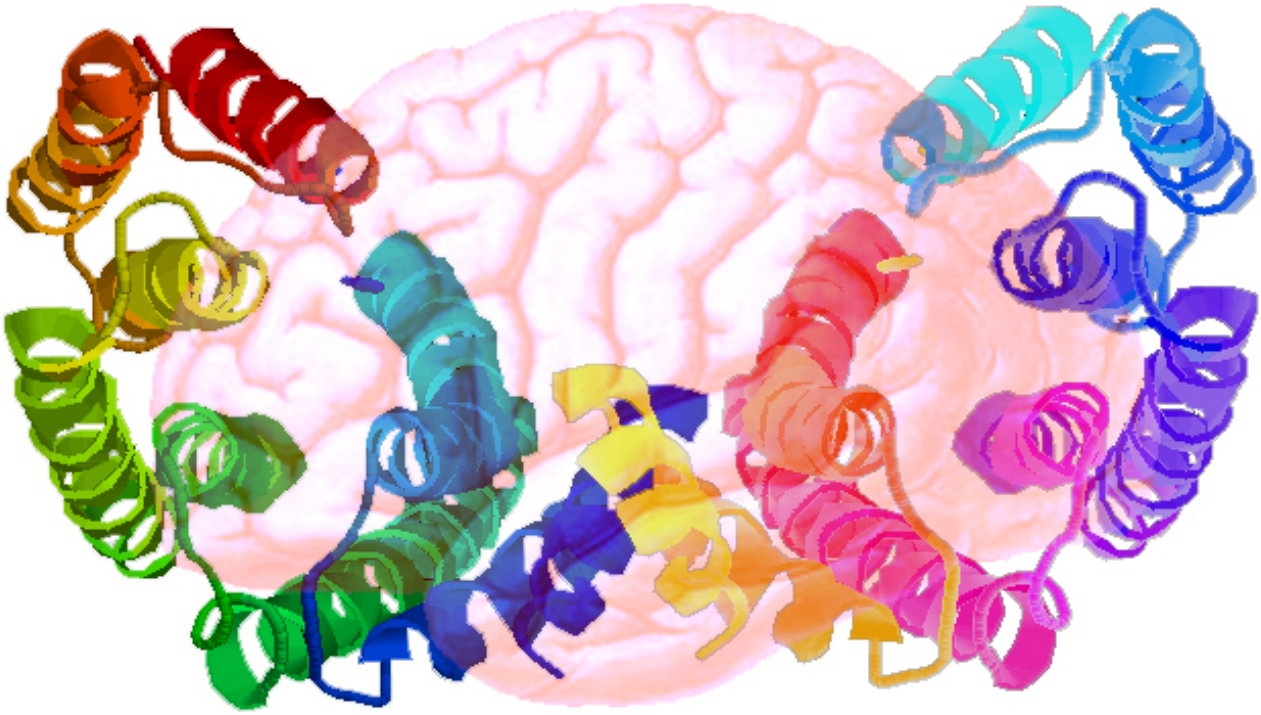
Novel 14-3-3 epsilon target proteins in *R. norvegicus* cortical neurons

Internship Report

Master Neurosciences



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Summary

All human behavior depends on the functioning of neurons. A network of proteins governs communication within neurons mediated by action potentials as well as communication between neurons, mediated in part by neurotransmitter release. In this study, an attempt was made to find and identify Mm14-3-3e interacting proteins in cytoplasm of R. norvegicus cortical neurons. To this end, recombinant Mm14-3-3eHis(6x) protein was expressed in E. coli and purified in order to use as bait in a pull-down assay. The pull-down assay was performed using a HisTrap HP column connected to an FPLC, and Mm14-3-3eHis(6x) targets were eluted with a specific high affinity peptide called R18. Eluted proteins were visualized on SDS-page gels, that were stained with coomassie blue. Confirmation of genuine 14-3-3e targets was obtained by applying an overlay onto the elutions. Matrix-assisted laser desorption/ ionisation-time of flight mass spectrometry was used for identification of the eluted proteins. Over 200 putative Mm14-3-3e target proteins were identified. ± 50 of these identified with high confidence. Among these proteins are a great number of novel Mm14-3-3e targets. A number of novel targets are involved in neurotransmitters release, indicating an important role for Mm14-3-3e in neuronal functioning. The specific actions of Mm14-3-3e within the protein network are topics for future research.

Introduction

A very complex, yet highly structured and organized neural network controls all human behavior. Numerous neurons function in this network as elemental integrators of electrical and chemical input. Electrochemical forces can cause a neuron to fire an action potential that travels down the axon, the primary output of a neuron. Upon reaching the end of an axon, the action potential triggers calcium influx, which activates many protein cascades involved in neurotransmitter release. The firing behavior depends on many factors, for instance conductance and sensitivity of ion channels to the membrane potential, and size and morphology of the neuron.
Neurotransmitter release, an important feature of the pre-synaptic site that can inhibit or excite the post-synaptic site, is a complicated process. It requires many proteins to exert their specific function within a network that controls a regulated secretory pathway in which vesicles undergo exocytose to release their contents in the synaptic cleft.
The firing behavior of neurons as well as neurotransmitter release is under control of regulatory proteins. Acting together in the cell to shape the properties of neurons, these proteins ultimately determine human behavior.
14-3-3, a protein that has been shown to interact with proteins involved in synaptic function is highly expressed in brain. It is a small protein (± 30 kD), with five highly conserved sequence blocks separated by less conserved blocks (1). The conserved sequences contain alpha helices that are important for dimerization and alpha helices that form an ampiphatic groove. Target proteins of 14-3-3 bind to this ampiphatic groove (Figure 1) of 14-3-3 and can contain one or more 14-3-3 binding motifs. In 14-3-3 target proteins two consensus sites have been identified: RS*X*p**S***X*P (mode 1), or R*XXX*p**S***X*P (mode 2), where *X* stands for any amino acid, and p**S** for phosphorylated Serine (2). However, it has been shown that 14-3-3 proteins bind to certain motifs that are completely unlike these consensus sites (3).
In human, seven known isoforms are present (β σ ε θ ζ γ η), which can form homo- or heterodimers. Dimerization gives 14-3-3 proteins some remarkable features. It allows binding of two different regions located on one protein, which results in a more stable binding, or a conformational change of the target protein. 14-3-3 dimers can also serve as scaffolding proteins, bringing two different proteins together, thereby altering their activity (4). Owing to a very rigid structure, 14-3-3 dimers can also serve to obscure trafficking signal sequences within certain proteins, influencing their cellular localization.

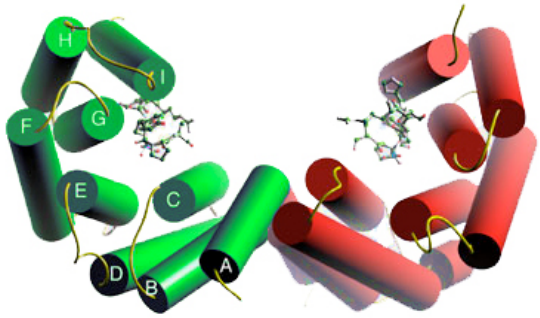


Figure 1 *– 3D-representation of a 14-3-3 dimer. The nine alpha helices are indicated by letters A-I. Two peptides are shown at the assumed docking site in the ampiphatic groove.*

