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Genetic engineering cures mice of neurological deficits: prospects for treating Angelman syndrome

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'Hopefully insight into the mechanisms of releasing the brake on CaMKII will put the search for a therapy for Angelman Syndrome into full speed.'

Our insight in the molecular and cellular basis of the neurological deficits associated with childhood developmental disorders gains rapid progress. Mouse models of these diseases have greatly contributed to that advancement. Typically, this research goes through a series of steps:

- Assessment as to whether a mouse mutant is a good model for the disease. This is typically determined by comparing the behavioral phenotype of the mouse (e.g., learning deficits, epilepsy or motor coordination impairments) with the neurological deficits associated with the disease. In addition, mouse brain pathology can be compared with the post-mortem and magnetic resonance imaging (MRI) findings of patient brains;
- Investigation of the underlying mechanism that is ultimately responsible for the neurological deficits. Even though considerable progress has been made in identifying molecular pathways subserving neuronal function, the precise functions of most genes is still unknown. A combination of molecular biology, electrophysiology and pharmacology can eventually elucidate the downstream target that is ultimately affected by the absence of the mutated gene;
- Testing whether an identified target is indeed directly responsible for the major symptoms of the disease;
- Testing whether the cognitive deficits are reversible or can at least be ameliorated in young or adolescent animals.

Obviously, the last point is of critical importance, since it ultimately determines whether a treatment of the disease is ever going to be feasible. Importantly, the prospects of reversing cognitive deficits become significantly more promising. For example, it has recently been shown that rescue of some or even all deficits can be achieved in mouse models for neurofibromatosis [1,2], fragile-X [3,4], Rett syndrome [5] and Down's syndrome [6].

It is interesting to note that genetic engineering allows us to test the reversibility of a disorder, without going through the second and third above-mentioned steps – thus without any knowledge regarding the underlying mechanism. This has recently been demonstrated for Rett syndrome, in which the authors used a genetic approach to switch the silenced gene back on, once the mice had matured [5]. Similarly, a genetic approach can also be extremely useful in testing the validity of a candidate target as being ultimately responsible for the major symptoms associated with the disorder (step 3). This has recently been accomplished using a mouse model for Angelman Syndrome (AS) [7].

AS is a severe neurological disorder affecting 1:15,000 children and is characterized by mental retardation, motor dysfunction, absence of speech, autism and epilepsy. The disorder is typically caused by a deletion of human chromosome 15q11–13 (reviewed in [8]). However, a subset of AS patients (10–15%) do not show the deletion in the *UBE3A* gene. This gene resides in the 15q11-13 region and encodes the E6 associated protein (E6-AP), which is a member of the E3A family of ubiquitin protein ligases. Thus, the loss of E6-AP is primarily responsible for causing the disease. The 15q11–13 region on chromosome 15 is imprinted, which means that the paternal copy of the *UBE3A* gene is silenced in certain cell types. In particular, hippocampal neurons and cerebellar Purkinje cells show exclusive expression of the maternally inherited copy [9]. Hence, a *de novo* mutation in the maternally inherited copy of the *UBE3A* gene will result in the complete loss of E6-AP expression in these brain areas, as it is encoded by the *UBE3A* gene. E6-AP is a ubiquitin protein ligase, and therefore involved in proteasome mediated degradation of target proteins. However, its target protein(s) and its precise role in neuronal function remain elusive.

As mentioned above, an important first step in unraveling the mechanism of a cognitive disorder is the creation of a mouse mutant and the

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demonstration that the mutant is a valid model for the disease. For AS, this was undertaken by the laboratories of Arthur Beaudet and David Sweatt. Mice carrying the maternally inherited *Ube3a*-null mutation showed epilepsy, motor coordination deficits, learning deficits and impaired hippocampal plasticity [10].

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For the second step, identifying the ultimate target responsible for the disease, David Sweatt and colleagues used a smart combination of electrophysiology, pharmacology and molecular biology. This led to the finding that the activity of calcium/calmodulin-dependent kinase Type 2 (CaMKII) was decreased by approximately 30% in the *Ube3a* mice [11]. Since CaMKII had been shown to be an essential kinase in synaptic function and learning (reviewed in [12,13]) this finding was of significant importance. However, the finding was also puzzling since heterozygous αCaMKII knockout mutants, which show a similar loss of total CaMKII activity, did not show any apparent learning and plasticity deficits. In addition, the protein levels of CaMKII in *Ube3a* mutants did not seem to be affected at all. The explanation to this puzzle came firstly from the important biochemical finding that CaMKII can inhibit itself *in vitro*, by autophosphorylation of the calcium/calmodulin binding site of the protein. This autophosphorylation at the threonines 305 and 306 interferes with the binding of calcium/calmodulin and, hence, renders the protein unresponsive to calcium signaling [13]. Secondly, by raising antibodies against this phosphorylation site, the laboratory of Alcino Silva showed that this self-inhibition also occurs *in vivo*, and more importantly, that a targeted mutation mimicking self-inhibited αCaMKII resulted in a severe dominant negative effect [14]. These mice showed severe learning deficits, and a severe loss of synaptic plasticity. Thus, even a moderate increase in the amount of self-inhibited CaMKII could potentially explain the phenotype of the *Ube3a* mice. Further analysis of the *Ube3a* mutants confirmed that the loss of kinase activity in these mice was indeed likely attributable to the increased self-inhibition of CaMKII in these mutants [11]. But did that mean that the ultimate cause of the deficits in *Ube3a* mice was discovered, even without knowing anything about the role of neuronal E6-AP?

