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CITATION

Heverin, Maura; Brennan, Gary P.; Koehler, Christian J.; Treumann, Achim; Henshall, David C. (2012): Proteomic analysis of 14-3-3 zeta binding proteins in the mouse hippocampus.. Royal College of Surgeons in Ireland. Journal contribution. <https://hdl.handle.net/10779/rcsi.10791254.v1>

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Original Article

Proteomic analysis of 14-3-3 zeta binding proteins in the mouse hippocampus

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Received June 1, 2012; accepted June 20, 2012; Epub June 23, 2012; Published June 30, 2012

Abstract: 14-3-3 proteins are ubiquitous molecular chaperones with important roles in brain development and neuronal function. Altered expression of 14-3-3 proteins has been reported in several neurologic and neurodegenerative disorders and identifying 14-3-3 binding proteins may provide important insights into the physiologic and pathophysiologic roles of these proteins. Particular interest has emerged on 14-3-3 zeta (ζ) in the setting of neuronal injury because reducing 14-3-3 ζ levels triggers an endoplasmic reticulum stress-like response in neurons and increases vulnerability to excitotoxicity. Here we examined the subcellular distribution of 14-3-3 ζ in the mouse hippocampus. We then used recombinant His-tagged 14-3-3 ζ to pull-down interacting proteins from the mouse hippocampus followed by identification by liquid chromatography-mass spectrometry. 14-3-3 ζ protein was present in the cytoplasm, microsomal compartment, nucleus and mitochondrial fractions of the mouse hippocampus. Recombinant 14-3-3 ζ eluted 13 known 14-3-3 binding partners, including three other 14-3-3 isoforms, and 16 other proteins which have not previously been reported to bind 14-3-3 ζ . The present study identifies potentially novel 14-3-3 ζ binding proteins and contributes to defining the 14-3-3 ζ interactome in the mouse brain.

Keywords: 14-3-3 interactome, apoptosis, epilepsy, hippocampus, mass spectrometry, proteomics

Introduction

14-3-3 proteins are a family of conserved acidic proteins of which there are seven different isoforms found in mammals (ζ , γ , η , ϵ , β , θ , σ) [1]. They function as molecular adapters which interact with key signaling molecules and thereby regulate various cell functions including metabolism, division, differentiation, autophagy and apoptosis [2-4]. 14-3-3 proteins are ubiquitously expressed, but are most abundant in the brain [5]. They are generally considered to be cytosolic proteins, although subcellular fractionation analyses of rat brain has shown they are also present in other compartments, including intracellular organelles such as mitochondria and the endoplasmic reticulum [6-8]. Knockout of 14-3-3 isoforms has demonstrated essential roles for some while loss of others appears to be adequately compensated by the remaining isoforms [9].

14-3-3 proteins have three main modes of action; altering conformation of their targets,

physically occluding structural features, and scaffolding [9]. By doing so, 14-3-3 proteins influence the functions of their targets, including modulating intracellular trafficking, blocking or activating enzymatic activity and influencing posttranslational modification. The identification of two binding motifs (RSXpSXP and RXY/FXpSP, where p denotes a phosphorylated serine and X denotes any amino acid) [10] provided critical insight into the mechanism of their binding, although many target proteins do not contain these motifs [9]. The advent of proteomic profiling has facilitated a large increase in the identification of 14-3-3 target proteins and to date over 200 different binding partners have been identified [11-15]. These include protein kinases, phosphatases, signaling molecules, adaptors/scaffolding, transcription factors, receptors and cytoskeletal proteins.

Aberrant expression or function of 14-3-3 proteins has been linked to several diseases including cancer [16] and neurodegeneration [5]. Particular interest has emerged on 14-3-3 ζ

(Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide). Subcellular fractionation of human hippocampus showed the ζ isoform to be particularly abundant, and one of a sub-set of isoforms present in the microsome-enriched fraction containing the endoplasmic reticulum and Golgi apparatus [7]. Altered expression of 14-3-3 ζ has been reported in Alzheimer's disease [17], spinocerebellar ataxia [18], autism spectrum and neuropsychiatric disorders [19]. Levels of 14-3-3 ζ were also found to be elevated in the microsomal fraction of the hippocampus from patients with temporal lobe epilepsy [7], and other data has emerged to suggest seizures alter the expression or function of 14-3-3 ζ [7, 20, 21]. The presence of 14-3-3 ζ in cerebrospinal fluid may also be a biomarker of seizure-induced brain injury [22].

