angela Maab, we may be able to study human cells.

I would like to thank all those who helped me with my research. Barbara, a post-doctoral student who has been working alongside me for five years, ran this project from end to end. I received funding from NIH and the Angelman Syndrome Foundation.

Document drafted by Ubiqus – Phone: +331.44.14.15.16 – http://www.ubiqus.fr – infofrance@ubiqus.com