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Role of hippocalcin in mediating Aβ toxicity☆

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia, and amyloid-β (Aβ) plaques and tau-containing tangles are its histopathological hallmark lesions. These do not occur at random; rather, the neurodegenerative process is stereotyped in that it is initiated in the entorhinal cortex and hippocampal formation. Interestingly, it is the latter brain area where the calcium-sensing enzyme hippocalcin is highly expressed. Because calcium deregulation is a well-established pathomechanism in AD, we aimed to address the putative role of hippocalcin in human AD brain and transgenic mouse models. We found that hippocalcin levels are increased in human AD brain and in Aβ plaque-forming APP23 transgenic mice compared to controls. To determine the role of hippocalcin in Aβ toxicity, we treated primary cultures derived from hippocalcin knockout (HC KO) mice with Aβ and found them to be more susceptible to Aβ toxicity than controls. Likewise, treatment with either thapsigargin or ionomycin, both known to deregulate intracellular calcium levels, caused an increased toxicity in hippocampal neurons from HC KO mice compared to wild-type. We found further that mitochondrial complex I activity increased from 3 to 6 months in hippocampal mitochondria from wild-type and HC KO mice, but that the latter exhibited a significantly stronger aging phenotype than wild-type. Aβ treatment induced significant toxicity on hippocampal mitochondria from HC KO mice already at 3 months of age, while wild-type mitochondria were spared. Our data suggest that hippocalcin has a neuroprotective role in AD, presenting it as a putative biomarker.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 26 million people worldwide [1]. Histopathologically, the AD brain is characterized by amyloid-β (Aβ) plaques and tau-containing neurofibrillary tangles in the brain [2,3]. At present, there is no cure for this debilitating disease [1]. The hippocampus is one of the earliest brain areas affected in AD, reflecting the fact that it is the formation of new memories that is impaired at an early stage of disease [4]. Furthermore, this is a brain area known to be a major target of excitotoxic damage, whereby neurons die because of excessive stimulation of glutamate receptors [5].

Hippocalcin is a protein that is highly expressed in the hippocampus [6]. It is a member of the neuronal-specific calcium sensor (NCS) family of proteins, and by employing a calcium-activated myristoyl switch it is translocated to the plasma membrane upon binding of calcium [7]. The expression pattern of hippocalcin differs from that of other known members of the protein family, with hippocalcin being mainly expressed in the hippocampus, while the other members are also widely expressed in other brain regions [8]. This expression pattern suggests that hippocalcin may have a unique function that is not shared by the other family members.

Hippocalcin is important for the homeostasis of intracellular calcium levels. By studying the interaction between muscarinic acetylcholine receptors and NMDA receptors (NMDARs), it was found that the stimulation of the muscarinic receptors caused long-term depression (LTD) of NMDAR-mediated synaptic transmission. This form of LTD involved the release of Ca^{2+} from intracellular stores and was expressed via the internalization of NMDARs. Interestingly, the data

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suggest that the molecular mechanism involves a dynamic interaction between hippocalcin itself, the clathrin adaptor molecule AP2, the postsynaptic density enriched protein PSD-95 and NMDARs. Specifically, the authors of the study proposed that hippocalcin binds to the SH3 domain of PSD-95 under basal conditions, and that it translocates to the plasma membrane upon sensing Ca^{2+}, thereby causing PSD-95 to dissociate from NMDARs, permitting AP2 to bind and initiating dynamin-dependent endocytosis [9]. Also, infusion of a truncated mutant form of hippocalcin that lacks the Ca^{2+} binding domains has been shown to prevent synaptically evoked LTD but had no effect on long-term potentiation (LTP) [10]. To better understand the function of this calcium sensor, knock-out mice have been generated and shown to display memory deficits in spatial recognition tasks, further suggesting that the protein has a role in memory formation [11]. Knock-out mice also show an impairment in the activation of Raf conducted by the Ras signaling pathway [12]. Physiological increments of Ca^{2+} are sensed by hippocalcin with a $K_{1/2}$ of 0.5 μM [13].

Hippocalcin has been found to exert a neuroprotective effect *in vitro*, such as in the spinal cord motor neuron-like cell line NSC-34 or the neuroblastoma cell line Neuro-2a, by interacting with NAIP (neuronal apoptosis inhibitory protein) or by promoting calcium extrusion [14,15]. There is also histopathological evidence for a role in neurodegeneration, because of the protein's localization to Lewy bodies, insoluble aggregates present in Parkinson's disease brains, thereby suggesting that hippocalcin may play a role in the pathogenesis of this condition [16]. In Huntington's disease, on the other hand, hippocalcin levels are decreased [17]. Together, this data supports the notion that hippocalcin is deregulated under neurodegenerative conditions.

The present study firstly aimed to determine the role of hippocalcin levels in AD brain and in a mouse model with amyloid plaque formation. We further addressed the protein's role in Aβ toxicity using primary neuronal cultures that have been established from hippocalcin knock-out and wild-type mice.

2. Materials and methods

2.1. Ethics statement

Human brain tissue was obtained from the Sydney Brain Bank, with approval from the Human Ethics Review Committee of the University of New South Wales. Written informed consent was obtained from donors or their next of kin for brain donation. The animal experiments were