To test whether the increased self-inhibition of CaMKII was indeed causing the cognitive deficits of the *Ube3a* mice, an αCaMKII-TT305/6VA mutant was used, in which the amino acids Thr305/306 were substituted by two nonphosphorylatable residues (Val and Ala). Homozygous loss of αCaMKII self-inhibition results in several behavioral impairments, but heterozygous αCaM-KII-TT305/6VA mice showed no discernible phenotype. Thus, crossing this mutation into mice carrying the *Ube3a* mutation might significantly decrease the amount of inhibited CaMKII and result in some improvement of the observed deficits. This project, jointly undertaken in the Weeber and Elgersma laboratory, turned out to be much more fruitful than could be hoped for [7]. The *Ube3a/αCaMKII-TT305/6VA* double mutants showed a decreased amount of inhibitory phosphorylation, and nearly restored levels of CaMKII activity. More importantly, the learning and motor deficits were completely rescued, and plasticity as measured in brain slices was back to normal. Epilepsy was still present in some of the double mutants, but the number of animals showing epilepsy was threefold reduced as compared with the *Ube3a* single mutants. Finally, it was noted that the moderate increase in bodyweight of the *Ube3a* mice was rescued. Although overweight is not known as a characteristic feature of the disease, a tendency to become obese with age has been reported [15], and obesity is present in the majority (85%) of AS children carrying the *UBE3A* mutation [16]. Furthermore, obesity is a common feature in children with a previously unrecognized form of AS [17]. Thus, even in that respect the *Ube3a* mouse turned out to be a good model, and the absence of increased bodyweight in the double mutants indicated that increased self-inhibition of CaMKII was also responsible for this aspect of the disease. This is an interesting finding, since αCaMKII is only expressed in neurons, indicating that the overweight has a neurological origin. Most likely this reflects changes in feeding behavior, since most children with the *UBE3A* mutation show a strong attraction to food [16].

The results obtained by the *Ube3a/CaMKII* double mutant clearly identify the increased amount of self-inhibited CaMKII as the direct cause of the neurological symptoms associated with AS. But where do we go from here? I believe that it is still very important to figure out the steps that link A6-EP to CaMKII, because knowledge of that pathway will give us the greatest chance of finding a treatment. Since

decreased self-inhibition rescues all behavioral phenotypes, it is likely that the link between E6-AP and CaMKII is rather direct. An obvious model is that E6-AP regulates a phosphatase that is responsible for dephosphorylating CaMKII. Indeed, protein phosphatase activity (PP1/PP2a) is reduced in the *Ube3a* mutant [11]. However, protein levels of these phosphatases are unaffected, indicating that these enzymes themselves are not a direct target for E6-AP mediated degradation. It is also puzzling why the reduced PP1/PP2a activity does not affect other kinases. So it remains to be seen whether the decreased phosphatase activity is indeed causing the increased level of self-inhibited CaMKII, and further work is required to solve this puzzle.

'These results identify the increased amount of self-inhibited CaMKII as the direct cause of the neurological symptoms associated with Angelman syndrome.'

Of course, even with all this data in place the ultimate question is whether we will be able to improve the cognitive impairments of the affected children. The complex regulation and involvement in many neuronal processes make CaMKII a very precarious target. Furthermore, how do we prevent the self-inhibition? Although we can (and should) test ways to increase CaMKII activity, it is also wise to take a few steps back and test if the behavioral phenotype of AS is actually reversible, at least in mice. This can be achieved by using the pharmacogenomics strategy, as described above for Rett syndrome. Even though many AS children show microcephaly by the age of 2 years, and sometimes some cerebral atrophy, there is still a reasonable possibility that the symptoms can be ameliorated to some extent. CaMKII is involved in processes that are extremely plastic, and therefore most likely reversible. Therefore, it is now time to take up this challenge. Hopefully insight into the mechanisms of releasing the brake on CaMKII will trigger the interest of pharmaceutical companies and will put the search for a therapy for AS into full speed.

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