

RESEARCH ARTICLE

Increased Expression of the Large Conductance, Calcium-Activated K⁺ (BK) Channel in Adult-Onset Neuronal Ceroid Lipofuscinosis

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Abstract

Cysteine string protein (CSP α) is a presynaptic J protein co-chaperone that opposes neurodegeneration. Mutations in CSP α (i.e., Leu115 to Arg substitution or deletion (Δ) of Leu116) cause adult neuronal ceroid lipofuscinosis (ANCL), a dominantly inherited neurodegenerative disease. We have previously demonstrated that CSP α limits the expression of large conductance, calcium-activated K⁺ (BK) channels in neurons, which may impact synaptic excitability and neurotransmission. Here we show by western blot analysis that expression of the pore-forming BK α subunit is elevated ~2.5 fold in the post-mortem cortex of a 36-year-old patient with the Leu116 Δ CSP α mutation. Moreover, we find that the increase in BK α subunit level is selective for ANCL and not a general feature of neurodegenerative conditions. While reduced levels of CSP α are found in some postmortem cortex specimens from Alzheimer's disease patients, we find no concomitant increase in BK α subunit expression in Alzheimer's specimens. Both CSP α monomer and oligomer expression are reduced in synaptosomes prepared from ANCL cortex compared with control. In a cultured neuronal cell model, CSP α oligomers are short lived. The results of this study indicate that the Leu116 Δ mutation leads to elevated BK α subunit levels in human cortex and extend our initial work in rodent models demonstrating the modulation of BK α subunit levels by the same CSP α mutation. While the precise sequence of pathogenic events still remains to be elucidated, our findings suggest that dysregulation of BK channels may contribute to neurodegeneration in ANCL.

Introduction

Cysteine string protein (CSP α) is a synaptic vesicle protein and molecular chaperone that is essential for neuroprotection. Mutations in CSP α , L115R and L116 Δ , cause adult neuronal ceroid lipofuscinosis (ANCL), a neurodegenerative disease characterized by the lysosomal accumulation of auto-fluorescent storage material, lipofuscin [1–3]. CSP α is comprised of an N terminal “J domain”, a hydrophobic stretch of residues followed by the characteristic cysteine string region and a C terminal domain thought to bind client proteins [4]. The mutations L115R and L116 Δ that cause ANCL are in the cysteine string region and disrupt anchoring of CSP α to synaptic vesicles [5], most likely leading to a loss-of-chaperone-function at the synaptic vesicle and a toxic gain-of-function of mis-localized CSP α .

The role of CSP α -mediated synapse protection in neurodegenerative diseases remains a central biological question. Recognition of the importance of CSP α in the defense against neurodegeneration has fueled the pursuit of strategies to reinforce CSP α 's neuroprotective activity. CSP α KO mice exhibit fulminant neurodegeneration that is activity-dependent and have a shortened lifespan [6,7]. In *Drosophila*, CSP α KO's are characterized by uncoordinated movements, shaking, temperature-sensitive paralysis and reduced lifespan [8]. In *C elegans*, CSP α null mutants show age-dependent sensorimotor defects, neurodegeneration and reduced lifespan [9]. Understanding the biochemical sequence of events underlying CSP α -mediated neuroprotection is critical in order to evaluate the efficacy and safety of therapeutics targeting CSP α . The assembly of CSP α with Hsc70 (heat shock cognate protein of 70kDa) and SGT (small glutamine rich tetratricopeptide repeat protein) to prevent synapse loss is an important feature of current models of the biochemical pathway underlying CSP α -mediated-neuroprotection [6,10–12]. As chaperone systems, in general, are responsible for the dynamic balance between promoting protein folding and directing proteins to degradation via the quality control machineries, the conformational work performed by the CSP α /Hsc70/SGT complex is likely important for maintaining the functional integrity of presynaptic protein clients.

We have recently reported that the expression of large conductance, calcium-activated K⁺ (BK) channels at the cell surface is regulated by CSP α [13,14]. BK channels are activated by both membrane depolarization and elevated intracellular Ca²⁺ levels and are central to neuronal excitability and neurotransmitter release. BK channel activity is regulated by a number of pre- and post-translational events and several conditions are further reported to influence channel expression at the plasma membrane, such as auxiliary BK β subunits, alternative splicing of the pore-forming α subunit and protein ubiquitination [15]. Our recent work has demonstrated that expression of the human mutations CSP α L115R or L116 Δ in a neuronal cell line, is associated with a significant elevation of BK channel density at the cell surface. To extend these observations, in the present study we have analyzed human post-mortem ANCL brain specimens by western blot. Expression of the pore-forming BK α subunit in ANCL and Alzheimer's disease (AD) was compared. Our data demonstrate that BK channel protein expression is higher in human post-mortem ANCL compared with age-matched control specimens. We further show that BK α subunit levels are not altered in brain cortical tissue from AD patients. These results suggest that dysregulation of BK α subunit expression is selective for the pathogenic cascade of events underlying ANCL.

Results

BK channel expression is elevated in ANCL

BK α subunit expression was evaluated in crude synaptosome fractions (P2) prepared from a post-mortem ANCL cortex sample obtained from a 36 year old male with the CSP α mutation

L1160 Δ and a control cortex sample derived from a 34 yr old male (Fig 1). A higher level of BK α subunit (~2.5 fold increase) was found in ANCL cortex compared with the control sample. No difference was detected in the cellular levels of β -actin. This increase in BK channel expression in human ANCL cortex is consistent with our previous work showing that BK α levels are higher in CSP α KO mice and neuronal cell lines expressing mutated forms of CSP α , including L116 Δ [13,14].

Neuronal Ceroid Lipofuscinoses (NCLs), also known as Batten Disease, are a group of at least 14 distinct genetic disorders characterized by lysosomal accumulation of auto-fluorescent lipofuscin in neurons and neurodegeneration in the CNS. ANCL is the only NCL type with dominant inheritance. It is a rare, autosomal dominant adult-onset, neurodegenerative disorder, and to date, less than 100 proven isolated or familial cases have been reported. To examine if the CSP α -related changes in synaptic protein machinery are involved in diseases other than ANCL, we investigated the expression of BK α subunit levels in post-mortem Alzheimer's disease (AD) cortex samples. Fig 2 shows AD cortex samples from a female of 64 years, a male of 64 years, a male of 71 years and a female of 76 years. No change in BK α subunit expression was observed in the four AD cortex specimens evaluated compared with age-matched controls. The detection of β -actin in the same samples is shown for reference. We also examined BK α subunit expression in a post-mortem sample from an individual with clinically suspected ANCL, who was negative for mutations in the gene *DnaJC5*, the gene coding for the CSP α protein. BK channel levels were not altered in this tissue compared with age-matched control. These data suggest that the increase in expression of the BK α channel in post mortem cortex is selectively associated with mutations in the gene *DNAJC5*.