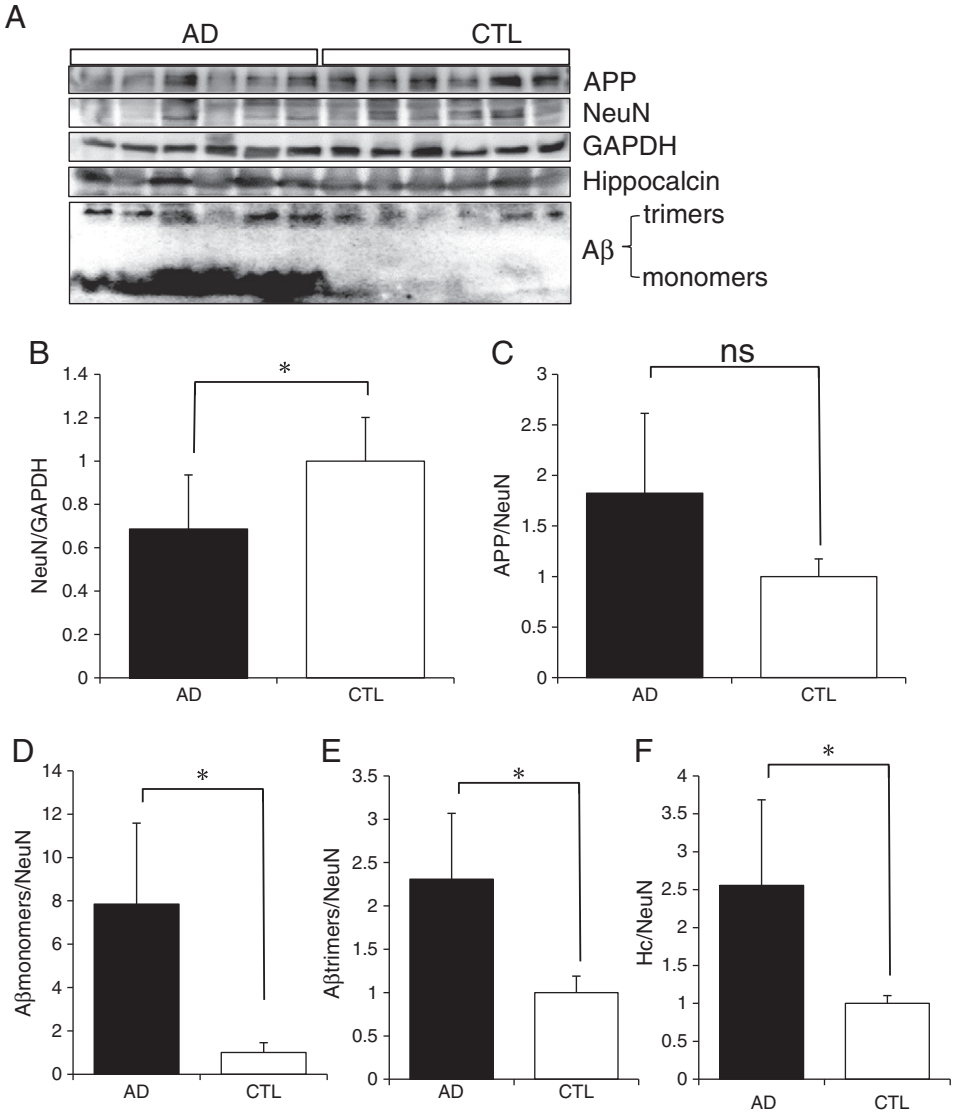

Fig. 1. Western blot analysis of human AD and control (CTL) hippocampal samples (A). Neuronal numbers are significantly decreased in AD compared to CTL samples (B). APP levels are comparable between AD and CTL brains (C), while there are significantly higher levels of Aβ monomers and trimers in the AD compared to the CTL brain samples (D, E). Hippocalcin levels are significantly higher in the AD brain compared to CTL (F). (*, $p<0.05$).

approved by the Animal Ethics Committee (AEC) of the University of Sydney (approval number K00/1-2009/3/4914).

2.2. Immunofluorescence staining of transgenic mice

Six and 18 month-old transgenic APP23 mice [18] and age-matched wild-type littermate controls were transcardially perfused with 1× PBS, followed by separation of the cerebral hemispheres. The left hemispheres were post-fixed in 4% paraformaldehyde overnight at 4 °C and the paraformaldehyde exchanged for 70% ethanol on the next day. Brains were then processed in a Shandon Excelsior processor (Thermo), followed by embedding in paraffin as described [19]. Three μm sections were rehydrated step-wise before antigen retrieval was performed in a microwave system (Milestone) in pre-warmed Tris/EDTA at pH 9.0 for 7 minutes at 120 °C and allowed to cool on the bench-top for 15 min. Sections were stained for Aβ with 6E10 (mouse, 1:500, Covance) and for hippocalcin (rabbit, 1:100, [20]), and counterstained with DAPI (Invitrogen) to visualize the nuclei. Alexa-Fluor secondary antibodies (Alexa-Fluor 488 and Alexa-Fluor 555, respectively, Invitrogen) were used for detection. Fluorescent pictures of the stained sections were taken on a Zeiss LSM 710 confocal microscope.

2.3. Protein extraction of transgenic mice

While the left hemisphere was used for immunofluorescence, the right hemisphere and the hippocampus were sub-dissected and snap frozen in liquid nitrogen for Western blotting. Protein extraction was performed on mouse hippocampal tissue using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate 1% Triton-X with protease inhibitors). Tissue was sonicated on ice to obtain a homogeneous suspension. The lysates were centrifuged at 17,000 ×*g* for

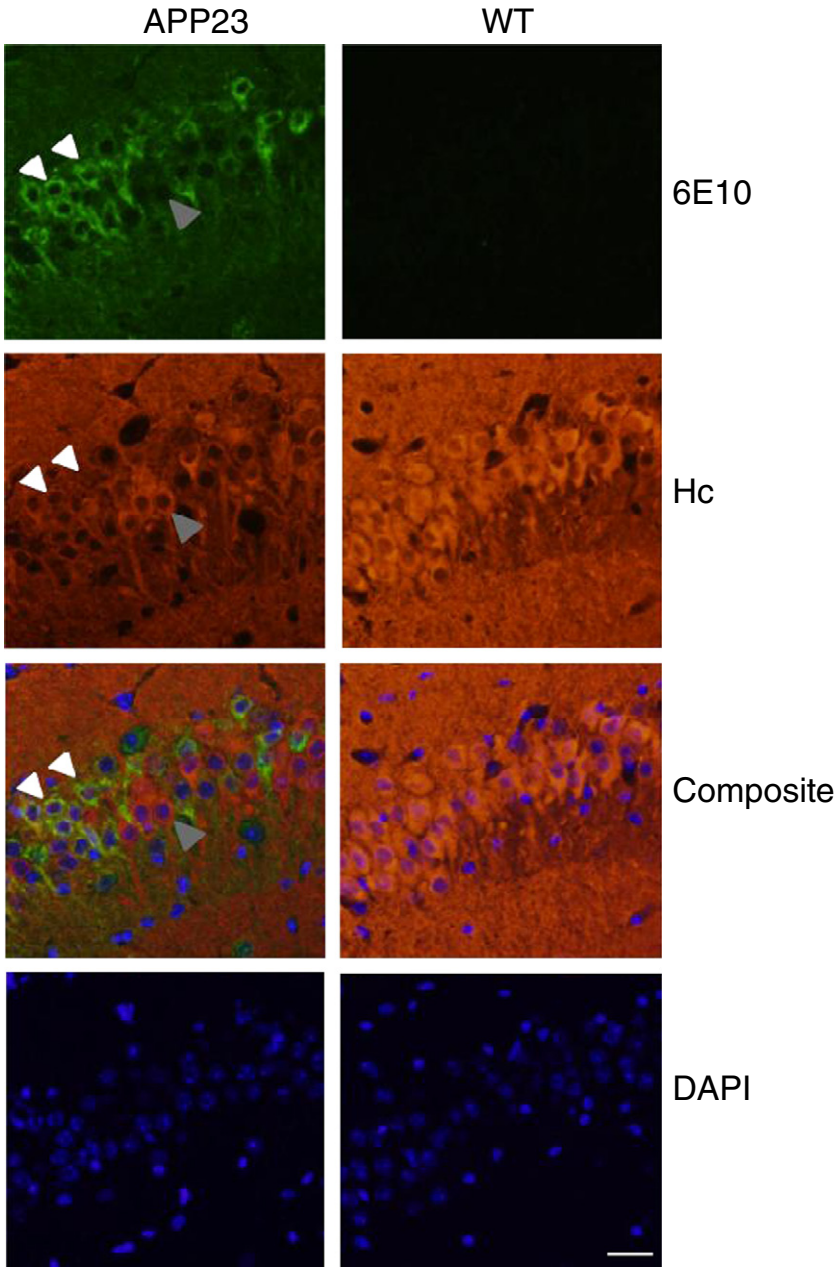

Fig. 2. Confocal fluorescence analysis of 18 months-old Aβ-plaque-forming APP23 and wild-type littermate controls (WT). Paraffin-embedded sections were stained with antibodies directed against Aβ/APP (6E10; green), hippocalcin (Hc; red) and DAPI (nuclear). Neurons that have high 6E10 reactivity show very low hippocalcin staining (white arrowheads), while neurons that have high hippocalcin levels reveal no 6E10 staining within the limits of detection (grey arrowhead). Scale bar, 50 μm.