In *Rattus norvegicus*, 14-3-3 dimers have been shown to be involved in stability and correct assembly of the $GABA_BRI/GABA_BRII$-complex (5,6), as well as in posttranslational trafficking of two pore domain acid sensitive K^+-channel (TASK) to the cell membrane (7). Nuclear distribution element-like (NUDEL), which in a complex with lissencephaly gene 1 (*Lis1*) regulates cytoplasmic dynein heavy chain function, is prevented from dephosphorylation by 14-3-3ε in *Mus musculus,* resulting in modification of it's affinity for other proteins in a conserved pathway that regulates nuclear movement through dynein activity (8). Similarly, binding of 14-3-3ζ prevents dephosphorylation of Rim1α, which is required for induction of cerebellar long-term potentiation. Alternatively, 14-3-3ζ induces conformational changes in Rim1α, which alters interactions with release machinery or cytoskeletal proteins (9). A large conductance calcium dependent potassium channel in *Drosophila melanogaster*, dSlo, is involved in regulating neuronal firing patterns and neurotransmitter release. Sensitivity of dSlo to voltage is modulated via Slob by 14-3-3ζ (10). Calcium/Calmodulin-dependent protein kinase kinase alpha (CaMKKα) can activate CaMKI and CaMKIV, involved in mRNA translation, cytoskeletal organization and axonal growth cone motility. This protein can either be directly inhibited by binding of 14-3-3γ to one site, or, by binding of 14-3-3γ to another site, it is prevented from dephosphorylation, which prolongs inhibition (11). Rad and Rem, proteins that can block surface expression of Voltage Dependent Ca^{2+} Channels (VDCCs) by binding it's β-subunit in cytosol or sequestering it into the nucleus, are distributed between cytoplasm and nucleus and the ration between these cellular compartments can be regulated by binding of 14-3-3ζ (12). Together with Serum- and Glucocorticoid-Induced Protein Kinase 1 (SGK1), the theta-isoform of 14-3-3 can form a

ternary complex with Tau, which results in the phosphorylation and translocation of microtubule associated Tau to the nucleus (13). As Tau is important in synaptic vesicle transport along microtubules, 14-3-3θ indirectly modulates synaptic function. Also, 14-3-3 has been reported to play a role in Alzheimer's disease (14), a disorder characterized by the presence of neurofibrillary tangles, which are in fact hyperphosphorylated aggregates containing paired helical filaments, 14-3-3 and Tau.

Presynaptic kinases are part of overlapping signal transduction cascades that are important in influencing the functional plasticity of the synapse. A 14-3-3ζ homolog may be interacting with one of these kinases to regulate the pool of readily releasable synaptic vesicles in the *Drosophila melanogaster* neuromuscular junction (15). A model for the regulated secretory pathway distinguishes between priming and releasing of synaptic vesicles. The priming stage includes docking of the vesicles at the synaptic membrane, a process for which a core complex is needed, consisting of vesicle protein synaptobrevin and plasmamembrane proteins syntaxin and SNAP-25. In this process, 14-3-3ζ acts in the early MgATP-dependent priming stage, thereby stimulating catecholamine release (16).

14-3-3 proteins exert their effect on a wide range of proteins, altering their activity, their localization or their affinity for target proteins. Neuronal firing behavior is the elemental determinant for control of behavior and occurs at synapses at the very end of neuronal axons. Many synaptic properties seem to be under control of 14-3-3 activities, however, 14-3-3 proteins also occupy a great number of roles in global cellular functioning. So far, it has not been studied which possible targets of 14-3-3 proteins reside in cytoplasm or synapses of neurons.

As described above, 14-3-3 proteins can interact with a great number of different proteins, and it is greatly expressed in brain. Still, even more proteins continue to be identified as targets of 14-3-3 proteins. Therefore, many proteins are expected to be found and identified as 14-3-3 interacting proteins in brain extracts.

The amino acid sequence of the 14-3-3ε isoform in *M. musculus* is 100% homologous to the 14-3-3ε isoform of *H. sapiens* (Figure 2). *R. norvegicus* is often used as a model for molecular brain functions of humans. In this study, recombinant *M. musculus* 14-3-3ε protein with an N-terminal histidine(6x) tag was constructed and purified utilizing FPLC to use as bait in a pull-down assay on cortical cytoplasm of *R. norvegicus*. By using a peptide with a high affinity for the binding groove of 14-3-3 proteins, i.e. R18 (amino acid sequence: PHCVPRDLSWLDLEANMCLP), many putative novel target proteins were competed of the recombinant Mm14-3-3ε-His(6x) and collected, for identification by LC-MS analysis. Confirmation of specific14-3-3 binding proteins was achieved by applying an overlay on different elutes using *X. laevis* 14-3-3ε(Bio).

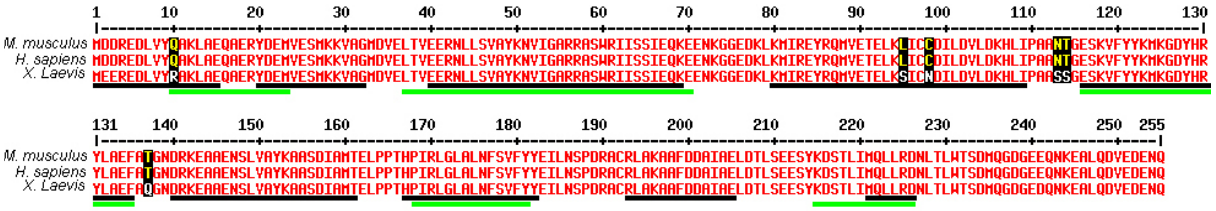


Figure 2 – *Alignment (17) of 14-3-3ε proteins in three different organisms. Black bars indicate alpha-helices, green bars conserved domains. M. musculus and H. sapiens are identical. Compared to X. laevis 14-3-3ε, six mismatches are present. Functional differences caused by these mismatches are expected to be low, since only two mismatches concern amino acids from different polar categories, of which the first one (position 10) lies in a helix that is important for dimerization, and the second one in a non-conserved outer helix (i.e. not part of the ampiphatic groove)(18).*

Experimental Procedures

Construct

To obtain recombinant Mm14-3-3ε-His(6x), Mm14-3-3ε was amplified from pcDNA3.1, a kind gift from dr. Aitken, by PCR, containing 10.0 µl 5xPhusion Buffer, 5.0 µl dNTP solution (2mM of each nucleotide), 1.25 µl forward primer Mm1433εFw (5'-ATC GGA TCC TCA TGG ATG ATC GGG AGG ATC TGG TG-3') (20µM), 1.25 µl of reverse primer (5'-ATC GGA TCC TCA CTG ATT CTC ATC TTC CAC ATC C-3') Mm1433εRv (20µM), 1.0 µl pcDNA3.1 and 0.5 µl Phusion DNA Polymerase (polymerase with proofreading activity). The primers contained BamH1 recognition sites at their flanks. After the PCR, 1 µl was put on 1% agarose gel for verification of a successful reaction. After verification, the DNA was precipitated by adding 1/10 volume of 3M NaAc pH 5.1 and 2.5 volumes of 96% ethanol to the mixture and left on ice for 20 minutes. 1 µl of glycogen was added for visualization of the pellet and the sample was centrifuged for 25 minutes at 20817g. The supernatant was discarded and the resulting pellet was washed with 70% ethanol. After centrifugation for 10 minutes at 20817g, the supernatant was discarded en the pellet was dissolved in 43.5 µl water (MilliQ). This was added to a digestion mix, which contained 1.0 µl of BamH1, 0.5 µl BSA (10 mg/ml) and 5.0 µl BamH1 buffer to reach a volume of 50 µl and incubated o/n at 37°C. At the same time, the pRSET vector was digested and dephosphorylated in a mix containing 1 µl BamH1, 1µl CIP, 0.5 µl BSA (10 mg/ml), 5.0 µl BamH1 (New England Biolabs) buffer, 20.0 µl pRSET and 22.5 µl water (MilliQ).

Separation of digested fragments was achieved by electrophoresis on 1% agarose gel. A DNA marker (O'RangeRuler 100bp +5000 bp, Fermentas) was used for confirmation of the size of the fragment. The fragment was cut out using a scalpel and extracted from the gel by using an Gel Extraction Kit (Qiagen). The extracted DNA (insert) was precipitated as described above and dissolved in 10 µl watrer (MilliQ). This was ligated at 4°C o/n into the pRSET vector. The ligation mix contained 1 µl of the linear pRSET vector, 4 µl of the precipitated insert, 1 µl T4 DNA Ligase buffer, 1 µl of T4 DNA Ligase and 3 µl MilliQ.

The resulted outcome was prepared for sequencing in a BigDye® Sequencing Reaction Setup (Applied Biosystems) v3.1, which contained 1 µl DNA, 4 µl Water (MilliQ), 1 µl primer (FWMm1433ε: 5'-ATC GGA TCC TCA TGG ATG ATC GGG AGG ATC TGG TG-3', or RVMm1433ε: 5'-ATC GGA TCC TCA CTG ATT CTC ATC TTC CAC ATC C-3', or FWT7: 5'-TAA TAC GAC TCA CTA TAG-3', or RVpRSET: 5'-CAG CAG CCA ACT CAG CT-3'), 3µl 2.5x Dilution buffer (200mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$), and 1 µl premix. The result was precipitated as described above to a volume of 20µl. The precipitated DNA was applied on an ABI3100 for sequencing.