CSP α KO mice appear normal at birth, but around postnatal day 20, they develop progressive neurodegeneration, followed by early lethality between days 40–80. However, CSP α heterozygote mice, which have reduced levels of CSP α , are asymptomatic [6]. The extent to which CSP α expression falls before initiating degenerative consequences is not yet determined, but would appear to be <50% of normal, based on survival data from heterozygous mice. CSP α is a heavily palmitoylated protein that migrates as a monomer of ~35 kDa following separation by SDS-PAGE. In cortical tissue from an ANCL patient with the L116 Δ mutation, CSP α levels were reduced compared with an age matched control (Figs 1 and 3), consistent with a report by Noskova et al [2]. Fig 3 shows that SNAP25 and dynamin 1, two proteins in the cellular CSP α pathway, are also reduced in ANCL cortex [16–19]. We also observed that mRNA levels for CSP α and SNAP25 were reduced compared with levels in the control brain tissues. The sample identified as clinically suspected ANCL, but negative for mutations in *DnaJC5*, did not have a similar reduction in CSP α at either the mRNA or protein levels.

CSP α is capable of forming oligomers. We and others have detected 70 kDa detergent-resistant CSP α dimers in rat brain [20] and various cell lines [21,22] and have shown that amino acids between 83 and 136 are important for CSP α self-association [23]. Whether CSP α oligomers display an altered chaperone specificity or chaperone-independent functions is not known. Mutant forms of CSP α (i.e. L115R and L116 Δ), exhibit a high potency to oligomerize [5,24]. To what extent ANCL pathology results from a reduction in monomeric CSP α versus the generation/action of CSP α oligomers is not clear. We asked if crude synaptosomal (P2) fractions would contain an abundance of the higher molecular weight CSP α oligomers. To resolve CSP α oligomers by SDS-PAGE, we first solubilized synaptosomes in 0.5% (v/v) Triton-X100/PBS at 4°C prior to incubation in Laemmli sample buffer at 37°C for 1 hour. The results in Fig 3 demonstrate that the crude synaptosome preparations from post-mortem ANCL have reduced levels of both CSP α monomer and oligomers compared with an age-matched control sample.

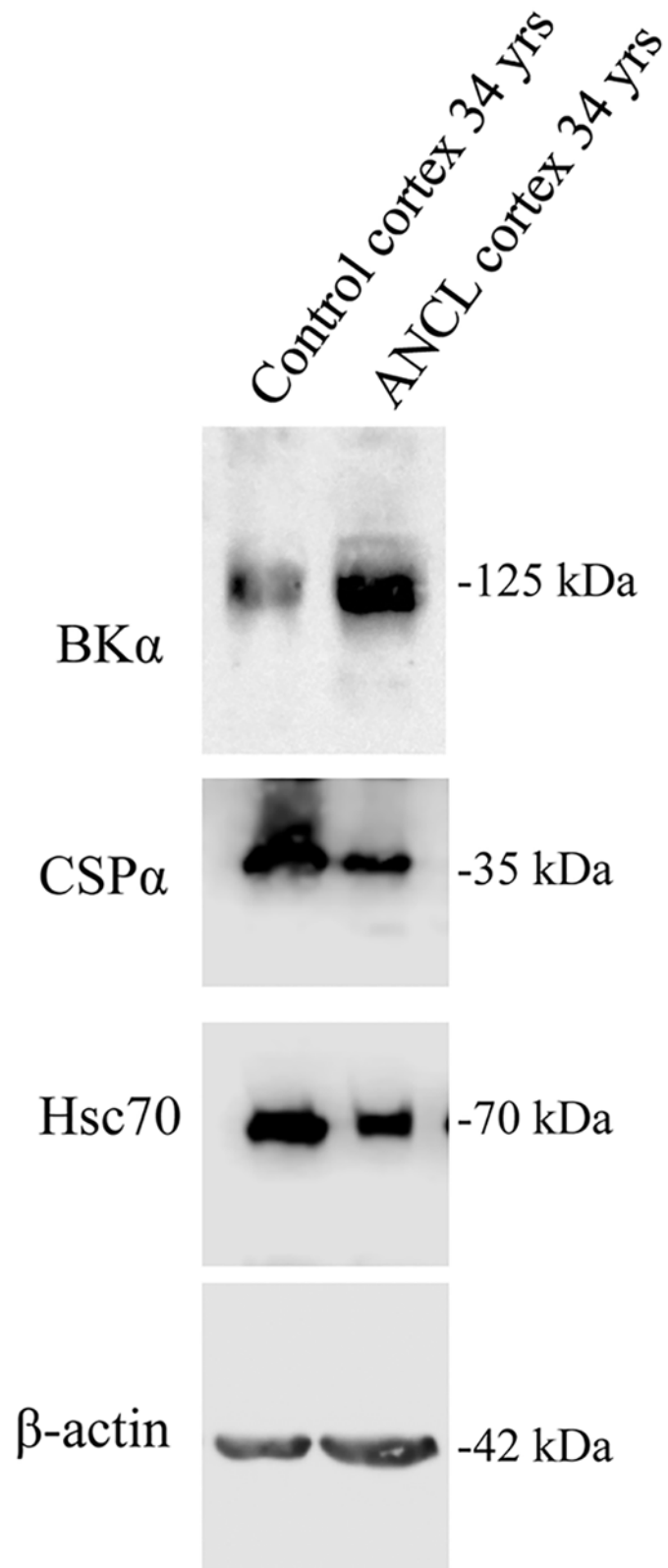


Fig 1. BK α channel expression is elevated in ANCL. Western analysis of BK α channel, CSP α , and Hsc70 detected in 20 μ g of crude synaptosome fraction prepared from human cortex Control (34 yrs) and ANCL.

(L 116 Deletion; 36 yrs) as indicated. Detection of β -actin on the same blot was used to verify equal loading between the various lanes.