The 14-3-3 ζ brain interactome has not been characterized. Several studies have characterized 14-3-3 interactomes, although the degree of overlap between studies has been quite low because of the various isoforms, models and platforms employed [9]. Approaches used include yeast two-hybrid screening [23], protein microarrays [15], and using tagged 14-3-3 to purify bound proteins [13]. Here we used a recombinant His-tagged 14-3-3 ζ to elute interacting proteins from mouse brain combined with tandem mass spectrometric (MS/MS) identification.

Methods

His-tagged 14-3-3 zeta

To identify novel 14-3-3 ζ interacting proteins in the hippocampus we used an immunoprecipitation-pull down approach. Recombinant human His-tagged 14-3-3 ζ was expressed and purified from *E. coli* clones from the hEx1 library (ImaGenes, Berlin, Germany) according to previous descriptions with modifications [24]. For purification, proteins were extracted in a lysis buffer, sonicated and centrifuged to remove any insoluble cellular debris. Supernatant was loaded onto a column, washed, and then bound protein eluted with buffer containing imidazole followed by desalting, and concentration, and fast performance liquid chromatography. Purified proteins were separated by SDS-PAGE and stained with Coomassie Blue to verify the size of the purified proteins against the expected

value. Protein concentrations were determined using the MicroBCA assay (Pierce, Rockford, IL) according to manufacturer's specifications.

Elution of 14-3-3 ζ -interacting proteins using His-14-3-3 ζ

C57BL/6 adult male mice (Harlan, U.K.) were euthanized and hippocampi were dissected, immediately frozen and stored at -70°C until use. Hippocampi were then homogenised in ice-cold lysis buffer (50 mM Tris-HCL, pH 8.0, 250 mM NaCl, 10 mM imidazole, 0.5 % NP-40, protease and phosphatase inhibitor cocktails). After 30 min, the lysate was centrifuged for 10 min at 8000 x g to remove unbroken cells. The resultant supernatant was collected and pre-cleared with 100 μ l cobalt beads for 2 h. At the same time, 40 μ g of His-tagged 14-3-3 ζ was incubated with 100 μ l of cobalt resin in 1 ml lysis buffer for 2 h, after which it was centrifuged and the resin washed with PBS. The cobalt resin now bound with His-tagged protein was incubated with the pre-cleared supernatant overnight. As a negative control supernatant was incubated with cobalt resin alone. The beads were then washed with lysis buffer without NP-40 four to five times before eluting the 14-3-3 complexes with 500 μ l of 250 mM imidazole. The eluate was precipitated with TCA, separated on 12 % SDS PAGE gels and stained with colloidal Coomassie.

Mass spectrometry

Gels were sliced into 10 bands for each lane. The bands were reduced, alkylated with iodoacetamide, and in-gel digested with trypsin (Promega). The samples were subjected to LC-MS/MS analysis as follows. Peptides were separated on a 75 μ m inner diameter x 15cm column (C18 Pepmap 100, 3 μ m, Dionex) at a flow rate of 200 nl/min delivered by an Ultimate 3000 nano-LC system (Dionex, U.K.) with the following solvent scheme: solvent A, 0.08 % formic acid; solvent B, 90 % acetonitrile in 0.08 % formic acid. Samples were loaded in solvent A, and peptides were eluted by 7 % solvent B for 3 min followed by a linear gradient to 50 % solvent B for 45 min and holding the column at 95 % solvent B for 5 min before re-equilibrating the system for 10 min using 7 % solvent B. Up to 3 peptide precursor ions were subjected to data-dependent acquisition of tandem mass spectra using an ion trap mass spectrometer (HCT Ultra,