Transformation of electro competent *E.coli* strain Top10 with recombinant Mm14-3-3ε-His(6x) construct

Electro competent high-copy *E.coli* cells (40 µl) were mixed with 1 µl of the constructed vector, transferred to a 0.1 mm electroporation cuvette. The electroporation unit was set to 25 µF and 200 Ω and the cells were electroporated at 1.5kV; optimal delay time should be 4.5 –5.0 ms. Immediately after the electroporation the cells were carefully mixed with 1 ml of 37°C SOC medium, which contained 20 mg Bacto Trypton, 5 mg Bacto Yeast Extract, 0.5 mg NaCl and 3.9 mg glucose. After transferring the cells to a 2 ml vial, the cells were incubated at 37°C for 30 minutes. After incubation, 100 µl of the E.coli suspension was plated out on a selective agar plate containing YT-medium and 50 µg/ml ampiciline. The remaining cells were spun down at 4000g for 2 minutes and plated out on the same plates. These plates were incubated o/n at 37°C. 48 colonies were screened for the construct with the insert in the right direction by performing a PCR containing 2.5 µl 10xPCR buffer, 2.5 µl dNTP solution (10 mM), 1.0 µl primer T7promotorFW (20 µM), 1.0 µl primer Mm1433εRv (20 µM), 0.1 µl Taq DNA Polymerase and 17.9 µl MilliQ. Ten positive colonies were cultured in 5 ml YT-medium containing 50 µg/ml ampicillin o/n at 37°C. From these cultures the plasmids were isolated using a Sigma Gen Elute Plasmid kit. Electro competent *E.coli* strain JM109, which is a strain suitable for protein synthesis, was transformed with the isolated construct. Transformation and confirmation by PCR was done in the same manner as with the TOP10 cells.

Expression and isolation of Mm14-3-3ε-His(6x) protein

One positive colony was cultured in 5 ml YT-medium, containing 50 µg/ml ampicillin o/n at 37°C. The overnight culture was then transferred to 500 ml YT-medium containing 50 µg/ml ampicillin and incubated for approximately 6 hours shaking at 155 RPM. The OD_{600nm} was measured, and a value of approximately 0.8, the cells were induced by addition of IPTG to a final concentration of 1mM. The suspension was incubated o/n at 37°C and shaking at 155 RPM, after which the

suspension was centrifuged at 6500g for 12 minutes. The resulting pellet was resuspended in 30 ml startbuffer, which contained 20 mM HEPES, 0.5M NaCl and 10 mM Imidazol at pH 7.5, and protease inhibitor PefaBloc was added to a final concentration of 1 mM. Lysozym was added to a final concentration of 1 mg/ml, and after stirring for 30 minutes, 40 µg DNaseI was added and the suspension was stirred for another 30 minutes.
The French Press was used for the completion of the lysis, and the resulting suspension was centrifuged at 100000 g for 30 minutes. The supernatant (cytosolic fraction) was filtered through a membrane filter with 0.2 µm pores using a 50 ml syringe.
An ÄKTA Explorer FPLC in combination with a Ni^{2+} chelating column (Amersham Biosciences; column volume: 1ml) was used to perform an FPLC in order to purify of Mm14-3-3ε-His(6x). Tubing and column were equilibrated with 30 ml startbuffer at a flowrate of 2 ml/min. The sample was applied into the sample loop and injected into the system at 0.5 ml/min. After injection, unbound sample was washed out with 10 ml startbuffer at 2 ml/min. Elution of bound sample was achieved by a 0 – 100% gradient of elution buffer (startbuffer containing additional 500 mM imidazole) in 30 minutes. The elute was collected in fractions of 1.5 ml. 3 µl of twelve fractions were spotted onto a nitrocellulose membrane and incubated with 20 ml PBS-T containing 1% BSA for 10 min. 40 µl of avidin-HRP was added and incubated for 30 minutes. The blot was washed 3 times 10 min with PBS -T and a final wash for ten minutes using TBS. The blot was developed by applying a mix of 20 ml TBS, 5 ml MeOH, 125 µl 2-chloro-4-naphtol (100 mg/ml) and 87,5 µl H_2O_2. This was incubated at room temperature in the dark for 20 min. Appropriate fractions were applied to a Western blot, using rabbit anti14-3-3β (Santa Cruz) (diluted 1:2000) as primary antibody and goat anti rabbit-HRP (diluted 1:2000) as secondary antibody, to visualize recombinant Mm14-3-3ε-His(6x). Fractions showing Mm14-3-3ε-His(6x) of the right size were pooled and desalted using a Millipore Centrifuge Filter membranes with NMWL of 5000 D, and centrifuging at 3220G. After spinning down to a volume of approximately 1 ml, 10 ml of 20 mM HEPES pH 7.5 was added to dilute remaining imidazole, and the sample was spun down to a volume of 1- 3ml.

Preparation of cortical fractions
Cortices of five rats were homogenized in 10 ml of 5 mM HEPES/NaOH, pH 7.4, by twelve strokes with a Teflon Dounce Homogenizer at 900 RPM on ice. Purification from cell debris was achieved by centrifugation of the sample at 1000 g for 10 min. at 4˚ C. Supernatant was centrifuged at 20000 g for 30 min. at 4˚ C. To the supernatant, containing cytoplasm and microsomal fraction, 20 ml of 5 mM HEPES/ NaOH, pH 7.4 was added and centrifuged at 100000 g for 60 min. at 4˚ C. The pellet, containing the microsomal fraction was resuspended in 2.1 ml 5 mM HEPES/NaOH pH 7.4.

Pulldown Assay
MgATP was added to a total cytoplasmic volume of 30 ml to give to a final concentration of 500 µM. The cytoplasm was precipitated on ice by addition of portions of 2.6 g $(NH_4)_2SO_4$ $^-$ until a final amount of 13.0 g was added followed by additional 20 minutes stirring on ice. The mixture was centrifuged at 12000 g for 20 min at 4°C. The pellet was resuspended in 10 ml buffer C, which contains 50 mM HEPES-KOH pH 7.5, 1 µM Cantharidin, 1 mM DTT and 1 tablet Roche Protease Inhibitor per 500 ml. After incubation for 10 min at 4°C, the sample was dialysed for one hour against 1000 ml 50 mM HEPES-KOH pH=7.5 and 1 µM Cantharidin at 4°C. Every hour, the dialysis buffer was refreshed. The sample was clarified by centrifugation at 33000 g for 20 min at 4°C, and to 15 ml supernatant 2 mM MgATP, 0.5 mM PefaBloc and 4 µg/ml Leupeptin was added. The mixture was incubated at 30°C for 10 minutes and subsequently cooled on ice. 150 mM NaCl and 6.0 mg recombinant Mm14-3-3ε-His(6x) was added to the mixture, which was mixed end-over-end for 60 min at 4°C. The mixture was run over a 1ml Ni^{2+} chelating column using an ÄKTA Explorer coupled to the column with buffer C with additional 50mM imidazole and 500mM NaCl as running buffer at a flow rate of 1 ml/min. The column was washed with running buffer until protein concentration was below 5 µg/ml. A mock elution consisted of injecting 1 ml running buffer into the system, of which elute was collected. An additional mock elution was carried out by the injection of 1 ml 500µM of a peptide that does not contain a consensus site for 14-3-3 (sequence: TFWTVPDET). Mock elutions were followed by elution of Mm14-3-3ε-His(6x) binding proteins. This was carried out by injecting 1 ml 500 µM R18 peptide (sequence: PHCVPRDLSWLDLEANMCLP) dissolved in running buffer into the system and collecting the elute. Following R18-elution, recombinant Mm14-3-3ε-His(6x) was eluted with 0.5 M imidazole in running buffer. The elutes were concentrated by applying Millipore Centrifuge Filter membranes with NMWL of 5000 D for the first mock and imidazole elution, and wit NMWL of 10000 D for the mock elution with a specific peptide and R18 elution, to a final volume of 200 µl.

