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Proteomic Response of the Brain to Hypoxic Stress in Marine Medaka Fish (*Oryzias melastigma*)

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Hypoxia is a serious issue that affects the marine environment, with a growing number of hypoxic "dead zones" occurring each year. Reports have indicated that hypoxia is detrimental to the reproductive function and sexual development of fish *via* the disruption of endocrine signaling in organs involved in the hypothalamus–pituitary–gonad axis, including the brain. While we previously reported that hypoxia induces transcriptome-wide alterations in the brain of marine medaka (*Oryzias melastigma*), whether these effects were reflected at the protein level remains unclear. Therefore, the present study used high-throughput proteomic sequencing along with bioinformatics analysis to assess the short-term and multi-generational effects of hypoxia on the brain proteome of *O. melastigma*. We identified 36,567 peptides and 7,599 proteins (1% false discovery rate in brain samples), with functions involved in cellular and metabolic processes such as signaling and reproductive processes as well as energy production and conversion. Furthermore, we determined that hypoxia resulted in the significant differential expressions of 33 upregulated and 69 downregulated proteins in the short-term exposure group and 24 upregulated and 52 downregulated proteins in the multi-generational exposure group. Pathway enrichment analysis of the deregulated proteins indicated that hypoxia could impair brain function by altering arachidonic acid metabolism, tight junctions, and adrenergic signaling under short-term hypoxic exposure and by altering p53 and PI3K–Akt signaling under multi-generational hypoxic exposure, which may lead to the onset of neurodegenerative disorders including Alzheimer's disease and amyotrophic lateral sclerosis. Ingenuity pathway analysis of the deregulated proteins showed that hypoxia affected common signaling pathways in the brain (e.g., integrin, paxillin, and epithelial adherens junction signaling) under both short-term and multi-generational exposures. Hypoxia also deregulated pathways specific to short-term exposure (including integrin-linked kinase, calcium, and

integrin signaling) and multi-generational exposure (including sphingosine-1-phosphate signaling, endocannabinoid neuronal synapse pathway, and endoplasmic reticulum stress pathway). Overall, our results provide additional insights into the mechanisms of hypoxia disrupting neuronal function at the protein level in marine medaka.

Keywords: hypoxia, brain, proteomic, fish, stress

INTRODUCTION

Hypoxia, at levels of dissolved oxygen (DO) <2.0 mg L^{-1}, is becoming increasingly prevalent in aquatic environments. Notably, the global number of hypoxic "dead zones" has been increasing exponentially since the 1960s (Diaz and Rosenberg, 2008; Galic et al., 2018). The occurrence of aquatic hypoxia is partly exacerbated by water pollution in the form of excess nutrients from agricultural and urban waste, which leads to eutrophication (Conley et al., 2009; Jenny et al., 2016). Decreased oxygen levels generally cause adverse effects on the physiology and behavior of marine biota (Pollock et al., 2007; Galic et al., 2018). For example, hypoxia reduced the swimming speed in fish such as the Atlantic cod and dogfish (Metcalfe and Butler, 1984; Schurmann and Steffensen, 1994). Hypoxia also delayed development and growth in zebrafish embryos (Kajimura et al., 2005) and reduced the spawning, fertilization, and hatching success rates in common carp (Wu et al., 2003).

Hypoxia and its induced protein, hypoxia-inducible factor 1 (HIF-1), have been linked with several neurodegenerative diseases (Zhang et al., 2011). For example, amyotrophic lateral sclerosis (ALS) is characterized by damaged motor neurons, and hypoxia may cause ALS pathogenesis by inducing motor neuron death through impairment of HIF-1-regulated vascular endothelial growth factor (VEGF) signaling (Skene and Cleveland, 2001). While VEGF regulates blood supply and angiogenesis and is normally upregulated in response to hypoxia, ALS-like symptoms develop upon loss of the hypoxia response element in the VEGF promoter, and people with impaired VEGF activity have higher susceptibility to ALS when working in environments with a high probability of hypoxic exposure (Oosthuyse et al., 2001; Skene and Cleveland, 2001; Vanacore et al., 2010). Another example is Alzheimer's disease, which is caused by neuron damage due to the aggregation of amyloid β (Aβ) peptides (Peers et al., 2009). Since hypoxia has been shown to increase the amyloid precursor protein (APP) levels, which are then converted to Aβ peptides (Peers et al., 2009; Zhang et al., 2011), it may be a driver of Alzheimer's disease. Hypoxia was also reported to influence the progression of Parkinson's disease, which is characterized by the loss of dopaminergic neurons (Zhang et al., 2011). For adult rat models of Parkinson's disease, exposure to intermittent hypoxic episodes was found to partially restore dopamine synthesis in the right hemisphere of the brain and increase serum antioxidant activity; therefore hypoxic conditioning may delay neurodegeneration in Parkinson's disease (Belikova et al., 2012).

Notably, multi-generational exposure to mild hypoxia can induce antioxidant responses in various neuronal cell types in the brain to promote survival and protection against oxidative stresses, such as through increased PI3K–Akt signaling and erythropoietin expression in astrocytes, increased MAPK signaling, and brain-derived neurotrophic factor expression in cortical neurons (Terraneo and Samaja, 2017; Terraneo et al., 2017). Cerebral blood and oxygen circulation is also affected by multi-generational hypoxia through increased angiogenesis and capillary density as well as elevated cerebral blood flow associated with increased nitric oxide (NO) synthesis and metabolism (Xu and LaManna, 2006; Terraneo and Samaja, 2017). In addition to antioxidant responses, multi-generational hypoxia simultaneously increases reactive oxygen species (ROS) production in the brain through the mitochondrial electron transport chain, thereby resulting in redox imbalance (Terraneo and Samaja, 2017; Terraneo et al., 2017). An overall increase in the ROS levels leads to brain damage, with neuron apoptosis occurring through the release of mitochondrial apoptotic proteins into the cytosol (Turrens, 2003; Coimbra-Costa et al., 2017).

The brain forms part of the hypothalamus–pituitary–gonad (HPG) axis, which is responsible for the regulation and production of hormones involved in reproductive function (Thomas and Rahman, 2009). Hypoxia has been reported to affect the hormone levels, reproductive development, and sex differentiation in fish (Wu et al., 2003; Yu and Wu, 2006). Moreover, hypoxia alters the proteomic profile of *Oryzias melastigma* testes in a transgenerational manner (Wang et al., 2016). We previously demonstrated that the transcriptomic changes in the brains of *O. melastigma* upon hypoxia exposure were related to brain function and development, including synaptic transmission and potassium ion transport (Lai et al., 2016). However, it remains unknown whether changes at the transcriptome level are reflected at the protein level. Therefore, in the present study, we identified changes to the brain proteome of *O. melastigma* in response to short-term and multi-generational hypoxia exposure.

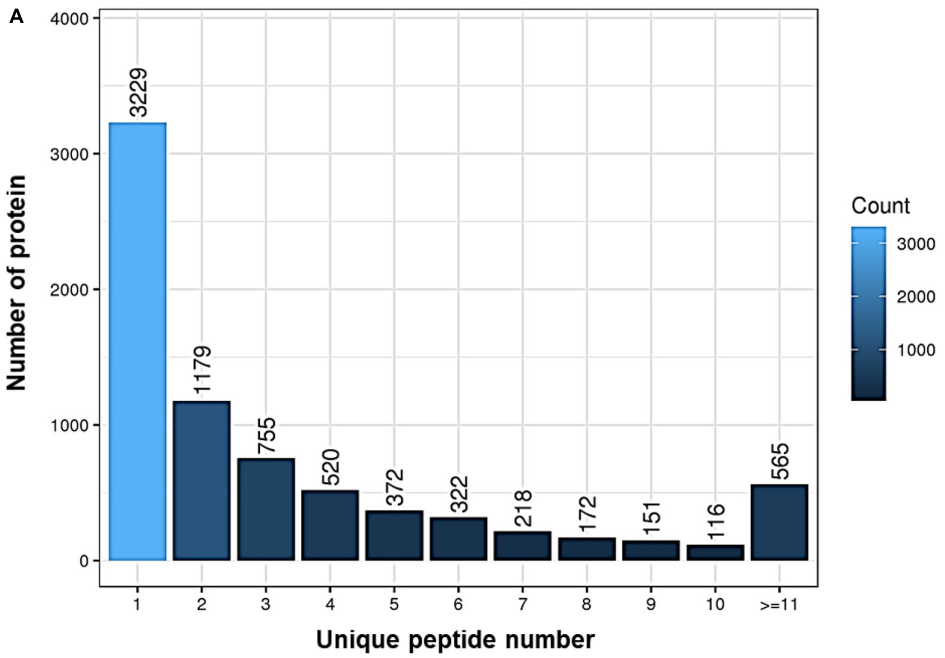
MATERIALS AND METHODS

Fish Maintenance and Hypoxia Exposure

Approval for the experimental setup involving fish was obtained from the Committee on the Use of Live Animals in Teaching and Research (CULATR, no. 2714-12) at the University of Hong Kong. *O. melastigma* were kept at 5.8 mg O_2 L^{-1}, 28 ± 2°C, pH 7.2, in a daily 14:10-h light/dark cycle, as previously described (Wang et al., 2016), until the start of the hypoxic/normoxic exposure experiment. For short-term exposure, sexually mature male *O. melastigma* were exposed to normoxic (5.8 ± 0.2 mg O_2 L^{-1}) or hypoxic (1.5 ± 0.2 mg O_2 L^{-1}) conditions, as

TABLE 1 | Proteome statistics in the brain of *Oryzias melastigma*.

