Perinatal Ischemia Alters Global Expression of Synaptosomal Proteins Critical for Neural Plasticity in the Developing Mouse Brain

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Abstract

Ischemic perinatal stroke (IPS) affects 1 in 2,300–5,000 live births. Despite a survival rate >95%, approximately 60% of IPS infants develop motor and cognitive impairments. Given the importance of axonal growth and synaptic plasticity in neurocognitive development, our objective was to identify the molecular pathways underlying IPS-associated synaptic dysfunction using a mouse model. IPS was induced by unilateral ligation of the common carotid artery of postnatal day 10 (P10) mice. Five days after ischemia, sensorimotor and motor functions were assessed by vibrissae-evoked forepaw placement and the tail suspension test respectively, showing evidence of greater impairments in male pups than in female pups. Twenty-four hours after ischemia, both hemispheres were collected and synaptosomal proteins then prepared for quantification, using isobaric tags for relative and absolute quantitation. Seventy-two of 1,498 qualified proteins were altered in the ischemic hemisphere. Ingenuity Pathway Analysis was used to map these proteins onto mo-

lecular networks indicative of reduced neuronal proliferation, survival, and synaptic plasticity, accompanied by reduced PKC α signaling in male, but not female, pups. These effects also occurred in the non-ischemic hemisphere when compared with sham controls. The altered signaling effects may contribute to the sex-specific neurodevelopmental dysfunction following IPS, highlighting potential pathways for targeting during treatment.

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Introduction

Ischemic perinatal stroke (IPS) is a significant cause of long-term neurologic and cognitive disability including cerebral palsy, epilepsy, neurodevelopmental impairments, and behavioral disorders [1, 2]. IPS thus constitutes a major long-term cost to society in terms of special education and health care [3]. The incidence of perinatal stroke is high, occurring in 1 of 2,300–5,000 live births [2, 4, 5]. The underlying mechanisms of perinatal stroke injury and repair remain poorly understood, partly due to the wide range of pathophysiology, from asymptomatic to hemorrhagic-encephalopathic

presentation [6]. Consequently, there remains a challenge to develop targeted and effective treatment approaches for the condition. Moreover, given the sex-dependent incidence and outcomes of IPS in humans, i.e., IPS results in more neurological deficits in male than female infants [7–9], understanding the underlying molecular mechanisms of long-term effects in IPS survivors is of major interest. This can provide an insight into the specific neurodevelopmental processes that are affected by IPS and may lead to novel strategies to prevent or mitigate the adverse long-term outcomes. Despite the recognition that neonatal brain injuries, including IPS, involve complex mechanistic cascades that can be detrimental to the developing brain [2, 10, 11], the major pathways and mechanisms underlying IPS-induced synaptic plasticity and dysfunction [12, 13] are poorly studied. Changes in specific synaptic proteins have previously been shown in the adult brain following ischemic stroke [14–17]. There is a report demonstrating acute changes in synaptic protein following neonatal hypoxia-ischemia injury [18], but comprehensive analyses of synaptosomal proteins altered by IPS in the developing brain remain limited.

One approach to the analysis of the synaptic proteome is through quantitative proteomic analysis using the isobaric tags for relative and absolute quantitation (iTRAQ) method [19–21]. Using the iTRAQ technique, the protein content and composition in synaptosomes can be quantified in the ischemic (ipsilateral [IL] to the ligated common carotid artery) and non-ischemic (contralateral [CL] to the ligated common carotid artery) hemispheres. Subsequently, the iTRAQ data can be functionally annotated to identify molecular networks using the knowledge-based Ingenuity Pathway Analysis (IPA) database (Qiagen, Redwood City, CA, USA), which is a collection of experimental findings from biomedical literature and third-party databases.

The objective of this study was to induce IPS in postnatal day 10 (P10) mouse pups, a developmental time point at which remarkable increases in axonal growth and dendritic density occur and is neurodevelopmentally equivalent to a full-term human infant [22]. A global proteomic approach was used to identify differentially expressed synaptosomal proteins and key molecular network changes between the ischemic and non-ischemic hemispheres in mouse pups. Elucidating the major pathways altered by IPS will likely uncover specific mechanisms that may underlie synaptic dysfunction and uncover potential targets for preventative and therapeutic strategies.

Materials and Methods

Animals

C57B6/J mice were kept in 14-h/10-h light/dark cycle with ad libitum access to food and water.

Induction of Ischemia

The procedure was performed as previously described [23] with modifications. Pups were sexed and randomized for ischemic or sham surgery. P10 pups from litters of 7–10 pups were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg, Phoenix, St. Joseph, MO, USA) and xylazine (10 mg/kg, LLOYD Laboratories, Shenandoah, IA, USA). A small midline incision was made in the neck to isolate the right common carotid artery, which was then permanently ligated using silk. The incision was closed with a 6–0 surgical suture (Ethicon, Somerville, NJ, USA). After recovering from anesthesia on a heat pad to prevent inadvertent therapeutic hypothermia, pups were reunited with nursing dams in their home cages. Sham-operated animals underwent incision and carotid artery isolation without ligation.

