DATASET BRIEF

Exploring the proteome of an echinoderm nervous system: 2-DE of the sea star radial nerve cord and the synaptosomal membranes subproteome

Catarina Ferraz Franco[1], *Romana Santos*[1,2] *and Ana Varela Coelho*[1]

[1] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal
[2] Unidade de Investigação em Ciências Orais e Biomédicas, Faculdade de Medicina Dentária, Universidade de Lisboa, Portugal

Received: August 27, 2010
Revised: November 25, 2010
Accepted: December 29, 2010

We describe the first proteomic characterization of the radial nerve cord (RNC) of an echinoderm, the sea star *Marthasterias glacialis*. The combination of 2-DE with MS (MALDI-TOF/TOF) resulted in the identification of 286 proteins in the RNC. Additionally, 158 proteins were identified in the synaptosomal membranes enriched fraction after 1-DE separation. The 2-DE RNC reference map is available via the WORLD-2DPAGE Portal (http://www.expasy.ch/world-2dpage/) along with the associated protein identification data which are also available in the PRIDE database. The identified proteins constitute the first high-throughput evidence that seems to indicate that echinoderms nervous transmission relies primarily on chemical synapses which is similar to the synaptic activity in adult mammal's spinal cord. Furthermore, several homologous proteins known to participate in the regeneration events of other organisms were also identified, and thus can be used as targets for future studies aiming to understand the poorly uncharacterized regeneration capability of echinoderms. This ''echinoderm missing link'' is also a contribution to unravel the mystery of deuterostomian CNS evolution.

Keywords:
2-DE / Animal proteomics / MALDI-TOF/TOF / Radial nerve cord / Sea star / Synaptosomal membrane

The echinoderms (Phylum Echinodermata), as invertebrate deuterostomes, are one of the closest living relatives to vertebrates (Phylum Chordata), as they both belong to the superphylum Deuterostomia. Several reasons make these exceptionally well-adapted organisms very interesting animals to be studied: (i) their spectacular regenerative capability, including the nervous system [1–3]; (ii) their phylogenetic proximity to chordates (Fig. 1A) highlights them as alternative animal models for neurobiology which can contribute with a ''missing link'' of extreme importance to draw new theories on brain evolution.

Echinoderms present a neural organization that distinguishes them from other deuterostomes (chordates and hemichordates). In the adult echinoderm, the nervous system does not present a cephalized region, being composed by five radial nerve cords (RNCs) that derive from the circumoral nerve ring, a pentagonal nervous center that surrounds the mouth [4–6] (Fig. 1B). The integrity of the radial nerves and the circumoral nerve ring was shown to be essential for the reconstruction of their external body parts and internal organs [7].

Here, we present the first proteomic characterization of the RNC of an echinoderm, the sea star *Marthasterias glacialis*, as well of the synaptosomal membranes (SMs) subproteome. This first proteomic characterization of the nerve cord of an echinoderm is a significant step to

Correspondence: Dr. Ana Varela Coelho, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República – EAN, 2780-157 Oeiras, Portugal
E-mail: varela@itqb.unl.pt
Fax: +351-21441-1277

Abbreviations: RNC, radial nerve cord; **SM,** synaptosomal membrane enriched fraction; **SV,** synaptic vesicles