	Total spectra	Spectra	Unique spetra	Peptide	Unique peptide	Protein
Identified number	1,235,781	218,746	157,189	36,567	30,345	7,599



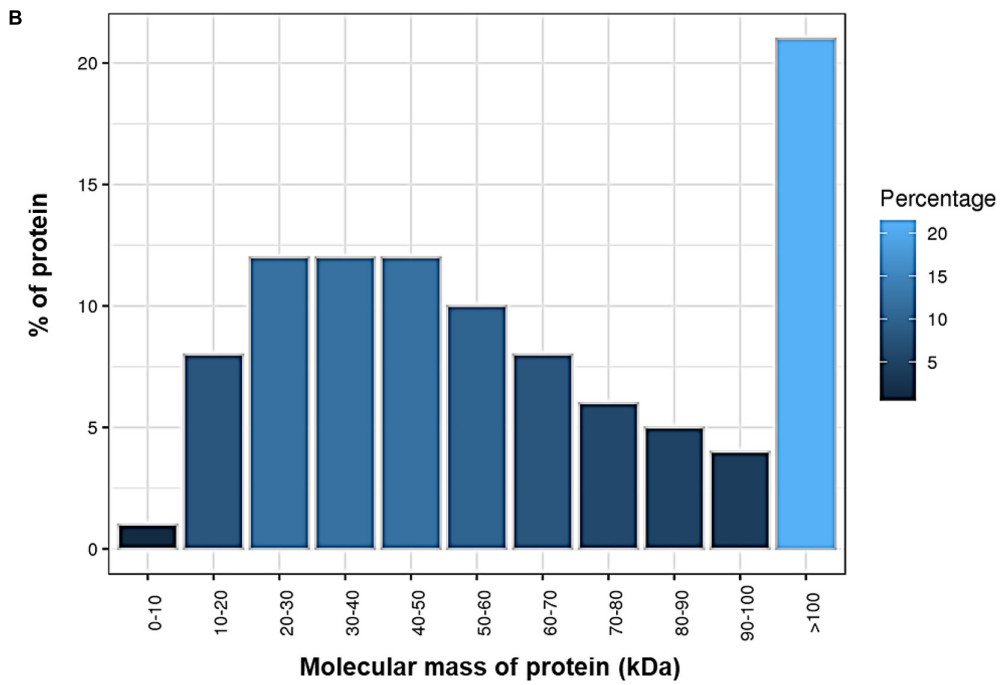


FIGURE 1 | Identification of proteins in the brain of *Oryzias melastigma* using isobaric tags for relative and absolute quantitation (iTRAQ) technology. **(A)** Number of proteins with different unique peptide numbers. **(B)** Percentages of proteins with different molecular masses.

described in Lai et al. (2016), for a period of 1 month. For multi-generational exposure, sexually mature male medaka fish were continuously exposed to either normoxic or hypoxic conditions for three generations. The progeny of every generation mated and continued to be exposed to hypoxia or normoxia, and the mate pairs (both males and females) were also kept in either normoxic or hypoxic conditions. The desired DO levels were achieved by continuously passing a constant ratio of premixed air and nitrogen through a 4-cm-wide gas-stripping column into a 300-L reservoir tank. A DO meter (YSI model 580) was used to monitor the DO levels twice per day. The temperature, salinity, pH, and ammonia levels were also kept constant and monitored daily throughout the exposure period.

Protein Extraction From Brain Tissues for iTRAQ

Total protein was extracted from the brain tissues of nine fish from each treatment group. Three brain tissues were pooled together into a single biological replicate, and three replicates were used for iTRAQ (isobaric tags for relative and absolute quantitation) analysis. Proteins were extracted using lysis buffer 3 [8 M urea, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM triethylammonium bicarbonate (TEAB)/Tris–HCl, 2 mM EDTA, and 10 mM dithiothreitol (DTT), pH 8.5] and two 5-mm magnetic beads. Proteins were released from the mixture using a TissueLyser (2 min, 50 Hz). The mixture was centrifuged at 25,000 × *g*, 4°C, for 20 min. The supernatant was transferred to a new tube, reduced using DTT (10 mM) at 56°C for 1 h, and then alkylated in the dark with iodoacetamide (IAM, 55 mM) at 25°C for 45 min. The supernatant was centrifuged at 25,000 × *g*, 4°C, for 20 min, and then the supernatant containing the proteins was collected. The Bradford assay was used to quantify the total protein concentration of the supernatant.

Protein Digestion and Peptide Labeling

The solution containing 100 μg protein in 8 M urea was diluted four times using TEAB (100 mM). The proteins were digested with Trypsin Gold (Promega, Madison, WI, United States) at a 40:1 protein/trypsin ratio at 37°C overnight. The digested peptides were desalted using a Strata-X C18 column (Phenomenex) and then vacuum-dried. The peptides were reconstituted in 30 μl of TEAB (0.5 M) and vortexed. The iTRAQ Reagent 8Plex Kit was used for iTRAQ labeling of the peptides following the manufacturer's instructions. The peptides were then pooled and passed through a Strata-X C18 column (Phenomenex) for desalting and afterward vacuum-dried.

Peptide Fractionation

The peptides were dissolved in 2 ml of buffer A [95% H_2O, 5% acetonitrile (ACN), pH adjusted to 9.8 with ammonia] and then loaded onto a high-pH RP column (particle size, 5 μm; Phenomenex). The peptides were passed through the column using a Shimadzu LC-20AB HPLC Pump system at a flow rate of 1 ml min^{-1} using the following gradient of buffer B (5% H_2O, 95% ACN, pH adjusted to 9.8 with ammonia): 5% for 10 min, then 5–35% for 40 min, followed by 35–95% for 1 min and then 95% for 3 min. The gradient of buffer B was then decreased to 5% within 1 min and then maintained at 5% for 10 min. The peptides were eluted in fractions separated at 1-min intervals while monitoring absorbance at 214 nm. A total of 20 fractions were collected and vacuum-dried.

High-Performance Liquid Chromatography and Mass Spectrometry Detection

For each peptide fraction, buffer A [0.1% formic acid (FA) and 2% ACN in H_2O] was added and the reconstituted fraction was centrifuged for 10 min at 20,000 × *g*. The supernatant was then loaded using the autosampler onto the C18 trap column of the LC-20AD nano-HPLC instrument (Shimadzu, Kyoto, Japan) at a rate of 5 μl min^{-1} for 8 min. The fraction was passed through the trap column and the peptide separation was performed on an self-packed 75-μm-wide analytical C18 column at a rate of 300 nl min^{-1} using a buffer B (0.1% FA and 2% H_2O in ACN) gradient going from 8 to 35% in 35 min, then up to 60% in 5 min. The gradient was kept at 80% for 5 min and then decreased to 5% within 1 min, and the column was then equilibrated for 10 min. Mass spectrometry was done using the TripleTOF 5600 System (SCIEX, Framingham, MA, United States) fitted with a Nanospray III source (SCIEX, Framingham, MA, United States), with a pulled-quartz tip emitter (New Objectives, Woburn, MA, United States), with the setting of the ion spray voltage at 2,300 V, curtain gas and nebulizer gas of 30 and 15, respectively, and the interface heater temperature set at 150°C. Data were collected using the Analyst 1.6 software (AB SCIEX, Concord, ON, United States) on high-sensitivity mode. With a MS1 accumulation time of 250 ms, the mass range for scanning set at 350–1,500 Da, and filtering for MS scans exceeding 150 counts per second with a charge state between 2 + and 5 + and dynamic exclusion of half the peak width (12 s), up to 30 product ion scans were selected for collision-induced dissociation (CID). The collision energy was adjusted for precursor ions for CID, and a Q2 transmission window at 100% for 100 Da was used for iTRAQ data collection.