Body Temperature Monitoring

Body temperature was monitored throughout the surgical procedure using a rectal temperature probe for neonatal mice (Physi-temp Instruments Inc., Clifton, NJ, USA). Pup temperature was taken again 24 h after surgery. No significant interaction between treatment and sex or difference between groups using 2-way ANOVA and post hoc Bonferroni-corrected *t* test were found (data not shown).

Vibrissae-Evoked Forepaw Placement Assessment

Five days after ischemia, pups were tested for sensorimotor function using a whisker-evoked forepaw placement as previously described [24]. In brief, pups were held by the neck skin to allow forepaw movement and at a 45° angle from the bench top. They were then slowly moved upward to stimulate their whiskers by brushing against the edge of the bench top. Ten trials were performed for each side and the frequency of IL forepaw placements onto the bench surface was recorded for each animal. Two-way ANOVA was used to analyze the mean differences among groups and, where appropriate, the post hoc Bonferroni-corrected *t* test was used to calculate mean between-group differences. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Tail Suspension Test

Five days after ischemia induction, pups were tested for motor function using a tail suspension test as previously described [24]. In brief, pups were suspended head down by their tails approximately 10–12 cm above the horizontal surface and then slowly lowered towards the surface. Mice normally respond by reaching toward the surface, with both forelimbs and hindlimbs extended and abducted, and dorsiflexion (C-shaped bending) of the body; any deviations from these stereotypical responses were considered abnormal (Supplemental Video Recordings 1, 2; for all online suppl. material, see www.karger.com/doi/10.1159/000499126). Behavioral testers were blinded to treatment groups. The χ^2 test for an association between ischemia and motor dysfunction was performed (GraphPad Prism).

2,3,5-Triphenyl-Tetrazolium Chloride Staining

Brain injury was assessed with 2,3,5-triphenyl-tetrazolium chloride (TTC) staining as previously described [25]. In brief, 24 h or 5 days after ischemia, brains were removed from the crania and sectioned at 2.0-mm thickness using an ice-cold mouse brain matrix (RWD Life Science, Shenzhen, China). Sections were stained with 2% TTC (diluted in PBS) at 37°C for 30 min in a 6-well plate, and subsequently fixed in 4% paraformaldehyde (in PBS) overnight at 4°C. Sections were then mounted onto a glass slide and covered with a glass coverslip and a drop of mineral oil. Images were captured using an iPhone 7 digital camera (Apple Inc.). The extent of brain injury was quantified as a ratio of infarct volume over total analyzed brain volume using Adobe Photoshop CS. Fisher's exact test for association between ischemia and brain injury was performed (GraphPad Prism).

Synaptosomal Preparation

Twenty-four hours after ischemia induction, pups were euthanized by decapitation for brain dissection. Following the removal of the cerebellum, the brain was bisected along the midline on an ice-cold metal block. Ischemic (IL) and non-ischemic (CL) hemispheres were isolated. Synaptosomes were prepared from each hemisphere following the protocol described previously [21, 26]. In brief, the ischemic and non-ischemic hemispheres were homogenized in ice-cold solution A (0.32 M sucrose, 1 mM NaHCO₃, 1 mM MgCl₂, and 0.5 mM CaCl₂) and centrifuged (1,400 g) at 4°C for 10 min. The soluble portion of the fraction was saved, while the pellet was resuspended with an additional 3 mL of solution A and centrifuged (710 g) for 10 min. The supernatant and the previously soluble portion of the fraction were combined and centrifuged (13,800 g) for 10 min. The pellet was collected and resuspended in 0.5 mL of ice-cold solution B (0.32 M sucrose, 1 mM NaHCO₃) and layered onto a sucrose gradient composed of 0.85 M, 1.00 M, and 1.20 M sucrose buffered in 1 mM NaHCO₃. This was centrifuged at 32,800 g for 120 min using a swing-bucket rotor (Beckman-Coulter, Indianapolis, IN, USA). The synaptosomal band above the 1.20 M sucrose layer was removed from the gradient, diluted 10-fold with solution B, and then centrifuged at 13,800 g for 30 min to obtain the synaptosomal protein pellet. The quality of synaptosomal proteins was validated by Western blot analysis of synaptic protein enrichment (online Suppl. Fig. 1A, B).

Protein Extraction

Synaptosomes from hemispheres of 3 pups were pooled for protein preparation. Four pooled (2 ischemic and 2 non-ischemic) synaptosomal protein isolates were generated. Synaptosomal protein was extracted following the protocol described previously [21]. In brief, solution B was aspirated from the synaptosomal pellet and replaced with 125 μ L of protein extraction buffer (7 M urea, 2 M thiourea, 0.4 M triethylammonium bicarbonate [TEAB] pH 8.5, 20% methanol, and 4 mM tris (2-carboxyethyl) phosphine [TCEP]) on ice. The samples were then resuspended and transferred to a PCT tube with a 100- μ L cap for the Barocycler NEP2320 (Pressure Biosciences, South Easton, MA, USA). The samples were cycled between 35 kpsi (30 s) and 0 kpsi (15 s) for a total of 40 cycles at 37°C. Each sample received 5 μ L of 200 mM methyl methanethiosulfonate (MTS, 8 mM final concentration) mixed by brief vortex, and then incubated for 15 min at room temperature. Each sample was then transferred to a 1.5-mL microfuge Protein

LoBind tube (Eppendorf, Hauppauge, NY, USA). Duplicates of each sample were taken to quantify protein concentration by Bradford assay.