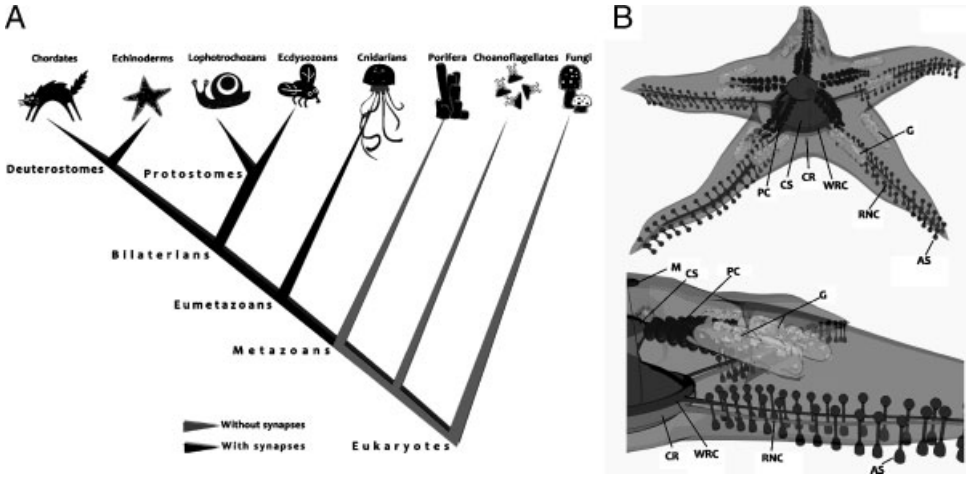

Figure 1. (A) Eukaryotes phylogeny. Phylogenic tree emphasizing the proximity between chordates and echinoderms. (B) Simplified diagrams of the anatomical organization of the sea star nervous system. Sea stars as all echinoderms have a radial symmetry, which means that each arm has an exact replica of all internal organs. Top: top view of an adult sea star (aboral side), and Down: lateral view of one sea star arm, show the organizational relationship between the nervous system and other internal organs. Nervous system: CR, circumoral nerve ring; RNC, radial nerve cord. Digestive system: CS, cardiac stomach; PC, pyloric caeca. Water vascular system: WRC, water ring canal; AS, ambulacrary system with tube feet; M, madreporite (controls entry of water into the water vascular system). Reproductive system: G, gonads.

withdraw them from the least studied metazoan nervous systems category and can be used as a starting point for future studies on neurobiology and organ regeneration and also to elucidate the role of this organism as a model animal, as the given results reveal an extensive homology between the echinoderm nervous system and the dorsal nerve cord of chordates.

Several adult specimens of the sea star *M. glacialis* (Linné, 1758) were collected at low tide on the west coast of Portugal (Estoril, Cascais) and kept at ''Vasco da Gama'' Aquarium (Dafundo, Oeiras) in open-circuit tanks with re-circulating seawater at 15°C and 33‰. They were fed ad libitum with a diet of mussels collected at the same site. Animals used for the experiments had similar sizes (21–26 cm measured between one arm tip and the most distant opposite one). Two RNCs were collected per animal as previously described [8].

Nerve cord proteome: For protein extraction, approximately 30 mg of the RNCs were homogenized with 100 μL of solubilization buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 60 mM DTE) containing a complete protease inhibitor cocktail (Sigma, Portugal) and centrifuged at 10 000 × *g* for 15 min at 4°C to remove cellular debris. The protein concentration was determined using the 2D Quant Kit™ (GE Healthcare, Portugal). RNC protein extracts were subjected to 2-DE using an IPGphor system (GE Healthcare), 11 cm Immobiline pH 3–11 non linear DryStrip loaded with 400 μg total protein and 1% v/v of ampholytes in the rehydratation buffer (GE Healthcare) with a minor adaptation of the first dimension IEF program due to high salt content of sea star RNC (a total of 24 kV h starting with several step&hold constant voltages for 5 h and with a maximum end voltage of 4000 V). After a two-step equilibration of the strips for reduction and alkylation, the second dimension was performed in a Ettan DaltSix (GE Healthcare). The 24-cm gels containing two IEF strips were stained with Coomassie Blue Colloidal [9], scanned with LabScan (GE Healthcare) and analyzed with ImageMaster Platinum software (version 5.0; GE Healthcare). To characterize the nerve cord proteome of *M. glacialis*, ten 2-DE gels were run and a total of ten biological replicates were used. The spots selected for protein identification were present at least in half of the 2-DE gels and had a relative spot volume (%vol) above 0.05%.

SM proteome: The SM fraction was isolated as previously described [10, 11], with minor adaptations to sea star nervous tissue. Briefly, three RNCs (±40 mg) were homogenized in ice-cold TEVP buffer (10 mM Tris-HCl pH 7.4; 5 mM NaF; 1 mM Na_3VO_4; 1 mM EDTA; 1 mM EGTA) containing 320 mM sucrose and a complete protease inhibitor cocktail (Sigma). After large debris removal (1000 × *g* for 10 min) the obtained supernatant was centrifuged at 10 000 × *g* for 20 min in order to obtain a crude synaptosomal fraction which was subsequently lysed by hypo-osmotic shock and centrifuged at 25 000 × *g* for 30 min to pellet the SMs. For protein extraction, the SMs were solubilised in a buffer containing SDS (1%, w/v) and DTT (50 mM) and heated up to 60°C for 10 min to ensure complete solubilization of large protein complexes and then diluted to 0.5% w/v SDS and incubated in 1-DE sample buffer [12]. The SMs protein extract were loaded (25 μg total protein per lane) on 10% w/v acrylamide gels and stained with Coomassie Blue Colloidal [9]. Then, two gel lanes were excised for in-gel digestion.