Bioinformatics Analysis and Protein Quantification

Using tandem mass spectrometry (MS/MS) data in MGF format as input, the peptides labeled with isobaric tags were quantified using IQuant software (Wen et al., 2014). The Mascot Percolator software package was used to rescore the peptide-spectrum matches (PSMs). PSMs were pre-filtered at a false discovery rate (FDR) of 1%, and then used for protein set assembly. Protein-level FDRs for the identified set of proteins were estimated using the picked protein FDR strategy (Savitski et al., 2015), and the identified proteins at a protein-level FDR < 0.01 were used for pathway, Clusters of Orthologous Groups (COG)/Eukaryotic Orthologous Group (KOG), and Gene Ontology (GO) analyses. Differentially expressed proteins (DEPs) were identified based on the filtering criteria of relative fold change > 1.2 and q-value < 0.05. The list of DEPs were used for additional GO,

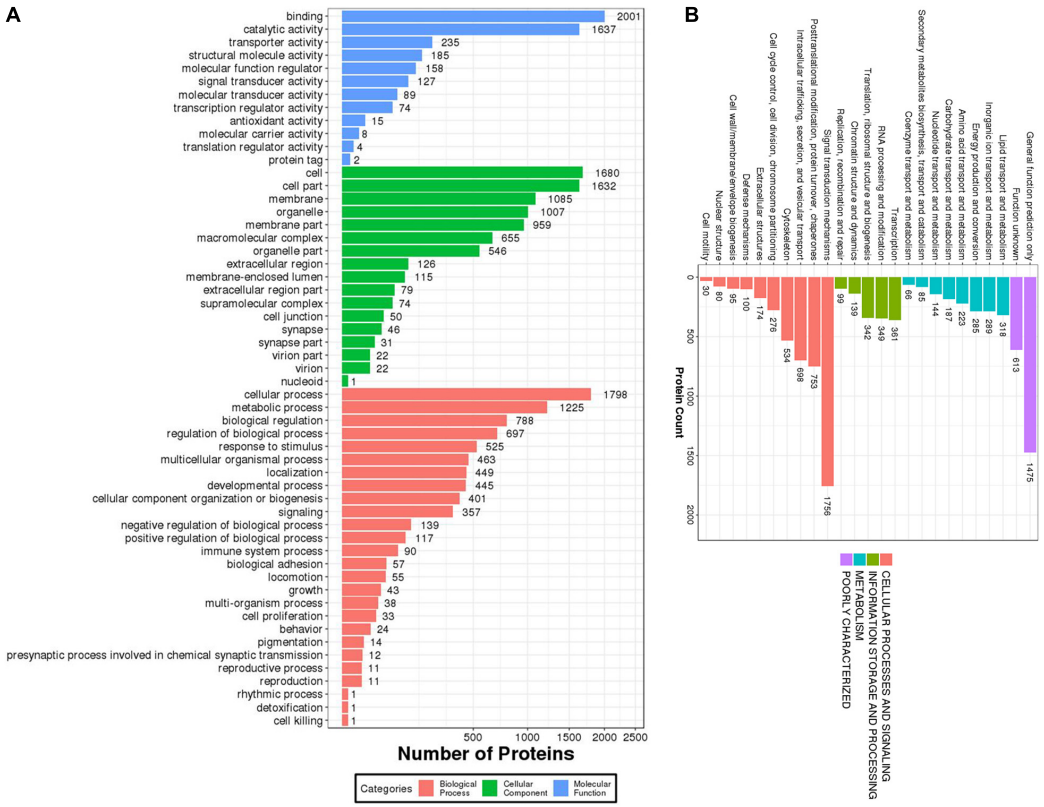


FIGURE 2 | Functional characterization of the identified proteins in the brain of *Oryzias melastigma*. **(A)** Gene Ontology (GO) annotation of the biological processes, cellular components, and molecular functions mediated by the identified proteins. **(B)** Eukaryotic Orthologous Group (KOG) annotation of the cellular processes and signaling, information storage and processing, and metabolism mediated by the identified proteins.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and COG/KOG analysis.

RESULTS

Protein Identification in the Brain of *O. melastigma*

By using the iTRAQ technology, a total of 1,235,781 spectra were generated, and 36,567 peptides and 7,599 proteins were identified at a FDR cutoff < 0.01 in the brain samples (**Table 1**). Our results demonstrated that 3,229 of the identified proteins originated from a single peptide (**Figure 1A**) and over 20% of the proteins had a molecular mass over 100 kDa (**Figure 1B** and **Supplementary Table 1**).

Classification of Identified Proteins in the Brain of *O. melastigma*

The proteins identified in the brain of *O. melastigma* were subjected to GO and KOG annotation to determine their functional roles in the brain. In GO annotation, the molecular functions, cellular components, and biological processes of the proteins were classified (**Figure 2A**). Our results indicated that the majority of these proteins were involved in binding and catalytic activity and were also responsible for structural molecule activity and signal transducer activity (**Figure 2A**). Many of the proteins were localized to the cell membrane, organelle, macromolecular complex, as well as the synapse (**Figure 2A**). In the analysis of biological processes, our results identified enrichment in terms of cellular and metabolic processes. Furthermore, these proteins also served roles in response to stimuli, developmental process, signaling, cell proliferation, and reproductive process (**Figure 2A**). Then, KOG annotation was conducted by matching with similar protein sequences of multiple reference genomes for functional and evolutionary genome analysis to cover three categories: (1) metabolism, (2) information storage and processing, and (3) cellular processing and signaling (**Figure 2B**). We identified the involvement of these proteins in the transport and metabolism of a large number of molecules, including lipids, inorganic ions, amino acids, carbohydrates, and coenzymes. Additionally, energy production and conversion were also highlighted (**Figure 2B**).

Effect of Hypoxia on Differential Protein Expression

A comparative proteomic analysis was conducted to determine the differential protein expression in hypoxic brains of *O. melastigma*. Our results indicate that short-term hypoxia