In-Solution Digestion and iTRAQ Labeling

Protein digestion and iTRAQ labeling were done following a previously described protocol [21]. Each sample (100 μ L) was brought to the same volume using 8 mM MMTS for the iTRAQ 4-plexes analysis. Ultrapure water was used to dilute each sample (4-fold). A 1:35 ratio of trypsin (Promega, Madison, WI, USA) was added and followed by a 16-h incubation at 37°C, a 30-min freeze at -80°C, and a dehydration in vacuo for the trypsin digestion process. Each sample was cleaned with a 4-mL Extract Clean C18 SPE cartridge (Grace-Davidson, Deerfield, IL, USA). Eluents were vacuum-dried and resuspended in dissolution buffer of the iTRAQ kit (final concentration 2 μ g/ μ L). Each 40 μ g sample was labeled following the manufacturer's protocol. After iTRAQ labeling, samples were cleaned using a 4-mL Extract Clean C18 SPE cartridge and dried in vacuo.

Offline Fractionation and Mass Spectrometry

Fractionation and mass spectrometry were performed as described previously [21]. In brief, the iTRAQ 4-plexes sample was resuspended in buffer A (10 mM ammonium formate pH 10 in 98% water and 2% acetonitrile) and fractionated offline by high pH reverse-phase chromatography using MAGIC 2002 HPLC (Michrom BioResources, Auburn, CA, USA) and C18 Gemini-NX column (150 $\times$ 2 mm ID, 5- μ m particle, 110 Å pore size [Phenomenex, Torrance, CA, USA]). Buffer B (10 mM ammonium formate pH 10 in 10% water and 90% acetonitrile) was used to maintain the flow rate (100 μ L/min) at a 5–35% gradient for 60 min and a 35–60% gradient for 5 min. Fractions were collected every 2 min and UV-light absorbance was read at 215 and 280 nm. Fractions containing peptide were divided into 2 equal groups labeled “early” and “late.” The first “early” fraction was concatenated with the first “late” fraction, and so on. Samples were then vacuum-dried and resuspended in loading solvent (98% water, 2% acetonitrile, and 0.01% formic acid). Aliquots (1–1.5 μ L) were run on a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The RAW data (Proteowizard files) were converted to the MzXML format using MSconvert software and to MGF files using the TINT raw-MGF converter. Collected data were analyzed using ProteinPilot™ 4.5 (AB Sciex, Foster City, CA, USA). Search parameters were: cysteine MMTS; iTRAQ 4-plexes (peptide-labeled); trypsin; instrument Orbi MS (1–3 ppm) Orbi MS/MS; bias corrections were applied to account for biological modifications including systematic errors in protein amount among samples; a thorough search effort; and local 5% false discovery rate analysis (with reversed database). A summary of the protein search and identification is shown in online Supplementary Figure 1C.

iTRAQ Data Treatment and Selection

The 4-plexes parameter was set up as follows: m/z 113 (IL1), m/z 114 (CL1), m/z 115 (IL2), and m/z 116 (CL2). The m/z 114 was used as denominator (control) to generate ratio values (IL/CL). Proteins with <3 distinct peptides (95% confidence) or error factor (EF) >2.0 were eliminated from the analysis. These selection criteria resulted in 1,498 identified proteins from the 4-plexes dataset. Differentially expressed proteins were identified using criteria (absolute [fold change] $\geq 1.5\times$; $p < 0.05$).

Fig. 1. P10 ischemia induced variable brain injury in mouse pups. Representative images of TTC stained brain sections from sham (**a, b**) and ischemic mouse pups showing injured ipsilateral hemispheres at 24 h (24h; **c, d**, arrows, white areas) and 5 days (5d; **e, f**, arrow, white areas) after ischemia. **g** Table showing the number of pups with or without brain injury assessed by TTC following ischemia. ** $p < 0.01$, * $p < 0.05$, Fisher's exact test for association between ischemia and brain injury.

Bioinformatics

IPA was used to identify networks, pathways, molecular and cellular functions, and behavioral and neurological dysfunctions using proteins from the iTRAQ datasets. Fisher's exact test was used to calculate the p value for the proteins in the datasets that mapped onto specific pathways and functions. IPA does this by using an algorithm based on molecular function, cellular function, and functional group.