Bruker). Tandem mass spectra were converted into peak lists using DataAnalysis (Bruker Daltonics) and searched against mouse ENSEMBL v.46 (<http://aug2007.archive.ensembl.org/index.html>) using X!Tandem (version 2007.07.01.2) [25] and the following parameters: parent ion mass tolerance: ± 300 ppm, fragment ion tolerance: 0.3 Da, tryptic cleavage specificity allowing for one missed cleavage site, fixed modification: carbamidomethyl, variable modifications: carbamidomethylated cysteines and N-terminal protein acetylation, refinement search parameters were set to the following additional modifications: deamidation of Asn and Gln, phosphorylation of Ser, Thr and Tyr, oxidation and dioxidation of Met and Trp, methylation of Cys, Asp, Asn, Glu, His, Lys and Arg, dehydration of Ser and Thr, carbamidomethylation of Lys, His, Asp and Glu, missed carbamidomethylation of Cys. All search results had a false positive rate of less than 1.5% [26]. All proteins that were identified with only one peptide were validated manually by comparing them against higher scoring entries gpmdb (gpmdb.thegpm.org).

Subcellular fractionation

Subcellular fractionation was undertaken using the sucrose technique as previously described with modifications [27]. Briefly, hippocampus ($n = 3$) from C57BL/6 mice was homogenized in 1 x M-SHE buffer (210 mM Mannitol, 70 mM Sucrose, 10 mM HEPES-KOH pH 7.4, 1 mM EDTA, 1 mM EGTA and a protease inhibitor cocktail) and then centrifuged twice at 1200 x g for 10 min. The pellet that remained contained the nucleus and unlysed cells and was stored on ice for further purification later. The post-nuclear supernatant was then centrifuged twice at 10,000 x g for 15 min and the resulting mitochondrial pellet was resuspended in a sucrose buffer (395 mM Sucrose, 0.1 mM EGTA, 10 mM HEPES-KOH pH 7.4) and purified through a percoll bilayer in gradient buffer (1.28 M Sucrose, 0.4 mM EGTA, 40 mM HEPES-KOH, pH 7.4) by centrifugation at 41,000 x g for 30 min. The crude cytosolic fraction was then centrifuged at 100,000 x g for 1 h to separate the microsomal and cytosolic fractions. The nuclear pellet was rehomogenised in 1 ml M-SHE buffer and centrifuged at 1000 x g for 10 min until a pellet bilayer was obtained, the supernatant was discarded and the opaque layer of the pellet was retained and resuspended in 1 ml TSE buffer

with 1 % NP-40. This step was repeated two more times until a monolayer pellet was obtained which was then suspended in 40 μ l of lysis buffer. Protein quantity per fraction was determined by BCA assay. Fraction quality was determined by immunoblotting with antibodies against markers for the cytoplasm (Bad, #9292, Cell Signaling Technology), mitochondria (Porin, ab34726, Abcam), nucleus (Lamin A/C, #2032, Cell Signaling Technology) and microsomes (protein disulfide isomerase (PDI), sc20132, Santa Cruz Biotechnology). Twenty micrograms of protein was then transferred to nitrocellulose membrane (Biorad), probed with antibodies against 14-3-3 ζ (sc1019, Santa Cruz Biotechnology) and visualised using HRP-conjugated secondary antibody (Millipore) and visualised using a FujiFilm Las4000 system by chemiluminescence.

Gene ontology

Gene ontology (GO) was undertaken for the identified proteins by uploading to the Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>) and categorized according to biological process, molecular function and cellular component, as described [28].

Results

Subcellular localization of 14-3-3 ζ in the mouse hippocampus

Although previous work reported the presence of 14-3-3 ζ in the endoplasmic reticulum-enriched microsomal fraction in the mouse [20], this work used organotypic cultures. To determine where 14-3-3 ζ localizes in the adult mouse brain we analyzed the nuclear, mitochondria, cytoplasm and microsomal fractions prepared from hippocampus extracted from adult C57BL/6 mice. Western blotting determined that each fraction was enriched for established markers and devoid of significant cross-contamination (**Figure 1A**). Thus, the Bcl-2 family protein Bad was exclusively in the cytoplasm, Porin was only in the mitochondrial fraction, Lamin A/C only in the nucleus, and PDI was found in the microsomal fraction (**Figure 1A**). Western blot analysis determined 14-3-3 ζ to be present in each fraction, being particularly abundant in the cytoplasm and the microsomes, while being present at somewhat lower levels in

