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Mass spectrometrical analysis of *phosphoprotein enriched in astrocytes of 15 kDa* in mouse hippocampi

Short Communication

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Summary. Phosphoprotein enriched in astrocytes of 15 kDa (PEA-15) is a small protein that was first identified as an abundant phosphoprotein in brain. PEA-15 was characterised so far at the immunochemical level and by a microsequencing attempt. In order to update characterisation of this important structure by advanced methodology unambiguously identifying proteins independent of antibody availability and specificity, we used a proteomic method for this purpose: Performing protein profiling in mouse hippocampi using two dimensional gel electrophoresis with subsequent mass spectrometrical (MS/MS) identification we detected this protein and demonstrate proteomic characterisation of PEA-15 (Q62048). This study enables further specific and unambiguous determination serving as an analytical tool.

Keywords: Astrocytic phosphoprotein PEA-15 – Two-dimensional gel electrophoresis – Hippocampus – Mouse

Abbreviations: 2-DE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight

Phosphoprotein enriched in astrocytes of 15 kDa (PEA-15) is a small protein that was first identified as an abundant phosphoprotein in brain astrocytes by two-dimensional gel electrophoresis and partial microsequencing (Araujo et al., 1993), and subsequently shown to be widely expressed in different tissues (lung, thymus, adrenal gland, heart, kidney and spleen) by Northern blot and in a variety of brain regions (cerebral cortex, olfactory bulb, striatum, hypothalamus, cerebellum and spinal cord) by immunochemistry with polyclonal affinity purified PEA-15 antibodies (Estelles et al., 1996). In addition it is detected in different cell types (astrocyte, oligodendrocyte, microglia, neuron, endothelial cell and fibroblast) by immunoblotting with polyclonal anti-PEA-15 and was shown to be highly conserved among mammals (Estelles et al., 1996; Danziger et al., 1995). It is composed of a N-terminal death effector domain and a C-terminal tail of irregular structure. PEA-15 is regulated by multiple calcium-dependent phosphorylation pathways that account for its different forms: a non-phosphorylated form in equilibrium with a mono and a biphosphorylated variety. This suggests that PEA-15 may play a major role in signal integration. Accordingly, it has been demonstrated to modulate signalling pathways that control apoptosis and cell proliferation. In particular, PEA-15 diverts astrocytes from TNFα-triggered apoptosis (Estelles et al., 1999; Kitsberg et al., 1999) and regulates the actions of the ERK MAP kinase cascade by binding to ERK and altering its subcellular localisation (Ramos et al., 2000; Formstecher et al., 2001). The three-dimensional structure of PEA-15 has been modelised and recently determined using NMR spectroscopy (Hill et al., 2002).

However, most studies on functional characterisation of PEA-15 have been carried out at the transcriptional or immunoreactivity level. Although it was separated by two-dimensional electrophoresis (2-DE) gel and partly identified by partial microsequencing (Araujo et al., 1993), updating of the characterisation by advanced protein chemical methods, independent on antibody availability and specificity, is mandatory.

The advent of 2-DE with in-gel digestion and subsequent mass-spectrometrical identification of protein spots along with high-throughput robot technology allows systematic analysis of large series of proteins with excellent sensitivity and specificity of identification, in particular when MS-MS is used, mainly in form of MALDI-TOF-TOF technology (Mann et al., 2001).

In order to provide an analytical tool to analyse PEA-thus forming the basis for further reliable proteinchemical work on signalling pathways in brain, we applied 2-DE and mass spectrometrical analysis. The use of high-throughput robotic matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF/TOF as a valuable and rapid tool warrants unambiguous identification (Fountoulakis, 2001).

Nine FVB/N mice, five months old, male, were sacrificed for the experiments by decapitation and hippocampi were dissected at $-20°C$ and kept at $-80°C$ until biochemical assays were performed. The freezing chain was never interrupted until use.