The excised gel spots and bands were digested as previously described [13] and peptides were resuspended in 5% v/v formic acid (Supporting Information 1). Prior to MS analysis, tryptic peptides from the 2-DE gel spots were desalted and concentrated on chromatographic microcolumns using GELoader tips packed with POROS R2 (20 μm bead size) and the directly eluted onto the MALDI plate using 0.5 μL of 5 mg/mL α-CHCA in 50% v/v ACN with 2.5% v/v of formic acid. The tryptic peptides from the SM fraction gel bands, were subjected to an additional sequential stepwise elution from microcolumns packed with increasing hydrophobic material (POROS R2 (20 μm bead size), R3 (20 μm bead size) and activated charcoal) [14, 15] in order to overcome the limiting separating ability of 1-DE gels.

MS/MS was performed using a MALDI-TOF/TOF 4800 plus mass spectrometer (Applied Biosystems, Foster City, CA, USA). Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems) and contaminant m/z peaks resulting from trypsin autodigestion were excluded when generating the peptide mass list used for database search.

A detailed description of the protein identification workflow is given in Supporting Information 1. To overcome the lack of a complete sea star genome information, which impairs the success of protein identification, two different protein identification algorithms were used: PARAGON® provided with the ProteinPilot software (version 3.0, revision 114732; Applied Biosystems) and MOWSE® from MASCOT (version 2.2; Matrix Science, Boston, MA, USA), and three different protein sequence databases were used for protein identification. UniProt (Release 2010_06; 11 384 898 entries; European Bioinformatics Institute) joined with the purple sea urchin *Strongylocentrotus purpuratus* predicted protein database (42 420 entries; December 2006; ftp://ftp.ncbi.nih.gov/genomes/Strongylocentrotus_purpuratus/protein); and the non-redundant database Uniref100 (release 2010_06; 10 246 365 entries). Protein identification files derived from MASCOT were converted to mzML files using PRIDE Converter tool [16] and are available in the PRIDE database [17] under accession number 15331. Uncharacterized/unknown proteins and all *S. purpuratus* proteins were further submitted to protein-protein BLAST searches against Swiss-Prot database using Basic Local Alignment Search tool available at NCBI web site (http://blast.ncbi.nlm.nih.gov/). The false discovery rate (FDR) for each 2-DE spot and 1-DE band was determined using PSPEP algorithm from Protein Pilot search engine, using concatenated database joined with the reversed decoy database. Identified proteins were selected if a false discovery rate $<1\%$.

A mean value of 403 spots was detected per 2-DE gel of RNC (Fig. 2A) and after analysis a total of 339 spots were selected and processed for protein identification by MS (RNC 2-DE annotated reference gel available via the WORLD-2DPAGE Portal displaying also relevant information on all identified spots including protein identification data). Using the two identification algorithms and the selected protein databases, 286 spots were successfully identified (Supporting Information Tables ST1 and ST2) representing 84% of the selected spots. Searches using MASCOT resulted in the identification of 112 proteins in the UniProt/*S. purpuratus* databases and 126 proteins in the UniRef100 protein database (Supporting Information 1). Searches with ProteinPilot combining UniProt/*S. purpuratus* and UniRef100 databases produced 139 protein identifications. Altogether, approximately 200 non-redundant protein identifications were achieved (Supporting Information Table ST1). This high yield of protein identification was only possible using a protein identification workflow that comprised different databases and search algorithms (Supporting Information 1).