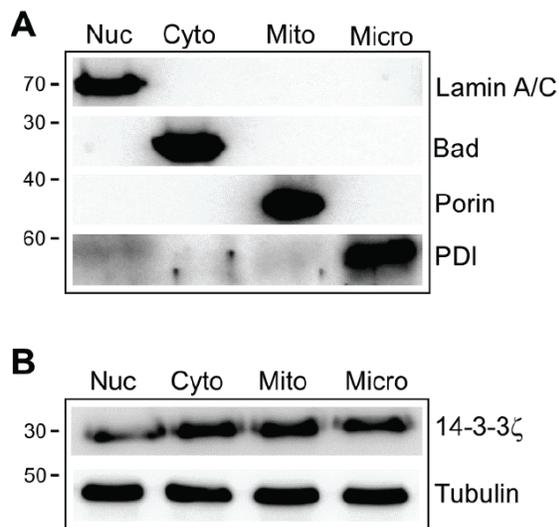


Figure 1. Subcellular location of 14-3-3 ζ in the mouse hippocampus. (A) Western blots validate the fraction purity using markers specific for each subcellular compartment; Lamin A/C for nuclear, Bad for cytoplasm, Porin for mitochondria and PDI for the microsomal fraction. (B) Representative western blot showing 14-3-3 ζ in each subcellular compartment. α -Tubulin is included as a guide to protein loading.

the nucleus and mitochondria (**Figure 1B**).

Isolation and identification of 14-3-3 ζ -binding proteins

Successful MS analysis of 14-3-3 ζ target proteins in the hippocampus depended on the expression and purification of the His-tagged recombinant human 14-3-3 ζ which was expressed and isolated from *E. Coli* and used in the subsequent pull-down experiments. Purity of the His-tagged protein was confirmed by 1D electrophoresis and Coomassie staining (**Figure 2A**) and was estimated to be 99% purified. Western blot analysis verified the specificity of the protein as being 14-3-3 ζ (**Figure 2B**). Faint bands detected using antibodies against other isoforms likely result from known cross-reactivity [6].

To identify 14-3-3 ζ -interacting proteins in the mouse hippocampus, the His-tagged 14-3-3 ζ was incubated with precleared protein lysate from mouse hippocampus. Proteins bound to 14-3-3 ζ were eluted, processed and identified by mass spectrometry. The experiment was performed in duplicate. In the first study a total of 111 proteins passed the criteria for detection

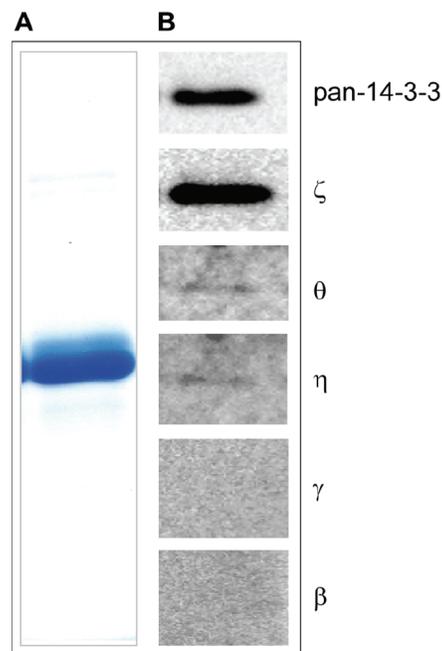


Figure 2. Characterization of recombinant His-tagged 14-3-3 ζ used for proteomics. (A) Coomassie stained gel of the purified protein. (B) Western blot analysis of the purified protein. The strongest band was seen when the protein was immunoblotted using antibodies against the zeta isoform of 14-3-3.