Sample preparation with hippocampus tissues and 2-DE was performed as reported (Shin et al., 2004). Within predicted location of PEA-15 in 2-DE gels, spots were excised with a spot picker (PROTEINEER sp^{TM}, Bruker Daltonics, Germany) and placed into 96-well microtiter plates. In-gel digestion and MALDI sample preparation were performed according to a standard protocol using PROTEINEER dp^{TM} (Bruker Daltonics) (Yang et al., 2004). Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, the gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/mL trypsin in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30°C. Peptide extraction was performed with 10 mL of 1% TFA in 5 mM OGP. Extracted peptides were directly applied onto a target (AnchorChip^{TM}; Bruker Daltonics) that was loaded with a-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) matrix (25 mg/mL solution in acetone – 0.1% TFA 97:3 v/v). The mass spectrometer used in this work was an Ultraflex^{TM} TOF/TOF (Bruker Daltonics), operated in the reflector mode for MALDI-TOF-peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF with a fully automated mode using the flexControl^{TM} software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 200 consecutive laser shots and spectra were interpreted with the aid of the Mascot Software (Matrix Science Ltd, London, UK). For protein search, a mass tolerance of 100 ppm and 2 missing cleavage sites were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification. Those samples which could not be identified by their PMF from MALDI-TOF were additionally analysed using LIFT-TOF/TOF-MS/MS from the same target. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of a jointly migrating parent and fragment ions in a timed ion gate, the ions were lifted by 19 kV to a high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could simultaneously be analysed in the reflector with high sensitivity. LIFT spectra were interpreted with the Mascot software. MS/MS tolerance of 0.5 Da and 1 missing cleavage sites were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification. Finally, database searches, through Mascot, using combined PMF and MS/MS datasets were performed *via* BioTools 2.2 software (Bruker) (Yang et al., 2004).

Araujo and coworkers (1993) demonstrated three spots of PEA-15 on 2DE as a non-phosphorylated, mono and a biphosphorylated form. In our system PEA-15 was represented by a single spot (non-phosphorylated form), which

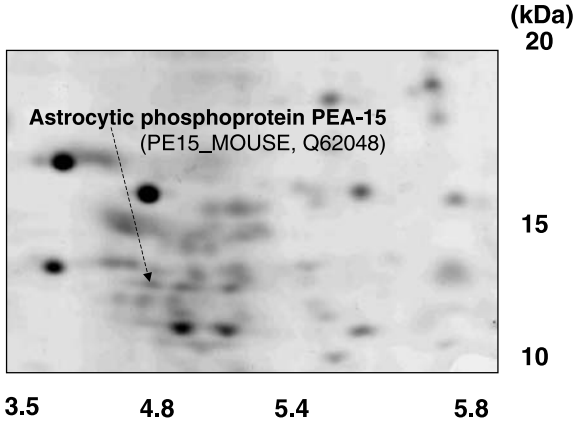


Fig. 1. Partial images of PEA-15 in 2-DE gels. The proteins were separated on an immobilised pH 3–10 NL gradient IPG strip, followed by separation on a gradient polyacrylamide gel. The gel was stained with Coomassie blue and spots were analysed by MALDI-TOF/TOF

Table 1. Protein identification and mass spectrometical details of PEA-15

Protein name	Theoretical/Observed		Matched peptides (Sequence Coverage/Score[a]/Expect)	Peptides for MS/MS (PMM)			
	pI	M_r (kDa)		Mr (obv)	Mr (expt)	Mr (calc)	Score[b]
Astrocyte Phosphoprotein PEA-15 (Q62048)	4.94/4.75	15.04/14.35	SEEITTGSAWFSFLESHNK LDKDNLSYIEHIFEISR **DNLSYIEHIFEISR**[c] DNLSYIEHIFEISR RPDLLTMVVDYR + methionine oxidation RPDLLTMVVDYR **RPDLLTMVVDYR** (36%/92/6e-007)	DNLSYIEHIFEISR 1735.85 RPDLLTMVVDYR 1493.74	1734.84 1492.74	1734.86 1492.77	47 19

Extracted proteins were separated by 2-DE and identified by MALDI-TOF/TOF, following in-gel digestion with trypsin

[a] Score is $-10 * \text{Log}(P)$, where P is the probability that the observed match is a random event (MASCOT, http://www.matrixscience.com)

[b] With MS/MS score is greater than 17 or 34, it indicates that this peptide is homolog or identity to MS results