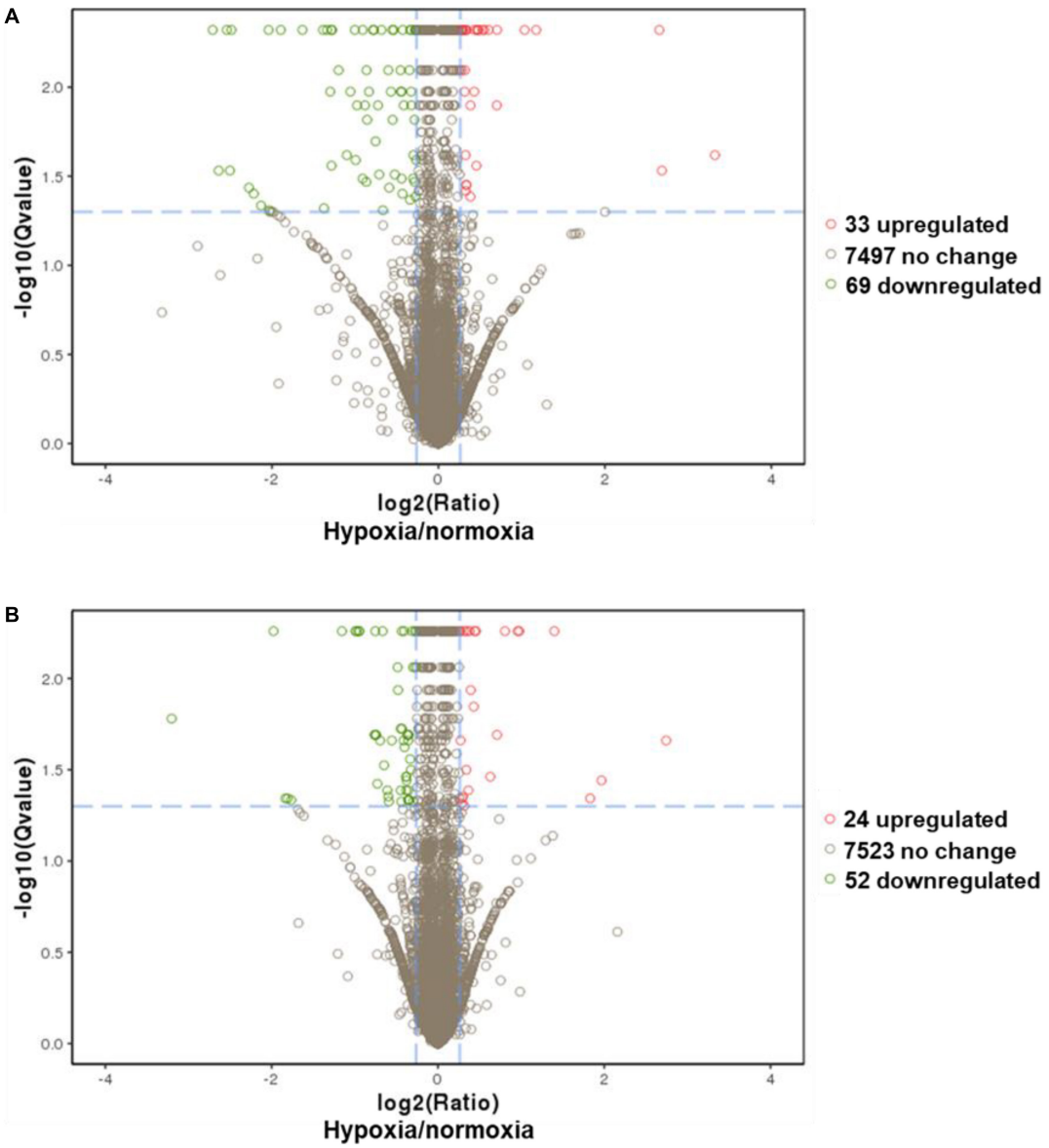


FIGURE 3 | Differential expressions of proteins in the brain of *Oryzias melastigma* caused by hypoxia. **(A)** Short-term hypoxia exposure causes the deregulation of protein expression. **(B)** Multi-generational hypoxia exposure causes the deregulation of protein expression. The *x*-axis represents the relative expression of proteins (hypoxia/normoxia). The *y*-axis represents the -log(*q*-value). The cutoffs for the determination of differentially expressed proteins are -log(*q*-value) >1.3 and hypoxia to normoxia ratio > 1.2. *Green bubbles* represent downregulated proteins and *red bubbles* represent upregulated proteins under hypoxia exposure.

exposure could lead to the deregulated expression of 33 upregulated and 69 downregulated proteins in the F0 generation (**Figure 3A** and **Table 2**). In the multi-generational exposure group, we observed the upregulation of 24 proteins and the downregulation of 52 proteins relative to the corresponding normoxic group (**Figure 3B** and **Table 3**). Then, we compared the identified differentially expressed proteins with our previous report of transcriptome data (Lai et al., 2016). We found five and seven common deregulated genes–proteins in short-term and multi-generational hypoxia exposures, respectively (**Table 4**).

Effects of Hypoxia on Neural Functions

To understand the neurotoxicological effects of hypoxia, the hypoxia-induced DEPs were subjected to GO and pathway enrichment analysis. Through GO enrichment analysis, we determined that short-term (**Figure 4A** and **Supplementary Table 2**) and multi-generational (**Figure 4B** and **Supplementary Table 3**) hypoxia exposures could lead to significantly different alterations of the biological processes and molecular functions ($p < 0.05$). For biological processes, short-term hypoxia exposure affected a wide range of lipid metabolic processes such as phospholipids, glycerolipids, glycerophospholipids,

TABLE 2 | Differentially expressed proteins in the brain of *Oryzias melastigma* following short-term hypoxia exposure.

Protein description	Ratio (hypoxia/normoxia)	*q*-value
Regulation of nuclear pre-mRNA domain-containing protein 2-like isoform X1	0.153	0.005
PREDICTED: UDP-GlcNAc:betaGal beta-1,3-*N*-acetylglucosaminyltransferase 9 isoform X2	0.16	0.029
Fast skeletal myosin heavy chain isoform mMYH-2	0.172	0.005
Troponin T, fast skeletal muscle isoforms-like isoform X4	0.176	0.029
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 isoform X2	0.178	0.005
Serine/threonine–protein phosphatase 2A regulatory subunit B″ subunit alpha isoform X1	0.206	0.037
Oxidative stress-induced growth inhibitor 2	0.215	0.04
Myosin-6-like	0.228	0.046
Insulinoma-associated protein 1	0.243	0.005
Histone-lysine *N*-methyltransferase 2B-like	0.245	0.049
PDZ and LIM domain 7	0.269	0.005
Troponin T, fast skeletal muscle isoforms-like isoform X7	0.322	0.005
Titin-like	0.383	0.005
Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	0.386	0.048
Myosin-binding protein C, fast-type-like isoform X5	0.395	0.005
Myosin light chain 3	0.406	0.011
Actin, alpha skeletal muscle	0.41	0.005
Fast skeletal myosin heavy chain isoform mMYH-11	0.411	0.028
Myomesin-2-like	0.413	0.005
Alpha-actinin-3	0.436	0.008
Titin-like	0.466	0.024
Integrin alpha-11-like	0.481	0.011
PREDICTED: nebulin	0.499	0.005
PREDICTED: serine protease 23-like	0.502	0.026
Titin-like	0.508	0.013
Glutathione S-transferase kappa 1	0.532	0.033
Natterin-like protein	0.533	0.005
Collagen alpha-1(XII) chain isoform X2	0.542	0.013
Creatine kinase U-type, mitochondrial	0.548	0.034
PREDICTED: myomesin-2-like isoform X2	0.55	0.008
Nuclear export mediator factor NEMF isoform X2	0.552	0.015
Myosin light chain 3, skeletal muscle isoform	0.561	0.011
Inositol monophosphatase 1-like	0.58	0.005
Complement C1q subcomponent subunit B-like	0.584	0.005
Glyceraldehyde-3-phosphate dehydrogenase	0.593	0.02
Nucleoside diphosphate kinase B-like	0.605	0.013
Nephrosin precursor	0.611	0.031
Collagen alpha-3(VI) chain	0.622	0.005
PREDICTED: dystrophin-like isoform X4	0.63	0.049
Centrosomal protein of 89-kDa isoform X2	0.659	0.008
Tropomyosin beta chain isoform X2	0.664	0.037
PREDICTED: BTB/POZ domain-containing protein KCTD5-like	0.673	0.011
Complement C1q subcomponent subunit B-like	0.681	0.005
Plexin-B1-like	0.683	0.015
Collagen alpha-1(X) chain-like	0.689	0.005
Complement component C3-1 precursor	0.695	0.031
Apolipoprotein Eb-like	0.729	0.008
KAT8 regulatory NSL complex subunit 1-like protein isoform X3	0.732	0.011
Protein cornichon homolog 3-like isoform X2	0.736	0.033
Cofilin-2-like	0.736	0.011
Serine/threonine–protein phosphatase 2A 55-kDa regulatory subunit B beta isoform isoform X2	0.741	0.04
Eosinophil peroxidase-like	0.743	0.005
PREDICTED: 40S ribosomal protein S27	0.749	0.013
Thiopurine S-methyltransferase isoform X1	0.786	0.008
CD81 antigen-like	0.79	0.043
Lipocalin-like	0.791	0.005
PREDICTED: peroxiredoxin-1	0.797	0.005
Epiplakin 1	0.797	0.011
mRNA turnover protein 4 homolog	0.798	0.013
Adenylosuccinate synthetase isozyme 2	0.81	0.033
Serine/threonine–protein kinase D1	0.812	0.024
Golgi-associated PDZ and coiled-coil motif-containing protein isoform X2	0.82	0.034
Vacuolar protein sorting-associated protein 37C-like isoform X3	0.82	0.015
Glycerol-3-phosphate dehydrogenase	0.823	0.005
Protein LEG1 homolog	0.825	0.005
LOW QUALITY PROTEIN: ubiquitin-like modifier-activating enzyme 6	0.826	0.041
Coiled-coil domain-containing protein 93	0.828	0.005
Trafficking protein particle complex subunit 5	0.83	0.026
Alcohol dehydrogenase class-3	0.831	0.005
Peroxisomal acyl-coenzyme A oxidase 1	1.206	0.005
Lysine–tRNA ligase isoform 2	1.212	0.005
Complexin-1	1.217	0.008
Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A-like	1.218	0.005
Uncharacterized protein C7orf57 homolog	1.219	0.005
Alpha-synuclein-like	1.229	0.005
Annexin A10	1.245	0.011
Reticulon-4 isoform X1	1.247	0.008
Prostaglandin E synthase 2	1.247	0.005
Fatty acid-binding protein, brain	1.254	0.024
60S ribosomal protein L7	1.256	0.005
Ankyrin repeat and MYND domain-containing protein 1	1.256	0.038
Uncharacterized protein LOC105356426	1.262	0.035
Bromo adjacent homology domain-containing 1 protein	1.262	0.035