(see Methods) and in the second experiment, 110 proteins were identified (data not shown). The common proteins among the two lists (29 in total) are shown in **Table 1**. Included in this list were 10 proteins that have previously been validated as 14-3-3 binding proteins. Many of these proteins were identified with high confidence (**Table 1**). Three 14-3-3 isoforms were also detected; β , θ and γ , which were most likely in hetero-dimeric formation with the zeta isoform. The remaining 16 proteins identified are potentially novel 14-3-3 ζ binding proteins (**Table 1**).

Gene ontology analysis of 14-3-3 ζ binding proteins

To explore the biological functions of the 14-3-3 binding proteins we used gene ontology software and analyzed biological process, molecular function and cellular component (**Figure 3**). Among biological processes, were synaptic transmission and protein transport, as well as protein kinase and neuronal migration (**Figure 3A**). For molecular function, binding was the

14-3-3 zeta proteomics

Table 1. Common 14-3-3 ζ -interacting proteins

	Gene name	Description	Mass (kDa)	rl	Log(e)	Motif
1	Tuba1a	Tubulin, alpha 1A	50.1	13	-118.0	Low 1
2	Kif5c	Kinesin family member 5c	109.2	13	-137.0	Med 1
3	Ywhab	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	28.1	8	-46.8	
4	Mark2	MAP/microtubule affinity-regulating kinase 2	86.3	9	-89.6	High 2
5	Mark3	MAP/microtubule affinity-regulating kinase 3	84.3	9	-95.5	Med 2
6	Mark1	MAP/microtubule affinity-regulating kinase 1	88.3	9	-77.8	Med 4
7	Nsf	Vesicle-fusing ATPase	82.5	7	-78.9	Low 1
8	Atp1a1	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	112.9	7	-80.0	Med 1
9	Klc2	Kinesin light chain 2	68.1	6	-71.2	Med 3
10	Rps18	Ribosomal protein S18	17.7	5	-25.8	Med 1
11	Trim32	Tripartite motif protein 32	72.0	5	-36.3	Med 2
12	Arhgef7	Rho guanine nucleotide exchange factor (GEF7)	82.8	5	-48.4	High 1
13	Psd3	Pleckstrin and Sec7 domain containing 3	111.1	7	-33.2	Med 3
14	Ywhaq	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	27.8	5	-46.9	
15	Lgj1	Leucine-rich repeat LGL family, member 1	63.6	3	-15.0	Low 4
16	Cfl1	Cofilin 1, non-muscle	24.7	4	-25.1	Med 1
17	EG433923	PREDICTED: similar to adenine nucleotide translocase	33.1	3	-26.9	
18	Slc25a12	Calcium-binding mitochondrial carrier protein Aralar1	74.5	3	-28.6	Low 5
19	Ywhag	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	28.3	3	-12.1	
20	Adam22	A disintegrin and metallopeptidase domain 22	99.5	2	-26.6	Low 8
21	Arf3	ADP-ribosylation factor 3	20.6	2	-11.4	0
22	Dmxl2	Dmx-like 2	322.4	7	-11.8	Med 4
23	Nckipsd	SH3 adapter protein SPIN90 (NCK interacting protein with SH3 domain)	78.5	1	-12.8	High 1
24	Ksr1	Kinase suppressor of Ras 1	96.7	1	-1.8	High 1
25	Slc25a3	Phosphate carrier protein, mitochondrial precursor (PTP)	39.6	1	-3.0	Med 1
26	Brsk2	BR serine/threonine kinase 2	73.1	1	-3.2	Med 1
27	Rps27a	Ribosomal protein S27a	17.9	1	-6.1	Med 1
28	Syn2	Synapsin II	63.3	1	-6.6	Med 1
29	Atp2b1	ATPase, Ca ²⁺ transporting, plasma membrane 1	134.7	1	-6.6	Med 1

Key. Proteins in light grey shading are known 14-3-3 interactors. Those in darker shading are other 14-3-3 isoforms. rl is the average number of peptides used to identify the protein. log(e) is log₁₀ of the probability that the assignment is a stochastic event. The motif score is the number of 14-3-3 consensus binding sites identified using the Scansite program at high, low and medium stringency.

most prominent category (lipid, ATP, ion and nucleotide) (**Figure 3B**). Finally, among cellular component the cytoplasm and plasma membrane, nucleus and synapse, and both Golgi and endoplasmic reticulum featured (**Figure 3C**).