[c] Bold letter indicates the peptides used for MS/MS analysis

is not a contradiction as this may be due to methodological and specimen differences. A single spot was identified with a theoretical Mw/pI of 15.04/4.94 and observed MW/pI values of 14.35 kDa and 4.75 (Fig. 1). Mass fingerprinting revealed 7 matching peptides (Table 1 and Fig. 2A) with a sequence coverage of 36% (Fig. 2B). MS/MS analysis unambiguously identified *astrocytic phosphoprotein PEA-15* by analysis of singly charged $[M+H]^+$ protonated molecules of m/z 1493.74 and 1735.85 (Fig. 2C and D) corresponding to peptides RPDLLTMVVDYR and DNLSYIEHIFEISR.

In conclusion, we have reliably and unambiguously identified and characterised PEA-15 in mouse hippocampus by a protein chemical rather than an immunochemical method, independent of antibody specificity and availability. And indeed, specific and reliable analysis of brain proteins is a major issue in neurochemistry and in the neurosciences and protein work should not rely on immunochemical analyses only. It is well known and documented that posttranslational modifications of proteins, in particular phosphorylation, change immunogenicity or even prevents detection of the protein at all.

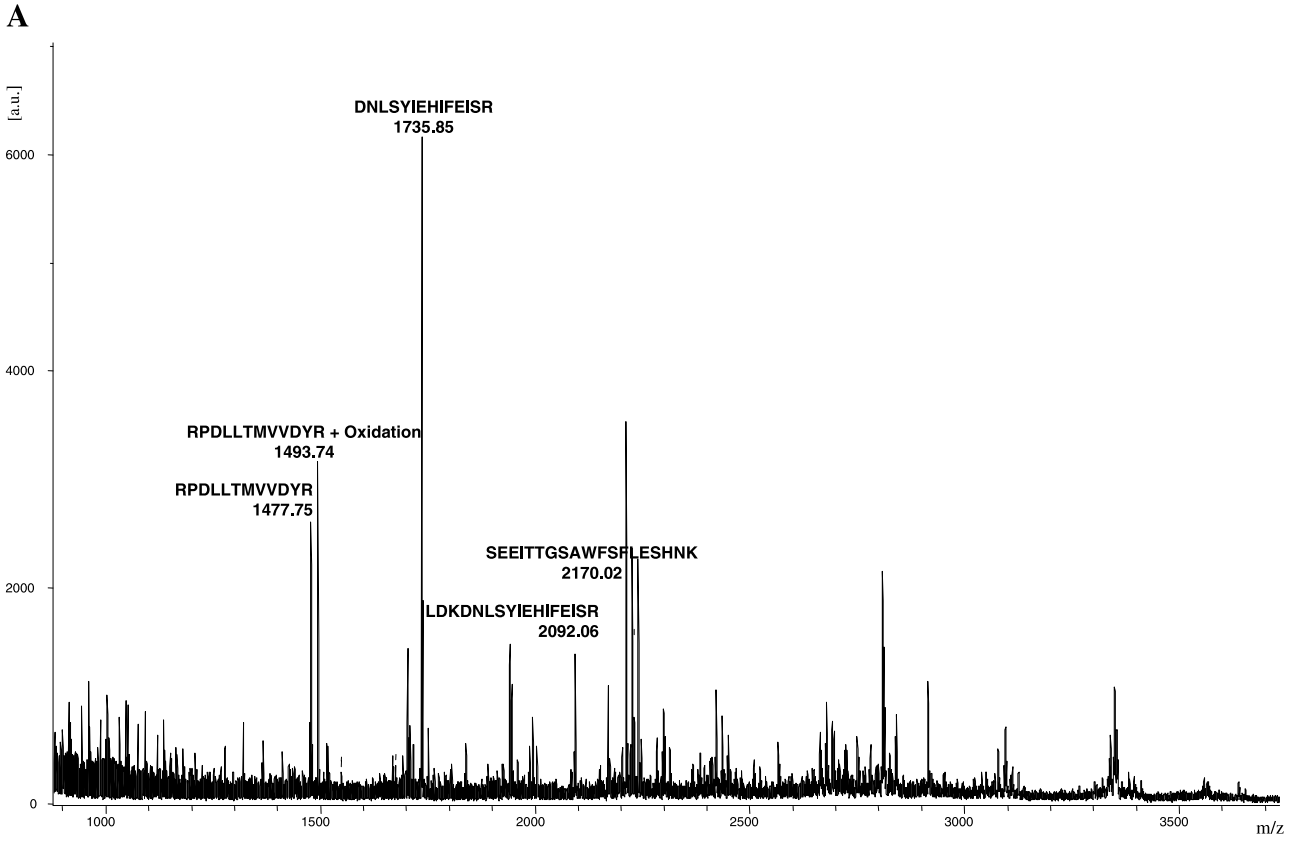
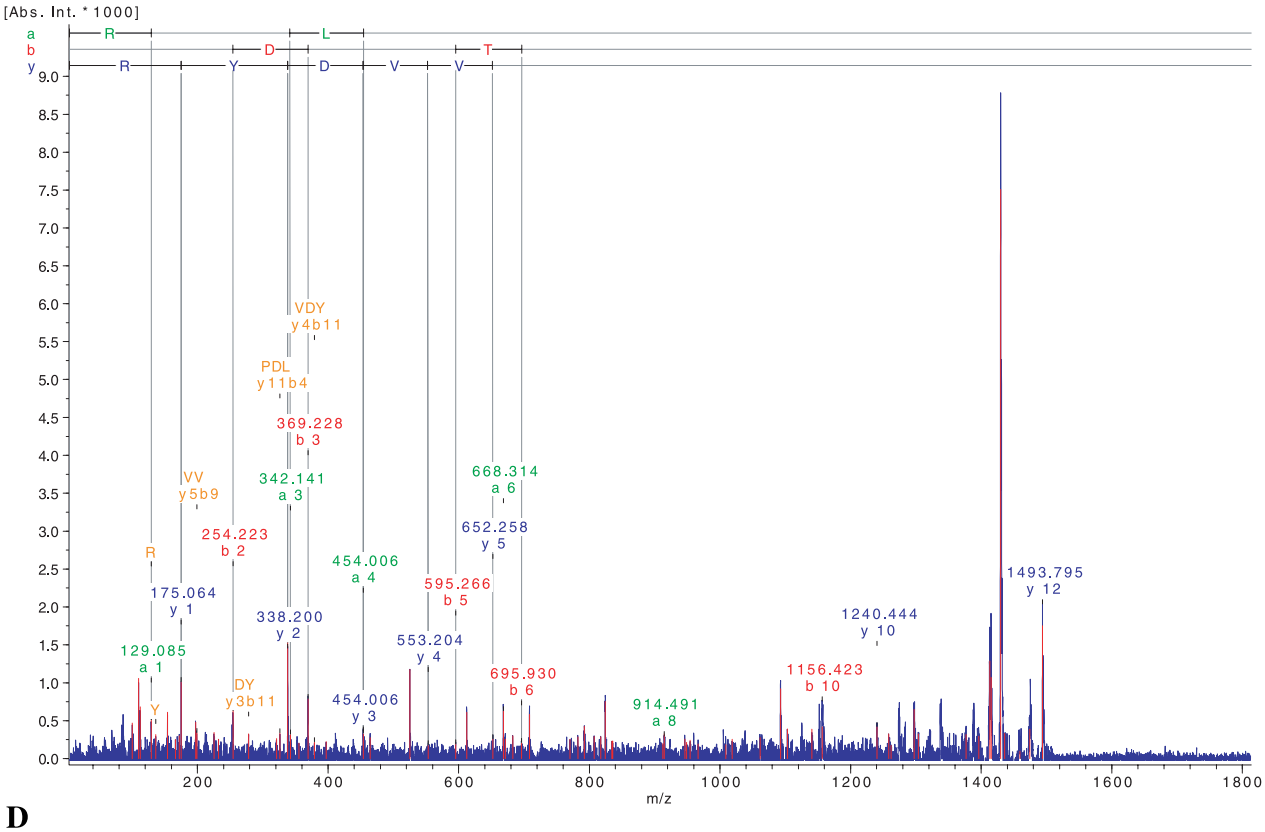