Polyacrylamide gel separation of eluted fractions.
Of each concentrated elution, 16 µl was loaded onto a 12% polyacrylamide gel. Proteins were fixed by rinsing the gel two times in 50% ethanol with 3% H_3PO_4 for 25 minutes. Subsequently, the gel was coomassie stained at room temperature o/n in a solution containing 34% methanol, 3% phosphoric acid, 150 g/l ammoniumsulphate and 1 g/l coomassie brilliant blue (G250). Destaining was done with reagent grade H_2O until background was reduced to slightly blue. The gel was scanned on a Bio-Rad Densitometer (GS- 800), using Pro-One Software.

Overlay
Of each elution appropriate volumes were evaporated so that 8-10 µg protein remained, which was resuspended in 20 µl of SDS loading buffer and loaded onto a 12% polyacrylamide gel. To serve as a negative control, recombinant fusion protein GST-R18 was also loaded. The proteins were blotted onto a PVDF membrane for 3 hours at 15 V, followed by overnight incubation with 5% Marvel Skimmed Milk in buffer A, which contained 25 mM Tris-HCl, pH 7.5 and 0.5 M NaCl. Subsequently, the buffer was replaced by buffer A with BSA (1mg/ml). Recombinant Xl14-3-3εBio was added in a 40 times dilution, except for the negative control, and the blot was incubated for 1 hour, followed by 6 times 10 minutes washing with buffer A. Avidine-HRP was added (1:5000 diluted) and the blot was incubated for 1 hour. After washing 3 times ten minutes with buffer A and 1 time ten minutes with TBS, the blot was developed using the ECL kit (Amersham Biosciences).

In gel and in volume digestion of eluted proteins.
Twenty pieces were cut from the gel and washed separately with 400 µl 50 mM NH_4HCO_3. The pieces were completely destained by vigorous shaking in 400 µl 50% acetonitrile in 50 mM NH_4HCO_3 several times. All gel pieces were dried in a SpeedVac. Trypsine solution (sequence grade, Promega) was added to the gel pieces as much as was absorbed by the gel pieces, after which the saturated gel pieces were covered in 50 mM NH_4HCO_3 and incubated o/n at 37º C.
Also, an in volume trypsine digestion was carried out by adding 20 µl Trypsin solution to 40 µl R18-elute, followed by o/n incubation at 37º C. After incubation, digestion mixes were put in a Speedvac evaporator (Eppendorf). Remaining peptides were stored at -20º C. LC-MS analysis was carried out by the proteomics department of the Center for Neurogenomics and Cognitive Research located at the VU, Amsterdam.

Results

Successful subcloning of Mm14-3-3ε from pcDNA3.1 into pRSET vector

Analysis of the PCR on the pcDNA3.1 construct was done by electrophoresis of 1 µl PCR-product on 1% agarose gel. A clear band of the right size was observed (Figure 3), confirming a successful reaction. After digestion of this PCR product, separation of DNA fragments was done on 1% agarose gel. Following ligation of the insert and the linear plasmid and subsequent transformation, a PCR was performed on 48 colonies, revealing some positive colonies (Figure 4). The colony corresponding to the indicated PCR-product was cultured in 5 ml YT-medium and subsequently the plasmid was isolated. This plasmid was then applied for transformation of E.coli strain JM109 as described earlier. A PCR on 16 of these JM109 colonies showed 14 positive colonies. One colony was chosen for protein expression.

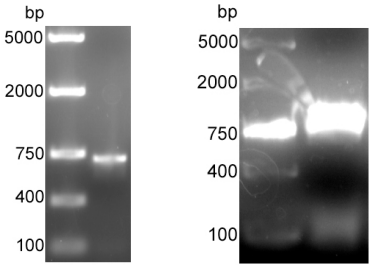
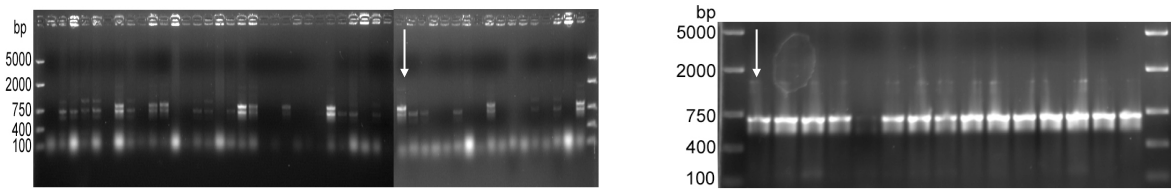


Figure 3 – *A: 1% agarose gel showing a clear band after electrophoresis of 1 µl product of the PCR on pcDNA3.1; B: 1% agarase gel from which the digested PCR-product was extracted.*



A *B*

Figure 4 – A: *1% agarose gel used to screen for positive colonies. The arrow indicates the colony that was used for transformation of E. coli strain JM109; B: 1% agarose gel used to screen for positive colonies. The arrow indicates the colony that was used for expression of recombinant Mm14-3-3εHis(6x)*

Recombinant Mm14-3-3ε-His(6x) expression and purification

Figure 5 shows the output of the Äkta Explorer FPLC for the purification of Mm14-3-3εHis(6x) using a HisTrap HP column. Confirmation of Mm14-3-3εHis(6x) containing fractions by western hybridization can be observed in Figure 6. Positive fractions were pooled and concentrated to a volume of 1-3 ml using the Millipore Centrifuge Filter membranes. Protein isolation yielded approximately 10 mg protein per 1000 ml cell culture.

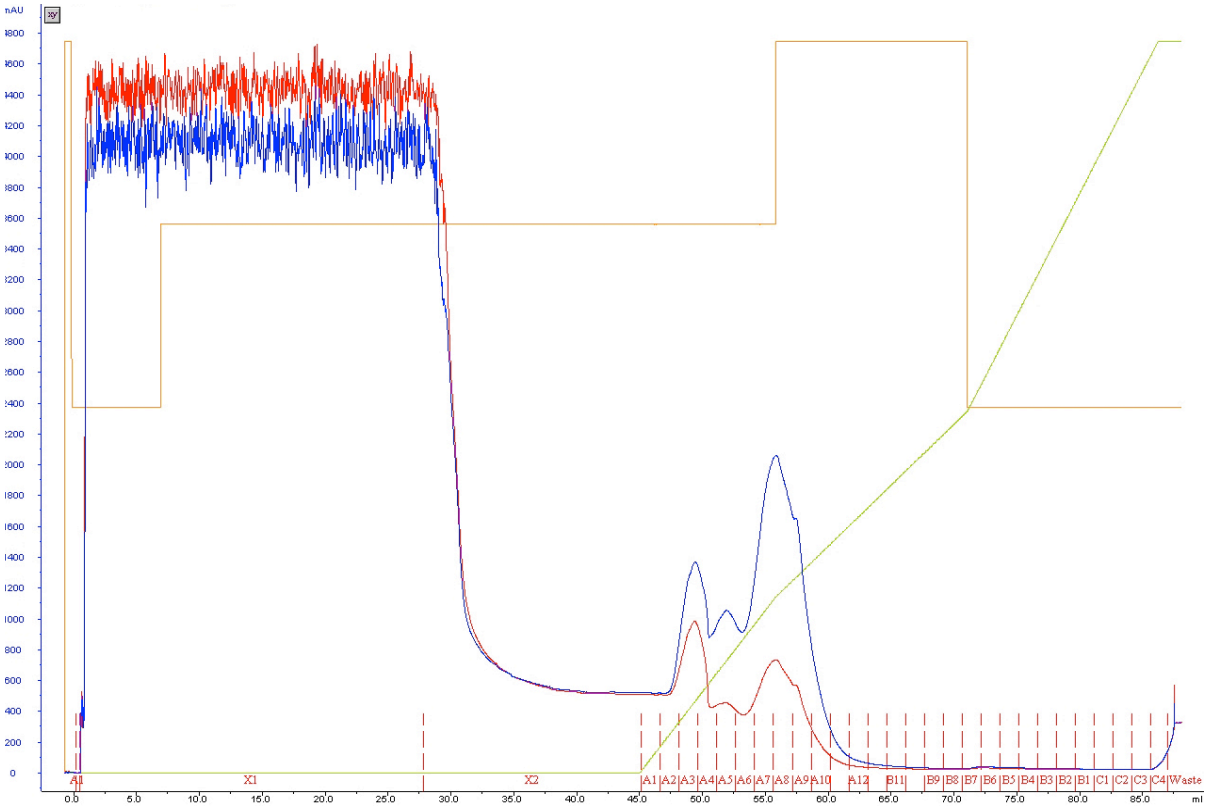


Figure 5 - *Output of the Äcta Explorer FPLC for purification of Mm14-3-3εHIS. Blue line: 280nm; Red Line: 254 nm; orange line: sample flow; green line: imidazole gradient (0 – 500 mM). A: Sample Load; B: a-specific binding proteins; C: Mm14-3-3εHIS tagged. Numbers below indicate collected fractions.*