20 min at 4 °C. The supernatant was retrieved and protein levels were determined using the Bradford assay. Samples were stored at −80 °C until further use.

2.4. Human tissue and protein extraction

Frozen human hippocampal brain tissue from 6 AD and 6 age- and gender-matched controls (age at death in years: AD 91±4, control 86±4; gender M/F: AD 4/2, control 2/4; post mortem delay (hrs): AD 10±2, control 8±8) was homogenized in 1:10 w/v of a Tris buffer solution (TBS) (2.5 mL of 20 mM Tris-Cl, 5 mM EDTA, 0.02% sodium azide) containing protease inhibitors (Complete Mini, Roche Diagnostics). Homogenates were centrifuged at 17,000 ×*g* at 4 °C for 2 h and the supernatant was retrieved to obtain the Tris buffer-soluble fraction. The pellet was re-suspended in TBS containing 2% SDS (2.5 mL of 20 mM Tris-Cl, 5 mM EDTA, 0.02% sodium azide, 2% SDS) and protease inhibitors (Complete Mini, Roche Diagnostics) and was centrifuged again at 17,000 ×*g* at 25 °C for 30 min to obtain the SDS-soluble fraction, containing membrane-bound, intracellular proteins. The supernatant was retrieved and protein levels were determined using the Bradford assay and stored at −80 °C until needed.

2.5. Western blotting

Forty μg of protein was separated on 10% Tris-Tricine SDS gels as described previously to visualize Aβ [21]. Proteins were transferred onto nitrocellulose membranes (Hybond, GE Healthcare). To enable an accurate comparison between the different proteins of interest, blots were cut at positions 55, 38 and 17 kDa using the protein molecular weight ladder (Fermentas, Catalogue number: SM-1811) as reference, and blocked, in parallel, with 5% skim milk in 1× TBST (Tris-Buffered Saline with 1% Tween 20) for an hour at room temperature. Primary antibodies were diluted in 5% milk/TBST and incubated overnight at 4 °C, using the following antibodies: 6E10 (directed against residues 1-16 of Aβ, 1:1000, Covance), GAPDH (1:5000, Millipore), NeuN (1:1000, Millipore) and Hippocalcin (1:1000) [20]. Blots were washed 3 times with 1× TBST (5 minutes wash each), and incubated with the respective alkaline phosphatase-

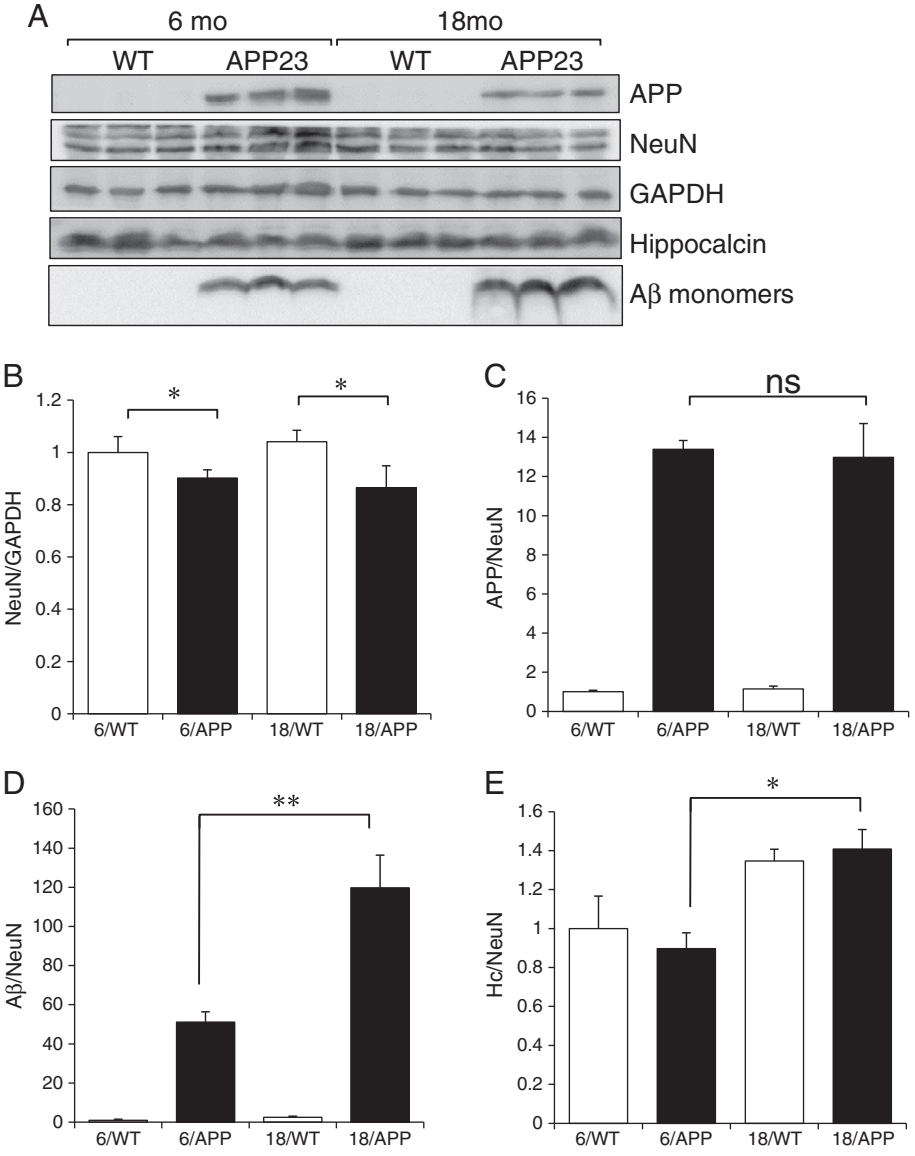

Fig. 3. Western blot analysis of 6 and 18 months-old APP23 transgenic and wild-type mice (A). There are significantly lower neuronal numbers as measured by NeuN levels between wild-type and transgenic mice at both ages (B), an indication of a neurodegenerative state. APP levels remain unchanged between 6 and 18 month-old APP transgenic mice (C), while Aβ levels are significantly higher in 18 month-old when compared to 6 month-old transgenic mice (D). Hippocalcin levels are significantly increased from 6 to 18 months of age in the transgenic animals (E). (*, $p<0.05$; **,$p<0.01$).

tagged secondary antibodies for 1 hour at room temperature. The blots were again washed 3 times with 1× TBST (5 minute washes each), after which they were developed with Immobilon Chemiluminescent Alkaline Phosphatase substrate (Millipore), and detected using the VersaDoc Model 4000 CCD camera system (BioRad). Densitometric analyses were performed using the ImageJ software (NIH).

