C2 Domain Function in Healthy and Diseased Brain

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Chapter 6
General Discussion

Scope

The aim of the work described in this thesis was to gain a better understanding of the function of C2 domains, a class of protein domains found in over 200 mammalian proteins, many of which are important for neuronal function. We applied various approaches and techniques to study four C2 domain proteins involved in synaptic vesicle release (Doc2B, Synaptotagmin-1 and Piccolo) and in neuronal development (CC2D2A).

Main findings

We investigated the direct interaction between Doc2B and phospholipid membranes, developing an innovative method to investigate the formation of a single fusion pore in real time, with concurrent real-time fluorescence imaging, microfluidics and force spectroscopy.

We have characterized mice carrying the genetic variation in the C2 domain of Pclo associated with Major Depressive Disorder. We could identify changes in certain cellular parameters, but not calcium-dependent lipid binding. Importantly, there were no changes observed at the behavioral level.

We studied mutations in CC2D2A that cause autosomal recessive mental retardation as well as Meckel and Joubert syndromes. We found that the C2 domain that is truncated in these ciliopathies does not normally bind phospholipid membranes, and that CC2D2A truncation resulting from mutations in its gene can lead to CC2D2A degradation.

In combination, these findings underscore the fact that although for many C2 domains calcium-dependent phospholipid binding is the main mode of action, there must be other modalities by which C2 domains exert their functions.

C2 domains in human disease

C2 domain mutations are associated with a large number of human diseases, for the most part related to the central nervous system function. This fact clearly indicates the importance of this class of domains in neuronal cell function, and indeed many C2 domain proteins have been implicated in membrane fusion dynamics. Otoferlin (containing six C2-domains) is the calcium sensor for synaptic vesicle exocytosis in the inner hair cell ribbon synapses and its absence or dysfunction was shown to cause deafness[1]. Dysferlin and myoferlin are two muscle cell proteins containing several C2 domains that bind calcium and membranes[2,3], and defects in either of these proteins result in muscular dystrophy both in humans and in mice[4-6]. We have studied disease-associated variants of C2 domains of CC2D2A and Piccolo, with the aim of understanding the mechanism of their involvement in human disease.

CC2D2A

An increasing number of reports suggest that cilia play an important role in neuronal cells. This is supported by the identification of brain ciliopathies, and discoveries of the important contribution of neuronal cilia to cerebellar development, hippocampal neurogenesis, and axon guidance (for a review, see[7]). Because mutations in CC2D2A observed in different families gave rise to different diseases with ciliopathies as a common hallmark, it's likely that CC2D2A is involved in ciliary formation or function. However, in our study we did not observe localization of CC2D2A at neuronal cilia. This does not exclude a role of CC2D2A in ciliary formation (at earlier stage than detectable by the ciliary marker we used), or that CC2D2A affects cilia while located elsewhere. There is at least one published report of a protein that causes a ciliopathy without localizing to the cilium (by interfering with ciliary protein processing in the mitochondria[8]).

A mutation in a related protein, CC2D1A (that has similar domain structure with CC2D2A, including a C2 domain in the C-terminal part of the protein), was found in nine consanguineous families with mental retardation[9,10]. As in the case of CC2D2A, the mutation produced a truncated protein product lacking the C2 domain[9]. Knockout studies in mice revealed that CC2D1A is essential for survival, and controls functional maturation of central synapses. It contains a C2 domain, but does not bind to calcium[11]. We showed that C2 domain of CC2D2A does not function via calcium or lipid binding, but its presence may be critical for CC2D2A stability. The similarities with CC2D2A suggest that both proteins may have similar function in brain development and in particular in synapse maturation. Further studies of the role of CC2D2A in synaptic maturation will benefit from implementation of CC2D2A knock-down in neurons. This will allow testing the consequences of CC2D2A absence and of expression of truncated (C2 domain deleted) versions of CC2D2A on a CC2D2A-free background, which is a better model in view of the autosomal recessive inheritance of the related diseases.