Discussion

The present study used recombinant His-tagged 14-3-3 ζ to identify potentially novel 14-3-3 ζ binding proteins in the mouse hippocampus. Using a proteomic LC-MS/MS approach we identified a number of previously known 14-3-3 binding proteins, including other 14-3-3 iso-

forms, and a series of potentially new 14-3-3 ζ binding proteins. We also included an analysis of the subcellular distribution of 14-3-3 ζ in the mouse hippocampus which revealed the presence of 14-3-3 ζ within the endoplasmic reticulum-containing microsomal fraction, supporting previous work. These studies identify additional 14-3-3 ζ interacting proteins which may be important in normal brain function or in disease.

Defining the 14-3-3 ζ interactome is an important goal if we are to fully understand the myriad functions and processes regulated by this member of the 14-3-3 family. Significant efforts

14-3-3 zeta proteomics

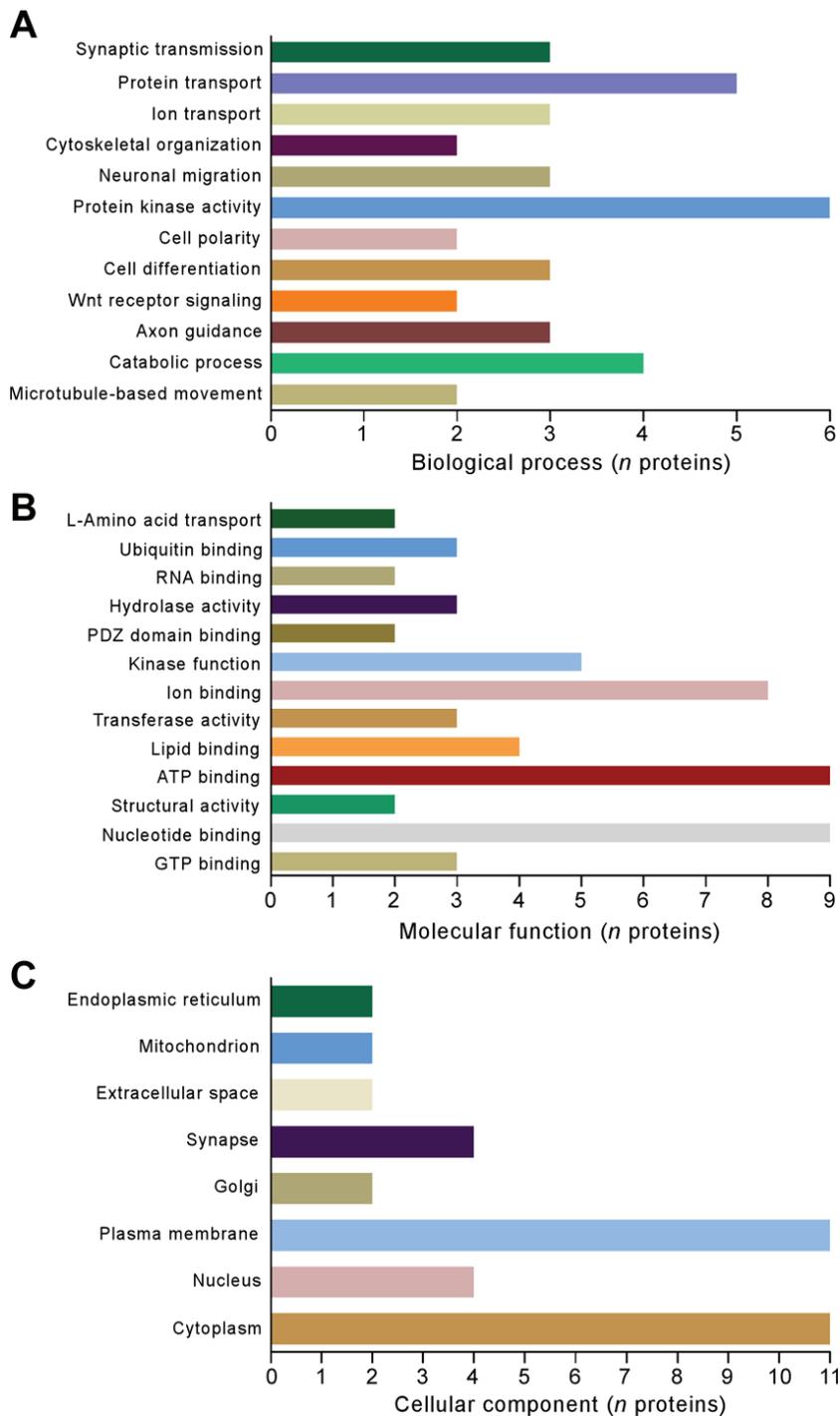


Figure 3. Gene ontology analysis of 14-3-3 ζ binding proteins. The DAVID bioinformatics tool was used to categorize the 14-3-3 ζ proteomics list according to GO processes (A) Biological process; (B) Molecular function and (C) Cellular component.

have been expended toward full characterization of the 14-3-3 interactome using proteomics

involved in cytoskeletal functions, members of which have previously been shown to interact

-based approaches [9] and the present work extends findings on other isoforms [12, 13]. Here, we used recombinant human His-tagged 14-3-3 ζ to elute 14-3-3 binding proteins from mouse hippocampus. Thus, our approach is most similar to that used in HeLa cells to characterize the 14-3-3 ζ interactome during interphase and mitosis [29]. The choice of 14-3-3 ζ as bait is particular apt given the implication of this isoform in neurodegenerative, neurologic and neuropsychiatric diseases [7, 17-19] and also because 14-3-3 ζ may function to oppose endoplasmic reticulum stress [20], an important trigger of apoptosis [30]. Altogether we identified 29 proteins, a number very similar to that reported for the 14-3-3 ζ interactome in HEK293 cells [12]. This included a number of known 14-3-3 ζ -interacting proteins, including three other 14-3-3 isoforms. In addition to other isoforms of 14-3-3, we identified proteins previously been identified as interacting with 14-3-3 including Kinesin light chain 2 [31], Adam22 [32] and Cofilin [33]. Taken together, these results provide confidence that the approach we used was suitable for identifying 14-3-3 ζ -interacting proteins.