2.6. Primary cultures

Primary cultures were prepared from embryonic day 16 (E16) hippocalcin-knockout and wild-type mice as described previously [22]. In brief, hippocampi and cortices were dissected and trypsinized in Hank's balanced salt solution (HBSS) with 0.0008% DNase (w/v). The preparation was gently shaken and centrifuged at 720 ×*g* for 3 minutes at room temperature to allow the brain pieces to settle at the bottom of the tube. The supernatant was aspirated and the remaining brain tissue was triturated 30 times in plating media (10% FBS in DMEM). Cell numbers were determined using a Neubauer haemocytometer (Marienfeld, Germany). 50,000 cells were plated per well. Cultures were placed in a 5% CO_2 incubator at 37 °C for 2 h to allow the cortical neurons to adhere to the poly-lysine pre-coated 48-well plates, after which the plating medium was removed and replaced with Neurobasal medium supplemented with 1% (v/v) B27 supplements (Gibco), 0.01% (v/v) 200 mM L-glutamine (Gibco) to minimize growth of microglia and astrocytes. Cultures were grown for 10 days in vitro (DIV) before treatment.

2.7. Treatment and cell viability of primary cultures

After 10 days in vitro (DIV), primary hippocampal and cortical neurons were treated with different concentrations of thapsigargin (Sigma), ionomycin (Sigma), Aβ42 (Bachem) or DMSO for 4 days. In addition, primary cultures were treated with Aβ42. Cell viability was assayed using MTT as described previously [23]. Background absorbance was subtracted from raw MTT absorbance readings and resulting values were normalized against DMSO vehicle treatments as described [24].

2.8. Mitochondrial analysis

Hippocampi (left and right hippocampi pooled for each animal) and cortices were dissected from five mice per group (3 months-old wild-type, 3 months-old hippocalcin knockout, 6 months-old wild-type, and 6 months-old hippocalcin knock-out). Mitochondria were isolated as described [25], and the protein concentration determined, followed by pooling of the samples within a group to obtain sufficient material for the mitochondrial analysis. Then, 100 μg of mitochondria in a volume of 20 μl was used for measurement of complex I activity per experiment. At least three measurements were performed per group. 100 μg of isolated mitochondria were solubilized in n-dodecyl β-D-maltoside (0.1 mg). NADH:hexaammineruthenium(III)-chloride (HAR) activity was measured at 30 °C in a buffer containing 2 mM Na+/MOPS, 50 mM NaCl, and 2 mM KCN, pH 7.2, using 2 mM HAR

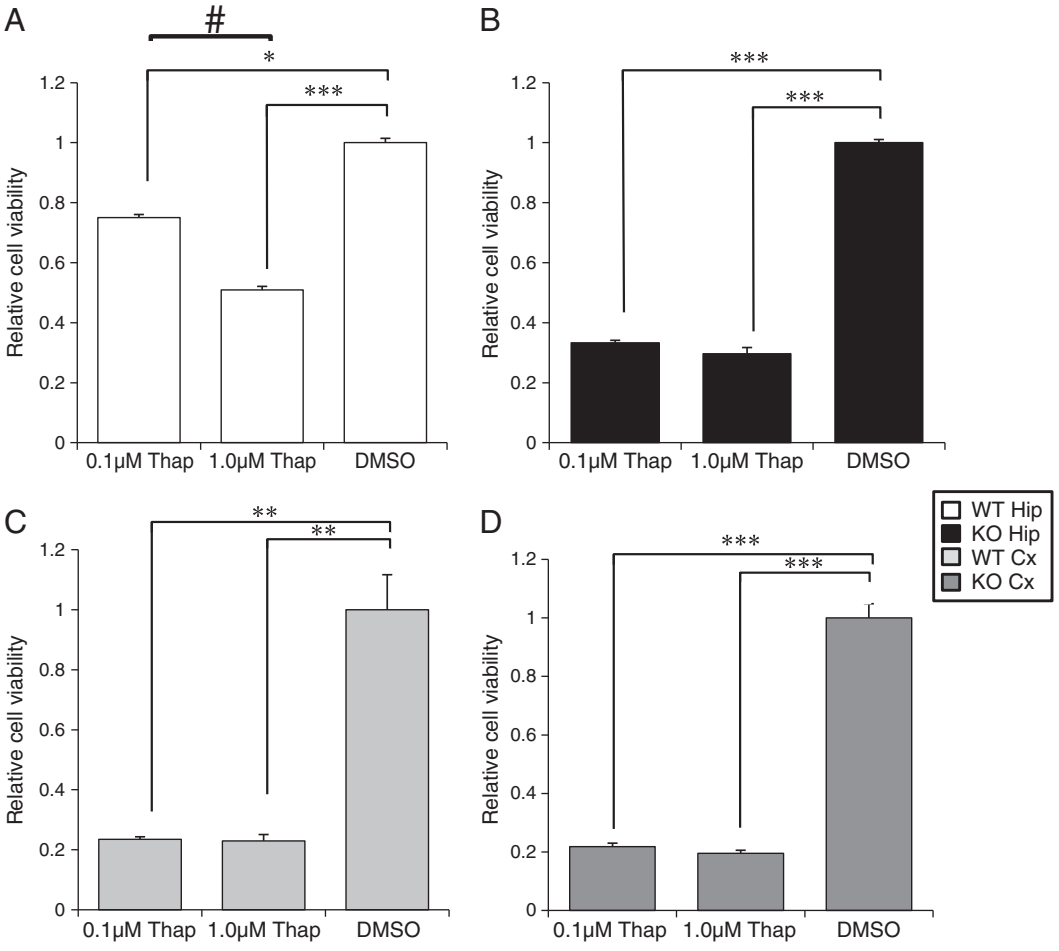

Fig. 4. MTT assay to determine cell viability after thapsigargin treatment of wild-type and hippocalcin knock-out cortical and hippocampal cultures. Wild-type hippocampal cultures were the most resistant to thapsigargin toxicity and treatment with 0.1 μM and 1.0 μM thapsigargin resulted in cell viabilities of 75% and 50% respectively (A). At the same concentrations, hippocalcin knock-out hippocampal cultures had a survival rate of 33% and 30%. (B) Wild-type and hippocalcin knock-out cortical cultures were not significantly different, with cell survival rates all at around 20% (C,D). (**,$p<0.01$; ***, $p<0.001$, #, $p<0.05$).