(Continued)

TABLE 2 | Continued

Protein description	Ratio (hypoxia/normoxia)	*q*-value
Microtubule-associated protein 2	1.269	0.005
G protein-regulated inducer of neurite outgrowth 1	1.305	0.041
Hemopexin precursor	1.306	0.013
PREDICTED: sodium- and chloride-dependent GABA transporter 2-like	1.348	0.011
Retinol-binding protein 1	1.351	0.005
39S ribosomal protein L11, mitochondrial isoform X2	1.372	0.028
Alpha-type globin	1.381	0.005
High mobility group nucleosome-binding domain-containing protein 3 isoform X1	1.388	0.005
Beta-synuclein	1.394	0.005
Band 3 anion exchange protein	1.441	0.005
Histone H1-like	1.465	0.005
Hemoglobin subunit beta-1-like	1.518	0.005
Sorcin	1.627	0.013
Carbonic anhydrase-like	1.63	0.005
Uncharacterized protein LOC101175432	2.051	0.005
Hemoglobin embryonic subunit alpha-like	2.258	0.005
Myosin heavy chain, fast skeletal muscle	6.295	0.005
PREDICTED: protocadherin gamma-C5-like isoform X6	6.431	0.029
Myosin heavy chain, fast skeletal muscle	10	0.024

and phosphatidylinositols. Additionally, phosphate-containing compound metabolic processes, including phosphorus and organophosphate, were interrupted by short-term hypoxia exposure (**Figure 4A** and **Supplementary Table 2**). Interestingly, multi-generational hypoxia exposure led to the alteration of the different acid biosynthetic processes such as carboxylic acids, organic acids, and cellular amino acids. Additionally, multi-generational hypoxia exposure could trigger a response to toxic substances and result in cell death and apoptosis. More importantly, multi-generational hypoxia exposure can dysregulate the immune system (**Figure 4B** and **Supplementary Table 3**). Through pathway enrichment analysis, we determined that short-term hypoxia exposure altered the arachidonic acid metabolism and tight junctions in the brain tissue ($p < 0.05$) (**Figures 4C,D**). Furthermore, this could lead to hypertrophic cardiomyopathy through the dysregulation of adrenergic signaling and to Alzheimer's disease ($p < 0.05$) (**Figures 4C,D**). On the other hand, multi-generational hypoxia exposure could lead to a larger number of alterations in the brain of *O. melastigma* ($p < 0.05$). This could trigger the development of neuronal diseases such as Alzheimer's disease and amyotrophic lateral sclerosis ($p < 0.05$) (**Figures 4E,F**). Additionally, multi-generational exposure can also lead to tuberculosis, atherosclerosis, and various cancers, which possibly occur through the dysregulation of certain signaling pathways, such as the p53 signaling pathway and the PI3K–Akt signaling pathway ($p < 0.05$) (**Figures 4E,F**). Taken together, our results suggest that short-term and multi-generational exposures could lead to different neural responses in the brains of fish.

TABLE 3 | Differentially expressed proteins in the brain of *Oryzias melastigma* following long-term hypoxia exposure.

Protein description	Ratio (hypoxia/normoxia)	*q*-value
Hemoglobin subunit beta-like	6.703	0.022
FRAS1-related extracellular matrix protein 2-like	3.911	0.036
Coiled-coil domain-containing protein 181	3.557	0.045
Choline transporter-like protein 5-B isoform X1	2.638	0.006
Parvalbumin beta-like	1.97	0.006
Desmoglein-2-like isoform X2	1.944	0.006
Replication factor C subunit 5	1.748	0.006
Tartrate-resistant acid phosphatase type 5	1.636	0.02
Transposable element Tc3 transposase	1.547	0.035
Actin-related protein 3	1.372	0.006
Cellular retinoic acid-binding protein 1	1.358	0.006
Ictacalcin-like isoform X2	1.347	0.014
Uncharacterized protein LOC101175432	1.314	0.012
Lipocalin-like	1.302	0.006
Isoamyl acetate-hydrolyzing esterase 1 homolog isoform X2	1.289	0.041
60S ribosomal protein L7	1.27	0.006
Glutathione S-transferase A-like isoform X3	1.266	0.032
Apolipoprotein C-I-like	1.242	0.006
Dihydropyrimidine dehydrogenase	1.232	0.044
RNA-binding protein 4.1-like	1.221	0.045
Calpain-1 catalytic subunit-like	1.22	0.049
Mothers against decapentaplegic homolog 7	1.208	0.022
Cysteine-rich protein 1	1.202	0.006
LOW QUALITY PROTEIN: betaine–homocysteine S-methyltransferase 1-like	1.201	0.006
PREDICTED: peroxiredoxin-1	0.832	0.006
Hippocalcin-like protein 1	0.828	0.006
Complement component C3-2 precursor	0.827	0.009
Protein phosphatase 1 regulatory subunit 14A	0.814	0.047
Parvalbumin, thymic CPV3-like	0.812	0.009
Stathmin	0.807	0.006
Integrator complex subunit 10 isoform X3	0.798	0.032
Reticulon-4 isoform X1	0.793	0.028
PREDICTED: voltage-dependent L-type calcium channel subunit alpha-1C isoform X16	0.785	0.046
A-kinase anchor protein 7	0.785	0.046
Transketolase-like	0.785	0.02
Complement component C3-1 precursor	0.781	0.022
Band 3 anion exchange protein-like	0.781	0.041
Kazrin	0.778	0.046
PREDICTED: collagen alpha-1(I) chain-like isoform X1	0.777	0.02
Rho-related GTP-binding protein RhoA-B	0.774	0.02
LOW QUALITY PROTEIN: protein capicua homolog	0.769	0.036
Neurofilament heavy polypeptide isoform X2	0.768	0.041
Mitochondrial 10-formyltetrahydrofolate dehydrogenase isoform 2	0.768	0.035
G protein-regulated inducer of neurite outgrowth 1	0.768	0.035
28S ribosomal protein S36, mitochondrial	0.757	0.024

(Continued)

TABLE 3 | Continued

Protein description	Ratio (hypoxia/normoxia)	*q*-value
Complement C1q subcomponent subunit B-like	0.755	0.006
PREDICTED: anoctamin-4 isoform X1	0.751	0.022
Zinc finger protein 518A-like isoform X2	0.741	0.019
Somatotropin	0.74	0.006
Cytochrome c iso-1/iso-2	0.737	0.047
LOW QUALITY PROTEIN: serine hydrolase-like protein 2	0.733	0.041
Pro-FMRFamide-related neuropeptide FF	0.733	0.019
PREDICTED: ADP-ribosylation factor 1	0.717	0.012
Fatty acid-binding protein, brain	0.714	0.009
Nectin-3-like protein	0.681	0.022
NAD kinase-like isoform X2	0.664	0.047
von Willebrand factor A domain-containing protein 7-like	0.661	0.044
PREDICTED: thymosin beta-4	0.656	0.041
Fatty-acid amide hydrolase 1	0.638	0.03
Putative sodium-coupled neutral amino acid transporter 10	0.63	0.006
Semaphorin-6D-like isoform X1	0.617	0.022
Caspase 3B	0.603	0.038
Protein lin-37 homolog	0.598	0.02
Tributyltin-binding protein type 1 precursor	0.594	0.02
Allograft inflammatory factor 1-like	0.593	0.006
Otolith matrix protein 1-like	0.589	0.02
Legumain	0.519	0.006
Integrin alpha-11-like	0.518	0.006
PREDICTED: sodium- and chloride-dependent GABA transporter 2-like	0.508	0.006
Clathrin interactor 1-like	0.502	0.006
Torsin-3A	0.449	0.006
Myomegalin-like isoform X1	0.294	0.046
Inositol polyphosphate 5-phosphatase K	0.286	0.045
PREDICTED: fizzy-related protein homolog isoform X1	0.28	0.045
Retrotransposable element Tf2 protein type 1	0.254	0.006
Solute carrier family 22 member 2-like	0.109	0.017

TABLE 4 | Common deregulated genes and proteins after short-term and multi-generational hypoxia exposures.

