Proteomics
Proteomics and Systems Biology
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Larval Zebrafish Proteome Regulation in Response to an Environmental Challenge

Kasper Langebeck-Jensen, Or D. Shahar, Erin M. Schuman, Julian D. Langer,* and Soojin Ryu**

Adaptation to the environment during development influences the life-long survival of an animal. While brain-wide proteomic changes are expected to underlie such experience-driven physiological and behavioral flexibility, a comprehensive overview of the nature and extent of the proteomic regulation following an environmental challenge during development is currently lacking. In this study, the brain proteome of larval zebrafish is identified and it is determined how it is altered by an exposure to a natural and physical environmental challenge, namely prolonged exposure to strong water currents. A comprehensive larval zebrafish brain proteome is presented here. Furthermore, 57 proteins that are regulated by the exposure to an environmental challenge are identified, which cover multiple functions including neuronal plasticity, the stress response, axonal growth and guidance, spatial learning, and energy metabolism. These represent candidate proteins that may play crucial roles for the adaption to an environmental challenge during development.

The ability of animals to adapt to local environments is key to survival. In all cells, including neurons, proteins are responsible for most cellular functions and responses to internal and external perturbations. The importance of successful adaptation to an environment is especially pronounced during early development. Early experiences may affect brain development resulting in long lasting physiological and behavioral modifications. The zebrafish larva is a good model to study the molecular correlates of adaptation in young animals because it can be manipulated and measured with modern molecular tools. However, to date, studies investigating the comprehensive brain proteome or synapse proteome have been performed on the brain of the adult zebrafish.[1,2] Recently, Nolte et al. used liquid chromatography mass-spectrometry (LC-MS) combined with stable heavy isotope labeling and identified 2159 brain proteins.[3] While such reports provide a good basis for adult zebrafish data, no studies to date have focused on the brain proteome of the larval zebrafish. Here, we present the zebrafish larvae brain proteome, comprising 5929 proteins, making it the most comprehensive zebrafish brain proteome published to date. Furthermore, we have identified 57 significantly altered proteins in larvae pre-exposed to an environmental challenge.

One challenging environment, which zebrafish larvae encounter in the wild, is strong water currents. An assay simulating this environment has recently been developed.[4,5]

We adopted the protocol of Castillo-Ramirez et al., and exposed 5 dpf old *Konstance wild type* (wt) zebrafish larvae to a water vortex flow for 9 hours (h) and then re-exposed them to the same stimulus 15 h late (**Figure 1**A). Larvae exposed to this protocol initially show an acute startle reaction at the onset of the vortex flow, which correlates with the initial fast displacement away from the center of the petri dish, that is, the source of the vortex flow.[4,5] Upon re-exposure to vortex flows, they display reduced startle reactions and reduced glucocorticoid reactivity.[4,5] We confirmed these results by comparing the mean distance to center at the onset of vortex flow between naïve and pre-exposed larvae. We observed that pre-exposed larvae show reduced startle reactions and therefore occupy a position that is, on average, at a shorter distance from the source of the vortex flow (Figure 1B,C). Further, similar to Castillo-Ramierez et al., we observed that upon re-exposure, pre-exposed larvae released significantly less cortisol, compared to naïve larvae (Figure 1D). Altogether, these results point toward an experience-dependent physiological and behavioral flexibility that could support adaptation to the environmental challenge.

To determine whether such adaptive processes could be supported by proteomic changes, we identified proteins regulated in

Dr. K. Langebeck-Jensen, Dr. O. D. Shahar, Prof. E. M. Schuman, Dr. J. D. Langer
Max Planck Institute for Brain Research
60438 Frankfurt, Germany
E-mail: erin.schuman@brain.mpg.de; Julian.langer@brain.mpg.de
Dr. J. D. Langer
Max Planck Institute of Biophysics
60438 Frankfurt, Germany
Prof. S. Ryu
University Medical Center
Johannes Gutenberg University Mainz
55131 Mainz, Germany
E-mail: soojin.ryu@uni-mainz.de
Prof. S. Ryu
German Resilience Center
Johannes Gutenberg University Mainz
55131 Mainz, Germany