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Fig 3 also shows that CSP α monomer levels were reduced in three of the four AD samples evaluated, relative to the age-matched controls; only the 64 yr old female did not show a reduction in CSP α . There is substantial variability in the extent of reduction in CSP α levels. Notably, CSP α levels were found to be decreased by 35% in the 68yr old control cortex compared with cortex of the 67, 59 and 36 yr controls. Chandra and colleagues have recently reported a decrease in CSP α levels in postmortem AD cortex [16]. Together these observations suggest that CSP α levels are generally reduced in subpopulations of AD patients and that an age-dependent, non-AD dependent reduction in CSP α levels may also occur. In contrast to our data in ANCL tissue and CSP α knockout mice, we did not observe an increase in BK α channels in AD specimens, indicating that a partial reduction in CSP α levels did not result in the increase in BK α subunit levels in AD post-mortem cortex. These findings are consistent with our observations in CSP α heterozygous mice, which have approximately half the normal level of CSP α , but normal BK channel expression and are asymptomatic [6]. Together these findings indicate that the observed increase in BK channels correlates with total CSP α deficiency, as observed in the CSP α knockout mice, or with the heterozygous CSP α mutation L116 Δ as seen in ANCL, but not with partial reductions in the expression of normal CSP α protein, as observed in heterozygous CSP α mice.

Time course of CSP α oligomer expression in CAD cells

Three obvious scenarios arise from the fact that CSP α oligomers are not abundant in crude synaptosome fractions (P2) prepared from post-mortem ANCL cortex; (1) CSP α oligomers accumulate during ANCL disease progression, but do not co-fractionate with CSP α monomers anchored to synaptosomes (2) CSP α oligomers are quickly cleared from neurons or (3) CSP α

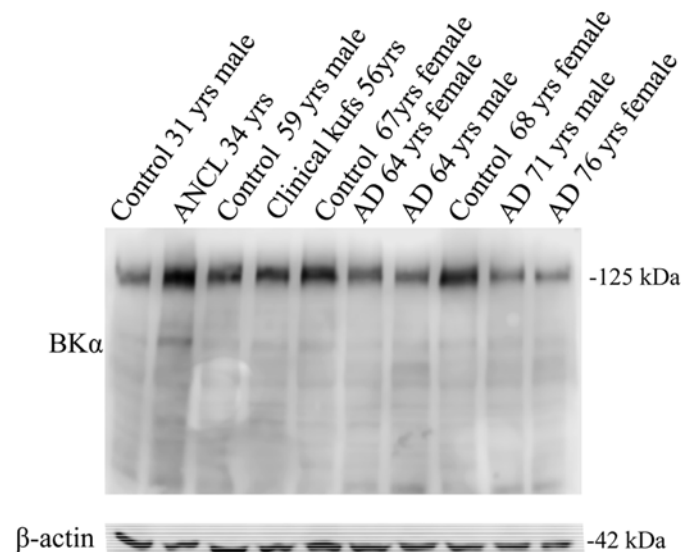


Fig 2. BK α channel expression is elevated in ANCL. Western blot analysis of BK α channel detected in 35 μ g of crude synaptosomes prepared from human cortex as indicated. Detection of β -actin on the same blot was used to verify equal loading amongst the various lanes. For the 10 human samples the BK α values were (left to right); 203000, 496000, 336000, 322000, 399000, 256000, 227000, 406000, 215000, 154000.

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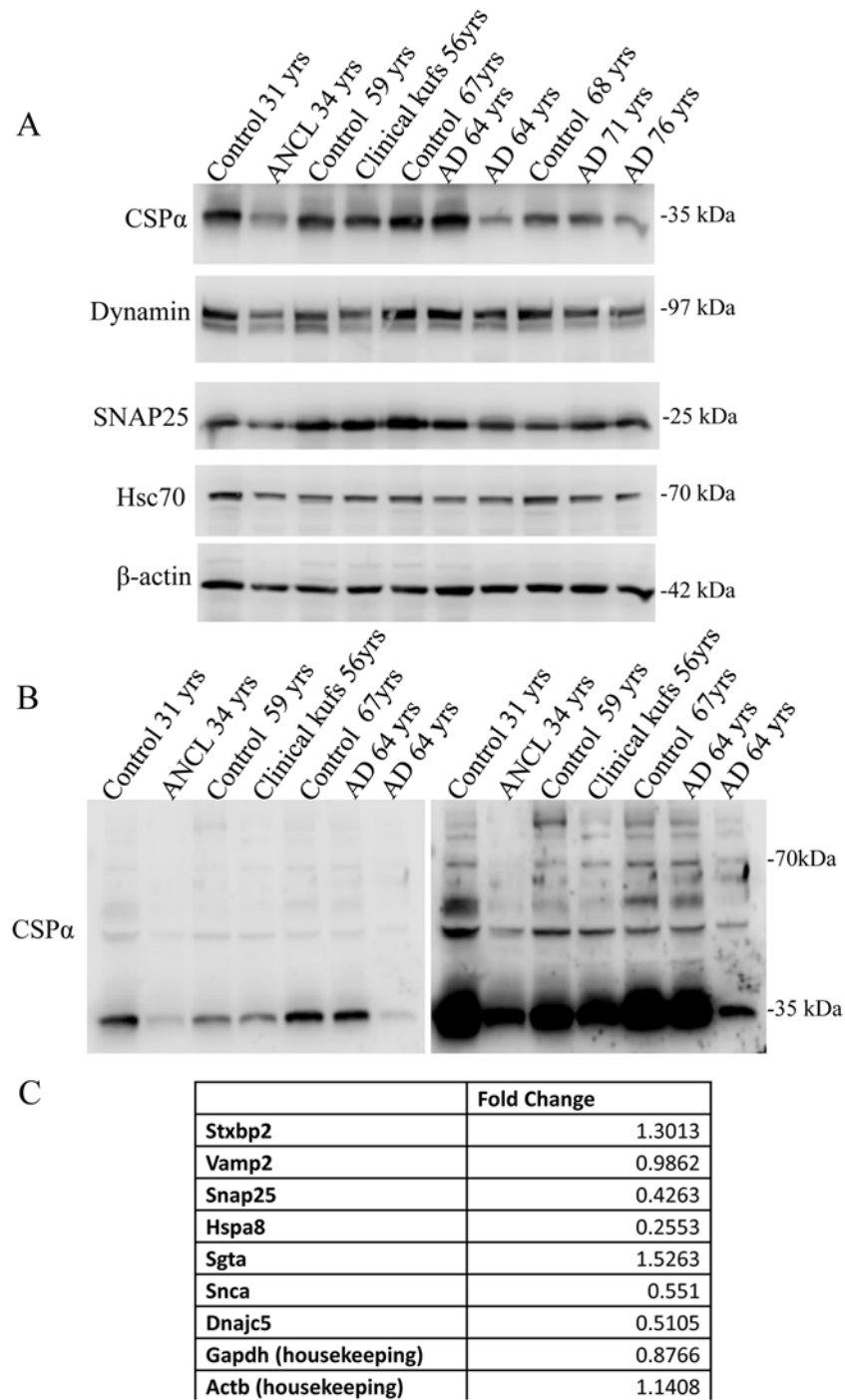