Short-term hypoxia	Multi-generational hypoxia
COCA1	PPA5
OSGI2	S6A13
SYUA	FAAH1
CO3	KAZRN
KPCD1	PEG10
	SEM6D
	CAC1C

Mechanisms Underlying the Neurotoxicological Effect of Hypoxia

To elucidate the possible molecular mechanisms behind the effects of hypoxia on brain toxicology in fish, ingenuity pathway analysis (IPA) was conducted. The canonical pathway analysis in IPA highlighted both similar and different responses of the brain to short-term and multi-generational hypoxia exposures. The results indicate that both short-term and multi-generational hypoxia exposures could lead to the alteration of integrin signaling, paxillin signaling, Parkinson's signaling, epithelial adherens junction signaling, and D-*myo*-inositol (1,4,5)-trisphosphate degradation and metabolism (**Table 5**). However, only integrin subunit alpha 11 (ITGA11) and inositol polyphosphate-5-phosphatase J (INPP5J) were found to be commonly deregulated under these two hypoxia treatments. Furthermore, certain pathways that serve important roles in brain function were observed to be unique to either short-term or multi-generational hypoxia exposure. Under short-term hypoxia exposure, ILK signaling, tight junction signaling, calcium signaling, integrin signaling, RhoA signaling, CXCR4 signaling, thrombin signaling, mTOR signaling, calcium transport I, protein kinase A signaling, and PAK signaling were associated with neurological diseases or functions and were highlighted in the analysis. These signaling pathways were controlled by a cluster of proteins including Actn3, MYH6, MYL2, MYL4, PPP2R3A, TNNT3, TTN, PRKD1, PRKD1, RPS27, and ANXA5 (**Table 6** and **Supplementary Table 4**). Under multi-generational hypoxia exposure, another group of pathways including anandamide degradation, GABA receptor signaling, the sumoylation pathway, pentose phosphate pathway, sphingosine-1-phosphate signaling, endocannabinoid neuronal synapse pathway, and endoplasmic reticulum stress pathway were altered. The DEPs involved in these pathways included FAAH, CACNA1C, SLC6A13, RFC5, RHOA, TKT, and CASP3 (**Table 7** and **Supplementary Table 5**). These data suggest that short-term and multi-generational hypoxia exposures could lead to both common and unique mechanisms of neurotoxicity in marine fish.

DISCUSSION

Hypoxia is a pressing global problem facing marine ecosystems, and it is believed that this problem cannot be solved in the near future due to global warming. Studies have demonstrated that hypoxia is an endocrine disruptor, affecting both male and female reproductive systems and interfering with the hormonal reproductive hormonal system through the HPG axis. As the core of the HPG axis, the brain was reported to be interrupted by hypoxia. We have previously shown that hypoxic stress induced transcriptomic changes in the brain of *O. melastigma*. However, studies of the hypoxic responses of proteomes in other organs and tissues of fish remain limited. For example, in the heart of goldfish, hypoxia deregulated five enzymes involved in glycolysis and gluconeogenesis, including fructose-1,6-bisphosphate aldolase (Imbrogno et al., 2019). Presently, we attempted to elucidate the proteomic changes of fish brains after short-term and multi-generational hypoxia exposures.

In the first part of our study, we identified proteins in the brain of *O. melastigma* for functional analysis, which demonstrated that these proteins contribute to important brain

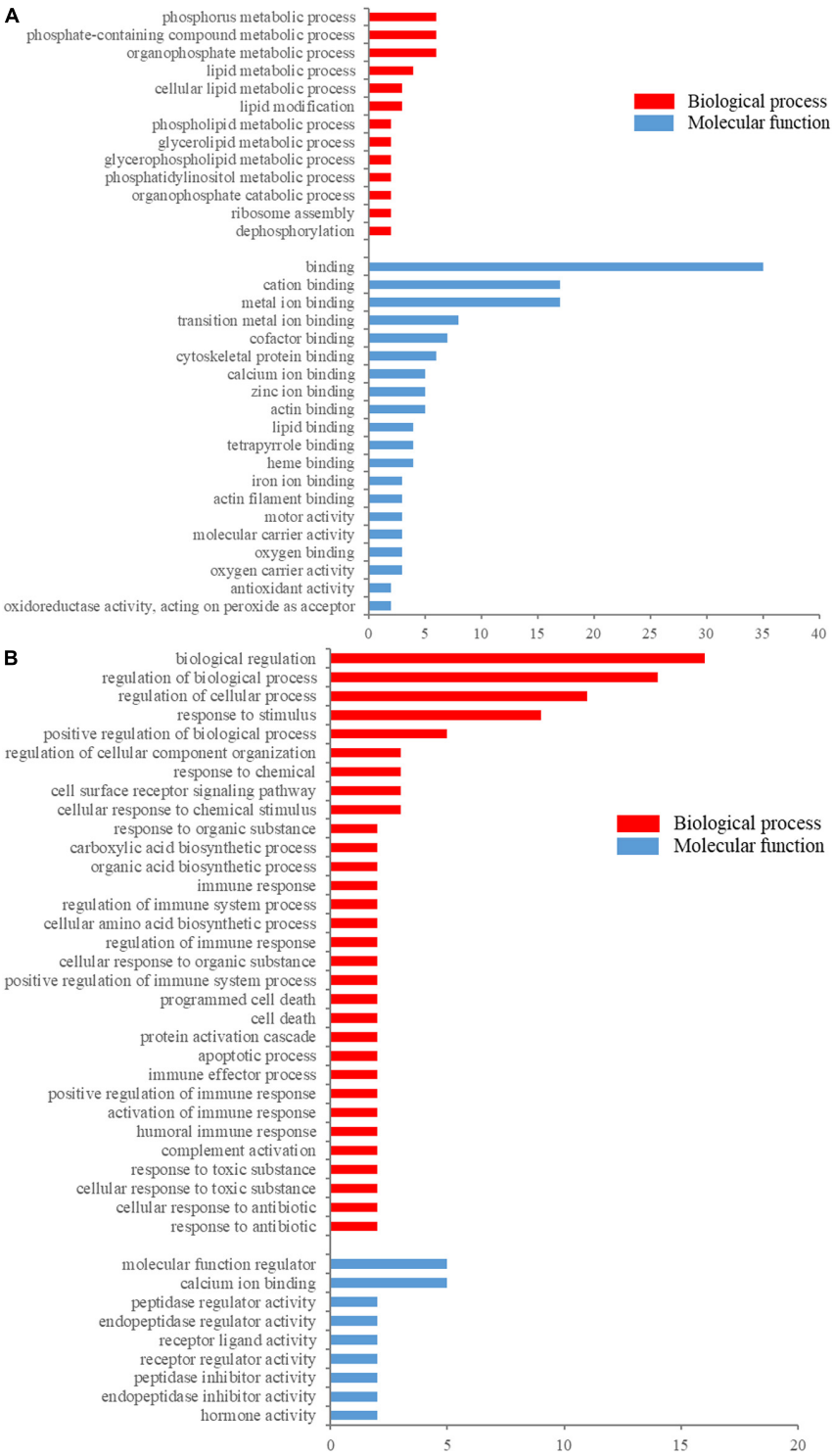
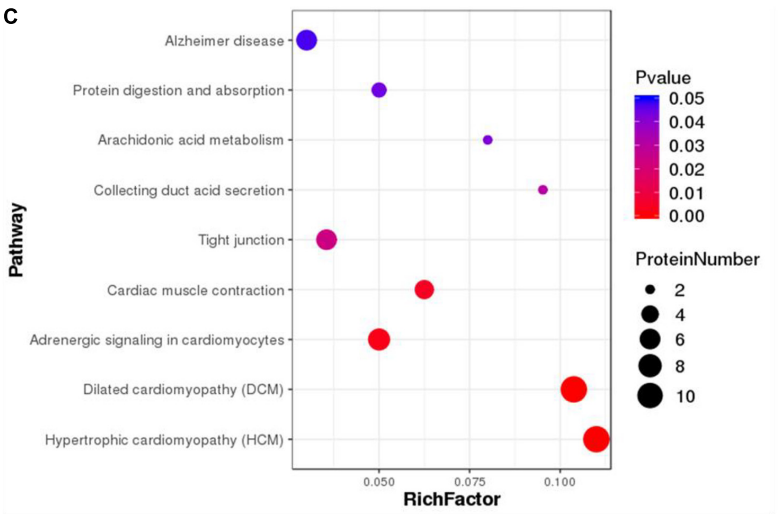