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/pmic.201900028

DOI: 10.1002/pmic.201900028

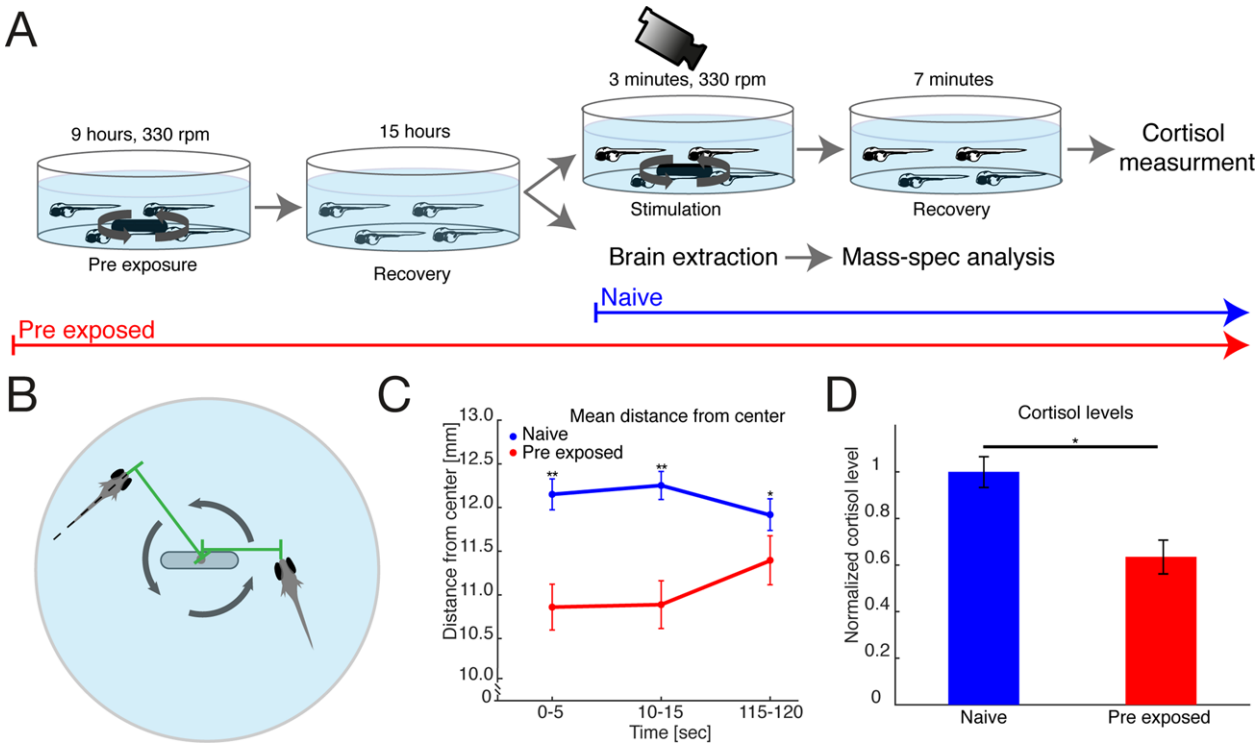

Figure 1. The position and cortisol levels of naïve and pre-exposed larvae during the stimulation assay. A) Schematic overview of the experimental procedures. B) A drawing showing examples of the water current induction (grey, center) from top and the measure used for distance from center (green). C) Plots indicating the mean distance from the center. $N = 44$ for naïve and 22 for pre-exposed. D) Bar plot showing the relative cortisol responses of naïve and pre-exposed larvae. The cortisol response of pre-exposed larvae treated in parallel with larvae used for MS showed a significant decrease in cortisol response. Y-axis: mean cortisol level of normalized to the cortisol level of naïve larvae, total $n = 11$ and 15 larvae for the naïve and pre-exposed respectively, three independent experiments were done. $*p < 0.05$, $**p < 1E^{-6}$ (Kruskal–Wallis).

response to the early exposure to water vortex assay by combining the assay with LC-MS analyses of the larval brain tissue (outlined in Figure 1A). In total, five or six biological replicates of 20 brains from pre-exposed larvae and naïve control siblings, respectively, were collected across two independent experiments and analyzed in three technical replicate LC-MS/MS runs. To identify proteins, we required peptide detection in a minimum of two out of three technical replicates. Our master dataset, comprising proteins from both the pre-exposed and naïve groups, contained a total of 5929 protein groups. We identified 2438 protein groups (44.01%) in all 11 samples and 3983 protein groups (72.04%) in at least six samples. In pre-exposed larvae, 2498 proteins species (47.75%) were detected in all five biological replicates and 3816 (70.92%) were detected in at least three biological replicates. In naïve larvae, 2599 protein groups (47.01%) were detected in all six biological replicates and 4079 protein groups (80.27%) were detected in at least three biological replicates. This suggests that protein identification between the two groups was consistent and comparable.