and 200 μM NADH as substrates to estimate the complex I content. To determine NADH-ubiquinone oxidoreductase activity, 100 μM n-decylubiquinone (DBQ) and 100 μM NADH were used as substrates and 5 μM rotenone as inhibitor, as described previously [26]. Oxidation rates of NADH were recorded with a Shimadzu Multi Spec-1501 diode array spectrophotometer (ε340-400 nm = 6.1 mM–1 cm^{-1}). Complex I activity was normalized to the complex I content of the mitochondrial preparation and is given as DBQ/HAR ratio. To determine the effect of Aβ, 2.2μl of fibrillar Aβ42 preparation (final assay concentration 5 μM) were added to the mitochondrial suspension and incubated for 15 min on ice [27], before measurement of complex I activity.

3. Results and Discussion

3.1. Hippocalcin levels vary with Aβ levels in human hippocampal brain samples

Hippocalcin is a neuronal protein that is highly expressed in the hippocampus [20]. Since this brain area is one of the brain structures where the degenerative process is initiated in AD, we performed Western blotting on protein extracts obtained from the human AD hippocampus and age- and gender-matched control brain samples (Fig. 1A). NeuN was used for quantification as it correlates well with the number of viable neurons (Fig. 1B) [28,29]. Western blotting showed that levels of Aβ monomers and trimers were significantly higher in AD brains than controls (both $p<0.05$) (Fig. 1D, E), while APP levels did not differ ($p=0.08$) (Fig. 1C), as previously observed [30]. NeuN levels were significantly lower in AD brains compared to controls, reflecting the neuronal loss that characterizes AD ($p<0.05$) [31]. Interestingly, levels of the neuronal protein hippocalcin [14,32] were significantly increased in AD brains compared to controls, suggesting that the calcium sensor is up-regulated in those neurons that survive the degenerative process, possibly as a protective mechanism against Aβ toxicity ($p<0.05$) (Fig. 1F). Together, our findings indicate that the surviving neurons in AD brains have higher levels of hippocalcin than neurons in healthy control brains. This suggests a neuroprotective role for hippocalcin.

3.2. Levels of hippocalcin and Aβ are inversely correlated in APP23 mouse brain

To validate the findings for hippocalcin observed in human tissue in mice, we investigated the expression of hippocalcin in Aβ-plaque-forming APP23 mice, an established AD mouse model [18]. The APP23 strain expresses human APP together with the AD pathogenic KM670/671NL double mutation (Swedish mutation). In these mice, Aβ levels increase with age, and by around 6 months, Aβ plaque formation is initiated. To correlate the hippocampal expression of hippocalcin with Aβ, we analyzed 6 and 18 month-old transgenic mice and wild-type littermate controls. We first co-stained sagittal sections with antibody 6E10 for Aβ and a hippocalcin-specific antiserum [20], and counterstained with DAPI to visualize the nucleus. 6E10 shows some cross-reactivity with full-length APP. The data reveal an inverse relationship between the Aβ/APP pathology and the staining intensity for hippocalcin in APP23 hippocampal neurons. At

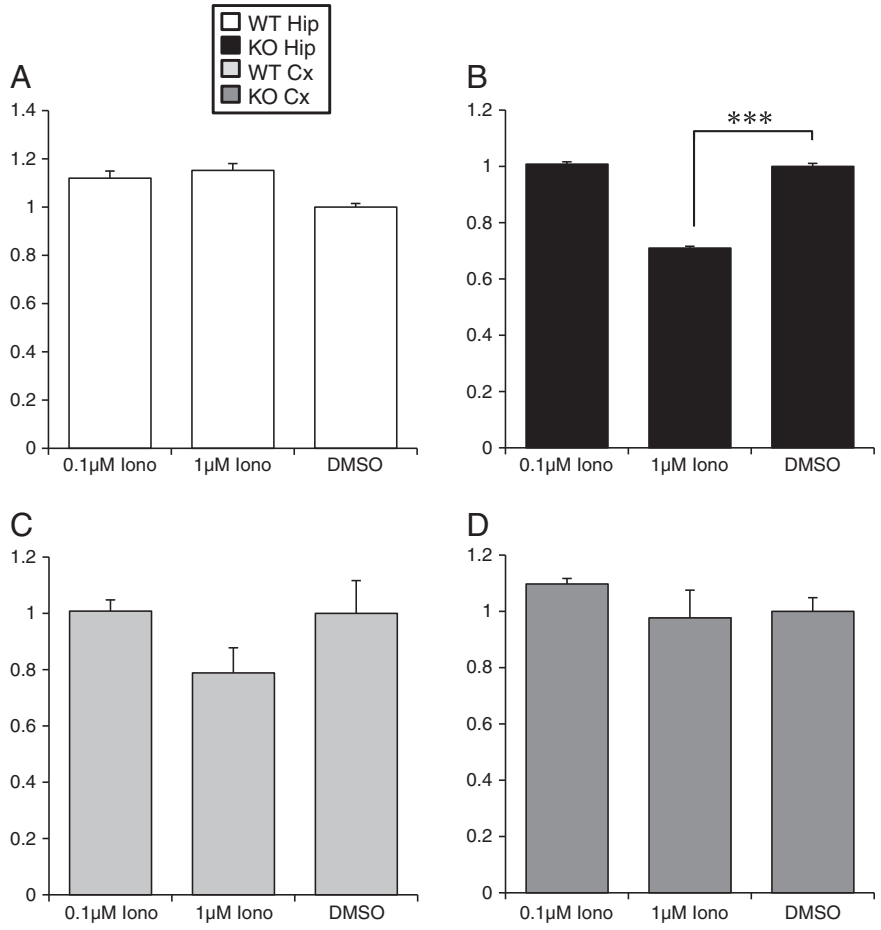

Fig. 5. MTT assay for cell viability after ionomycin treatment of wild-type (A, C) and hippocalcin knock-out (B, D) cortical (C, D) and hippocampal cultures (A, B). Ionomycin treatment was not toxic to all neuronal preparations except for hippocalcin knock-out hippocampal neurons (cell survival ~70%, 5B). (***, $p<0.001$).