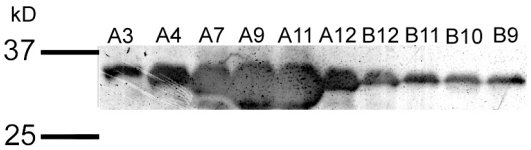


Figure 6 - *Western blot of indicated fractions from the purification of Mm14-3-3ε-His(6x). Ab: T7-HRP(dilution: 1:5000), developed with ECL development kit (Amersham Biosciences).*

Pull down assay with Mm14-3-3ε-His(6x) protein yields many putative targets

The Äkta Explorer output for the pull-down assay can be observed in Figure 7. In Figure *8*, 12% polyacrylamide gels that were coomassie blue stained are shown. Many proteins were eluted with the R18 peptide (*R18*), whereas only one double band is visible in the first mock elutions (*Mock and Mock1)*. As the molecular mass of this double band is about 33 kD, it is very likely the recombinant Mm14-3-3ε-HIS(6x), possibly in both a phosphorylated and non-phosphorylated state. This leakage can often be seen using certain amounts of His(6x)-tagged proteins on this column. The second mock elution (*Mock 2*) that was applied by injecting an a specific peptide can be seen at *C*. Only a small number of proteins were eluted that were not eluted without any peptide (*Mock1*). These could be proteins that were a specifically bound to Mm14-3-3εHIS(6x). However, there is also the possibility that due to a disruption of the Mm14-3-3εHIS(6x) binding to the column, an additional amount of Mm14-3-3εHIS(6x) leaked of the column. Only the band that was estimated to be 76 kD cannot be readily identified in the R18 elution. Other bands are visible in the R18, and since the thick band of approximately 33 kD indicates the additional amount of recombinant Mm14-3-3εHIS(6x), these bands could still be genuine targets. Confirmation of presence of Mm14-3-3εHIS(6x) was achieved by eluting the column with imidazole (*Imidazole*). In addition to the recombinant Mm14-3-3εHIS(6x), proteins that were not eluted by the R18 peptide can be seen in this lane.

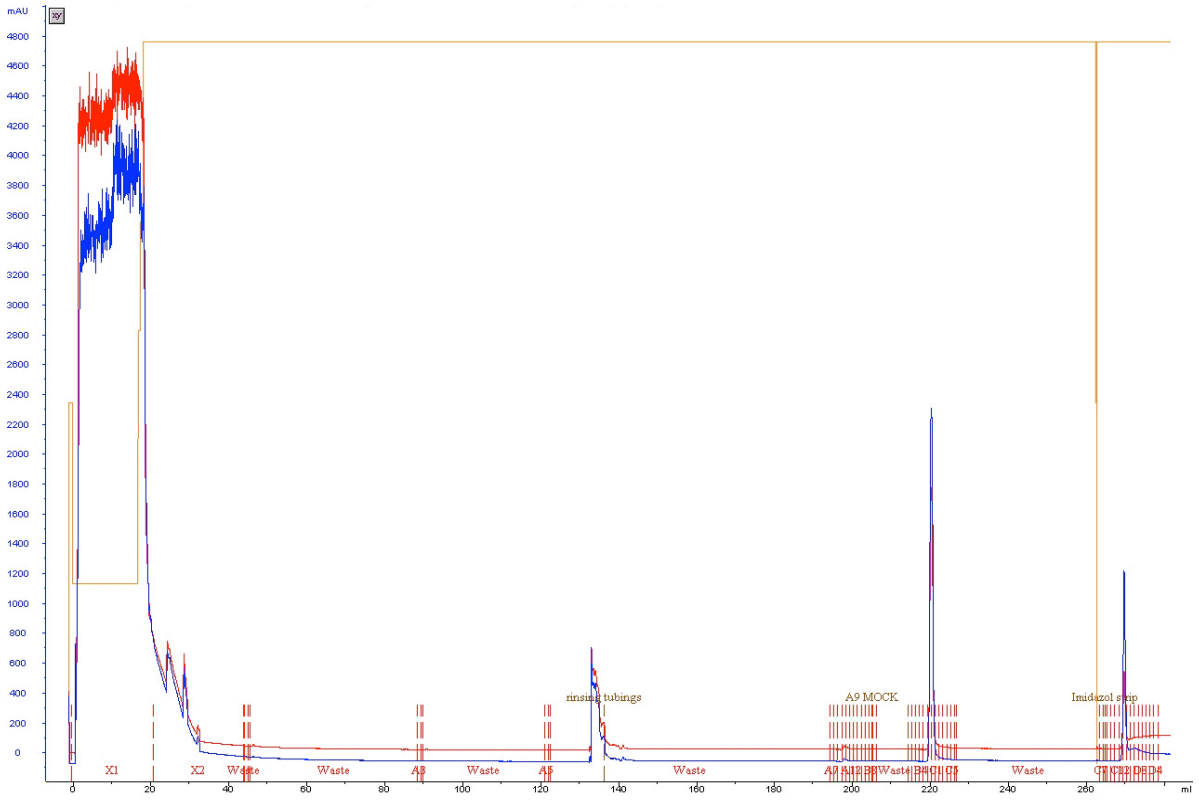


Figure 7 - *Output of the Äcta Explorer FPLC for a pull-down assay. Blue line: 280nm; Red Line: 254 nm; orange line: sample flow. A: Sample Load; B: mock elution; C: Elution with R18 peptide; D: Elution with imidazole*

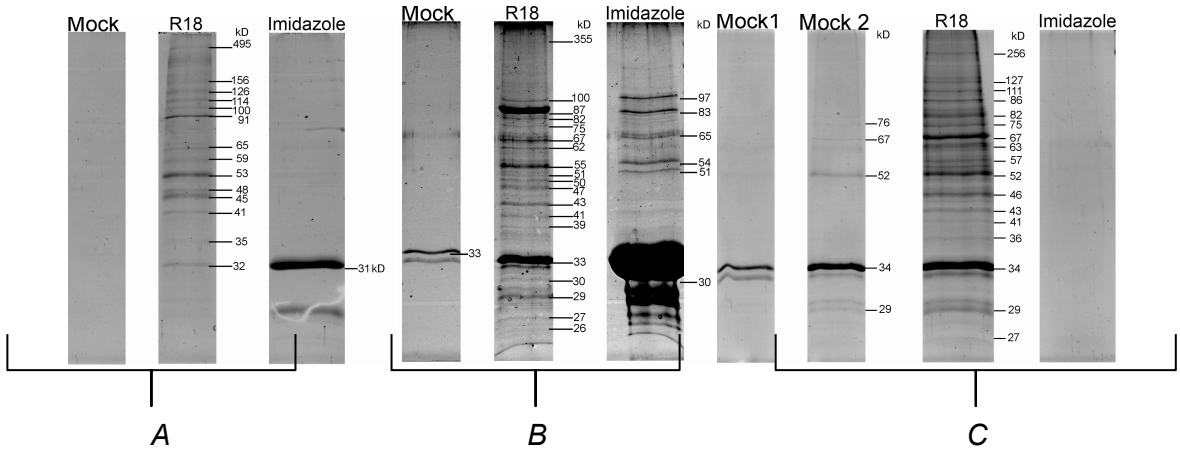


Figure 8 – *From three pull-down experiments (A, B and C) the indicated elutions were loaded onto a 12% polyacrylamide gel and Coomassie stained. In experiment C an additional mock was carried out using a peptide with an a-specific sequence.*

Confirmation of Mm14-3-3εHIS(6x) binding proteins can be achieved by applying an overlay

In Figure 9, an overlay applied on the R18 elution of the first polyacrylamide gel is shown. From this picture it becomes clear that specific targets of Xl14-3-3εBio were eluted. As described in the introduction, functional binding differences of Xl14-3-3ε and Mm14-3-3ε are expected to be low, since mismatches with only minor variability are present between these two proteins. An explanation for the low number of positive targets that are visible in the overlay compared to the coomassie blue stained protein gel in Figure 8, is that the amount of protein loaded onto the gel was not sufficient enough to properly visualize more proteins, as can be inferred from the high background to signal ratio.

