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PROTEOME ANALYSIS REVEALS PROTEIN CANDIDATES INVOLVED IN EARLY STAGES OF BRAIN REGENERATION OF TELEOST FISH

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Abstract—Exploration of the molecular dynamics underlying regeneration in the central nervous system of regeneration-competent organisms has received little attention thus far. By combining a cerebellar lesion paradigm with differential proteome analysis at a post-lesion survival time of 30 min, we screened for protein candidates involved in the early stages of regeneration in the cerebellum of such an organism, the teleost fish *Apteronotus leptorhynchus*. Out of 769 protein spots, the intensity of 26 spots was significantly increased by a factor of at least 1.5 in the lesioned hemisphere, relative to the intact hemisphere. The intensity of 9 protein spots was significantly reduced by a factor of at least 1.5. The proteins associated with 15 of the spots were identified by peptide mass fingerprinting and/or tandem mass spectrometry, resulting in the identification of a total of 11 proteins. Proteins whose abundance was significantly increased include: erythrocyte membrane protein 4.1N, fibrinogen gamma polypeptide, fructose-biphosphate aldolase C, alpha-internexin neuronal intermediate filament protein, major histocompatibility complex class I heavy chain, 26S proteasome non-ATPase regulatory subunit 8, tubulin alpha-1C chain, and ubiquitin-specific protease 5. Proteins with significantly decreased levels of abundance include: brain glycogen phosphorylase, neuron-specific calcium-binding protein hippocalcin, and spectrin alpha 2. We hypothesize that these proteins are involved in energy metabolism, blood clotting, electron transfer in oxidative reactions, cytoskeleton degradation, apoptotic cell death, synaptic plasticity, axonal regeneration, and promotion of mitotic activity.

Key words: *Apteronotus leptorhynchus*, injury, proteomics, regeneration, wound healing.

INTRODUCTION

In most adult vertebrates, and particularly in mammals, the regenerative capacity of tissue and organs after injury is very limited or absent. A notable exception is the ability of mammals and other vertebrates to regenerate hepatocytes after liver injury (for reviews see Fausto, 2000; Michalopoulos and DeFrances, 2005). Comparative studies have demonstrated that the regenerative potential is much more pronounced in anamniotes than in amniotes (for review see Stocum, 2006). Well-known examples of the regenerative potential of anamniotes are urodele amphibians, which are capable of regrowing their limbs after amputation (for reviews see Brockes, 1997; Nye et al., 2003), and teleost fish, which have retained in adult stages of development not only the capacity to regrow a number of peripheral organs, such as fins (for review see Akimenko et al., 2003) or heart (for review see Raya et al., 2004), but also the ability to replace lesioned portions of their central nervous system by generating de novo neurons and glial cells (for reviews see Zupanc and Zupanc, 2006a; Becker and Becker, 2008; Zupanc, 2008, 2009; Fleisch et al., 2011; Sîrbulescu and Zupanc, 2011; Zupanc and Sîrbulescu, 2011). The latter phenomenon is commonly referred to as neuronal regeneration.

Experiments employing a cerebellar lesion paradigm in the teleost fish *Apteronotus leptorhynchus* have shown that the neural tissue is restored within a few weeks following the injury (Zupanc et al., 1998). This is mediated by a cascade of events, including: elimination of damaged cells through apoptosis (Zupanc et al., 1998); removal of cellular debris by the phagocytic action of microglia/macrophages (Zupanc et al., 2003); generation of new cells from adult stem cells (Zupanc and Ott, 1999), followed by their recruitment to the site of injury by radial glia-guided migration (Clint and Zupanc, 2001); differentiation of these cells and their integration into existing neural circuits (Zupanc and Ott, 1999); and promotion of cellular survival (Zupanc et al., 2006; Zupanc and Zupanc, 2006b).

In a first attempt to identify the multitude of proteins involved in some of these processes, we have employed differential proteome analysis 3 days after a cerebellar lesion (Zupanc et al., 2006), a time point characterized by a marked increase in cell proliferation and recruitment (Zupanc and Ott, 1999). Out of nearly 800 protein spots revealed by 2D gel electrophoresis, 53 spots exhibited significant intensity differences after application of a lesion, compared to intact controls. This result hints at a tremendous number of molecular signals that direct the individual steps of the entire regenerative process.

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Abbreviations: BVA, biological variance analysis; DIA, differential in-gel analysis; DIGE, difference gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; MALDI-TOF, matrix assisted LASER desorption/ionization time-of-flight; MHC, major histocompatibility complex; MS, mass spectroscopy; MS/MS, tandem mass spectrometry; PMF, peptide mass fingerprinting; SDS, sodium dodecylsulphate.

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There is evidence that the first steps in the regenerative process are initiated very shortly after the traumatic insult. For example, the first apoptotic cells can be detected at the lesion site as soon as 5 min after the injury (Zupanc et al., 1998). To reveal potential molecular signals involved in these early stages of regeneration, in the present study we have performed differential proteome analysis on tissue collected 30 min after application of a lesion to the cerebellum of *A. leptorhynchus*. Eleven of the proteins that exhibited significant changes in abundance after the lesion could be identified, and their potential role in the regenerative process is discussed in the present paper.

EXPERIMENTAL PROCEDURES

Animals

Brown ghost knifefish (*A. leptorhynchus*; Gymnotiformes, Teleostei) were supplied by a tropical fish importer (Aquarium Glaser, Rodgau, Germany). The fish were kept in 45 and 300 l tanks at temperatures of approximately 27 °C, a water conductivity of 150–250 μS/cm, and a pH of 7, and exposed to a 12-h light/12-h dark photoperiod.