Our study also identified 16 potentially novel 14-3-3 ζ interacting proteins in the mouse hippocampus. This included Kif5c, a member of the kinesin family involved in cytoskeletal functions, members of which have previously been shown to interact

with 14-3-3 ζ [29, 34]. We also detected Nsf, a vesicle-fusing ATPase [35]. Although an interaction with 14-3-3 ζ has not been reported previously, this protein functions in synaptic transmission and 14-3-3 isoforms are known to regulate several proteins with ATPase activity [9]. Two ribosomal proteins were eluted by 14-3-3 ζ in our study which is consistent with findings reported for the HeLa cell 14-3-3 ζ interactome [29]. Rps27a is involved in ubiquitin metabolism and although no previous study has linked this gene to 14-3-3 ζ , 14-3-3 ζ is involved in ubiquitin-regulation of proteins [36] and 14-3-3 proteins are themselves targets for ubiquitin-dependent regulation [37]. Similarly, Trim proteins are known to target 14-3-3 for ubiquitin-mediated degradation [38] and here we identified Trim32 as a potentially novel 14-3-3 ζ -interacting protein. Another detected protein was Arhgef7 which has not previously been reported to bind 14-3-3, although members of the Rho guanine exchange factor family have been shown to be regulated by 14-3-3 [39]. Nckipso (SPIN90) is another protein associated with neuronal function, expressed in dendritic spines where it promotes spine density [40]. Relatively little is known about the function of Slc25a3 in the brain but it has been reported to control the release of pro-apoptotic protein from mitochondria [41]. We detected 14-3-3 ζ in the mitochondrial fraction of mouse hippocampus and our study may therefore have identified a novel apoptosis-regulatory protein with which 14-3-3 can interact. Given the association between changes to 14-3-3 ζ and human temporal lobe epilepsy it is notable that among the novel proteins was Lgi1, a gene deleted in certain epilepsies [42]. Slc25a12 was also detected, a gene whose absence results in global hypomyelination and seizures [43]. A further potential link to epilepsy comes from the identification of 14-3-3 ζ as a binding protein for a common anti-epileptic drug [44].