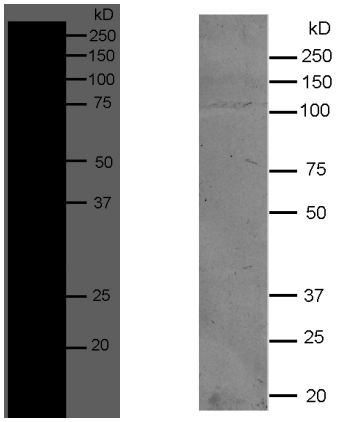


Figure 9 - *An overlay was carried out on a concentrated sample of the first R18 elution and subsequent hybridization with Xl14-3-3εBio and avidine-HRP followed. A negative control (D) was obtained by loading GST- R18 and omitting hybridization with Xl14-3-3Bio. Development was done with the ECL kit. Estimated molecular masses (kD) are shown at the right of each lane.*

LC-MS-MS analysis

LC-MS-MS analysis on the in volume digestion of the first R18 elution (Figure 8 *A*) yielded over 200 proteins. From these proteins, only the ones that were identified on the basis of 2 or more peptides can be seen in Table 1(next page). Analysis of the in gel digested proteins is expected to reveal a greater number of proteins with a high confidence interval, since the gel was cut in small pieces, thereby increasing the concentration of separate proteins, and hence, detectability. At the time of writing, these date were underway.

Table 1 –*List of proteins that were identified based on 2 or more peptides. Putative 14-3-3 binding sites were retrieved using the Minimotif Miner tool (19)*

Protein Name	Accession Nr	Protein MW	Putative 14-3-3 binding site(s)
(P04691) Tubulin beta chain (T beta-15)	TBB1_RAT	50387,1	1
(P69897) Tubulin beta-5 chain	TBB5_RAT	50095,1	1
(P47942) Dihydropyrimidinase related protein-2 (DRP-2) (Turned on after division, 64 kDa protein) (	DPYL2_RAT	62637,7	2
(P68370) Tubulin alpha-1 chain (Alpha-tubulin 1)	TBA1_RAT	50787,9	1
(P15146) Microtubule-associated protein 2 (MAP 2) (MAP-2)	MAP2_RAT	202743,7	-
(Q9WTT6) Guanine deaminase (EC 3.5.4.3) (Guanase) (Guanine aminase) (Guanine aminohydrolase) (GAH	GUAD_RAT	51554,0	-
(P09606) Glutamine synthetase (EC 6.3.1.2) (Glutamate--ammonia ligase) (GS)	GLNA_RAT	42981,6	2
(P62260) 14-3-3 protein epsilon (14-3-3E) (Mitochondrial import stimulation factor L subunit) (MSF	1433E_RAT	29326,5	*
(Q62950) Dihydropyrimidinase related protein-1 (DRP-1) (Collapsin response mediator protein 1) (CRM	DPYL1_RAT	62498,7	2
(Q8VHK2) Caskin-1 (CASK interacting protein 1)	CSKI1_RAT	150882,1	6
(Q9Z272) ARF GTPase-activating protein GIT1 (G protein-coupled receptor kinase-interactor 1) (GRK-i	GIT1_RAT	85919,5	-
(Q63537) Synapsin-2 (Synapsin II)	SYN2_RAT	63701,9	3
(P01946) Hemoglobin alpha-1 and alpha-2 chains	HBA_RAT	15358,8	-
(P11517) Hemoglobin beta chain, minor-form	HBB2_RAT	15955,3	-
(P63259) Actin, cytoplasmic 2 (Gamma-actin)	ACTG_RAT	42107,9	0
(P68035) Actin, alpha cardiac (Alpha-cardiac actin)	ACTC_RAT	42334,0	-
(Q62951) Dihydropyrimidinase related protein-4 (DRP-4) (Collapsin response mediator protein 3) (CRM	DPYL4_RAT	61617,3	-
(P97571) Calpain 1, large [catalytic] subunit (EC 3.4.22.52) (Calcium-activated neutral proteinase)	CAN1_RAT	82751,5	5
(O55081) Retinoblastoma-like protein 2 (130 kDa retinoblastoma-associated protein) (PRB2) (P130) (R	RBL2_RAT	129219,6	7
(P42264) Glutamate receptor, ionotropic kainate 3 precursor (Glutamate receptor 7) (GluR-7) (GluR7)	GRIK3_RAT	104859,2	2
(Q9JIS1) Regulating synaptic membrane exocytosis protein 2 (Rab3-interacting molecule 2) (RIM 2)	RIMS2_RAT	176716,1	7
(P81128) Glucocorticoid receptor DNA binding factor 1 (GAP-associated protein p190)	GRLF1_RAT	173981,1	6
(P02688) Myelin basic protein S (MBP S)	MBP_RAT	21414,9	1
(Q04940) Neurogranin (Ng) (Protein kinase C substrate 7.5 kDa protein) (RC3)	NEUG_RAT	7719,6	-
(P63018) Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	HSP7C_RAT	71055,3	2
(P19491) Glutamate receptor 2 precursor (GluR-2) (GluR-B) (GluR-K2) (Glutamate receptor ionotropic,	GRIA2_RAT	99252,4	1
(P68511) 14-3-3 protein eta	1433F_RAT	28234,1	*
(O35832) Serine/threonine-protein kinase PCTAIRE-3 (EC 2.7.1.37) (PCTAIRE-motif protein kinase 3)	PCTK3_RAT	52077,1	3
(P47709) Rabphilin-3A (Exophilin 1)	RP3A_RAT	76640,0	2
(P62632) Elongation factor 1-alpha 2 (EF-1-alpha-2) (Elongation factor 1 A-2) (eEF1A-2) (Statin S1)	EF1A2_RAT	50764,4	1
(Q63716) Peroxiredoxin 1 (EC 1.11.1.-) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide r	PRDX1_RAT	22323,4	-
(P70712) Kynureninase (EC 3.7.1.3) (L-kynurenine hydrolase)	KYNU_RAT	52892,4	1
(P70615) Lamin B1	LAM1_RAT	66662,7	2
(Q63180) ADAM 7 precursor (A disintegrin and metalloproteinase domain 7) (Epididymal apical protein	ADAM7_RAT	91812,4	3
(O55043) Rho guanine nucleotide exchange factor 7 (PAK-interacting exchange factor beta) (Beta-Pix)	ARHG7_RAT	73607,3	3
(Q9WV87) NADPH oxidase homolog 1 (NOX-1) (NOH-1) (NADH/NADPH mitogenic oxidase subunit P65-MO	NOX1_RAT	65932,4	2
(P70618) Mitogen-activated protein kinase 14 (EC 2.7.1.37) (Mitogen-activated protein kinase p38alp	MK14_RAT	41392,0	1
(O35276) Neuropilin-2 precursor (Vascular endothelial cell growth factor 165 receptor 2)	NRP2_RAT	105199,1	5
(P33272) Cytochrome P450 2B12 (EC 1.14.14.1) (CYPIIB12)	CP2BC_RAT	56046,2	1
(P97523) Hepatocyte growth factor receptor precursor (EC 2.7.1.112) (Met proto-oncogene tyrosine ki	MET_RAT	156180,6	7
(P97837) Disks large-associated protein 2 (DAP-2) (SAP90/PSD-95-associated protein 2) (SAPAP2) (PSD	DLP2_RAT	123581,7	10
(Q63679) Testis specific protein A (Zinc finger protein TSGA)	TSGA_RAT	137542,4	7
(O08839) Myc box dependent interacting protein 1 (Bridging integrator 1) (Amphiphysin-like protein)	BIN1_RAT	64721,4	3
(Q64535) Copper-transporting ATPase 2 (EC 3.6.3.4) (Copper pump 2) (Wilson disease-associated prote	ATP7B_RAT	157430,5	3
(Q63603) Dedicator of cytokinesis protein 9 (Cdc42 guanine nucleotide exchange factor zizimin 1) (T	DOCK9_RAT	83542,0	-
(P23565) Alpha-internexin (Alpha-Inx)	AINX_RAT	56252,7	3
(O88778) Bassoon protein	BSN_RAT	420146,6	22
(P06536) Glucocorticoid receptor (GR)	GCR_RAT	88640,9	6
(Q64565) Alanine--glyoxylate aminotransferase 2, mitochondrial precursor (EC 2.6.1.44) ((R)-3-amino	AGT2_RAT	57869,1	3
(O88806) Protein-arginine deiminase type I (EC 3.5.3.15) (Peptidylarginine deiminase I) (PAD-R11)	PADI1_RAT	74552,0	2
(Q9WVC7) A-kinase anchor protein 6 (Protein kinase A anchoring protein 6) (PRKA6) (mAKAP)	AKAP6_RAT	256759,3	16
(P41562) Isocitrate dehydrogenase [NADP] cytoplasmic (EC 1.1.1.42) (Oxalosuccinate decarboxylase) (	IDHC_RAT	47046,7	-
(P29995) Inositol 1,4,5-trisphosphate receptor type 2 (Type 2 inositol 1,4,5-trisphosphate receptor	ITPR2_RAT	310055,3	3
(P55161) Nck-associated protein 1 (NAP 1) (p125Nap1) (Membrane-associated protein HEM-2)	NCKP1_RAT	130092,9	4
(Q63207) Coagulation factor X precursor (EC 3.4.21.6) (Stuart factor)	FA10_RAT	55598,6	4
(P31421) Metabotropic glutamate receptor 2 precursor (mGluR2)	MGR2_RAT	97480,0	4
(P48721) Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Pe	GRP75_RAT	74082,9	-