Western Blot Analysis

Western blot was used to quantify the levels of selected proteins chosen from 72 differentially expressed proteins. Protein lysates ($n = 4-7$ /group) prepared from ischemic (IL), non-ischemic (CL), and sham control hemispheres were separated using a 4-12% Bis-Tris gel (Novex, Life Technologies, Carlsbad, CA, USA), and transferred to a nitrocellulose membrane (Pierce, Rockford, IL, USA). The membranes were blocked using a blocking buffer for fluorescence Western blotting (Rockland, Gilbertsville, PA, USA) and sealed in a plastic bag. The membranes were treated with primary

antibodies and then with fluorescent secondary antibodies. Primary antibodies included: CaMKII α (1:2,000, Abcam, Cambridge, MA, USA), MAP-2 (1:2,000, Abcam), MAPK (1:1,000, Cell Signaling, Danvers, MA, USA), phospho-PKC α (1:1,000, Cell Signaling), PKC α (1:1,000, Cell signaling), PSD-95 (1:2,000, Abcam), α -synuclein (1:1,000, Abcam); and β -actin (1:1,000, Sigma, St. Louis, MO, USA). Secondary antibodies included: DyLight 700 anti-mouse (1:12,500 $\times$, Rockland) and DyLight 800 anti-rabbit (1:12,500 $\times$, Rockland). The membranes were then analyzed by near-infrared fluorescence using the Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE, USA). The protein of interest was normalized against β -actin and quantified using ImageStudio software (Li-Cor). Representative Western blot images are shown in online Supplementary Figure 2. One-way ANOVA was used to analyze the mean differences among groups, and where appropriate, the post hoc Tukey t test was used to calculate the mean difference between groups. Statistical analyses were performed using GraphPad Prism.

Fig. 2. Impaired sensorimotor and motor function in mouse pups 5 days after ischemia. **a** Graph showing reduced frequency of forelimb extension following vibrissae stimulation contralateral to ligation in male pups compared to sham controls. Values are mean $\pm$ SEM. $n = 12$ –13 males and 10 females. ANOVA, significant difference between groups with *** $p < 0.001$, post hoc Bonferroni-corrected t test. **b–d** Abnormal motor function in mouse pups 5 days after ischemia. Representative still images of a mouse pup showing an attempt of dorsiflexion (**b**, curved line) and hindlimb hemiparesis (**c**, arrow) ipsilateral to the ligation. **d** Table showing results of tail suspension test of ischemic pups performed 5 days after ligation. χ^2 test for an association between ischemia and motor dysfunction with significant differences between sham and ligated groups with ** $p < 0.01$, * $p < 0.05$. IL, ipsilateral hemisphere; CL, contralateral hemisphere.

Results

Perinatal Ischemia Induces IL Brain Injury Accompanied by Sensorimotor and Motor Impairments

TTC staining was used to visualize brain injury following ischemia induced by ligation of the right common carotid artery in P10 mouse pups. Assessments at 24 h and 5 days after ischemia showed brain injury IL to the ligation in both male and female pups when compared with sham-operated pups. The extent of injury ranged from no visible injury to about 27% of total brain volume, with injury still appreciable 5 days after ischemia (Fig. 1).

To further demonstrate the functional consequence of perinatal ischemia, 5 days after ischemia, the sensorimotor and motor functions were assessed by vibrissae-evoked forelimb placement and the tail suspension analysis, respectively. Ischemic male pups showed a lower frequency of forepaw placement CL to the ligation side when compared with sham controls (Fig. 2a; $p = 0.032$ sex difference). In addition, ischemic male pups showed greater attempts of dorsiflexion of the body, favoring the ligated hemisphere (Fig. 2b, d). A significant number of ischemic male pups showed hindlimb hemiparesis IL to the ligation when compared to the sham control group (Fig. 2c, d).

Fig. 3. IPA-mapped differentially expressed synaptosomal proteins in P10 mouse pups 24 h after ischemia onto molecular networks associated with decreased (**a**) and increased (**b**) functions or diseases. Red shapes, upregulated; green shapes, downregulated; blue shapes, predicted inhibition; orange shapes, predicted activation; blue arrows, activation; orange arrows, inhibition; yellow arrows, findings inconsistent with state of downstream molecule; gray arrows, effects not predicted.

Perinatal Ischemia Suppresses Interactive Gene Networks Implicated in Neuronal Development and Survival

Proteomic analysis showed that 72 of 1,498 qualified proteins (4.8%) were differentially expressed (absolute [fold change] $\geq 1.5\times$; $p < 0.05$) between the ischemic and non-ischemic hemispheres (Table 1), including developmentally relevant synaptic proteins (GAP43, synapsin II, and CASK) and energetically important mitochondrial proteins (Cox7c, SLC25A5, and NDUFA4). IPA mapped

these differentially expressed proteins onto networks that predict reduced neuronal proliferation and synaptic plasticity concomitant with increased phosphorylation of protein, perinatal death, and neuronal cell death (Fig. 3). Using IPA, differentially expressed synaptosomal proteins were also used to predict altered activities of upstream regulators (Table 2). Inhibition of inflammatory regulators (NFE2L2 and ADORA2A) and activation of neuronal growth and differentiation regulators (ESRRa and mTOR) were the significant perinatal ischemic ef-

Table 1. Differentially-expressed synaptosomal proteins in ischemic hemisphere 24 h after unilateral common carotid artery ligation of P10 mice