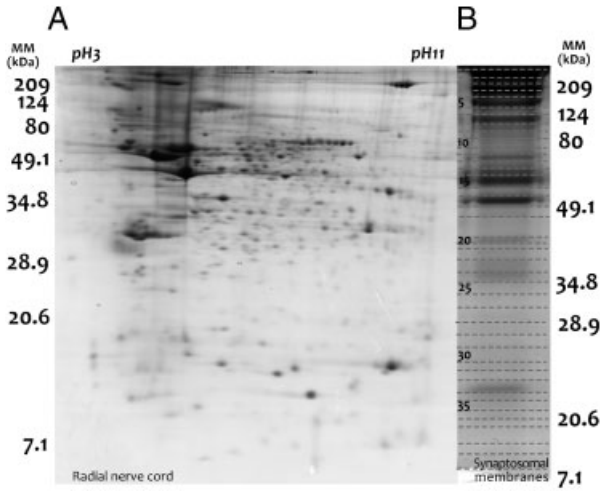

Figure 2. (A) 2-DE gel of the sea star *M. glacialis* RNC: Sea star nerve cord proteins were separated by p*I* using non-linear 3–11 11-cm IEF strips. For second dimension 12.5% SDS-PAGE gels were used to separate proteins according to their molecular masses. The complete annotated 2-DE gel image is present in Supporting Information Table ST2. (B) Synaptosomal-enriched fraction: 1-DE separation of the SM proteins using a 10% SDS-PAGE gel. The SM protein lanes were sliced for further processing as indicated in the figure (horizontal lines).

Since proteomic analysis of whole tissues is often unsuitable for the study of low-abundance proteins, a nerve subcellular fractionation was employed based on mammal nervous tissues protocols, in order to improve the proteomic characterization of sea star RNC through the enrichment in proteins specific of nerve physiological functions. Visible bands, mainly on the higher mass region of the gel, were excised for protein identification, while the remaining lane was sliced in order to identify, possible unstained low abundant proteins (Fig. 2B). The stepwise elution of the retained peptides using increments of ACN minimized the ion suppression effect and greatly increased the number of peptides detected by MALDI-TOF/TOF mass spectrometry. This fractionation resulted in the identification of 158

proteins, of which 38 are proteins that were not identified in the intact nerve (Supporting Information Tables ST3 and ST4).

Since a limited number of sea star proteins are deposited on the available protein sequence databases, the present study is a homology-driven proteomic characterization of *M. glacialis* RNC. STRAP software [18] was used to fully annotate the identified proteins using the UniProt gene ontology information. Three independent sets of ontology were used in the annotation: the biological function in which the protein participates (Fig. 3A), their subcellular location (Fig. 3B) and their molecular functions (Fig. 3C). However, since cellular pathways focus on physical and functional interactions between proteins rather than merely taking the gene-centric view of GO-based analyses, a pathway analysis using DAVID functional annotation tools (http://david.abcc.ncifcrf.gov/home.jsp) [19] was also performed and therefore a more comprehensive overview of the relevant functions enrolled by this tissue is also presented (Supporting Information Tables ST1 and ST3). The comparison of the subcellular localization and function of the identified synaptosomal proteins with the proteins identified in the intact nerve 2-DE gels showed that although the SM fractionation procedure was originally optimized for mammalian nerve tissues, it was also effective on echinoderms nerve tissues. As shown in Fig. 3A, among the identified proteins with known localization, approximately 15% of the proteins identified in the SM fraction are membrane-associated proteins, in contrast with the 6% found in the intact nerve cord. Nevertheless, some nuclear proteins (11%) persisted in the SM fraction. As for mitochondrial proteins they were totally absent in the SM fraction. In terms of biological functions (Fig. 3B), after the abundant constitutive proteins as expected due to the high cellular abundance of these classes [20] (cytoskeleton, 23%, and metabolic pathways, 6%), the majority of the identified proteins in the RNC are involved in synaptic vesicles (SVs) and protein transport (24%) or are G-protein modulators (15%). Proteins having a molecular transducing activity are also highly represented in the SM-enriched fraction with 12% as well as calcium binding proteins (10%). Proteasome proteins, which are known to regulate some pre-synaptic protein functions [21], were also found in the SM representing 6%.

The possible homology between the echinoderms nervous system and the chordate CNS is neither new nor