Our gene ontology analysis extended insights beyond the proteomic work. As would be expected, prominent in the analysis were proteins associated with the cytoplasm and synapse. This is consistent with the original work identifying these as important sites of 14-3-3 localization [6] and work since that has linked 14-3-3 functions to neurotransmission [5]. Proteins known to reside within the Golgi apparatus and the endoplasmic reticulum also featured in the cellular component category in agreement with

the established roles of 14-3-3 in trafficking proteins and in endoplasmic reticulum stress [45]. Predictably, binding dominated the category of biological and molecular function. However, other categories were also found, including ubiquitin and kinase function, which have been emphasized in 14-3-3 proteomic and other work previously [29, 46, 47]. Also, among molecular function were neuronal migration, a process previously linked to 14-3-3 ζ [48, 49]. Notably, Cheah et al recently reported that mice lacking 14-3-3 ζ show aberrant development of the hippocampus with migratory defects of pyramidal cells and granular neurons as well as abnormal synaptic input on pyramidal neurons [19].

The present study also included a subcellular analysis of 14-3-3 ζ in the mouse hippocampus. Previous work on this isoform has been undertaken using rat brain [6] and human material [7]. 14-3-3 ζ like other members of the family is thought to be most abundant in the cytosol, but the protein is also present in the synaptic compartment [6], in mitochondria and in the microsomal fraction which contains the endoplasmic reticulum [7, 20]. Our data are consistent with these reports and we found 14-3-3 ζ in the cytoplasm, mitochondria and microsomes. In addition, we report 14-3-3 ζ in the nuclear compartment of the mouse hippocampus. This localization is consistent with roles for 14-3-3 proteins in regulating trafficking and function of nuclear proteins [50, 51].

Some caveats should be considered in the present study. The list of proteins is probably a significant underestimate of the 14-3-3 ζ interactome in the mouse hippocampus. Foremost is the limitation of sensitivity and detection, particularly taking into account the small size and the resulting small amounts of material to be analyzed in this study. We cannot assume that proteins eluted by 14-3-3 ζ represent direct binding to 14-3-3 ζ rather than extraction as part of a multi-protein complex or their presence as un-specific binders, although the latter was significantly mitigated in the design of our experiment through the use of precleared lysates. Beyond this, we selected only those proteins detected in both experiments, which increases confidence in the proteins we identified being bona fide 14-3-3 ζ -interacting proteins in the mouse hippocampus but we have probably missed others. Another consideration is our use of human re-

combinant 14-3-3 ζ as bait to elute 14-3-3 ζ binding proteins. Presumably, only those proteins freely available for binding and under the conditions in our pull-down study could be identified. Many proteins already complexed to endogenous 14-3-3 ζ would be missed. Further insights may be obtained if experiments used subcellular fractions rather than whole cell lysates, given the variable presence of 14-3-3 ζ between compartments in the mouse hippocampus. Also, because the lysates would contain both glial and neuron populations, we do not know in which cell population these interactions occur. Finally, future studies must include individual validation experiments to confirm the specificity of the interactions and validate these proteins as bona fide targets of 14-3-3 ζ .

In conclusion, this study shows the presence of 14-3-3 ζ in four subcellular compartments in the mouse hippocampus. We characterized the 14-3-3 ζ interactome in this tissue and present a novel list of potential interacting proteins which were previously unknown. Together, our studies expand our knowledge about the potential targets of 14-3-3 ζ which may contribute to our understanding of the physiological and disease-related roles of this protein in the brain.

Acknowledgements

This study was funded by Science Foundation Ireland award 04/IN3/B466 and 08/IN1/B1875 and by the Programme for Research in Third Level Institutions (PRTL cycle 3). The authors would like to thank Jorin Pang and Ina Woods for technical support.

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14-3-3 zeta proteomics

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