Gene name	Symbol	Ischemic/ non-ischemic		Location	Type(s)
		log ₂ (FC)	p value		
abl-interactor 2	ABI2	-1.265	0.047	cytoplasm	other
acyl-CoA oxidase 1, palmitoyl	ACOX1	1.261	0.039	cytoplasm	enzyme
alanyl-tRNA synthetase	AARS	-1.149	0.004	cytoplasm	enzyme
aldehyde dehydrogenase 1 family, member L1	ALDH1L1	-1.130	0.027	cytoplasm	enzyme
aldo-keto reductase family 1, member A1 (aldehyde reductase)	AKR1A1	-1.310	0.015	cytoplasm	enzyme
aldolase A, fructose-bisphosphate	ALDOA	-1.256	0.000	cytoplasm	enzyme
asparaginase-like 1	ASRGL1	-1.288	0.049	cytoplasm	enzyme
ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1	ATP5F1	1.261	0.032	cytoplasm	transporter
ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G	ATP5L	1.376	0.022	cytoplasm	transporter
ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1	ATP6V1E1	-1.157	0.036	cytoplasm	transporter
ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	ATP6V1D	-1.155	0.044	cytoplasm	transporter
ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	ATP6V1C1	-1.131	0.034	cytoplasm	transporter
ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	ATP6V1B2	-1.079	0.050	cytoplasm	transporter
ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	ATP6V1A	-1.076	0.037	plasma membrane	transporter
calcium/calmodulin-dependent protein kinase II alpha	CAMK2A	-1.227	0.003	cytoplasm	kinase
calcium/calmodulin-dependent serine protein kinase (MAGUK family)	CASK	-1.128	0.043	plasma membrane	kinase
CaM kinase-like vesicle-associated	CAMKV	-1.224	0.004	other	kinase
contactin 2 (axonal)	CNTN2	1.240	0.000	plasma membrane	other
copine VI (neuronal)	CPNE6	-1.230	0.016	plasma membrane	transporter
cytochrome c oxidase subunit VIIc	Cox7c	1.443	0.048	cytoplasm	other
cytoplasmic FMR1 interacting protein 2	CYFIP2	-1.198	0.014	cytoplasm	other
dimethylarginine dimethylaminohydrolase 1	DDAH1	-1.243	0.017	cytoplasm	enzyme
dynamin 1	DNM1	-1.080	0.034	cytoplasm	enzyme
erythrocyte membrane protein band 4.1-like 1	EPB41L1	-1.279	0.001	plasma membrane	other
erythrocyte membrane protein band 4.1-like 2	EPB41L2	-1.216	0.007	plasma membrane	other
FK506 binding protein 1A, 12 kDa	FKBP1A	-1.489	0.042	cytoplasm	enzyme
G protein regulated inducer of neurite outgrowth 1	GPRIN1	-1.163	0.007	plasma membrane	other
G-protein signaling modulator 1	GPSM1	-1.447	0.046	cytoplasm	other
glutamate-ammonia ligase	GLUL	-1.162	0.003	cytoplasm	enzyme
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	-1.320	0.000	cytoplasm	enzyme
growth associated protein 43	GAP43	-1.277	0.026	plasma membrane	other
guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	GNB2L1	1.216	0.040	cytoplasm	enzyme
heterogeneous nuclear ribonucleoprotein K	HNRNPK	1.700	0.006	nucleus	other
lipid phosphate phosphatase-related protein type 3	LPPR3	-1.189	0.040	other	phosphatase
MAP-kinase activating death domain	MADD	-1.179	0.045	cytoplasm	other
MARCKS-like 1	MARCKSL1	-1.179	0.035	cytoplasm	other
microtubule-associated protein 2	MAP2	-1.257	0.001	plasma membrane	other
microtubule-associated protein 6	MAP6	-1.433	0.001	cytoplasm	other
myosin VA (heavy chain 12, myosin)	MYO5A	-1.070	0.036	cytoplasm	enzyme
myosin, heavy chain 10, non-muscle	MYH10	-1.069	0.028	cytoplasm	other
NCK-associated protein 1	NCKAP1	-1.178	0.003	plasma membrane	other
NDUFA4, mitochondrial complex associated	NDUFA4	1.548	0.034	cytoplasm	enzyme
optic atrophy 1 (autosomal dominant)	OPA1	1.608	0.030	cytoplasm	enzyme
OTU domain, ubiquitin aldehyde binding 1	Otub1	-1.217	0.045	cytoplasm	enzyme
peptidylprolyl isomerase A (cyclophilin A)	PPIA	-1.394	0.003	cytoplasm	enzyme
phosphofructokinase, liver	PFKL	-1.151	0.048	cytoplasm	kinase
phosphorylase, glycogen; brain	PYGB	-1.117	0.001	cytoplasm	enzyme
platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45 kDa)	PAFAH1B1	-1.226	0.000	cytoplasm	enzyme
protein kinase C, alpha	PRKCA	-1.181	0.015	cytoplasm	kinase
protein kinase C, beta	PRKCB	-1.262	0.010	cytoplasm	kinase
protein kinase C, gamma	PRKCG	-1.283	0.002	cytoplasm	kinase
protein phosphatase 2, regulatory subunit A, alpha	PPP2R1A	-1.175	0.021	cytoplasm	phosphatase
RAB11B, member RAS oncogene family	RAB11B	-1.147	0.025	cytoplasm	enzyme
RAB6A, member RAS oncogene family	RAB6A	-1.220	0.025	cytoplasm	enzyme
ras homolog family member T1	RHOT1	1.277	0.042	cytoplasm	enzyme
RAS protein activator like 1 (GAP1-like)	RASAL1	-1.250	0.015	cytoplasm	other
ribosomal protein L10	RPL10	1.224	0.030	cytoplasm	other
ribosomal protein L12	RPL12	1.284	0.021	nucleus	other
ribosomal protein L3	RPL3	1.322	0.015	cytoplasm	other
ribosomal protein L31	RPL31	1.295	0.023	cytoplasm	other
ribosomal protein S13	RPS13	1.254	0.026	other	other
ribosomal protein S25	RPS25	1.477	0.046	cytoplasm	other
septin 7	SEPT7	-1.133	0.039	cytoplasm	other
signal recognition particle receptor, B subunit	SRPRB	1.259	0.001	cytoplasm	other
solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	SLC25A5	1.349	0.004	cytoplasm	transporter
synapsin II	SYN2	-1.175	0.013	plasma membrane	other
talin 2	TLN2	-1.161	0.041	nucleus	other
translin	TSN	-1.312	0.032	nucleus	other
tubulin, alpha 4a	TUBA4A	-1.242	0.017	cytoplasm	other
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	TYWHAG	-1.227	0.005	cytoplasm	other
v-ral simian leukemia viral oncogene homolog A (ras-related)	RALA	-1.165	0.025	cytoplasm	enzyme
vesicle amine transport 1-like	VAT1L	-1.303	0.019	other	enzyme