Fig. 2. **A** MALDI-TOF MS analysis of PEA-15. Two trypsin peaks $[M+H]^+$ (m/z 842.50 and 2211.10) were used for internal calibration. **B** Sequence of PEA-15 was shown and bold letter indicated the peptides matched. **C**, **D** MALDI-TOF MS/MS analysis of PEA-15. MS/MS analysis unambiguously identified *astrocytic phosphoprotein PEA-15* by analysis of singly charged $[M+H]^+$ protonated molecules of m/z 1493.74 (**C**) and 1735.85 (**D**) corresponding to peptides RPDLLTMVVDYR and DNLSYIEHIFEISR

C


MS/MS spectra of m/z 1493.7430
[Abs. Int. * 1000]
a
b
y
R
L
D
T
R
Y
D
V
V
VDY
y4b11
PDL
y11b4
369.228
b 3
VV
y5b9
342.141
a 3
668.314
a 6
652.258
y 5
254.223
b 2
R
454.006
a 4
595.266
b 5
175.064
y 1
1493.795
y 12
338.200
y 2
1240.444
y 10
553.204
y 4
129.085
a 1
695.930
b 6
1156.423
b 10
DY
y3b11
454.006
y 3
914.491
a 8
Y
m/z

D

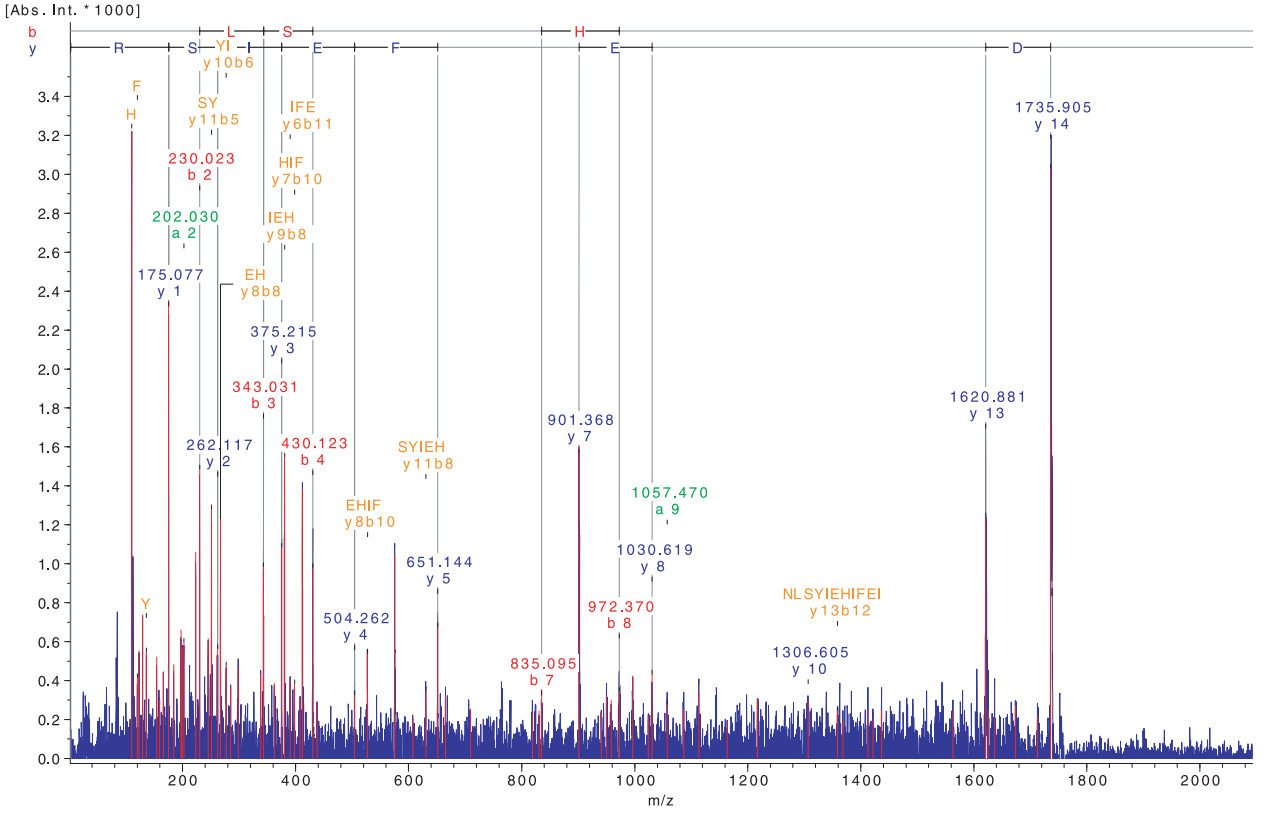

MS/MS spectra of m/z 1735.8450
[Abs. Int. * 1000]
b
y
L
S
H
R
S
YI
y10b6
I
E
F
E
D
F
H
SY
y11b5
IFE
y6b11
1735.905
y 14
230.023
b 2
HIF
y7b10
202.030
a 2
IEH
y9b8
175.077
y 1
EH
y8b8
375.215
y 3
343.031
b 3
1620.881
y 13
901.368
y 7
262.117
y 2
430.123
b 4
SYIEH
y11b8
1057.470
a 9
EHIF
y8b10
1030.619
y 8
651.144
y 5
NLSYIEHIFEI
y13b12
Y
504.262
y 4
972.370
b 8
1306.605
y 10
835.095
b 7
m/z

Fig. 2 C, D

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References

Araujo H, Danziger N, Cordier J, Glowinski J, Chneiweiss H (1993) Characterization of PEA-15, a major substrate for protein kinase C in astrocytes. J Biol Chem 268: 5911–5920

Danziger N, Yokoyama M, Jay T, Cordier J, Glowinski J, Chneiweiss H (1995) Cellular expression, developmental regulation, and phylogenic conservation of PEA-15, the astrocytic major phosphoprotein and protein kinase C substrate. J Neurochem 64: 1016–1025