To assess the functional characteristics of the proteins identified in the brain proteome, we performed an enrichment analysis for proteins enriched within zebrafish anatomical terms using the Database for Annotation Visualization and Integrated Discovery (DAVID).[6] 2382 of the 5929 proteins had assigned zebrafish anatomical terms. The top 15 enriched terms included ten terms that were directly related to brain expression (Table S1, Supporting Information). The terms with the highest enrichment scores include the brain regions: optic tectum, alar plate midbrain region, central nervous system, cranial ganglion, midbrain, and cerebellum. We found three terms related to eye, which are probably derived from eye tissues that were co-purified (Table S1, Supporting Information). Taken together, we observed a clear enrichment of brain-specific terms, confirming the successful purification of brain tissue using our experimental approach. Next, we examined which biological processes we could monitor within the master proteome. We performed a GO annotation analysis using Panther[7] and searched against the zebrafish *Danio rerio* reference genome database (ZFIN).[8] We identified proteins that were significantly enriched within a number of terms (**Figure 2**). Enrichment of the terms synaptic transmission and neurotransmitter secretion suggested that we detected some neuron-specific proteins. Additionally, enrichment of the terms chromatin organization along with terms involving translation and mRNA splicing, processing, and localization, indicated the detection of proteins associated with gene regulation.

To investigate the brain proteome changes in pre-exposed larvae, we compared the abundance of brain proteins between pre-exposed and naïve larvae using Label-free Quantitation (LFQ)–based analysis. We identified 57 proteins that exhibited a significant difference in abundance. Out of these, 41 were more abundant in pre-exposed animals, and 16 were less abundant (**Figure 3**). Next, we tested if there was an enrichment