Table 2. Top 5 upstream regulators induced by ischemia in P10 mouse CNS

Upstream regulator	Molecule type	Predicted activation state	Activation z score	<i>p</i> value of overlap	Target molecules in dataset
ESRRα	transcription regulator	activated	2.24	4.79×10^{-6}	ACOX1, ALDOA, ATP5F1, Cox7c, PFKL
ADORA2A	G-protein coupled receptor	inhibited	-1.63	1.80×10^{-6}	ATP6V1B2, CAMKV, GAPDH, HNRNPK, PPP2R1A, RAB6A
mTOR	kinase		-1.13	4.74×10^{-7}	AKR1A1, ATP5F1, GAP43, Otub1, PYGB, RAB11B, RPL12
NFE2L2	transcription regulator	inhibited	-1.89	7.71×10^{-5}	AKR1A1, ALDOA, GNB2L1, NCKAP1, PAFAH1B1, PRKCB, SYN2
HDAC4	transcription regulator			5.77×10^{-6}	CAMK2A, CYFIP2, PRKCA, PRKCB, SYN2

Table 3. Top 5 causal network regulators altered by ischemia in P10 mouse CNS

Master regulator	Molecule type	Participating regulators	Predicted activation state	Activation z score	<i>p</i> value of overlap	Network bias-corrected <i>p</i> value	Target molecules in dataset
CD38	enzyme	CBL, CD38, CD3E, GF1R, ITK, MAP2K1, Mapk, MTOR, PPARG, RXRA, SERPINE1, SIRT1, TGFB1, TP53	inhibited	-2.20	9.88×10^{-9}	0.001	ACOX1, AKR1A1, ALDH1L1, ALDOA, ATP5F1, ATP5L, ATP6V1D, CYFIP2, FKBP1A, GAP43, GAPDH, GLUL, MYH10, NDUFA4, Otub1, PFKL, PRKCA, PRKCB, PRKCG, PYGB, RAB11B, RACK1, RPL12, RPS25, VAT1L
MAP2K1	kinase	CDKN1B, Creb, CTNNB1, ELK1, ERK, HIF1A, INSR, MAP2K1, MAPK3, MAPK7, MAPK8, MAPK9, MTOR, NFE2L2, NR3C1, PPARG, RPS6KA3, SMAD3, TP53	inhibited	-2.27	9.89×10^{-9}	0.001	ABI2, AKR1A1, ALDH1L1, ALDOA, ATP5F1, ATP5L, ATP6V1D, CYFIP2, FKBP1A, GAP43, GAPDH, GLUL, MARCKSL1, MYH10, NCKAP1, NDUFA4, Otub1, PAFAH1B1, PRKCA, PRKCB, PRKCG, PYGB, RAB11B, RACK1, RPL12, RPS25, SYN2, TUBA4A
DRD3	G-protein coupled receptor	AKT1, DRD3, ELK1, FOXO1, HMOX1, MAPK1, MAPK3, MTOR, NFE2L2, TP53, TSC2	inhibited	-2.29	4.56×10^{-8}	0.001	AKR1A1, ALDOA, ATP5F1, ATP6V1D, CYFIP2, FKBP1A, GAPDH, GLUL, MYH10, NCKAP1, NDUFA4, Otub1, PAFAH1B1, PRKCA, PRKCB, PRKCG, PYGB, RAB11B, RACK1, RPL12, RPS25, SLC25A5, SYN2
Gsk3	group	Akt, ELK1, ERK1/2, FLI1, FOXO1, Gsk3, IGF1R, MAP2K1, MAP2K2, MAPT, MMP2, MTOR, NFE2L2, NMDA Receptor, PRKAA1, PRKCD, SLC29A1, STAT1, TP53, TSC2	activated	2.27	1.29×10^{-8}	0.001	AKR1A1, ALDH1L1, ATP5F1, ATP5L, ATP6V1D, CAMK2A, CYFIP2, DDAH1, FKBP1A, GAP43, GAPDH, GLUL, MAP2, MYH10, NDUFA4, Otub1, PAFAH1B1, PPP2R1A, PRKCA, PRKCB, PRKCG, PYGB, RAB11B, RACK1, RPL12, RPL3, RPS25, SYN2
VEGFα	growth factor	c-Src, CAV1, ERK1/2, FOXO1, IGF1R, Jnk, JUN, MAP2K1, MAPK14, MAPK9, MMP2, MMP9, MTOR, NFE2L2, NR3C2, PRKCG, RTN4, SRC, SRC (family), STAT6, TGFB1, TP53, VEGFA	activated	2.35	2.01×10^{-8}	0.001	ACOX1, ALDH1L1, ALDOA, ATP5F1, ATP5L, ATP6V1D, CYFIP2, GAP43, GLUL, MARCKSL1, MYH10, NCKAP1, NDUFA4, Otub1, PAFAH1B1, PPP2R1A, PRKCB, PRKCG, PYGB, RAB11B, RPL12, RPS25, SYN2, TUBA4A, VAT1L, YWHAG