Fig 3. CSPα expression is lower in ANCL and some AD patients. (A) Western analysis of CSPα, Dynamin 1, SNAP25, Hsc70 and β-actin detected in synaptosome fractions prepared from human cortex as indicated. For the 10 human samples the CSPα values were 671000, 459000, 583000, 530000, 684000, 690000, 351000, 432000, 397000, 342000. SNAP25 values were 2540000, 1640000, 2880000, 3040000, 3210000, 2530000, 2460000, 2050000, 2430000, 2140000, dynamin values were 3290000, 2160000, 3060000, 2580000, 3410000, 3500000, 3010000, 3240000, 2530000, 2300000 (B) Immunoblot showing CSPα monomer expression (left panel) and longer exposure showing high molecular weight CSPα oligomers (right panel) (C) mRNA levels for the indicated proteins (fold change compared to control brain tissue).

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oligomers are more abundant in brain regions other than cortex. We therefore examined the rate of clearance of the high molecular weight CSP α oligomers following transient transfection of the murine CNS-derived catecholamine (CAD) cell line. Fig 4 shows the expression of myc-tagged CSP α , CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ at 24 hrs, 48hrs and 72hrs following transfection. As expected, myc-tagged wild type CSP α is expressed as unpalmitoylated (26kDa), a palmitoylated monomer (35kDa) and a dimer (70kDa) species in CAD cells. CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ are primarily expressed as the unpalmitoylated 26kDa species and high molecular weight CSP α oligomers. CSP α oligomers do not build up in CAD cells and both monomer and high molecular weight oligomers are significantly cleared three days post transfection. The expression of endogenous Hsc70 is shown for reference. Taken together, while CSP α oligomers have been consistently documented and extensive oligomerization is observed with the CSP α mutants, L115R and L116 Δ , the oligomers are not retained for a longer window of time in CAD cells compared with the CSP α monomer (Fig 4) and are not particularly abundant in crude synaptosomal fractions from ANCL cortex (Fig 3).

We then investigated if mutant CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ oligomerized in the presence of wild type CSP α . Fig 5A shows that two days following transfection, CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ but not CSP $\alpha_{HPD-AAA}$, were associated with increased levels of high molecular weight CSP α oligomers both in the absence and presence of wild type CSP α . The 35 kDa myc-CSP α monomer was reduced in the presence of CSP α_{L115R} and CSP $\alpha_{L116\Delta}$, consistent with a recent report showing that wild type and mutant CSP α co-oligomerize, leading to a decrease in functional chaperone activity [24]. Very low levels of the 35kDa CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ in CAD cells indicate that these mutants can be posttranslationally modified. These observations were confirmed utilizing an anti-CSP α polyclonal antibody generated to the C terminus of CSP α (Fig 5B). Note that low levels of the 35kDa & 70kDa species of endogenous CSP α are found in CAD cells (lanes 1&9).

Next we ‘titrated’ the effect of CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ on FLAG-tagged CSP α . CAD cells were transfected with 0.75 μ g of flag-tagged CSP α DNA encoding in the presence and absence of 0.25, 0.5, 0.75 and 1 μ g of DNA encoding myc-tagged CSP α mutants. Fig 6 demonstrates that CSP α oligomers were detected at low (0.25 μ g) expression of CSP α_{L115R} and CSP $\alpha_{L116\Delta}$ and observed to increase at high (1 μ g) expression of the CSP α mutants. Increasing the proportion of mutant CSP α also decreased the proportion of posttranslationally-modified FLAG-tagged CSP α (35kDa). CSP $\alpha_{L116\Delta}$ oligomerization was lower than that found for CSP α_{L115R} ,

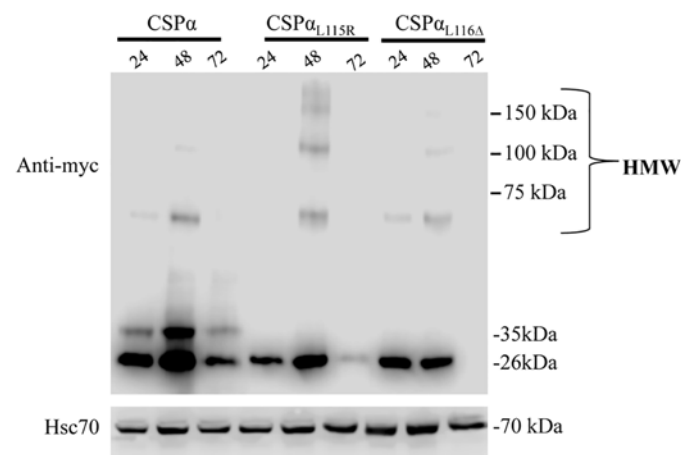


Fig 4. Time course of oligomer expression in CAD cells. Immunoblotting of myc-tagged CSP α , CSP α_{L115R} or CSP $\alpha_{L116\Delta}$ 24, 48 and 72 hrs post-transfection. 30 μ g of CAD cell lysates were evaluated and endogenous Hsc70 detection is shown for reference. Results are representative of 4 separate experiments.