6 months, there was no 6E10 reactivity detectable (data not shown). At 18 months, confocal microscopy revealed that wild-type CA1 pyramidal neurons had a uniformly strong reactivity for hippocalcin, while in the APP23 hippocampus, there were two types of cells revealing an inverse relationship between hippocalcin and 6E10 reactivity: there were CA1 neurons that showed a strong hippocalcin reactivity and none for 6E10 (grey arrowhead), and others that showed a strong staining for 6E10 reactivity that were hippocalcin-negative (white arrowheads) (Fig. 2). Intracellular Aβ has previously been correlated with neuronal loss and found to be a good predictor for synaptic dysfunction and neuronal loss in AD [33,34]. Further, increased levels of intracellular calcium were found to promote the formation of oligomeric forms of Aβ [35,36]. Interestingly, increased calcium levels also promote the aggregation of α-synuclein that forms aggregates in Parkinson's disease [37]. As it is established that hippocalcin protects neurons by enhancing calcium extrusion [14], the inverse relationship in hippocampal neurons expressing hippocalcin and 6E10 reactivity suggests that Aβ may cause reduced levels of the calcium sensor hippocalcin preventing it from conferring neuroprotection. In APP23 mice there are less plaques in the hippocampal formation compared to the cortex, despite comparable expression levels in the two brain areas [38]; whether this is because hippocalcin confers initial protection from plaque formation in the hippocampus, which is not available to the cortex, where it is not expressed, remains to be determined.

3.3. Hippocalcin protein levels in APP23 mice are higher than in wild-type mice and increase with age

We next performed Western blotting on protein extracts obtained from brains of 6 and 18 months-old APP23 transgenic mice and wild-type littermate controls (Fig. 3A). We quantified levels of the neuronal nuclear protein NeuN that reflects numbers of viable neurons [28,29]. We found that NeuN levels were significantly decreased in APP23 transgenic compared to wild-type hippocampi at both 6 and 18 months of age ($p<0.05$ each) (Fig. 3B), reflecting the neurodegenerative state of the transgenic mice as determined previously [38,39]. APP levels in the APP23 hippocampus were not significantly different for the two age groups (Fig. 3C), suggesting that they do not change with aging. Aβ levels however were significantly higher in 18 months-old APP23 transgenic mice compared to 6 month-olds (Fig. 3D), as reported previously [40]. Since there was only a change in Aβ, but not APP levels, any putative change in hippocalcin levels can therefore be attributed to levels of Aβ rather than APP in these mice. We found that hippocalcin levels were increased in 18- compared to 6 months-old APP23 transgenic mice ($p<0.05$) (Fig. 3E), in a pattern similar to that seen for Aβ. In contrast, hippocalcin levels in wild-type controls did not show significantly different hippocalcin levels upon aging, further indicating that it is Aβ that alters hippocalcin levels in the APP23 mice. Together with the immunohistochemical data, this indicates that hippocalcin is altered in APP

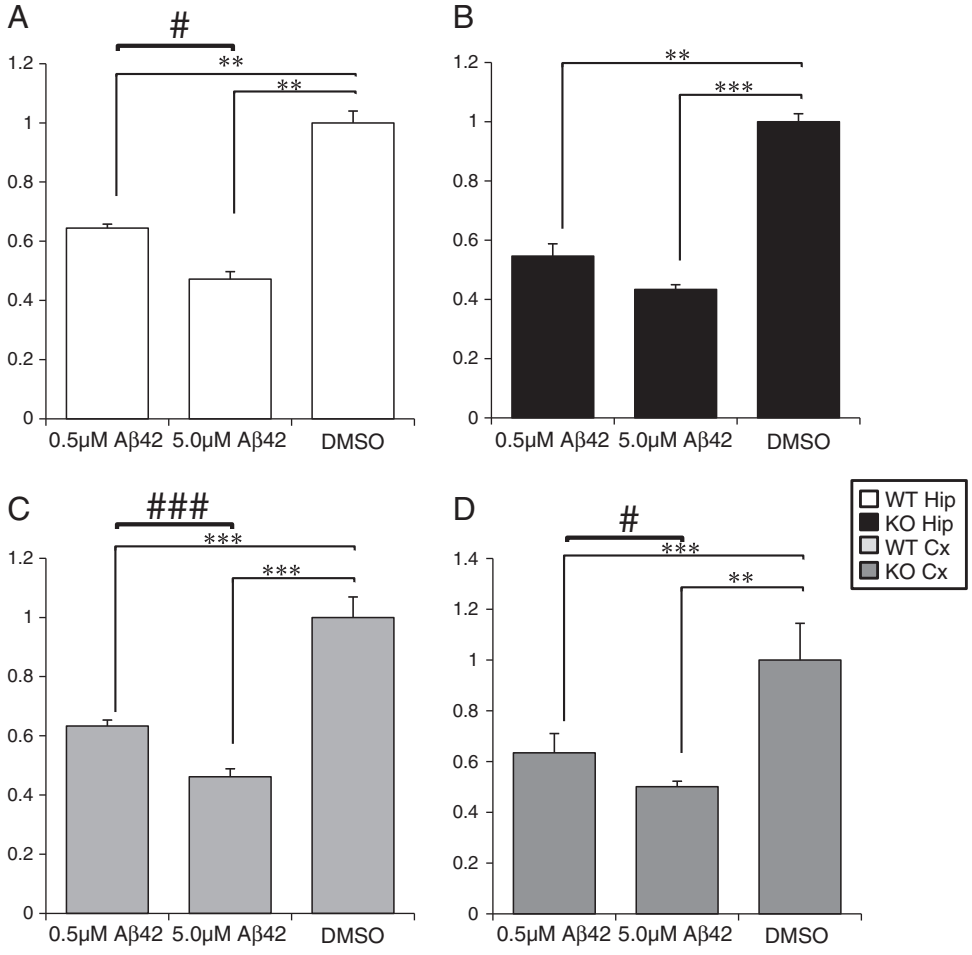

Fig. 6. MTT assay for cell viability after Aβ42 treatment of wild-type (A,C) and hippocalcin knock-out (B,D) cortical (C,D) and hippocampal cultures (A,B). Aβ42 treatment induced significant cell toxicity in all neuronal cultures. Treatment with Aβ42 at 0.5 μM led to a survival rate of 65% in wild-type hippocampal neurons (A), 54% in hippocalcin knock-out neurons (B), and 63% in wild-type and hippocalcin knock-out cortical neurons (C,D). At 5 μM, cell viabilities of all neuronal preparations were below 50%. (**, $p<0.01$; ***, $p<0.001$; #, $p<0.05$; ###, $p<0.001$).

mutant mice. This may suggest that neurons upregulate hippocalcin to counteract the toxicity of Aβ as addressed below.

3.4. Hippocalcin knock-out neurons are more sensitive to calcium deregulation than wild-type neurons

Numerous studies indicate a neuroprotective effect of calcium-binding proteins (Ca2+ binding proteins) in neurodegenerative diseases [41]. To better understand the putative neuroprotective role of hippocalcin in calcium deregulation, primary cortical and hippocampal neuronal cultures were obtained from embryonic day 16 (E16) hippocalcin-knockout ([11] and wild-type mice. After 10 days *in vitro* (DIV), the neurons were treated with varying concentrations of thapsigargin (Fig. 4) and ionomycin (Fig. 5), both of which are known to raise intracellular calcium levels although via distinct mechanisms [42,43]. Thapsigargin has been shown to raise the intracellular calcium concentration by preventing cells from directing calcium into the endoplasmic reticulum, thereby depleting intracellular calcium stores [44]. Ionomycin affects extracellular calcium transport into cells by increasing the calcium permeability of the plasma membrane [45].