fects observed. Analysis of causal network regulators also indicated increased activity of VEGFa and Gsk3 accompanied by decreased activity of CD38, MAP2K1 and DRD3 (Table 3).

Perinatal Ischemia Reduces the Expression of Synaptic Proteins in Signaling Pathways Critical for Synaptic Development

IPA mapped differentially expressed proteins onto specific canonical signaling pathways, indicating decreased activity in 4 important clusters: chemokine signaling, cAMP response element-binding protein (CREB) signaling, neuregulin signaling, and α -adrenergic signaling (Fig. 4a). Given the documented role of CREB signaling in neonatal models of hypoxic-ischemic (HI) brain injury [27, 28], the differentially expressed synaptosomal proteins within the CREB signaling pathway were chosen for validation using Western blot (Fig. 4b, c). These particular proteins were also chosen because of their important role in multiple signaling pathways (e.g., CREB and mTOR). Total PKC α and its phosphorylated levels were lower in the ischemic hemisphere in male, but not female, pups (Fig. 4c). The sham-operated group of mice was used as a control and showed that ischemia induced a significant reduction of the analyzed synaptic proteins even in the non-ischemic (CL) hemisphere of mouse pups (Fig. 4c).

Discussion

Approximately 60% of IPS survivors develop long-term neurological and cognitive disabilities [1–3]. Molecular mechanisms, at the global proteomic level, that underlie IPS-associated neurodevelopmental disorders remain understudied. Our proteomic analysis of synaptosomal proteins from an IPS mouse model identified molecular pathways/networks that are critical for neuronal survival and function. The results corroborate previously documented effects of ischemic stroke on dendritogenesis [29, 30], neuronal death, and neuronal proliferation [31–36]. In addition, they implicate, for the first time, cellular dysfunctions including neuronal differentiation, synaptogenesis, and protein phosphorylation (Fig. 3) as potential contributing factors to the neurodevelopmental deficits following perinatal ischemic brain injury.

The finding of increased phosphorylation of protein following neonatal ischemia highlights a common molecular mechanism underlying the pathophysiology of

brain injury, as phosphorylation of protein kinases was demonstrated in both adult and perinatal models of HI brain injury [37–41]. The cellular effects of increased protein phosphorylation remain to be established in this IPS model but are likely to regulate neuronal death and survival as observed in previous studies on HI brain injury [40–43]. Moreover, our finding of reduced α -adrenergic and CREB signaling, activities which are regulated by protein phosphorylation and play critical roles in neuronal survival by regulating brain-derived neurotrophic factor [33, 44–46], further underscores the appropriate regulation of neuronal survival and death as a part of the repair mechanism following perinatal brain injury [27, 47–50]. Our perinatal ischemic brain injury model also showed lowered expression and signaling of PKC α , CaMKII α , and MAP-2, which play critical roles in long-term potentiation and depression, indicating impaired synaptogenesis and neuronal connectivity in the developing ischemic brain [51, 52]. Reduced synaptogenesis and synaptic plasticity as predicted by IPA following perinatal ischemia are consistent with previous findings of decreased myelination [29, 30, 53, 54] which affects processing speed and neuronal differentiation [34–36]. These effects likely result in compromised neuronal connectivity. Alternatively, they may simply reflect a developmental pause in axonal and synaptic growth to minimize aberrant connectivity immediately following ischemia. Additional analyses to determine whether these signaling changes remain permanent or recover later after ischemia will be highly relevant.