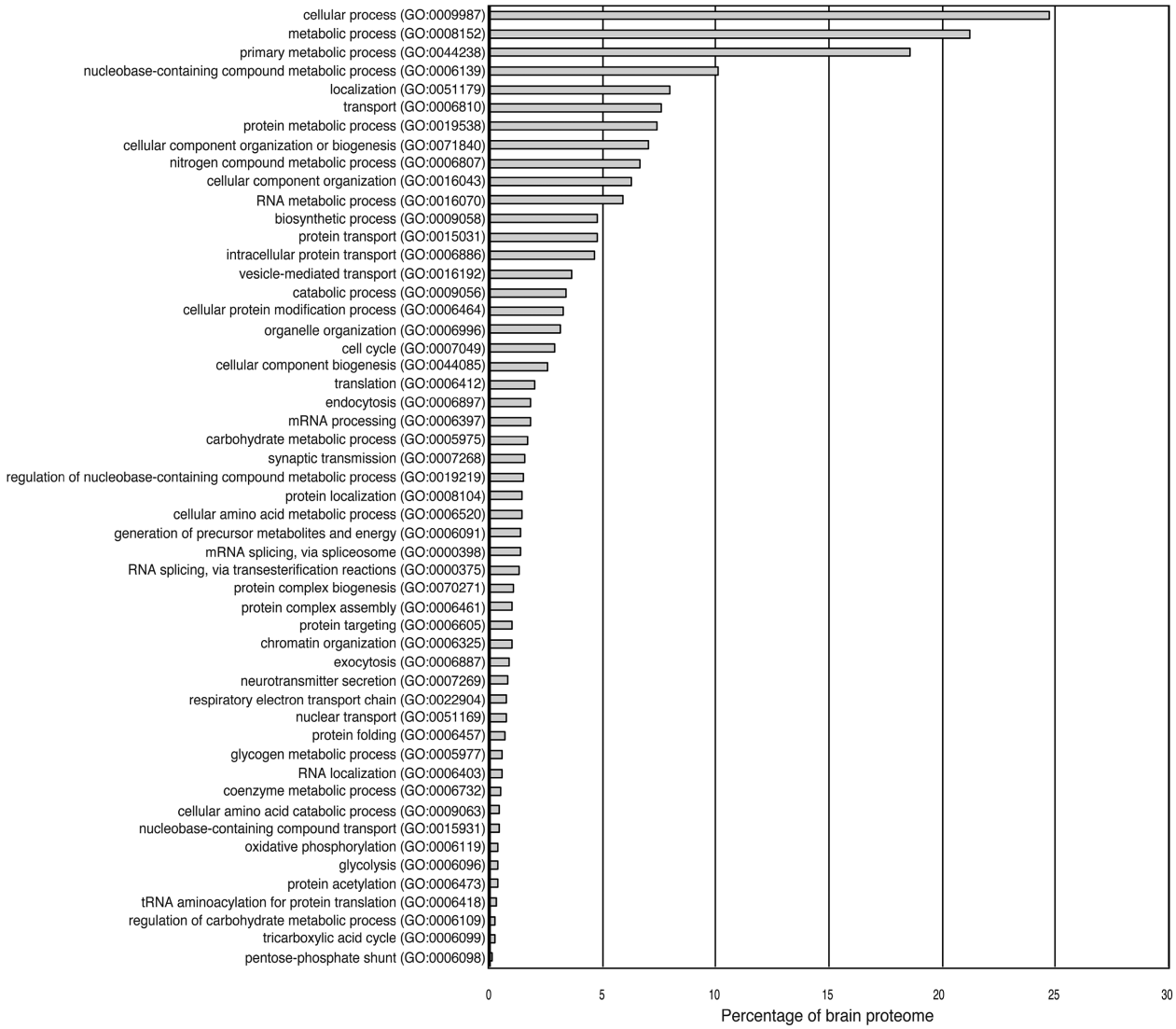

Figure 2. GO analysis of master proteome showing significantly enriched terms for biological roles and the percentage of the larval brain proteome enriched within them. Panther analysis, p-value < 0.05.

of biological roles related to the environmental stimulus within the 57 regulated proteins using a DAVID functional annotation analysis. Since the zebrafish database contains only limited behavioral information, we performed the test using *Mus musculus* protein orthologs and the corresponding database. We identified enriched proteins associated with terms such as learning, learning and memory, and behavior (Table S2, Supporting Information). Further, we observed enrichment for terms involved in brain development, namely "regulation of organelle organization," "tube morphogenesis," and "chordate embryonic development." In addition to the GO analysis, we manually categorized the 57 regulated proteins based on functional information from multiple species (using pubmed.org and www.genecards.org). The proteins were divided to four groups: "neuronal plasticity," "general neuronal role," "general role," and "uncharacterized" (Table S3, Supporting Information). Fourteen proteins were assigned to "neuronal plasticity" and 15 proteins to "general neuronal role," making a total of 29 proteins that were previously described to have neuronal functions. 17 proteins were described to play a more general role and 11 proteins have yet to be described.

Several stress response proteins were down-regulated in pre-exposed animals, including Heat Shock Protein alpha (Hsp90aa) and Methyl-CpG-binding-protein 2 (Mecp2). In rats, HSP90A plays a role in generating the pulsatile gene expression induced by glucocorticoid receptor activation.[9] MECP2 is implicated in the Retts syndrome and is involved in experience-dependent epigenetic programming in mammals.[10] Early life stress has been shown to cause hypomethylation in CpG residues that serve as DNA-binding sites for MECP2 and induce phosphorylation of MECP2.[11]

Pre-exposed larvae showed adjustment in locomotion, startle response, and glucocorticoid reactivity,[4,5] (Figure 1B–D). Hence, we hypothesized that proteins involved in spatial learning might be regulated in response to prolonged exposure to water vortex flows. Indeed, we identified Asic1b, Kcnip3a, and Aplp2 as significantly altered proteins. Mammalian orthologs of these