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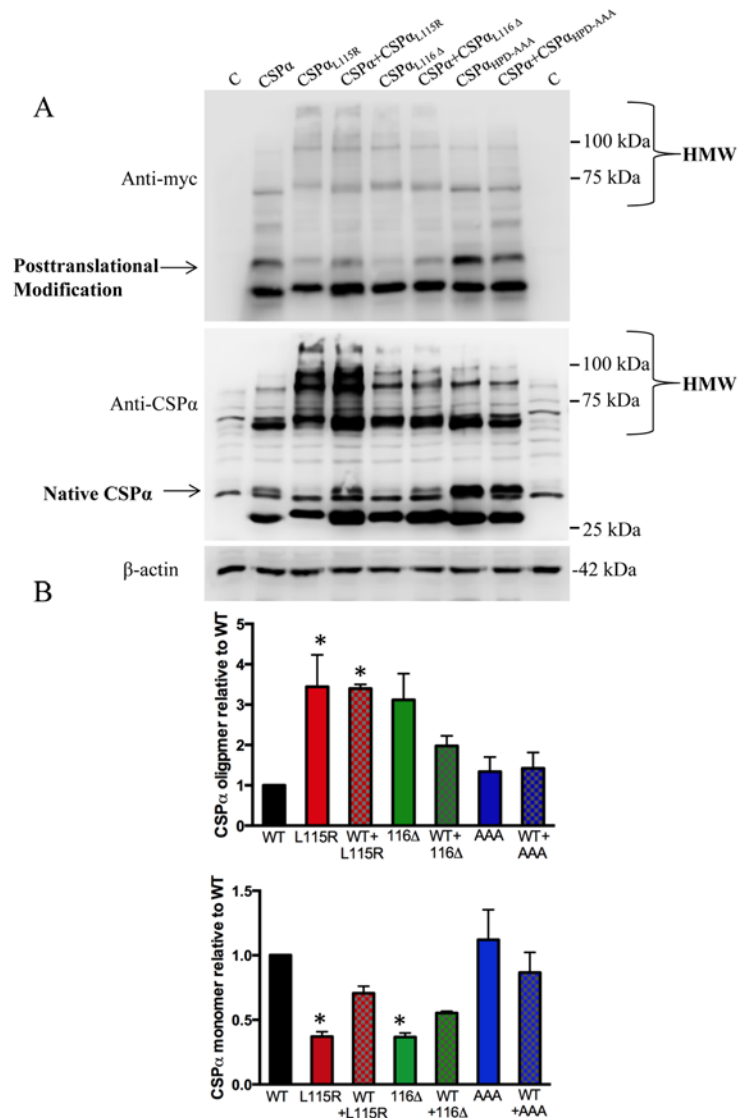


Fig 5. CSPα oligomerization is greater in CSPα_{L115R}, CSPα_{Δ116} mutants. (A) CAD cells were transiently transfected with 0.75 μg cDNA encoding myc tagged wild type CSPα or 1.0 μg cDNA encoding either CSPα_{L115R}, CSPα_{L116Δ} or CSPα_{HPD-AAA} as indicated and lysed 48 hours post-transfection. CSPα monomer and oligomers were detected in CAD cell lysates by western blot with anti-myc (upper panel) and anti-CSPα (middle panel). Detection of β-actin was used to verify equal loading amongst the various lanes. (B) Quantification of CSPα high molecular weight oligomers (starting from 70kDa) CSPα monomers. *p < 0.01. Results are from 4 independent experiments.

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consistent with previous results [24]. Taken together, these data show that increasing the mutant:WT CSPα ratio, correlates with a loss of monomer and an increase in oligomer formation.

Cellular CSPα-BK channel complexes

CSPα is a presynaptic synaptic vesicle protein that regulates BK channels, most likely by interacting with presynaptic BK channels. We have previously determined that the mutant CSPα_{HPD-AAA} as well as the human disease-associated CSPα mutations CSPα_{L116Δ} and CSPα_{L115R} are capable of increasing BK channel cell surface expression and current density

[13]. While $CSP\alpha_{L116\Delta}$ and $CSP\alpha_{L115R}$ increase BK current at the membrane, the increase is not as large as that observed with $CSP\alpha_{HPD-AAA}$. These observations indicate that BK channels are trafficked to the surface and are functional in the presence of mutant $CSP\alpha$'s. We have also previously demonstrated that when BK channels are expressed at high levels, wild type $CSP\alpha$ reduces BK channel expression in a dose- and time-dependent manner without altering BK β

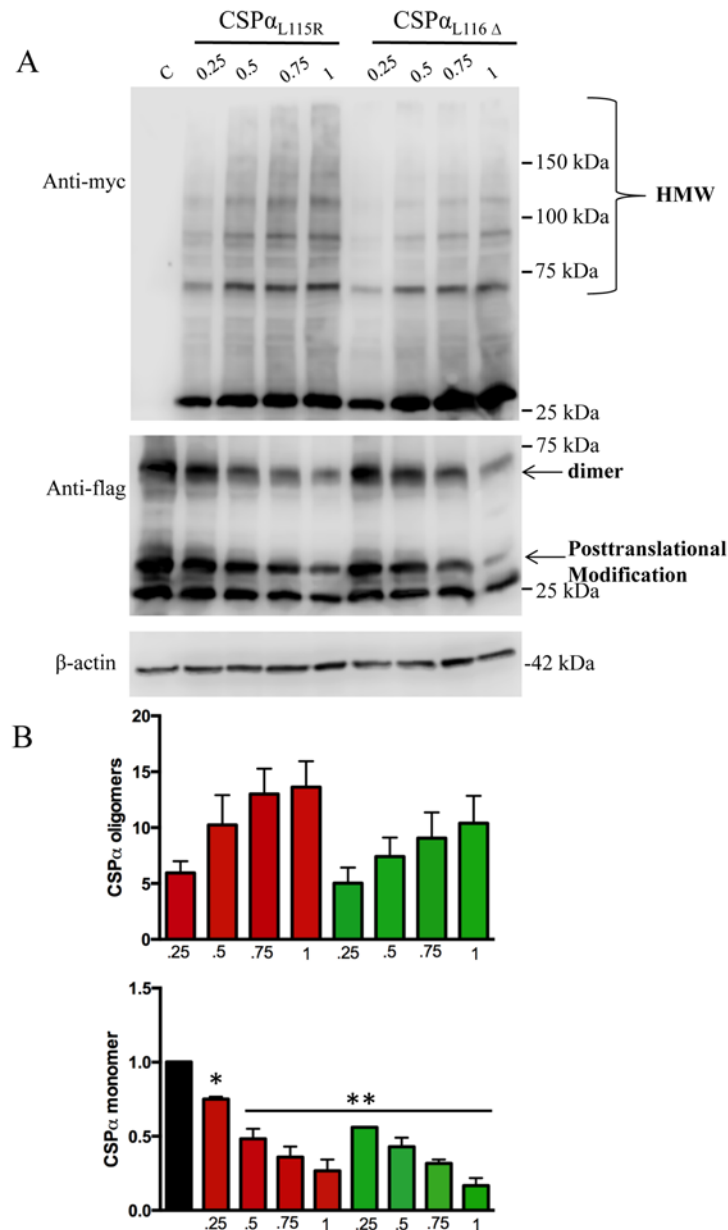


Fig 6. Oligomerization of $CSP\alpha_{L115R}$, $CSP\alpha_{\Delta 116}$ mutants is concentration-dependent. CAD cells were transfected with 0.75 μ g of flag-tagged WT $CSP\alpha$ and 0.25, 0.5, 0.75, 1 μ g of either $CSP\alpha_{L115R}$, or $CSP\alpha_{L116\Delta}$ as indicated and lysed 48 hours post transfection. Mutant $CSP\alpha$ was detected with anti-myc (upper panel) and WT $CSP\alpha$ was detected with anti-flag (lower panel). Detection of β -actin was used to verify equal loading amongst the various lanes. **(B)** Quantification of $CSP\alpha$ high molecular weight oligomers (starting from 70kDa) and $CSP\alpha$ monomers. Differences between $CSP\alpha_{L115R}$ and $CSP\alpha_{L116\Delta}$ oligomers are not significant, * $p < 0.02$; ** $p < 0.001$. Results are from 3 independent experiments.