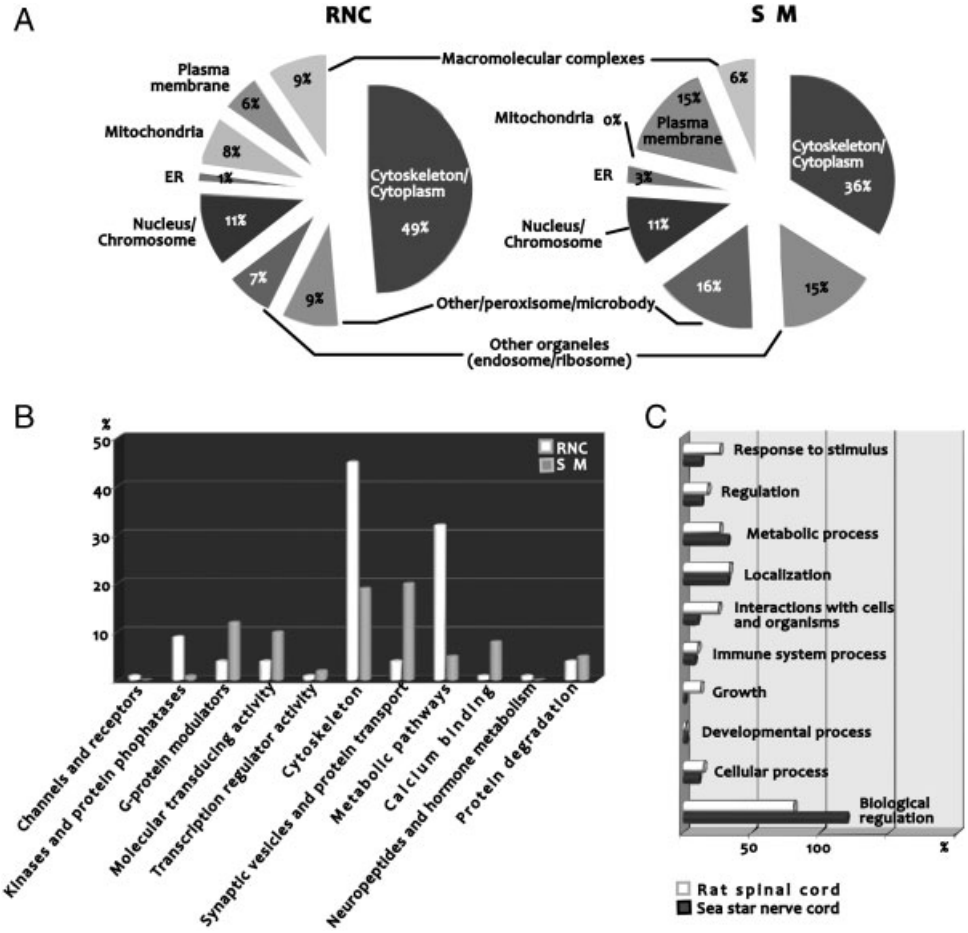

Figure 3. Gene ontology annotations of the identified sea star *M. glacialis* nerve cord proteins. Cellular localization (A) and biological function (B) of the identified proteins in the RNC (NC) and SMs-enriched fraction (SM). (C) Homology of rat spinal cord and sea star RNC proteins according to their biological function. Biological function distribution of the identified proteins in spinal cord of a vertebrate [25] and the RNC of an echinoderm, the sea star *M. glacialis*.

consensual [22–24]. It is still an issue of great debate since the major approaches to support these hypotheses rely mainly on information provided by comparative anatomy and morphological studies. In an effort to further clarify this persisting question, the biological functions of the identified proteins in sea star nervous system were compared with the described proteins from the spinal cord of a chordate [25]. In order to do so, all accession numbers of the rat spinal cord and sea star nervous tissue proteins were manually uploaded into STRAP software [18],] which was then used to annotate the identified proteins of both organisms according to gene ontology. This analysis revealed an important homology between the biological functions of the proteins described for rat and sea star nervous systems (Fig. 3C). Nevertheless, since this study is the first characterization of an echinoderm RNC proteome, more detailed and thorough studies should be performed before drawing new theories on CNS evolution.

A functional overview of the identified proteins in *M. glacialis* nervous system clearly highlights the functional complexity of echinoderms nervous system.