Twelve fish were used in this study. Post-mortem gonadal inspection demonstrated that 4 of these fish were male, and 8 were female. Total length ranged between 100 and 124 mm (mean: 113 mm; median: 113 mm) and body weight between 2.1 and 4.7 g (mean: 3.3 g; median: 3.3 g), indicating that all the individuals can be regarded as adults. The gonadosomatic index (gonad weight divided by body weight) of the fish ranged between 0.0009 and 0.0031 in males (mean: 0.0017; median: 0.0014) and 0.0049 and 0.0167 in females (mean: 0.0082; median: 0.0072). This suggests that most of the individuals of this seasonally breeding species were not reproductively active at the time of inspection.

Application of cerebellar lesions

Mechanical lesions were applied to the dorsalmost subdivision of the cerebellum, the corpus cerebelli, as described previously (Zupanc et al., 1998; Zupanc and Ott, 1999). Individual fish were deeply anesthetized by immersion into aquarium water containing approximately 2% ethyl carbamate ('urethane'; Sigma–Aldrich, Munich, Germany) and locally anesthetized on the head with 2% lidocaine solution (Caesar & Loretz, Hilden, Germany). Guided by landmarks on the fish's head, a 3-mm-deep stab wound was created with a sterile scalpel (No. 11, Feather). The path of the lesion ran parallel to the midline of the brain through the dorsal molecular layer of the corpus cerebelli and the adjacent granular layer. The lesion was positioned roughly halfway between the midline and the lateral edge of the granular layer of the corpus cerebelli. The fish were transferred to isolation tanks for a post-lesion survival time of 30 min. All experiments were carried out in accordance with the regulations of the relevant German law, the *Deutsches Tierschutzgesetz* of 1998.

Isolation of cerebellar tissue

The fish were killed by immersion into an overdose of 3-aminobenzoate methanesulfonate (MS-222; Sigma–Aldrich) dissolved in aquarium water. Their heads were cooled with pieces of crushed ice and the skulls were opened. A block was dissected out of corpus cerebelli in the lesioned hemisphere such that an approximately 0.5 mm thick rim of tissue surrounded the path of lesion. A tissue block of similar size was removed from the equivalent area in the intact hemisphere. These pieces of tissue were immediately frozen in isopentane at −45 °C, pooled with the corresponding tissue blocks of the other fish, and stored at −80 °C until further use.

Sample preparation

The pooled tissue samples from the lesioned and unlesioned hemispheres, respectively, were dissolved in a lysis buffer consisting of 7 M thiourea, 2 M urea, 4% CHAPS, 1% dithiothreitol, and 0.5% pharmalytes 3–10 (Amersham Biosciences, Freiburg, Germany). After dissolving in lysis buffer, the samples were sonicated 10 times for 1 s each at 60 W and centrifuged at 12,000*g* for 10 min. The protein content of the supernatant was measured using the Bradford assay system (Sigma–Aldrich).

Protein labeling

The proteins were labeled with the CyDye DIGE Fluors using the Ettan DIGE system (Amersham Biosciences, Freiburg, Germany). For analysis of proteins from the intact and lesioned hemispheres, 50 μg protein were labeled with 400 pmol of Cy3 and Cy5, respectively. For the internal standard, 25 μg protein from each of the two types of tissue were labeled with 400 pmol Cy2. After 30 min incubation at 4 °C, the reaction was stopped by adding 1 μl of a 1 mM L-lysine solution, followed by incubation in the dark at 4 °C for 10 min. Finally, the three labeled extracts were pooled and mixed on a shaker for 5 min at 20 °C.

Two-dimensional gel electrophoresis

Protein extracts were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), running the pooled samples from the lesioned and unlesioned hemispheres in triplicate. For isoelectric focusing (first dimension), a linear 18-cm pH 4–7 immobilized pH gradient (IPG) strip (Immobiline DryStrip pH 4–7, Amersham Biosciences, Freiburg, Germany) was rehydrated overnight. Isoelectric focusing was conducted at 38,700 V × h, using the Multiphor II system (Amersham Biosciences, Freiburg, Germany).

For the second dimension, the IPG strip was equilibrated in a 1% solution of dithiothreitol and 4% sodium dodecylsulphate (SDS) for 10 min, followed by 10 min in a 4% solution of iodoacetamide and 4% SDS. The IPG strip was embedded onto a SDS PAGE gel with 0.5% (w/v) agarose in electrode buffer (Laemmli, 1970), and a protein ladder (Serva, Heidelberg, Germany) was added. Gels were run at 13 °C and a maximum of 2000 V × h overnight.

Computerized analysis of protein spots

The labeled gels were digitized using an Ettan DIGE Imager (GE Healthcare, Munich, Germany) at a resolution of 100 μm per pixel. The resulting images were analyzed using the Differential In-gel Analysis (DIA) and Biological Variance Analysis (BVA) modules of the DeCyder 2D software (versions 5.0 and 6.5, Amersham Biosciences/GE Healthcare, Germany). The DIA module was employed to perform multi-dye co-detection of protein spots within each gel, and to quantify the relative abundance of protein spots in intact and lesioned samples, compared to the internal standard. The calculated standardized abundance values were further normalized to a log-mean value of zero for each of the two experimental conditions and for each of the three gels in order to correct for intrinsic differences between dye intensities. The automatic gel-to-gel matching of spots and subsequent calculation of average fold changes in protein abundance levels between the Cy5 (lesioned) and Cy3 (intact) labelings were performed using the BVA module of DeCyder. The matching of all protein spots showing average increases or decreases of more