Estelles A, Yokoyama M, Nothias F, Vincent JD, Glowinski J, Vernier P, Chneiweiss H (1996) The major astrocytic phosphoprotein PEA-15 is encoded by two mRNAs conserved on their full length in mouse and human. J Biol Chem 271: 14800–14806

Estelles A, Charlton CA, Blau HM (1999) The phosphoprotein protein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis. Dev Biol 216: 16–28

Formstecher E, Ramos JW, Fauquet M, Calderwood DA, Hsieh JC, Canton B, Nguyen XT, Barnier JV, Camonis J, Ginsberg MH, Chneiweiss H (2001) PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. Dev Cell 1: 239–250

Fountoulakis M (2001) Proteomics: current technologies and applications in neurological disorders and toxicology. Amino Acids 21: 363–381

Hill JM, Vaidyanathan H, Ramos JW, Ginsberg MH, Werner MH (2002) Recognition of ERK MAP kinase by PEA-15 reveals a common docking site within the death domain and death effector domain. EMBO J 21: 6494–6504

Kitsberg D, Formstecher E, Fauquet M, Kubes M, Cordier J, Canton B, Pan G, Rolli M, Glowinski J, Chneiweiss H (1999) Knock-out of the neural death effector domain protein PEA-15 demonstrates that its expression protects astrocytes from TNFalpha-induced apoptosis. J Neurosci 19: 8244–8251

Mann M, Hendrickson RC, Pandey A (2001) Analysis of proteins and proteomes by mass spectrometry. Annu Rev Biochem 70: 437–473

Ramos JW, Hughes PE, Renshaw MW, Schwartz MA, Formstecher E, Chneiweiss H, Ginsberg MH (2000) Death effector domain protein PEA-15 potentiates Ras activation of extracellular signal receptor-activated kinase by an adhesion-independent mechanism. Mol Biol Cell 11: 2863–2872

Shin JH, London J, Le Pecheur M, Hoger H, Pollak D, Lubec G (2004) Aberrant neuronal and mitochondrial proteins in hippocampus of transgenic mice overexpressing human cu/zn superoxide dismutase 1. Free Radic Biol Med 37: 643–653

Yang JW, Czech T, Lubec G (2004) Proteomic profiling of human hippocampus. Electrophoresis 25: 1169–1174

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