Synaptic transmission in echinoderms: Up to date no voltage-gated ion channels (VGIC) molecules have been described on echinoderms nervous tissues and our study is the first evidence that several of these channels, which are specially crucial in chemical synapses, are present. Evidence of membrane potentials generated by K^+; Ca^{2+} and Na^+ permeability is given, namely by the identification of a potassium channel, sodium/potassium-transporting ATPase and also several calcium-dependent proteins (e.g. Calmodulin; Calpain), showing evidence for calcium-based action potentials. Proteins responsible for the turnover of the neutransmitters glutamate and choline were also found. Several Rab GTPases, a multigene family that mediates targeting of intracellular vesicles to membranes, were also identified in the SM-enriched fraction (Supporting Information Table ST3). Among these is the Rab GDP dissociation inhibitor α, which in vertebrates is predominantly present in brain and neural/sensory tissues and Rab-3D, which is the major isoform that binds to SVs. The identified vesicle fusing ATPase is required for vesicle-mediated transport and is a cellular component of dendritic shaft and postsynaptic density. Electrical synapses (or neuronal gap junctions) are relatively simple compared to chemical ones and enable rapid impulse propagation [26]. However, these proteins appear to be encoded by distinct gene families unequally distributed among different animal phyla [27]. BLAST searches within the genome of the purple sea urchin failed to find representative genes of any of these proteins [28] and in agreement also no Gap junction proteins were identified in our study, which can be one more hint on the similarity between the RNC of echinoderms and spinal cord of chordates since the synaptic activity of adult mammal spinal cords relies essentially on chemical transmission. Other proteins involved in protein trafficking and transport among different compartments, as well as clathrin, one of the major proteins of SV, were also identified.

Neurogenesis and regeneration: One of the most interesting echinoderm capabilities is their amazing ability to fully regenerate body parts upon a traumatic injury, a natural trait also extended to their nervous system [1–3], a process that is at the present time far from being understood. Regeneration is seen at some point to be a recapitulation of the embryogenic pathways. Several proteins involved in neurogenesis with functions of axonal guidance, dentrite morphogenesis and neuron growth have been identified, namely calreticulin, dihydropyrimidinase and protein enabled. Proteins belonging to the *Wnt* signaling pathway, described as involved in the regeneration of the thickened wound epithelia in *Amphiura filiformis* [29], were also identified.

Sensory perception: Echinoderms lack evident light-sensitive organs; however, they respond to light, photoperiod and lunar cycles. Several proteins responsible for sensory perception were identified which further highlights the functional complexity of the echinoderm nervous system.

In summary, the many newly identified proteins in the nerve cord of *M. glacialis* are of extreme importance and highlight the potential of echinoderms as models to study CNS itself and its regeneration ability. The use of these animals as model systems, given their simpler morphology, easy manipulation, complex nervous system, can be a promising way to understand the molecular mechanisms involved in regeneration, which can then be transposed to find regeneration targets to be studied in other model organisms, namely mammals.

Protein identification data are available in the PRIDE database, accession number 15331.

This work was supported by Fundação para a Ciência e Tecnologia through a PhD grant to Catarina Franco (SFRH/BD/29799/2006), a research contract by the Ciência 2008 program to Romana Santos and a project grant (PTDC/MAR/104058/2008). We also acknowledge Dr. Patrick Flammang for scientific advice, Vasco da Gama Aquarium (Dafundo, Oeiras, Portugal), namely Dr. Fátima Gil and Miguel Cadete, for sea stars maintenance, Henrique Braga for the help with mussels collection and Dr. Renata Soares for paper revision.

The authors have declared no conflict of interest.

References

[1] Thorndyke, M. C., Patruno, M., Moos, C., Beesley, P., Cellular and molecular bases of arm regeneration in brittlestars, in: Barker, M. (Ed.) *Proceedings of the 10th International Echinoderm Conference*, Dunedin, 2000. AA Balkema, Rotterdam 2001, pp. 323–326.

[2] Thorndyke, M., Carnevali, C., Regeneration neurohormones and growth factors in echinoderms. *Can. J. Zool.* 2001, *79*, 1171–1208.

[3] Dupont, S., Thorndyke, M., Bridging the regeneration gap: insights from echinoderm models. *Nat. Rev. Genet.* 2007, *8*.

[4] Ruppert, E. E., Barnes, R. D., *Invertebrate Zoology*, 6th Edn, Saunders College Publishing, Philadelphia 1994.

[5] Smith, J. E., On the nervous system of the starfish *Marthasterias glacialis* (L.). *Philos. Trans. R. Soc. Lond.* 1936, *227*, 111–173.

[6] Smith, J. E., *Structure and Function in the Nervous Systems of Invertebrates*. T.H., Horridge, G.A. Bullock. W.H. Freeman and Co., San Francisco 1965, pp. 1519–1558.