Discussion

The 14-3-3 proteins are involved in a great number of molecular functionalities of cells. Since 14-3-3 proteins are abundantly expressed in brain tissue, it is remarkable that no attempt has been made so far to find and identify novel targets. In the present study, a method was applied that is able to pull down many different cytosolic proteins from cortical cytosol in *R. norvegicus* that interact directly with 14-3-3ε or are pulled down via complexes that have an interaction with 14-3-3ε.

Here, a great number of proteins were identified as putative targets for the *M. musculus* 14-3-3 epsilon isoform in cortical cytoplasm of *R. norvegicus*. This was achieved by specifically eluting the targets using the R18 peptide that has high affinity for the 14-3-3 binding groove.

A number of proteins active or involved in fundamental neuronal functioning are among the identified ones. The 62 kD dihydropyrimidinase related protein (DRP-2), which has been reported to have high homology with *M. musculus* UNC-33, has been associated with a repair mechanism of neurons and axonal growth (20). As this protein is abundantly expressed in brain during development, it is thought to play an important role in correct development of the neural system (21). The present pull down assay was performed on cortices of adult rat, which could imply that certain proteins that interact with DRP-2 during development were not found as a result of altered expression levels. Therefore, a functional meaning for the role of 14-3-3 acting on DRP-2 during development is difficult to infer from these results. However, in the adult brain, DRP-2 still plays a role in regulation of glutamatergic transmission. DRP-2 is cleaved by calpain-1 upon stimulation of NMDA-type glutamate receptors. It was found that the calpain cleaved product of DRP-2 abolishes surface expression of the NMDA receptor subunit 2A, which determines the specificity of the receptor function (22). As a result, neurons become resistant to the glutamatergic agonist NMDA. The present study has identified both calpain and DRP-2 as 14-3-3 targets, and the Minimotif Minter Tool reveals five and two putative 14-3-3 binding motifs respectively. As 14-3-3 often acts as a scaffolding protein, its function in this process seems straightforward: bringing together a protease and a substrate. Unfortunately, no experimental data on this hypothesis exists to date. Another important finding is that in brains from patients suffering from Alzheimer's disease, DRP-2 is present in neurofibrillary tangles, at the cost of the cytosolic pool, causing a deterioration of neural regeneration. Paired helical filaments, of which neurofibrillary tangles are composed, contain hyperphosphorylated tau, which may destabilize microtubules. It was shown that DRP-2 does not directly interact with tau (23), a protein that is also present in neurofibrillary tangles. As described earlier, certain 14-3-3 isoforms are able to bind tau. Since 14-3-3 proteins are also present in neurofibrillary tangles, it could be speculated that 14-3-3 acts as an adapter between these two proteins. However, following the indication that DRP-2 can also bind directly to microtubules (24) it could be argued that 14-3-3 and DRP-2 colocalize in neurofibrillary tangles in a complex, without a direct binding.

In addition, a protein that is assumed to play an important role in synaptic vesicle release was picked up by the pull down assay: rabphilin-3a, a 76.6 kD protein that contains two putative non-consensus 14-3-3 binding sites that were found using the Minimotif Minrer Tool (19). Experimental data (25) has shown that 14-3-3 is able to bind to a rabphilin residue that includes $RANS_{274}VQ$ motif, in which the Serine is phosphorylated. The phosphorylated state of this Serine, which is crucial for binding of 14-3-3, depends on high K^+-induced membrane depolarization. Other work has indicated that rabphilin is a regulator of neurotransmitter release (26). It has been proposed to act in a complex with rab3 as a fine-tuning mechanism important for transition of primed vesicles to Ca^{2+}-responsive vesicles. This fine-tuning is implicated in short-term synaptic plasticity, a process that mediates the ease with which a pre-synaptic site releases neurotransmitter and hence the ability of a pre-synaptic action potential to evoke a post-synaptic action potential. In this process, 14-3-3 could be involved in determining the destination of rabphilin, as rabphilin was shown to have a lower affinity for vesicles when phosphorylated (27). Interaction with rab3 was not affected by *in vitro* phosphorylation of rabphilin. Rabphilin could be a vesicle controller by allowing only those vesicles to reach the plasma membrane that are mature. This could be achieved by binding of rabphilin to messenger proteins that are specific for a mature state via 14-3-3. The phosphorylation dependent nature of this partnership between rabphilin and the 14-3-3 causes it to be a depolarization dependent one; one that could support an important role in synaptic plasticity. The 14-3-3 proteins have been encountered at various studies that were conducted to elucidate the workings of synapses. Here, a strong indication is presented that 14-3-3 is indeed involved in synapse-specific pathways.

The 150.9 kD protein caskin-1 is a vertebrate specific CASK interacting protein. CAKI, a *drosophila* homolog of CASK, was shown to

function as a regulator for vesicle release. In CAKI knockouts, miniature end-plate potentials recordings showed an increase in spontaneous vesicle release, while vesicles itself contained equal amounts of neurotransmitter, as deduced from evoked post-synaptic currents (28). Caskin-1 competes with another protein, called Mint-1, for binding to CASK. In experiments using Mint-1 knock-out mice, it was found that deletion of Mint-1 increased the release probability within GABAergic synapses, implicating a down regulating role on CASK, since knocking out CASK also showed an increased release probability. In this light, binding of caskin-1 to CASK in itself could be of little importance. Rather, caskin-1 could be acting as a transient blocking mechanism for one or more CASK-binding proteins. Currently, little is known about the functional meaning of caskin-1 binding to CASK. Analysis of caskin-1 with the Minimotif Miner tool (19) revealed 6 putative 14-3-3 binding sites of which two consensus sites. As caskin-1 seems to be non-essential for general neuronal function, caskin-1 may be regarded as part of a process that shapes and/or enhances synaptic transmission, raising the question whether 14-3-3 interaction might also only serve as aid in fine-tuning of synaptic transmission.