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channel subunit expression [14]. Fig 7A shows that WT $CSP\alpha$ limits the $CSP\alpha_{HDP-AAA}$ -induced increase in BK channel expression, but does not influence the $CSP\alpha_{L116\Delta}$ and $CSP\alpha_{L115R}$ -induced increases in BK channel expression. We speculate that the reason why the 0.75:1 ratio of WT $CSP\alpha$: $CSP\alpha_{L115R}$ or WT $CSP\alpha$: $CSP\alpha_{L116\Delta}$ does not block the increase in BK channel expression may be a consequence of oligomerization of WT $CSP\alpha$ by the $CSP\alpha_{L115R}$ and $CSP\alpha_{L116\Delta}$ mutants (Fig 6). Chaperones are known to bind and unbind client proteins with fast

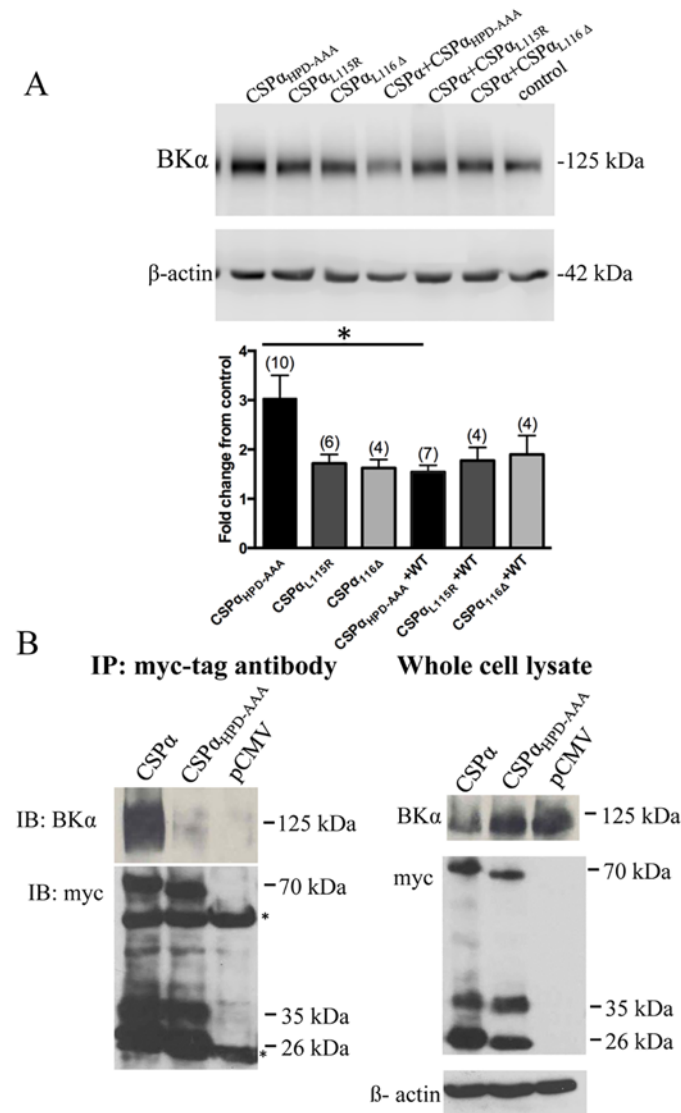


Fig 7. BK channel co-immunoprecipitates with $CSP\alpha$ but not $CSP\alpha_{HDP-AAA}$. (A) CAD cells were transiently transfected with 1.0 μ g cDNA encoding either $CSP\alpha_{L115R}$, $CSP\alpha_{L116\Delta}$ or $CSP\alpha_{HDP-AAA}$ in the presence and absence of 0.75 μ g cDNA encoding myc tagged wild type $CSP\alpha$ and lysed 48 hours post-transfection. Western analysis and quantification of BK channel is shown; * $p < 0.05$. Detection of β -actin on the same blot was used to verify equal loading between the various lanes. (B) CAD cells were transiently co-transfected with 1 μ g BK channel and 0.75 μ g myc-tagged $CSP\alpha$, 0.75 μ g myc-tagged $CSP\alpha_{HDP-AAA}$ and 0.75 μ g pCMV (negative control). 0.7 mg of soluble cell lysate was subjected to immunoprecipitations with anti-myc monoclonal followed by Western blot analysis with anti-BK channel polyclonal and anti-myc monoclonal. The 55 kDa and 26kDa represent the heavy and light chain of the monoclonal myc-tag antibody. The right panel shows total cellular protein (input). Data are representative of three experiments.

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kinetics, making the identification of chaperone complexes challenging. Since we were unable to capture stable CSP α -BK channel complexes from wild-type mouse brain using a classic immunoprecipitation strategy [13], we investigated the nature of the CSP α -BK channel association in CAD cells co-transfected with BK channel and either myc-tagged CSP α or myc-tagged CSP $\alpha_{\text{HPD-AAA}}$. Fig 7B shows that WT CSP α , but not CSP $\alpha_{\text{HPD-AAA}}$, co-immunoprecipitates with BK channels. The pCMV vector control shows that BK channels are not detectable in the immunoprecipitate when CSP α is not co-transfected. Total lysates are shown in the right-hand panel. Taken together, these results indicate that CSP α and BK channel can associate, while this is less likely for CSP $\alpha_{\text{HPD-AAA}}$ mutant and BK channels. Such observations support the idea that CSP $\alpha_{\text{HPD-AAA}}$ cannot function to limit BK channel expression.

The J protein network is compromised in ANCL

Finally, we asked if the expression of select J proteins is altered in crude synaptosomes from post-mortem ANCL cortex. CSP α is a member of a large J protein family that is central to cellular protein homeostasis pathways [25,26]. While pathological neurodegeneration in ANCL is directly correlated with mutations in CSP α , the extensive synapse dysfunction associated with disease progression may be expected to compromise other members of the chaperone network. Exhaustion of molecular chaperones would render many cellular pathways vulnerable, triggering a generalized collapse of proteostasis. Reductions were found in DnaJA2 (26%) and DnaJA3 (25%) levels in post-mortem ANCL cortex synaptosomes (Fig 8). These observations suggest that disease progression in ANCL may eventually lead to an impairment of the J protein network.