Piccolo

Based on findings from a Genome-Wide Association Study (GWAS)[12], we created and characterized a knock-in mouse model carrying the PCLO Serine-Alanine missense variant rs2522833, which showed suggestive association with MDD in human population studies. Our findings revealed a slight but significant increase in the synaptic expression of the Piccolo protein relative to a synaptic vesicle marker VAMP2. In addition, EPSPs evoked by single action potentials in cultured hippocampal neurons from $Pclo^{SA/SA}$ mice were increased. The differences in the levels of Piccolo protein and the synaptic transmission were in the same range (30% change),

as might be expected from a small variation in amino acid sequence. We excluded modification of the calcium-dependent membrane binding function of the C2A domain of Piccolo. It is possible that the trafficking of Piccolo to the synaptic terminals is altered as a result of the variation.

It was clear that none of the observed cellular changes translated into a detectable behavioral phenotype in $Pclo^{SA/SA}$ mice. Indeed, the analysis of $Pclo^{SA/SA}$ mouse behavior consistently showed no significant differences in general health, spontaneous or challenged home cage behavior, coordination, anxiety, learning, memory, fear response or despair. This lack of behavioral phenotype may relate to the multifactorial nature of MDD and the small effect size of the rs2522833 variation, and thus need for other genetic and/or environmental factors for the depression phenotype to manifest itself.

Our result brings up a question of when the appropriate time is to start modeling a disease-associated mutation in mice. With the knowledge that complex disorders are not caused by a single genetic factor, how do we decide to pursue detailed characterization of the effect of a single gene in an animal model? There are also related considerations on adjusting significance thresholds or matching data groups in GWAS studies in order to tease out the most likely candidates that would otherwise not meet the standard criteria of significance.

One answer could be that rather than a single gene, we should look for groups of genes that function in the same biological pathway in analyzing GWAS data[13]. While single gene variations found in complex neuropsychiatric disorders can produce behavioral phenotypes in rodents, identification of more than one gene belonging to the same or related cellular function gives more certainty in pursuing such projects. In the case of autism spectrum disorders, characterization of mouse models of Neuroligin, Neurexin and Shank family genes was preceded by identification and confirmation of the involvement of synapse formation and maintenance pathways[14]. For Major Depression, so far there is substantial genetic and functional evidence for the role of Serotonin Transporter, 5HTT[15], brain-derived neurotrophic factor, BDNF[16], and Tryptophan hydroxylase, TPH2[17] genes. To the best of our knowledge, there is no direct evidence of cross-talk between Piccolo and the serotonin transport (5HTT), synthesis (TPH2), or BDNF pathways.

Normal function of C2 domains

C2 domain heterogeneity

In our work we attempted to take advantage of the shared propertied of C2 domains, in order to apply the same principles and methods to the study of different proteins. We observed substantial differences already in the production of recom-

binant C2 domains of different proteins from bacterial culture. Even proteins with a high degree of homology (such as Synaptotagmin-1 and Doc2B) exhibited different yields and stabilities. Interestingly, the third member of the Doc2 protein family, Doc2C, which shares 43% homology (amino acid identity) with Doc2B, does not bind calcium[18]. Also, in our hands the C2 domain of CC2D2A did not show calcium-dependent lipid binding. Thus, the general definition of C2 domains as calcium- and membrane-binding units[19] does not cover the full range of C2 domain properties. Additional properties of C2 domains are probably mediated by protein-protein interactions (which we investigated for the Piccolo C2A domain).

Another aspect of C2 domain function that appears to vary between proteins is the nature of the change that occurs upon calcium binding. While conformational changes are thought to predominate e.g. in the Piccolo C2A domain[20], electrostatic changes are considered to have a major contribution to membrane penetration and membrane fusion activity of Synaptotagmin-1 [21]. These conformational and electrostatic changes might affect membrane penetration, but potentially also modulate C2 domain cooperativity (see below), or protein interactions.