[7] Carnevali, C., Bonasoro, F., Welsch, U., Thorndyke, M. C., Arm regeneration and growth factors in crinoids. Mooi, R., Telford, M. (Eds.), *Echinoderm*, Balkema, Rotterdam, 1998, pp. 145–150.

[8] Moss, C., Hunter, A., Thorndyke, M., Patterns of bromodeoxyuridine incorporation and neuropeptide immunoreactivity during arm regeneration in the starfish *Asterias rubens*. *Phil. Trans. R. Soc. Lond.* 1998, *353*, 421–436.

[9] Neuhoff, V., Harold, N., Ehrhardt, W., Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988, *9*, 255–262.

[10] Singh, O. V., Yaster, M., Xu, J., Guan, Y. et al., Proteome of synaptosome-associated proteins in spinal cord dorsal horn after peripheral nerve injury. *Proteomics* 2009, *9*, 1241–1253.

[11] Dunah, A. W., Standaert, D. G., Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. *J. Neurosci.* 2001, *21*, 5546–5558.

[12] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680–685.

[13] Santos, R., Costa, G. C., Franco, C. F., Alves, P. G. et al., First insights into the biochemistry of the tube foot adhesive from the sea urchin *Parentrotus lividus* (Echinoidea, Echinodermata). *Mar. Biotechnol.* 2009, *11*, 686–698.

[14] Larsen, M. R., Cordwell, S. J., Roepstorff, P., Graphite powder as an alternative or supplement to reversed-phase material for desalting and concentration of peptide mixtures prior to matrix-assisted laser desorption/ionization-mass spectrometry. *Proteomics* 2002, *2*, 1277–1287.

[15] Gobon, J., Nordhoff, E., Migorodskaya, E., Ekman, R., Roepstorff, P., Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 1999, *34*, 105–116.

[16] Barsnes, H., Vizcaino, J. A., Eidhammer, I., Martens, L., PRIDE converter: making proteomics data-sharing easy. *Nat. Biotechnol.* 2009, *27*, 598–599.

[17] Vizcaíno, J. A., Côté, R., Reisinger, F., Foster, J. M. et al., A guide to the Proteomics Identifications Database proteomics data repository. *Proteomics* 2009. *9*, 4276–4283.

[18] Bhatia, V. N., Perlman, D. H., Costello, C. E., McComb, M. E., Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Anal. Chem.* 2009, *81*, 9819–9823.

[19] Da Wei Huang, D. W., Sherman, B. T., Lempicki, R. A., Systematic and integrative analysis of large genelists using DAVID bioinformatics resources. *Nat. Protoc.* 2009, *4*, 44–57.

[20] Petrak, J., Ivanek, R., Toman, O., Cmejla, R. et al., Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* 2008, *8*, 1744–1749.

[21] Burré, J., Volknandt, W., The synaptic proteome. *J. Neurochem.* 2007, *101*, 1448–1462.

[22] Hagg, E. S., Echinoderm rudiments, rudimentary bilaterians, and the origin of the chordate CNS. *Evol. Dev.* 2005, *7:4*, 280–281.

[23] Nielsen, C., Homology of echinoderm radial nerve cords and the chordate neural tube?. *Evol. Dev.* 2006, *8:1*, 1–2.

[24] Hagg, E. S., Reply to Nielsen. *Evol. Dev.* 2005, *8:1*, 3–5.

[25] Gil-Dones, F., Alonso-Orgaz, S., Avila, G., Martin-Rojas, T. et al., Na optimal protocol to analyze the rat spinal cord proteome. *Biomark. Insights* 2009, *4*, 135–164.

[26] Zoidl, G., Dermietzel, R., On the search for the electrical synapse: a glimpse at the future. *Cell Tissue Res.* 2002, *310*, 137–142.

[27] Hervé, J. C., Phelan, P., Bruzzone, R., White, T. W., Connexins, innexins and pannexins: bridging the communication gap. *Biochim. Biophys. Acta* 2005, *1719*, 3–5.

[28] Burke, R. D., Angerer, L. M., Elphick, M. R., Humphrey, G. W. et al., A genomic view of the sea urchin nervous system. *Dev. Biol.* 2006, *300*, 434–460.

[29] Rychel, A. L., Swalla, B. J., Regeneration in Hemichordates and Echinoderms. In: Rinkevich, B., Matranga, V. (Eds.), *Stem Cells in Marine Organisms*. Springer, Netherlands, 2009, pp. 245–265.