FIGURE 4 | Continued

functions. These functions include signal transducer activity, which is important for the functioning of neurotransmitters and hormones in regulating neuronal functions (Cooper, 2000). One possible outcome is the onset of chronic neurodegenerative diseases. For example, Alzheimer's disease was highlighted in our results. More importantly, we identified some proteins that were associated with reproductive processes, including WBP2 N-terminal like, double-strand break repair protein,



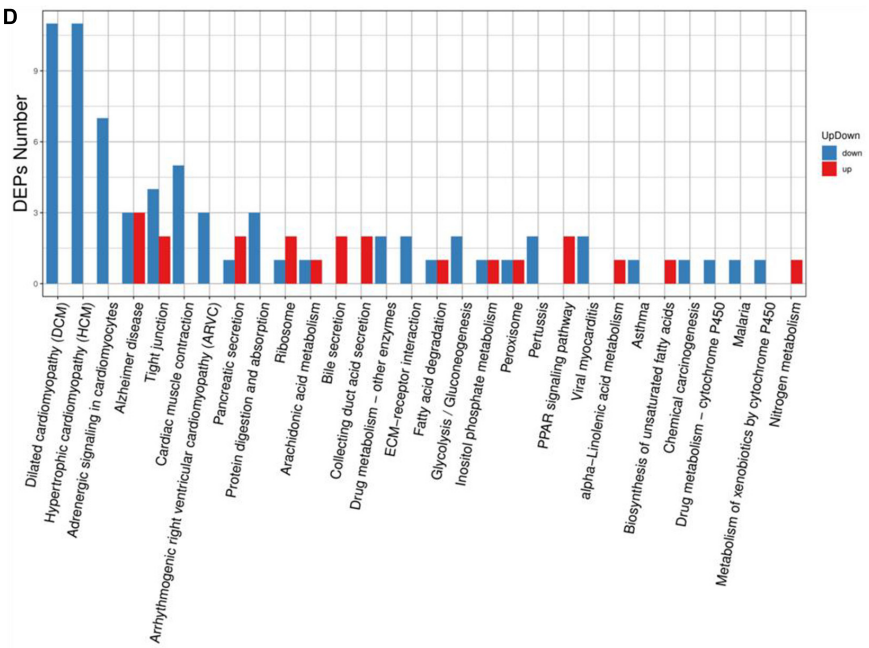


FIGURE 4 | Continued

TABLE 5 | Common deregulated canonical pathways in the brain of *Oryzias melastigma* caused by short-term and multi-generational hypoxia exposures.

Canonical pathway	Short-term	Long-term
Integrin signaling	Actn3, ITGA11, MYL2, TTN	ARF1, ITGA11, RHOA
Paxillin signaling	Actn3, ITGA11	ARF1, ITGA11
Parkinson's signaling	SNCA	CASP3
D-*myo*-inositol (1,4,5)-trisphosphate degradation	IMPA1, INPP5J	INPP5J
Epithelial adherens junction signaling	Actn3, MYH6, MYL2, MYL4	CLINT1, RHOA
Superpathway of D-*myo*-inositol (1,4,5)-trisphosphate metabolism	IMPA1, INPP5J	INPP5J

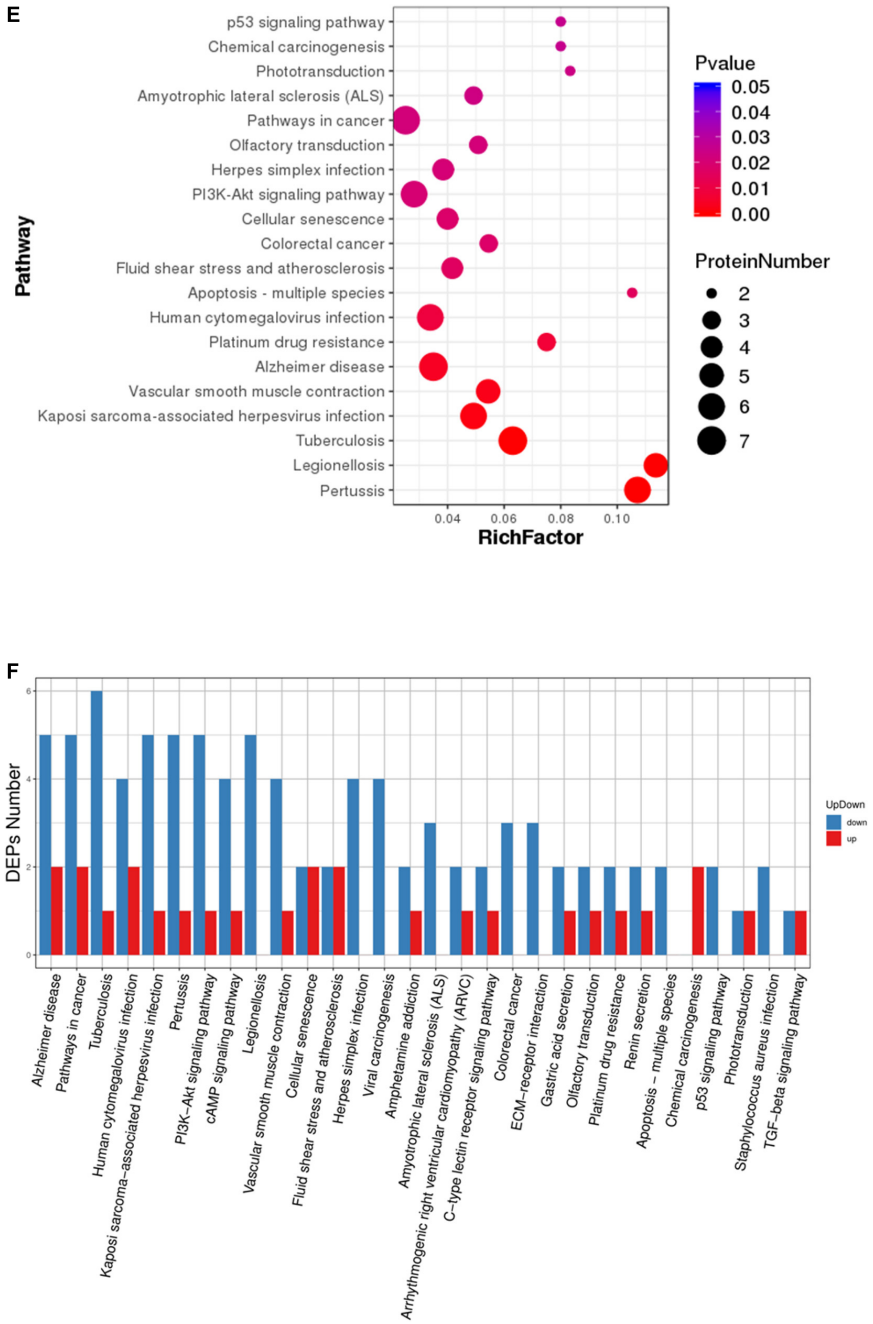


FIGURE 4 | Functional distortion in the brain of *Oryzias melastigma* caused by short-term and multi-generational hypoxia exposures. Gene ontology analysis of differentially expressed proteins obtained from **(A)** short-term hypoxia exposure and **(B)** multi-generational hypoxia exposure. *Red bars* represent biological processes and *blue bars* represent molecular functions. Pathway enrichment analyses including **(C)** rich factor analysis and **(D)** Koto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to determine alterations of the pathways caused by short-term hypoxia exposure. The effects of multi-generational hypoxia exposure are shown using **(E)** rich factor analysis and **(F)** KEGG pathway analysis.

male-enhanced antigen 1, spindlin, and synaptonemal complex protein 1. Of the identified proteins, WBP2 N-terminal like was reported to serve an important role in spermatozoa and oocyte activation (Hamilton et al., 2018; Gonzalez-Castro et al., 2019). The other identified reproductive protein, double-strand break repair protein, is responsible for generating either non-crossovers or crossovers during the process of homologous recombination in meiosis (Gray and Cohen, 2016). We also identified the male-enhanced antigen 1 (mea1) in the brain of medaka. Notably, the mea1 protein was reported to be expressed specifically in elongated spermatids and residual bodies, which suggests a functional role in

TABLE 6 | Unique deregulated canonical pathways in the brain of *Oryzias melastigma* caused by short-term hypoxia exposure.