Discussion

ANCL is a rare neurodegenerative disease caused by L115R and L116 Δ mutations in the synaptic vesicle protein CSP α . The pathogenesis of ANCL is not established. In this study we investigated BK α channel expression in ANCL post-mortem cortex with a L116 Δ mutation. Our previous work demonstrated that the presynaptic chaperone CSP α limits BK channel density and that ectopic expression of CSP α_{L115R} and CSP $\alpha_{\text{L116}\Delta}$ results in elevation of BK channel expression in neuronal cell culture models [13,14]. We have also shown that CSP α KO mice have a 2.5 fold increase in BK channel levels in the brain [13]. Here we show that BK α subunit levels are elevated in crude synaptosomes from human ANCL cortex. Thus, we conclude that the pathological sequence of events in ANCL involves elevations in BK channel levels, which may contribute to the dysregulation of neuronal excitability.

The neural chaperone network that maintains the balance between protecting functional proteins and preventing accumulation of mis-folded proteins is elaborate. CSP α is a pivotal element of the presynaptic proteostasis machinery [6]. Biochemical analysis of CSP α KO mice has identified SNAP25 and dynamin1, proteins critical to synaptic vesicle recycling, as CSP α -protein clients [16–19,27]. The decrease in SNAP25 and dynamin1 levels in CSP α KO mice suggest that these client proteins are degraded rather than refolded and retained in this model [27]. In contrast, BK channel levels increase in CSP α -KO mice, suggesting that either delivery of the channel to the synapse is increased or removal of the channel is decreased implicating BK channel proteostasis in the cellular pathway of CSP α -mediated neuroprotection. Other proteins that interact with CSP α and might be involved in CSP α 's neuroprotective activity have been identified, including voltage dependent Ca²⁺ channels, G α_s , syntaxin, rab3, synaptotagmin [28–37].

The cysteine string region is a heavily palmitoylated region that anchors the chaperone, CSP α , to synaptic vesicles [38]. Mutations of CSP α , L115R and L116 Δ , have been shown to

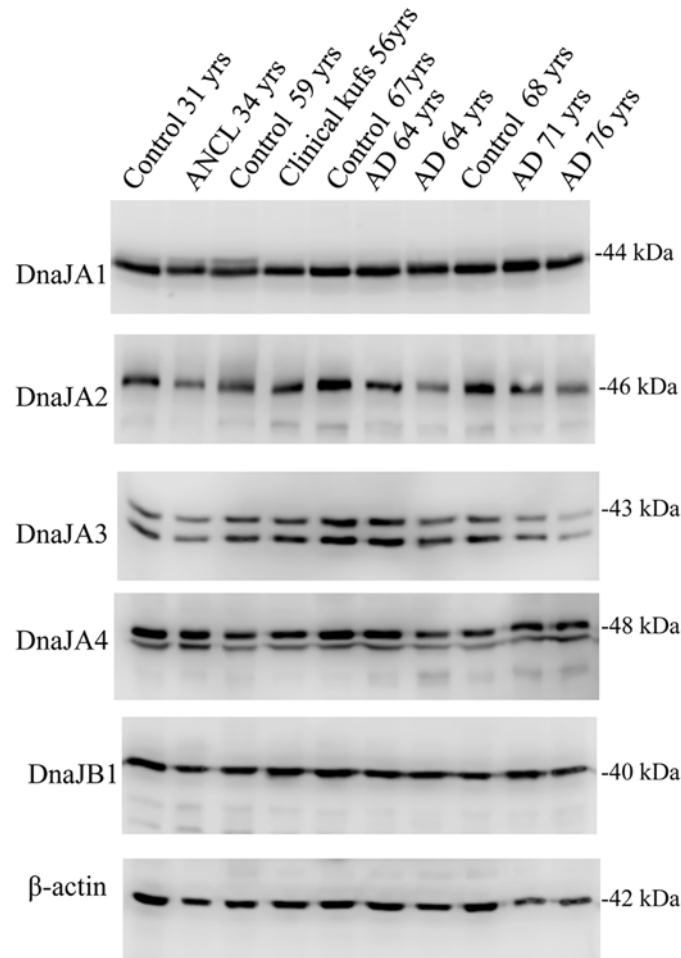


Fig 8. Expression of DnaJA1, DnaJA2, DnaJA3, DnaJA4 and DnaJB1, in human cortex samples. Western analysis of the indicated proteins in synaptosome-enriched fractions prepared from human cortex. For the 10 human samples the DnaJA2 values were: 77500, 57900, 85900, 78500, 98100, 69700, 52500, 83800, 64300, 56400. The DnaJA3 values were: 298000, 224000, 356000, 416000, 554000, 509000, 375000, 342000, 234000, 181000.

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interfere with palmitoylation and to promote a high potency to oligomerize [5,24]. CSP α oligomers with and without palmitoyl groups have been identified [5,24]. In contrast to the CSP α monomer, high molecular weight CSP α oligomers do not activate Hsc70 ATPase for conformational work [24]. Protein oligomerization and buildup of protein aggregates is a common event in several neurodegenerative diseases, nonetheless, we were unable to detect an overabundance of oligomers. In fact, both monomeric and oligomeric species of CSP α are reduced in ANCL synaptosomes. In cell culture, CSP α oligomers were reduced 3 days following transfection. This temporal pattern is similar to the expression of ectopically expressed CSP α monomer and thus it appears that CSP α oligomers do not progressively build up. Recent reports demonstrate that oligomers are poly-ubiquitinated [24] consistent with our observations that neurons effectively clear CSP α oligomers. Furthermore, in CAD neuroblastoma cells, wild type CSP α reverses the CSP $\alpha_{\text{HPD-AAA}}$ -induced increase but not the CSP $\alpha_{\text{L116}\Delta}$ and CSP α_{L115R} -induced increase in BK channel levels. These observations indicate that at the ratio of 0.75WT:1mutant; mutations in the J-domain (i.e. CSP $\alpha_{\text{HPD-AAA}}$) but not mutations in the cysteine string region (i.e. CSP $\alpha_{\text{L116}\Delta}$ or CSP α_{L115R}) are compensated.