Nonetheless, our findings uncover potentially novel molecular networks to explain the impaired neurocognitive development in perinatal ischemia survivors. Our findings are in line with a recent analysis of altered synaptosomal proteins due to neonatal hypoxia-ischemia, which is likely a more severe neonatal brain injury model than our ischemia model [18]. In particular, the significant decrease in synaptic proteins at 24 h following a transient increase of these proteins immediately following HI, that was observed in Shao et al. [18], suggests that our models of ischemia only and hypoxia-ischemia share common pathways responsible for neuronal injury and repair. Our findings of ischemic effects on the CL (non-ischemic) hemisphere are also consistent with models of HI brain injury [55].

The discovery of predicted dysfunction of upstream regulators (e.g., ESR α and mTOR) suggests that these factors are important targets for therapeutic manipulation to mitigate the long-term neurodevelopmental effects of IPS. While data are limited, emerging evidence

Fig. 4. IPA-annotated canonical signaling pathways altered in P10 ischemic mouse brains. **a** Reduced activity (blue) of 4 unique clusters of signaling pathways with a z score ≤ -2.0 in the ipsilateral (IL) versus contralateral (CL) hemispheres. Dots indicate a nonsignificant (z score > -2.0) activation state. **b** Altered expression of molecules within the CREB signaling pathway (green, downregulated; red, upregulated). **c** Validation of synaptic proteins by Western

blotting. Graphs show Western blot quantification of selected proteins in both male and female pups normalized to the male sham control (Sh) group. Values are mean $\pm$ SEM, $n = 4-7$ /group, significant difference between groups with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with the post hoc Tukey t test. p-PKC α , phosphorylated PKC α .

links estrogen receptor and mTOR signaling to the risk of ischemic stroke and recovery [56–59]. Interestingly, given the sex-dependent incidence and outcomes of neonatal ischemic stroke in humans [7–9], our findings implicate estrogen receptor signaling and estrogen-related receptor signaling as major mechanisms that confer neuroprotection in females, lessening their vulnerability to adverse outcomes following IPS [60, 61]. While measuring estrogen-related activity was beyond the scope of our study, the prediction of increased ESRR α signaling can inform future investigations of the crosstalk between ESRR α and the PKC/mTOR/AKT/MAP-2 signaling pathways in the etiology of neonatal ischemic stroke [62, 63]. Likewise, the targeting of inflammatory and epigenetic regulators is emerging as a novel neuroprotective strategy against brain injury in early postnatal life [64–69]. In particular, therapeutic strategies targeting CD38, GSK3, and NFE2L2 signaling will likely deliver favorable outcomes, given the activation of microglia in models of neonatal brain injury [31, 53, 70–72]. Finally, the decreased dopamine D3 receptor (DRD3) signaling in our perinatal ischemic brain injury model is a new finding (Table 3). Activation of DRD3 signaling is known to facilitate protective effects from ischemic-reperfusion injury in the renal tissue [73]. An additional study of DRD3 signaling may lead to new insights into the role of DRD3 signaling in IPS pathophysiology.

Our study has 2 limitations. First, the inconsistent outcomes of brain injury induced by ischemia likely resulted in an underestimation of differentially expressed synaptosomal proteins, thereby limiting the scope of the findings. We were not able to address this problem as it was necessary to pool synaptosomes from 3 mouse pups to generate sufficient proteins for iTRAQ analysis. This limitation can be overcome in future studies when advancements in mass spectrometry technology allow a higher level of protein resolution and a lower protein quantity requirement, thus enabling iTRAQ analysis at the level of individual animal. Second, the analysis targeted the alteration of synaptosomal proteins within 24 h after ischemia, which aims to identify changes that occur during the critical period for instituting therapeutic interventions. It remains unknown whether these changes persist into adulthood. Such an analysis is also beyond the scope of this study but can be addressed in the future.

In conclusion, despite the inherent limitations, the proteomic analysis of synaptosomal proteins in a model of IPS corroborated previously implicated mechanisms in neonatal HI brain injury and uncovered novel molecular networks implicated in synapse formation and neuro-

transmission. This study adds to the body of work explaining the pathophysiology of mild to moderate HI brain injury during the neonatal period [74–76]. Moreover, the observation that IPS causes more neurological deficits in males, in both mice and humans [7–9], highlights that mouse models of IPS accurately reflect clinical IPS phenomena. As such, studies on mice can help elucidate the molecular pathways underlying the clinical manifestations of IPS. Finally, the findings underscore therapeutic approaches that aim to enhance synaptic plasticity as a treatment for the long-term neurological dysfunctions following IPS [77], and highlight novel signaling networks for potential combinatorial therapeutic strategies for IPS-associated neurodevelopmental effects.

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Statement of Ethics

The University of Minnesota Institutional Animal Care and Use Committee approved all experiments in this study according to the Ethical and Responsible Animal Care in Research Guidelines.

Disclosure Statement

The authors declare that there are no conflicts or financial interests.

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Author Contributions

A.K.B. performed ischemic surgery, collected synaptosomes, and prepared the manuscript. M.M.B. assisted in ischemic surgery, provided postsurgical care, and collected body temperature and behavioral data. T.M. performed and analyzed behavioral data. J.J.G. isolated proteins and performed Western blot analysis. R.R. provided intellectual discussion. P.V.T. conceptualized and supervised the study, performed surgery, prepared the manuscript, and had the primary responsibility for the final content. All authors reviewed and approved the final draft.

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