Why would you need more than one C2 domain?

For many C2 domains the main function is related to phospholipid membrane association. There is an important distinction between proteins containing a single C2 domain (such as protein kinase C, PKC, and phospholipase C, PLC), where the C2 domain serves to localize the protein to the site of its enzymatic activity, versus two or more tandem C2 domains (Synaptotagmins, Doc2), where C2 domains can produce membrane deformation. Multiple tandem C2 domains were also shown to mediate calcium sensing for membrane fusion, for example in ferlin family proteins (otoferlin[1], dysferlin[6] and myoferlin[5]). Also for this class of proteins, the fusion mechanism co-depends on SNARE protein assembly[22]. It has been suggested that the presence of two or more C2 domains in a protein represents a coincidence detection mechanism, to enhance the membrane-binding function of one C2 domain upon calcium signal, where activation of one domain leads to activation of the other domain. In this model, close proximity of two different lipid binding partners (phosphatidylserine (PS) and PIP_2) on the membrane determines the binding site by the double-C2 domain protein[23]. While one of the two C2 domains (C2A for Syt-1 and DOC2B) binds to PS, the other (C2B for both Syt-1 and DOC2B) binds to both PIP_2 and PS. On the other hand, Synaptotagmin-1 C2 domains were shown to interact with each other in the presence[24,25] and in the absence of calcium[26]. Such cooperativity between adjacent C2 domains might explain in part the specific properties of tandem-C2 domain proteins, such as the ability of double, but not single C2 domains in a protein to enhance phospholipid membrane fusion.

It has been shown that two tandem C2 domains are necessary to bridge membranes[21,27]. It was thus suggested that the function of double C2 domain proteins such as Synaptotagmin-1 is to connect opposing membranes, pulling them into close proximity to facilitate the initiation of the fusion reaction by the SNARE complex[28]. An alternative mechanism proposed that double C2 domain sensors reduce the energy barrier for fusion by generating positive membrane curvature[29,30]. Visual evidence for membrane curvature induction by Synaptotagmin-1 and Doc2B is based on electron microscopy of protein-liposome mixtures after staining with uranyl acetate. However, the physiological relevance of this phenomenon remained controversial without supporting evidence from live imaging.

Our results with immobilized phospholipid bilayers revealed that C2AB domains directly induced membrane coupling events between opposing membranes. As expected for Ca^{2+} sensor proteins, this activity was dependent on Ca^{2+}, phosphatidylserine and protein concentration. Membrane coupling events were accompanied by the formation of membrane stalks, resistant to high forces and with membrane (but not luminal contents) continuity, suggesting that hemifusion had occurred. Thus, while SNARE complex assembly undoubtedly plays a crucial role in driving membrane fusion, our data demonstrate that tandem C2 domains participate meaningfully in Ca^{2+}-secretion coupling by direct membrane binding, lowering the energy barrier in the presence of Ca^{2+} signals.

In our experiments with immobilized bilayers, we did not include PIP_2 or SNARE proteins. The new method can be applied extensively in future research, where the inclusion of these components, other accessory proteins or comparisons between different Ca^{2+} sensors can potentially reveal a wealth of information about C2 domain-mediated membrane fusion.

SNARE-mediated membrane fusion

Synaptotagmin-1 and Doc2B are neuronal calcium sensors for synaptic vesicle fusion (together with Synaptotagmin-2[31] and Synaptotagmin-7[32]), and their interplay with the SNARE complex machinery is one of the central questions in understanding the regulation of synaptic vesicle release. While there is overwhelming evidence of the key role the SNARE complex plays in membrane fusion[33,34], the role of Doc2B and Synaptotagmins in the fusion reaction is less clear. We know that the C2AB domains of Syt-1 and Doc2B are necessary to encode the strict Ca^{2+} dependence of vesicle fusion rates in secretory systems[30,35,36].