A diverse number of human neurodegenerative disorders are caused by mutations in members of the J protein family DnaJC29 in addition to DnaJC5 (CSP α), for example DnaJB2, DnaJC6, DnaJC13, DnaJC19 [39,40]. Loss of co-chaperone activity and/or gain of additional modes of chaperone activity almost certainly underlies the pathophysiology of these different diseases. Additionally, the buildup of misfolded proteins in common neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease implies that chaperone activity is compromised, however a comprehensive understanding of the identity of the specific defective chaperone(s) in these diseases is not clearly established. It is clear that CSP α confers synapse protection [6] and that synaptic function is compromised in the absence of CSP α [6,17–19,27,41]. While partial reductions in CSP α (e.g. CSP α heterozygote mice) are not pathogenic, the reduction in CSP α monomer together with the assembly of CSP α into oligomers (e.g. ANCL), does result in neurodegeneration.

In conclusion, we provide evidence that the L116 Δ mutation of the presynaptic chaperone CSP α , increases BK α subunit expression in crude synaptosomes from post-mortem cortex. Cell surface expression of BK channels is subject to elaborate regulatory mechanisms. BK channels are positioned to influence synaptic transmission and excitability. Our work points to the dysregulation of BK channels in ANCL. Together, these data suggest that while reinforcement of CSP α co-chaperone activity may prove effective therapy for ANCL, effectively overcoming the oligomerizing and sequestering activity of wild type CSP α by the human CSP α mutations remains an important consideration.

Materials and Methods

Preparation of fractions from human cortex

This study of the brain tissues was conducted according to protocols approved by the Institutional Review boards of the New York State Institute for Basic Research in Developmental Disabilities and the Institutional Review Board of Massachusetts General Hospital. The brain tissue with DNAJC5 deletion was from an individual that was previously published [42]. The specimen from individual with ANCL negative for DNAJC5 mutation was obtained from the Human Brain and Spinal Fluid resource Center in Los Angeles, CA. The rest of the tissues were obtained from the Brain Bank for Developmental Disability and Aging at the New York State Institute for Basic Research in Developmental Disabilities. For all tissues written consent for research participation was obtained from the affected individual or from next of kin. The study was conducted according to principles of the Declaration of Helsinki.

Briefly, frozen human cortex samples were homogenized in 0.7 mls of ice cold 0.32M sucrose, 10mM HEPES, 1 mM EGTA, 0.1 mM EDTA and 0.3 mM PMSF with 20 up and down strokes using a plastic mini homogenizer. The homogenate was centrifuged at 4°C for 5 min at 700 x g and the supernatant (S1) collected. The S1 supernatant was then spun for 15 min at 22,000 x g and the resulting supernatant (S2) was discarded. The pellet (P2) was washed by re-suspension buffer and then re-centrifuged at 22,000 x g. The final pellet, representing washed-crude synaptosomes, was re-suspended in 0.4 ml of buffer.

Cell culture

CAD (CNS catecholaminergic derived) mouse neuroblastoma cells stably expressing BK α subunit were seeded into 6 well plates and grown in DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.5mg/ml zeocin, as previously described [13]. For transient transfection, CAD cells were washed in PBS and transiently transfected using with the indicated amount of cDNA and 6 μ l of Lipofectamine-2000 (Invitrogen) per dish. Reagents were mixed in 0.2 ml of Opti-MEM medium and then diluted to a total volume

of 1 ml with DMEM. After 6 hours the medium was replaced with DMEM/F12 supplemented with 1% fetal bovine serum, 1% penicillin/streptomycin and 0.5mg/ml zeocin. Cells were lysed in 40 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM Na₃ VO₄, 0.1% SDS, 1% (v/v) Triton X-100, 0.5 mM PMSF and protease inhibitor (Sigma) at 4°C for 1 hour. Lysates were centrifuged at 10000 x g for 5 minutes at 4°C and the supernatant (soluble fraction) was collected and stored at -70°C. Protein concentration of the soluble CAD cell lysate was determined using the Pierce BCA protein assay.

Immunoblotting

Proteins were separated by SDS-PAGE and electrotransferred from polyacrylamide gels to nitrocellulose membrane (0.2 μm pore size). Membranes were blocked in tris-buffered saline (TBS) containing 0.1% Tween 20, 1% BSA and then incubated with primary antibody overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase-coupled secondary antibody for ~2 h at room temperature. Bound antibodies on the membranes were detected by incubation with the LiCor WesternSure chemiluminescence reagent (Mandel) and exposure to Cdigit, LiCor. The chemiluminescent signals were quantified using image studio digits software (Mandel). Primary antibodies were obtained as follows: BK monoclonal, c-myc monoclonal, flag monoclonal and dynamin monoclonal (BD Biosciences). SNAP25 monoclonal (Sternberger monoclonals). Syntaxin monoclonal, Hsc70 monoclonal, and β-actin monoclonal (Sigma-Aldrich). DnaJA2 monoclonal was from US Biologicals, DnaJA1 monoclonal and DnaJA4 monoclonal was from Abnova. DnaJB1 polyclonal was from Enzo Life Sciences. DnaJA3 monoclonal was from Pierce, Thermo scientific. Anti-DnaJC5 polyclonal was prepared as previously described [20].

Quantitative PCR

Total RNA was isolated from frozen brain tissues using Trizol reagent (Life technologies) and RNeasy kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time RT-PCR was done using custom RT² profiler PCR Array Format A (Qiagen) according to the manufacturer's protocol. Data analysis was done using ΔΔCt method and automated software from Qiagen.

Co-Immunoprecipitation

CAD cells were transiently transfected with cDNA and 24 hrs later were lysed in 1 ml 1% v/v Triton X-100 in PBS and protease inhibitor (complete, EDTA-free, Sigma). Lysates were centrifuged at 15,000 rpm for 15 min at 4°C, the supernatant collected and precleared with Protein A/G-coupled agarose beads (50% w/v slurry). 5μg of anti-myc monoclonal was incubated overnight at 4°C with the precleared lysates. Following the addition of 20μl of Protein A/G coupled beads (50% slurry) and a subsequent 2 hr incubation at 4°C, the lysates were centrifuged at 5,000 rpm for 2 min. Pellets were washed thrice with 0.1% v/v Triton X-100 in PBS. Proteins were eluted from agarose beads with 2X Laemmli sample buffer and separated on SDS-PAGE.

Statistics

All values are presented as the mean ±SEM. Calculations were performed using GraphPad Prism 6 software.

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Author Contributions

Conceived and designed the experiments: MV JEAB. Performed the experiments: JD STB ND JEAB. Analyzed the data: APB JEAB. Contributed reagents/materials/analysis tools: EA ND. Wrote the paper: JEAB APB ND MV.

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