We compared hippocampal and also cortical cultures from wild-type and hippocalcin knockout mice and treated them for 2 days with the two compounds, followed by an MTT assay to assess cell viability. Treatment with 0.1 μM of thapsigargin revealed that wild-type hippocampal neurons were the most resistant, with a survival rate of about 75% (Fig. 4A). Wild-type cortical neurons had a survival rate of less than 25% ($p<0.01$ versus non-treated counterparts). Hippocalcin knock-out hippocampal and cortical neurons had a survival rate of 33% and 21%, respectively ($p<0.01$ versus non-treated counterparts) (Fig. 4B–D). At 1.0 μM thapsigargin, cell viability of wild-type hippocampal neurons was about 50% (Fig. 4A), while for the knockout hippocampal neurons, it was 30% (Fig. 4B–D). We did not find significant differences in cell viability between wild-type and hippocalcin knock-out cortical cells (between 20 and 30%) (Fig. 4C, D), which is not surprising since hippocalcin is only expressed at very low levels in the cortex [8]. Together, our findings reveal that hippocalcin protects hippocampal neurons from the toxic effects exerted by thapsigargin.

To further assess the neuroprotective effects of hippocalcin, primary hippocampal and cortical cultures from hippocalcin knock-out and wild-type mice were treated with different concentrations of ionomycin. Treatment with 0.1 μM ionomycin was not toxic to any treatment groups (Fig. 5). However, at 1 μM, ionomycin was toxic only to hippocalcin-knockout hippocampal neurons ($p<0.001$) (Fig. 5B), while wild-type cortical neurons, as well as hippocalcin knock-out hippocampal and cortical neurons were spared (Fig. 5A, C, D). Together, this supports the notion that calcium homeostasis is critical in determining neuronal survival. Our data indicate that hippocalcin may play an important role in the maintenance of calcium homeostasis in the hippocampus.

3.5. Hippocalcin knock-out neurons are more sensitive to Aβ toxicity

Since hippocalcin levels are consistently up-regulated in human AD as well as in transgenic APP23 mice, the putative neuroprotective role of hippocalcin in Aβ toxicity was further investigated by determining cell viability of primary hippocampal and cortical cultures from hippocalcin knockout and wild-type mice, after treatment with varying concentrations of Aβ (Fig. 6).

This revealed a dose-dependent toxicity and cell viability that was decreased for all cell types by treating the cultures with Aβ concentrations between 0.5 and 5 μM ($p<0.05$ to $p<0.001$) (Fig. 6A, C, D), except for the hippocalcin knock-out hippocampal neurons, for which at 0.5 μM of Aβ, the cells already revealed the lowest viability of all cultures (Fig. 6B). At 0.5 μM Aβ, the survival rate of all cell cultures was around 65% (Fig. 6A, B, D), except for hippocalcin-

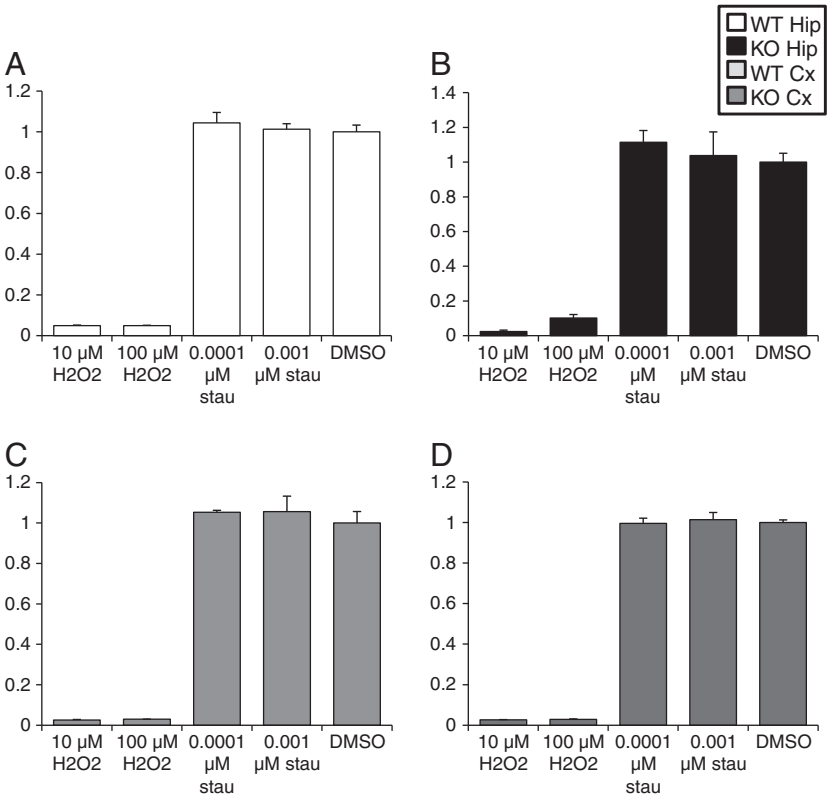

Fig. 7. MTT assay for cell viability after hydrogen peroxide (H_2O_2) and staurosporine (stau) treatments of wild-type (A,C) and hippocalcin knock-out (B,D) cortical (C,D) and hippocampal cultures (A,B). Cell viabilities of hippocalcin knock-out neurons were not significantly different from the wild-type neuronal preparations after exposure to H_2O_2 and staurosporine, indicating that hippocalcin knock-out neurons are not selectively sensitive to toxic effects exerted by H_2O_2 and staurosporine.

knockout hippocampal neurons, where it was below 55% (Fig. 6B). This indicates that hippocalcin-knockout hippocampal neurons are more susceptible to Aβ toxicity. However at 5 μM Aβ, the survival rates of all cell types were around 45%, obliterating any neuroprotective effects of hippocalcin.

Aβ is known to exert its toxicity via a myriad of mechanisms [46–48]. To determine whether the toxicity observed after exposure to Aβ is specific to Aβ rather than being a general consequence of cell death, wild-type and hippocalcin-knockout neurons were also exposed to varying concentrations of hydrogen peroxide as a source of oxidative stress, as well as staurosporine, a non-selective protein kinase inhibitor that exerts toxicity by interfering with many toxicity pathways [49]. Importantly, for both treatments, the viability of both hippocalcin knock-out hippocampal and cortical cells did not differ from their wild-type counterparts (Fig. 7), indicating that the susceptibility of hippocalcin knock-out hippocampal neurons to Aβ toxicity is specific. Together, these data suggest that hippocalcin has a critical function at an early stage of Aβ-induced toxicity in AD.