An ongoing debate on the role of Synaptotagmin-1 and Doc2B in synaptic vesicle fusion offers two main mechanisms of action in relation to the SNARE complex: they act as a clamp, releasing the block imposed on the SNARE complex when calcium

levels reach their threshold of sensitivity, or they change the shape of membranes to reduce the energy necessary for fusion. The calcium induced stimulation of fusion by Syt-1 was shown to be at least in part due to a change in the interaction of sensor with the SNARE complex[22,37,38]. There is also ample evidence of Syt-1 and Doc2B acting directly on membranes, and inducing membrane curvature[21,29,30].

The clamping mechanisms and the active membrane modifying mechanisms of action of Doc2B and Syt-1 are not mutually exclusive, and can be tested separately using *in vitro* assays. With our Optical Tweezers based method, we have focused on direct interaction of Doc2B with the phospholipid membranes, and cannot provide any conclusions on the potential clamping function of Doc2B or Syt-1. We provide evidence that C2AB domains of Doc2B can stimulate hemifusion of SNARE-free phospholipid membranes containing phosphatidylserine. Our findings confirm that Doc2B does play an active role as inducer of the phospholipid membrane fusion reaction, and is not a mere accessory protein. Moreover, we show for the first time that C2 domain proteins are capable of inducing membrane hemifusion without the SNARE complex. In living systems, we suggest that this activity contributes to the fusion reaction along with the SNARE proteins, by membrane remodelling and stimulation the formation of the hemifusion intermediate state.

Perspective and future directions

As mentioned above, there is a substantial number of human diseases caused by mutations in C2 domains. There are also several GWAS proposing association of sequence variations in C2 domains with susceptibility to different disorders, e.g. cancer or hypertention[39,40]. All this should encourage us to invest more resources in expanding our understanding of the function of these domains. So far a lot of attention has been focused on teasing out calcium-dependent phosphlipid membrane interactions of C2 domains. It's possible that C2 domains are involved in functions beyond calcium sensing, membrane anchoring and shaping, for example through interaction with other proteins. Indeed, some of the C2 domains in proteins fundamental for brain function (e.g. CC2D2A) do not bind calcium. For the calcium-sensing C2 domains, the functional defect produced by mutation is not necessarily caused by calcium binding defects (e.g. we found that MDD-associated variant of Piccolo had normal calcium-dependent lipid binding, while some of the synaptic functions were compromised). This suggests that more attention should be dedicated to investigating other ways that C2 domains exert their functions, and our suggestion is to start by mapping and characterizing the protein interactors of C2 domains.

The optical tweezers based method we have developed to investigate the interaction of Doc2B C2 domains with the phospholipid membrane provides an exciting

pathway to collect data on membrane fusion, hemifusion, and fusion pore formation using a variety of proteins and conditions. We can envision for example comparing how the C2AB domains of Doc2B and Synaptotagmin-1 induce (hemi)fusion at different calcium concentrations, or how phospholipid membrane composition affects their fusogenic properties. It can also be used to test various C2 domain mutants, and visualizing their "behavior" and position in relation to the fusion pore by tagging proteins with a fluorescent marker. This method is also amenable to assessing a more complex system, with several protein components (e.g. SNARE proteins, complexin, Munc-13, etc), or different lipid composition on the two membrane surfaces (reproducing synaptic vesicle – plasma membrane topology).

An important feature of the method is the possibility to investigate fusion at single "vesicle" level, measuring and imaging discreet fusion events instead of an average of a population of vesicles/liposomes. This provides an advantage over other cell-free systems such as reconstituted fusion assays[41].

The powerful combination of force spectroscopy with fluorescence imaging in our method enables concurrent quantification of the force and visualization of membrane dynamics. This may lead to a broader application of the system, to characterize the forces involved in the membrane fusion reaction, and the critical and rate-limiting steps in this process.

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