Ingenuity canonical pathways	Molecules	Studies related to brain functions
ILK signaling	Actn3, MYH6, MYL2, MYL4, PPP2R3A	Pan et al., 2014; Hussain and Macklin, 2017
Tight junction signaling	MYH6, MYL2, MYL4, PPP2R3A	Luissint et al., 2012; Tietz and Engelhardt, 2015
Calcium signaling	MYH6, MYL2, MYL4, TNNT3	Heizmann, 1993; Kawamoto et al., 2012; Bading, 2013
RhoA signaling	MYL2, MYL4, TTN	Govek et al., 2005; Gross et al., 2007; Stankiewicz and Linseman, 2014
CXCR4 signaling	MYL2, MYL4, PRKD1	Jiang et al., 2013; Cheng et al., 2017; Bonham et al., 2018
Thrombin signaling	MYL2, MYL4, PRKD1	Wang and Reiser, 2003; Ben Shimon et al., 2015; Krenzlin et al., 2016
mTOR signaling	PPP2R3A, PRKD1, RPS27	Graber et al., 2013; Lee, 2015; Ryskalin et al., 2017
Calcium transport I	ANXA5	Lin and Way, 1984; Heizmann, 1993; Bading, 2013
Protein kinase A signaling	MYL2, MYL4, PRKD1, TTN	Gervasi et al., 2007; Huang et al., 2011; Dagda and Das Banerjee, 2015
PAK signaling	MYL2, MYL4	Kreis and Barnier, 2009; Civiero and Greggio, 2018

TABLE 7 | Unique deregulated canonical pathways in the brain of *Oryzias melastigma* caused by multi-generational hypoxia exposure.

Ingenuity canonical pathways	Molecules	Studies related to brain functions
Anandamide degradation	FAAH	Vogel et al., 1993; Weidenfeld et al., 1994; Scherma et al., 2019
GABA receptor signaling	CACNA1C, SLC6A13	Owens and Kriegstein, 2002; Luján et al., 2005; Wu and Sun, 2015
Sumoylation pathway	RFC5, RHOA	Schorova and Martin, 2016; Peters et al., 2017; Bernstock et al., 2018
Pentose phosphate pathway	TKT	Brekke et al., 2014; Bouzier-Sore and Bolaños, 2015
Sphingosine-1-phosphate signaling	CASP3, RHOA	Prager et al., 2015; Grassi et al., 2019
Endocannabinoid neuronal synapse pathway	CACNA1C, FAAH	Castillo et al., 2012; Augustin and Lovinger, 2018
Endoplasmic reticulum stress pathway	CASP3	Raghubir et al., 2011; Hashimoto and Saido, 2018; Tan et al., 2018

spermiogenesis (Ohinata et al., 2002). Spindlin, a maternal effect factor associated with the meiotic spindle during the meiosis of oocytes (Zhang et al., 2015), is responsible for the oocyte-to-embryo transition in carp fish (Sun et al., 2010). Synaptonemal complex protein 1 (SYP-1), which is localized to the short arms of chromosomes, serves a role in recruiting the chromosomal passenger complex and phosphorylated histone H3 to the short arm of chromosomes during meiosis I, thereby promoting the disjunction of chromosomes in meiosis I (Sato-Carlton et al., 2017).

After elucidating the functions of the identified proteins, we investigated the adverse effects of both short-term and multi-generational hypoxia exposures. Upon comparing the results, we observed some common deregulation of the cell signaling pathways, including integrin signaling, paxillin signaling, and Parkinson's signaling, that are related to neural functions and diseases caused by both short-term and multi-generational hypoxia exposures. Integrins are a class of transmembrane receptors involved in cell adhesion to the extracellular matrix (ECM) (Giancotti, 1999) and are expressed in various brain sections, including the hippocampus and cortex (Pinkstaff et al., 1999). As receptors, integrins play a crucial role in the pathophysiology of many brain diseases *via* signal transmissions from the ECM to the cell. Notably, aberrations in integrin signaling within the brain may impair the synaptic activity in brain disorders such as schizophrenia and epilepsy (Wu and Reddy, 2012). Paxillin is an adaptor protein for focal adhesions that coordinates the integrin-mediated rearrangements of the cytoskeleton (Rashid et al., 2017). In human cancer research, paxillin was identified as an oncogene that promotes cell migration and invasion in gliomas (Chen et al., 2016). Additionally, hypoxia could induce the invasive potential of glioblastomas through the HIF-2α–EPHB2–paxillin axis, thereby suggesting a possible interaction between hypoxia and paxillin signaling (Qiu et al., 2019). Parkinson's disease is a progressive neurodegenerative disorder (Beitz, 2014). Recently, it has been reported that oxidative stress may play a role in Parkinson's disease (Islam, 2016). Moreover, numerous studies on signaling in Parkinson's disease models have demonstrated a link between hypoxia-inducible factors and Parkinson's disease (Kim et al., 2020; Zhang et al., 2020). Therefore, the results of our study further suggest hypoxia as a possible cause of Parkinson's disease.

Apart from the common effects, both short-term and multi-generational hypoxia exposures could lead to unique neural alterations. Notably, the metabolisms of various classes of lipids (e.g., phospholipids, glycerophospholipids, and phosphatidylinositols) were found to be affected by short-term hypoxia exposure. Phospholipids (glycerophospholipids and sphingolipids) are major structural components of the plasma membrane of various cell types of the brain, including neurons and glial cells (Adibhatla and Hatcher, 2007; Kosicek and Hecimovic, 2013). Neural membrane function can be affected by their glycerophospholipid composition (Farooqui et al., 2000). Imbalances of phospholipid metabolism have previously been reported in cases of Alzheimer's disease (Kosicek and Hecimovic, 2013). Furthermore, short-term hypoxia exposure also affected arachidonic acid metabolism in the fish brain. Adequate levels of arachidonic acid, a polyunsaturated fatty acid, are required for normal cerebral functions and structures, including synaptic signaling and neurotransmitter release (Ojeda et al., 1989; Kaufmann et al., 1996;

Contreras and Rapoport, 2002). Altered brain arachidonic acid metabolism has previously been associated with several neurological, neurodegenerative, and brain diseases, including schizophrenia and Parkinson's and Alzheimer's disease (Müller et al., 2002; Teismann et al., 2003; Esposito et al., 2008). Unlike the responses to short-term hypoxia exposure, multi-generational hypoxia exposure could lead to the alteration of the carboxylic acid metabolism in the fish brain. Carboxylic acids are a class of organic acids characterized by a carboxyl (-COOH) functional group. Various carboxylic acids have been reported to play important roles in neural functions. For example, 5-methoxyindole-2-carboxylic acid can mitigate brain damage from stroke (Sumien et al., 2019), while losartan carboxylic acid, the potent AT1 blocker metabolite of losartan, improved motor coordination and enabled central nervous system protection from an increase in locomotor activity (Subudhi et al., 2018). Apart from the deregulation of organic acid metabolism, long-term hypoxia exposure could also alter certain cell signaling pathways in the brain, such as PI3K–Akt signaling and p53 signaling. PI3K–Akt is an important signaling pathway in cell proliferation and cell cycle control (Yu and Cui, 2016). It has been reported that aberrations in PI3K–Akt signaling may be involved in the pathogenesis of Alzheimer's disease (Gabbouj et al., 2019) and brain malformation (Jansen et al., 2015). Notably, an Alzheimer's disease mouse model demonstrated that the activation of PI3K–Akt signaling could mitigate neurodegeneration and memory impairment (Ali et al., 2017). For example, the transcription factor p53, a mediator of cellular response to stress, is required for normal brain development (Contreras et al., 2018). More importantly, p53 is the key mediator of autophagy and apoptosis, processes that are known to be dysregulated in ALS and Parkinson's and Alzheimer's diseases (Ghavami et al., 2014). Interestingly, some cancer studies reported that the p53 and Akt pathways were linked to the modulation of apoptosis under hypoxic conditions (Leszczynska et al., 2015; Yun and Glazer, 2015). In line with the above results, ALS, which is characterized by the deterioration of motor neurons (McCombe et al., 2020), was also highlighted in our analysis. Therefore, multi-generational hypoxia exposure could result in behavioral disorders in fish *via* neurodegeneration. Due to the harmful effects of this potential interaction, future studies should be conducted to address this issue.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://www.ebi.ac.uk/pride/archive/projects/PXD022579.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Use of Live Animals in Teaching and Research (CULATR, #2714-12) at the University of Hong Kong.

AUTHOR CONTRIBUTIONS

KL and RK contributed to the conception, organization, and execution of the research project. SW and NT contributed to the experimental setup and sample preparation. YT, JZ, WT, XL, TC, and DA contributed to the bioinformatic analysis and statistical analysis. RW contributed to manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.618489/full#supplementary-material

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