Synaptic vesicles are described to be divided into three functional different pools: the release-ready pool, which comprises synaptic vesicles that are docked to the presynaptic plasma membrane , the proximal pool, which is situated near the active zone via a cytomatrix of filaments, and the reserve pool, which lies further away from the active zone and are kept in position by microfilaments (29). It is also described that vesicles in the reserve pool are tethered to the microfilaments by short fine filaments, which are composed of synapsin. This binding is regulated by phosphorylation. The cytomatrix, in which the proximal pool of synaptic vesicles is located contains two large proteins that are anchored very tightly to the cytoskeleton: Piccolo and Bassoon. This anchorage makes is quite difficult to study the functions of these protein. Yet, is has been hypothesized that Bassoon is able to undergo some interaction with synaptic vesicles (30). Synapsin and Bassoon were both identified as 14-3-3ε interacting proteins. Influence of 14-3-3 seems not be restricted to mediating pathways that are directly the release-ready synaptic vesicle pool, but extends to the pathways that are governing the proximal synaptic vesicle pool and reserve pool.

This study has indicated that 14-3-3 fulfills an important role in synaptic functioning by interacting with many synapse-specific proteins. Still, it is important to keep in mind that in this study homogenized cortical neurons were used, which could implicate that some false positive targets were identified. To investigate whether these targets interact *in vivo*, further research is needed. Also, it would be interesting to investigate functional isoform specificity in cortical cytoplasm, and assess the level of redundancy between the different isoforms. For a great number of proteins that have some function in the synapse many questions remain unanswered. Without doubt, it could be argued that elucidating the functional role of 14-3-3 for these proteins could answer at least some of these questions.

From the identified putative 14-3-3 target proteins, only a number of proteins and their proposed functions are described here. Extensively describing all proteins that were identified would go beyond the intention of this study. It becomes clear from the enormous number of proteins that are somehow functionally connected to 14-3-3 proteins, classical studies on protein-protein interactions will not be sufficient to elucidate the many putative functions of 14-3-3 proteins described here. An important emerging approach is to use computer simulated networks of known proteins and their binding partners to model the molecular machinery within (parts of) the cell as a whole (31).

In conclusion, Mm14-3-3ε interacts with a great number of proteins. Some of these act as central units in a network from which all human behavior emerges, others activate, modify or control these central proteins. So, by acting not only on the central units, but in a great number of different pathways, Mm14-3-3ε determines to a great extent the behavior of humans.

References

1. Berg D, Holzmann C, Riess O. 14-3-3 proteins in the nervous system. Nat Rev Neurosci 2003;4(9):752-62.
2. Tzivion G, Avruch J. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 2002;277(5):3061-4.
3. van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. Bioessays 2001;23(10):936-46.
4. Yaffe MB. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett 2002;513(1):53-7.
5. Couve A, Kittler JT, Uren JM, Calver AR, Pangalos MN, Walsh FS, Moss SJ. Association of GABA(B) receptors and members of the 14-3-3 family of signaling proteins. Mol Cell Neurosci 2001;17(2):317-28.
6. Brock C, Boudier L, Maurel D, Blahos J, Pin JP. Assembly-dependent surface targeting of the heterodimeric GABAB Receptor is controlled by COPI but not 14-3-3. Mol Biol Cell 2005;16(12):5572-8.
7. Rajan S, Preisig-Muller R, Wischmeyer E, Nehring R, Hanley PJ, Renigunta V, Musset B, Schlichthorl G, Derst C, Karschin A and others. Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. J Physiol 2002;545(Pt 1):13-26.
8. Toyo-oka K, Shionoya A, Gambello MJ, Cardoso C, Leventer R, Ward HL, Ayala R, Tsai LH, Dobyns W, Ledbetter D and others. 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller-Dieker syndrome. Nat Genet 2003;34(3):274-85.
9. Simsek-Duran F, Linden DJ, Lonart G. Adapter protein 14-3-3 is required for a presynaptic form of LTP in the cerebellum. Nat Neurosci 2004;7(12):1296-8.
10. Zhou Y, Schopperle WM, Murrey H, Jaramillo A, Dagan D, Griffith LC, Levitan IB. A dynamically regulated 14-3-3, Slob, and Slowpoke potassium channel complex in Drosophila presynaptic nerve terminals. Neuron 1999;22(4): 809-18.
11. Davare MA, Saneyoshi T, Guire ES, Nygaard SC, Soderling TR. Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3. J Biol Chem 2004;279(50):52191-9.
12. Beguin P, Mahalakshmi RN, Nagashima K, Cher DH, Ikeda H, Yamada Y, Seino Y, Hunziker W. Nuclear sequestration of beta-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. J Mol Biol 2006;355(1):34-46.
13. Chun J, Kwon T, Lee EJ, Kim CH, Han YS, Hong SK, Hyun S, Kang SS. 14-3-3 Protein mediates phosphorylation of microtubule-associated protein tau by serum- and glucocorticoid-induced protein kinase 1. Mol Cells 2004;18(3): 360-8.
14. Umahara T, Uchihara T, Tsuchiya K, Nakamura A, Iwamoto T, Ikeda K, Takasaki M. 14-3-3 proteins and zeta isoform containing neurofibrillary tangles in patients with Alzheimer's disease. Acta Neuropathol (Berl) 2004;108(4):279-86.
15. Broadie K, Rushton E, Skoulakis EM, Davis RL. Leonardo, a Drosophila 14-3-3 protein involved in learning, regulates presynaptic function. Neuron 1997;19(2):391-402.
16. Chamberlain LH, Roth D, Morgan A, Burgoyne RD. Distinct effects of alpha-SNAP, 14-3-3 proteins, and calmodulin on priming and triggering of regulated exocytosis. J Cell Biol 1995;130(5):1063-70.
17. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 1988;16(22):10881-90.
18. Wang W, Shakes DC. Molecular evolution of the 14-3-3 protein family. J Mol Evol 1996;43(4):384-98.
19. Balla S, Thapar V, Verma S, Luong T, Faghri T, Huang CH, Rajasekaran S, del Campo JJ, Shinn JH, Mohler WA and others. Minimotif Miner: a tool for investigating protein function. Nat Methods 2006;3(3):175-7.
20. Fountoulakis M, Hardmaier R, Schuller E, Lubec G. Differences in protein level between neonatal and adult brain. Electrophoresis 2000;21(3):673-8.
21. Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. J Neurochem 2002;82(6):1524-32.
22. Bretin S, Rogemond V, Marin P, Maus M, Torrens Y, Honnorat J, Glowinski J, Premont J, Gauchy C. Calpain product of WT-CRMP2 reduces the amount of surface NR2B NMDA receptor subunit. J Neurochem 2006.
23. Yoshida H, Watanabe A, Ihara Y. Collapsin response mediator protein-2 is associated with neurofibrillary tangles in Alzheimer's disease. J Biol Chem 1998;273(16):9761-8.
24. Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H and others. CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. Nat Cell Biol 2002;4(8):583-91.
25. Sun L, Bittner MA, Holz RW. Rim, a component of the presynaptic active zone and modulator of exocytosis, binds 14-3-3 through its N terminus. J Biol Chem 2003;278(40):38301-9.
26. Deak F, Shin OH, Tang J, Hanson P, Ubach J, Jahn R, Rizo J, Kavalali ET, Sudhof TC. Rabphilin regulates SNARE-dependent re-priming of synaptic vesicles for fusion. Embo J 2006;25(12):2856-66.
27. Foletti DL, Blitzer JT, Scheller RH. Physiological modulation of rabphilin phosphorylation. J Neurosci 2001;21(15): 5473-83.
28. Zordan MA, Massironi M, Ducato MG, Te Kronnie G, Costa R, Reggiani C, Chagneau C, Martin JR, Megighian A. Drosophila CAKI/CMG protein, a homolog of human CASK, is essential for regulation of neurotransmitter vesicle release. J Neurophysiol 2005;94(2):1074-83.
29. Garner CC, Kindler S, Gundelfinger ED. Molecular determinants of presynaptic active zones. Curr Opin Neurobiol 2000;10(3):321-7.
30. Dresbach T, Hempelmann A, Spilker C, tom Dieck S, Altrock WD, Zuschratter W, Garner CC, Gundelfinger ED. Functional regions of the presynaptic cytomatrix protein bassoon: significance for synaptic targeting and cytomatrix anchoring. Mol Cell Neurosci 2003;23(2):279-91.
31. Grant SG. Synapse signalling complexes and networks: machines underlying cognition. Bioessays 2003;25(12): 1229-35.