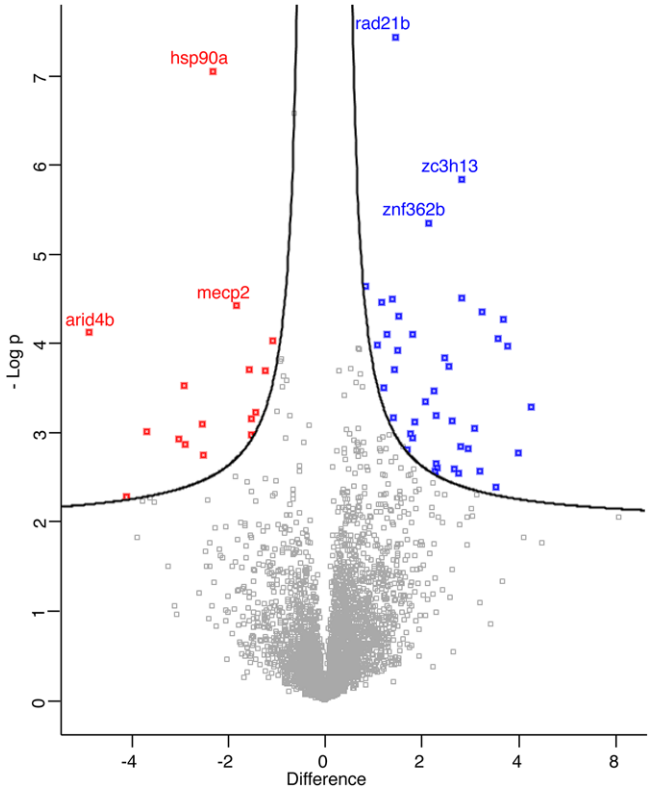

Figure 3. Quantitative analysis of the protein abundances. Volcano plot showing the differences in protein abundance between behaviorally adapted and naïve larvae. The 57 proteins above the asymptotic curves exhibited a significant difference in abundance, with 16 less abundant (red) and 41 more abundant proteins. Each dot represents one protein group, *x*-axis = fold difference between behaviorally adapted and naïve groups, asymptotic line = significance cutoff defined by a false discovery rate of 0.05 and an s0 value of 0.15.

proteins have been shown to play a role in experience-dependent behavioral alteration.[12–14] Since neurite growth is crucial for the formation of new neuronal connections during learning, we examined whether any of the 57 regulated proteins are known to be involved in neurite growth and guidance. We identified proteins that are known to play a role in axonal growth or guidance in zebrafish or mice: Olfm1,[15] Rab33a,[16] Fmn2b,[17] and Ntn1b.[18] These four proteins were up regulated in pre-exposed larvae.

Comparing our dataset to previously published brain proteomes for adult zebrafish, we found that our dataset constitutes the most comprehensive zebrafish brain proteome, published to date for zebrafish. We detected more than double the number of proteins identified in earlier studies.[3,19–21] To test the coverage of the larval brain proteome against the adult brain, we compared our dataset to the adult zebrafish brain proteome published by Nolte et al. In this study the authors identified 2159 brain-expressed proteins.[3] Our dataset overlapped with ≈85% of the Nolte et al. brain proteome. The remaining ≈15% of proteins not covered by our dataset are likely missing due to the differences in age and genetic background of the zebrafish used to generate the two proteomes.

In summary, in this study, we defined the zebrafish larval brain proteome and examined how the larval brain proteome was altered by exposure to an environmental challenge. We identified 5929 brain proteins, more than doubling the number of identified proteins detected in adult zebrafish,[3] and identified 57 proteins that were significantly changed in pre-exposed larvae. These proteins covered multiple functions including general brain functions, neuronal plasticity, the stress response, axonal growth and guidance, spatial learning, and the energy metabolism. This larval brain proteome provides a valuable reference proteome for future studies. The next steps would be to determine which of the regulated proteins are crucial for the adaptation and to investigate protein regulation on a long-term basis. In addition, cell-type specific labeling, visualization, and quantification of selected proteomes will provide more detailed understanding of proteome remodeling in the zebrafish brain in response to environmental challenges.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank Dr. Luis Castillo-Ramirez and Dr. Rodrigo De Marco for sharing information before publication and providing valuable comments. The authors thank Jan Glüsing, Hans Siegert, Gabriele Wexel, and Anett-Yvonn Loos for expert fish care. O.D.S was funded by an EMBO Long-Term Fellowship, and the Marie Sklowdoska-Curie grant 628003. S.R. is funded by the Johannes Gutenberg University Medical School, BMBF (grant number 01G1404), and DFG CRC 1193. E.M.S. is funded by the Max Planck Society, an Advanced Investigator award from the European Research Council (grant agreement no 743216), DFG CRC 1080, DFG CRC 902, DFG CRC 1193.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

adaptation, brain, environmental challenge, proteomics, zebrafish

Received: January 16, 2019
Revised: May 2, 2019

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