3.6. Hippocalcin knock-out neurons show an age-dependent deregulation of complex I activity and vulnerability to Aβ insult

Since MTT measurements (Figs. 4–7) are an indicator of mitochondrial health [24,50], we next determined whether mitochondrial functions are impaired in the hippocalcin knock-out hippocampus. We found that between 3 and 6 months of age, mitochondrial complex I activities go up in both wild-type and hippocalcin knock-out hippocampi (Fig. 8A), whereas an age-dependent decline is present in wild-type and hippocalcin knock-out cortices (Fig. 8B). Of note, in both brain regions the lack of hippocalcin induced a stronger aging phenotype. At 3 months of age, the basal activity of complex I is about 10-fold higher in cortical compared to hippocampal mitochondria in wild-type, but only about 3-fold higher in hippocalcin knock-out mice demonstrating a deregulation of mitochondrial activity. At 3 months, hippocampal wild-type mitochondria showed no sensitivity for Aβ, in contrast to hippocampal hippocalcin knock-out mitochondria where Aβ induced a significant reduction of complex I activity (Fig. 8C). No sensitivity to Aβ could be detected in cortical mitochondria from 3 months-old wild-type as well as hippocalcin knock-out animals (Fig. 8D), in contrast to wild-type mitochondria which were spared. At 6 months sensitivity to Aβ was strongly enhanced in hippocampal as well as in cortical mitochondria from hippocalcin knock-out mice (Fig. 8C, D). We conclude that the highest sensitivity to Aβ, i.e. reduction of complex I activity, is most pronounced in the hippocampus of hippocalcin knock-out mice where usually the highest level of hippocalcin is found, further supporting the notion that hippocalcin has a critical function in neuroprotection against Aβ toxicity.

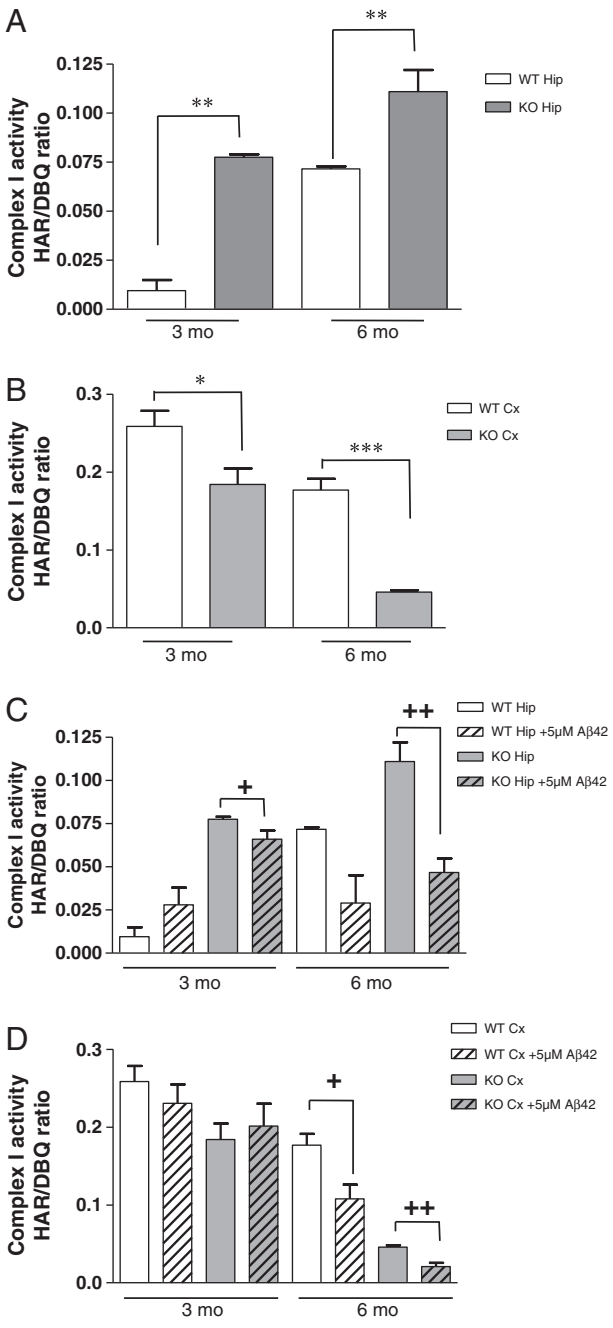

Fig. 8. Mitochondrial complex I activity (DBQ/HAR ratio) in the hippocampus (A,C) and cortex (B,D) from 3- and 6 months-old wild-type (WT) and hippocalcin knock-out (HC KO) mice. Complex I activity increased from 3 to 6 months in hippocampal mitochondria from WT as well as HC KO mice, but HC KO mitochondria exhibited a significantly stronger aging phenotype than WT mice (A) (Two-way ANOVA: aging effect $p = 0.0003$, KO effect $p = 0.0446$). In cortical mitochondria (B) complex I activity decreased from 3 to 6 months in both mice groups. Again, HC KO mitochondria exhibited a significantly stronger aging phenotype than in WT (Two-way ANOVA: aging effect $p = 0.001$, KO effect $p = 0.002$). Aβ42 treatment induced significant toxicity on hippocampal mitochondria from HC KO mice already at 3 months of age (C), whereas WT mitochondria were spared (C). Sensitivity to Aβ42 treatment was significantly increased at 6 months (C,D). A-D: ($^{*}p > 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{+}p < 0.05$; $^{++}p < 0.01$).

4. Conclusions

Alzheimer's disease (AD) is characterized by widespread excitotoxicity, especially in the hippocampus, one of the brain structures known to degenerate earliest in AD [51]. Hippocalcin is a neuron-specific calcium sensor protein that is highly expressed in the hippocampus and has previously been suggested to be neuroprotective [14,32]. In this study, we found that those hippocampal neurons that survive the degenerative process in AD increase hippocalcin expression, indicating a neuroprotective attempt to promote neuronal survival. We also found that hippocalcin levels were affected by Aβ levels. Our findings suggest that neurons that are capable of up-regulating hippocalcin may receive some protection, as indicated by our Western blot finding.

To investigate the neuroprotective role of hippocalcin, we had treated embryonic neuronal primary cultures. In this experimental paradigm, hippocalcin-knock out hippocampal cultures showed significantly more toxicity in the presence of thapsigargin and ionomycin, indicating that hippocalcin plays an important neuroprotective role in calcium-related neuronal injury in the hippocampus. This has significant implications for other calcium-related disorders such as stroke and epilepsy [52]. Employing the same experimental system, we could also show that hippocalcin confers neuroprotection against Aβ toxicity, and this was reflected by the observed age-dependent changes in mitochondrial activities. Collectively, our study indicates that hippocalcin

levels are deregulated by Aβ. They further present hippocalcin as a biomarker and possible therapeutic target for AD.

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